Serine Palmitoyltransferase Regulates \textit{de Novo} Ceramide Generation during Etoposide-induced Apoptosis*

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The \textit{de novo} pathway of sphingolipid synthesis has been identified recently as a novel means of generating ceramide during apoptosis. Furthermore, it has been suggested that the activation of dihydroceramide synthase is responsible for increased ceramide production through this pathway. In this study, accumulation of ceramide mass in Molt-4 human leukemia cells by the chemotherapy agent etoposide was found to occur primarily due to activation of the \textit{de novo} pathway. However, when the cells were labeled with a substrate for dihydroceramide synthase in the presence of etoposide, there was no corresponding increase in labeled ceramide. Further investigation using a labeled substrate for serine palmitoyltransferase, the rate-limiting enzyme in the pathway, resulted in an accumulation of label in ceramide upon etoposide treatment. This result suggests that the activation of serine palmitoyltransferase is the event responsible for increased ceramide generation during \textit{de novo} synthesis initiated by etoposide. Importantly, the ceramide generated from \textit{de novo} synthesis appears to have a distinct function from that induced by sphingomyelinase action in that it is not involved in caspase-induced poly (ADP-ribose)polymerase proteolysis but does play a role in disrupting membrane integrity in this model system. These results implicate serine palmitoyltransferase as the enzyme controlling \textit{de novo} ceramide synthesis during apoptosis and begin to define a unique function of ceramide generated from this pathway.

It is increasingly apparent that sphingolipids, and in particular ceramide, are important mediators in regulating the response to stress of a cell. The agents that induce ceramide generation include physiological factors, such as tumor necrosis factor and the Fas ligand, as well as therapeutic agents, such as chemotherapy drugs and radiation. Many of these agents induce ceramide generation via the hydrolysis of sphingomyelin by the activation of one or more sphingomyelinases. Additional studies, however, have begun to implicate ceramide generated from the \textit{de novo} pathway of sphingolipid synthesis as having a signaling function (1–8).

Studies of \textit{de novo} sphingolipid biosynthesis have been advanced by the realization that a class of fungal metabolites known as fumonisins share structural similarities with the sphingoid backbone. During investigation of the effects of fumonisin on sphingolipid metabolism in hepatocytes, it was observed that the synthesis of complex sphingolipids was significantly inhibited. It was also determined that the primary site of action of fumonisin was dihydroceramide synthase (9), an enzyme in the \textit{de novo} pathway that catalyzes the N-acylation of sphinganine to produce dihydroceramide.

Because ceramide has been implicated as a regulatory molecule in apoptosis, more recent studies have used fumonisins to investigate the potential role of ceramide from the \textit{de novo} pathway in this process. These studies have demonstrated that fumonisin is able to attenuate apoptosis induced by daunorubicin, camptothecin, tumor necrosis factor-\(\alpha\), and phorbol ester (1, 3, 5, 6). Because fumonisin inhibits dihydroceramide synthase, it has generally been assumed that this is the regulatory step in the \textit{de novo} pathway during apoptosis.

In this study, we have used the chemotherapy agent etoposide to activate \textit{de novo} ceramide synthesis in Molt-4 human leukemia cells. In preliminary experiments, we were unable to find any evidence for activation of dihydroceramide synthase, and we became interested in determining the regulatory point in \textit{de novo} ceramide synthesis under apoptotic conditions with etoposide. Using intact cell radiolabeling techniques and cell-free enzyme assays, we determined that serine palmitoyltransferase, the initial and rate-limiting enzyme in the pathway, is activated during apoptosis and governs the production of ceramide.

We were also interested in elucidating a regulatory function for ceramide generated \textit{de novo} in apoptosis, and our present studies demonstrate that it has its predominant effects on membrane-related events in apoptosis. Importantly, unlike short-chain ceramide or ceramide generated from sphingomyelinase action, this ceramide is dissociated from caspase activation. This study provides the first evidence that serine palmitoyltransferase is a regulated enzyme during apoptosis and provides further evidence that ceramide generated \textit{de novo} functions as a regulatory molecule in mediating membrane-related apoptotic events.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Materials—}Ceramide standards were derived from phospholipase C hydrolysis of brain sphingomyelin and were purchased from Avanti Polar Lipids (Alabaster, AL). [\(\gamma\text{-}^{32}\text{P}\)]ATP (3000 Ci/mmol) and [9,10-\(\text{H}\)]palmitic acid (43 Ci/mmol) were from NEN Life Science Products. [\(\text{H}\)]Sphinganine was synthesized as described previously (10). [9,10-\(\text{H}\)]palmitoyl CoA (60 Ci/mmol) and L-[\(\text{H}\)]serine (20 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). A rabbit polyclonal antibody raised to an epitope in the automodification domain of human poly(ADP-ribose) polymerase (PARP)1 was a gift of

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2 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; RT, reverse transcription; PCR, polymerase chain reaction.
Dr. Guy Poirier of Laval University (Ste Foy, Quebec, Canada.

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RESULTS

Induction of de Novo Synthesis of Ceramide by Etoposide—

The chemotherapy agent etoposide initiates an apoptotic response by inhibiting topoisomerase II, resulting in single-stranded DNA breaks (15). Through unknown mechanisms, this insult by etoposide results in the release of cytochrome c from the mitochondria (16), activation of caspases (17), DNA fragmentation (18), and subsequent cell death (reviewed in Ref. 19). Because ceramide has been shown to be an inducer of these events (20–22), we were interested in determining whether etoposide elevated ceramide levels and, if so, in determining the origin of ceramide. As demonstrated in Fig. 1, treatment with etoposide resulted in a nearly 3-fold elevation of ceramide after 6 h. In the presence of fumonisin, an inhibitor of de novo ceramide synthesis, etoposide-induced ceramide accumulation was decreased by nearly 75% after 6 h.

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Radiolabeling of Cells with a Serine Palmitoyltransferase Substrate but Not a Dihydroceramide Synthase Substrate Results in Increased Incorporation of Radiolabel into Ceramide upon Etoposide Treatment—

To determine whether induction of de novo ceramide generation was occurring by activation of dihydroceramide synthase as previously reported using daunorubicin (1), cells were radiolabeled with [3H] sphinganine, the substrate for this enzyme (Fig. 2), and simultaneously treated with 10 μM etoposide. Using this method and measuring [3H]ceramide over a 9-h time course with etoposide, no increase in incorporation of label into ceramide was observed (Fig. 3A). This result suggests that neither dihydroceramide synthase nor the subsequent enzyme in de novo synthesis, dihydroceramide desaturase, is responsible for increased synthesis of ceramide.

In a similar experiment using a 6 h time period of etoposide treatment in the absence or presence of fumonisin, the incorporation of [3H] sphinganine into [3H] ceramide was significantly inhibited (Fig. 3B), consistent with the reported inhibition of dihydroceramide synthase by fumonisin (23). The lack of an increase in de novo ceramide synthesis using a dihydroceramide synthase substrate indicated a prior step in the pathway as the site of activation. Cells were therefore radiolabeled with [3H] palmitate, a precursor to the serine
palmitoyltransferase substrate palmitoyl CoA, and simultaneously treated with etoposide. An increase of over 3-fold in \(^{3}H\)ceramide was observed by 9 h (Fig. 4A), suggesting that serine palmitoyltransferase, the rate-limiting enzyme in de novo synthesis of sphingolipids, is activated during de novo synthesis of ceramide under apoptotic stress. Moreover, both fumonisin and cycloserine, an inhibitor of serine palmitoyltransferase (24), inhibited the incorporation of \(^{3}H\)palmitate into \(^{3}H\)ceramide during a 6-h time period of etoposide treatment (Fig. 4B).

**Regulation of Serine Palmitoyltransferase Activity**—Previously, it had been demonstrated that during irradiation of keratinocytes, RNA levels of LCB2, a serine palmitoyltransferase subunit, were up-regulated (25). Therefore, in order to understand the regulation of serine palmitoyltransferase activity during etoposide-induced apoptosis, we initially determined mRNA levels of the LCB1 and LCB2 subunits of serine palmitoyltransferase by RT-PCR. No increases in the message level of either subunit were observed. In contrast, the serine palmitoyltransferase RNA was degraded by 6 h of etoposide treatment (Table I). Because up-regulation of RNA was not responsible for increased enzyme activity, in vitro enzyme assays for serine palmitoyltransferase were conducted using microsomes from either control- or etoposide-treated cells. Over a 6-h course of treatment, serine palmitoyltransferase activity was elevated early (by 0.5 h) and sustained throughout the treatment (Fig. 5). These results rule out etoposide-induced activation of serine palmitoyltransferase by up-regulation of RNA and suggest activation by covalent modification or allosteric regulation of the enzyme.

**Dissociation of de Novo Synthesized Ceramide from Caspase Activation**—Because we and others have previously demonstrated that short-chain ceramide induces PARP proteolysis by caspase activation (21, 26) and because etoposide had previously been demonstrated to cause PARP proteolysis (18), we were interested in determining whether the de novo generation of ceramide by etoposide was also instrumental in this caspase-mediated event. In the presence of fumonisin, etoposide-induced de novo ceramide generation returned to basal levels yet PARP proteolysis was unaffected (Figs. 4B and 6). This result demonstrates that de novo ceramide generation is not necessary for the activation of a PARP-cleaving caspase(s) in this cell system. In addition, we have also examined the effect of fumonisin on lamin B cleavage by etoposide, reportedly occurring by caspase-6 (27), and found no inhibition (data not shown). Additional studies using the chromogenic caspase substrates YVAD-pNA, DEVD-pNA, and IETD-pNA were also conducted. These peptides function as substrates for group I, group II, and group III caspases, respectively (28). Whereas etoposide induced both DEVD-pNA and IETD-pNA peptidase activity, fumonisin was without an inhibitory effect (data not shown). These results are consistent with prior studies demonstrating etoposide activation of caspase-3 (of which DEVD-pNA is a substrate), and caspase-6 (of which IETD-pNA is a substrate) in human leukemia cells (29). Furthermore, they provide evidence for the dissociation of de novo generated ceramide from caspase activation and serve to distinguish its function from
both ceramide generated from sphingomyelinases and from exogenous short-chain ceramides, both of which have been implicated in activation of caspases (21, 26, 30).

**Effect of Fumonisin on Cell Death—**Finally, we were interested in determining whether the inhibition of de novo generated ceramide by fumonisin protected cells from death. Because noticeable cell death did not occur during the first 6 h of treatment with etoposide, we assayed cell death at 9 or 24 h after treatment and observed nearly 15 and 75% cell death, respectively, as determined by trypan blue staining (Fig. 7). When the cells were treated during this period with either fumonisin or zVAD-fmk, a pancaspase inhibitor, cell death was approximately 7% after 9 h and 30% after 24 h. Interestingly, when fumonisin and zVAD-fmk were added together, near complete protection from cell death was observed. These results suggest that ceramide from de novo synthesis and caspases contribute to independent pathways of death during etoposide-induced apoptosis.

**DISCUSSION**

The results from this study provide the first evidence that the initial and rate-limiting enzyme in the de novo pathway of
Sphingolipid biosynthesis, serine palmitoyltransferase, is activated during apoptosis. They also provide further support for a regulatory role in apoptosis of ceramide generated from the de novo pathway.

Currently, very little is known about the function of serine palmitoyltransferase other than its role in sphingolipid synthesis for housekeeping functions, but studies in Saccharomyces cerevisiae have begun to define a role for the enzyme in stress response signaling. A yeast strain lacking serine palmitoyltransferase activity has been identified that is unable to grow unless supplemented with sphingoid bases (31). Suppressors of this mutation have also been isolated that are able to grow at ambient temperature but are unable to survive hyperosmolar or heat stress (32). The suppressor strains could be rescued, however, either by transfection with the serine palmitoyltransferase or by supplying exogenous sphingoid bases (33), thus implicating serine palmitoyltransferase in both heat and osmotic stress responses.

In mammalian cells, it has been demonstrated that sphingoid bases are capable of down-regulating serine palmitoyltransferase (34) and that the activity of the enzyme progressively increases during the differentiation process of neuronal cells in culture (35). Moreover, a recent report indicated that 48 h after UV irradiation of keratinocytes, mRNA levels for the LCB2 subunit of serine palmitoyltransferase were up-regulated 1.7-fold and that this corresponded to a 1.5-fold increase in enzyme activity (25).

Our results suggest that caution should be used in interpreting data obtained with the dihydroceramide synthase inhibitor, fumonisin. An inhibition of ceramide accumulation with fumonisin, although implicating de novo synthesis, does not imply that dihydroceramide synthase is the regulatory enzyme. Such a result would also be consistent with a regulatory role of any of the preceding enzymes in the pathway. In two recent studies of phorbol ester- and daunorubicin-induced apoptosis mediated by de novo ceramide synthesis, in vitro assays of dihydroceramide synthase indicated that the V_max was increased 1.6–1.7-fold after treatment (1, 5). However, in our intact cell [3H]sphinganine labeling experiment using etoposide or using an in vitro enzyme assay (data not shown), we found no evidence that dihydroceramide synthase is activated during de novo synthesis in response to etoposide.

The enzymes of the de novo pathway (Fig. 2) leading to the production of ceramide reside on the endoplasmic reticulum (36). In studies using hepatocytes, it was determined that the specific activity of serine palmitoyltransferase is considerably less than that of other enzymes in the pathway, including ketosphinganine reductase and dihydroceramide synthase. This fact, in conjunction with the observation of low sphingoid base levels in cells, has led to the conclusion that serine palmitoyltransferase is activated during apoptosis, thereby suggesting the involvement of de novo synthesis.

In fact, our results suggest that caution should be used in interpreting data obtained with the dihydroceramide synthase inhibitor, fumonisin. An inhibition of ceramide accumulation with fumonisin, although implicating de novo synthesis, does not imply that dihydroceramide synthase is the regulatory enzyme. Such a result would also be consistent with a regulatory role of any of the preceding enzymes in the pathway. In two recent studies of phorbol ester- and daunorubicin-induced apoptosis mediated by de novo ceramide synthesis, in vitro assays of dihydroceramide synthase indicated that the V_max was increased 1.6–1.7-fold after treatment (1, 5). However, in our intact cell [3H]sphinganine labeling experiment using etoposide or using an in vitro enzyme assay (data not shown), we found no evidence that dihydroceramide synthase is activated during de novo synthesis in response to etoposide.

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These studies with etoposide do not exclude the possibility that other inducers of de novo synthesis and/or other cell types regulate additional enzymes in the de novo pathway. Also, the results do not exclude the possibility of additional functions for de novo generated ceramide in the apoptotic response. However, it is clear that care should be exercised in defining the main sites of biochemical regulation in the de novo pathway using a combination of enzymatic and labeling studies. Care should also be exercised in determining what specific aspects of apoptosis are regulated (or not regulated) by the de novo pathway.

The results from the trypan blue experiment suggest that de novo ceramide generated during apoptosis exerts a key regulatory function in effecting membrane damage. Furthermore, the additive and protective effect of fumonisin and a caspase inhibitor on cell death provide evidence that ceramide from de novo synthesis and caspases are activating independent pathways in apoptosis and suggest the model proposed in Fig. 8.

In summary, the results from this study begin to define serine palmitoyltransferase as an important regulatory step in the de novo pathway of ceramide synthesis during apoptosis. Moreover, they dissociate this pathway from a role in caspase activation and implicate it in mediating membrane-related events in apoptosis.

Acknowledgment—We thank Dr. Ala Bielawska for the synthesis of [3H]sphinganine.

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J. Biol. Chem. 2000, 275:9078-9084.
doi: 10.1074/jbc.275.12.9078

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