Production of Ceramides Causes Apoptosis during Early Neural Differentiation in Vitro*

Received for publication, January 31, 2000, and in revised form, May 25, 2000
Published, JBC Papers in Press, June 20, 2000, DOI 10.1074/jbc.M000714200

Thomas Herget‡§, Christina Esdar‡¶, Silke A. Oehrlein†, Michael Heinrich‡, Stefan Schütze§, Alfred Maelicke‡, and Gerhild van Echten-Deckert**

* This work was supported in part by Deutsche Forschungsgemeinschaft Grants He 2557.1-2, Ma 599.1-1, and SFB 415 (to S. S.) and by the Naturwissenschaftlich-Medizinische Forschungszentrum of the University of Mainz (to T. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Axxima Pharmaceuticals AG, Am Klopferspitz 19, 82152 Martinsried, Germany. Tel.: 49 89-740 165-0; Fax: 49 89-740 165-20; E-mail: herget@axxima.com.
‡ Recipient of a postgraduate fellowship from the Fonds der Chemischen Industrie.
§ To whom correspondence should be addressed: Axxima Pharmaceuticals AG, Am Klopferspitz 19, 82152 Martinsried, Germany. Tel.: 49 89-740 165-0; Fax: 49 89-740 165-20; E-mail: herget@axxima.com.
¶ Recipient of a postgraduate fellowship from the Fonds der Chemischen Industrie.

To investigate signal transduction pathways leading to apoptosis during the early phase of neurogenesis, we employed PCC7-Mz1 cells, which cease to proliferate and begin to differentiate into a stable pattern of neurons, astroglial cells, and fibroblasts upon incubation with retinoic acid (RA). As part of lineage determination, a sizable fraction of RA-treated cultures die by apoptosis. Applying natural long-chain C_{16}-ceramides as well as membrane-permeable C_{12}C_{0}-ceramide analogs caused apoptosis, whereas the biologically nonactive C_{2}-dihydroceramide did not. Treating PCC7-Mz1 stem cells with a neutral sphingomyelinase or with the ceramidase inhibitor N-oleoylthanolamine elevated the endogenous ceramide levels and concomitantly induced apoptosis. Addition of RA caused an increase in ceramide levels within 3–5 h, which reached a maximum (up to 3.5-fold of control) between days 1 and 3 of differentiation. Differentiated PCC7-Mz1 cells did not respond with ceramide production and apoptosis to RA treatment. The acidic sphingomyelinase contributed only weakly and the neutral Mg^{2+}-dependent and Mg^{2+}-independent sphingomyelinases not at all to the RA-mediated production of ceramides. However, ceramide increase was sensitive to the ceramide synthase inhibitor fumonisin B_{1}, suggesting a crucial role for the de novo synthesis pathway. Enzymatic assays revealed that ceramide synthase activity remained unaltered, whereas serine palmitoyltransferase (SPT), a key enzyme in ceramide synthesis, was activated –2.5-fold by RA treatment. Activation of SPT seemed to be mediated via a post-translational mechanism because levels of the mRNAs coding for the two SPT subunits were unaffected. Expression of marker proteins shows that ceramide regulates apoptosis, rather than differentiation, during early neural differentiation.

Neural cells die at all developmental stages and for many different reasons. Successful removal of cells with minimum disturbance of the surrounding tissue is achieved by a process called programmed cell death or apoptosis. In contrast to the striking heterogeneity of cell death induction pathways, the execution of the death program often involves identical molecules and is associated with characteristic morphological and biochemical changes (1). It has been reported that, during brain development, 20–80% of all neurons are eliminated by apoptosis (2). The reasons for this massive neuronal cell death are discussed in the context of, for example, correction of erroneous projections, creation of pathways for axonal outgrowth, numerical limitations imposed by mechanisms involving successive cell doubling, and transient functions of the eliminated neurons (reviewed in Refs. 2 and 3). Although some of these hypotheses have received experimental support in individual systems, none appear to be generally applicable, and it is unlikely that a single explanation exists. Neurons seem to be produced in excess to allow competition for contacts with their cellular partners and thus adjust their numbers to provide sufficient enervation of their targets, a process called the "neurotrophic strategy" (reviewed in Ref. 4).

During recent years, a novel type of neuronal programmed cell death has become evident. In several systems, it was demonstrated that neuronal cells die very early during neurogenesis and, in some cases, well before the period of target contact (5, 6). During chick development, cells die in the early neural tube (embryonic days 2–3) shortly after becoming post-mitotic or even during the cell cycle (7). Preventing this programmed cell death by administering caspase inhibitors blocks neural tube closure (8). Also, widespread programmed cell death was discovered to commence after embryonic day 10 in the murine cerebral cortex (5). Interestingly, the majority of dying cells were found within zones of proliferating cells rather than in regions of post-mitotic cells. At present, neither the exact biological function nor the specific mechanisms regulating this new form of neuronal cell death during pattern formation are known. A satisfactory biochemical analysis of this cell death during early neurogenesis is hampered in vivo due to the lack of specific markers for dying cells and of sufficient material, to problems of drug application, and to the fact that development of neuronal cells is not synchronized. To evade these limitations, it was desirable to establish and to employ an appropriate in vitro cell system.

We reported earlier that the teratocarcinoma cell line PCC7-Mz1 adequately mimics early steps of neural development (9–11), including the processes of determination, differentiation, and apoptosis (12). Following incubation with all-trans-retinoic acid (RA),1 PCC7-Mz1 cells cease proliferation and differenti-
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Ceramide production was independent of sphingomyelinase and ceramide synthase action, but was due to activation of serine palmitoyltransferase. Interestingly, ceramide causes only apoptosis, but not neural determination or differentiation. Furthermore, we isolated subclones of PCC7-Mz1, which were unable to produce ceramides upon RA treatment and therefore did not undergo programmed cell death in response to RA.

MATERIALS AND METHODS

Cell Culture—The mouse embryonic carcinoma cell line PCC7-Mz1 is a subclone of the PCC7-S-AzadR (clone 1009) cell line. Culture conditions, growth characteristics, and the RA-induced differentiation pattern have been described previously (9, 10, 12).

PCC7-Mz1 cells were grown in plastic tissue culture flasks in Dulbecco's modified Eagle's medium (Flow, Meckenheim, Germany) supplemented with 12.5% fetal calf serum (batch 148, Roche Molecular Biochemicals, Mannheim, Germany) at 37 °C in humidified air and 10% CO2. RA-resistant cells were selected by culturing PCC7-Mz1 cells in the presence of 0.1 μM RA. Proliferating cell colonies were isolated by ring cloning, and individual clones (R-clones) were established. Stock cultures of R-clones were cultivated in the presence of 0.01 μM RA, which was omitted for the experimental cultures.

Prior to induction of differentiation, PCC7 cells were seeded at a density of 1.75 × 10^4 cells/cm^2 in plastic culture dishes. For differentiation, cultures were treated with 0.1 μM RA (final concentration; Sigma, Munich, Germany) 1 day after plating. 24 h later, the culture medium was replaced by Dubecco's modified Eagle's medium supplemented with 12.5% fetal calf serum, 0.1 μM RA, and 1 mM dibutyryl cAMP (Roche Molecular Biochemicals).

Treatment with fumonisin B1 and desipramine (Sigma) was carried out 1 h before adding RA. C16-ceramide (Sigma) was applied according to the method described by Ji et al. (26). Briefly, C16-ceramide was dissolved in ethanol/dodecane (98:2, v/v; Sigma), thoroughly mixed with the medium, and added to PCC7-Mz1 cells plated the day before in a 96-well plate (5 × 10^4 cells/well). The final concentration of the solvent was <0.5% ethanol and 0.01% dodecane.

Cell Viability Assay—For quantification of the degree of cell death in cell culture, we employed the viability assay based on the reduction of tetrazolium salt to formazan by mitochondrial dehydrogenase activity (27). The assay was performed in 96-well microtiter plates (Falcon, Heidelberg, Germany) as described previously (12), but WST-1 (Roche Molecular Biochemicals) was used instead of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The light absorbance at 405 nm of the medium including all factors but without cells was determined and subtracted from the absorption readings with cells. At least eight wells/sample point were analyzed. Each experiment was repeated three times.

Genomic DNA Analysis—Cells were cultivated in 6-cm dishes, and genomic DNA was isolated as described previously (12). Briefly, cells in the medium were collected by centrifugation (2000 × g, 10 min, 4 °C), and the pellet was resuspended in 20 μl of cell lysis buffer (0.5% Triton X-100, 20 mM EDTA, and 5 mM Tris-HCl, pH 8). Adherent cells were washed once with ice-cold phosphate-buffered saline (PBS), detached with a rubber policeman, and centrifuged. This cell pellet was also resuspended in 20 μl of cell lysis buffer. The lysed cells were immediately incubated with 0.5 mg/ml proteinase K (Sigma) and then with 0.5 mg/ml RNase A (Roche Molecular Biochemicals) at 50 °C for 1 h each. The reactions were stopped by heat treatment at 70 °C for 10 min. The samples were kept at 56 °C, and 15 μl of prewarmed sample buffer (1% low-melt agarose, 10 mM EDTA, 0.25% bromphenol blue, and 40% sucrose) was added. The samples were then loaded onto a 1.4% agarose gel. After electrophoresis, the DNA band pattern was visualized under UV light using ethidium bromide.

Preparation of Cell Lysates and Western Blot Analysis—Western blot analyses were performed as described previously (12, 28, 29). To prepare extracts of apoptotic cells, detached PCC7-Mz1 cells in the supernatant were collected by centrifugation (2000 × g, 10 min) and resuspended in 150 μl of lysis buffer (50 mM Heps, pH 7.5, 0.2 μM NaCl, 1% Triton X-100, 0.4 mM EDTA, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 100 μg/ml leupeptin, 100 μg/ml aprotinin, 10 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, and 0.1 mM sodium ortho-vanadate). Adherent cells were washed twice with ice-cold PBS and scraped off the dish with 200 μl of lysis buffer using a rubber policeman. The lysed cells were centrifuged at 20,000 × g for 10 min at 4 °C. Protein concentrations of the supernatants were determined using
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the SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Immobilon P, Millipore Corp., Bedford, MA) by semidy blotting. The membranes were blocked with PBS/Trition (0.1% Triton X-100 in phosphate-buffered saline, pH 7.2) supplemented with 5% nonfat dry milk powder (Nutricia, Heidelberg, Germany), dried samples were subjected to mild alkaline hydrolysis (0.1 M KOH in methanol for 1 h at 37 °C) to remove glycerophospholipids. 500 μl of chloroform/methanol (95:5, v/v) was added, the reaction was stopped by extraction of lipids with 1 ml of chloroform/methanol (2:1, v/v) and 250 μl of H2O. After phase separation by centrifugation, radioactivity in 100 μl of the aqueous phase was measured by scintillation counting using 5 ml of scintillation fluid (Zinsser, Frankfurt, Germany).

Determination of Ceramide Levels—Cellular ceramide levels were estimated by the diacylglycerol kinase assay and by employing densitometric analysis of TLC plates.

DAG assay and ceramide quantification—Total cellular ceramide levels were quantified by the diacylglycerol (DAG) kinase assay as 32P incorporated upon phosphorylation of ceramide to cerophosphate by diacylglycerol kinase (30). PCC7-Mz1 cells were plated in 25-cm tissue culture plates with cupric reagent as described previously (32). The plates were visualized and quantified by charring of the ceramide analysis, the solvent system dichloromethane/methanol/acetic acid (75:25:5, v/v/v) as solvent and migrated as a single spot at 37 °C.) to remove glycerophospholipids. 500 μl of chloroform/methanol (95:5, v/v) was added, the reaction was stopped by extraction of lipids with 1 ml of chloroform/methanol (2:1, v/v) and 250 μl of H2O. After phase separation by centrifugation, radioactivity in 100 μl of the aqueous phase was measured by scintillation counting using 5 ml of scintillation fluid (Zinsser, Frankfurt, Germany).

Ceramide Synthase Assay—Day 1 after plating, PCC7-Mz1 cells were washed three times with ice-cold PBS, scraped off the culture dish with 1 ml of PBS, and pelleted by centrifugation (1000 g for 10 min) to obtain a single-cell suspension. The cells were washed twice with ice-cold PBS and harvested in glass tubes. After centrifugation (1000 × g, 5 min, 4 °C), lipids were extracted with 1 ml of chloroform/methanol/hydrochloric acid (1:100:1, v/v/v), 170 μl of buffered saline solution (335 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, and 10 mM Hepes, pH 7.2), and 30 μl of 100 mM EDTA. The lipids of the organic phase were transferred to a new glass tube and dried under a stream of N2. Lipid extracts were then subjected to mild alkali hydrolysis (0.1 M KOH in methanol for 1 h at 37 °C) to remove glycerophospholipids. 500 μl of chloroform, 270 μl of buffered saline solution, and 30 μl of 100 mM EDTA were added. After drying the organic phase with N2, lipids were separated by TLC. After centrifugation, radioactivity in 100 μl of the aqueous phase was measured by scintillation counting using 5 ml of scintillation fluid (Zinsser, Frankfurt, Germany).

Serine Palmitoyltransferase Activity—The enzymatic activity of serine palmitoyltransferase was measured using [14C]serine and palmitoyl-CoA as substrates (34, 35). The assay mixture contained 0.1 M Hepes, pH 7.4, 5 mM dithiothreitol, 10 mM EDTA, 50 μM pyridoxal 5’-phosphate, 1.2 mM L-[3H]serine (1.6 μCi), 0.15 mM palmitoyl-CoA, and 100–150 μg of cell protein in a total volume of 100 μl. After incubation for 10 min at 37 °C, reactions were terminated by addition of chloroform/methanol (5:3, v/v), and the microsomal membrane pellet was resuspended in 1.0 ml of homogenization buffer. For assaying ceramide synthase (sphinganine N-acetyltransferase) activity, sphinganine and palmitoyl-CoA were used as substrates (33). The reaction mixture (in a total volume of 1.0 ml) contained 20 mM Hepes, pH 7.4, 2 mM MgCl2, 20 μM of fatty acid-free bovine serum albumin (Sigma), 20 μM sphinganine (BIOMOL Research Labs Inc.), 70 μM unlabeled palmitoyl-CoA (Amer sham Pharmacia Biotech), 3.6 μM (0.4 μCi) [1-14C]palmitoyl-CoA (55 kBq/μmol), 50 mM NaF, and 100 μM phenylmethylsulfonyl fluoride. After incubation for 1 h at 37 °C, lipids were extracted and separated by TLC (CHCl3, methanol, and 3.5 nm ammonium hydroxide, 15:1:5, v/v/v), and the production of radiolabeled dihydroceramide was determined by scanning of the TLC plates using a Fujix-1000 Imager (Raytest).

Northern Blot Analysis—For RNA preparation, cells were seeded at a density of 1.75 × 104 cells/cm2 in plastic dishes. At the indicated times after treatment with 0.1 μM RA, cells were washed with ice-cold PBS and harvested. Total RNA was extracted using the RNeasy Total RNA kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. 10 μg of total cellular RNA was separated on 37% formaldehyde-containing 1.2% agarose gels and transferred onto Biodyne B membranes (Pall, Hamburg). After prehybridization, filters were incubated with cDNA fragments labeled by random priming in the presence of [32P]dCTP. The probes were a 807-base pair EcoRI fragment of mLICB and a 739-base pair HindIII-PstI fragment of mLIC2 (36). The final washing conditions were 0.2% SSC and 0.2% SDS at 60 °C for 45 min (37). Blots were exposed to Kodak X-AR5 films with intensifier screens at −70 °C for 2 days.

RESULTS

Ceramide-induced Apoptosis in PCC7-Mz1 Cells—Incubation of PCC7-Mz1 stem cells with the classical morphogen RA causes both apoptosis and neural differentiation in a dose- and time-dependent manner (12). Within 24 h, RA (0.1 μM) induced the formation of apoptotic bodies, the DNA of which showed typical DNA laddering (Fig. 1A). The cleavage of genomic DNA into multimeric fragments of ~180–200 base pairs is a hall-
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To assess whether ceramide is involved in mediating the death signal originating from RA, we exposed cultures of PCC7-Mz1 stem cells to synthetic, short-chain, membrane-permeable ceramides. After 24 h of incubation with C2-ceramide (N-acetylsphingosine) and C2-ceramide (N-hexanoylsphingosine), 10 μM each, genomic DNA of detached cells was isolated and analyzed by agarose gel electrophoresis. Both ceramides caused DNA laddering (Fig. 1A) in a dose-dependent manner, whereby C2-ceramide was slightly more efficient than C2-C2-ceramide. Furthermore, C2/C2-ceramide activated within hours apoptosis-related cysteine/aspartate-specific proteases (caspases), demonstrated by cleavage of poly(ADP-ribose) polymerase, a typical substrate for this family of enzymes (data not shown). Thus, apoptotic cell death by ceramide was confirmed by DNA laddering and the activation of caspases. Ceramide application did, however, not induce expression of neuronal markers (data not shown).

Since DNA laddering is difficult to quantify, we employed a viability assay for precise estimation of the degree of cell death. The tetrazolium salt WST-1 becomes reduced to the purple-blue formazan by the dehydrogenase activity of the mitochondria of living cells (27). We added RA and C2 and C6-ceramides to PCC7-Mz1 cells for 24 h and evaluated the viability by the WST-1 assay. 10 μM C2 and C2-ceramides caused a loss of viability of 23.1 ± 5.2 and 20.6 ± 4.3%, respectively, which was similar to 0.1 μM RA (23.5 ± 4.5%) (Fig. 1A, middle panel). The concentrations for the half-maximal effect (EC50) of cell death were 17 ± 2.5 and 30 ± 4.3 μM for C2 and C2-ceramides, respectively (n = 3), and thus were relatively low compared with other cell systems (see Ref. 20). Adding C2-ceramide (10 μM) and RA (0.1 μM) together had a more than additive effect, with a loss of viability of 56.5 ± 9.4% (n = 3). These results imply a potential coupling of RA- and ceramide-activated apoptotic pathways.

Due to insolubility in aqueous solution, a mixture of C16-ceramides up to a final concentration of 100 μM did not activate the suicide program in PCC7-Mz1 stem cells (Fig. 1A, right panel; and data not shown). However, when C16-ceramides (1 μM) was dispersed in a solvent mixture of ethanol and dodecane (98:2, v/v) prior to addition to the culture medium, a final concentration of 1 μM C16-ceramide efficiently caused the death of 85% of the culture (EC50 = 0.5 ± 0.2 μM) (Fig. 1A, right panel). Thus, natural long-chain ceramides, when delivered properly, are even more effective in induction of apoptosis than the short-chain C2/C6-ceramide analogs.

In addition to applying synthetic ceramides, we also used compounds from endogenous sources that caused an increase in cellular ceramide levels, which mimics a rather more physiological situation. Incubation of PCC7-Mz1 cells with the neutral sphingomyelinase from S. aureus, which cleaves sphingomyelin of the plasma membrane and thus increases the cellular ceramide level, was likewise able to induce apoptosis (EC50 = 1 ± 0.25 units/ml; n = 3) in PCC7-Mz1 stem cells. Thus, all ceramide-generating agents tested efficiently caused apoptosis.

To compare the kinetics of ceramide- and RA-induced cell death, PCC7-Mz1 stem cell cultures were treated with RA (0.1 μM), C2-ceramide (10 μM), C2-ceramide (10 μM), and the biologically less active C2-dihydroceramide (10 μM). Dihydroceramide is a naturally occurring ceramide that lacks the 4,5-trans double bond, but retains the stereochemical configuration; and the uptake and metabolism are very similar to those of α-erythro-ceramide (38). DNA of detached cells was analyzed after 3, 6, 12, 18, 24, and 48 h and compared with solvent-treated (0.05% Me2SO) control cultures (Fig. 1B). Both the C2-dihydroceramide- and solvent-treated cultures showed only little, spontaneous DNA fragmentation, whereas the biologically active C2/C2-ceramides caused pronounced DNA fragmentation already after 18 h. It is noteworthy that, at that time point, RA-induced DNA degradation was hardly visible, but became obvious just a few hours later at -24 h of RA incubation.

RA-induced Generation of Ceramide—We investigated whether RA, which acts by binding to nuclear receptors, fulfills some of its effects during early neurogenesis by generation of the second messenger ceramide. PCC7-Mz1 cells were incu-
bated with RA (0.1 μM) and solvent (0.05% Me₂SO) for several time periods. At the indicated time points, cellular lipids were extracted, mildly hydrolyzed with alkaline, and phosphorylated with the diacylglycerol kinase in the presence of [γ-32P]ATP; and phosphorylated ceramide was separated by TLC (Fig. 2A, lower panel). The amount of phosphorylated ceramide was quantified using a phosphorimager and calculated per mg of protein. The examination revealed that, after a lag period of 3 h, the endogenous level of ceramide increased ∼3 ± 0.8-fold within 24 h. Ceramide levels remained at an elevated plateau for 3 days before they declined to near basal levels 5 days after RA-induced differentiation (Fig. 2A, upper panel). To prove that the drastic attenuation of ceramide levels within 1 day was due to RA treatment and not to unfavorable culturing conditions, PCC7-Mz1 cells were incubated with the corresponding amount of solvent (0.05% Me₂SO) as a control. This administration had a minor effect and increased ceramide levels merely by 50% after 24 h (Fig. 2A, upper panel). Therefore, we conclude that the rapid increase in cellular ceramide levels is specific for RA action and parallels the RA-induced processes of neural determination and differentiation.

The increase in ceramide levels by RA treatment was confirmed by a method alternative to the DAG kinase assay, viz. ceramide was visualized by charring densitometry (32, 39). Lipids of PCC7-Mz1 cells treated for 24 h with RA (0.1 μM) or with solvent (0.05% Me₂SO) were isolated, separated by HPTLC, and visualized by charring the plates (Fig. 2B, right panel). The various lipids were identified with the help of standards run in parallel, and their relative amounts were determined by laser scanning of the TLC plate. The level of cholesterol, which remained constant, served as endogenous reference. The analysis revealed an increase in ceramide levels after a 24-h RA treatment to 175 ± 50% compared with control cells (Fig. 2B, left panel). The amount of ceramide increased from 3.0 to 5.23% of total lipids. Accordingly, we confirmed, by a different method, that RA treatment induces ceramide generation in PCC7-Mz1 stem cells.

We addressed the question whether elevated cellular ceramide levels are a consequence of the apoptotic process or a regulator of the process itself. Since DNA fragmentation, as shown in Fig. 1, is a quite late event in the course of apoptosis, an earlier biochemical event, i.e. activation of caspase-3, was investigated. Characteristic for caspase-3 activation is the cleavage of the 32-kDa proenzyme into fragments of 11 and 20 kDa, which can easily be detected by Western blot analysis. Fig. 2C demonstrates that active caspase-3 was not detectable during the first 8 h of RA treatment, i.e. during the phase of commitment to apoptosis (12) and when ceramide levels start to rise (Fig. 2A). As a control, we investigated extracts of cells incubated for 24 h with RA, which clearly expressed the 20-kDa fragment (indicated on Fig. 2C). We conclude that RA-induced production takes place during the initial phase of RA-induced apoptosis.

**RA-induced de Novo Synthesis of Ceramide—**Since the cellular level of ceramide appears to determine the degree of apoptosis, we tried to further increase the RA-induced increase in ceramide levels by inhibiting the catabolism of ceramide. Treating PCC7-Mz1 cells for 24 h with 500 μM N-oleylethanolamine (nOE), which blocks the ceramide-hydrolyzing enzyme ceramidase, enhanced the endogenous level of ceramide by ∼3.5-fold compared with untreated control cells (Fig. 3A) and efficiently promoted apoptosis (Fig. 3B). Thus, the effects of nOE on ceramide levels and apoptosis were similar to those of 0.1 μM RA. Furthermore, treating PCC7-Mz1 cells with nOE and RA together had an enhancing effect neither on ceramide levels nor on apoptosis in comparison with the efficacy of each compound alone (Fig. 3, A and B). Therefore, it was conceivable that RA, as nOE, may augment the ceramide level by reducing the turnover of ceramide.

![Figure 2](image_url)
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Inhibition of de novo ceramide synthesis blocks apoptosis, whereas inhibition of ceramide metabolism induces apoptosis. A, effects of fumonisin B, and N-oleoylthanolamine on ceramide levels. PCC7-Mz1 cells were plated in 25-cm culture dishes and, on the following day, pretreated with the ceramide synthase-specific inhibitor fumonisin B, (25 mM) as indicated. After 1 h, 0.1 mM RA was added; and 24 h later, ceramide levels were estimated by the DAG kinase assay as described under "Materials and Methods." Phosphorylated ceramide was detected by autoradiography and quantified utilizing a phosphoimager. The increase in the ceramide level by RA treatment (−RA, −FB,) was drastically reduced by inhibiting the ceramide synthase with fumonisin B, (25 mM) and N-oleoylthanolamine (500 mM), an inhibitor of ceramidase, induced a 3.6-fold rise in the ceramide level without RA (−RA, +nOE). This increase was not further enhanced by additional RA application (−RA, +nOE). B, effects of fumonisin and N-oleoylthanolamine on viability. PCC7-Mz1 stem cells were treated for 24 h with fumonisin B, and N-oleoylthanolamine in the presence or absence of 0.1 mM RA as shown. Then, viability of cultures was measured employing the WST-1 test and compared with solvent-treated control cells (−RA, −FB, −nOE, =100%). Fumonisin B, at a concentration of 25 mM did not influence viability on its own, but almost completely prevented the RA-induced apoptosis. N-Oleoylthanolamine (500 mM) itself caused a loss of viability in the presence and absence of RA. The results are representative of three independent experiments.

Additionally, ceramides can be produced by two major pathways, viz. either by de novo synthesis or by hydrolysis of membranous sphingomyelin (14). To discriminate between these two pathways, the use of specific inhibitors has proven to be very helpful. Fumonisins belong to a recently discovered group of mycotoxins structurally related to sphinganine and sphingosine (40). In rat hepatocytes, fumonisin B, (FB,) was shown to block a crucial step of de novo ceramide synthesis, catalyzed by sphingosine N-acyltransferase (ceramide synthase) (41). Treating PCC7-Mz1 cells with 25 mM FB, for 24 h by its own did not alter the cellular ceramide level (Fig. 3A) or cause DNA fragmentation (data not shown) or alteration of viability (Fig. 3B). Adding FB, 1 h before induction of neural differentiation by RA completely blocked the RA-induced increase in ceramide levels within 24 h, as shown by the DAG kinase assay (Fig. 3A). The same result was independently obtained when applying the HPTLC charring method for ceramide detection (data not shown). Remarkably, the number of cells dying was drastically reduced, and the viability increased dose-dependently from 79% (only RA) to 91% (10 mM FB, plus RA; data not shown) and 97% (25 mM FB, plus RA) of untreated control cells (Fig. 3B). These results demonstrate that RA caused a significant elevation in ceramide levels arising from de novo biosynthesis and that interruption of this pathway efficiently prevents RA-induced apoptosis. Accordingly, ceramide production seems largely to mediate the RA-induced death signal during early neurogenesis.

Ceramide Synthase and Serine Palmitoyltransferase Activities—We investigated the effect of RA on enzymes of the de novo ceramide synthase pathway and whether FB, indeed obstructs ceramide synthase activity. Therefore, PCC7-Mz1 cells remained untreated or were incubated with RA in the presence or absence of FB,. After 24 h, cells were harvested, and proteins were extracted and analyzed for ceramide synthase activity (Fig. 5A). The levels of in vitro produced 14C-labeled dihydroceramide were independent of treating the cells with RA and indicate that the increase in ceramide levels after RA treatment was not due to an augmentation of ceramide synthase activity. As anticipated, fumonisin B, (25 mM) efficiently attenuated ceramide synthase action in the presence and absence of RA (Fig. 5A).

The long-chain base sphinganine originates from the condensation of serine and palmitoyl-CoA to 3-oxosphinganine (3-ketosphinganine, 3-dehydroxyphinganine) catalyzed by the key enzyme of sphingolipid biosynthesis, serine palmitoyltransferase (SPT). To evaluate the effect of RA on SPT, we analyzed SPT activity in extracts of untreated or RA-treated PCC7-Mz1 cells. SPT activity in extracts was measured by adding the radioactive SPT substrate [14C]serine. Lipids were then extracted and separated by TLC, and the synthesized 3-ketosphinganine was detected by fluorography. Fig. 5B shows that SPT activity was 2.1-fold (100 μg of protein) and 3.2-fold (150 μg of protein) enhanced in extracts of RA-treated cells. Taken together, these results (Figs. 3 and 4) demonstrate that RA-
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Fig. 4. A, effect of RA treatment on sphingomyelinase activity. Cultures of PCC7-Mz1 cells remained untreated (light-gray bars) or were treated for 5 h (dark-gray bars) or 24 h (black bars) with 0.1 μM RA. 10 μg of protein from total cell extracts was analyzed for neutral magnesium-dependent (neutral, +Mg²⁺), neutral magnesium-independent (neutral, −Mg²⁺), and acid sphingomyelinase (acidic) activities employing a mixed-micelle assay system as described under “Materials and Methods.” Desipramine concentrations represented as a percentage of untreated control cultures (100%). Desipramine concentrations were the means ± S.E. of three experiments with six wells/condition each.  

B, effect of desipramine on RA-induced apoptosis. PCC7-Mz1 cells were treated with the indicated concentrations of desipramine for 1 h and then incubated for 24 h in the absence (light-gray bars) or presence (black bars) of 0.1 μM RA. Viability was evaluated by the WST-1 test and is represented as a percentage of untreated control cultures (100%). Desipramine concentrations >10 μM were cytotoxic. The mean ± S.E. of three experiments with six wells/condition each is depicted. C, consequence of desipramine treatment on acidic sphingomyelinase (aSMase) activity. Cells grown in 10-cm dishes were treated as described for B, and the activity of the cellular sphingomyelinase was measured as described under “Materials and Methods.” Desipramine concentrations >1 μM inhibited the acidic sphingomyelinase in the absence (light-gray bars) and presence (black bars) of 0.1 μM RA. Values are the means ± S.E. of three experiments with six wells/condition each.

induced changes in ceramide levels are mediated by an increase in de novo synthesis caused by enhanced SPT activity.

Expression of Serine Palmitoyltransferase—Since RA acts, after binding to nuclear receptors, directly on the transcription of many genes containing hormone-responsive elements (42, 43), we studied whether the increase in de novo ceramide synthesis is due to the up-regulation of mRNAs coding for enzymes in this pathway. Murine SPT consists of two subunits, the catalytically active mLCB2 and the regulatory mLCB1, which are highly expressed in brain (36). Total RNA was isolated from PCC7-Mz1 stem cells and from cultures treated for 1 and 2 days with RA and was subjected to Northern blot hybridization analysis. The radioactively labeled mLCB1 (Fig. 6A) and mLCB2 (Fig. 6B) cDNA probes detected transcripts of 2.9 and 2.3 kilobases in length, respectively. Both mRNAs were constitutively expressed at a constant ratio and to similar degrees. Therefore, transcription of these SPT genes does not seem to be regulated by RA receptors.

Differentiating Cells Do Not Respond to RA and Ceramide—The finding that RA induced ceramide generation in PCC7-Mz1 cells and caused a sustained increase in the endogenous ceramide level for 3 days (Fig. 2) prompted us to examine whether RA and ceramide can still exert apoptotic effects on differentiating cells. PCC7-Mz1 stem cells (d0) and PCC7-Mz1 cells 1, 2, 3, 5, and 7 days after induction of differentiation with RA were treated for 24 h either again with 0.1 μM RA or with 10 μM C₆-ceramide or remained untreated as controls. The viability was determined by the WST-1 assay, and the results are shown in Fig. 7A. Whereas stem cell cultures lost ~20% of their viability by the RA and ceramide treatment, differentiating
cells at all time points (d1–d7) became completely resistant to both treatments. It is plausible that the ability to respond to ceramide is a prerequisite for RA-induced apoptosis.

To further investigate the link between RA-induced cell death and production of ceramide, we analyzed four independently isolated PCC7 subclones (RII/1, RII/2, RII/26, and RII/28) that are resistant to RA. These R-clones lost the potential to differentiate upon RA treatment, but retained a stem cell-like characteristic demonstrated by constant DNA synthesis, inability to express marker proteins, and failure to develop morphologically.

We investigated whether R-clones respond with apoptosis upon RA treatment. Neither a prounced DNA laddering as seen in PCC7-Mz1 cells (Fig. 1A) nor loss of viability was observed upon an 24-h RA treatment of R-clones (Fig. 1B). These findings indicate that the capability to respond to RA with differentiation is connected with the ability to accomplish apoptosis. Addition of 10 μM C6-ceramide, but not of 10 μM dihydroceramide or 0.1 μM RA, produced a pronounced DNA laddering in all R-clones (Fig. 1B). The EC50 values of the four R-clones, evaluated by the WST-1 test, for induction of the programmed cell death were 23, 20, 17, and 22 μM C6-ceramide and thus in the same order of magnitude as the original PCC7-Mz1 clone (17 ± 2.5 μM) (n = 3). This means that R-clones, which neither fully differentiate nor die upon RA treatment, are still able to respond with apoptosis, but not with morphological differentiation (data not shown) upon exogenously applied ceramide. Hence, we investigated whether the R-clones were still capable of generating ceramide after RA treatment. Addition of 0.1 μM RA to the R-clones did not produce a significant rise in the endogenous ceramide levels, whereas, in parallel, the levels of PCC7-Mz1 control cells increased ~2.6-fold, as shown by the DAG kinase assay (Fig. 1B, right panel). Therefore, it is likely that the failure of R-clones to respond with ceramide production is responsible for their resistance to RA-induced apoptosis. Accordingly, the block of the RA-induced signal is upstream of ceramide generation, but downstream or at the level of the retinoic acid receptors and their targets.

Fig. 6. Northern blot analysis of serine palmitoyltransferase.

Total cellular RNA was prepared from PCC7-Mz1 stem cells (day 0) and from cells incubated with 0.1 μM RA for 1 and 2 days as indicated. 10 μg of RNA was separated by formaldehyde-agarose gel electrophoresis and analyzed by Northern blotting using radioactively labeled probes of the two SPT subunits, mLCB1 (A) and mLCB2 (B). The positions of 18 S and 28 S rRNAs are shown. Both SPT subunits were constitutively expressed during PCC7-Mz1 differentiation. Loading of equal amounts of RNA onto the gel is demonstrated by the ethidium bromide (Et. Bromide)-stained gel (C). kb, kilobases.

Fig. 7. Effect of RA and ceramide treatment on PCC7-Mz1 and RA-resistant PCC7 cell lines. A: RA and C6-ceramide (C6-Cer.) induce apoptosis in PCC7-Mz1 stem cells, but not in differentiating cells. Cells were seeded in a 96-well microtitrter plate and remained untreated (day 0; stem cells) or were differentiated for 1, 2, 5, and 7 days, as indicated, with 0.1 μM RA. Cultures were then treated for 24 h with 0.1 μM RA (dark-gray bars), with 10 μM C6-ceramide (black bars), or with solvent (0.05% Me2SO) (light-gray bars; set as 100%); and viability was estimated by the WST-1 assay. The values depicted represent the means ± S.E. of eight values. Neither RA nor C6-ceramide was able to efficiently induce apoptosis in differentiating PCC7-Mz1 cells between 1 and 7 days after RA treatment. B: left panel, ceramide induces apoptosis in the RA-resistant cell line RII/26. The cell line PCC7-RII/26, a PCC7 clone that was selected based on its inability to differentiate upon incubation with RA, was treated with 0.1 μM RA, 10 μM C6-ceramide, or 10 μM C6-dihydroceramide (dhC2) or was left untreated (control [Co]). After 24 h, cells from the medium were harvested, and the genomic DNA was separated on a 1.4% agarose gel. C6-ceramide, but not RA, caused DNA laddering in PCC7-RII/26 cells. The results presented are typical for three further RA-resistant cell lines. The marker (M) was as in Fig. 1. Right panel, RA-resistant cell lines do not produce ceramide upon RA treatment. 1 day after plating PCC7-Mz1 cells and the RA-resistant cell line PCC7-RII/26 in 25-cm dishes, cultures were incubated for 24 h with 0.1 μM RA (black bars). The endogenous ceramide levels were determined, and the levels of untreated cells (light-gray bars) were set as 1. The data show the means of two experiments performed in duplicate. Whereas the ceramide level of PCC7-Mz1 cells increased ~2.7-fold after RA incubation, the rise in the RA-resistant clones was maximal at 1.3-fold (black bars). Since the results of all R-clones (RII/1, RII/2, RII/26, and RII/28) were identical, we depict here only one of them (RII/26).
Ceramide Mediates the Death Signal during Early Neural Differentiation—We have previously shown that treating proliferating stem cells of the teratocarcinoma cell line PCC7-Mz1 with physiological concentrations of retinoic acid promotes both development of neuroectodermal derivatives and apoptosis (9–12). In the present study, this model system for neurogenesis served to dissect signal transduction pathways determining the fate of survival and death. We show that an increase in the cellular ceramide level is responsible for initiating early apoptosis. 1) Incubation of PCC7-Mz1 stem cells with membrane-permeable C\textsubscript{14} and C\textsubscript{16}-ceramides, but not with non-membrane-permeable C\textsubscript{16}-ceramides or the biologically less active C\textsubscript{14} dihydroceramide, caused apoptosis in a dose- and time-dependent manner. 2) Apoptosis was triggered by adding bacterial sphingomyelinase to release ceramide from sphingomyelin of the cytoplasmic membrane and by applying the ceramide-specific inhibitor N-oleylethanolamine. Both procedures caused an increase in the ceramide pool by utilizing endogenous sources. 3) PCC7-Mz1 stem cells, but not differentiating PCC7-Mz1 cells (>d1), responded to both RA treatment and exogenously added C\textsubscript{14}/C\textsubscript{16}-ceramides in an apoptotic manner, implying that RA- and ceramide-activated pathways are interconnected. 4) C\textsubscript{14}/C\textsubscript{16}-ceramides caused significant DNA laddering after 18 h and thus several hours earlier than RA did (after 24 h), which may be due to a belated RA-mediated ceramide production. 5) Within a few hours of RA treatment, well before activation of caspases, the ceramide concentration steadily increased (up to 3-5-fold) and reached a maximal plateau after 24 h before declining after d3. These kinetics match perfectly the time course of apoptotic activity, which is high in the initial phase of neural differentiation, but ceases afterward when a stable neuronal network starts to develop (>d3) (12). Additionally, we previously showed that at least 2 h of RA treatment are necessary to induce apoptosis (12). This period may be necessary to increase production of ceramide, which became obvious 3 h after RA treatment. 6) Inhibition of de novo synthesis of ceramide by blocking the ceramide synthase with fumonisin B\textsubscript{1} abrogated the RA-induced increase in ceramide levels and, concomitantly, apoptosis. However, inhibition of ceramide production did not affect neuronal differentiation.

Regulation of Ceramide Production—Ceramide is produced in various cell types in response to stimulation by several agonists with outcomes as diverse as cell proliferation, differentiation, growth arrest, and apoptosis (13, 46–48). However, reports describing links between RA signaling and ceramide production were rare. In GH4C1 cells, RA concentrations able to inhibit cell proliferation caused a significant and prolonged increase in cellular ceramide content as a result of enhanced sphingosine N-acylation (49). In a recent study, treatment of the neuroblastoma cell line Neuro2a with RA to induce differentiation prompted activation of sphingomyelinase and of the de novo ceramide synthesis pathway (44). Elevated levels of ceramide caused morphological differentiation of Neuro2a cells, i.e., formation of neurites, but not cell death, seemingly contrasting with our results. However, one has to bear in mind the status of differentiation of the Neuro2a neuroblastoma cells and of the PCC7-Mz1 embryonic carcinoma cells used here. Although neuroblastoma cells are already committed to become neurons, the PCC7-Mz1 stem cells are multipotent and able to differentiate into the various derivatives of the neuroectoderm (9, 11). We show that the lipid messenger ceramide is involved in regulating the degree of apoptosis in the very early phase of neural determination. At later stages, also reflected by neuroblastoma cells, ceramide may play a mediator role in the maturation of neurons. In this context, it is noteworthy that exogenous C\textsubscript{14}/C\textsubscript{16}-ceramides are unable to promote cell death in PCC7-Mz1 cells when cell lineage determination is complete, i.e. after 1 day of RA treatment (11). This resistance may be in part due to the up-regulation of the anti-apoptotic Bcl-2 protein during neural differentiation (12). Indeed, Bcl-2 overexpression in PCC7-Mz1 stem cells provides resistance to both RA- and C\textsubscript{14}/C\textsubscript{16}-ceramide-induced apoptosis (data not shown), implying that Bcl-2 acts as a signal transduction component downstream of ceramide production, as was previously shown in leukemia cells (50).

There are several cellular modes of increasing ceramide levels. Ceramide concentrations may arise either by hydrolysis of membrane-inserted sphingomyelin or by de novo synthesis at the endoplasmic reticulum. RA increased slightly the activity of the acidic (but not the neutral) SMase in PCC7-Mz1 cells after only 24 h, yet blocking acid SMase in PCC7-Mz1 cells had no effect on RA-induced apoptosis. The RA-induced increase in the
ceramide level in PCC7-Mz1 cells was nearly completely abolished by inhibiting de novo synthesis with fumonisin B1. Enzymatic assays revealed that the activity of the ceramide synthase was unaffected, but the activity of SPT, a rate-limiting enzyme in de novo ceramide biosynthesis (51), was enhanced (∼2–3-fold) in RA-incubated cultures. This increase matches very precisely the rise in cellular ceramide concentrations. We investigated whether RA, which binds nuclear receptors, controls the expression of the recently cloned serine palmitoyltransferase genes (36). However, RA does not exert its effect by increasing levels of the two SPT mRNAs during RA-induced neural differentiation. Therefore, we envisage a RA-mediated post-translational mechanism regulating the activity of this enzyme.

Another potential mechanism of regulating the sphingomyelin cycle is by inhibiting enzymes that metabolize ceramides like glycosylceramide synthase and ceramidase (sphingolipid-elin cycle is by inhibiting enzymes that metabolize ceramides enzyme.

post-translational mechanism regulating the activity of this enzyme. Controls the expression of the recently cloned serine palmitoyltransferase genes (36). However, RA does not exert its effect by increasing levels of the two SPT mRNAs during RA-induced neural differentiation. Therefore, we envisage a RA-mediated post-translational mechanism regulating the activity of this enzyme.

Apoptosis during Early Neural Development—Apoptosis that occurs at very early stages in nervous system development may shape the gross morphology and/or create a permissive environment for the growth of axons (2). However, it is less clear why individual neural precursor cells die at early phases of their development. One reason for abolishing neural precursor cells is the adjustment of an optimal cell density for accomplishing neurogenesis. We showed that the degree of RA-induced cell death is indeed dependent on the cell density of PCC7-Mz1 cultures and that this cell death is not due to lack of neurotrophic factors (12). Another reason may be to eliminate unwanted precursor cells, i.e. cells with inappropriate phenotypes. Thereby, apoptosis may play a crucial role in pattern formation. Consistent with this possibility, dying cells are observed in four distinct regions of the caudal neural tube of the developing chick embryo (7). These dying cells either became recently post-mitotic or are even still within the cell cycle, and this programmed cell death coincides with cellular differentiation and cell lineage formation of the neural tube along the dorsal-ventral axis. This concept is reflected in our model system of neural differentiation presented here. RA induced ceramide production and apoptosis in PCC7-Mz1 cells only within the first 24 h, the period of determination. At later stages of development, when mainly neuronal maturation processes occur (10, 11), cells became insensitive to both RA and ceramide treatment. This finding is in line with the observation that the cell lineage of a multipotent PCC7-Mz1 cell is determined within the first 24 h of RA treatment. Only within this time window can the fate of a cell be shifted by the presence of other cells or when the cells are plated on laminin-coated surfaces (11).

An analogous phenomenon was observed during the early development of the mouse limb. Apoptosis increases in sites of naturally occurring cell death upon treatment of the embryo with RA. This effect is under tight regulation in that there is a specific temporal sensitivity of the cells to RA (54). Whether RA induces ceramide production in the dying cells of the developing limb remains to be elucidated.

Here we describe that, during very early neural differentiation, apoptosis (but not neurogenesis) is caused by an increase in ceramide levels due to activation of SPT. The PCC7-Mz1 system will allow the function of this distinct cell death, which might serve to control cell density and/or lineage determination, to be revealed by cell biological, genetic, and biochemical methods.

Acknowledgments—We thank Dr. E. Lang for establishing RA-resistant PCC7 clones and Dr. D. Dornmann and N. Düwelhenke (all from University of Mainz) and M. Homoer (University of Bonn) for experimental assistance. We are grateful to Prof. W. Stoffel (University of Cologne) for providing long-chain base cDNAs and discussing results.

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