2003

BCL-2 Down Regulating Ceramide De Novo Pathway

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by

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A thesis submitted to the faculty of Medical University of South Carolina in partial fulfillment of the requirements for the degree of Masters of Science in the College of Graduate Studies

Department of Molecular and Cellular Biology and Pathobiology

2003

Approved by:

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Abstract

Ceramide is a sphingolipid second messenger that is involved in apoptosis and other cellular responses to stress. Ceramide generation occurs primarily by two methods: hydrolysis of sphingomyelin by sphingomyelinases or by de novo synthesis, which is initiated on the surface of the endoplasmic reticulum by serine palmitoyltransferase (SPT). The de novo synthesis of ceramide is activated in response to retinoic acid, chemotherapeutic agents like etoposide or daunorubicin, and TNF-_. We have shown previously that SPT is activated in response to chemotherapy and governs de novo ceramide production. To determine factors that regulate SPT and the de novo pathway we have investigated this pathway in cells over-expressing Bcl-2.

Bcl-2 is a 26-kDa integral membrane oncoprotein that is capable of suppressing apoptosis. It prevents many of the morphological and biochemical changes observed in apoptosis. It was shown that Bcl-2 interrupts apoptosis by different mechanisms such as forming heterodimers with Bax, affecting Ca^{+2} flux, or preventing the release from the mitochondria of apoptotic activators like cytochrome c and AIF. We herein demonstrate a caspase-dependent induction of de novo ceramide in etoposide-induced apoptosis and its inhibition by Bcl-2.

Acknowledgements

I would like to thank my advisory committee members; Dr. Julio Barredo, Dr. Yusuf Hannun, Dr. Inderjit Singh, and Dr. Yi-Te Hsu. I would like to extend special thanks to my committee chairperson Dr. David Perry for his guidance and support. For the two years I have spent in Dr. Perry's laboratory he has given me the latitude to think, analyze, and interpret data, to compose a comprehensive research. I am also grateful for the time he has spent with me teaching me the technical details for experiments. I will always remember the long hours we spent on the tedious DGK assay until he made sure I mastered it. Dr. Perry's mission was hard because as a MD with no previous laboratory experience it was difficult for me to achieve the transition from clinical thinking to bench environment and basic science methodology. I could have not achieved this without his patience and supervision. I am also grateful to Nick Luong from Dr. Perry's laboratory for all the help he gave me. Finally, I want to thank my wife Tharwa Bilbeisi for her enormous support and kindness during these extremely busy two years.

Introduction

Sphingolipids

Sphingolipids are a diverse group of compounds that differ from glycerolipids in having a sphingoid base instead of glycerol as the common structural component (***figure 1***). Structurally, the most basic sphingolipids are the sphingoid or long-chain bases (lcb) sphinganine and sphingosine. These can be acylated through their amino group to produce dihydroceramide or ceramide, respectively. Finally, the complex sphingolipids are formed by the addition of polar head groups to ceramide.

The key sites on sphingolipid molecules are located on carbons 1-5 of the sphingoid backbone. These functional groups confer on sphingolipids their important biological functions and specificity. C-1 and C-3 are the locations of hydroxyl groups and the C-3 hydroxyl group serves as the site for polar headgroup addition. C-2 is the site of the amino group through which the fatty acid is attached, and C-4/C-5 is the location of the trans double bond, which confers specificity. Importantly, dihydroceramide which differs from ceramide only in that it is fully saturated at C-4/C-5 is without signaling function in all the systems tested (***figure 1***).

Ceramide generation

Ceramide can be generated from three sources. One of these is by sphingomyelin hydrolysis, which was the first route identified for generation of a signaling pool of ceramide. A second source of ceramide generation is through the de novo pathway which has long been known to provide substrates for complex sphingolipids biosynthesis and in recent years has been observed to supply its own signaling pool of ceramide. The third source of ceramide is the salvage pathway in which sphingosine from catabolic processes is reacylated to form ceramide (reference 5).

Ceramide fate

After ceramide is generated from the de novo pathway, it can be subject to a number of metabolic modifications. Some of the simplest of these include phosphorylation of the C-1 hydroxyl group to produce ceramide-1-phosphate or, alternatively, acylation at the same position to produce 1-O-acylceramide. Both of these are metabolic routes that have not been shown to be quantitatively significant.

Other more quantitatively important modifications of the 1-OH group include the addition of a phosphorylcholine headgroup to yield sphingomyelin or the addition of glucose to yield glucosylceramide. A final important metabolic route is the catabolism of ceramide by ceramidase to from a free fatty acid and sphingoid base (reference 6).

De novo biosynthesis (*Figure 2*)

In our laboratory we focused our attention on the de novo pathway with special interest in its regulators and modulators. The enzymes of the de novo pathway are oriented on the cytosolic face of the endoplasmic reticulum (ER) as shown by Mandon et al (reference 7) in 1992. The pathway starts with the condensation of serine and palmitoyl CoA resulting in an 18 carbon sphingoid backbone. This initial reaction is mediated by serine palmitoyl transferase (SPT), which will be discussed in more detail later. The product of this condensation reaction is 3-ketosphinganine, which undergoes reduction by ketosphinganine reductase to yield sphinganine (dihydrosphingosine) which is, in turn, acylated by ceramide synthase (dihydroceramide synthase) to give dihydroceramide. Finally, the double bond is added by

dihydroceramide desaturase to yield ceramide. So the de novo biosynthesis can be summarized in three stages: the synthesis of a long-chain base, the formation of ceramide by N-acylation, and finally, the transport of ceramide to the Golgi for headgroup addition.

Why is the de novo pathway important?

The short answer to the above question is because it is an important source of ceramide. Ceramide is implicated in different cell functions like proliferation, cell cycle regulation, senescence, and apoptosis. For all these reasons de novo biosynthesis is vital for cellular functions in signaling. It is also an essential pathway since sphingoid bases are required for cell growth although it's unclear if this is related to a signaling or structural integrity event. Finally, because the de novo pathway is involved in generating signaling molecules that program cellular events, it is implied that the pathway has tightly controlled regulatory mechanisms.

Apoptosis

A- *Chemotherapy induced apoptosis* (reference 9 for more review)

De novo ceramide synthesis can induce apoptosis in response to different chemotherapeutic agents. Etoposide can activate apoptosis via de novo ceramide synthesis in Molt-4 human T-leukemia cells (reference 8). This was through SPT activation through post-translational modulation since SPT mRNA levels did not change. Also, daunorubicin in the P388 murine leukemia cell line and U937 human monoblastic leukemia cells induced the de novo pathway and programmed cell death by activating dihydroceramide synthase (reference 10). This conduct was not limited to hematological cancers but also included many of the solid tumors cell lines; camptothecin in 4B1 (also called L929) mouse fibroblasts, taxol in MCF-7 breast cancer cells, and gemcitabine in A549 lung cancer cell line (references 11-13).

It should be noted that induction of de novo ceramide does not obligate an apoptotic response. Akao et al (reference 14) demonstrated this when they treated LNCaP prostate cancer cells with camptothecin (CPT-11). Here the cell death caused by CPT-11 was not because of increased de novo ceramide generation. De novo ceramide is synergistic to

radiation therapy as is the case when treating LNCaP cells with 12-O-tetradecanoylphorbol acetate (TPA) or radiation. Neither agent induced apoptosis alone (reference 15).

The ceramide-induced apoptosis is gaining more attention because of new challenges to old notions regarding how chemotherapy induces programmed cell death. It was shown in murine mammary cell lines that cell cycle arrest and apoptosis caused by taxol are not synonymous processes (reference 12). Similar doubts were also cast on daunorubicin-induced apoptosis mechanisms because it was shown that apoptosis could be induced under conditions where no free drug is available intra-cellularly (reference 16).

B-Fatty acids induced apoptosis

Research has shown that de novo synthesis induced apoptosis can be activated by applying fatty acids like palmitate or stearate directly or by creating conditions that increase their availability to the de novo pathway by means of inhibiting beta-oxidation with agents like etomoxir that inhibits carnitine palmitoyltransferase (CPT-I) (reference 17). This data was further substantiated using reverse methodology by Shimabukuro et al (reference 11). They were able to inhibit apoptosis by inhibiting fatty acyl-CoA

synthase or enhancing free fatty acids beta-oxidation using triacsin C or troglitazone, respectively.

C-Retinoic acid (RA) induced apoptosis

PCC7 teratocarcinoma cell line responds to all-trans RA by differentiation and apoptosis. In this system, retinoic acid activated SPT leading to an increase in de novo ceramide generation (reference 19). Treating the GH4C1 rat pituitary cell line with all-trans RA also induced de novo ceramide but through increasing sphingosine N-acylation (reference 20). Other RA derivatives like the synthetic RA, N- (4-Hydroxyphenyl) retinamide (4-HPR) produce a similar response in CHL90 neuroblastoma cells by activating both SPT and dihydroceramide synthase (reference 21).

D- Cytokines induced apoptosis

TNF-alpha induced cell death alone or with cycloheximide aid in MCF-7 or bovine cerebral endothelial cell line (BCECs) cells respectively. In both cases this induced apoptosis was through activating the de novo synthesis pathway (references 74 and 75). In MCF-7 cells sphingomyelin hydrolysis was also activated.

E- BcR cross linking in B-cells

In the Ramos cell line (Epstein-Barr negative Burkitt's lymphoma cells) BcR cross-linking induced cell death via activating de novo ceramide generation (reference 22)

F- Cannabinoid-mediated apoptosis

Both tetrahydrocannabinol (THC) and the CB2 selective agonist JWH-133 increased de novo ceramide synthesis causing cell death in glioma cells (references 24 and 25). It is apparent from all of the data collected that different stress agents can induce de novo ceramide in a variety of systems and the consequence of this induction is often apoptosis.

The nervous system

It is estimated that about 20-80% of neurons die during brain development. This is due to the neurotrophic strategy where excess neurons are made to maximize enervation capacity, then, those without synaptic connections expire (reference 19). Ceramide from both de novo and sphingomyelin hydrolysis has been implicated in nervous system apoptosis. De novo biosynthesis is also involved in neuronal differentiation, which was shown by retinoic acid experiments in PCC7-Mz1 teratocarcinoma or Neuro2a neuroblastoma cell lines (references 19 and 26). There is mounting evidence exhibiting clear involvement of de novo ceramide synthesis in nervous system pathology. This includes a wide range of diseases like neurodegenerative etiology. This is the case of Batten's disease where

photoreceptors die by programmed cell death (reference 27). It was found by Rylova et al that the gene responsible for the disease, CLN3 is a negative regulator of de novo ceramide biosynthesis (reference 28). De novo ceramide in the nervous system is an important factor in other pathological processes like the inflammatory process in response to cytokines like TNF-alpha (reference 74) and cannabinoid mediated effects through their CB1 or CB2 receptors (reference 24). More interesting are the new reports identifying SPT mutations as the culprit in hereditary sensory neuropathy type 1 (references 29 and 30). This is considered the first pathology of sphingolipid metabolism that does not involve a lipid storage disease.

The immune system

De novo ceramide is responsible for activated induced cell death (AICD) in B-cells. This process is believed to control B-cell populations and functions to protect the body from autoimmune diseases (reference 22). The CB2 receptor is a cannabinoid receptor that is restricted to brain tumors and immune system cells. The CB2 selective agonist JWH131 inhibited glioma cell growth by activating de novo ceramide. However there may be also a down side for ceramide activation by inhibiting the immune system

response to malignant cells due to the presence of CB2 receptors (reference 25).

Epidermal barrier integrity (references 31-33)

Holleran et al. demonstrated that by disrupting the epidermal barrier using chemical, physical or pathological methodology, the biosynthesis pathway for sphingolipids is activated via inducing SPT activity. The epidermal recovery was sequential with an increase in cholesterol first, followed by sphingolipids later. Farrell et al. was able to show that UVB irradiation will increase SPT activity in keratinocytes by up regulating the LCB2 (2.3KB) mRNA (reference 33).

Other functions and pathologies

De novo biosynthesis is implicated indirectly in a wide range of non-nervous system diseases. This is very clear from the occurrence of the harmful effects of fumonisin B in humans and animals, which are, associated with an increase in the sphingoid base levels in urine and plasma samples (references 34 and 35). These diseases include equine leukoencephalopathy, porcine pulmonary edema, and murine hepatotoxicity. In humans fumonisin B is implicated

in endemic esophageal cancer in South Africa (reference 34) and endemic nephropathy in Croatia and other parts of Eastern Europe (reference 36). The de novo pathway is also being implicated in atherosclerosis, obesity and diabetes (references 37-39 and 18). Other functions of de novo ceramide biosynthesis are cell cycle progression via dephosphorylating Rb protein (reference 40), serine/arginine-rich (SR) protein phosphorylation which regulates alternative splicing (references 13 and 41), and modulating drug resistance (reference 42).

De novo synthesis modulators

Inhibitors

1) SPT inhibitors: myriocin, beta-chloro-alanine, beta-fluoro-alanine, and L-cycloserine. Sphingoid bases or their homologues are also important inhibitors for de novo sphingolipid biosynthesis, and may play an important role in the endogenous regulation of SPT activity and sphingolipid biosynthesis. The sphingoid base inhibition effect is dose and time dependent, SPT specific, and stereo specific (references 43-45). The

full-length wild type leptin receptor (OB-Rb) also inhibits SPT activity (reference 39).

2) Dihydroceramide synthase (N-acylsphingosine transferase) inhibitors:

Fumonisin B compounds are the most widely known and they inhibit dihydroceramide synthase by competitive inhibition with its substrates. As shown in ***(figure 3)*** (reference 35) there is a great resemblance of fumonisin B to the sphingoid bases. Fumonisin B can inhibit both the salvage pathway as well as the de novo by blocking N-acylation of sphingosine and sphinganine.

3) Indirect inhibition of de novo ceramide can be achieved through manipulating fatty acid oxidation or by regulating enzymes that degrade the de novo synthesis metabolites. This regulation occurs with 1-methylthiodihydroceramide (1-MSDH-cer) which inhibits de novo synthesis by activating sphinganine kinase, leading to degradation of sphinganine (reference 46).

Activators

1) SPT activators include; chemotherapeutic agents like etoposide, all-trans retinoic acid or synthetic

retinoid like 4-HPR, UV irradiation, and fatty acids (references 8, 18-21, and 33).

2) Dihydroceramide synthase activators: daunorubicin, 4-HPR, and TPA (references 10, 21, 15, and 47).

Paradox and complexity in studying ceramide

The study of ceramide is at times complicated by contradictory results. These difficulties are due to specificity in sphingolipid functions. This specificity can be system directed, agent specific, or related to the cell status like maturity or differentiation. This applies to ceramide generated from sphingomyelin or de novo pathways. Since our interest here is de novo we will use examples related to the de novo pathway. First, LNCaP prostate cancer cells are sensitive to TPA in the confluent status but resistant in the plateau phase (references 47 and 15). The second example is that mature Ramos B-lymphoma cells respond to BcR cross-linking differently from immature WEH231 murine B-lymphoma cell line (references 22 and 23). Third, the PCC7 teratocarcinoma response to all-trans retinoic acid is dependent on differentiation status (reference 19). Fourth, agent specificity: while etoposide induced de novo via SPT activation in Molt-4 human T-leukemia cell line, other agents like GW 1843 (reference

50) induced a sphingomyelin source of ceramide. Fifth, is dose and time differences; this is evident in the case of daunorubicin and the U937 human leukemia cell line (references 10 and 16). Bose et al. was able to induce de novo ceramide by activating dihydroceramide synthase (reference 10) while Jaffrezou showed neutral sphingomyelinase activation (reference 16). It should be noted that Bose et al. applied 10 micro molar daunorubicin for 12 hours and Jaffrezou used only 1 micro molar for 4 hours.

Bcl-2 Oncogene

Bcl-2 was first identified at the chromosomal breakpoint of t (14; 18) in human B-cell lymphomas. This translocation brings the Bcl-2 gene under the control of the actively transcribed Ig gene on chromosome 14. The Bcl-2 family now includes pro-apoptotic proteins like BAX, BAD, and BID, as well as anti-apoptotic members like Bcl-2 and Bcl-X_L (reference 51). The anti-apoptotic members are found in the mitochondrial, endoplasmic reticulum and nuclear membranes, while the pro-apoptotic are located in the cytosol or cytoskeleton. All the anti-apoptotic members are composed of four Bcl-2 homology (BH) domains, BH1-BH4, with the carboxy-terminal the hydrophobic domain that anchors them to the membranes (reference 52). The various methods

used to execute their anti- or pro-apoptotic functions include: channel forming, homo- or hetero-dimerization, or binding to proteins that are involved in programmed cell death (reference 53). In the case of Bcl-2 it prevents cytochrome c release and inhibits poly (ADP-ribose) polymerase (PARP) protease activation (reference 54 and 55). Bcl-2 was also found to interrupt downstream the ceramide pathway in apoptosis without interfering with its generation (references 56-58).

Serine Palmitoyltransferase

The SPT enzyme is the rate-limiting enzyme that catalyzes the first step in sphingolipid biosynthesis. Most of SPT activity is localized to the microsomal fraction (reference 61). SPT enzyme belongs to a subfamily of the pyridoxal5'-phosphate utilizing enzymes. This subfamily is called the alpha-oxoamine synthases, which catalyze the transfer of the acyl group from an acyl-CoA to the alpha-carbon of an amino acid.

In mammalian cells the SPT enzyme consists of two subunits, LCB1 and LCB2, with stoichiometry of 1:1 (reference 62). Both LCB1 and LCB2 subunits are required for SPT activity (references 63 and 64). However, Weiss and Stoffel

demonstrated that in human embryonic kidney cells (HEK 293) the over expression of LCB2 increases SPT activity (reference 65). According to Hanada this might be due to forming complexes between the endogenous LCB1 and the over expressed LCB2 (reference 63). In yeast SPT is necessary for growth (references 66-68). Activity also requires both LCB1 and LCB2 subunits (references 68-70). Dunn et al. isolated an 80-amino acid protein (Tsc3p) that may be part of the SPT complex in yeast (reference 71).

In eukaryotes the SPT subunits are hydrophobic, membrane-associated proteins with potential transmembrane helices, while in bacteria the SPT enzyme is a water-soluble homodimeric protein (reference 72). Although it was first identified in the late sixties, SPT has been gaining renewed interest due to its implication signaling and human disease. Nicholson et al. (reference 30) observed mutations in SPT of patients with hereditary sensory neuropathy type I and postulated that these cause an increase in SPT activity leading to the production of more de novo ceramide. Predicting this will lead to neural degeneration and more apoptosis. However, the recent paper by Dunn et al. (reference 73) showed that similar mutations in yeast have inhibitory effect on SPT. This discovery will

represent more challenges to researchers and further the impetus for studying SPT.

Others and we have demonstrated that cellular stress agents can activate SPT and lead to upregulation of de novo ceramide and apoptotic responses. We also know that these agents due not interact directly with SPT. Therefore, we were interested in identifying endogenous upstream regulators of SPT in the de novo pathway.

One lead came from earlier data demonstrating the integral relationship between ceramide, caspases, and bcl-2 (reference 58). This work demonstrated that although bcl-2 prevents apoptosis and the activation of executioner caspases, it does not interfere with ceramide production from agents that turn on sphingomyelinase activation. As de novo ceramide production differs spatially in the cell from that produced by sphingomyelinases, we set out to determine if bcl-2 was capable of regulating de novo ceramide production and if caspases were involved in this process.

Materials and Methods

Materials: ceramide standards were purchased from Avanti polar lipids. [Gamma-^{32}P]ATP (3000 Ci/mmol) and [9, 10-^{3}H] palmitic acid (43 Ci/mmol] were purchased from NEN. Etoposide was purchased from sigma, and z-VAD.fmk peptide from Calbiochem. GW 554869a was obtained from Dr. Yusuf Hannun's lab.

Cell culture: Molt-4 T-leukemia cells from Zhang et al were used for experiments (reference 57). These cells had been transfected with pMEP4 vector with and without full- length murine bcl-2. Cells were maintained at 37°C and 5% CO_2 in RPMI medium with 10% fetal calf serum (FCS).

Ceramide mass measurements: Molt-4 cells were seeded at 5x10^{5}/ml in a 6-well plate (3mls/well). After the desired treatment, ceramide mass was determined using the diglyceride kinase assay (DGK) as (reference76). In summary 5x10^{5} cells (equals 1 ml and 2 mls left for lipid phosphate determination) were used for the DGK assay. These cells were lysed in a mixture of chloroform and methanol (1:2) and lipids were extracted as prescribed by the Bligh and Dyer method (reference 77) then solubilized in mixed micelles for 30 minutes at 37°C. Three _g of membranes from

E.coli overexpressing DGK followed by the addition of three μCi of [gamma-^{32}P] ATP in the presence of 1mM carrier ATP and kept at room temperature. Thirty minutes later the reaction was quenched with chloroform/methanol (1:2) and the lipids extracted again based on the Bligh and Dyer method. The chloroform was evaporated and the lipid pellet was suspended in 40 _l of chloroform/ methanol (4:1) and resolved on TLC plates. The solvent system used was chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1). After autoradiography, the ceramide location was identified by iodine-staining of a ceramide standard and radioactivity was quantified by liquid scintillation spectrometry. Ceramide mass was quantified based on the standard curve and normalized to lipid phosphate.

Lipid phosphate determination: Lipids extracted from 10^6 Molt-4 cells were concentrated to remove the chloroform. Then the lipid pellets were incubated at 180°C overnight in 0.6 ml of 10 N H_2SO_4/70%$HClO_4$/H_2O (9:1:40). Then, 0.6 ml of H_2O, 0.5 ml of 0.9% ammonium molybdate, and 0.2 ml of 9.0% freshly made ascorbic acid were added and incubated for 30 minutes at 45°C. Inorganic phosphate was detected by absorbance at 820 nm and quantified based upon a standard curve of K_2HPO_4.

Radiolabeling of cells: Molt-4 cells were seeded at $5x10^5$/ml (3mls/well in a 6-well plate). 1 _Ci/ml of [^{3}H] palmitate was added with 10 micromolar of etoposide at the same time. At the desired time point cells were lysed in a mixture of chloroform and methanol (1:2) and lipids were extracted according to the Bligh and Dyer method. The chloroform phase was evaporated in a speed vacuum device and the lipid pellets were suspended in 40 _l of chloroform/methanol (4:1) and resolved on TLC plate. The solvent system used was chloroform/methanol/2N NH_4OH (40:10:1). After chromatography, the ceramide standard was identified using iodine vapors. Then, TLC plates were sprayed with enhancer, and radioactivity was visualized by autoradiography after 48-72 hours at -80°C. The radioactive spots corresponding to the ceramide standard were scraped and quantified by liquid scintillation spectrometry.

Results and discussion

Our investigations were predicted on data we had previously established showing that the topoisomerase inhibitor and chemotherapy agent etoposide caused a several-fold elevation of ceramide mass in Molt-4 cells (***Figure 10***). Importantly, it was also demonstrated that the dihydroceramide synthase inhibitor, fumonisin, inhibited this increase in ceramide suggesting its generation from the de novo pathway (reference 8). The involvement of de novo ceramide synthesis was further substantiated by showing that etoposide indirectly activated the rate-limiting enzyme in the pathway, serine palmitoyltransferase (reference 8).

As a starting point in looking for upstream regulators of de novo ceramide production and for reasons we stated at the conclusion of the introductory section, we proceeded to utilize Molt-4 cells transfected with a control vector or vector containing the murine bcl-2 oncogene. Overexpression of murine bcl-2 predominantly in a heavy membrane fraction in these cells had earlier been confirmed (reference 57). Upon treatment of both cell lines with etoposide over a 12-hour time course, we observed near total abatement of the ceramide response in the bcl-2 over-expressing cells compared to the vector control cells (***figure 4***).

Importantly, ceramide mass was not diminished by an inhibitor of the neutral sphingomyelinases implicated in ceramide generation in apoptotic signaling. A Glaxo Wellcome (G.W) compound inhibits this enzyme and TNF-signaling responses (reference 79). It was without effect in our cells (***figure 6***) providing further support that etoposide-induced ceramide is from the de novo pathway. We also investigated the impact of bcl-2 on ceramide generation as assessed by a [^{3}H]-palmitate pulse-labeling study. Palmitate is rapidly incorporated into palmitoyl CoA, a substrate for the first enzyme in the de novo pathway, and the radiolabel in the fatty acid chain is incorporated into the sphingoid backbone. Further metabolism in the de novo pathway results in the production of ceramide whose degree of radiolabeling we assessed by thin layer chromatography and liquid scintillation counting. This pulse, rather than equilibrium, approach results in radiolabeled ceramide that is reflective of de novo synthesis rather than from constitutive or agonist-induced turn over of sphingomyelin.

The period of pulse-labeling was begun at the same time of etoposide treatment, and over the course of 12 hours, we observed a steady increase in ceramide generation up to two-fold after 12 hours (***figure 5***). As we observed in

figure 4 with the mass measurements, corresponding inhibition in the bcl-2 over-expressing cells was also observed in the ceramide radiolabeling assay.

That bcl-2 inhibited the ceramide response to etoposide as assessed by both fumonisin-sensitive mass measurements and radiolabel analysis provides added confidence that bcl-2 is functioning as a regulator of the de novo pathway. These results also differentiate regulation of the de novo and sphingomyelinases mediated pathways of ceramide generation as ceramide production by the latter is not inhibited by bcl-2 (reference 58).

Bcl-2 is a membrane protein located primarily in the mitochondria. It is an established regulator of apoptosis and is thought to mediate its inhibitory effects predominantly through regulating mitochondria events. In this regard, it prevents the release of cytochrome c from the mitochondria, subsequent apoptosome formation, and the activation of executioner caspases. Because we observed inhibition of de novo ceramide synthesis in bcl-2 overexpressing cells and because bcl-2 inhibits activation of both caspase-9 and downstream executioner caspases, we hypothesized that caspases might be involved in up-regulating de novo synthesis.

To test our hypothesis, we treated Molt-4 cells with etoposide +/- varying doses of the pancaspase inhibitor, zVAD. zVAD inhibited both mass ceramide accumulation (***figure 7***) and radiolabeled ceramide accumulation (***figure 8***) in etoposide-treated vector control cells. Correspondingly, the diminished increase of ceramide mass observed in the Molt-4 bcl-2-transfected cells was not affected by zVAD (***figure 9***).

These results allow us to draw several conclusions. First, they further implicate the mitochondria as a coordinating center for stress response. In this case, the DNA damage induced by etoposide is sensed in an unknown manner by the mitochondria leading to apoptotic signaling events. In instances were bcl-2 is upregulated, it is unclear if the mitochondria still sense the damage or if the damage is sensed but unable to be acted upon due to the presence of bcl-2. Second, the results demonstrate the necessity of caspase activation in de novo ceramide synthesis. Identification of the caspase(s) involved in this role awaits further study and will be assessed using caspase isozyme-specific inhibitors. This is crucial because there is a growing body of evidence indicating that pan-caspase inhibitors like z-VAD-fmk, z-YVAD-fmk, and Z-DEVD-fmk inhibit other cysteine proteases like cathepsins

B,H,L,S,K,F,V,X and C (reference 80). Some of these cysteine proteases especially cathepsin B are involved in the apoptotic process (references 81-84) which forces us to take data from pan caspase inhibitors with caution and emphasizes the importance of future caspase isozyme-specific inhibitor .Third, the results open to discussion the role of de novo ceramide in etoposide-signaling. Ceramide has been shown in many instances to act further upstream in stress signaling and coordinating stress responses. The fact that we observe ceramide generation in a distal site (on par with the activation of executioner caspases) in the dogmatic apoptosis cascade might suggest that it is acting in more of an executioner phase. Fourth and finally, although we know from prior work that serine palmitoyltransferase activation in this system drives de novo ceramide generation, we do not know if caspase activation is necessary for its activation or if it is necessary for progression through the de novo pathway downstream of serine palmitoyltransferase.

In conclusion, these results have identified a novel role for Bcl-2 in inhibiting apoptotic responses and have identified caspases as regulators of de novo ceramide synthesis.

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Figure legends

Figure 1. **Ceramide:** demonstrating the resemblances and differences among the three molecules: Ceramide, dihydroceramide and diacylglycerol(DAG). DAG and ceramide have opposing cellular functions and dihydroceramide is an inert molecule.

Figure 2. **The de novo pathway**

Figure 3. **Fumonisins:** there are significant similarities between fumonisins and sphingoid bases and they act as competitive inhibitors of dihydroceramide synthase.

Figure 4. **Effects of Bcl-2 on ceramide production:** Molt-4 cells transfected with Bcl-2 (solid and open circles) or control vector (solid and open squares) were treated with 10__ etoposide (solid symbols) or vehicle control (open symbols) for 0-12 hours. At the indicated time points, the cells were harvested, and ceramide mass was quantified using diglyceride kinase assay and normalized to lipid phosphate. The open circles or squares are for control BCL-2 or vector cells, respectively.

Figure 5. **Effects of Bcl-2 oncogene on de novo ceramide production after etoposide treatment:** Molt-4 leukemia cells transfected with Bcl-2 oncogene (solid circles) or control vector (open squares) were treated with

10__ etoposide and [^{3}H]-palmitate (1_Ci/ml) for 0-12 hours. At the indicated time interval, cells were harvested and [^{3}H]-ceramide quantified. Results represent the fold increase in ceramide at each point in comparison to time-matched control.

Figure 6. **Sphingomyelinase inhibition using GW 554869a:** Molt-4 cells transfected with Bcl-2 (scored bars) or control vector (solid bars) were treated with 10__ etoposide for 12 hours. Concurrent with the addition of etoposide, GW 554869a using different concentrations was added. At hour 12, the cells were harvested and ceramide mass was quantified using DGK assay. The results were normalized to lipid phosphate.

Figure 7. **Effect of caspase inhibition on etoposide-induced ceramide:** Molt-4 cells transfected with control vector were treated in a dose-dependent manner with the pancaspase inhibitor N-benzyloxycarbonyl-val-ala-asp-fluromethylketone (z-VAD) for 30 minutes. Then, 10__ etoposide was added and cells incubated for 12 hours before harvesting. Mass ceramide was quantified using DGK assay and normalized to lipid phosphate.

Figure 8. **Effect of caspase inhibition on Etoposide-induced de novo ceramide:** Molt-4 cells transfected with control vector were treated in a dose dependant manner with z-VAD for 30 minutes. Then, 10__ etoposide and [^{3}H]-palmitate (1_Ci/ml) were added simultaneously for 12 hours. At the end of 12 hours cells were harvested and [^{3}H]-ceramide quantified.

Figure 9. **Effect of Caspase inhibition on eoposide-induced ceramide in Bcl-2 cells:** Molt-4 cells transfected with Bcl-2 oncogene were treated in a dose-dependent manner with z-VAD for 30 minutes. Then, 10__ etoposide was added and cells incubated for 12 hours before harvesting. Mass ceramide was quantified using DGK assay and normalized to lipid phosphate.

Figure 10. **Time course for the induction of de novo Synthesis by etoposide:** Molt-4 cells were treated for 0-6 hours with 10 μM etoposide in the presence (solid square) or absence (open square) of 50 μM fumonisin.

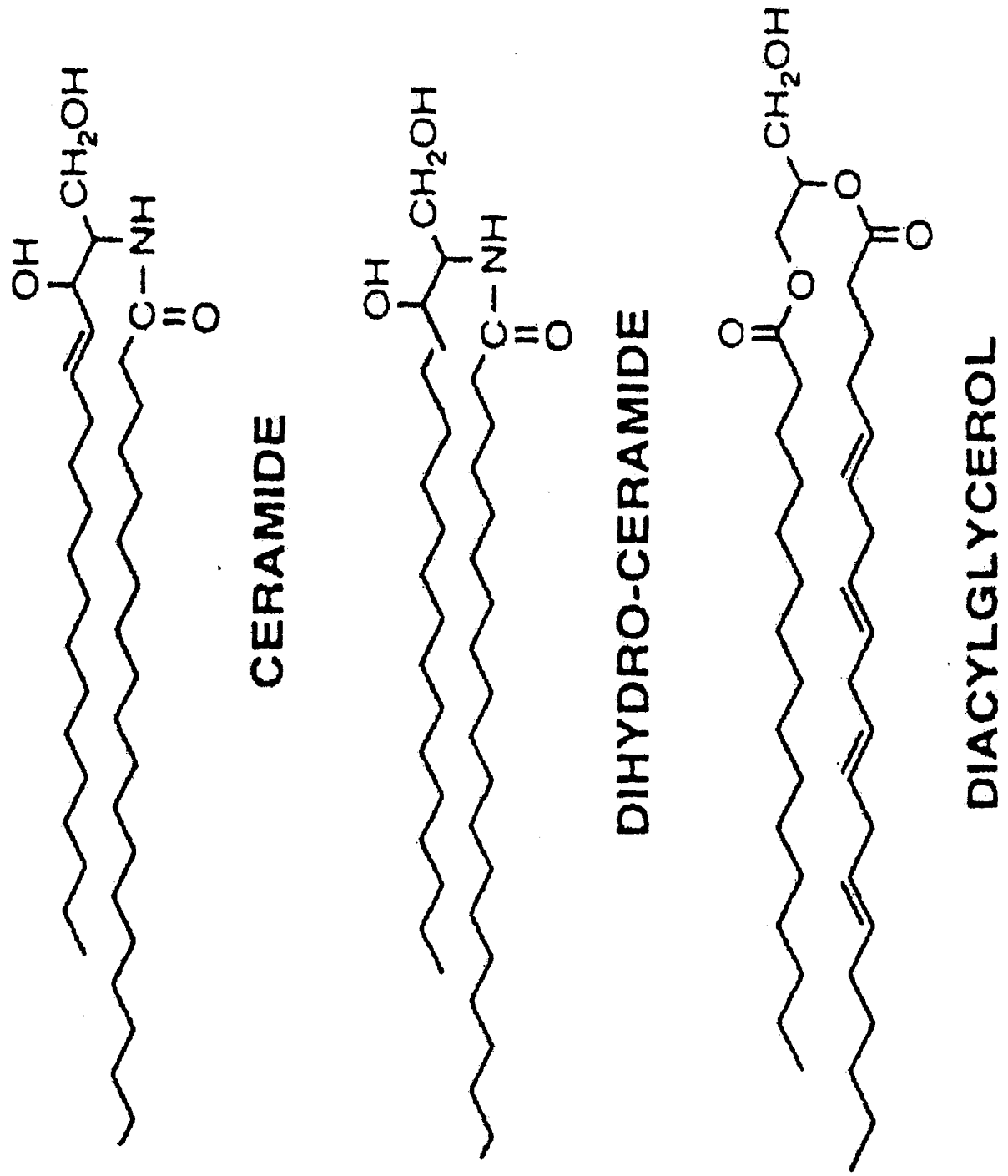


Figure (1)

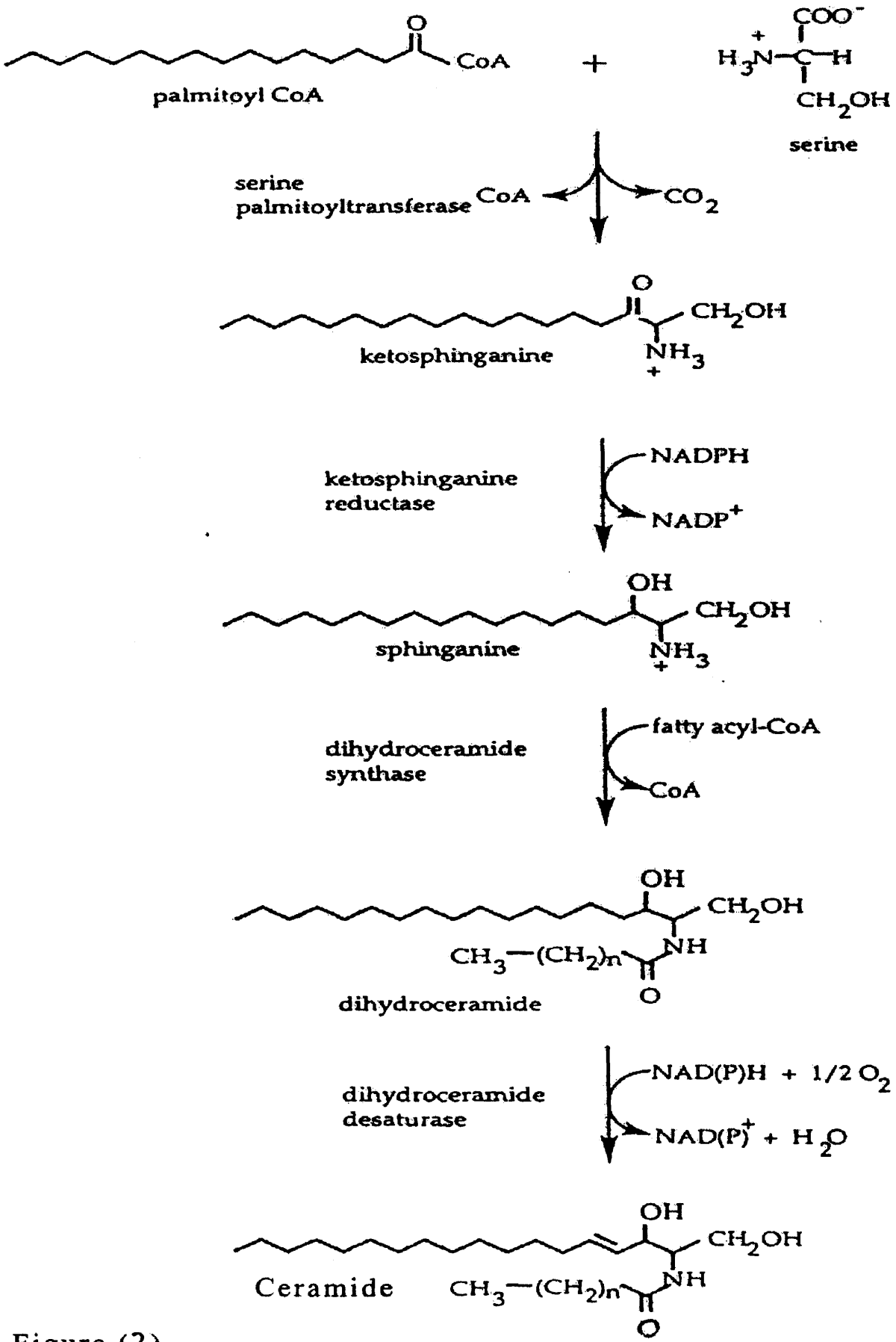
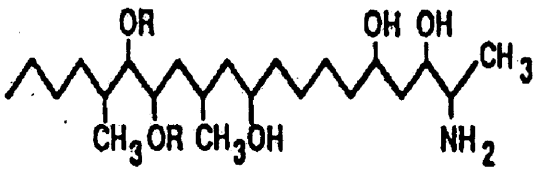


Figure (2)

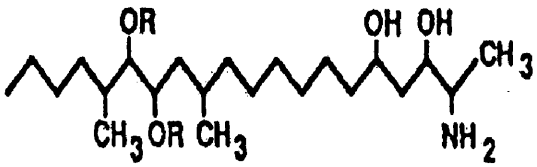
From J. Biol . CHem.,2000

Sphinganine

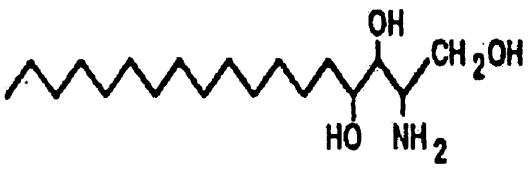


Fumonisin B_1

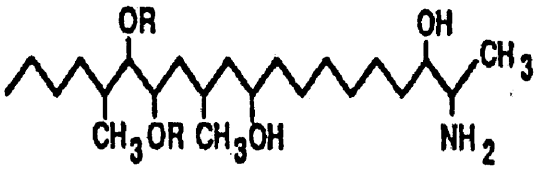
Sphingosine



Fumonisin B_2



4-Hydroxysphinganine

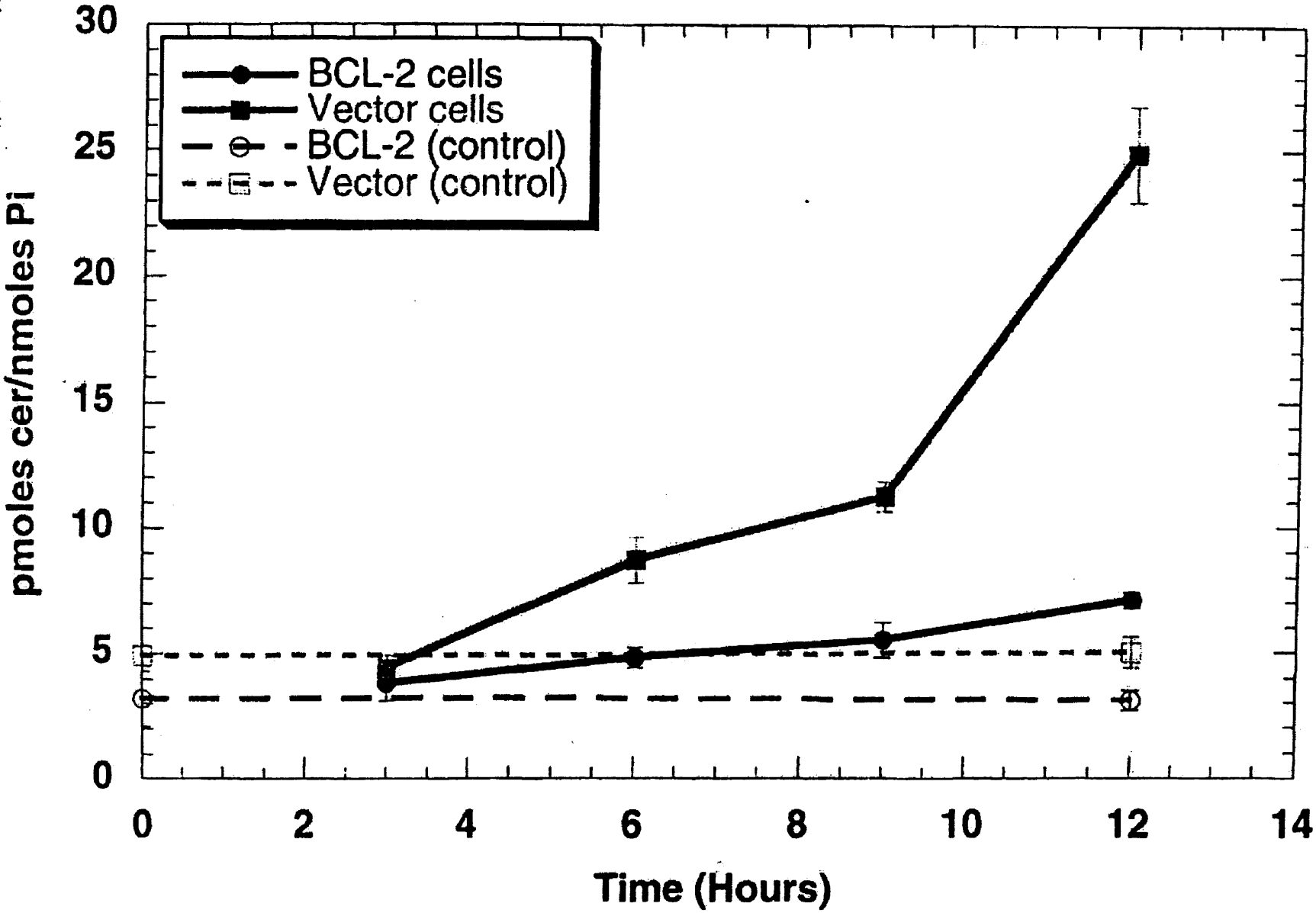


Fumonisin B_3

$R = COCH_2CH(COOH)CH_2COOH$

Figure (3) Advances in Lipid research

Figure (4)


BCL-2 cells
Vector cells
BCL-2 (control)
Vector (control)
pmoles cer/nmoles Pi
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20
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Time (Hours)

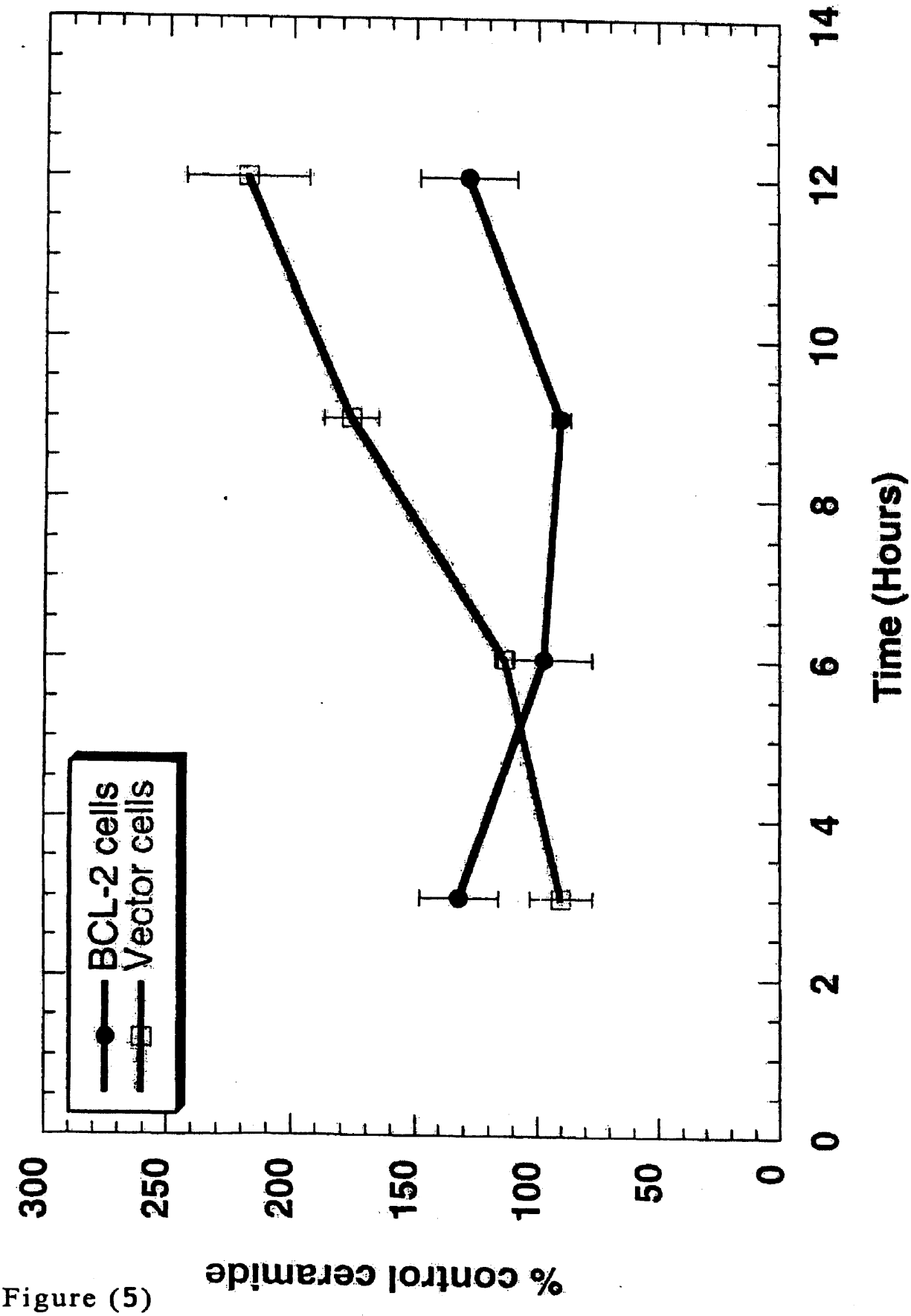
% control ceramide
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
Time (Hours)
BCL-2 cells
Vector cells

Figure (5)

p moles Cer/ n moles Pi
120
100
80
60
40
20
0
BCL-2
VECTOR
Control
Vehicle
1 micro M.
3 micro M.
10 micro M.
20 micro M.
G.W55 4869a

Figure (6)

Pmoles cer/nmole Pi
60
50
40
30
20
10
0
Vector cells
Vehicle only
Etoposide only
1 micro M.
3 micro M.
10 micro M.
30 micro M.
50 micro M.
100 micro M.
z-VAD doses

Figure (7)

Ceramide DPM
4 10^4
3.5 10^4
3 10^4
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
Vector cells
Vehicle only
Etoposide only
10 micro M.
50 micro M.
100 micro M.
z-VAD doses

Figure(8)

Pmoles cer/nmoles Pi
60
50
40
30
20
10
0
Bcl-2 cells
Vehicle only
Etoposide only
10 micro M.
50 micro M.
100 micro M.
z-VAD doses

Figure (9)

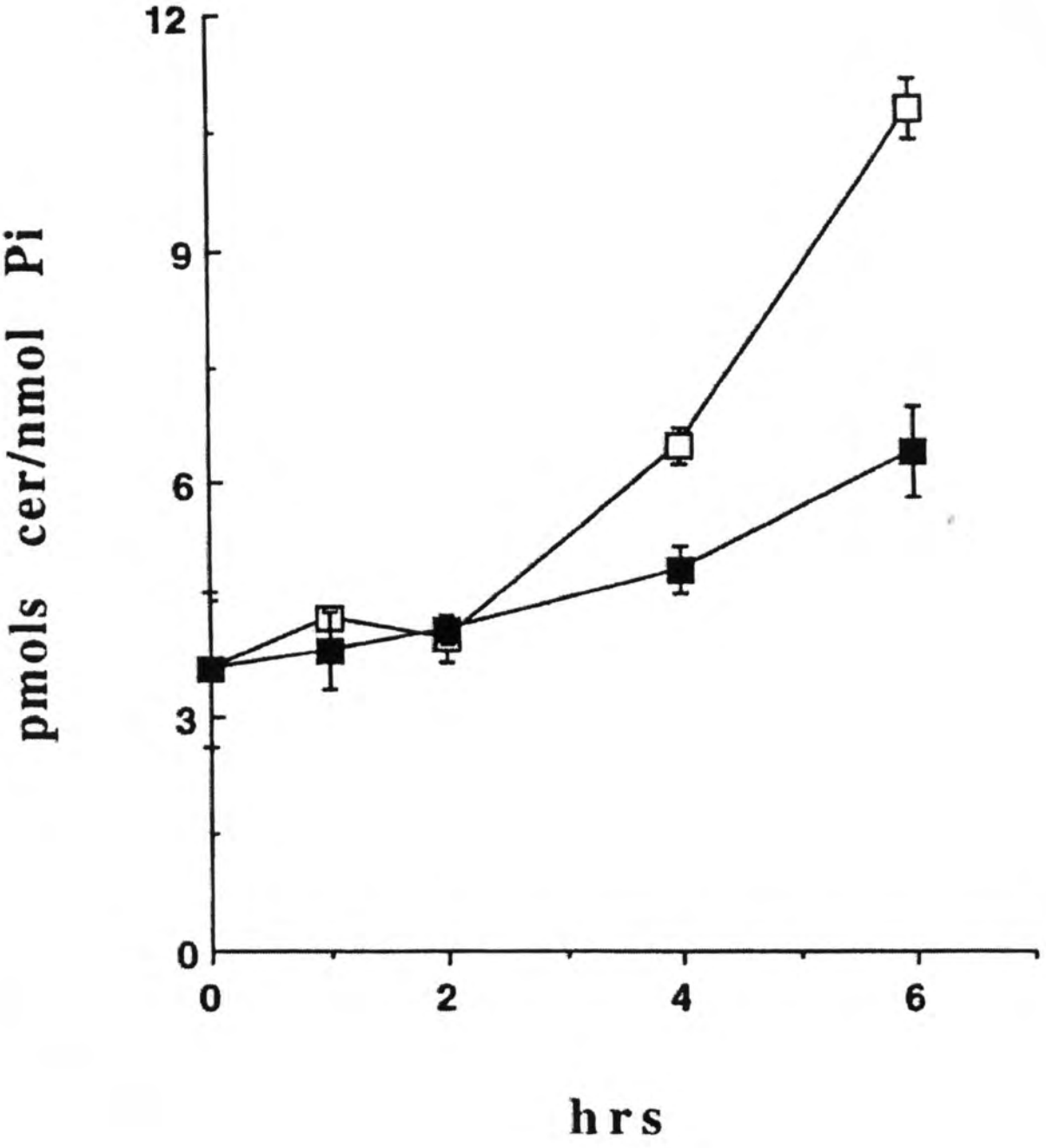

Figure (10)