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Alkaline Ceramidase 2 (ACER2) and Its Product Dihydrosphingosine Mediate the Cytotoxicity of *N*-(4-Hydroxyphenyl)retinamide in Tumor Cells*

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Increased generation of dihydrosphingosine (DHS), a bioactive sphingolipid, has been implicated in the cytotoxicity of the synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4-HPR) in tumor cells. However, how 4-HPR increases DHS remains unclear. Here we demonstrate that 4-HPR increases the expression of ACER2, which catalyzes the hydrolysis of dihydroceramides to generate DHS, and that ACER2 up-regulation plays a key role in mediating the 4-HPR-induced generation of DHS as well as the cytotoxicity of 4-HPR in tumor cells. Treatment with 4-HPR induced the accumulation of dihydroceramides (DHCs) in tumor cells by inhibiting dihydroceramide desaturase (DES) activity, which catalyzes the conversion of DHCs to ceramides. Treatment with 4-HPR also increased ACER2 expression through a retinoic acid receptor-independent and caspase-dependent manner. Overexpression of ACER2 augmented the 4-HPR-induced generation of DHS as well as 4-HPR cytotoxicity, and 4-HPR-induced death in tumor cells, whereas knocking down ACER2 had the opposite effects. ACER2 overexpression, along with treatment with GT11, another DES inhibitor, markedly increased cellular DHS, leading to tumor cell death, whereas ACER2 overexpression or GT11 treatment alone failed to do so, suggesting that both ACER2 up-regulation and DES inhibition are necessary and sufficient to mediate 4-HPR-induced DHS accumulation, cytotoxicity, and death in tumor cells. Taken together, these results suggest that up-regulation of the ACER2/DHS pathway mediates the cytotoxicity of 4-HPR in tumor cells and that up-regulating or activating ACER2 may improve the anti-cancer activity of 4-HRR and other DHC-inducing agents.

The synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4-HPR),[2] also known as fenretinide, has both chemopreventive and chemotherapeutic properties against different types of cancers (1–6). 4-HPR effectiveness in anti-cancer chemoprevention and chemotherapy is due to its induction of growth inhibition (7), apoptotic death (8, 9), and/or autophagic cell death (10) in premalignant and malignant cells. 4-HPR exerts its antiproliferative and pro-apoptotic effects mainly through retinoic acid receptor (RAR)-independent mechanisms, which have not been fully understood.

An increasing number of studies suggest that 4-HPR modulates the metabolism of sphingolipids (11–13) (Fig. 1), which have been implicated in cell differentiation, growth arrest, and cell death (14). Using a less specific method of quantification, 4-HPR was initially found to increase cellular ceramides (7, 15), which are well known pro-apoptotic molecules. However, using a more sensitive and specific method, electrospray ionization mass spectrometry (ESI/MS/MS), recent studies demonstrated that 4-HPR in fact decreases ceramides in various tumor cell lines while increasing DHCs, ceramide analogues lacking the double bond in their sphingoid base moiety (11–13). 4-HPR inversely regulates cellular ceramides and DHCs by inhibiting DHC desaturase (DES) activity of converting DHCs to ceramides, a key step of the biosynthesis of ceramides (11–13).

Wang *et al.* (13) demonstrated that 4-HPR, in addition to increasing DHCs, also markedly increases DHS, the precursor of DHCs, and phosphorylated DHS or DHS-1-phosphate (DHS1P), in tumor cells. The same study also showed that inhibiting the conversion of DHS to DHS1P with dimethylsphingosine (DMS), an inhibitor of sphingosine kinases responsible for the phosphorylation of DHS, further augments the 4-HPR-induced increase in DHS, leading to increased cytotoxicity of 4-HPR in tumor cells (13). These findings suggest that increased DHS may contribute to the cytotoxicity of 4-HPR. Wang *et al.* (13) further revealed that treatment with exogenous DHS, but not a cell-permeable short-chain DHC (D-e-C_6-DHC), induces cytotoxicity in tumor cells, supporting the view that increased DHS but not DHC may mediate the cytotoxicity of 4-HPR. This underscores the importance of understanding the mechanism by which 4-HPR induces the generation of DHS in tumor cells.

DHS is mainly synthesized *de novo* from serine and palmitoyl-CoA through the action of serine palmitoyltransferase

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[2] The abbreviations used are: 4-HPR, *N*-(4-hydroxyphenyl)retinamide; RAR, retinoic acid receptor; ESI, electrospray ionization; DHC, dihydroceramide; DHS, dihydrosphingosine; DHS1P, DHS-1-phosphate; DES, DHC desaturase; DMS, dimethylsphingosine; SPT, serine palmitoyltransferase; ACER2, alkaline ceramidase 2; PARP, poly(ADP-ribose) polymerase; SCC, squamous cell carcinoma; TET, tetracycline; ET, ethanol; qPCR, quantitative PCR; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SPH, sphingosine; AC, acid ceramidase; NC, neutral ceramidase; MNE, mean normalized expression; ER, endoplasmic reticulum.

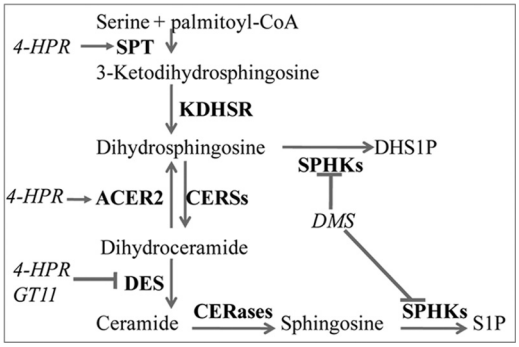

FIGURE 1. **Metabolism of dihydroceramides and dihydrosphingosine.** *KDHSR*, keto-dihydrosphingosine reductase; *SPHK*, sphingosine kinase; *CERSs*, (dihydro)ceramide synthase; *CERases*, ceramidase.

(SPT) and 3-keto-dihydrosphingosine reductase (*KDHSR*) (Fig. 1). Once synthesized, DHS is acylated by the action of (dihydro)ceramide synthases to form DHCs. 4-HPR has been shown to increase SPT activity (16), so it may increase cellular DHS by activating SPT. We previously demonstrated that increasing expression of the alkaline ceramidase 2 (ACER2), a Golgi ceramidase that catalyzes the hydrolysis of ceramides, elevates both cellular sphingosine and DHS (17). Our more recent studies demonstrated that *in vitro* the human alkaline ceramidase 3 (ACER3), a ACER2 homologue, also catalyzes dihydroceramides with unsaturated long acyl chains ($C_{18:1}$ or $C_{20:1}$), so called unsaturated long-chain dihydroceramides, to generate DHS (18). These results suggest that DHS can also be generated from the hydrolysis of DHCs via the action of alkaline ceramidases.

In this study we explore the role of ACER2, ACER3, and their homologue, the human alkaline ceramidase 1 (ACER1) in mediating the 4-HPR-induced generation of DHS and the cytotoxicity of 4-HPR in tumor cells. We show that 4-HPR up-regulates the expression of ACER2 but not ACER3 or ACER1 in HeLa cervical tumor cells and that ACER2 up-regulation increases the conversion of DHC into DHS. We also show that ACER2 overexpression enhances not only the 4-HPR-induced generation of DHS in cells but also the cytotoxicity of 4-HPR in tumor cells, which is associated with increased apoptotic cell death, whereas knocking down ACER2 has opposite effects. Moreover, we find that ACER2 overexpression, along with treatment with GT11, a different DES inhibitor, induces DHS accumulation and cell death in tumor cells. These results suggest that inverse regulation of ACER2 and DES is an important mechanism by which 4-HPR exerts its cytotoxic and apoptotic effects in tumor cells.

EXPERIMENTAL PROCEDURES

Reagents—The anti-ACER2 antibody was previously raised against a peptide located at the carboxyl terminus of ACER2 in our laboratory (17). Anti-GM130 antibody was from BD Biosciences. Antibodies against poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3 were from Cell Signaling Technology (Danvers, MA). *O*-Phthalaldehyde was from FLUKA (Milwaukee, WI). Pan-RAR antagonist LG100815 was from Ligand Pharmaceuticals, Inc. (San Diego, CA). Minimal essential medium, fetal bovine serum (FBS), trypsin-EDTA, Ca^{2+}-free phosphate-buffered saline, penicillin/streptomycin solution, blasticidin, Zeocin, and G418 were purchased from Invitrogen. D-e-$C_{18:1}$, $C_{20:1}$, and $C_{20:4}$-DHC were synthesized in the Lipidomics Core Facility at Medical University of South Carolina. Other sphingolipids used in this study were purchased from Avanti Polar Lipids (Alabaster, AL). The dihydroceramide desaturase inhibitor *N*-[(1*R*,2*S*)-2-hydroxy-1-hydroxymethyl-2-(2-tridecyl-1-cyclopropenyl)ethyl]octanamide (GT11) was from Matreya LLC (Pleasant Gap, PA). Other unlisted chemicals were purchased from Sigma.

Cell Lines and Culture Conditions—T-REx-HeLa cells (Invitrogen) and HSC-1 skin squamous cell carcinoma cells were cultured in minimal essential medium supplemented with 10% FBS, a 1% penicillin/streptomycin solution, and 10 μg/ml blasticidin. The T-REx-HeLa derivative cell lines AC-TET-ON, NC-TET-ON, ACER1-TET-ON, and ACER2-TET-ON were cultured in minimal essential medium supplemented with 10 μg/ml blasticidin and 50 μg/ml Zeocin as described in our previous study (19). The cell line SCC-ACER2-TET-ON was generated in this study using the same strategy for the generation of other TET-ON cell lines. Briefly, SCC1 cells, a human oral squamous cell carcinoma (SCC) cell line, was first stably transfected with pcDNA6/TR (Invitrogen) according to the manufacturer's instructions. SCC1 cells stably expressing pcDNA6/TR were then transfected with pcDNA4-ACER2 to generate the cell line ACER2-TET-ON-SCC. In all the TET-ON cell lines, gene expression is induced by adding tetracycline (TET) but not the vehicle ethanol (ET) to medium.

RNA Interference (RNAi)—A control siRNA (siCON, 5′-UAAGGCUAUGAAGAGAUACUU-3′ (sense)/5′-GUAUCUCUUCAUAGCCUUAUU-3′ (antisense)), ACER2-specific siRNA (siACER2, 5′-UGACCGAGCUUUCUGCGAGUU-3′ (sense)/5′-CUCGCAGAAAGCUCGGUCAUU-3′), and ACER3-specific siRNA (siACER3–1, 5′-UGGGAUCCUGGUGCUUCCA-3′ (sense)/5′-UGGAAGCACCAGGAUCCCA-3′ (antisense)) were synthesized in Dharmacon, Inc. (Chicago, IL). siRNA transfection was performed with Oligofectamine (Invitrogen) as described in our previous study (17).

Quantitative Polymerase Chain Reaction (qPCR)—Total RNA was isolated from cells using RNeasy kits (Qiagen) according to the manufacturer's instructions. Five μg of total RNA from each cell sample were reverse-transcribed into cDNA as described (20). Quantitative PCR was performed on an iCycler system (Bio-Rad, Inc.) using the primer pairs 5′-TGATGCTTGACAAGGCACCA-3′/5′-GGCAATTTTTCATCCACCACC-3′ for ACER1, 5′-AGTGTCCTGTCTGCGGTTACG-3′/5′-TGTTGTTGATGGCAGGCTTGAC-3′ for ACER2, 5′-CAATGTTCGGTGCAATTCAGAG-3′/5′-GGATCCCATTCCTACCACTGTG-3′ for ACER3, and 5′-CAATGTTCGGTGCAATTCAGAG-3′/5′-GGATCCCATTCCTACCACTGTG-3′ for β-actin. The standard reaction volume was 25 μl, including 12.5 μl of iQ™ SYBR Green Supermix (Bio-Rad), 10 μl of cDNA template, and 2.5 μl of a primer mixture. The initial PCR step was 3 min at 95 °C followed by 40 cycles of a 10-s melting at 95 °C and a 45-s annealing/extension at 60 °C. The final step was 1 min of incubation at 60 °C. All reactions were performed in triplicate. qPCR results were analyzed using

Q-Gene software which expresses data as mean normalized expression (MNE) (21). MNE is directly proportional to the amount of mRNA of the target gene (*ACER1*, *ACER2*, or *ACER3*) relative to the amount of mRNA of the reference gene (β-actin).

MTT Assay—Cell viability was determined using an *in vitro* toxicology assay kit based on 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, Inc.) according to the manufacturer's instructions.

Fluorescence-activated Cell Sorting (FACS) Analysis—DNA fragmentation and cell cycle profiles were analyzed by FACS on a FACStarplus flow cytometer (BD Biosciences) according to the manufacturer's instructions.

Protein Concentration Determination—Protein concentrations were determined with BSA as a standard using a BCA protein determination kit (Pierce) according to the manufacturer's instructions.

Western Blot Analysis—Proteins were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes, which were then analyzed by Western blot using various antibodies. Protein band density was determined by densitometry performed on a ChemiImager 4400 system (Alpha Innotech, San Leandro, CA) according to the manufacturer's instructions.

Lipid Extraction—Total lipids were extracted from cells according to the Bligh-Dyer method (22).

(Dihydro)ceramidase Activity Assay—(Dihydro)ceramidase activity was determined by the release of SPH or DHS from the hydrolysis of ceramides or DHCs, respectively, as described (17). Briefly, a substrate was dispersed into a buffer containing 0.3% Triton X-100 by water bath sonication. The lipid-detergent mixture was boiled for 30 s and chilled on ice immediately to form homogeneous lipid-detergent micelles, which were mixed on ice with an equal volume of microsomes suspended in the same buffer but with no Triton X-100. Microsomes were prepared from cells as described in our previous study (20). Enzymatic reactions were initiated by incubating the substrate-enzyme mixtures at 37 °C for 20 min. The reactions were stopped by adding the same volume of methanol to the reaction mixtures. An internal standard (D-e-C_{17}-SPH, C17SPH) was added to the reaction mixtures, which were completely dried on a Savant SpeedVac system (Thermo Fisher Scientific Inc., Waltham, MA). The amounts of SPH or DHS in the enzymatic reactions were determined by HPLC. Both reaction time and amounts of enzyme were within the linear range.

ACER2 Promoter Cloning and Activity Assays—The 1882-base pair DNA fragment upstream of the ACER2 translation initiation site (ATG) was amplified by PCR from human genomic DNA, which was isolated from the whole blood of disease-free sources (Clontech) using the primers 5′-cggggtaccatggaatctcgctctgtcaccc-3′ (forward) and 5′-cccaagcttggccactccggggcattggagcag-3′ (reverse). This DNA sequence was cloned into a promoter reporter vector pGL3 (Promega) to generate an ACER2 promoter construct, pGL3-ACER2P. This construct or the control vector pGL3 was co-transfected with the plasmid phRL-TK (Promega) into HeLa cells using Effectene (Qiagen) according to the manufacturer's instructions. pGL3-ACER2P contains the firefly luciferase gene and phRL-TK the *Renilla* luciferase gene, which serves as an internal control for normalization. ACER2 promoter activity was determined using a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Promoter activity was expressed as the ratio of the firefly luciferase activity (average relative light units) to the Renilla luciferase activity (average relative light units).

HPLC Analysis for SPH and DHS—The amount of SPH and DHS in enzymatic reactions or lipid extracts of cells was determined by high performance liquid chromatography (HPLC) after its derivatization with *O*-phthalaldehyde as described (23). The dry enzymatic reaction mixtures were treated for 30 min at 37 °C with 0.125 M methanolic KOH to hydrolyze phospholipids. After Bligh-Dye extraction (22), SPH and DHS in the enzymatic reactions were derivatized with *O*-phthalaldehyde before being analyzed by HPLC.

ESI/MS/MS Analysis for Sphingolipids—Sphingolipids were determined by ESI/MS/MS as described (24). Briefly, cells were harvested after being washed with ice-cold 25 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. Fifty μl of a mixture (1 μM) of internal sphingolipid standards including C17SPH, C17SPH-1-phosphate, D-e-C_{16}-ceramide (d17:1/16:0), and D-e-$_{18}$-ceramide (d17:1/18:0) was added to each cell pellet sample before lipid extraction with 4 ml of the ethyl acetate/isopropyl alcohol/water (60/30/10%;v/v) solvent system. After centrifugation, 1 ml of lipid extracts from each sample was used for determination of total phospholipids, and the remainder was used for ESI/MS/MS. The lipid extracts were dried under a stream of nitrogen gas. For ESI/MS/MS, dried lipid extracts of each sample were dissolved in 100 μl of acidified (0.2% formic acid) methanol and injected on the HP1100/TSQ 7000 LC/MS system and gradient-eluted from the BDS Hypersil C8, 150 × 3.2 mm, 3-μm particle size column with 1.0 mM methanolic ammonium formate, 2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and sphingolipid standards were collected and processed using the Xcalibur software. Quantitative analyses of endogenous sphingolipids were based on calibration curves generated by spiking an artificial matrix with known amounts of the target analyte synthetic standards and an equal amount of the sphingolipid standard. The target analyte/sphingolipid standard peak area ratios were compared with the calibration curves using a linear regression model. Levels of the particular sphingolipid were normalized to phospholipids and expressed as sphingolipids/phospholipids (pmol/μmol).

Quantification of Phospholipids—The total amount of phospholipids (P) in lipid extracts was determined by quantifying inorganic phosphate (Pi) released from the acidic hydrolysis phospholipids as described (25).

Statistical Analysis—Student's *t* test was applied for statistical analysis using the software GraphPad Prism. Values of $p < 0.05$ were considered significant and are marked with an *asterisk* (*).

RESULTS

ACER1 and ACER2 Catalyze the Generation of DHS in Vitro and in Cells—As mentioned earlier, ACER3 can hydrolyze certain DHC species *in vitro*. To investigate whether its homologues ACER1 and ACER2 can also catalyze the hydrolysis of DHCs, we determined whether overexpression of ACER1 or

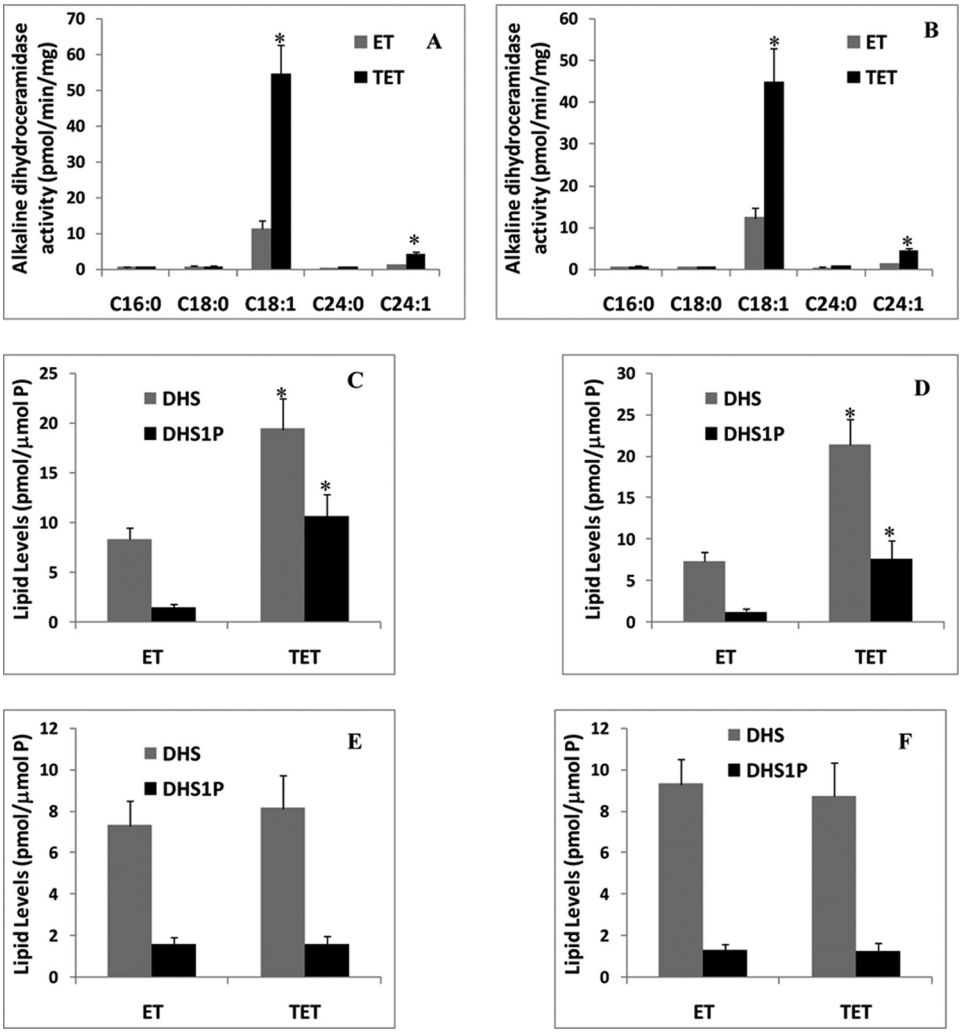

FIGURE 2. **ACER1 and ACER2 catalyze the hydrolysis of dihydroceramides into dihydrosphingosine *in vitro* and in cells.** *A* and *B*, microsomes were isolated from ACER1-TET-ON cells (*A*) or ACER2-TET-ON cells (*B*) grown in the presence of ET or TET (10 ng/ml), and alkaline (dihydro)ceramidase activity on various DHCs was determined in isolated microsomes. *C–F*, ACER1-TET-ON (*C*), ACER2-TET-ON (*D*), AC-TET-ON (*E*), or NC-TET-ON (*F*) cells grown in the presence of ET or TET were treated with DMSO or 4-HPR (10 μM) for 6 h before DHS and DHS1P were analyzed by ESI/MS/MS. Data represent the mean values ± S.D. of three independent experiments. *, $p < 0.05$ *versus* control (ET).

ACER2 increased *in vitro* (dihydro)ceramidase activity on various DHC substrates. To do this, we used stable T-REx-HeLa cell lines ACER1-TET-ON and ACER2-TET-ON, which were generated in the course of previous studies, and overexpress ACER1 and ACER2, respectively, under the control of an inducible CMV-TET-ON promoter system (17, 19). In these cells the ectopic expression of ACER1 or ACER2 is induced by TET (10 ng/ml) but not by ET, the vehicle control. This was confirmed by Western blot analysis (data not shown). When D-e-$C_{18:1}$-DHC or D-e-$C_{24:1}$-DHC was used as a substrate, microsomes isolated from ACER1-TET-ON cells grown in the presence of TET had higher (dihydro)ceramidase activity than microsomes isolated from ACER1-TET-ON cells grown in the presence of ET, whereas no difference was seen when D-e-$C_{16:0}$-DHC, D-e-$C_{18:0}$-DHC, or D-e-$C_{24:0}$-DHC was used as a substrate (Fig. 2*A*), suggesting that ACER1 catalyzes the hydrolysis of DHCs with unsaturated acyl-chains, termed unsaturated DHCs. Similar to ACER1 overexpression, ACER2 overexpression also increased microsomal (dihydro)ceramidase activity on D-e-$C_{18:1}$ or $C_{24:1}$-DHC but not D-e-$C_{16:0}$-, $C_{18:0}$-, or $C_{24:0}$-DHC (Fig. 2*B*), suggesting that, like ACER1, ACER2 also catalyzes the hydrolysis of unsaturated DHCs but not saturated DHCs.

We previously demonstrated that ACER2 overexpression increased cellular DHS (17), suggesting that ACER2 can hydrolyze DHCs to generate DHS in cells. However, it remains unclear whether ACER1 also catalyzes the hydrolysis of DHCs in cells. To this end, we investigated whether ACER1 overexpression increased cellular DHS. ESI/MS/MS showed that the levels of DHS in ACER1-TET-ON cells grown in the presence of TET (10 ng/ml) were 2-fold higher than those in the cells grown in the presence of ET (Fig. 2*C*), suggesting that ACER1 also catalyzes the hydrolysis of DHCs to generate DHS in cells. ACER1 overexpression also increased cellular phosphorylated DHS, DHS1P (Fig. 2*C*). We also confirmed our previous finding that ACER2 overexpression increased the generation of both DHS and DHS1P in cells (Fig. 2*D*).

In addition to the alkaline ceramidases, the human acid ceramidase (AC) (26) or rat neutral ceramidase (NC) (27) was also shown to catalyze the hydrolysis of DHCs *in vitro*, although with low efficiency. However, it remains unclear whether they also catalyze the generation of DHS in cells. To address this issue, we determined whether overexpression of AC or NC increased cellular DHS. We previously generated the T-REx-HeLa-based cell lines AC-TET-ON and NC-TET-ON, which overexpress AC and NC, respectively, under the control of the CMV-TET-ON promoter system (17). In these cell lines, overexpression of AC or NC is induced by TET but not ET. This was confirmed by Western blot analysis (data not shown). ESI/MS/MS demonstrated that TET-induced expression of AC or NC did not increase cellular DHS or DHS1P (Fig. 2, *E* and *F*), suggesting that unlike the alkaline ceramidases, neither AC nor NC catalyzes the generation of DHS in cells.

4-HPR Increases ACER2 Activity and Protein—Because the alkaline ceramidases have the ability to regulate the generation of DHS in cells, we investigated whether 4-HPR induced the generation of DHS by increasing activity or expression of these enzymes in HeLa cells. First, we determined whether 4-HPR increased alkaline (dihydro)ceramidase activity on D-e-$C_{18:1}$-

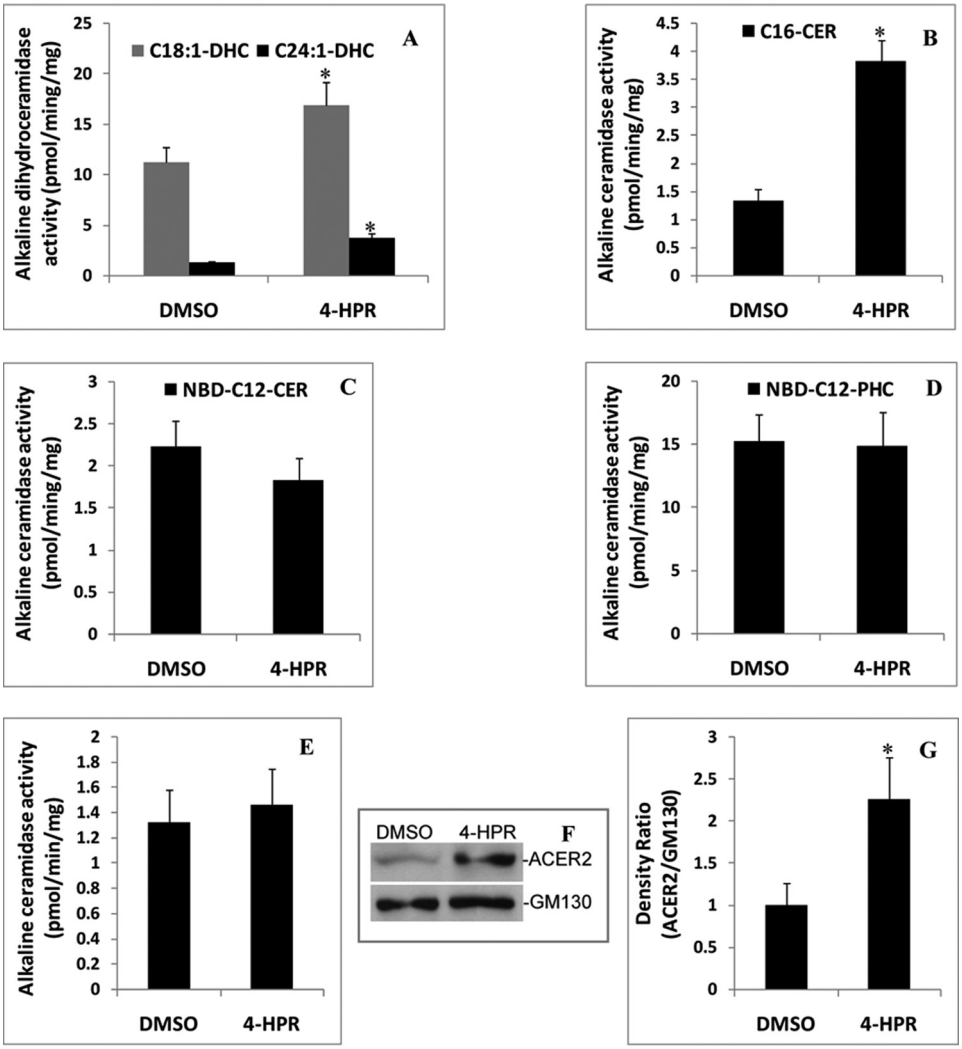

FIGURE 3. **4-HPR increases both ACER2 mRNA and protein.** *A*, T-REx-HeLa cells were treated with 4-HPR (10 μM) or DMSO for 48 h before microsomes were prepared. Microsomal alkaline ceramidase activity on D-e-$C_{18:1}$-ceramide (*$C_{18:1}$-DHC*) or D-e-$C_{24:1}$-DHC (*$C_{24:1}$-DHC*) were then determined by the HPLC method. *B–D*, microsomal preparations as in *A* were measured for alkaline ceramidase activity on 150 μM D-e-C_{16}-ceramide (*B*), D-e-C_{12}-NBD-ceramide (*C*), or D-ribo-C_{12}-NBD-phytoceramide (*D*). *E*, alkaline ceramidase activity in microsome-isolated untreated T-REx-HeLa cells was measured in assay buffer containing DMSO or 4-HPR (10 μM). *F* and *G*, T-REx-HeLa cells were treated with 4-HPR (10 μM) or DMSO for 48 h before microsomes were prepared and analyzed by Western blot using anti-ACER2 antibody (*F*), and the protein density was estimated by densitometry (*G*). The density ratio of ACER2 to GM130 in ACER2-TET-ON cells treated with DMSO was arbitrarily set at 1. Image datum represents one of two independent experiments with similar results. Numerical data represent mean values ± S.D. of *3* independent experiments. *, $p < 0.05$ *versus* control (DMSO).

DHC or D-e-$C_{24:1}$-DHC. We measured alkaline ceramidase activity in microsomes isolated from T-REx-HeLa cells treated with DMSO or 4-HPR (10 μM). Compared with treatment with DMSO, treatment with 4-HPR increased microsomal alkaline dihydroceramidase activity on D-e-$C_{18:1}$-DHC and D-e-$C_{24:1}$-DHC by 120 and 250%, respectively (Fig. 3*A*), suggesting that 4-HPR increases alkaline (dihydro)ceramidase activity on unsaturated DHCs. To distinguish which alkaline ceramidase activity was increased by 4-HPR, we determined microsomal alkaline ceramidase activity on natural or synthetic ceramide substrates specific for each of the alkaline ceramidases. We previously demonstrated that ACER1 hydrolyzes D-e-C_{12}-NBD-ceramide but not D-e-C_{16}-ceramide (28), whereas the opposite is true with ACER2 (29), and that ACER3 (but not ACER1 or ACER2) hydrolyzes D-ribo-C_{12}-NBD-phytoceramide (28, 30) with moderate but significant activity on D-e-C_{12}-NBD-ceramide. Compared with treatment with DMSO, 4-HPR caused a 3-fold increase in microsomal alkaline ceramidase activity on D-e-C_{16}-ceramide (Fig. 3*B*) without affecting the activity on D-e-C_{12}-NBD-ceramide (Fig. 3*C*) or D-ribo-C_{12}-NBD-phytoceramide (Fig. 3*D*), suggesting that 4-HPR increases ACER2 activity but not ACER1 or ACER3 activity.

To determine whether the ACER2 alkaline ceramidase activity increase is due to an activation of pre-existing ACER2 or an increase in ACER2 protein in cells, we determined whether 4-HPR affected *in vitro* ACER2 activity and its protein content in cells. Compared with DMSO, 4-HPR, when added to the assay buffer, did not alter alkaline ceramidase activity in microsomes isolated from T-REx-HeLa cells (Fig. 3*E*), suggesting that 4-HPR does not activate pre-existing ACER2. Western blot analyses with anti-ACER2 antibody showed that compared with treatment with DMSO, treatment with 4-HPR increased ACER2 protein in microsomes of T-REx-HeLa cells (Fig. 3, *F* and *G*), suggesting that 4-HPR increases ACER2 protein in cells.

4-HPR Increases ACER2 Expression through a Caspase-dependent Manner—To determine whether the increase in ACER2 protein is due to an increase in ACER2 mRNA, we measured ACER2 mRNA levels in T-REx-HeLa cells treated with DMSO or 4-HPR. qPCR demonstrated that compared with treatment with DMSO, treatment with 4-HPR increased ACER2 mRNA levels in T-REx-HeLa cells (Fig. 4*A*). In agreement with the finding that 4-HPR does not affect ACER3 activity, 4-HPR treatment did not alter ACER3 mRNA levels in T-REx-HeLa cells (Fig. 4*B*). We previously demonstrated that ACER1 is not expressed in HeLa cells (28). Here, we also found that ACER1 mRNA was undetectable in T-REx-HeLa cells, a HeLa derivative, and that 4-HPR treatment did not induce ACER1 mRNA expression (data not shown). These results suggest that 4-HPR increases ACER2 mRNA levels in T-REx-HeLa cells.

The ACER2 mRNA increase could be due to an increase in ACER2 mRNA stability or ACER2 transcription. To investigate which scenario would be relevant, we determined whether 4-HPR increased ACER2 transcription by determining its promoter activity. We cloned the putative ACER2 promoter, a

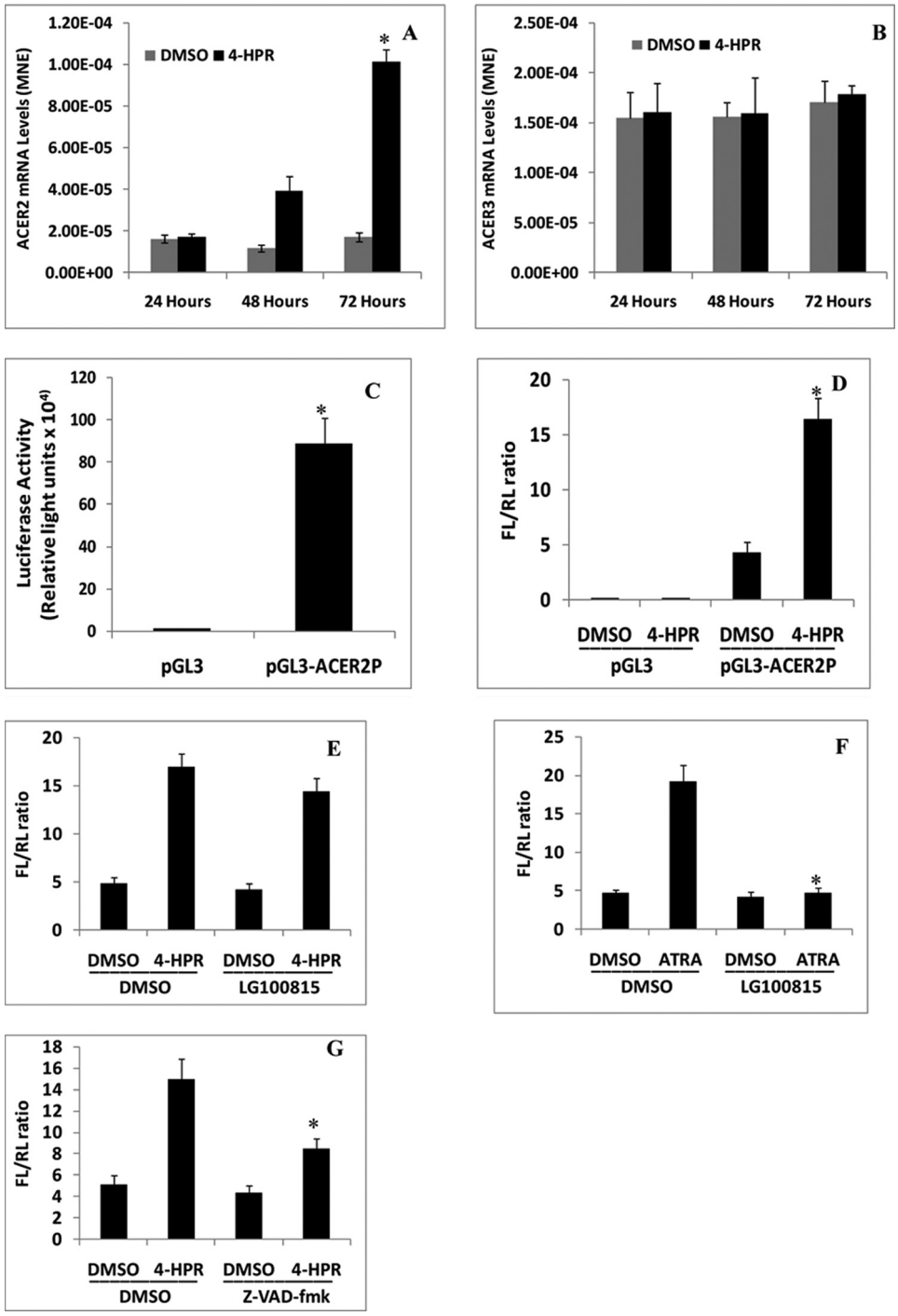

FIGURE 4. **4-HPR increases ACER2 transcription by activating caspases.** *A* and *B*, T-REx HeLa cells were treated with DMSO or 4-HPR (12.5 μM) for various time periods before ACER2 mRNA (*A*) or ACER3 mRNA (*B*) was analyzed by qPCR. *C*, the same amount of the pGL3-ACER2P or pGL3 DNA was transfected into T-Rex HeLa cells for 24 h before ACER2 promoter activity was determined by luciferase activity assays. *D*, T-REx HeLa cells were co-transfected with phRL-TK and pGL3-ACER2P or pGL3 for 24 h before being treated with 4-HPR (12.5 μM) or DMSO. Twenty-four hours post-4-HPR or DMSO treatment, ACER2 promoter activity was determined. *FL*, firefly luciferase activity encoded by pGL3 or pGL3-ACER2P; *RL*, *Renilla* luciferase activity encoded by phRL-TK. *E* and *F*, T-REx HeLa cells co-transfected with pGL3-ACER2P and phRL-TK were treated with 4-HPR (12.5 μM) (*E*), ATAR (1 μM) (*F*), or DMSO in the presence of the pan-RAR antagonist LG100815 (10 μM) or DMSO for 24 h before ACER2 promoter activity was determined. *G*, T-REx HeLa cells co-transfected with pGL3-ACER2P, and phRL-TK were treated with 4-HPR (12.5 μM) or DMSO in the presence of benzyloxycarbonyl-VAD-fluoromethyl ketone (50 μM) or DMSO for 24 h before ACER2 promoter activity was determined. Data represent the mean values ± S.D. of three independent experiments. *, $p < 0.05$ *versus* control.

1.9-kb DNA fragment upstream of the translation initiation site of ACER2 into a promoter reporter vector pGL3. The resulting ACER2 promoter construct pGL3-ACER2P or the control vector was transfected into T-REx HeLa cells. Transfection with pGL3-ACER2P markedly increased luciferase activity compared with transfection with pGL3 (Fig. 4*C*), suggesting that the ACER2 promoter is functional in T-REx HeLa cells.

To determine whether 4-HPR increases ACER2 transcription, we determined whether treatment with 4-HPR increased ACER2 promoter activity. T-REx cells transfected with pGL3 or pGL3-ACER2P were treated with 4-HPR (12.5 μM) or DMSO for 24 h before ACER2 promoter activity was measured. Treatment with 4-HPR increased ACER2 promoter activity in T-REx HeLa cells transfected with pGL3-ACER2P but not in cells transfected with pGL3, compared with treatment with DMSO, the vehicle control (Fig. 4*D*), suggesting that 4-HPR increases ACER2 transcription.

We previously demonstrated that ACER2 expression is increased in T-REx cells by all-*trans* retinoic acid (19), a natural retinoid that regulates gene expression through RARs. Like all-*trans* retinoic acid, 4-HPR can also up-regulate gene expression through a RAR-dependent manner, although its biological effects are mainly through RAR-independent mechanisms (31). These observations prompted us to determine whether 4-HPR increases ACER2 promoter activity by activating RAR/retinoic X receptors. We found that treatment with a pan-RAR antagonist (LG100815) only moderately inhibited 4-HPR-stimulated ACER2 promoter activity (Fig. 4*E*) while abolishing the all-*trans* retinoic acid-stimulated ACER2 promoter activity in T-REx HeLa cells (Fig. 4*F*), suggesting that 4-HPR increases ACER2 expression mainly through a-RAR-independent mechanism.

Because 4-HPR has also been shown to up-regulate gene expression by activating caspases, we then determined whether caspase activation mediates 4-HPR-induced expression of ACER2. T-REx HeLa cells transfected with pGL3-ACER2P were treated with 4-HPR or DMSO in the presence or absence of benzyloxycarbonyl-VAD-fluoromethyl ketone, a

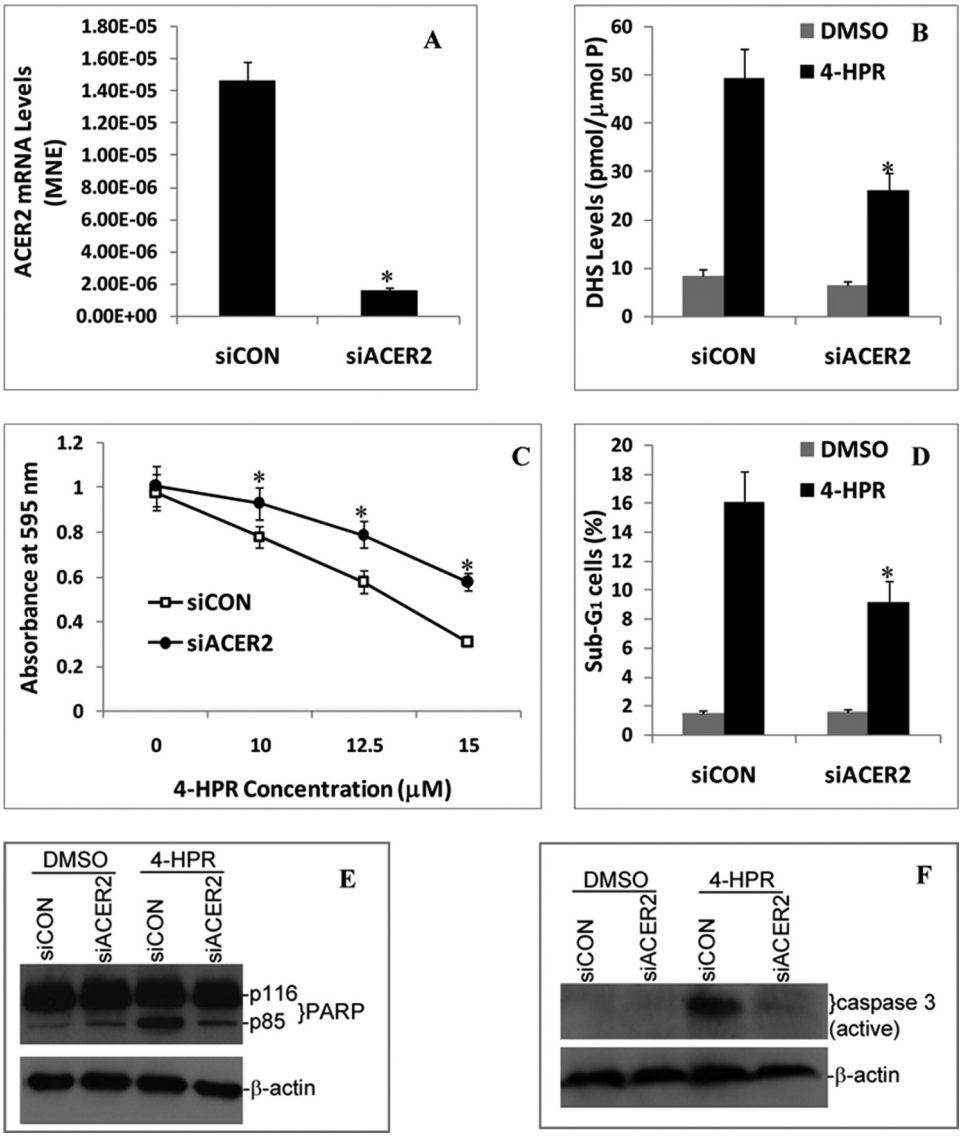

FIGURE 5. **ACER2 knockdown inhibits DHS generation, cytotoxicity, and cell death in tumor cells in response to 4-HPR treatment.** *A*, T-REx-HeLa cells were transfected with siCON (5 nM) or siACER2 (5 nM) for 48 h before ACER2 mRNA was analyzed by qPCR. *B*, T-REx-HeLa cells transfected with siCON or siACER2 were treated with 4-HPR (10 μM) or DMSO for 6 h before DHS levels were determined by HPLC. *C*, T-REx-HeLa cells transfected with siCON or siACER2 were treated with 4-HPR at indicated concentrations or DMSO (0 μM 4-HPR) for 48 h before cell viability was determined by MTT assays. *D–F*, T-REx cells transfected with siCON or siACER2 were treated with 12.5 μM 4-HPR or DMSO for 48 h before the percentage of cells with sub-G_1 DNA (sub-G_1 cells) was determined by FACS (*D*) or Western blot analyses of PARP cleavage (*E*) and caspase-3 activation (*F*). Images represent at least two independent experiments with similar results. Numeral data represent the mean values ± S.D. of *3* independent experiments. *, $p < 0.05$ *versus* control (siCON).

pan-caspase inhibitor. Treatment with benzyloxycarbonyl-VAD-fluoromethyl ketone significantly inhibited a 4-HPR-induced increase in ACER2 promoter activity (Fig. 4*G*), suggesting that 4-HPR increases ACER2 expression by activating caspases.

ACER2 Knockdown Inhibits the Generation of DHS, Cytotoxicity, and Cell Death in Tumor Cells in Response to 4-HPR Treatment—After finding that 4-HPR up-regulated ACER2 and that ACER2 has the ability to hydrolyze DHCs to generate DHS in cells, we investigated whether ACER2 up-regulation mediates the 4-HPR-induced generation of DHS in cells. We determined whether knocking down ACER2 by RNAi inhibited the 4-HPR-induced generation of DHS in cells. T-REx-HeLa cells were transfected with a control siRNA (siCON) or siACER2, an ACER2-specific siRNA that has been validated to knock down ACER2 efficiently in our previous study (19). qPCR analyses confirmed that compared with transfection with siCON, transfection with siACER2 caused a marked decrease in ACER2 mRNA levels in T-REx-HeLa cells (Fig. 5*A*). HPLC analyses found that ACER2 knockdown significantly inhibited the 4-HPR-induced increase in DHS in T-REx-HeLa cells (Fig. 5*B*), suggesting that ACER2 up-regulation is important for 4-HPR-induced generation of DHS.

Because DHS has been implicated in the cytotoxicity of 4-HPR toward tumor cells (13), inhibiting the 4-HPR-induced generation of DHS may reduce the cytotoxicity of 4-HPR. To test this idea, we determined whether ACER2 knockdown decreased the cytotoxicity of 4-HPR. MTT assays demonstrated that treatment with 4-HPR at each concentration reduced the viability of T-REx-HeLa cells transfected with siACER2 less effectively than the viability of T-REx-HeLa cells transfected with siCON (Fig. 5*C*), suggesting that ACER2 knockdown decreases the cytotoxicity of 4-HPR toward tumor cells.

Both DHS and 4-HPR have been implicated in cell death, so we then determined whether ACER2 knockdown reduced the cytotoxicity of 4-HPR by inhibiting cell death. FACS analysis was performed to determine whether ACER2 knockdown affected 4-HPR-induced increase in the sub-G_1 DNA content or DNA fragmentation, a hallmark of cell death. It was found that ACER2 knockdown substantially inhibited the 4-HPR-induced increase in the percentage of cells with sub-G_1 DNA content (sub-G_1 cells) (Fig. 5*D*), suggesting that ACER2 knockdown inhibits the 4-HPR-induced cell death in tumor cells. To further confirm this notion, Western blot analyses were performed to determine whether ACER2 knockdown inhibited the 4-HPR-induced cleavage of PARP and the 4-HPR-induced activation of caspase-3, two additional hallmarks of cell death. We found that ACER2 knockdown significantly inhibited both the 4-HPR-induced cleavage of PARP (Fig. 5*E*) and activation of caspase-3 (Fig. 5*F*), supporting that ACER2 knockdown inhibits the 4-HPR-induced cell death in tumor cells.

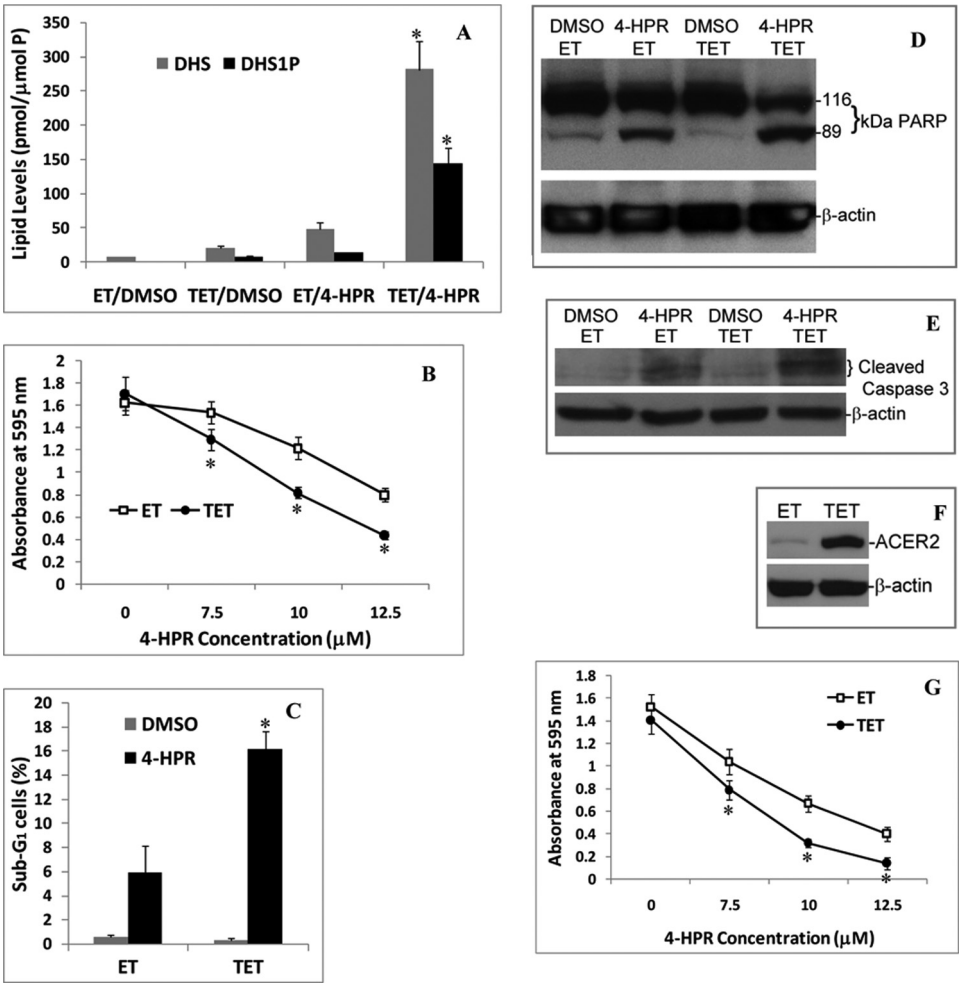

FIGURE 6. **ACER2 overexpression increases the generation of DHS, cytotoxicity, and cell death in tumor cells in response to 4-HPR treatment.** *A*, ACER2-TET-ON cells grown in the presence of ET or TET (10 ng/ml) were treated with DMSO or 4-HPR for 6 h before levels of DHS and DHS1P were analyzed by ESI/MS/MS. *B*, ACER2-TET-ON cells grown in the presence of ET or TET (10 ng/ml) were treated with DMSO (0 4-HPR) or 4-HPR at various concentrations for 48 h before cell viability was determined by MTT assays. *C*, ACER2-TET-ON cells grown in the presence of ET or TET (10 ng/ml) were treated with 4-HPR (10 μM) or DMSO for 48 h before the percentage of sub-G_1 cells was determined by FACS analysis. *D* and *E*, ACER2-TET-ON cells grown in the presence of ET or TET (10 ng/ml) were treated with 4-HPR or DMSO for 48 h before Western blot analyses were performed using anti-PARP antibody (*D*) or antibody specific for cleaved caspase-3 (*E*). *F*, ACER2-TET-ON-SCC cells were grown in the presence of TET (10 ng/ml) or ethanol for 48 h before ACER2 expression was determined by Western blot. *G*, ACER2-TET-ON-SCC cells grown in the presence of ET or TET were treated with 4-HPR at indicated concentrations for 48 h before cell viability was assessed by MTT assays. Image data represent at least two independent experiments with similar results. Numerical data represent the mean values ± S.D. of 3 independent experiments. *, $p < 0.05$ *versus* control (ET/DMSO or ET).

ACER2 Overexpression Enhances DHS Generation, Cytotoxicity, and Cell Death in Tumor Cells in Response to 4-HPR Treatment—After finding that ACER2 knockdown inhibits the 4-HPR-induced DHS generation, cytotoxicity, and cell death in tumor cells, we determined whether ACER2 overexpression has the opposite effects. First, we determined whether ACER2 overexpression enhances the 4-HPR-induced generation of DHS in tumor cells. ACER2-TET-ON cells grown in the presence of ET or TET (10 ng/ml) were treated with 4-HPR (10 μM) or DMSO for 6 h before ESI/MS/MS was performed to measure cellular DHS and DHS1P. ACER2 overexpression or 4-HPR treatment alone caused a severalfold increase in cellular DHS, whereas 4-HPR treatment along with ACER2 overexpression caused a greater than 30-fold increase in cellular DHS (Fig. 6*A*). ACER2 overexpression or 4-HPR treatment alone also caused a severalfold increase in cellular DHS1P, whereas ACER2 overexpression along with 4-HPR treatment caused a greater than 80-fold increase in cellular DHS1P (Fig. 6*A*). These results suggest that ACER2 overexpression enhances the 4-HPR-induced generation of DHS and DHS1P in tumor cells.

To determine whether ACER2 overexpression enhanced the cytotoxicity of 4-HPR by promoting cell death, MTT assays were performed to determine cell viability in ACER2-TET-ON cells subjected to different treatments. The results showed that ACER2 overexpression significantly enhanced the 4-HPR-induced decrease in the viability of T-REx HeLa cells (Fig. 6*B*). FACS analysis was then performed to determine DNA fragmentation in ACER2-TET-ON cells grown in the presence of ET or TET and treated with DMSO or 4-HPR. It was found that ACER2 overexpression markedly augmented the 4-HPR-induced increase in the percentage of sub-G_1 T-REx-HeLa cells, suggesting that ACER2 overexpression indeed enhances 4-HPR-induced cell death in tumor cells (Fig. 6*C*). Consistently, Western blot analyses demonstrated that ACER2 overexpression markedly augmented both 4-HPR-induced cleavage of PARP (Fig. 6*D*) and activation of caspase-3 (Fig. 6*E*), supporting the view that ACER2 overexpression sensitizes tumor cells to 4-HPR-induced cell death.

To determine whether the role of ACER2 in mediating 4-HPR-induced generation of DHS and the cytotoxicity of 4-HPR is general or cell type-specific, we tested whether ACER2 overexpression can further enhance the effects of 4-HPR on DHS generation and cytotoxicity in a different tumor cell line, SCC1 oral squamous cell carcinoma cells. We generated a SCC1 stable cell line, ACER2-TET-ON-SCC, using the same Tet-ON expression system as used for ACER2 expression in T-Rex HeLa cells as described in our previous study (17). In ACER2-TET-ON-SCC cells, ACER2 expression was induced by TET but not ET (Fig. 6*F*). MTT assays showed that treatment with 4-HPR at each concentration reduced the viability of ACER2-TET-ON-SCC cells grown in the presence of TET more effectively than that of ACER2-TET-ON cells grown in the presence of ET (Fig. 6*G*), suggesting that ACER2 overexpression also increases the cytotoxicity of 4-HPR in different tumor cell types.

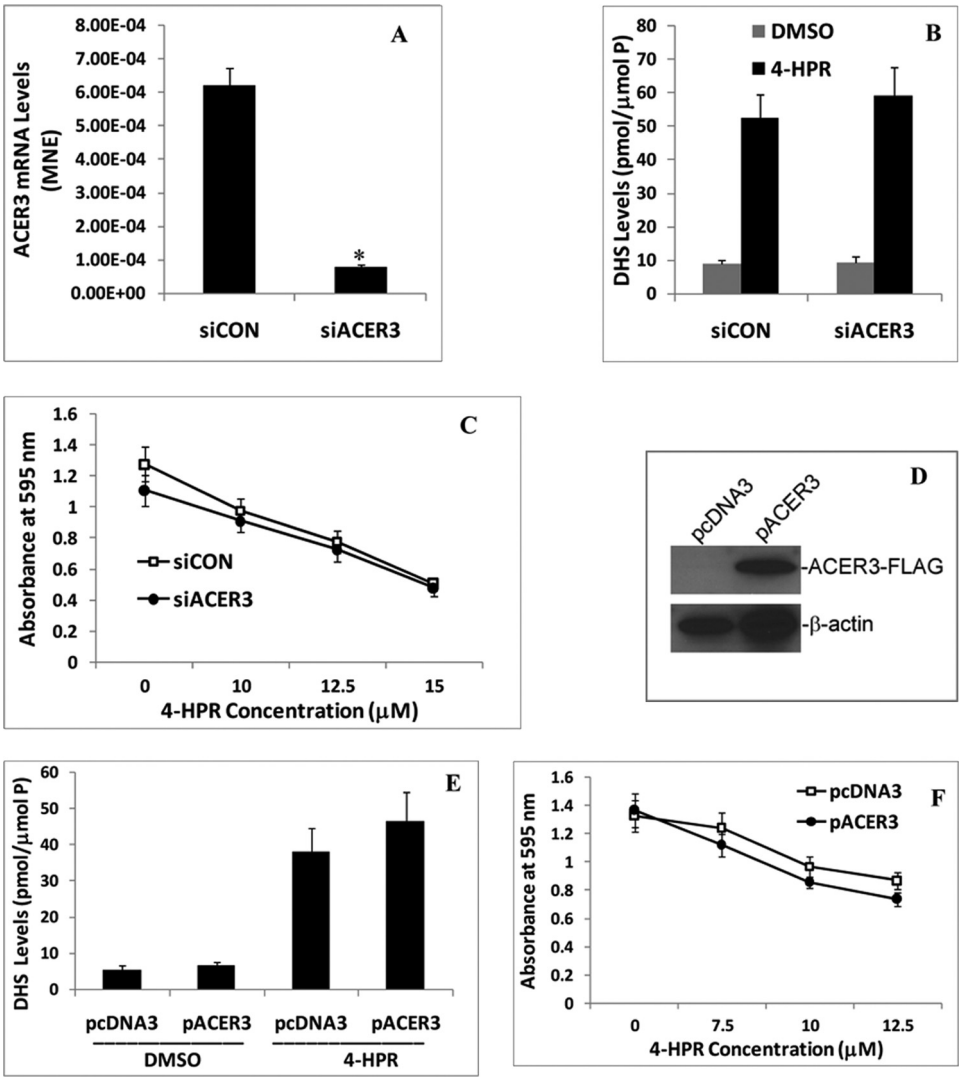

FIGURE 7. **ACER3 expression does not affect 4-HPR-induced DHS generation and cytotoxicity of 4-HPR.** *A*, T-REx-HeLa cells were transfected with siCON (5 nM) or siACER3(5 nM) for 48 h before ACER3 mRNA was analyzed by qPCR. *B*, T-REx-HeLa cells transfected with siCON or siACER3 were treated with 4-HPR (10 μM) or DMSO for 6 h before DHS levels were determined by HPLC. *C*, T-REx-HeLa cells were transfected with siCON or siACER3 and were treated with either 4-HPR at indicated concentrations or DMSO for 48 h before cell viability was determined by MTT assays. *D*, HSC-1 cells were transfected with pcDNA3 or pcDNA3-ACER3 (pACER3) for 48 h before Western blot analysis with anti-FLAG antibody as described in our previous study (18). *E*, HSC-1 cells transfected with pcDNA3 or pACER3 were treated with 4-HPR (12.5 μM) or DMSO for 24 h before DHS levels were determined by ESI/MS/MS. *F*, HSC-1 cells transfected with pcDNA3 or pACER3 were treated with DMSO or 4-HPR at the indicated concentrations for 48 h before MTT assays. Data represent the mean values ± S.D. of three independent experiments. *, $p < 0.05$ *versus* control (siCON).

ACER3 Overexpression Enhances 4-HPR-induced Generation of DHS and the Cytotoxicity of 4-HPR in HSC1 Cutaneous Squamous Cell Carcinoma Cells—Although ACER3 expression is not up-regulated by 4-HPR, we found that ACER3 expression is much higher than ACER2 expression in resting T-REx-HeLa cells (Figs. 3*D* and 4*B*). This promoted us to determine whether the high constitutive expression of ACER3 was important in mediating the 4-HPR-induced generation of DHS and the cytotoxicity of 4-HPR in these cells. T-REx-HeLa cells were transfected with siCON or siACER3, an ACER3-specific siRNA that has been proven to knock down ACER3 efficiently in our previous study (18). qPCR analyses confirmed that compared with transfection with siCON, transfection with siACER3 markedly decreased ACER3 mRNA levels in T-REx-HeLa cells (Fig. 7*A*). Interestingly, HPLC analyses demonstrated that ACER3 knockdown slightly increased rather than decreased the 4-HPR-induced generation of DHS in T-REx-HeLa cells (Fig. 7*B*). MTT assays demonstrated that ACER3 knockdown did not affect the 4-HPR-induced decrease in the viability of T-REx-HeLa cells (Fig. 7*C*).

We then determined whether ACER3 overexpression has any effect on the 4-HPR-induced generation of DHS and the cytotoxicity of 4-HPR. Because we previously demonstrated that HSC-1 cells, a skin SCC cell line, express very low levels of ACER3, we determined whether ACER3 overexpression enhanced DHS generation and cytotoxicity in this cell line in response to treatment with 4-HPR. HSC-1 cells were transiently transfected with pcDNA3 or pcDNA3-FLAG-ACER3 as described in our previous study (18). Western blot analysis demonstrated that FLAG-tagged ACER3 (ACER3-FLAG) was expressed in HSC-1 cells transfected with pcDNA3-FLAG-ACER3 (pACER3) but not with pcDNA3 (Fig. 7*D*). HSC-1 cells transfected with pcDNA3 or pcDNA3-ACER3 were treated with DMSO or 4-HPR (12.5 μM) for 24 h before DHS levels were determined by ESI/MS/MS. Compared with transfection with pcDNA3, transfection with pcDNA3-ACER3 only slightly increased the 4-HPR-induced generation of DHS in HSC-1 cells (Fig. 7*E*). MTT assays demonstrated that ACER3 overexpression only moderately enhanced the 4-HPR-induced decrease in cell viability (Fig. 7*F*). These results suggest that unlike ACER2, ACER3 has a limited role in mediating the 4-HPR-induced generation of DHS and the cytotoxicity of 4-HPR in tumor cells.

ACER2 Overexpression and DES Inhibition Induce Cell Death of Tumor Cells—In addition to ACER2 up-regulation, 4-HPR has been shown to inhibit DES activity (11–13). This prompted us to determine whether both ACER2 up-regulation and DES inhibition are required and sufficient to mediate the 4-HPR-induced cytotoxicity in tumor cells. ACER2-TET-ON cells grown in the presence of ET or TET were treated with GT11, another DES inhibitor (33, 34), or DMSO, the vehicle control. MTT assays showed that ACER2 overexpression or GT11 treatment alone caused little or no

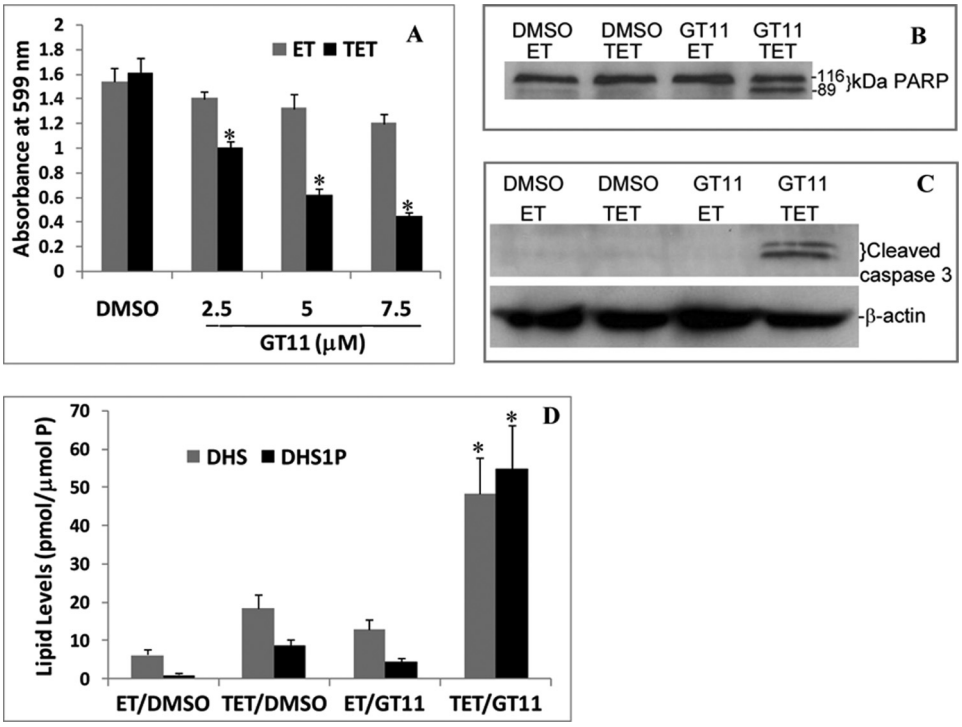

FIGURE 8. **ACER2 up-regulation along with DES inhibition induces cell death in tumor cells.** *A*, ACER2-TET-ON cells were grown in the presence of ET or TET (10 ng/ml) for 24 h before they were treated with various concentrations of GT11 or the vehicle control, DMSO, and incubated for 48 more hours. Cell viability was determined by MTT assays. *B* and *C*, ACER2-TET-ON cells grown in the presence of ET or TET (10 ng/ml) were treated with GT11 (5.0 μM) or DMSO for 48 h before PARP cleavage (*B*) or caspase-3 activation (*C*) was analyzed by Western blot. *D*, ACER2-TET-ON cells grown in the presence of ET or TET (10 ng/ml) were treated with GT11 (5.0 μM) of DMSO for 24 h before DHS and DHS1P were analyzed by ESI/MS/MS. Image data represent three independent experiments with similar results. Numerical data represent the mean values ± S.D. of three independent experiments. *, $p < 0.05$ *versus* control (siCON).

decrease in cell viability, whereas ACER2 overexpression along with GT11 treatment markedly decreased cell viability (Fig. 8*A*), suggesting that both ACER2 up-regulation and DES inhibition are required and sufficient to induce cytotoxicity.

To determine whether ACER2 up-regulation along with GT11 treatment induces the cytotoxicity due to increased cell death, we determined whether ACER overexpression along with DES inhibition caused PARP cleavage and caspase-3 activation. A Western blot demonstrated that ACER2 overexpression along with GT11 treatment caused a marked increase in both the cleavage of PARP and the activation of caspase-3, whereas ACER2 overexpression or GT11 treatment alone had no such effects (Fig. 8, *B* and *C*), suggesting that ACER2 up-regulation along with DES inhibition induces cell death in tumor cells.

To determine whether both ACER2 up-regulation and DES inhibition induced cytotoxicity and cell death by increasing the generation of DHS in cells, we determined the levels of DHS in ACER2-TET-ON cells treated with GT11 or DMSO. ESI/MS/MS analyses showed that treatment with GT11 or ACER2 overexpression alone increased DHS levels by 2- and 4-fold, respectively, in T-REx-HeLa cells, whereas GT11 treatment along with ACER2 overexpression caused a greater than 14-fold increase in cellular DHS (Fig. 8*D*). We also found that ACER2 up-regulation and GT11 treatment synergistically increased cellular DHS1P (Fig. 8*D*). These results suggest that ACER2 up-regulation and DES inhibition synergistically increase cellular DHS.

DISCUSSION

There were several important findings in this study. First, we demonstrated that DHS, which was thought to be only synthesized *de novo*, can also be derived from the hydrolysis of DHCs through the action of the alkaline ceramidases, such as ACER1 and ACER2, thus revealing a novel pathway for the generation of DHS in cells. Second, we showed that 4-HPR increases the generation of DHS and its phosphate DHS1P in tumor cells by inversely regulating DES activity and ACER2 expression. Third, we proved that DHS generated by the inverse regulation of DES and ACER2 mediates the 4-HPR-induced cytotoxicity and cell death in tumor cells. Finally, we demonstrated that increasing ACER2 expression markedly enhances the cytotoxicity of 4-HPR in different tumor cell types by enhancing the 4-HPR-induced generation of DHS. These results strongly support the notion that up-regulating or activating the ACER2/DHS pathway can effectively kill tumor cells and may be instrumental in cancer therapy.

DHS has long been known to be a bioactive lipid that mediates proliferation inhibition and cell death in tumor cells (13, 35–37) but how its generation is regulated in cells has not been fully understood. It was thought that DHS is only synthesized through the *de novo* (anabolic) pathway involving two sequential enzymatic steps catalyzed by SPT and ketodihydrosphingosine reductase, although its unsaturated analogue, sphingosine, is only generated from the hydrolysis of ceramides via the action of ceramidases. Recently, we demonstrated that ACER3, one of the three human alkaline ceramidases that we identified, can catalyze the hydrolysis of unsaturated long-chain dihydroceramides as efficiently as unsaturated long-chain ceramides (18). In this current study we demonstrated that ACER1 and ACER2, the homologues of ACER3, also catalyzed the hydrolysis of certain DHC species to generate DHS. Consistent with their *in vitro* activity of hydrolyzing certain DHC species, ACER1 or ACER2 overexpression increased DHS levels in cells, suggesting that both ACER1 and ACER2 also catalyze the hydrolysis of DHCs to generate DHS in cells. These findings clearly suggest that like SPH, DHS can also be derived from a catabolic pathway.

Different ceramidases have different capabilities to control the generation of DHS in cells. Our recent studies demon-

strated that ACER3 overexpression, in contrast to ACER1 or ACER2 overexpression, only slightly increased DHS in cells (18). In this study we found that although ACER3 is highly expressed in T-REx-HeLa cells, its knockdown did not affect cellular DHS under a resting condition or in response to 4-HPR treatment. Moreover, ACER3 overexpression only slightly increased the 4-HPR-induced generation of DHS and the cytotoxicity of 4-HPR in HSC-1 cells that express very low levels of endogenous ACER3. These observations suggest that ACER3 expression has a minimum role in controlling the generation of DHS in cells. The different capabilities of the alkaline ceramidases to generate DHS may be brought about by the discrepancies in their substrate specificity. ACER3 only hydrolyzes unsaturated long-chain DHCs (D-e-$C_{18:1}$ and $C_{20:1}$-DHC), whereas both ACER1 and ACER2 hydrolyze the D-e-$C_{24:1}$-dihydroceramide, an unsaturated very long-chain DHC, in addition to unsaturated long-chain DHCs. Because D-e-$C_{24:1}$-DHC is a major mammalian dihydroceramide species whereas D-e-$C_{18:1}$ and D-e-$C_{20:1}$-DHC are minor DHC species, tumor cells are much more abundant in the DHC substrates of ACER1 or ACER2 than in the DHC substrates of ACER3. This explains why ACER1 and ACER2 are more potent than ACER3 in catalyzing the generation of DHS in cells.

Although the human AC (26) or rat NC (27) was also shown to catalyze the hydrolysis of DHCs *in vitro*, we found that overexpression of the human AC or NC does not increase cellular DHS, suggesting that they may have a limited role in the generation of DHS in cells. It is noteworthy that although ACER1 has the ability to generate DHS in cells, its expression is limited to certain cell types, such as epidermal keratinocytes (28). Indeed, we found that ACER1 mRNA was undetectable in T-REx-HeLa tumor cells (data not shown). These observations suggest that ACER2 is a major ceramidase responsible for the generation of DHS from the hydrolysis of DHC in tumor cells.

DHC is synthesized in the ER, whereas ACER2 is localized to the Golgi complex. Increasing ACER2 expression markedly enhanced the 4-HPR-induced generation of DHS, suggesting that DHC is transported from the ER to the Golgi complex. Consistent with this view, Wang *et al.* (13) demonstrated that 4-HPR markedly increases the levels of DHC-containing sphingomyelins or dihydrosphingomyelins, which are synthesized mainly in the Golgi complex. The transport of ceramides from the ER to the Golgi complex is mediated mainly by the ceramide transfer protein (CERT), which can also transfer DHC from the ER to the Golgi complex (38). This explains why ACER2 can readily access to cellular DHC accumulated by treatment with 4-HPR.

In many tumor cell types, DHS is kept to a minimum under resting conditions, likely due to its high cytotoxicity. As mentioned earlier, once synthesized in the ER, DHCs are immediately converted to ceramides by the action of DES. In many cell types, DHCs are much less abundant than ceramides, likely due to high DES catalytic efficiency and/or expression. Moreover, ACER2 is expressed at low levels in most cell types (17). Therefore, DHS is scarce in tumor cells, likely due to both limited availability of DHCs and low dihydroceramidase activity of converting DHCs into DHS in cells.

4-HPR has been shown to inhibit DES activity, resulting in accumulation of DHCs in cells (13). We demonstrated that 4-HPR also up-regulated the expression of ACER2 by activating caspases. Therefore, 4-HPR increases the generation of DHS by increasing both the availability of DHCs and the ACER2-catalyzed hydrolysis of DHCs into DHS in cells. This is supported by the finding that treatment with GT11, another DES inhibitor (33, 34), slightly increased DHS in cells expressing low endogenous ACER2 but markedly increased DHS in cells overexpressing ACER2.

4-HPR cytotoxicity has been linked to the modulation of the metabolism of sphingolipids. An increase in ceramides was initially thought to mediate the cytotoxicity of 4-HPR (15, 16). Later, it was found that 4-HPR in fact decreases ceramides but increases the precursors of ceramides, DHCs (11–13), thus, excluding the role of ceramides. A more recent study found that 4-HPR increases DHS in addition to DHCs (13). Unlike ceramides, DHCs have been suggested to have no cytotoxicity in tumor cells, so an increase in DHCs is unlikely to mediate the cytotoxicity of 4-HPR in tumor cells. This is supported by the finding that treatment with a short-chain ceramide, D-e-C_2 or C_6-ceramide, induces cytotoxicity in various tumor cell types (39–42), whereas treatment with D-e-C_2-DHC or D-e-C_6-DHC fails to do so (13, 36). By contrast, treatment with either the natural DHS (D-e-DHS) or synthetic DHS (L-t-DHS) has been shown to potently induce cytotoxicity in various tumor cell types (36, 43). These observations suggest that DHS rather than DHC mediates 4-HPR-induced cytotoxicity.

This notion is supported by two lines of evidence provided in this study. First, we demonstrated that treatment with GT11 failed to induce cytotoxicity in tumor cells expressing a low level of endogenous ACER2 due to a moderate increase in cellular DHS. By contrast, treatment with GT11 induced a marked increase in not only DHS but also cytotoxicity in tumor cells overexpressing ACER2. Second, we showed that ACER2 overexpression enhanced not only the 4-HPR-induced generation of DHS but also the cytotoxicity of 4-HPR in both T-REx HeLa and SCC1 tumor cells. It is noteworthy that ACER2 up-regulation along with DES inhibition also mediates the 4-HPR-induced generation of DHS1P. Because DHS1P, similar to its analogue S1P, has been shown to promote cell proliferation and survival (44), its increase is unlikely to contribute to the 4-HPR-induced cytotoxicity. Based on these observations, we conclude that an increase in DHS due to the inverse regulation of DES and ACER2 activity mediates 4-HPR-induced cytotoxicity in tumor cells.

4-HPR has been shown to induce both growth arrest (7, 45) and cell death (9, 46) in tumor cells. We demonstrated that ACER2 overexpression enhanced the 4-HPR-induced cell death of tumor cells, whereas ACER2 knockdown had the opposite effect. ACER2 overexpression along with DES inhibition by GT11 also induced the death of T-REx HeLa tumor cells. These results suggest that an increase in DHS due to the inverse regulation of DES and ACER2 mediates 4-HPR-induced cell death in tumor cells. However, it is noteworthy that knocking down ACER2 only partially inhibited the effect of 4-HPR. There are two explanations for this incomplete effect. First, ACER2 expression was not completely inhibited by siRNA. Second, 4-HPR induces the cytotoxicity probably

through other mechanisms in addition to the DHS increase by ACER2 up-regulation.

In addition to 4-HPR, several anti-cancer molecules have been shown to increase DHCs in cells by inhibiting DES activity or increasing SPT activity. Signorelli *et al.* (47) demonstrated that treatment with resveratrol increases DHCs in gastric cancer cell line HGC-27 cells by inhibiting DES activity. Schiffmann *et al.* (48) showed that treatment with celecoxib, a cyclooxygenase-2 inhibitor, significantly increases DHCs as well as DHS in several tumor cell lines by both inhibiting DES activity and increasing SPT activity. Jiang *et al.* (32) found that treatment with r-tocopherol, the major form of dietary vitamin E, also markedly increases DHCs and DHS in prostate cancer cells (LNCaP and PC-3) and A549 lung cancer cells by activating SPT. Based on our finding that increasing the ACER2-mediated conversion of DHCs into DHS sensitizes tumor cells to cell death, we predict that increasing ACER2 expression would also enhance the cytotoxicity of these DHC-inducing agents. Therefore, up-regulating or activating the ACER2/DHS pathway may improve the anti-cancer efficacy of various anti-cancer agents.

In conclusion, we demonstrated that 4-HPR increases ACER2 expression in cervical tumor cells. ACER2 up-regulation in conjunction with DES inhibition plays an important role in mediating both the 4-HPR-induced generation of DHS and the cytotoxicity of 4-HPR. DHS generated by ACER2 contributes to 4-HPR-induced cell death. Our *in vitro* studies suggest that increasing ACER2 activity or its expression may improve the anti-tumor efficacy of 4-HPR or other DHC-inducing anti-cancer agents *in vivo*.

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