Elevated ceramide is downstream of altered calcium homeostasis in low serum-induced apoptosis

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Jayadev, Supriya, J. Carl Barrett, and Elizabeth Murphy. Elevated ceramide is downstream of altered calcium homeostasis in low serum-induced apoptosis. Am J Physiol Cell Physiol 279: C1640–C1647, 2000.—Two immortalized cell lines, sup (+) and sup (−), derived from mutagenized Syrian hamster embryo cells, were used to study the relationship and temporal order between calcium and ceramide signals during apoptosis. The early preneoplastic cells, termed sup (+), suppress tumorigenicity when hybridized with tumor cells, whereas later-stage sup (−) cells do not. In reduced serum conditions, sup (+) cells cease proliferating and undergo apoptosis; in contrast, sup (−) cells continue slow growth and undergo necrosis. In sup (+) cells, decreased endoplasmic reticulum (ER) calcium occurs 4 h after low serum treatment and precedes apoptosis. Significant elevations in ceramide are observed 16 h after reduced serum treatment of sup (+) cells but are not found in sup (−) cells. Inhibiting ER calcium depletion in low serum-treated sup (+) cells by treating with high levels of calcium prevents both ceramide generation and apoptosis. Conversely, inducing ER calcium depletion in sup (−) cells by treating with low serum plus thapsigargin results in elevated ceramide levels and apoptosis. Furthermore, C2-ceramide treatment induced apoptosis of sup (−) cells in low serum, a condition that does not normally cause apoptosis. C6-ceramide treatment did not induce apoptosis in either sup (+) or sup (−) cells in 10% serum but did cause G2/M arrest. These studies show that ceramide production is downstream of ER calcium release.

A ROLE FOR CERAMIDE in apoptosis was first defined in U-937 cells where it was shown that tumor necrosis factor-α produced an early elevation in ceramide before apoptosis (23). Obeid and coworkers (23) found that exogenous ceramide treatment could induce apoptosis independent of other apoptotic stimuli. The effect of ceramide was found to be specific in that a closely related lipid molecule (dihydroceramide) and other lipid second messengers [diacylglycerol (DAG) and sphingosine] were unable to induce apoptosis. Since these initial studies, other inducers of apoptosis have also been associated with ceramide production, including Fas (5, 37), ultraviolet (40) and ionizing radiation (6), heat shock (43), oxidative stress (9), and nutrient reduction (11).

Two different kinetics of ceramide elevation have been observed in response to apoptotic stimulation of cells. Several studies have suggested that ceramide elevation occurs early in the apoptotic process, with the peak response evident within 5–30 min (10, 14, 19). Other studies, using different cell types and stimuli, have shown ceramide elevations occurring many hours after treatment, paralleling late events of apoptosis (11, 37). In fact, in another model of serum restriction-induced apoptosis, the MOLT-4 cell system, ceramide elevations were found to occur 24–96 h after treatment, concurrent with apoptosis (11).

Recent studies with a number of cell lines also implicate early changes in endoplasmic reticulum (ER) calcium with the induction of apoptosis. Baffy et al. (2) first reported that induction of apoptosis after interleukin (IL)-3 withdrawal from an IL-3-dependent cell line was associated with loss of calcium from ER stores. Other groups, including our own, have since confirmed that perturbation of ER-compartmentalized calcium is important for induction of apoptosis (3, 16, 26, 28).

To investigate mechanisms involved in apoptosis, we used two immortalized lines derived after mutagenesis of Syrian hamster embryo (SHE) cells, sup (+) and sup (−) cells. The early preneoplastic cells, termed sup (+), suppress tumorigenicity when hybridized with tumor cells, whereas the later-stage sup (−) cells have lost the ability to suppress tumors. In reduced serum conditions, sup (+) cells cease proliferating and undergo apoptosis, whereas sup (−) cells continue slow growth and undergo necrosis. This is consistent with previous studies that have illustrated that early neoplastic cells often show an increased susceptibility to apoptosis that is lost in later-stage preneoplastic cells (26). We were interested in investigating the changes that occur during neoplastic progression that render the cells less susceptible to apoptosis. We have previously demonstrated differences in calcium homeostasis in sup (+) vs. sup (−) cells. When treated with low serum, the sup (+) lineage responds with an early decrease in ER calcium followed by apoptosis; whereas, the sup (−)
lineage retains normal ER calcium levels and does not undergo apoptosis (26). If sup (+) or sup (−) cultures are treated with pharmacological agents that reduce ER calcium, both lineages undergo apoptosis. Conversely, in the sup (+) lineage, if depletion of ER calcium stores is prevented, then low serum-induced apoptosis is also prevented. These studies imply that a decrease in ER calcium is an important step needed to transduce the low serum-induced apoptotic response.

Recent studies have suggested an interaction between calcium signals and ceramide signals (38, 44). Prompted by these findings, we investigated whether ceramide elevations occur during low serum-induced apoptosis of sup (+) cells. We report here that ceramide elevations occur after 16–24 h of low serum treatment and follow the early calcium changes. If we pharmacologically decrease ER calcium, ceramide is elevated. Thus modulation of ER calcium levels leads to downstream effects on ceramide levels. Furthermore, we find that, although addition of C6-ceramide can induce apoptosis in 10% serum, it is not sufficient to induce apoptosis in 10% serum. Thus, in the absence of other stressors, ceramide appears to signal cell cycle arrest rather than death.

MATERIALS AND METHODS

Materials. RNase A was obtained from 5Prime-3Prime, and C6-ceramide and C6-dihydoceramide were purchased from Biomol Research Laboratories. All other chemicals were acquired from Sigma/Aldrich. Institute for Biological Research (IBR) medium was obtained from Life Technologies, and FCS was obtained from Intergen.

Cell lines and cell culture. Two SHE-derived lineages, originally immortalized via asbestos mutagenesis, were used in these studies (15, 27). The sup (+) lineage represents an early stage of tumorigenesis that has lost a senescence gene(s) but retains tumor suppressor capability. The sup (−) lineage represents a later stage of tumor progression that has lost both senescence and tumor suppressor genes. During normal passage, cells were maintained in Dulbecco’s modified IBR medium containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a 37°C incubator with 10% CO2-90% air. Cell lines were routinely tested and found negative for mycoplasma.

DNA fragmentation analysis. Cells were seeded in 100-mm plates at a density of 5 × 105 cells/plate and were grown for 24 h. Adherent cells were washed with calcium- and magnesium-free PBS (CMF-PBS), and 10 ml of the appropriate treatment medium were added. After 24 h, cells were harvested by scraping into treatment media and pelleted. The cell pellets were washed with CMF-PBS and pelleted and lysed in 50–100 μl of lysis buffer (10 mM EDTA, 50 mM Tris, pH 8, 0.5% sodium lauryl sarcosine, and 0.5 mg/ml proteinase K). Lysates were incubated at 50°C for 2 h, RNase was added, and the lysates were incubated at 37°C for a further 30 min. DNA was extracted and analyzed for fragmentation as described previously (12, 26).

Lipid quantification. Cells were seeded in 100-mm plates at a density of 5 × 105 cells/plate and were grown for 24 h. Adherent cells were washed with CMF-PBS, and 10 ml of the appropriate treatment media were added. After 4–24 h (see legends for Figs. 1–8), cells were harvested by scraping into treatment media, pelleted, and then washed with CMF-PBS. Cell pellets were resuspended in 3 ml of chloroform-methanol (1:2), and lipids were extracted via the method of Bligh and Dyer (4). Lipids, dried under nitrogen, were resuspended in 100 μl of chloroform, 20 μl duplicates were used for phosphate measurements (1), and 20-μl duplicates were used in the DAG kinase (DGK) assay (25, 39). Phosphorylated lipids were spotted on TLC plates, and plates were developed in chloroform-acetone-methanol-acetic acid-water (10:4:3:2:1). The ceramide-phosphate and phosphatidic acid spots were scraped into scintillation fluid and counted on a Packard Minaxiβ liquid scintillation counter. Ceramide-phosphate (representative of ceramide levels) and phosphatidic acid (representative of DAG levels) were quantitated using external ceramide and DAG standards, accordingly. Both were normalized against measured phosphate of the same samples.

Some recent studies have questioned the validity of the DGK assay for measurement of ceramide (8, 13, 41, 42). In a recent response to these concerns, Perry and Hannun (24) pointed out that the DGK assay is a sound method for measuring ceramide if done under proper conditions. Specifically, the accurate use of the DGK assay requires that the enzyme (DGK) be used in vast excess. The commercially available, lyophilized DGK enzyme is too expensive to use in excess, and this enzyme is sensitive to extraneous lipids that can be present in the cellular lipid extract. The work presented in the current study used DGK generated from a membrane preparation of bacteria that overexpressed the enzyme. With the use of the recombinant enzyme, it is possible to get >90% conversion of ceramide to ceramide-phosphate. The DGK assay when used under these conditions is valid because 1) it provides quantification of total ceramide mass, and 2) it allows for processing of numerous samples simultaneously. This method has been extensively validated in previous studies (24).

Cell cycle analysis. Cells were seeded in 100-mm plates at a density of 5 × 105 cells/plate and were grown for 24 h. Adherent cells were washed with CMF-PBS, and 10 ml of the appropriate treatment media were added. After 16 h, cells were harvested by scraping into treatment media and analyzed as previously described (11). Briefly, cell pellets were completely resuspended in 1 ml of CMF-PBS, 4 ml absolute ethanol was added to fix cells, and suspensions were stored at −20°C. On the day of analysis, the fixation buffer was removed, the cells were resuspended in 1 ml CMF-PBS, RNA was removed using 2 mg/ml DNase-free RNase, and cells were stained with 10 mg/ml propidium iodide for 30 min. Cells were analyzed using a Becton-Dickinson FACSort flow cytometer. Flow cytometric analyses were performed using an argon laser at 488 nm, and apoptotic cells were evident as shrunken cells containing <2 N DNA.

Statistical analyses. In comparing two groups, statistical significance was determined by Student’s t-test. For multiparameter comparisons, statistical significance was determined by ANOVA, adjusting for multiple comparisons using Fisher’s test for significance. A value of P < 0.05 was considered to be significant.

RESULTS

Changes in ceramide are not observed after 4 h of low serum treatment. Previous studies using sup (+) and sup (−) cells have shown that low serum treatment results in different end points in the two lineages (27). Previous studies also have documented the time course and mode of death of the sup (+) cells in low serum (27). In 10% serum, both sup (+) and sup (−) cells proliferate, and cell number more than doubles within...
In low serum conditions, sup (+) cells cease proliferating and undergo apoptosis (27). By 24 h, the effects of low serum on sup (+) cells are evident as a decrease in cell number (Fig. 1). In contrast, the sup (−) line responds to low serum by decreasing growth rate and death via necrosis. Thus, after 24 h of low serum treatment, sup (−) cell numbers remain similar to the baseline (time 0) numbers (Fig. 1).

Previous studies also showed a correlation between an early decrease in ER calcium stores and the ability to undergo apoptosis (12, 26). Thus sup (+) cells treated with low serum for 4 h show ~50% less ER calcium compared with control sup (+) cells in 10% serum (26). At this time point, cell number was not affected (Fig. 1), suggesting that calcium changes precede terminal events of apoptosis. No change in ER calcium was observed in low serum-treated sup (−) cells. However, pharmacological reduction of ER calcium stores, independent of serum reduction, was sufficient to induce apoptosis of both the sup (+) and sup (−) lineages. Conversely, prevention of store reduction was able to prevent apoptosis of the sup (+) population in response to low serum treatment (12, 26).

Recent literature has suggested a link between ceramide and calcium signals (38, 44). To determine the relationship between ER calcium and ceramide during low serum-induced apoptosis, we examined whether ceramide elevations could be observed at a time when we observe decreased ER calcium in sup (+) cells. As shown in Fig. 2, ceramide levels were measured at 4 h in sup (+) and sup (−) cells after treatment with 10 or 0.2% (low) serum conditions. No significant difference was observed in either the sup (+) or the sup (−) populations after treatment with low serum. Thus at a time when calcium changes were already apparent in cells destined to undergo apoptosis [in sup (+) cells treated with low serum], a parallel increase in ceramide was not apparent. This suggests that ceramide is probably not responsible for the reduction in ER calcium that occurs at 4 h after low serum treatment of sup (+) cells (26).

Ceramide elevations occur after protracted low serum treatment. Depending on the system, ceramide generation has been found to occur with two different kinetics; some agents produce ceramide elevation within seconds to minutes (14, 32), and others produce an elevation in ceramide only after an extended time, i.e., 24–96 h (11, 37). We therefore examined whether changes in ceramide occur downstream of calcium changes. We find that ceramide elevation occurs late in apoptosis in hamster cells. In contrast to the data at 4 h, 24 h of low serum treatment resulted in an ~70% elevation in ceramide in sup (+) cells compared with control, 10% serum-treated cells (Fig. 3). This elevation in ceramide was not evident in the sup (−) population, which does not undergo apoptosis after 24 h of low serum treatment. These findings are consistent with the hypothesis that ceramide elevation occurs exclusively in cells bound for apoptosis. Furthermore, the late kinetics of ceramide elevation places ceramide downstream of ER calcium changes during the apoptotic process.

ER calcium changes are upstream of ceramide elevations. An elevation in ceramide at 24 h is consistent with a role for ceramide downstream of decreased ER calcium in the apoptotic response. To substantiate that ceramide is indeed part of the signaling cascade leading from ER calcium store depletion to apoptosis, it is necessary to show a causal relationship between depletion of ER calcium stores and elevation of ceramide. To assess this, we used thapsigargin to pharmacologically deplete ER calcium stores. Our previous studies showed that thapsigargin, within 48 h, induced apop-

![Fig. 1. Cell number as a function of time in 10 and 0.2% serum expressed as relative cell number. Data are means (n = 3 measurements) ± SE. A time course of cell number at later times has been published previously (27).](http://ajpcell.physiology.org/)

![Fig. 2. Low serum does not cause an early elevation in ceramide. Cells were treated for 4 h with 10 or 0.2% (low) serum as indicated. After treatment, cells were harvested, and lipids were extracted as described in MATERIALS AND METHODS. No elevation in ceramide was observed in the apoptosis-destined population: the sup (+) cells treated with low serum. Data are presented as percent control; values in bars are means of 7 separate measurements; bars represent means ± SE.](http://ajpcell.physiology.org/)
Fig. 3. Ceramide elevation is observed late in apoptotic cells. Cells treated for 24 h with 10 or 0.2% serum were harvested, and lipids were extracted as described in MATERIALS AND METHODS. A significant (P < 0.006) elevation in ceramide levels is observed in sup (+) cells induced to undergo apoptosis, e.g., after low serum treatment. No increase in ceramide is apparent in the nonapoptosing sup (−) cells in low serum. Values in bars are means of 10–11 separate measurements; bars represent means ± SE.

Fig. 4. Endoplasmic reticulum (ER) calcium changes can modulate ceramide elevations. Cells were treated for 16 h in 10% or low (0.2%) serum conditions with thapsigargin or ± 3 mM calcium. Treatments that caused a decrease in ER calcium (0.2% and 10% + thapsigargin for sup (+); 0.2% + thapsigargin for sup (−)) resulted in an elevation of ceramide. Conversely, exogenous calcium treatment (which prevents apoptosis) prevented ceramide elevation in the low serum-treated sup (+) population. Data are presented as pmol of ceramide normalized to total lipid phosphate. Values in bars are means of 4 separate measurements; bars represent means ± SE. *Significantly different (P < 0.005) from sup (+), 10%; †significantly different (P < 0.0005) from sup (+), 0.2%, but not different from sup (+), 10%; ¥significantly different (P < 0.0005) from sup (−), 10% and sup (−), 0.2%.

Fig. 5. Ceramide induces apoptosis in sup (−) cells. Sup (−) cells were treated with 0.2% (low) serum ± 10, 20, or 50 μM C6-ceramide for 24 h. After treatment, cells were harvested, and DNA was extracted and analyzed as described in MATERIALS AND METHODS. Low serum alone is not sufficient to induce apoptosis of sup (−) cells; however, cotreatment with 10–50 μM ceramide could induce apoptosis of these cells. Lane 1, sup (−), 0.2%; lane 2, sup (−), 0.2% + 10 μM C6-ceramide; lane 3, sup (−), 0.2% + 20 μM C6-ceramide; lane 4, sup (−), 0.2% + 50 μM C6-ceramide.
trations (10–50 μM) that induced apoptosis of sup (+) cells in low serum was unable to induce apoptosis of sup (+) cells in 10% serum conditions. Similar findings were also observed in sup (−) cells in 10% serum conditions (see Table 1). Because high serum conditions could potentially bind up treatment lipid, the question of treatment effectiveness arose in the 10% serum studies. Although 10–50 μM C6-ceramide appeared to have no effect on apoptosis, it did in fact affect cell number. As shown in Fig. 7A, 10–50 μM C6-ceramide, concentrations that did not induce apoptosis, significantly decreased the proliferative capacity of cells in 10% serum conditions. This antiproliferative effect appears specific because the closely related lipid C6-dihydroceramide had little effect on cell number at even the highest concentrations (Fig. 7B). These results suggest that ceramide does indeed have an effect in 10% serum conditions; however, C6-ceramide treatment alone seems unable to induce apoptosis independent of other stressor(s). In contrast to C6-ceramide treatment, thapsigargin treatment (which depletes ER calcium) was able to induce apoptosis of sup (+) and sup (−) cells in 10% serum conditions (26). Thus it appears that depletion of ER calcium is sufficient to induce apoptosis; however, addition of C6-ceramide is not sufficient to signal apoptosis. These findings lend further support to the hypothesis that ceramide is downstream of calcium in the apoptotic process.

C6-ceramide induces cell cycle arrest in 10% serum. Cell cycle analysis showed that ceramide treatment in the presence of 10% serum led to a pronounced arrest of cells in the G2/M phase of the cell cycle (Table 1). While control cultures had ~17–19% of the population in G2/M, C6-ceramide-treated cells showed as many as 28–33% of cells in G2/M. The G2/M arrest is apparent in both the sup (−) and sup (+) cell lineages and is specific to C6-ceramide. Thus the closely related lipid dihydroceramide, which differs from ceramide only by the lack of a double bond between carbons-4 and -5 of the sphingoid backbone, had little effect on cell number or on cell cycle.

One possible explanation for the difference in the effects of ceramide in 10% vs. low serum conditions is the level of DAG present in cells. A previous study using the MOLT-4 cell line showed that the effect of ceramide can depend on the amount of DAG present in the cell (11). If ceramide elevation occurred when DAG levels were high, then cells underwent cell cycle arrest (in the case of the MOLT-4 cells, G0/G1 arrest). However, if DAG levels were not elevated when ceramide was added to cells, then cells underwent apoptosis. Based on these observations, we looked at the DAG levels in sup (+) and sup (−) cells in 10% vs. low serum conditions (Fig. 8). If the hamster cells exhibited the same characteristics as the MOLT-4 system, then we would expect that DAG levels would be high only under

Fig. 6. Ceramide is not sufficient to induce apoptosis of sup (+) cells in 10% serum. Sup (+) cells were treated with 10 or 0.2% (low) serum and 10, 20, or 50 μM C6-ceramide for 24 h. After treatment, cells were harvested, and DNA was extracted and analyzed as described in MATERIALS AND METHODS. Ceramide at all concentrations was unable to induce apoptosis in 10% serum conditions. Ceramide did not affect apoptosis induced by low serum treatment. Lane 1, sup (+), 10%; lane 2, sup (+), 10% + 10 μM C6-ceramide; lane 3, sup (+), 10% + 20 μM C6-ceramide; lane 4, sup (+), 10% + 50 μM C6-ceramide; lane 5, sup (+), 0.2%; lane 6, sup (+), 0.2% + 50 μM C6-ceramide.

Fig. 7. Ceramide inhibits proliferation of cells in 10% serum. Cells were treated with 10% serum ± 10, 20, or 50 μM C6-ceramide or with 50 μM C6-dihydroceramide for 24 h, and cells were counted. A: both sup (+) and sup (−) cells responded to ceramide treatment by a decrease in cell number compared with the time-matched control. B: ceramide’s effect was selective, because the closely related lipid, dihydroceramide, had little effect on cell number. [Ceramide], ceramide concentration.
CERAMIDE, CALCIUM, AND APOPTOSIS

Table 1. Ceramide treatment in 10% serum causes cell cycle arrest

<table>
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<th>G2/M</th>
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<td>sup (+)</td>
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<td>66.5</td>
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<td>C6-dihydroceramide (50 μM)</td>
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<td>63.9</td>
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<td></td>
<td>C6-ceramide (50 μM)</td>
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<td>56.0</td>
<td>10.5</td>
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</table>

Data are expressed as the percentage of cells in apoptosis or stage of the cell cycle. Cells were treated as indicated. After treatment, cells were harvested, and cell cycle analysis was done as described in MATERIALS AND METHODS. Ceramide treatment in 10% serum conditions does not induce significant levels of apoptosis and at higher concentrations (50 μM) may in fact decrease basal levels of apoptosis. Ceramide does induce G2/M arrest of cells as marked by a significant shift of cells out of the S phase and into the 2 N population.

conditions where C6-ceramide induces cell cycle arrest, e.g., in 10% serum. In fact, this was not the case. DAG levels were lower in the sup (-) cells compared with the sup (+) population both in 10% and in low serum conditions. However, 10% serum-treated cells did not exhibit elevated DAG levels relative to low serum-treated cells. These data suggest that, at least in the hamster cells, apoptosis vs. cell cycle arrest is not solely determined by the ratio of ceramide to DAG. Thus another signal must impinge on the ceramide signal to determine whether C6-ceramide leads to an apoptotic end point or a cell cycle arrest end point.

DISCUSSION

The current study begins to define a relationship between calcium and ceramide in apoptotic signaling. Several groups have reported that an early, sustained increase in cytoplasmic free calcium ([Ca2+]i) is a necessary precedent to cell death (20, 34). Others have reported that a decrease (2, 26, 31) or no detectable change in cytoplasmic calcium (30) correlates with apoptosis. We have shown previously that low serum-induced apoptosis in sup (+) cells is not accompanied by a measurable increase in [Ca2+]i (26). Because of the long time course needed for initiation of apoptosis, it is difficult to rule out a transient or localized change in [Ca2+]i, that could be missed. [Ca2+]i is only measured at discrete times because the calcium indicator used to measure [Ca2+]i, (fura 2) leaks out of these cells in ~1 h. In contrast to the inconsistent reports regarding the role of cytoplasmic calcium in apoptosis, a number of studies suggest that changes in ER calcium are important in the regulation of apoptosis (2, 16, 26). We have shown previously (12, 26) that a decrease in ER calcium occurs before apoptosis of sup (+) cells. Furthermore, we found that if we block the decrease in ER calcium by raising extracellular calcium, we block apoptosis. However, because released ER calcium must, at least transiently, enter the cytosol, it is difficult to distinguish whether it is the decrease in ER calcium per se or the transient or localized increase in cytoplasmic calcium that is important for signaling apoptosis.

However, because of the reproducibility of the decrease in ER calcium and because we can block the decrease in ER calcium, and consequently apoptosis, by raising extracellular calcium, we use altered ER calcium as a marker of altered calcium homeostasis.

Although a number of studies have shown that sphingosine and other mitogenic lysosphingolipids can modulate calcium homeostasis, only a few studies have shown an interconnection between ceramide and intracellular calcium. These studies postulate a role for ceramide upstream of calcium changes (17, 21). Lepple-Wienhues et al. (17) have reported that addition of C2-ceramide or C6-ceramide to T lymphocytes blocks store-operated calcium entry currents measured by patch clamp. Based on these studies, we initially hypothesized that an increase in ceramide might be responsible for the altered calcium homeostasis that occurs during apoptosis of sup (+) cells. We have previously shown that the sustained decrease in ER calcium observed before apoptosis was secondary to a decrease in store-operated calcium entry (26). We therefore hypothesized that an increase in ceramide might precede alterations in calcium homeostasis after low serum stimulation of sup (+) cells. However, we found instead that an increase in ceramide occurs well after the observed alterations in calcium. In fact, the current study is unique in suggesting a converse interaction between calcium and ceramide. Based on temporal relationships and pharmacology, we present data showing that changes in calcium homeostasis occur before changes in ceramide during apoptosis of sup (+) cells. Changes in calcium homeostasis, as evidenced by decreased ER calcium, occur after 4 h of low serum

![Fig. 8. Diacylglycerol (DAG) levels do not change with low serum treatment. Cells treated for 16 h with 10 or 0.2% (low) serum were harvested, and lipids were extracted as described in MATERIALS AND METHODS. Although sup (-) cells show less DAG than sup (+) cells, no difference in DAG levels is observed in 10% vs. low serum conditions. Thus differences in DAG cannot account for the differential effectiveness of C6-ceramide in 10% serum vs. low serum. Values in bars are means of 4 separate measurements; bars represent means ± SE. [DAG], DAG concentration.](http://apjcp.cellphysiology.org/10.22032-32-247)
treatment. In contrast, an elevation in ceramide is evident only after 16 h. Temporally therefore, a decrease in ER calcium precedes ceramide elevation during low serum-induced apoptosis of sup (+) cells. Furthermore, pharmacological manipulation of ER calcium levels leads to a parallel modulation of ceramide levels. When ER calcium levels are depleted with thapsigargin treatment, ceramide elevation is observed in sup (+) cells. Conversely, when ER calcium levels are prevented from decreasing [e.g., with high calcium treatment in conjunction with low serum in sup (+) cells], ceramide levels remain at basal levels. Blocking the decrease in ER calcium blocks both the rise in ceramide and apoptosis. However, raising extracellular calcium in sup (+) cells treated with low serum does not block cell death; rather, it shifts cell death from apoptotic to necrotic in conjunction with blocking the rise in ceramide. We also find that sup (-) cells in low serum, a condition that does not result in apoptosis, can be made to undergo apoptosis by the addition of C16-ceramide. It has been suggested that C16-ceramide might act more like a lysophospholipid and have effects not related to the specific effects of ceramide (8). Although one must always be cautious in using pharmacological analogs, numerous studies have shown the validity of using C16-ceramide as an analog of ceramide (see Ref. 24). Furthermore, in this study we show that inactive C16-dihydroceramide did not mimic the effects of C16-ceramide, thereby reducing the probability of nonspecific effects.

The data in this manuscript are consistent with data suggesting that changes in ceramide occur late in apoptosis (33, 35, 36, 41). There are several reports suggesting that activation of initiator caspases is involved in the generation of ceramide. Tepper et al. (36) report that generation of ceramide is blocked by zVAD-fmk, an inhibitor of caspase 9, an initiator caspase, but ceramide generation is not blocked by DEVD-CHO, an inhibitor of the effector caspases. These authors suggest that generation of ceramide is associated with the activation of effector caspases or the propagation of apoptosis. Interestingly, Nakagawa et al. (22) recently reported that ER stress, such as that induced by treatment with thapsigargin, causes activation of caspase 12, a caspase that is also inhibited by zVAD-fmk.

Having ordered the pathway to show that the changes in calcium precede changes in ceramide raises the question of how altered ER calcium might influence ceramide levels. It is intriguing that the enzymes involved in de novo synthesis of ceramide are located at the ER membrane (7, 18). Thus it is possible that the level of regulation derives from the availability of calcium to the bio-synthetic enzymes responsible for ceramide generation.

Another complexity of the ceramide signal arises from the differential effects that occur, depending on the context of the signal. Unlike the decrease in ER calcium, addition of C16-ceramide alone does not result in apoptosis in sup (+) or sup (-) cells in 10% serum. Only when additional stress is present (e.g., in low serum conditions) does C16-ceramide treatment lead to apoptosis. In the absence of an additional stress signal, C16-ceramide treatment results in cell cycle arrest. This result is similar to another model system of serum deprivation-induced apoptosis, the MOLT-4 cell line, in which the ability of ceramide to induce apoptosis rather than cell cycle arrest depends on the level of DAG present in the cell (11). When DAG levels were high, ceramide treatment led to cell cycle arrest (in the case of the MOLT-4 cells, G0/G1 arrest). However, when DAG levels were not elevated, ceramide treatment resulted in apoptosis. It has been suggested (29) that ceramide induces apoptosis by stimulation of a phosphatase that dephosphorylates Bcl-2 and that DAG opposes the action of ceramide by stimulation of protein kinase C-α-dependent phosphorylation of Bcl-2. Thus a high DAG would be expected to oppose ceramide-induced apoptosis. However, we found that the DAG level in 10% serum, conditions where exogenous ceramide treatment led to cell cycle arrest, was not significantly different from that measured in low serum (conditions where ceramide treatment led to apoptosis). Thus, unlike the MOLT-4 system in which the ratio of ceramide to DAG determines apoptosis vs. cell cycle arrest, in the hamster model the additional signal modulating the effect of ceramide remains to be determined. It is possible that the additional stress signal could modify the pathways that metabolize ceramide and thereby alter its effect, as has been suggested in other systems (35).

The data in this study suggest that changes in ceramide occur after alterations in calcium homeostasis and further show that inhibition of altered calcium homeostasis blocks the rise in ceramide and apoptosis. A role for ceramide in apoptosis is supported by the observation that addition of ceramide to sup (-) cells in low serum can trigger apoptosis. The data show that an elevation in ceramide is a late event and is downstream of altered calcium homeostasis in apoptotic signaling.

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