Induction of Carnitine Palmitoyltransferase I Augments Sphingolipid Synthesis and Palmitate-induced Apoptosis*

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To identify cell death-induced genes, we employed a subtractive hybridization approach and isolated a cDNA encoding a mouse homolog of carnitine palmitoyltransferase I (CPT I), an enzyme that resides at the outer mitochondrial membrane and facilitates passage of long-chain fatty acids into mitochondria for β-oxidation. Induced expression of CPT I mRNA was observed upon programmed cell death in the murine hematopoietic cell lines LyD9 and WEHI-231. To elucidate the role of CPT I in programmed cell death, we examined the effects of long-chain fatty acids and found that the addition of palmitate or stearate to cultured cells led to activation of a death program with a morphology resembling that of apoptosis. Other naturally occurring fatty acids, including myristate and palmitoleate, had no effect. Since both palmitate and stearate are sphingolipid precursors, the effect of these fatty acids on sphingolipid metabolism was tested. Our results indicate that apoptosis induced by palmitate or stearate is correlated with de novo synthesis of ceramide. Inhibition of CPT I by etomoxir enhanced palmitate-induced cell death and led to a further increase in ceramide synthesis.

Superfluous cells frequently arise during development of multicellular organisms (1–4). In addition, cells damaged by mutational events may emerge anytime in life and lead to cancer. Efficient removal of these cells is important to maintain morphogenesis and homeostasis and is achieved by an intrinsic mechanism resulting in cellular suicide. This death appears to be genetically controlled and has been termed programmed cell death. Tight regulation is essential since both excessive cell death and insufficient cell death will disrupt tissue integrity and may have fatal consequences for the organism. Cells undergoing the death program can often be recognized by a number of distinct morphological changes that have been described as apoptosis (5, 6).

Several genes regulating programmed cell death have been identified, and some insight into their function has been obtained (7). Induced expression of a short polypeptide encoded by the reaper gene, for example, precedes apoptosis in Drosophila melanogaster. Inactivation of reaper suppresses virtually all programmed cell death in Drosophila (8). The reaper polypeptide appears to have homology to the death domains found in the cytoplasmic region of several mammalian cell-surface receptors, including CD95 and TNFR1 (9). Members of the interleukin 1-converting enzyme family of cysteine proteases also play an important role in the regulation of cell death. These enzymes promote apoptosis by converting inactive precursor polypeptides into their active form (10). The BCL-2 protein, on the other hand, often extends cell survival by inhibiting apoptosis (11, 12). Several BCL-2 homologs have been isolated, but their function remains vague.

Analysis of signal transduction pathways has pointed toward sphingolipid metabolites as intracellular mediators of programmed cell death. Ceramide, in particular, has been shown to incite antiproliferative responses and apoptosis (13, 14). Several stimuli such as tumor necrosis factor-α, γ-interferon, and ionizing radiation initiate sphingomyelin hydrolysis, thereby generating ceramide (15–17). Downstream targets of ceramide action may include the retinoblastoma gene product p130 and some protein kinases (18, 19). Palmitoyl-CoA, an important precursor of sphingolipids synthesized de novo, is also a substrate for carnitine palmitoyltransferase I (CPT I), which resides at the outer mitochondrial membrane, and, together with CPT II and a carnitine/acylcarnitine translocase, play an important role in the regulation of cell death. These enzymes promote apoptosis by converting inactive precursor polypeptides into their active form (20).

In this report, we demonstrate that two saturated fatty acids, palmitate and stearate, induce de novo synthesis of ceramide and programmed cell death. We show that inhibition of CPT I activity enhances both sphingolipid synthesis and palmitate-induced cell death.

EXPERIMENTAL PROCEDURES

Materials—bcl-2β cDNA was a kind gift from Dr. D. Loh. Triacsin C and ISP1 were kindly provided by Dr. S. Omura (Kitsato Institute, Tokyo, Japan) and Dr. T. Kawasaki (Kyoto University, Kyoto, Japan), respectively. Etomoxir (2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylic acid) was purchased from ASAT AG (Zug, Switzerland). Fumonisin B1 and C2-ceramide were from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). L-[3-14C]Serine and the sn-1,2-diacylglycerol assay system were from Amersham Corp. Other chemicals were from Sigma and Wako Pure Chemical Industries (Osaka, Japan).

Cell Culture—The murine cell lines LyD9 (21) and WEHI-231 (22) were cultured in RPMI 1640 medium (Life Technologies, Inc.) as described (23). Long-chain fatty acids were dissolved in 30 mM KOH and gently heated when required. Fresh preparations were used for induction of cell death. Triacsin C and C2-ceramide were dissolved in ethanol and stored at −20 °C at concentrations of 2 and 40 μM, respectively. ISP1 was in methanol (100 μM). The final concentration of alcohol in cell

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1 The abbreviations used are: CPT I, carnitine palmitoyltransferase I; IL-3, interleukin-3; DTTP, diethylthiometiaminothiopentacetic acid; DAG, diacylglycerol.
ulture was ≤0.1%. Fumonisin B₁ and etomoxir were dissolved in water (10 mM each). Reagents dissolved in water were prepared immediately before use as cell culture additives.

Construction of cDNA Libraries and Differential Screening—Total cellular RNA was extracted by centrifugation through a cesium chloride cushion in the presence of guanidium isocyanate (24). Poly(A)⁺ RNA was purified by oligo(dT) column chromatography (24). The subtractive hybridization procedure was carried out as described (25), except that first-strand cDNA for library construction was generated using poly(A)⁺ RNA extracted from LyD9 cells that had been deprived of IL-3 for 6 h. For subtracted library construction, the Cr₊₀ value (normalized for the presence of high salt) (25) was driven to 6565 mol/s/liter. The Cr₊₀ value of a subtracted cDNA probe used for library screening reached 8307 mol/s/liter. This probe was radiolabeled to a specific activity of 2.5 × 10⁶ dpm/µg. For plaque hybridizations, a sodium dodecyl sulfate-based hybridization solution (26) was used to improve the signal-to-noise ratio. Positive clones were picked; inserts were subcloned into a Bluescript® KS plasmid vector (Stratagene); and their nucleotide sequence was determined.

Cloning, Northern Analysis, and Sequencing of Mouse Liver CPT I—Full-length cDNA was isolated from an ordinary LyD9 cDNA library, generated by standard procedures (24). Northern hybridizations were carried out as described (24). Nucleotide sequences were determined using a Model 373 DNA Sequence® (Applied Biosystems, Inc.).

Detection of Programmed Cell Death—DNA ladder formation was detected as described previously (23). Nuclear fragmentation was analyzed on a FACScan® flow cytometer (Becton Dickinson) employing the DNA ladder formation after addition of palmitate (palm.; 50 mM) or of palmitoyl-CoA (50 mM) and etomoxir (eto.; 200 µM) in LyD9 and WEHI-231 (Wehi-231) cells. For DNA preparations, 5 × 10⁶ cells were collected. Fragmented DNA was extracted (23) and separated on a 2% agarose gel. HindIII-digested Bluescript® plasmid served as size markers, with fragment sizes ranging from 1380 to 250 base pairs.

RESULTS

Induced Expression of Mouse Liver CPT I upon Programmed Cell Death—The murine IL-3-dependent hematopoietic precursor cell line LyD9 undergoes apoptosis after IL-3 deprivation (21, 23). Apoptosis in LyD9 cells is inhibited by actinomycin D and therefore requires de novo RNA synthesis. To isolate cell death-associated genes, we screened a subtracted LyD9 cDNA library enriched for cDNAs induced after IL-3 withdrawal. Differential screening led to the isolation of a clone whose expression was enhanced expression of CPT I suggested an involvement of fatty acid metabolism in programmed cell death. We intended to test whether substrates of CPT I, like palmitoyl-CoA, affect apoptosis. Because palmitoyl-CoA is not cell-permeable, we tested the effect of palmitate, the direct precursor of palmitoyl-CoA,
on cell survival. As shown in Fig. 2A, palmitate induced cell death in LyD9 cells as effectively as C₂- ceramide, a cell-permeable ceramide analog previously shown to cause apoptosis (13). After the addition of palmitate, mitotic activity ceased. The following cell death was accompanied by cellular shrinkage, nuclear condensation, and nuclear fragmentation, a morphology typically observed in cells undergoing apoptosis. Another long-chain fatty acid, stearate, had similar effects. Other saturated fatty acids with carbon chain lengths ranging from C₄ to C₁₄ were tested; however, none of them could induce apoptosis. Furthermore, unsaturated fatty acids with carbon chain lengths ranging from C₁₀ to C₂₀, including several branched fatty acids, had no effect on cell viability. Representative data from experiments using laurate, myristate, palmitate, palmitoleate, and stearate, which have a similar chemical structure, are shown in Fig. 2A. Cell death induction by palmitate was also observed in WEHI-231 cells (Fig. 2B), but not in bcl-2-transfected WEHI-231 cells (data not shown). As expected, induced expression of CPT I mRNA was found also when cell death was triggered by palmitate (Fig. 1).

**Impact of Lipid Metabolism Inhibitors on Apoptosis Induced by Palmitate**—To determine whether CPT I activity can modulate palmitate-induced cell death, we blocked the enzyme using etomoxir, a highly specific irreversible CPT I inhibitor (32, 33). The drug was added to LyD9 cell cultures at concentrations that did not compromise viability (Fig. 3A). When etomoxir was applied together with palmitate, a dramatic increase in DNA ladder formation (Fig. 2B) and nuclear fragmentation (Fig. 3B) was observed. Indeed, concentrations of palmitate that were not effective alone caused substantial cell death in combination with etomoxir, as judged by the incidence of DNA ladder formation events (Fig. 3B). Similar results were obtained using another cell line, WEHI-231 (data not shown). Thus, inhibition of CPT I by etomoxir appears to reduce the threshold for palmitate-induced apoptosis.

Long-chain fatty acids are not available as CPT I substrates until they are activated by acyl-CoA synthetase. The active form of etomoxir is its CoA ester, which is also formed by this enzyme (32). Therefore, both palmitate and etomoxir depend on acyl-CoA synthetase activity to gain access to the mitochon-
drial CPT system. Acyl-CoA synthetase activity is inhibited by a number of competitive inhibitors, with triacsin C being the most potent member of this group (34, 35). The addition of nontoxic concentrations of triacsin C to LyD9 cells grown in the presence of palmitate and etomoxir significantly reduced the occurrence of nuclear fragmentation (Fig. 3C). Thus, palmitoyl-CoA and/or etomoxir-CoA are necessary for apoptosis induction.

We speculated that inhibition of CPT I by etomoxir might prevent the entrance of palmitoyl-CoA into mitochondria and lead to its accumulation in the cytoplasm. Since palmitoyl-CoA is a precursor of sphingolipid synthesis, we considered the possibility that inhibition of CPT I leads to augmented production of ceramide. The initial step in ceramide synthesis is the formation of 3-ketodihydrosphingosine from palmitoyl-CoA and L-serine. This reaction is catalyzed by serine palmitoyltransferase, which is inhibited by the sphingosine analog ISP1 at picomole concentrations (36). The addition of ISP1 to LyD9 cells cultured with palmitate and etomoxir markedly reduced nuclear fragmentation (Fig. 3C). Similar data were obtained using fumonisin B1, which suppresses ceramide synthetase activity (37), the final step in de novo synthesis of ceramide.

These results suggest that de novo synthesized sphingolipids may be mediators of apoptosis induced by simultaneous treatment with palmitate and etomoxir. De Novo Synthesis of Ceramide and Sphingomyelin upon Palmitate-induced Apoptosis—To verify that the addition of palmitate causes de novo synthesis of ceramide, LyD9 cells were metabolically labeled with [14C]serine. After lipid extraction, samples were analyzed by TLC. The amount of de novo synthesized sphingolipids was compared among samples treated with various combinations of the inhibitors described above. The results shown in Fig. 4 confirmed that palmitate addition to cultured cells leads to de novo synthesis of ceramide. An ~3-fold increase in radiolabeled ceramide was observed in cells treated with 100 μM palmitate within 3 h as compared with cells treated with carrier alone. The additional use of etomoxir led to a further rise by a factor of 2. The use of palmitate and etomoxir in combination with either ISP1 or fumonisin B1 caused a reduction in de novo synthesized ceramide. ISP1 blocked ceramide synthesis almost completely, whereas fumonisin B1 was less effective, but still reduced ceramide build-up to a level similar to that seen in untreated cells (Fig. 4). Of note, the amounts of ceramide synthesized in the presence of the various reagents generally correlate with the extent of cell death observed (Fig. 3).

To further validate the notion that palmitate-induced apoptosis is mediated by de novo synthesized sphingolipids and that CPT I confers a protective effect on this kind of cell death, it was important to establish that cell membrane-derived sphingomyelin does not contribute to ceramide production after treatment of cells with palmitate. Total cellular ceramide con-
TABLE I

| Incubation | +Palmitate (50 μM) | +Stearate (50 μM) |
|------------|-------------------|------------------|
|            | −Etomoxir (200 μM) | +Etomoxir (200 μM) |
| h          | %                  | %                |
| 8          | 13.34              | 56.15            |
| 16         | 48.67              | 91.60            |
| 24         | 61.32              | 98.21            |

Samples treated with carrier alone for 24 h were 5.92%. Samples treated with etomoxir (200 μM) alone for 24 h were 4.17%.

Role of CPT I in Palmitate-induced Apoptosis

Induction of programmed cell death by palmitate and stearate in LyD9 cells

Fluorescence-activated cell sorting analysis was performed on cells cultured for 8, 16, and 24 h in the presence of the additives indicated. The numbers indicate the relative frequency of subdiploid DNA as illustrated in Fig. 3.

In this report, we describe an experimental system that allows the simultaneous measurement of ceramide phosphatide derived from ceramide and of phosphatidic acid derived from DAG. The results substantiate our previous data that palmitate treatment leads to ceramide production that is enhanced in the presence of etomoxir (Fig. 5A). DAG levels were also increased in the presence of palmitate and etomoxir (Fig. 5B). More important, the use of ISP1 in addition to palmitate and etomoxir caused a decrease in ceramide phosphate to levels well below those seen in cells treated with carrier alone, indicating that, in fact, all ceramide generated under these conditions is derived from de novo synthesis. Therefore, sphingomyelin residing in the cell membrane compartment does not appear to be released.

Different Effects of CPT I Inhibition on Sphingolipid Synthesis after Challenge with Palmitate and Stearate—Palmitoyl-CoA and stearoyl-CoA are important components of ceramide and other sphingolipids. To test whether both fatty acids have similar effects on de novo sphingolipid synthesis, stearate-treated LyD9 cells were metabolically labeled with [14C]serine as described above. The ceramide increase detected after apoptosis induction by stearate was almost indistinguishable from that seen after palmitate treatment (Fig. 4). However, in contrast to the observations made after cell death induction with palmitate, inhibition of CPT I by etomoxir did not lead to an additional increase in ceramide levels when stearate was used to induce apoptosis (Fig. 4). In agreement with these findings, etomoxir could not accelerate stearate-induced cell death as efficiently as it did when palmitate was used (Table I).

DIFFERENTIAL EFFECTS OF CPT I INHIBITION ON SPHINGOLIPID SYNTHESIS

In this report, we describe an experimental system that allows the investigator to study programmed cell death mediated by de novo synthesis of sphingolipids. Ceramide production is induced by culturing cells of hematopoietic origin in the presence of palmitate and is further enhanced by simultaneous administration of the CPT I inhibitor etomoxir. The use of the pharmacological inhibitors fumonisin B1 and ISP1, which interfere with ceramide synthesis, rescues cells from apoptosis (Fig. 3). Increased de novo synthesis of ceramide may ultimately lead to increased levels of other sphingolipids, such as sphingosine and sphingomyelin. The data presented here do not exclude the possibility that sphingolipids other than de novo-synthesized ceramide are directly involved in the cell death observed. Increased levels of sphingomyelin, for example, may be a target for sphingomyelinase and in turn be degraded to ceramide again.

Sphingolipid metabolism has been shown to play an important role in the regulation of programmed cell death (38). We show that CPT I, a component of the mitochondrial CPT enzyme complex, can protect from palmitate-triggered cell death by down-regulation of sphingolipid synthesis. This conclusion is based mainly on three observations. First, cell death induced by palmitic acid was accelerated after inhibition of CPT I by etomoxir (Fig. 3 and Table I). Second, an ~2-fold increase in de novo synthesized ceramide was observed in cells treated with etomoxir and palmitic acid as compared with cells treated with palmitic acid alone (Fig. 4). The palmitate-mediated build-up of ceramide is blocked in the presence of ISP1 and, to a lesser extent, fumonisin B2. As a result, total cellular ceramide levels drop (Fig. 5). Cell membrane-derived sphingomyelin does not appear to be utilized to replenish ceramide levels because, in the presence of ISP1, ceramide levels drop below those seen in cells treated with carrier alone. The various aspects of sphingolipid metabolism are restricted to different cellular compartments (39). Sphingomyelin within the cell membrane may not be released without the appropriate signals. Third, the sphingolipids produced after simultaneous treatment with palmitate and etomoxir were the cause of apoptosis because inhibition of sphingolipid synthesis by ISP1 and fumonisin B2 blocked the death program (Fig. 3). It is important to stress that the amounts of ceramide produced in the presence of the various inhibitors correlate well with the extent of cell death observed (Figs. 3 and 4 and Table I). ISP1-dependent depletion of intracellular sphingolipid levels has recently been shown to cause cell death in the interleukin-2-dependent cytotoxic T-cell line CTLL-2 (40). As suggested by Nakamura et al. (40), this may be due to a cell lineage-specific sensitivity toward sphingolipid depletion since viability in several other cell lines tested by the authors, including another interleukin-2-dependent cell line (F7), was not compromised. In our hands, neither LyD9 nor WEHI-231 cell growth was affected by 100 nM ISP1.

The protection from apoptosis afforded by CPT I inhibition after death induction with palmitate is likely to be the consequence of long-chain fatty acyl-CoA clearance from the cytoplasm. The CPT enzyme complex effectively transfers activated long-chain fatty acids into the mitochondrial matrix, where they serve as fuel for β-oxidation. Sphingolipid precursors, like palmitoyl-CoA, are subject to removal from the cytoplasm by this mechanism. Cells that express high amounts of CPT I per se are therefore expected to withstand palmitate-induced death. Indeed, hepatoma cell lines that are known to be rich in mitochondria were resistant to palmitate-induced apoptosis (data not shown).

In addition to palmitate, only stearate was able to induce apoptosis and led to increased ceramide synthesis. However, inhibition of CPT I by etomoxir did not enhance sphingolipid synthesis when stearate was used for cell death induction. Since palmitoyl-CoA and stearoyl-CoA are important components of ceramide and both are substrates for CPT I, the reason for this difference was not immediately obvious. However, the two activated fatty acids enter the sphingolipid synthesis pathway at different points. While palmitoyl-CoA, the substrate for serine palmitoyltransferase, constitutes the major non-amide-linked fatty acid component of ceramide, stearate is the predominant amide-linked fatty acid component of ceramide (41). The addition of the stearoyl group to the ceramide backbone is one of the last steps in ceramide synthesis, whereas the condensation of serine and palmitoyl-CoA is the first dedicated step in ceramide synthesis. It may be that an accumulation of palmitoyl-CoA as a result of CPT I inhibition immediately influences ceramide metabolism, while the accumulation of stearoyl-CoA without sufficient palmitoyl-CoA resources cannot effectively boost ceramide synthesis. Therefore, the available amount of palmitoyl-CoA may be rate-limiting in de novo synthesis of ceramide. Moreover, stearoyl-CoA may be replaced by other long-chain fatty acids like palmitoyl-CoA to form the
amide-linked component of ceramide, whereas the high substrate specificity of serine palmitoyltransferase prevents the use of another long-chain fatty acyl-CoA instead of palmitoyl-CoA.

Inhibition of CPT I by etomoxir boosts cell death also in WEHI-231 cells (Fig. 2B), which have been shown to accumulate ceramide after cross-linking of surface Ig (42). Ceramide has been reported to incite a stress-activated protein kinase. Of note, a <2-fold increase in intracellular ceramide levels was sufficient to induce stress-activated protein kinase and apoptosis (19). Other protein kinases may also be activated by increased ceramide levels (43, 44).

CPT I association with programmed cell death was initially discovered based on enhanced expression of its mRNA in LyD9 cells deprived of IL-3 (Fig. 1). However, apoptosis in LyD9 cells induced by growth factor withdrawal was only slightly accelerated in the presence of etomoxir (data not shown). Moreover, de novo synthesis of ceramide after IL-3 deprivation was only marginal (Fig. 4). Therefore, the mitochondrial CPT system may play an additional, yet to be elucidated role in the lipid metabolism of programmed cell death. CPT I isoforms with overlapping tissue-specific expression have been characterized (45). CPT I isoform switching has recently been shown to take place in development (46). Proteins with CPT activity are also associated with peroxisomes and microsomes (47). Murthy and Pande (48) have described a microsomal stress-regulated protein containing CPT activity. Additional analysis of the mitochondrial CPT system in the context of the cellular stress response will be required to further define the apoptosis-related aspects of this multienzyme complex.

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