Expression of Neutral Sphingomyelinase Identifies a Distinct Pool of Sphingomyelin Involved in Apoptosis*

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The activation of sphingomyelinase and generation of ceramide have been implicated as important regulatory pathways in cell growth and apoptosis. Bacterial sphingomyelinase has been used in many cell systems to mimic the activation of endogenous sphingomyelinase. These studies, however, have been complicated by the inability of exogenously applied bacterial sphingomyelinase to perform many of the effects of short chain cell-permeable ceramides, indicating that there may be a distinct signal transducing pool of sphingomyelin not accessible to exogenous sphingomyelinase or that endogenous ceramide is not sufficient to induce these changes. We cloned the Bacillus cereus sphingomyelinase gene by polymerase chain reaction and subcloned it into a mammalian expression vector under the control of an inducible promoter. Upon stable transfection and induction of B. cereus sphingomyelinase, there were increases in neutral sphingomyelinase activity, cellular ceramide levels, cleavage of the death substrate poly(ADP-ribose)polymerase, and cell death. In contrast, exogenously applied B. cereus sphingomyelinase, despite causing higher elevations in ceramide levels, was unable to induce poly(ADP-ribose)polymerase cleavage or cell death. These results support the existence of a signal transducing pool of sphingomyelin that is distinct from the pool accessible to exogenous sphingomyelinase.

It is now well appreciated that ceramide, the product of sphingomyelin hydrolysis, may play an important role in apoptosis (1) and other cellular responses including terminal differentiation (2), cell cycle arrest (3), and cellular senescence (4). Evidence to support this comes from studies using cytokines (such as TNFα (5), interleukin 1β (6, 7), and nerve growth factor (8)) or chemotherapeutic agents (such as vincristine (9), cytostatic arabinoside (10), and daunorubicin (11)), which are known to induce apoptosis, demonstrating that these agents induce elevations of intracellular ceramide. In addition, exogenously applied short chain cell-permeable ceramides are able to mimic these inducers and cause apoptosis. Finally, studies with inhibitors of ceramide metabolism, such as PDMP (12) and D-MAPP (13), show that these compounds also cause apoptosis, probably as a consequence of elevating ceramide levels.

Sphingomyelinase has been implicated as the cause of elevation of intracellular ceramide levels whereby neutral sphingomyelinase activity has been demonstrated to increase in response to TNFα as well as other inducers of apoptosis (3, 6, 10, 14, 15). However, other sources of ceramide generation have been proposed, including de novo synthesis of ceramide (11) as well as sphingomyelin hydrolysis by acid pH optimum sphingomyelinase (16).

Bacterial sphingomyelinase from Bacillus cereus functions at neutral pH (17) and thus has been considered a useful tool in tissue culture experiments to induce elevation of cellular ceramide levels in an attempt to mimic the biological effects of activation of cellular sphingomyelinase (18–21). This approach has the advantage of generating ceramide in the membrane at physiologically relevant concentrations and avoids some of the drawbacks of using exogenous ceramides. However, we have found that in many of these experiments, exogenously applied bacterial sphingomyelinase lacks substantial activity in inducing apoptosis and other responses. This suggests one of two possibilities: 1) that ceramide is not sufficient to induce these responses or 2) that there are distinct intracellular compartments where sphingomyelinase is activated leading to localized ceramide generation, thus implying that certain pools of ceramide are the biologically relevant or active pools.

In this study, we wanted to examine these possibilities and to determine whether exogenously applied bacterial sphingomyelinase is biologically different from activation of “cellular” sphingomyelinase, thus yielding distinct pools of ceramide. To achieve this goal, we stably transfected Molt-4 leukemia cells with B. cereus sphingomyelinase under the control of an inducible promoter. We demonstrate that we could induce apoptosis only upon induction of transfected sphingomyelinase activity and not when bacterial sphingomyelinase was used exogenously. These data support the hypothesis that ceramide is biologically active and that the generation of ceramide intracellularly is distinct from ceramide generated at the outer leaflet of the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials

B. cereus was obtained from American Type Culture Collection (ATCC 14579). Restriction endonucleases, T4 DNA ligase, the Klenow fragment of DNA polymerase, and RNA molecular weight markers were obtained from Boehringer Mannheim. Nick translation kit was purchased from Life Technologies, Inc. Taq polymerase was purchased from Perkin-Elmer Corp. Nitrocellulose and Zeta-probe blotting membranes were purchased from Bio-Rad. Anti-PARP anti-serum was purchased from Enzyme Systems Products (Dublin, CA). G418, RPMI 1640 medium, and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. Hygromycin B was purchased from Calbiochem.

n-erythro-2-N-myristoylaminol-1-phenyl-1-propanol; PARP, poly(ADP-ribose)polymerase; IPTG, isopropyl-β-D-thiogalactoside; FBS, fetal bovine serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumin; mU, milliunits.
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**Methods**

**PCR Amplification of Genomic DNA from B. cereus and DNA Sequencing**—Genomic DNA was isolated from *B. cereus* cultured overnight at 30 °C in brain heart infusion culture medium (Sigma). For PCR amplification, the sense and antisense sequences used were 5′-CCTCTAGATGGAGGTAGAATAAGGCG-3′ and 5′-AACAACTTCTAGAATTAGTCG-3′, respectively. PCR amplification was performed at a denaturing temperature of 94 °C for 1 min followed by annealing at 37 °C for 1 min, and extension was at 72 °C for 2 min for a total of 30 cycles. The amplified fragment (1000 base pairs) was separated by electrophoresis with a 0.8% agarose gel and purified by phenol extraction. For sequence confirmation, the purified insert was subcloned into the vector pBS (Stratagene), and its sequence was determined using the version 2.0 DNA Sequencing Kit (U. S. Biochemical Corp.). The 1-kilobase fragment was then removed from pBS and blunt-ended by the Klenow fragment, NotI linkers were added, and the fragment was then cloned into a NotI-linearized pOP13CAT vector (Lac Switch Inducible Mammalian Expression System from Stratagene). The resultant plasmid was named Bacterial Sphingomyelinase-13 (BSM-13).

**Transfection**—To obtain stable transfectants, Molt-4 cells, grown in RPMI 1640 containing 10% FBS and 25 mM HEPES, were first transfected by electroporation with p3SS vector (10 μg for 1 × 106 cells in 50 ml) using the Bio-Rad electroporation apparatus. Hygromycin (0.3 mg/ml) was added to the cells 48 h later and maintained as a selective pressure. The expression of the Lac repressor was monitored by Western blot with a polyclonal antibody (Stratagene). Upon obtaining a stable transfectant of the Lac repressor protein, the BSM-13 plasmid was then introduced by electroporation. In addition to hygromycin, selection pressure was further achieved with geneticin (0.2 mg/ml). The expression of the 1-kilobase fragment was examined by Northern blot.

**IPTG Induction**—Stably transfected cells (5 × 105 cells/ml) were rested for 2 h in RPMI containing 2% FBS and 25 mM HEPES prior to the addition of desired concentrations of IPTG. At the indicated times, cells were removed and assayed for viability (by trypan blue exclusion), ceramide content, sphingomyelinase activity and PARP proteolysis.

**Ceramide Measurement**—The level of ceramide in the transfected cells was determined by the *Escherichia coli* diacylglycerol ceramide assay system essentially as described (22).

**Sphingomyelinase Activity Assay**—For determining the activity of the neutral, magnesium-dependent sphingomyelinase, cells (2 × 106) were washed three times with ice-cold PBS and disrupted by freezing and thawing (three times in methanol-dry ice bath) in 400 μl of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM EGTA, 1 mM sodium vanadate, 10 mM B-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 20 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin. The lysate was centrifuged for 10 min at 1,000 × g at 4 °C, and the supernatant (post nuclear homogenate) was centrifuged for 60 min at 100,000 × g at 4 °C. The resulting pellet (acidic sphingomyelinase) was resuspended in 200 μl of lysis buffer. An aliquot of the membrane preparation was incubated for 30 min at 37 °C with [14C]sphingomyelin (100,000 dpm, 10 nmol) in a mixed micelle assay containing 100 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 0.1% Triton X-100 (final volume, 100 μl). The radiolabeled product was extracted as described (3), and the radioactivity was determined by liquid scintillation counting. To determine the acid sphingomyelinase activity, membranes were prepared from cells using a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride. The activity of acid sphingomyelinase was determined using a mixed micelle assay containing 100 mM sodium acetate, pH 5.0, and 0.1% Triton X-100.

**Western Blot for PARP Proteolysis**—Transfected cells (5 × 106) were pelleted, resuspended in 133 μl of PBS and 23 μl of 5 × SDS-electrophoresis sample buffer, and boiled immediately. 15 μl of the cellular lysate was subjected to electrophoresis with a 6% SDS-polyacrylamide gel. Western blots were performed exactly as described in Ref. 23.

**RESULTS**

We initially set out to evaluate the ability of exogenous bacterial sphingomyelinase to generate ceramide and to induce apoptosis. Molt-4 leukemia cells were treated with *B. cereus* sphingomyelinase and evaluated for ceramide generation and for cell death. Fig. 1A shows that exogenously administered sphingomyelinase induced significant increases in cellular ceramide levels. Surprisingly, however, *B. cereus* sphingomyelinase failed to induce cell death in these cells at concentrations of up to 300 mU/ml and for as long as 24 h (Fig. 1B).

**Cellular sphingomyelinase activity has been demonstrated to increase in response to inducers of apoptosis and is believed to be the cause of intracellular ceramide generation. Exogenously used bacterial sphingomyelinase, despite causing elevation of ceramide levels, apparently did not induce cell death. Linardic and Hannun have demonstrated that exogenous bacterial sphingomyelinase could not reach the so called signaling pool of sphingomyelin, which they suggested was on the inner leaflet of the plasma membrane (21). We therefore elected to stably transflect bacterial sphingomyelinase into cells and study its effects on ceramide generation and cell death when it was an integral part of the cell membrane. To do so we harvested genomic DNA from *B. cereus* and used it as a template to amplify the *B. cereus* sphingomyelinase gene by the polymerase chain reaction using the oligonucleotides described under “Experimental Procedures.” We then subcloned the gene and sequenced it to demonstrate no PCR-induced mutations. The gene was cloned into the eukaryotic lac-operator-containing vector pOP13CAT from the Lac Switch inducible mammalian expression system (see “Methods”). This vector was then used to transflect Molt-4 cells that had already been stably transfected with the eukaryotic Lac repressor-expressing vector p3SS.

We then showed that sphingomyelinase activity was repressed in the presence of the Lac repressor. Upon induction by IPTG, this repression was removed, and enzyme activity was expressed. Sphingomyelinase activity was significantly increased within 1 h of IPTG treatment and peaked at 250% of control levels by 4 h (Fig. 2A). The induced sphingomyelinase activity was a neutral pH optimum membrane activity and was linear with protein concentration (Fig. 2B). Acidic sphingomyelinase activity did not change upon IPTG induction (Fig. 2C). Concomitant with the increase in neutral sphingomyelinase activity, there was an increase in cellular ceramide levels such that ceramide levels were induced by IPTG (Fig. 2D) and were significantly higher than levels in vector transfected cells (Fig. 2E), indicating that the increase due to sphingomyelinase induction and not a consequence of IPTG treatment.

Because sphingomyelinase activity was increased and cellular ceramide levels were elevated in response to induction of the bacterial sphingomyelinase gene, we next evaluated its biological effects. Fig. 3 demonstrates that as early as 2 h after IPTG induction of sphingomyelinase activity, there was 20% cell death as measured by trypan blue exclusion. By 8 h, close to 60% of the cells were unable to exclude trypan blue as...
compared with less than 10% of vector transfected cells.

To demonstrate if the cell death occurring in response to induction of *B. cereus* sphingomyelinase was apoptotic, we assayed for cleavage of the known death substrate PARP (24). Recently we have demonstrated that ceramide induces PARP cleavage in Molt-4 cells (23), an effect also seen with chemotherapeutic agents and considered as an indicator of apoptosis. Fig. 4 shows that upon ceramide treatment, PARP was cleaved in Molt-4 cells (23), an effect also seen with chemotherapeutic agents. Induction of transfected sphingomyelinase, when applied exogenously to cells, fails to induce many of the biologic effects normally seen in association with activation of endogenous sphingomyelinase or induced by cell-permeable ceramides. On the other hand, upon stable transfection of bacterial sphingomyelinase into cells and induction of gene expression, we demonstrate the activation of a functional sphingomyelinase protein and a significant elevation of ceramide levels. Although the levels of ceramide achieved by inducing bacterial sphingomyelinase intracellularly are lower than those achieved by using exogenous bacterial sphingomyelinase, it appears that the ceramide generated from transfected sphingomyelinase but not that generated from exogenous sphingomyelinase leads to apoptosis.

Interestingly, several studies to date have demonstrated conflicting reports on the ability of exogenously applied bacterial sphingomyelinase to induce cellular effects. For example, Ji et al. have demonstrated that bacterial sphingomyelinase can mimic TNF and Cer-ceramide (albeit very modestly) and induce tyrosine phosphorylation of a 23-kDa nuclear protein (25). Raines et al. have demonstrated that bacterial sphingomyelinase can mimic TNFα and induce MAP kinase activation in...
HL60 cells (20). Sasaki et al. have also demonstrated that bacterial sphingomyelinase induces MAP kinase activity, as does exogenous use of ceramide in NIH 3T3 cells (26); however, in most studies, exogenous ceramides do not appear to activate MAP kinase and preferentially activate jun kinase (or stress-activated protein kinase) (27). Santana et al. have shown that bacterial sphingomyelinase as well as exogenous ceramide can partially mimic TNFα action in granulosa cells and cause inhibition of P-450 aromatase activity (28). Tamura et al. have demonstrated that bacterial sphingomyelinase can mimic NigF and inhibit neurite outgrowth in PC12 cells, but the effects of ceramides were not evaluated (19). Riboni et al. have demonstrated that bacterial sphingomyelinase as well as exogenous ceramide can mimic retinoic acid and cause inhibition of cell proliferation, differentiation, and stimulation of neurite outgrowth in neuroblastoma cells (29). On the other hand, Walev et al. have demonstrated only selective cytotoxic effects of bacterial sphingomyelinase on monocytic cells but not on human granulocytes, fibroblasts, lymphocytes, or erythrocytes (30); ceramide effects were also not tested. In our hands bacterial SMase failed to induce apoptosis in U937 cells, a monocytic leukemia cell line. Borchardt et al. demonstrated that bacterial sphingomyelinase caused elevation of ceramide levels but failed to inhibit growth of human T-cells (31). This resembles the failure of bacterial sphingomyelinase to induce growth arrest and cell death that we see. Again, these studies may be due to differential responses of different cell types or due to differential responses of different intracellular pathways to several potential pools of ceramide generation. Clearly there is a difference in cellular responses between exogenously applied bacterial SMase and short chain cell-permeable ceramides, particularly on modulation of growth arrest and apoptosis.

There are several implications to these results. First, ceramide generated by activation of transfected sphingomyelinase appears to be sufficient to induce cellular effects of growth suppression. Second, these results support the existence of different intracellular pools of ceramide, such that when cells are treated with bacterial sphingomyelinase, it is able to cleave the sphingomyelin from the outer leaflet of the plasma membrane, but apparently there remains a part of sphingomyelin that is not accessible to exogenously applied sphingomyelinase. In fact, this is supported by evidence from HL60 cells (21) and in fibroblasts (32) where the pool of sphingomyelin that is hydrolyzed in response to inducers (such as vit D3 or TNFα) appears to be on the inner leaflet of the plasma membrane (or another compartment distinct from the outer leaflet pool of sphingomyelin) and not accessible to exogenous sphingomyelinase. This pool appears to be the signal transducing pool because when sphingomyelinase is transfected into cells, it is able to induce its cellular effects, implying that ceramide is generated intracellularly.

If exogenous sphingomyelinase cleaves sphingomyelin and generates ceramide as seen by our data, then how come that ceramide appears to be biologically inactive? Several possibilities emerge. First, it is possible that the ceramide generated in the cell membrane is unable to flip and enter the cell, despite evidence to the contrary (33). Second, ceramide may be metabolized quickly prior to its reaching the cellular compartment where it performs its biological activity. Third, the molecular species of this ceramide is distinct from the signaling ceramide. Finally, the “signaling” ceramide is generated and is active in a compartment distinct from the plasma membrane or in a specialized compartment within the plasma membrane. Obviously, extensive further investigation is required to sort out these possibilities.

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