Identification of Arachidonic Acid as a Mediator of Sphingomyelin Hydrolysis in Response to Tumor Necrosis Factor α*

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Supriya Jayadev, Corinne M. Linardic, and Yusuf A. Hannun†
From the Departments of Medicine and Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

A sphingomyelin (SM)-signaling cycle has been described in human leukemia-derived HL-60 cells (Oka- zaki, T., Bell, R. M., and Hannun, Y. A. (1989) J. Biol. Chem. 264, 19076–19080). Activation of the cycle by tumor necrosis factor α (TNFα) occurs rapidly, with peak levels of approximately 30% SM hydrolysis observed within 45–60 min. The mechanisms by which TNFα induces this SM turnover remain largely unexplored. In this study, arachidonate (AA) was investigated as a potential mediator of TNFα effects on SM turnover. In HL-60 cells, 30 nM TNFα stimulated the release of AA within 5–10 min. In turn, AA stimulated SM hydrolysis and concomitant ceramide generation within 20 min of addition to cells. Other fatty acids, notably oleate, mimicked the effects of AA on SM hydrolysis, but the methyl ester and alcohol analogs of fatty acids were inactive. Diacylglycerol, a candidate mediator of TNFα responses, failed to stimulate SM hydrolysis even at concentrations as high as 300 μM. Moreover, in an in vitro assay, AA activated a cytosolic sphingomyelinase dose dependently, with 10–100 μM AA inducing 3–4-fold activation, thus suggesting a direct effect of AA on sphingomyelinase. Melittin, a potent phospholipase A2 activator, induced SM hydrolysis at concentrations as low as 35 nM. However, unlike AA, melittin was unable to stimulate sphingomyelinase activation in an in vitro assay system. Finally, exogenous addition of AA also produced antiproliferative effects reminiscent of ceramide effects. Thus, a role for the phospholipase A2/AA pathway in mediating TNFα induction of the SM cycle is supported by multiple lines of evidence. These studies begin to elucidate a mechanism of TNFα signaling and identify a close relationship between glycerophospholipid and sphingolipid signaling. AA, therefore, may be pivotal to understanding the sphingomyelin-signaling cascade.

Sphingolipids play important roles in cell regulation, in cell contact response, and as markers of cell differentiation and transformation. Recently, a role for sphingolipids in signal transduction has been suggested (1). A sphingomyelin (SM)1 cycle has been described in which activation of a neutral sphingomyelinase (SMase) leads to the breakdown of SM and the generation of phosphocholine and ceramide. Ceramide modulates a number of downstream events (phosphatase activation (2, 3), protein phosphorylation (4, 5), down-regulation of the c-myc protooncogene (6), and apoptosis (7)) and, therefore, may represent a novel lipid second messenger. In human promyelocytic leukemia-derived HL-60 cells, multiple inducers of monocytic cell differentiation (1,25-dihydroxyvitamin D3 (1), γ-interferon (8), and tumor necrosis factor α (TNFα) (8)) have been shown to stimulate this SM cycle.

SM turnover and ceramide generation in response to TNFα occurs within minutes of stimulation; however, the sequence of events linking receptor stimulation and SMase activation remains unknown. In a number of cell systems, interaction of TNFα with its membrane receptors (p75 and p55) has been found to activate phospholipase A2 and to induce release of AA from phosphatidylcholine and phosphatidylethanolamine pools (9–11). In the MC3T3-E1 cell line, AA release and production of lysophospholipids were found to occur within 10 min of TNFα stimulation (12). Thus, temporally, AA could represent an intervening signaling molecule involved in transducing the TNFα signal to SM.

In this study, we investigated AA as a mediator of TNFα-induced SM hydrolysis. We report that TNFα stimulates rapid release of AA in HL-60 cells, and AA release preceded TNFα-stimulated SM hydrolysis. Exogenous addition of AA induced SM hydrolysis and reproduced effects of ceramide on cell growth. In contrast, addition of a cell-permeable diacylglycerol (DAG) had no effect on either SM turnover or cell growth. These studies identify AA as a potential mediator of TNFα-induced SM hydrolysis and argue against a role for DAG in regulation of the SM cycle. The implications of these studies on regulation of the SM cycle are discussed.

EXPERIMENTAL PROCEDURES

Materials

HL-60 cells were purchased from ATCC (Rockville, MD). RPMI 1640 and fetal calf serum were purchased from Life Technologies, Inc. TLC plates were purchased from Fisher. ITS supplement, Streptomyces sp. sphingomyelinase, ceramide, protease inhibitors, melittin, dioctanoylglycerol, and all fatty acids other than arachidonate were purchased from Sigma. Arachidonic acid was purchased from Biomol Research Laboratories, Plymouth Meeting, PA. [3H]Choline chloride and [3H]arachidonic acid were purchased from DuPont NEN. TNFα was a gift from Knoll Pharmaceuticals, Whippany, NJ.

Methods

Cell Culture

HL-60 cells were maintained between passages 20 and 60 at 37 °C in a 5% CO2 incubator. For general maintenance, cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. During metabolic radiolabeling periods, cells were maintained in serum-free RPMI 1640 supplemented with ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite). For treatment, cells were maintained in RPMI 1640 supplemented with ITS and 25 mM HEPES. Time-matched controls were run concurrently.

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† To whom correspondence should be addressed: Duke University Medical Center, Dept. of Medicine, Box 3355, Durham, NC 27710. Tel.: 919-684-1444; Fax: 919-681-2653.
1 The abbreviations used are: SM, sphingomyelin; AA, arachidonic acid; DAG, diacylglycerol; diC4, dioctanoylglycerol; ETI, eicosatrynoic acid; PC, phosphatidylcholine; TNFα, tumor necrosis factor α; SMase, sphingomyelinase; PBS, phosphate-buffered saline; ITS, insulin, transferrin, sodium selenite.
Sphingomyelin Quantitation

HL-60 cells grown to a density of 0.5–0.8 × 10⁶ cells/ml were washed with PBS and resuspended at 0.2 × 10⁶ cells/ml in ITS-supplemented media. These cells were incubated for 72 h in the presence of 0.6 µC/ml (80 C/mmol) [3H]choline chloride. Post-labeling, cells were washed with PBS, resuspended at 0.5 × 10⁶ cells/ml in HEPES and ITS-supplemented media, and rested for 2–4 h. Cells were then treated as indicated and harvested. Cell pellets were resuspended in 3 ml of chloroform/methanol (1:2), and a standard Bligh and Dyer extraction was used to recover lipids (13). Lipids dried under nitrogen were resuspended in 80 µl of chloroform, 20-µl duplicates were set aside for phosphate determinations (14), and 20 µl was used for SM measurement by one of two methods as follows.

**TLC Method**—Lipid was spotted on TLC plates, and plates were developed in chloroform/methanol/acetic acid/water (50:30:8:3). Plates were sprayed with En³Hance (DuPont NEN) and exposed to film for 48–72 h. The SM and phosphatidylcholine (PC) spots were scraped into scintillation fluid and counted in a scintillation counter (LKB Wallac, Turku, Finland). To account for variability between samples, SM was normalized by dividing SM counts/min by either total lipid phosphate nanomoles or PC counts/min.

**Bacterial SMase Method**—As a means of making SM quantitation more efficient, we sought to develop an assay that could assess SM levels with greater ease and rapidity. The previously employed methodology relies upon TLC separation of labeled SM from labeled PC. Since the lipid pools were labeled with tritium, it took a protracted exposure to film before lipid spots could be visualized, scraped, and counted. Furthermore, when poor TLC separation resulted, scraping could lead to large errors in SM quantification. In order to bypass these problems, the new assay relies on complete cleavage of recovered cellular SM by bacterial sphingomyelinase to release labeled phosphorylcholine. The liberated [3H]phosphorylcholine is then recovered with the aqueous phase of a Folch extraction (15); thus, quantitation of the label in the aqueous phase yields a measure of the SM levels. The methodology was as follows. Cellular lipid was dried and resuspended in 100 µl of assay buffer to yield a final concentration of 100 µM Tris-HCl, pH 7.4, 6 mM MgCl₂, 0.1% Triton X-100, 1–10 nmol of phospholipid, and 1 unit/ml Streptomyces sp. sphingomyelinase. Reaction mixtures were incubated for 2 h at 37°C. Reactions were stopped by addition of 1.5 ml of chloroform/methanol (2:1). The Folch extraction was completed by addition of 200 µl of water. SM was quantitated by drying and counting the upper, aqueous phase, and PC was quantitated by drying and counting the lower, organic phase. SM was normalized using phosphate measurements and using PC measurements. Blank reactions contained no SMase, and assays were linear with respect to time and lipid.

Optimization studies illustrated that the above conditions yielded maximal SM hydrolysis (100%) without accompanying PC hydrolysis (<5%) (Fig. 1). Furthermore, a comparison of the TLC and enzymatic methods demonstrated that the enzymatic method was comparable with the TLC method of SM quantitation (see inset of Fig. 4A).

Ceramide Quantitation

HL-60 cells grown to a density of 0.5–0.8 × 10⁶ cells/ml were washed with PBS and resuspended at 0.2 × 10⁶ cells/ml in ITS-supplemented media. Cells were grown for 48 h in serum-free media and then washed with PBS, seeded at 0.5 × 10⁶ cells/ml in HEPES and ITS-supplemented media, and rested for 2–4 h. Cells were then treated as indicated and harvested, and the lipids were extracted. Lipids, dried under nitrogen, were resuspended in 120 µl of chloroform, 20-µl duplicates were set aside for phosphate measurements, and 60 µl was used in the diacylglycerol kinase assay (16, 17). The final reaction mixture of 100 µl contained 72 µM imidazole, 12.5 mM MgCl₂, 2 mM diithiothreitol, 1 mM EDTA, 0.4 mM diethylamino or pentaacetic acid, 5 mM dioleoylphosphatidylglycerol, 50 µM β-ocytolglucose, 50 µM LiCl, 50 µg/ml DAG kinase, and 1 mM ATP (4.5 µCi). Phosphorylated lipids were extracted and spotted on TLC plates. Plates were developed in chloroform/acetone/methanol/acetic acid/water (10:3:3:1:1). The ceramide-phosphate spots were scraped into scintillation fluid and counted. Ceramide-phosphate was normalized using phosphate measurements and quantitated using external ceramide standards.

**Sphingomyelinase Isolation**

Inducer was preincubated with approximately 100 µg of cytosolic protein for 10 min at 37°C. [3H]Labeled sphingomyelin (prepared via the method of Stoffel et al. (19)) was added as a mixed micelle with Triton X-100 (final assay conditions were 10 nmol of SM, 2 × 10⁶ cpm, 0.1% Triton X-100, and either 0.1 mM Tris-HCl, pH 7.4, 5 mM MgCl₂ (for assaying neutral enzyme activity) or 0.1 mM sodium acetate, pH 5.0 (for assaying acidic enzyme activity)). The reaction was incubated at 37°C for 15 min and then stopped via addition of 1.5 ml of chloroform/methanol (1:2). The upper phase from a Bligh and Dyer extraction was counted and represented the SMase activity.

**Arachidonic Acid Release Studies**

HL-60 cells grown to a density of 0.5–0.8 × 10⁶ cells/ml were washed with PBS and resuspended at 0.2 × 10⁶ cells/ml in ITS-supplemented media. Cells were incubated for 24 h in the presence of 0.5–2.0 µCi/ml [3H]arachidonic acid (100 C/mmol). Post-labeling, cells were washed with PBS, seeded at 0.5 × 10⁶ cells/ml in HEPES and ITS-supplemented media, and rested for 2–4 h. Cells were then treated with 30 nM TNFα or KBr-bovine serum albumin vehicle for varying times and subsequently pelleted. The resultant supernatant was collected and counted to determine levels of released label.

**Proliferation Studies**

HL-60 cells in HEPES and ITS-supplemented media were seeded into 6-well Costar plates at a density of 0.2 × 10⁶ cells/ml. Cells were rested for 2 h, and the indicated treatments were performed. Cell counts were made at the indicated times, and trypan blue dye exclusion was used to ascertain viability (>90% viability was always apparent).

**RESULTS**

TNFα Induces SM Turnover—TNFα, an inducer of monocytic differentiation of HL-60 cells, is able to induce time- and dose-dependent sphingomyelin hydrolysis in HL-60 cells (8). An increase in product, i.e. ceramide, paralleled the observed decrease in SM levels (8, 20). Depending upon the batch of HL-60 cells utilized, however, the time course of the TNFα effect varied. In order to establish the kinetics of the TNFα effect on SM hydrolysis in the cells utilized for the following studies, an initial time course study was performed. Similar to previous findings, SM turnover of approximately 10% could be observed as early as 15 min following treatment of cells (Fig. 2). Maximal effects of up to 30% SM hydrolysis were observed 45–60 min after treatment with 30 nM TNFα. Thus, TNFα was able to modulate production of the putative second messenger ce-
TNFα or vehicle (Krebs-Ringer HEPES with 0.1% bovine serum albumin) from three separate experiments. The mechanism by which TNFα regulates SM levels, a role for AA in TNFα-induced ceramide turnover with maximal hydrolysis of SM of approximately 35-40% began to induce cellular toxicity effects. Thus, the time frame of the peak and trough correlated approximately 15-20% SM hydrolysis within 10 min of treatment with 30 pm TNFα addition, and peak levels were attained within minutes (up to 35% by 10 min) of TNFα stimulation.

The ability of TNFα to induce AA release in a time frame preceding SM hydrolysis was assessed in HL-60 cells. Time course studies with TNFα showed that TNFα induced release of AA and metabolites within minutes of stimulation (Fig. 3). Levels of 15-25% above control were observed within 5 min of TNFα addition, and peak levels of 35-40% were observed within 10-20 min. The rapid rise in arachidonate release was followed closely by a decrease in arachidonate to levels below baseline; the significance of this finding is undetermined at present. The time frame of the peak and trough correlated extremely well with an hypothesized role for AA in TNFα-induced ceramide turnover, whereas peak AA levels were attained within minutes (up to 35% by 10 min) of TNFα stimulation.

AA induces SM turnover—We then investigated the effects of exogenous AA on SM turnover in HL-60 cells. Within 10 min of treatment with arachidonic acid, as much as 10-25% SM hydrolysis was seen (inset of Fig. 4A). The observed SM turnover was both time- and dose-dependent. Approximately 35% SM hydrolysis was evident by 30 min, and as much as 55% SM hydrolysis was apparent within 55 min of treatment with 15 μM AA (Fig. 4A). Doses as low as 5-10 μM were able to elicit approximately 15-20% SM hydrolysis within 10 min of treatment, while 30 μM AA induced as much as 30% decrease in SM (Fig. 4B). Higher doses of AA correspondingly produced greater SM turnover with maximal hydrolysis of SM of approximately 40-50% (data not shown). However, concentrations of 30 μM AA and greater began to induce cellular toxicity effects. Thus, the mechanism by which TNFα regulates the level of SM within cells, therefore, became a key point of interest.

TNFα liberates AA in HL-60 Cells—In light of the short time course in which TNFα modulates the SM levels of HL-60 cells, a rapid course of events must precede sphingomyelinase activation. In a variety of cell systems, arachidonic acid and/or eicosanoid metabolites have been shown to be liberated within minutes of TNFα stimulation (10-12). Thus, to determine the mechanism by which TNFα regulates SM levels, a role for arachidonate was examined. Studies were pursued at three levels. First, the ability of TNFα to liberate AA in HL-60 cells was examined. Second, the impact of AA on the SM cycle was determined, and, finally, the ability of AA to mimic ceramide-induced biology was examined.

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lower concentrations of AA were employed for subsequent studies.

Sphingomyelin hydrolysis in response to AA should manifest also as a change in ceramide levels within cells; therefore, ceramide measurements were performed to corroborate the observed effects of AA on SM levels. As expected, the observed decreases in SM levels in AA-treated cells correlated with increases in ceramide levels. Within 20 min of treatment with 10 μM AA, ceramide levels increased approximately 20% (Fig. 5). Furthermore, ceramide levels continued to rise approximately 2.5-fold over 90 min (up to 8 pmol of ceramide/nmol of phosphate). Thus, SM hydrolysis and ceramide generation induced by AA appear to be similar to that induced by TNFα, albeit with earlier kinetics.

AA-induced SM Turnover Exhibits Specificity—Specificity of AA in inducing SM hydrolysis was investigated using the following: 1) a variety of fatty acids, 2) a non-metabolizable analog of AA, and 3) a methyl ester and an alcohol of AA. All fatty acids examined (oleic acid, linoleic acid, linolenic acid (Fig. 6A), palmitic acid, and myristic acid (data not shown)) were able to induce SM turnover with potencies similar to AA. Within 30 min of treatment, 10 μM oleic, linoleic, and linolenic acids were able to induce 10–12% SM hydrolysis. Similarly, 10 μM palmitic and myristic acids were able to induce approximately 15% SM hydrolysis within 10 min of treatment. On the other hand, the methyl ester and alcohol analogs of AA were inactive. Treatment of HL-60 cells with 10 μM arachidonate methyl ester or arachidonoyl alcohol for 30 min produced no effective decrease in SM levels (Fig. 6A). Over a time course through which AA proved effective, both the methyl ester and the alcohol of AA (10 μM) were unable to induce SM turnover (data not shown).

The non-metabolizable AA analog, eicosatriynonic acid (ETI), was found to be as effective as AA itself in stimulating SM hydrolysis (Fig. 6B). ETI was able to produce 15–20% SM hydrolysis following 20–30 min of treatment, and ETI did not significantly enhance AA-induced SM hydrolysis when added in conjunction with AA. These studies suggest that AA may be a direct regulator of SMase without a requirement for further metabolism.

The Phospholipase A2/AA Pathway But Not the DAG/PKC Pathway Modulates SM Turnover—A role for the arachidonate pathway does not preclude involvement of alternate signaling cascades. In fact, TNFα has been shown to cause early elevation of DAG in U937 cells (21), suggesting activation of the protein kinase C pathway. Thus, to determine the specificity of lipid second messengers in modulating SM levels, a cell-permeable analog of DAG was evaluated. At a concentration of 10 μM, dioctanoylglycerol (diC8) failed to induce SM hydrolysis over 10–60 min (data not shown). Furthermore, higher concentrations of diC18, which had previously been found to stimulate acid (lysosomal) SMase activity in U937 cells (22), did not stimulate SM hydrolysis within this cell system (Fig. 7). In fact, reminiscent of the effects of the phorbol ester phorbol 12-myristate 13-acetate, diC8 produced an increase in SM levels at concentrations in which arachidonate induced SM hydrolysis. Phorbol 12-myristate 13-acetate has been shown to induce elevations of SM levels within 0.5–3 h in HL-60 cells (8), strongly ruling out an effect of PKC on SM hydrolysis.

On the other hand, melittin, a known activator of phospholipase A2 (23, 24), was able to stimulate SM hydrolysis in a dose- and time-dependent manner. Similar to AA, melittin was able to induce rapid decreases in SM levels; 500 ng/ml (176 nM) melittin produced 30% SM hydrolysis within 10 min of stimulation (Fig. 8A). At a dose of 100 ng/ml (35 nM), melittin was able to produce a significant decrease (15–20%) in SM levels (Fig. 8B).

AA Activates SMase in Vitro—The above studies raised the possibility that AA may regulate SMase directly. Therefore, the ability of AA to stimulate sphingomyelinase activity in a cell-free system was investigated. Neutral enzyme activity exhibit-
Arachidonic Acid as Mediator of Sphingomyelin Hydrolysis

**Fig. 7. Effect of dioctanoylglycerol on SM hydrolysis.** [3H]Choline-labeled HL-60 cells were treated at time 0 with the indicated concentrations of diC8 or ethanol vehicle. Following 20 min of treatment, cells were harvested, lipids were extracted, and SM was quantitated. The results shown are averages of triplicates and are representative of several experiments.

**Fig. 8. Time course and dose response of melittin-stimulated SM hydrolysis.** [3H]Choline-labeled HL-60 cells were treated at time 0 with melittin or vehicle. At the indicated times, cells were harvested, lipids were extracted, and SM was quantitated as described under "Methods." The results shown are representative of several experiments. A, time course of SM hydrolysis following treatment with 500 ng/ml melittin. B, dose response of SM hydrolysis at 15 min following melittin treatment.

**Fig. 9. Effect of AA on in vitro SMase activity.** Cytosolic enzyme activity was assayed following pretreatment of cells with the indicated concentrations of AA or ethanol vehicle. Following the 10-min pretreatment, labeled SM was added, and enzyme activity was assessed as the amount of label liberated from SM. A, dose response of AA-induced neutral SMase activity. B, effect of 30 μM AA on acid (pH 5.0) and neutral SMase activity.

AA exhibited a dose-dependent increase in response to AA (Fig. 9A). Doses of 10–30 μM AA stimulated sphingomyelinase up to 3-4-fold above basal levels, and higher doses stimulated activity up to 7-fold higher than basal levels (data not shown). Under the same conditions, AA failed to stimulate acidic sphingomyelinase activity above basal levels (Fig. 9B).

In contrast, melittin's effects on SM levels did not translate to modulation of SMase. Melittin was unable to stimulate neutral SMase activity in the in vitro assay (Fig. 10). Thus, melittin, unlike AA, is an indirect modulator of neutral sphingomyelinase activity, regulating the SM cycle through activation of endogenous phospholipase A₂.

AA Exhibits an Antiproliferative Effect—Finally, the ability of AA to modulate the SM cycle was assessed at the biological level. Exogenously added ceramide exhibits an antiproliferative effect in HL-60 cells (8). This led to the hypothesis that elevations in endogenous ceramide mediate the antiproliferative effects of TNFα. If AA transduces the effects of TNFα on SM hydrolysis, then, it was predicted, AA would exert an antiproliferative effect as well. Thus, the ability of AA to modulate growth of HL-60 cells was investigated. At concentrations of 10 μM, AA produced an early and reversible inhibitory effect on the growth of cells (Fig. 11). AA was able to decrease growth as much as 50% as early as 18 h. This growth inhibition was transient, and cells were able to recover and grow to control levels by 72 h following arachidonate treatment; however, the ability of AA to mimic early growth inhibitory effects analogous to ceramide further substantiates a role of arachidonate in modulating SM/ceramide signaling.
Arachidonic Acid as Mediator of Sphingomyelin Hydrolysis

**Fig. 10. Effect of melittin on in vitro neutral SMase activity.** Cytosolic enzyme activity was assayed following pretreatment of cells with 35 nM (100 ng/ml) melittin or vehicle. Following the 10-min pretreatment, labeled SM was added, and enzyme activity was assessed as the amount of label liberated from SM.

**Fig. 11. Effects of AA on cell proliferation.** HL-60 cells were treated at time 0 with 10 μM AA or ethanol vehicle. At the indicated times, cell numbers were determined. The results shown are averages of quadruplicate counts and are representative of results from three separate experiments.

**DISCUSSION**

We have found that SM turnover induced by TNFα occurs rapidly, peaking within 30–60 min following treatment of HL-60 cells. In assigning intermediary steps to this signaling cascade, a rapidly activated mechanism must be postulated. The phospholipase A2/AA pathway was studied as a candidate for mediating the effects of TNFα on SM hydrolysis, and multiple lines of evidence supported this hypothesis. First, TNFα caused early release of AA and metabolites. Second, addition of AA to cells resulted in a rapid decrease in SM levels and concomitant increase in ceramide levels. Third, the time course of AA-induced SM hydrolysis was more rapid than was observed with TNFα. These temporal relations fit well with the postulated role of AA transducing the TNFα signal to SM. Fourth, the inability of 1) dioctanoylglycerol, and 2) the methyl ester and alcohol analogs of AA to reproduce the same effects suggested that the AA response was specific. Other fatty acids, however, were able to mimic this effect of AA. Since other fatty acids are not metabolized to the same eicosanoid products as AA, these results suggest that the released fatty acid, e.g. arachidonate, and not a metabolite was responsible for stimulating SM turnover. This was corroborated by the ability of the non-metabolizable AA analog ET1 to induce SM hydrolysis. Fifth, melittin, a potent phospholipase A2 activator, was able to stimulate SM hydrolysis in vivo, reminiscent of AA. Sixth, the early antiproliferative response elicited by AA correlated with the biological effects of ceramide on HL-60 cells.

Finally, the regulation of SM hydrolysis by AA in cells translated to a direct stimulation of sphingomyelinase activity by AA in a cell-free system. AA was able to stimulate a neutral SMase (but not an acidic SMase) in cytosol fractions. The AA-stimulated enzyme activity was significant, with 10–30 μM AA exhibiting 3–4-fold stimulation above base line. In contrast, melittin, which was able to mimic AA-induced SM hydrolysis, was completely inert in the in vitro SMase assay. Thus, melittin is an indirect activator of SMase, working through the activation of a phospholipase A2 to yield AA, and AA appears to be a potent direct activator of neutral, cytosolic SMase activity.

From these studies, a scheme of signaling events emerged, as depicted in Fig. 12. The TNFα trimer interacts with either TNF receptor α, TNF receptor β, or both to stimulate a phospholipase A2 activity. The subsequent release of arachidonate from membrane phospholipid pools leads to the activation of a neutral sphingomyelinase. The consequence of enzyme activation is the generation of the novel second messenger, ceramide. The generated ceramide, in turn, mediates a number of biological activities, including inhibition of cell proliferation (2–4, 6).

In a number of cell systems, fatty acids have already been described to have antiproliferative effects (25–27). In human U937 leukemia cells and mouse L929 fibrosarcoma cells, AA has been implicated in mediating the cytolytic effects of TNFα (28, 29). Based on the scheme in Fig. 12, ceramide may be implicated as a key messenger molecule involved in transducing these effects of arachidonic acid. In fact, ceramide has been recently implicated as a mediator of TNFα-induced cytosis, which occurs through programmed cell death (apoptosis) (7). Thus, AA-induced ceramide generation could represent a novel pathway by which serial production of lipid mediators regulates cell growth and metabolism. These studies illustrate the complexity of signaling through lipids. They begin to identify a cascade of lipid second messengers that couple glycerol phospholipid and sphingolipid metabolism.

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Arachidonic Acid as Mediator of Sphingomyelin Hydrolysis

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