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Ceramide formation during heat shock: A potential mediator of αB-crystallin transcription

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ABSTRACT **Ceramide has been identified as a potential second messenger that may mediate cell differentiation and apoptosis after exposure to hormonal agonists such as 1α,25-dihydroxyvitamin D_3, tumor necrosis factor α, or γ-interferon. The secondary cellular events that follow ceramide generation remain undefined. We report that in NIH WT-3T3 cells, ceramide induces an enhancement of gene transcription of αB-crystallin, a small heat shock protein. The levels of αB-crystallin, as measured by Northern blot and immunoblot analyses, were increased by the addition of an exogenous short-chain ceramide, *N*-acetylsphingosine, or by increasing endogenous intracellular ceramide by inhibition of glucosylceramide synthase. Similar effects were not seen in the expression of the closely related gene, *Hsp25*. To ascertain whether ceramide-mediated gene transcription was a feature of the heat shock response, cell ceramide was measured in heat shocked cells and observed to be elevated 2-fold immediately upon the return of cells to 37°C. Thus ceramide formed after heat shock treatment of 3T3 cells may mediate the transcription events associated with the cell stress response.**

Ceramide has been identified as a putative cellular messenger based on its formation after agonist-stimulated activation of sphingomyelinase. Such agonists include 1,25-dihydroxyvitamin D_3 (1), tumor necrosis factor α (TNF-α) (2), γ-interferon (3), nerve growth factor (4), interleukin 1 (5), and fMet-Leu-Phe (6). Once formed ceramide may induce cell differentiation (1), senescence (7), or apoptosis (8). The mechanisms by which ceramide induces these cellular changes are presently undefined. Possibilities include the activation of a ceramide-dependent kinase (9), a stress-activated protein kinase (10), or the activation of a cellular phosphatase (11).

A potential role for ceramide in gene transcription has recently been proposed. In this model, ceramide is released after TNF-α-induced activation of an acidic sphingomyelinase and stimulates transcription factor NF-κB translocation from the cytosol to the nucleus (12). However, this hypothesis has recently been challenged since TNF-α-induced signaling can occur in cells where ceramide levels can be dissociated from NF-κB activation (13).

Cell ceramide levels are increased in the presence of the ceramide analog *threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (14). PDMP inhibits glucosylceramide synthase and mimics many of the cellular effects of ceramide, including growth arrest (14, 15). In the course of attempting to clone glucosylceramide synthase, a clone was identified that encodes the small heat shock protein αB-crystallin. This protein is structurally similar to murine Hsp25 and has chaperone-like activity (16). We therefore studied the association between cell ceramide levels and αB-crystallin formation. We report herein that the transcription and translation of αB-crystallin are induced by ceramide. We also report that heat shock results in the formation of ceramide.

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MATERIALS AND METHODS

Materials. D-*threo*-PDMP was synthesized as described (17). *N*-Acetylsphingosine (C_2-ceramide) was from Matreya (Pleasant Gap, PA); receptor-grade human recombinant insulin-like growth factor I (IGF-I) was from Baxter Scientific Products (McGaw Park, IL). Rabbit anti-human αB-crystallin polyclonal antibody was from Novocastra Laboratories (Newcastle upon Tyne, U.K.).

Cell Culture. The NIH WT-3T3 mouse fibroblast cell line, which overexpress IGF-I receptors, was provided by Alan Saltiel (Parke–Davis Laboratories). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (Intergen, Purchase, NY), penicillin (100 units/ml), streptomycin (100 μg/ml), and Geneticin (GIBCO; 1 μg/ml) under 5% CO_2/95% air in an incubator at 37°C. Unless otherwise indicated, cells released by trypsin/EDTA treatment were seeded at 2×10^6 cells per 150-mm^2 Petri dish in 15 ml of serum-free DMEM supplemented with 10 nM IGF-I, penicillin (100 units/ml), and streptomycin (100 μg/ml) for 24 h. The cells were then washed twice with phosphate-buffered saline, 15 ml of fresh serum-free DMEM containing IGF-I was added in the presence of PDMP or short-chain ceramides, and culture was continued at 37°C as indicated. Heat shock treatment of NIH 3T3 cells was performed by incubating cells at 42.5°C for 1 h and then at 37°C for the time indicated after replacement with fresh medium preheated to 37°C.

Construction of a PCR Library by Using Degenerate Primers to a UDP-Hexose Binding Domain. PCR library construction was performed by a modification of the procedure of Wilks (18). NIH WT-3T3 cells were treated with 40 μM PDMP for 6 h as indicated above and total cellular RNA was isolated by using the TriReagent (Molecular Research Center, Cincinnati) according to the manufacturer's instructions. The total RNA was dissolved in diethyl pyrocarbonate-treated water and treated with RNase-free DNase I from Boehringer Mannheim at 1 unit of DNase I per 50 μg of total RNA for 30 min and then extracted with phenol/chloroform three times and precipitated with ethanol. After air-drying briefly, the pellet was dissolved in diethyl pyrocarbonate-treated water and quantitated spectrophotometrically.

Reverse transcription was performed by using 50 mM Tris·HCl/6 mM $MgCl_2$/40 mM KCl/1 mM dithiothreitol/all four dNTPs (each at 1.5 mM)/$(dT)_{12-17}$ (GIBCO/BRL; 2.5 ng/μl)/Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL; 10 units/μl)/4 μg of total RNA at 42°C for 2

Abbreviations: PDMP, *threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; IGF-I, insulin-like growth factor I; TNF-α, tumor necrosis factor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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h. After synthesis of first-strand DNA, PCR was performed by placement of 2 μl of the first-strand cDNA into a fresh tube containing 48 μl of 40 mM KCl/50 mM Tris·HCl, pH 8.0/all four dNTPs (each at 1.5 mM)/6 mM $MgCl_2$/50 pmol of 3′ primers [5′-TCT(G/C)CAAA(G/C)A(A/T/G)(T/A)GG(A/G)A(T/C)(T/G)CC-3′] and 50 pmol of 5′ primers [5′-CA(A/G)AATGA(C/T)CT(G/C/T)CTTGG-3′]. Denaturation was performed at 94°C for 40 sec, annealing at 37°C for 3 min, and primer extension at 55°C for 3 min. Eighty cycles were performed and fresh *Taq* polymerase was added at the beginning of the first 40 cycles and the second 40 cycles. Cloning was performed by using TA cloning kit from Invitrogene. Ligation and transformation were performed by the protocol provided with the kit. The ligation reaction mixture was incubated at 12°C overnight and contained 5 μl of sterile water, 1 μl of 10× ligation buffer, 2 μl of resuspended TA vector/1 μl of product from degenerated PCR, and 1 μl of T4 DNA ligase. Transformation was carried out by addition of TA cloning ligation reaction (or diluted pUC18 test plasmid for control) directly into the DH5α competent cells.

For restriction analysis and DNA sequencing analysis of recombinant clones, colonies were grown overnight in 5 ml of LB broth with ampicillin (50 μg/ml) at 37°C with shaking. Then plasmids were purified and digested with *Eco*RI. Recombinant clones of the expected size were sequenced with Sequenase version 2.0 (United States Biochemical) by the company's instructions. DNA sequences were searched in the GenBank data base by using BLAST.

Northern Blot Analysis. Northern blot analyses were performed as described (15). Asynchronously growing 3T3 cells were treated as indicated above. The total cellular RNA was isolated by using the TriReagent by the manufacturer's instructions. The isolated total RNA was dissolved in diethyl pyrocarbonate-treated water and quantitated by spectrometry as well as quantitative Tris acetate/EDTA-agarose gel electrophoresis. Equal amounts of total RNA were denatured in formamide and formaldehyde sample buffer, fractionated on a 1% agarose/formaldehyde gel, and then transferred to nitrocellulose membranes. The RNA was then hybridized to an [α-^{32}P]dCTP-randomly labeled 114-bp mouse αB-crystallin cDNA fragment. After overnight hybridization, the membranes were washed under high-stringency conditions, and the hybridization bands were visualized by autoradiography. For assessment of RNA loading, the membranes were stripped and rehybridized with a 139-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment as described (15) and subsequently restripped and probed with rat Hsp27 cDNA fragments labeled with [α-^{32}P]dCTP (provided by Michael Walsh, University of Michigan). All DNA fragments were labeled by using the Promega Prime-a-Gene labeling kit. The hybridization bands on the autoradiograms were quantitated by video imaging as described (15).

Immunoblot Analysis with Anti-αB-Crystallin and Anti-Murine Hsp25. The induction of αB-crystallin and murine Hsp25 was analyzed on immunoblots with anti-αB-crystallin and anti-murine Hsp25 antibodies (Stressgen, Victoria, BC, Canada), respectively. Cells were grown in low-serum DMEM (0.5% fetal bovine serum) as indicated above and treated with 15 μM *N*-acetylsphingosine for the indicated treatment time. Prior to cell harvesting, cell culture dishes were washed with ice-cold phosphate-buffered saline and directly lysed or stored at −80°C and lysed later with 2% (wt/vol) SDS/1% Nonidet P-40/100 mM 2-mercaptoethanol/100 mM phenylmethylsulfonyl fluoride. Cellular lysates were gathered by scraping and centrifugation at 16,000 × *g* for 5 min at 4°C. Protein concentrations were determined by Pierce Coomassie Plus assay reagent. Equal amounts of protein from the cell soluble lysate fraction in 1× SDS sample buffer (2% SDS/0.1 M Tris/1% bromphenol blue) were loaded on a 10% SDS/PAGE gel and separated. Proteins were then electrophoretically transferred onto a nitrocellulose membrane. The blot was then stained with Ponceau's solution and then blocked with 5% (wt/vol) nonfat milk/0.02% sodium azide/0.05% Tween 20 for 4 h. The blot was incubated with a 1:10,000 dilution of rabbit anti-Hsp25 antibody or with a 1:1000 dilution of rabbit anti-αB-crystallin antibody in blocking buffer at room temperature overnight and then washed three times in phosphate-buffered saline containing 0.05% Tween 20. The blot was subsequently incubated with a secondary horseradish peroxidase-conjugated anti-rabbit IgG (1:3000 dilution), rewashed with phosphate-buffered saline/Tween 20, and incubated with 20 ml of enhanced chemiluminescence detection reagent (Amersham). Blots were visualized by autoradiography.

Ceramide Determination. Ceramide was assayed as described (15).

RESULTS

Our original goal was to clone and sequence a glucosylceramide synthase based on two observations. (*i*) A conserved

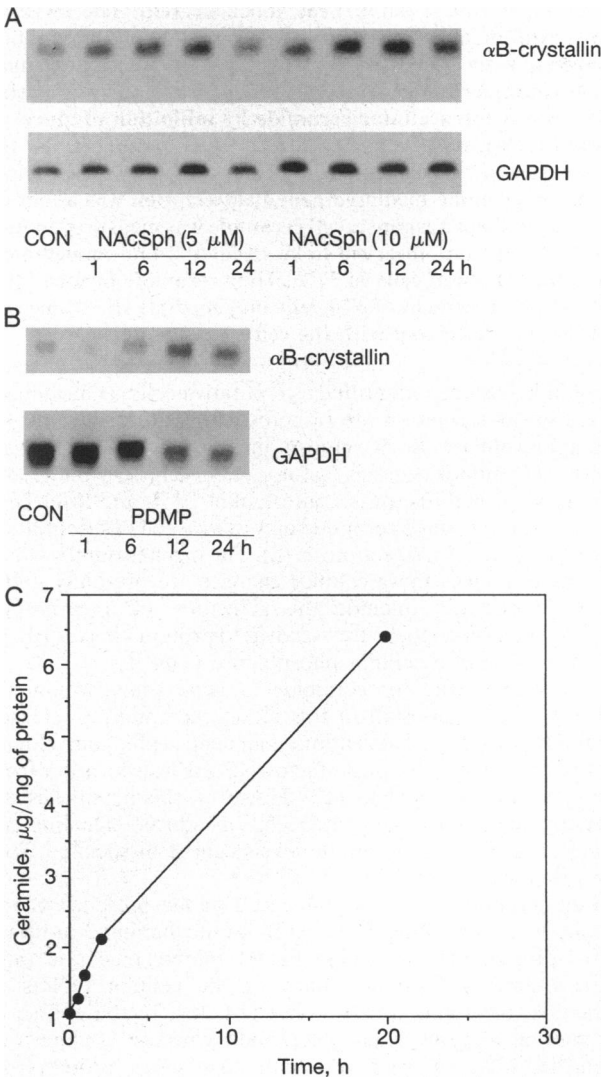

FIG. 1. Association between αB-crystallin mRNA and cell ceramide levels. (*A*) Time- and concentration-dependent changes in αB-crystallin mRNA after incubation of NIH 3T3 cells with *N*-acetylsphingosine (NAcSph; 5 and 10 μM). (*B*) Induction of αB-crystallin in 3T3 cells by PDMP. 3T3 cells were grown in defined medium in the presence of 10 nM IGF-I (time 0; CON) and then treated with 40 μM PDMP for 1, 6, 12, or 24 h. (*C*) Ceramide levels from cells treated with PDMP as in *B*.

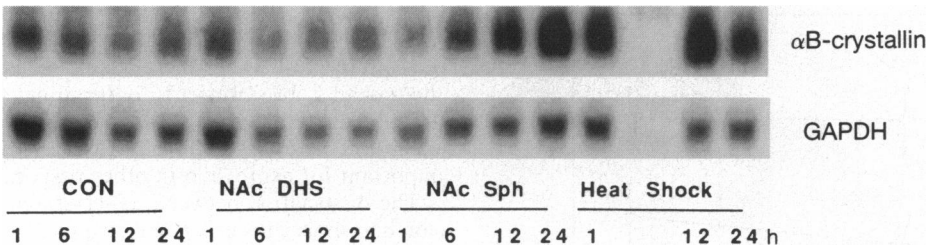

FIG. 2. Northern blot analysis of αB-crystallin mRNA after exposure of NIH 3T3 cells to *N*-acetylsphingosine (NAc Sph) or *N*-acetyldihydrosphingosine (NAc DHS) for the periods indicated.

UDP-hexose binding domain is present within several glycosyltransferases. (*ii*) Incubation of cultured cells with PDMP results in the transcriptionally dependent induction of the glucosylceramide synthase (19). By using degenerate primers designed to encode the UDP-hexose binding site, a ceramide- and PDMP-inducible clone was identified. On sequencing, this clone was found to be identical to αB-crystallin. When we incubated NIH 3T3 cells with a cell-permanent short-chain ceramide, *N*-acetylsphingosine, a time- and concentration-dependent increase in αB-crystallin transcription was observed (Fig. 1*A*).

To determine whether endogenous cell ceramides containing fatty acyl moieties of normal chain length were also active in stimulating transcription, we incubated cells with PDMP (Fig. 1 *B* and *C*). PDMP treatment resulted in a more gradual accumulation of ceramide. Under these conditions the αB-crystallin mRNA peaked at 12 h and was still detectable at 24 h.

The ability of ceramide to induce an increase in αB-crystallin mRNA was structurally specific (Fig. 2). The saturated form of ceramide, *N*-acetyldihydrosphingosine, had no effect on αB-crystallin mRNA levels. In contrast, *N*-acetylsphingosine induced αB-crystallin mRNA expression to levels comparable to those seen with heat shock.

The ceramide effect was specific for αB-crystallin. Hsp25 is a structurally similar protein with a high degree of homology at the amino acid and gene levels. The exposure of cells to 42.5°C for 1 h resulted in detectable increases in both αB-crystallin and Hsp25 mRNA (Fig. 3*A*). However, only αB-crystallin and not Hsp25 mRNA was detected after treatment with *N*-acetylsphingosine. The elevation of the cell temperature resulted in the formation of both αB-crystallin and Hsp25 protein (Fig. 3*B*). But only the protein level of αB-crystallin was increased after *N*-acetylsphingosine treatment.

To ascertain whether endogenous ceramides would be formed as part of the heat shock response, 3T3 cells were incubated at 42.5°C for 1 h and then at 37°C. Ceramide levels doubled after heat treatment and remained elevated for 6 h after the return to 37°C (Fig. 4).

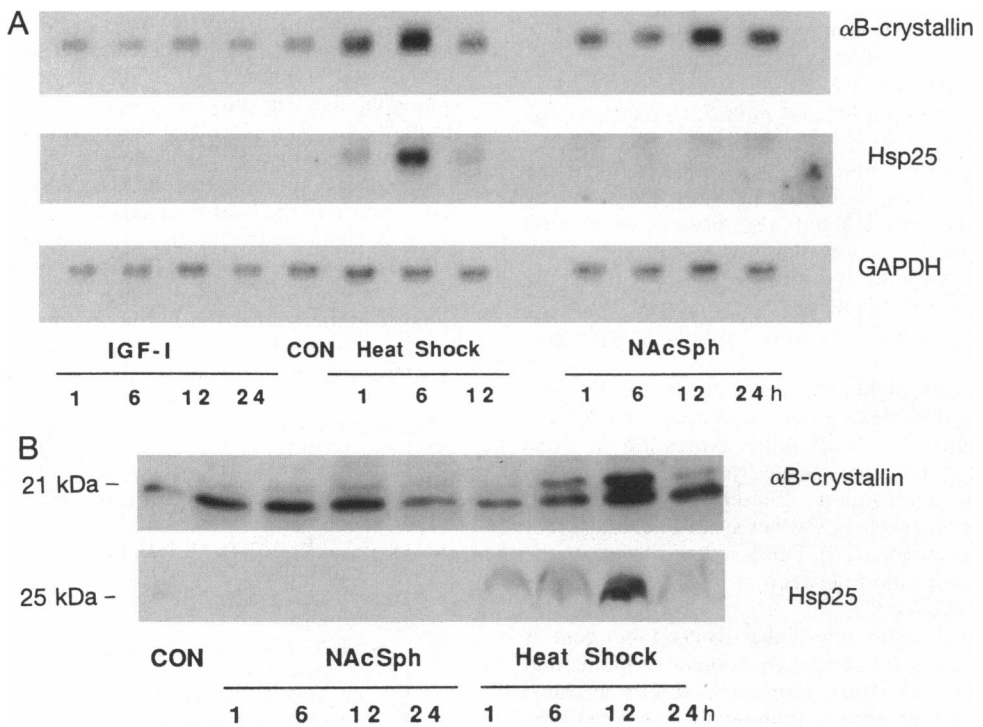

FIG. 3. (*A*) Northern blot analysis of αB-crystallin, Hsp25, and GAPDH in 3T3 cells after heat shock or exposure to *N*-acetylsphingosine (NAc-Sph). The mRNA in control cells grown in 10% fetal bovine serum and cells changed to defined medium containing 10 nM IGF-I and harvested at 1, 6, 12, and 24 h were compared to those heat-shock-treated for 1, 6, or 12 h or treated with 15 μM *N*-acetylsphingosine for 1, 6, 12, or 24 h, as indicated. Heat shock treatment times refer to time after the return of cultures to 37°C. αB-Crystallin mRNA was induced after both heat shock and *N*-acetylsphingosine treatment; Hsp25 mRNA was induced only after heat shock. (*B*) Immunoblots of αB-crystallin and Hsp25 proteins in 3T3 cells after *N*-acetylsphingosine and heat shock treatment. Total soluble cellular proteins (10 μg per lane) from 3T3 cells exposed to 15 μM *N*-acetylsphingosine for 1, 6, 12, or 24 h or heat-shocked at 42.5°C for 1 h and then allowed to recover for 1, 6, 12, or 24 h were separated and analyzed on immunoblots with anti-αB-crystallin or anti-Hsp25.

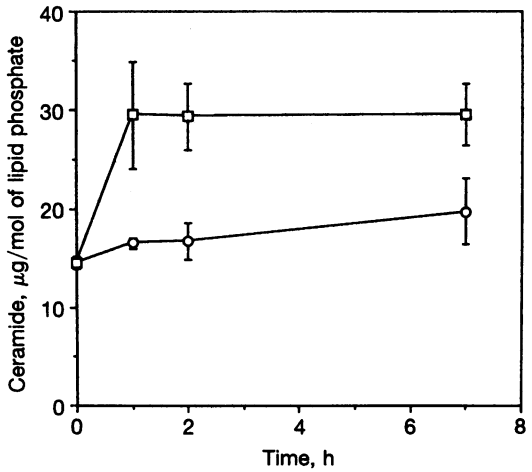

FIG. 4. Ceramide levels in 3T3 cells after heat shock treatment. 3T3 cells were heat-shocked at 42.5°C for 1 h and then returned to medium at 37°C (□). Time 0 refers to cells prior to heat shock. The 1-h time point denotes cells harvested immediately after placement at 37°C. Control cells (○) had medium replaced in an identical manner to those that were heat-shocked. The data represent the mean ± SD of three determinations.

DISCUSSION

Two interesting and potentially important observations were made in the present study. (*i*) Increased cell ceramide resulting from the exogenous addition of *N*-acetylsphingosine or inhibition of glucosylceramide synthase is associated with increased formation of the small heat shock-related protein αB-crystallin. The effects of ceramide are specific in that both transcription and translation of the homologous product Hsp25 are unaffected under similar conditions but are rapidly induced by heat shock. (*ii*) Heat shock treatment results in the rapid and pronounced formation of ceramide.

αB-Crystallin is a ubiquitous and multifunctional protein. The α-crystallins are members of a superfamily of small heat shock proteins and share many of the structural and functional properties of the small heat shock proteins (20). They not only have chaperone activity (21) but also possess autokinase activity (22) and confer thermostability to cells (23). αB-Crystallin associates with the cytoskeleton and may inhibit the assembly of intermediate filaments (24). Thus, αB-crystallin, like ceramide, may play an active role in cellular morphogenesis.

αB-Crystallin is also highly expressed in several diseases, most often degenerative diseases of the central nervous system. The significance of αB-crystallin expression in these disorders is unclear but may relate to increased protein degradation or the aggregation of intermediate filaments, which typifies disorders such as Werner syndrome, a disorder of premature cell senescence (25). This is noteworthy given the recently reported association between elevated cell ceramide levels and cell senescence (7).

The transcriptional regulation of the αB-crystallin gene is complex, demonstrating tissue-specific control elements and altered expression to cell stress. Expression studies in transgenic mice utilizing a transgene containing the fragment of the αB-crystallin gene from positions −661 to +44 fused to the bacterial chloramphenicol acetyltransferase gene revealed tissue-specific expression in some but not all tissues where αB-crystallin is normally expressed (26). These experiments suggested that the regulatory elements for αB-crystallin expression are located in the 5′ flanking sequence for lens and skeletal muscle but lie outside of this region for expression in kidney and brain.

An enhancer important for the expression of αB-crystallin in skeletal muscle cells has been identified between positions −427 and −259. Four functional elements were discovered in the enhance by DNase I footprinting and established by additional studies. One element, MRF, appears to be muscle specific. The other elements, αBE-1, αBE-2, and αBE-3, are important for expression in other tissues.

The dissociation between αB-crystallin and Hsp25 expression in response to ceramide is striking. Goldman and coworkers (27) have extensively studied the regulation of αB-crystallin and Hsp27 in astrocytes in response to physiological stress. In this system both heat shock and transition metals were able to induce transcription of both genes. However, TNF-α, a known activator of sphingomyelinase and ceramide formation, only induced formation of αB-crystallin. These observations are thus consistent with those reported in the present study.

Recently, ceramide has been identified as a potential activator of NF-κB, causing translocation from the cell cytosol to the nucleus (12). However, no NF-κB site has been identified within the αB-crystallin gene. Ceramide may also cause the activation of cellular kinases, JNK kinases, which phosphorylate the amino-terminal regions of c-jun (28) and ATF2 (29). The murine homolog of this kinase, SAP kinase, is activated after cell stress responses, including treatment of cells with sphingomyelinase (10). A consensus DNA motif for AP1 has been identified at position +1068 within the first intron. In addition, three cAMP-response element motifs have been identified including one at position −550. However, the functional significance of these sites and their relation to SAP/JNK kinase activation remain to be determined.

The mechanism by which heat shock results in the rapid increase in cell ceramide levels is unclear. In reports of agonist-stimulated ceramide formation, sphingomyelin hydrolysis appears to be the major source of ceramide. Other metabolic pathways may be involved including dephosphorylation of ceramide 1-phosphate, acylation of sphingosine, or activation of a glycanase that liberates ceramide from glycosphingolipids. The physical properties of sphingomyelin are unique (30). The transition temperature of conversion of *N*-palmitoylsphingosine phosphorylcholine from a gel phase to a liquid-crystalline lamellar phase occurs at 41.3°C, close to the temperature of the heat-stressed cells. This transition temperature is significantly lower than that observed for related phospholipids such as *N*-palmitoyldihydrosphingosine phosphorylcholine (47.8°C). One might speculate that significant changes in membrane fluidity in domains enriched in certain species of sphingomyelin occur with heat shock resulting in greater ceramide formation via the sphingomyelinase pathway.

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