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N-(4-Hydroxyphenyl)retinamide Elevates Ceramide in Neuroblastoma Cell Lines by Coordinate Activation of Serine Palmitoyltransferase and Ceramide Synthase[1]

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ABSTRACT

The retinoid *N*-(4-hydroxyphenyl)retinamide (4-HPR; fenretinide) is cytotoxic to a variety of cancer cell lines, and we previously showed an association between ceramide generation and 4-HPR cytotoxicity for neuroblastoma cell lines (B. J. Maurer *et al.*, J. Natl. Cancer Inst. (Bethesda), *91:* 1138–1146, 1999). Here we determine whether the increased ceramide mediated by 4-HPR in the CHLA-90 human neuroblastoma cell line results from *de novo* ceramide synthesis. Treatment of CHLA-90 with 4-HPR for 2 h, in the presence of [^{3}H]palmitic acid, caused sequential formation of [^{3}H]sphinganine (220% over control) and [^{3}H]ceramide (160% over control), with sphinganine returning to baseline at 4 h, and ceramide continuing to increase (215% over control). 4-HPR treatment did not accelerate cellular decay of sphingomyelin. Preincubation of cells with either L-cycloserine, an inhibitor of serine palmitoyltransferase (SPT), or fumonisin B_1, an inhibitor of ceramide synthase, retarded ceramide formation in response to 4-HPR treatment, although sphinganine was still generated when 4-HPR and FB_1 were present. Data from *in vitro* enzyme assays using microsomes showed that preexposure of intact cells to 4-HPR resulted in a time (175% over control; 6 h)- and dose-dependent increase (173% over control; 10 μM) in SPT activity as well as a time (265% over control)- and dose-dependent increase (215% above control; 10 μM) in ceramide synthase activity. Our results show that 4-HPR-mediated ceramide generation is derived from the *de novo* synthetic pathway by coordinate activation of SPT and ceramide synthase. Knowledge of these biochemical events is of utility when downstream modulators of ceramide metabolism are used to heighten the cytotoxic response to chemotherapy.

INTRODUCTION

Sphingolipids and, in particular, ceramide play a unique role in regulating cytotoxic response to chemotherapy drugs such as anthracyclines (1–3), Taxol (4), etoposide (5), and 4-HPR[3] (fenretinide). We recently reported that 4-HPR, which is cytotoxic to a variety of cancer cell lines, significantly increased ceramide levels and elicited mixed apoptosis/necrosis in neuroblastoma cell lines (6) and that L-cycloserine, an inhibitor of SPT, blocked 4-HPR-induced ceramide generation and reduced 4-HPR-related cytotoxicity (7). It was also shown that expression of p53 protein was not affected by 4-HPR and that death proceeded via necrosis when a caspase inhibitor prevented apoptosis (6). Furthermore, we have recently shown that modulators of ceramide metabolism strongly synergize 4-HPR cytotoxicity in a variety of solid tumor cell lines (7). Because 4-HPR may form the basis for a novel, p53-independent chemotherapy operating through the ceramide cycle, it is important to elucidate the biochemical mechanisms by which 4-HPR generates ceramide. In addition, because the cytotoxicity of ceramide in response to ceramide-generating drugs such as doxorubicin, SDZ PSC 833, and Taxol can be enhanced by the addition of modulators of ceramide metabolism (3, 8–13), understanding the mechanisms of action of 4-HPR may point to similar mechanisms for other drugs and provide a rational basis for selecting certain agents for combination chemotherapy trials.

In this study, we have used 4-HPR, a synthetic retinoid that has been shown to inhibit tumor growth in a variety of animal cancer models and act as a chemopreventive against human cancers (reviewed in Ref. 6), to activate ceramide formation in CHLA-90, a multidrug-resistant human neuroblastoma cell line established at time of tumor relapse after myeloablative therapy (14). In response to anthracyclines, ceramide can be generated *de novo* through ceramide synthase (1) or via activation of sphingomyelinase (2). Here we show that exposure of intact cells to 4-HPR, followed by lysis for isolation of microsomes, elicited activation of the *de novo* enzymes of ceramide synthesis, SPT and ceramide synthase, as assayed under cell-free conditions. This work provides strong evidence that enzymes governing ceramide production *de novo* are induced by 4-HPR.

MATERIALS AND METHODS

The human neuroblastoma cell line CHLA-90 (14) was maintained in Iscove's modified Dulbecco's medium (BioWhittaker, Walkersville, MD) containing 20% fetal bovine serum (Gemini BioProducts, Calabasas, CA) and other additives as described previously (14). 4-HPR was kindly provided by R. W. Johnson Pharmaceuticals (Spring House, PA). [9,10-^{3}H]Palmitic acid (51 Ci/mmol) was from DuPont New England Nuclear (Boston, MA), and [4,5-^{3}H]sphinganine (60 Ci/mmol) and L-[^{3}H]serine (20 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO). FB_1 and L-cycloserine were products of Biomol (Plymouth Meeting, PA). Sphinganine (D-erythro-dihydrosphingosine in pure form) was purchased from Matreya, Inc. (Pleasant Gap, PA). All other lipids were purchased from Avanti Polar Lipids (Alabaster, AL), and TLC Silica Gel G plates (250 μm) were from Analtech (Newark, DE).

Cell radiolabeling with [^{3}H]palmitic acid, lipid extraction, and analysis of [^{3}H]ceramide by TLC were conducted as described previously (6, 11). Sphinganine was resolved from other lipids by TLC in a solvent system containing chloroform/methanol/ammonium hydroxide (70:20:4, v/v), and sphingomyelin was separated using chloroform/methanol/acetic acid/water (60:30:7:3, v/v). Lipids were visualized in iodine vapor. Standards were cochromatographed, and spots were scraped and analyzed for tritium by LSC (11).

SPT was assayed with [^{3}H]serine as described previously (15), using CHLA-90 microsomes (100 μg of protein). Enzyme reactions were conducted at 37°C for 7 min. The product, 3-ketosphinganine, was isolated by differential solvent extraction (15) and quantitated by LSC. Ceramide synthase was assayed using [^{3}H]sphinganine, 100 μg of microsomal protein, and 100 μM palmitoyl-CoA as described previously (16). The assay was run at 37°C for 40 min. Under the reaction conditions used, kinetics were linear with regard to protein and time. The product, [^{3}H]dihydroceramide, was separated by TLC and quantitated by LSC (11).

To test whether 4-HPR had a direct influence on SPT activity, microsomes

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[3] The abbreviations used are: 4-HPR, *N*-(4-hydroxyphenyl)retinamide; LSC, liquid scintillation counting; FB_1, fumonisin B_1; SPT, serine palmitoyltransferase; TLC, thin-layer chromatography.

(100 μg of protein) were preincubated for various times (10–60 min) with drug (1–10 μM) at either 4°C or 37°C before initiating the enzyme incubation at 37°C. The SPT assay was conducted as described above.

RESULTS

Previously, we demonstrated that treatment of CHLA-90 cells with 4-HPR causes an increase in ceramide and induction of mixed apoptosis/necrosis (6). To assess the biochemical pathway of the 4-HPR response, the metabolism of ceramide precursors and enzyme activation parameters were examined in this neuroblastoma cell line. The experiment in Fig. 1 shows that treatment of cells with 4-HPR caused time-dependent, sequential generation of [^{3}H]sphinganine and [^{3}H]ceramide, measuring 220% and 160% over control, respectively, at 2 h (Fig. 1, ▨). At 4 h, cellular [^{3}H]sphinganine levels returned to baseline, whereas [^{3}H]ceramide continued to increase (215% over control; Fig. 1, ■). The inclusion of FB_1, an inhibitor of ceramide synthase, retarded 4-HPR-induced ceramide formation, whereas sphinganine production continued (Fig. 2). As shown in Fig. 2*A*, FB_1 alone inhibited *de novo* ceramide production and reduced 4-HPR-induced ceramide formation by 65%. Sphinganine, which by 4 h is converted to ceramide (see Fig. 1), reached a level that was 185% above control (1000 *versus* 1850 cpm) when FB_1 and 4-HPR were both present (Fig. 2*B*). The addition of L-cycloserine (an inhibitor of SPT) alone reduced cellular *de novo* ceramide synthesis >50% and also blocked 4-HPR-induced ceramide formation (Fig. 3).

The above-mentioned data indicate that 4-HPR may impact both SPT and ceramide synthase activities. Because of difficulties encountered in determining whether ceramide synthase is activated by 4-HPR if substrate is not available (as in Fig. 3, when L-cycloserine is present, retarding the formation of sphinganine, the substrate for ceramide synthase), we supplied CHLA-90 cells with an exogenous source of sphinganine (50 μM) in the culture medium. This method not only provides substrate for the enzyme being investigated but also obviates the use of inhibitors and the use of [^{3}H]palmitic acid, which can enter the ceramide metabolic pathway via SPT, as in Fig. 1. With the use of high specific activity [^{3}H]sphinganine (60 Ci/mmol), 4-HPR treatment of cells failed to enhance the cpm of tritium incorporated into intracellular ceramide (Fig. 4*A*). The slight decrease observed in the amount of [^{3}H]ceramide produced is likely due to dilution of [^{3}H]sphinganine radiospecific activity with intracellular sphinganine generated by 4-HPR exposure over the short 2-h period. In contrast, when cells are supplied with sufficient sphinganine to negate label dilution by intracellular *de novo* generated sphinganine, the inclusion of 4-HPR enhanced the cpm of tritium incorporated into intracellular ceramide (Fig. 4*B*). Based on radiospecific activity (7000 cpm/nmol), 4-HPR elicited an increase in ceramide mass from 0.9 nmol (control) to 1.3 nmol (Fig. 4*B*). These data demonstrate that 4-HPR stimulates ceramide synthase activity. Examination of sphingomyelin metabolism using cells that had been prelabeled with [^{3}H]palmitic acid for 24 h showed that subsequent 4-HPR treatment (10 μM) failed to activate [^{3}H]sphingomyelin decay over a 6-h exposure period (data not shown). Collectively, these experiments show that 4-HPR accelerates ceramide formation in intact cells through *de novo* synthesis by first activating SPT and forming sphinganine, with subsequent activation of ceramide synthase to generate ceramide.

To determine whether enzyme activation could be initiated in whole cells and detected at the cell-free level, CHLA-90 cells were preincubated with 4-HPR before subcellular fractionation and *in vitro* assaying of SPT and ceramide synthase. Pretreatment of cells with

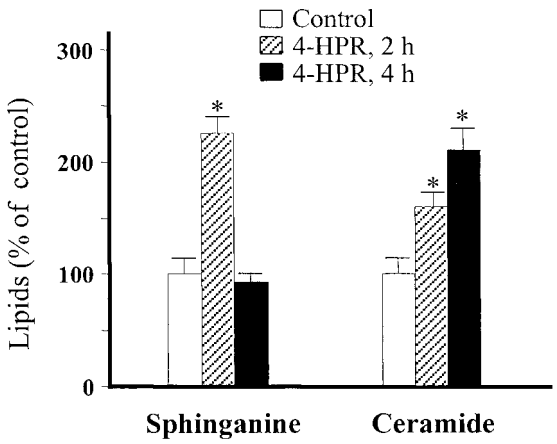

Fig. 1. Effect of 4-HPR on sphinganine and ceramide metabolism in CHLA-90 cells. CHLA-90 cells cultured in 6-well plates were treated without (control) or with 10 μM 4-HPR for the times indicated in medium containing [^{3}H]palmitic acid (1.0 μCi/ml). Radiolabeled sphinganine and ceramide were resolved from the total cell lipid extract by TLC and quantitated by LSC. *, $P < 0.01$. Distribution of lipid tritium in control and 4-HPR-treated cells, respectively: sphinganine, 0.12% and 0.23% at 2 h and 0.21% and 0.19% at 4 h; ceramide, 2.5% and 3.5% at 2 h and 1.3% and 2.8% at 4 h.

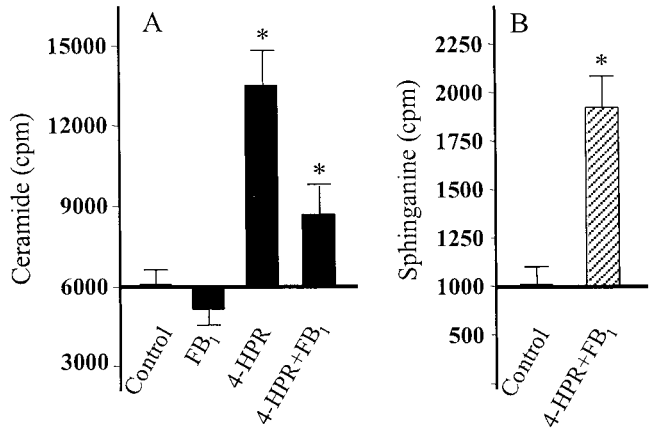

Fig. 2. Influence of ceramide synthase inhibitor FB_1 on 4-HPR-induced ceramide and sphinganine generation. *A*, ceramide formation. CHLA-90 cells cultured in 6-well plates were treated with FB_1 (50 μM), 4-HPR (10 μM), or both agents for 6 h in medium containing [^{3}H]palmitic acid. *B*, sphinganine formation. Cells were pretreated with FB_1 for 30 min before the addition of 4-HPR for 4 h. Radiolabeled ceramide and sphinganine were resolved from total cell lipids by TLC and quantitated by LSC. *, $P < 0.01$. The *Y* axis represents cpm of ceramide or sphinganine per 500,000 cpm of total lipid tritium.

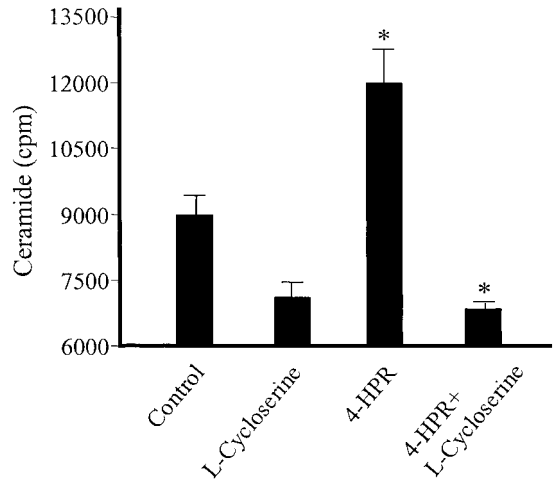

Fig. 3. Effect of SPT inhibitor L-cycloserine on 4-HPR-induced ceramide generation. CHLA-90 cells cultured in 6-well plates were treated with L-cycloserine (5 mM), 4-HPR (10 μM), or both agents in medium containing [^{3}H]palmitic acid for 4 h. Radiolabeled ceramide was resolved from the total cell lipids by TLC and quantitated by LSC. *, $P < 0.01$. The *Y* axis represents cpm of ceramide per 500,000 cpm of total lipid tritium.

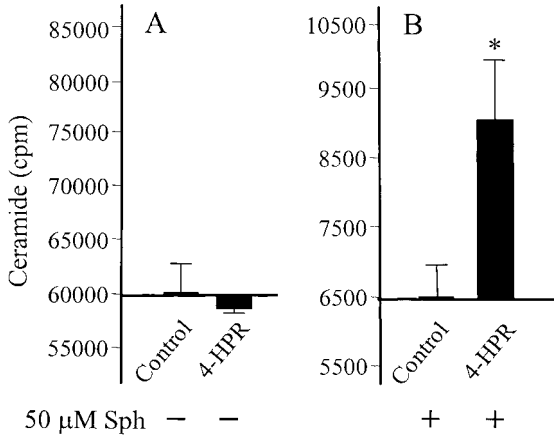

Fig. 4. Assay of ceramide synthase activity in intact cells. CHLA-90 cells cultured in 6-well plates were treated without (control) or with 10 μM 4-HPR for 2 h in medium containing 0.5 μCi/ml [^{3}H]sphinganine (60 Ci/mmol, 91,000 cpm/nmol) and supplemented without (*A*) or with (*B*) 50 μM unlabeled sphinganine (7000 cpm/nmol) in the culture medium. L-Cycloserine was not present in the incubations. Radiolabeled ceramide was resolved from total cell lipids by TLC and quantitated by LSC. *, $P = 0.02$. *Sph*, sphinganine.

4-HPR resulted in time- and dose-dependent increases in the activities of both enzymes (Fig. 5). The time courses of SPT and ceramide synthase activation were biphasic (Fig. 5, *A* and *C*), with an early peak at 30 min and a secondary increase 2–6 h after the addition of 4-HPR. Maximal activation of SPT and ceramide synthase was attained at 6 h (175% over control) and 4 h (270% over control), respectively. Dose-response experiments with 4-HPR showed that maximal activation of SPT and ceramide synthase was attained at a pretreatment concentration of 10 μM (Fig. 5, *B* and *D*), with activity measuring 175% and 220% above control, respectively.

4-HPR was also added to the *in vitro* SPT assay to determine whether there was a direct effect on enzymatic production of sphinganine. Preexposure of microsomes in the complete cell-free system minus substrate to 5 μM 4-HPR for 10, 30, and 60 min at 37°C, followed by substrate addition, resulted in [^{3}H]sphinganine production that was 112, 120, and 117% above control, respectively. All controls were preincubated at 37°C for the designated times but without 4-HPR.

DISCUSSION

Synthetic retinoids such as 4-HPR hold promise as novel agents in cancer therapy. Many synthetic retinoids act thought receptors; however, the primary cellular target of 4-HPR remains unidentified. Previously, we showed that 4-HPR cytotoxicity is linked to ceramide generation (6) and that the addition to 4-HPR of modulators of ceramide metabolism, such as safingol, tamoxifen, and 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), results in a synergistic increase in cell kill (7). Results from this study provide definitive evidence that 4-HPR activates SPT, the rate-limiting enzyme in the *de novo* pathway of sphingolipid biosynthesis, and ceramide synthase, an end point catalyst of ceramide production. It could be argued that increased ceramide results from surplus sphinganine, the substrate of ceramide synthase, through the initial activation of SPT by 4-HPR and not by activation of ceramide synthase; however, several points oppose this mechanism. First, ceramide synthase activity was markedly enhanced in cell-free assays using microsomes from whole cells that had been pretreated with 4-HPR (Fig. 5). This connotes the existence of an enzyme activation sequence initiated in intact cells. Secondly, when cells were given an exogenous supply of [^{3}H]sphinganine at a concentration of 50 μM, the amount of [^{3}H]ceramide synthesized was 40% greater (0.9 *versus* 1.3 nmol) in cells exposed to 4-HPR, indicating a ceramide synthase-mediated event (Fig. 4*B*).

The time frame for activation of SPT and ceramide synthase by 4-HPR in microsomes isolated from pretreated cells was biphasic in nature. This is similar to our previous findings of a biphasic generation of ceramide over time in intact neuroblastoma cells, although the early ceramide peak was attained at 6 h (6). Using etoposide-treated Molt-4 cells, Perry *et al.* (5) have likewise demonstrated biphasic activation of SPT in microsomes, with the early peak occurring at 30 min, similar to the results of our study (Fig. 5). The production of ceramide via retinoic acid-elicited SPT activity in an embryonic cell line was shown to be preceded by a posttranslational event (17); however, we cannot speculate as to the mechanism of SPT activation by 4-HPR. The short time frame for activation suggests a posttranslational avenue. The possibility exists that 4-HPR directly stimulates SPT activity because our preliminary results show that preincubation of microsomes with 4-HPR produces heightened formation of sphinganine.

The role of ceramide in chemotherapy-induced apoptosis has been reviewed recently (18, 19). A number of agents have been shown to boost cellular ceramide levels either through *de novo* synthesis or by degradation of sphingomyelin (reviewed in Ref. 19), contributing to apoptosis. With specific regard to retinoids, retinoic acid treatment of embryonic carcinoma cells causes apoptosis that is preceded by a multifold increase in ceramide levels (17). The rise in ceramide was sensitive to FB_1, and enzymatic assays revealed that SPT and not ceramide synthase was activated. Our finding that 4-HPR causes coordinate activation of both enzymes is unique. This may be either a cell type-specific response or indicative of a structure-activity relationship dependent on the phenyl amide grouping present on 4-HPR as opposed to the carboxylic acid function of all-*trans* retinoic acid. Also within the realm of chemotherapy, SPT has been shown to

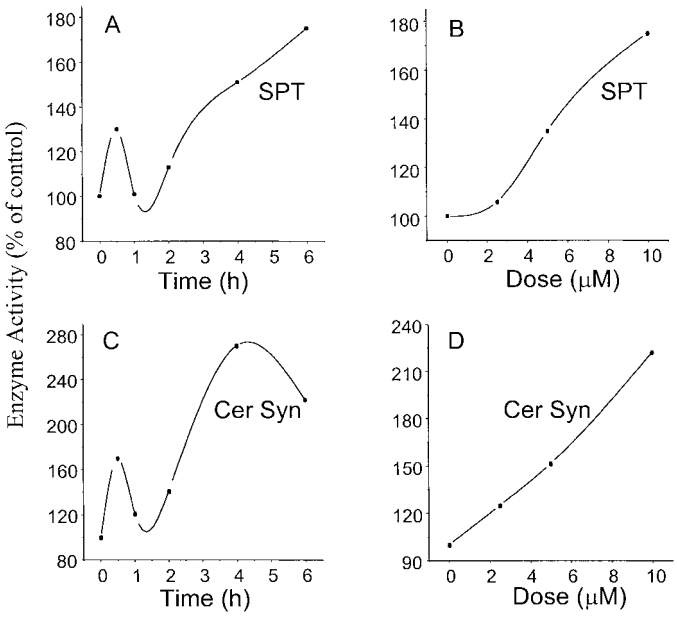

Fig. 5. Time course and dose-response effects of 4-HPR on cellular SPT and ceramide synthase activities measured *in vitro*. Cells grown in 10-cm dishes were treated with 4-HPR when approximately 80% confluent for the times and at the concentrations indicated. Microsomes were isolated from cell lysates, and SPT and ceramide synthase activities were assayed *in vitro* as described in "Materials and Methods." *A* and *C*, cells pretreated with 10 μM 4-HPR for the indicated times before lysis and assay. *B* and *D*, cells pretreated for 6 h with 4-HPR at the indicated dose before lysis and assay. These experiments were repeated three times, and the results were consistent. Data in this figure (*single points*) represent one such experiment.

regulate ceramide generation during etoposide-induced apoptosis in leukemia (5), further defining the novel function of ceramide in drug action. In breast cancer cells, Taxol induces ceramide generation and apoptosis through a pathway sensitive to inhibition of *de novo* ceramide synthesis (4), and in rhabdomyosarcoma cells, tumor necrosis factor-induced apoptosis is preceded by a multiphasic increase in ceramide potentiated via neutral sphingomyelinase, ceramide synthase, and acid sphingomyelinase activation (16).

Knowledge of sentinel cellular targets of agents like 4-HPR will provide valuable information aiding in the production of more specific drugs for cancer treatment. Targeting ceramide metabolism has been shown to be an effective route of cancer cell destruction, both genetically, through gene transfection (20–22), and with drugs that augment ceramide levels or ceramide cytotoxicity (7–13). Although these encouraging results stem from laboratory work, clinical application is on the horizon for treatment of neuroblastoma (7).

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