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Communication

Ceramide Activates the Stress-activated Protein Kinases*

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Tumor necrosis factor α (TNFα) activates the stress-activated protein kinases (SAPKs, also known as Jun nuclear kinases or JNKs) resulting in the stimulation of AP-1-dependent gene transcription and induces the translocation of NFκB to the nucleus resulting in the stimulation of NFκB-dependent gene transcription. A potential second messenger for these signaling pathways is ceramide, which is generated when TNFα activates sphingomyelinases. We show that treatment of HL-60 human promyelocytic cells with exogenous sphingomyelinase leads to rapid stimulation of JNK/SAPK activity, an effect not mimicked by treatment with phospholipase A_2, C, or D. Further, JNK/SAPK activity is stimulated 2.7- and 2.8-fold, respectively, in cells exposed to C_2-ceramide (5 μM) or TNFα (10 ng/ml). The prolonged stimulation of this kinase activity by C_2-ceramide is similar to that previously reported for TNFα. In contrast, the related mitogen-activated protein kinases ERK1 and ERK2 are weakly stimulated following TNFα treatment (1.5-fold) and are inhibited by C_2-ceramide treatment. TNFα also potently stimulates NF-κB DNA binding activity and transcriptional activity, but these effects are not mimicked by addition of C_2-ceramide or sphingomyelinase to intact cells. Furthermore, TNFα, sphingomyelinase, and C_2-ceramide induce c-*jun*, a gene that is stimulated by the ATF-2 and c-Jun transcription factors. These data suggest that ceramide may act as a second messenger for a subset of TNFα's biochemical and biological effects.

TNFα[1] is a multifunctional cytokine involved in inflammation, infection, and cancer (1, 2). TNFα has potent biological effects on cultured cells, including induction of apoptosis and differentiation (3, 4). TNFα signals via unknown second messengers leading to activation of NFκB (5, 6) and JNKs/SAPKs (7–9) and a weaker stimulation of MAP kinase activity (7, 8). Each of these pathways leads to activation of a specific set of transcription factors; JNK/SAPK phosphorylates and activates c-Jun (10, 11) and ATF-2 (12), NF-κB translocates to the nucleus where it functions as a transcription factor, and MAP kinase phosphorylates and activates Elk-1 (13, 14) and other transcription factors.

TNFα stimulates sphingomyelinase activity, which results in the generation of ceramide (15, 16). Treatment of cells with the cell-permeable C_2-ceramide mimics TNFα in the induction of apoptosis (4), differentiation (15), and in the activation of a ceramide-activated protein phosphatase activity similar to PP2A (17, 18). A ceramide-activated protein kinase that is proline-directed has been described, but its specific target(s) is unknown (19). Thus, many of the biological and biochemical effects of TNFα are mimicked by ceramide treatment, but the molecular links between TNFα-induced ceramide production and transcriptional activation are not clear.

EXPERIMENTAL PROCEDURES

Reagents—All reagents were obtained from Sigma unless otherwise specified. Recombinant human TNFα (for HL-60 cells) and murine TNFα (for L929 cells) were obtained from R&D Systems, Minneapolis, MN. Sphingomyelinase (*Bacillus cereus*) was obtained from Boehringer Mannheim. (2*S*,3*R*)-D-*erythro*-*N*-Acetylsphingosine (D-e-C_2) was prepared by acetylation of (2*S*,3*R*)-sphingosine as described (20). DL-*erythro*-Dihydro-C_2-ceramide was prepared by acetylation of DL-*erythro*-dihydrosphingosine (20) (obtained from Sigma). Aliquots were resuspended at 20 mM in ethanol before use. Radionucleotides were obtained from ICN Biomedicals (Irvine, CA). [γ-^{32}P]ATP used for *in vitro* kinase assays was >4000 Ci/mmol.

Tissue Culture—HL-60 cells were maintained at a density of $<1 \times 10^6$ cells/ml for less than 1 month before discarding. Cells were cultured in RPMI 1640 (Life Technologies, Inc.) with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasas, CA) in a humidified atmosphere of 5% CO_2, 95% air. For treatment with agonists, cells were resuspended in serum-free medium as described (18) at a density of 250,000 cells/ml and incubated for at least 2 h before treatment. L929 fibrosarcoma cells (21) were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (Gemini).

Extract Preparation and in Vitro Kinase Assays—Whole cell extracts were prepared as described previously (22). For assessment of JNK/SAPK activity, 50 μg of extract was used in a solid state *in vitro* kinase assay as described (23) with GST-c-Jun (10) as the substrate. For assessment of MAP kinase activity, whole cell extracts were immunoprecipitated with antisera to ERK2, which cross-reacts with ERK1 (SC-94, Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were collected with protein A/G-agarose (Oncogene Science, Uniondale, NY), washed extensively, and used in an *in vitro* kinase assay with 50 μM ATP (5 μCi of [γ-^{32}P]ATP), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, 25 mM β-glycerophosphate, 10 mM *p*-nitrophenyl phosphate for 20 min at 30 °C. 2 μg of GST-ElkC (a glutathione *S*-transferase fusion protein containing the C terminus of Elk-1, which contains multiple MAP kinase phosphorylation sites) was used as substrate (14, 24). Proteins were fractionated on 12.5% SDS-polyacrylamide gel electrophoresis and subjected to autoradiography.

Gel Mobility Shift Assays—NF-κB gel mobility shift assays were performed as described previously (3). Antibodies used in binding assays (1 μl/reaction) were obtained from Santa Cruz Biotechnology.

Plasmids and Transfection Analysis—Transient transfection of L929 cells was performed by calcium phosphate coprecipitation as described previously (25). Jun-luciferase consists of −1.1 to +740 of human c-*jun*

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[1] The abbreviations used are: TNFα, tumor necrosis factor α; JNK, Jun nuclear kinase; SAPK, stress-activated protein kinase; MAP, mitogen-activated protein; PP2A, protein phosphatase 2A; D-e-C_2, (2*S*,3*R*)-D-*erythro*-*N*-acetylsphingosine; GST, glutathione *S*-transferase; MEK, MAP kinase kinase.

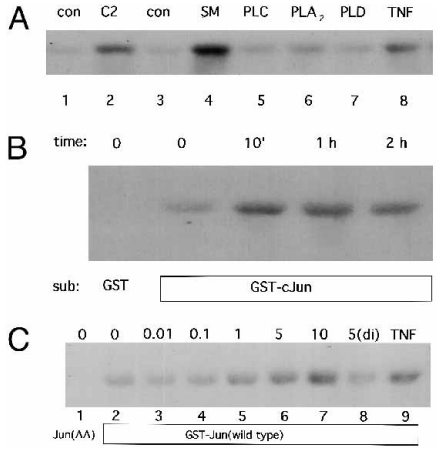

FIG. 1. **Stimulation of JNK/SAPK activity by sphingomyelinase (*SM*) and D-e-C_2-ceramide.** *A*, HL-60 cells were cultured as described (18) and treated with D-e-C_2-ceramide (*C2*, 5 μM, *lane 2*) or ethanol only (*con*, *lane 1*) or the enzymes indicated (300 milliunits/ml, *lanes 4–7*) or vehicle (*con*, *lane 3*) or TNFα (10 ng/ml, *lane 8*) for 20 min. The single prominent band visible following 10% SDS-polyacrylamide gel electrophoresis and autoradiography represents phosphorylated GST-c-Jun. Equal substrate loading was confirmed in all cases by Coomassie Blue staining of the gels prior to autoradiography. *PL*, phospholipase. *B*, time course of JNK/SAPK induction following D-e-C_2-ceramide addition (5 μM) to HL-60 cells. The substrate (*sub*) for the kinase assays was GST-cJun or GST alone as control. *C*, response of SAPK activity to D-e-C_2-ceramide treatment. All cells received the indicated concentration in an equal volume of ethanol or ethanol alone 5(di), 5 μM dihydro-C_2-ceramide. Extracts were prepared after 10 min of treatment. The substrate for the kinase assay was wild-type GST-cJun (*lanes 2–9*) or GST-cJun(AA) (*lane 1*) in which Ser-63 and Ser-73 are mutated to Ala (10).

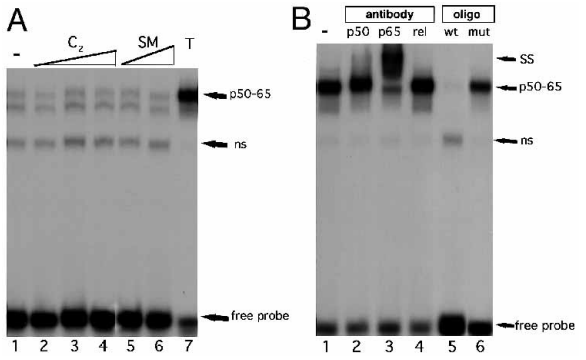

FIG. 2. **TNFα, but not sphingomyelinase or D-e-C_2-ceramide, activates NF-κB binding activity.** *A*, HL-60 cells were treated with vehicle, 0.1, 1, or 10 μM C_2-ceramide (*lanes 1–4*), or with 10 or 100 milliunits/ml sphingomyelinase (*SM*) (*B. cereus,* Boehringer Mannheim) (*lanes 5* and *6*) or recombinant TNFα (*T*, 10 nM, *lane 7*) for 30 min prior to nuclear extract preparation and gel mobility shift analysis using a consensus NF-κB binding site probe. *ns*, nonspecific shifted complex; *p50–65*, heterodimeric NF-κB. *B*, extracts from TNFα-treated HL-60 cells were used in binding assays, with antibodies to p50 (*lane 2*), p65 (*lane 3*), or c-Rel (*lane 4*), or with a 50-fold excess of unlabeled wild-type (*wt*, *lane 5*) or mutant NF-κB binding site (*mut*, *lane 6*) included in the reaction. After incubation on ice for 1 h, products were resolved on non-denaturing polyacrylamide gels. *SS*, supershifted NF-κB; *ns*, nonspecific shifted complex (not competed by cold excess oligonucleotide).

(26) linked to luciferase. Gal-ElkC (13) is cotransfected with 5 × Gal-luciferase as described (25). (KB)3-luciferase consists of three consensus NF-κB sites (27) linked to luciferase. Luciferase assays were performed as previously described (28).

Northern Blot Analysis—Total RNA was prepared from HL-60 cells and electrophoresed as described previously (29). After blotting to nitrocellulose, the RNA was probed with a *Hin*dIII-*Pst*I fragment of human c-*jun* (30) labeled by random priming (Stratagene, San Diego, CA) with [α-^{32}P]dCTP (3000 Ci/mmol, ICN, Irvine, CA). Final wash stringency was 0.1 × SSC, 0.1% SDS at 55 °C. The blot was stripped and reprobed with a glyceraldehyde-phosphate dehydrogenase cDNA probe (pHcGAP, ATCC 57090) to assess sample loading.

RESULTS AND DISCUSSION

We investigated the role of ceramide in TNFα-stimulated signal transduction by comparing the effects of TNFα, sphingomyelinase, and C_2-ceramide on JNK/SAPK activity, NFκB activity, and MAP kinase activity. Exogenous bacterial sphingomyelinase catalyzes the hydrolysis of cell membrane sphingomyelin and the formation of ceramide when added to intact HL-60 human myelocytic leukemia cells (31, 32). Treatment of cells with exogenous sphingomyelinase potently activates JNK/SAPK activity, while equivalent amounts of the lipases phospholipase C, phospholipase A_2, or phospholipase D do not (Fig. 1*A*). Exogenously added C_2-ceramide or TNFα stimulates JNK/SAPK activity in HL-60 cells to comparable levels (Fig. 1*A*). The activation of JNK/SAPK activity by C_2-ceramide is first detected in cell extracts after 10 min (Fig. 1*B*). In contrast to agents that transiently stimulate JNK/SAPK activity, such as epidermal growth factor and phorbol esters (8), ceramide elicits a prolonged activation (more than 2-fold over background levels) at least 2 h after stimulation. The kinetics of JNK/SAPK activation by ceramide parallel those of TNFα stimulation (data not shown and Ref. 8).

Increases in JNK/SAPK activity are observed at C_2-ceramide concentrations of 1 μM and above (Fig. 1*C*). 5 μM C_2-ceramide stimulates JNK/SAPK activity 2.7-fold over untreated cell levels (average of seven experiments), while TNFα (10 ng/ml) stimulates JNK activity 2.8-fold (average of four experiments). Uptake of C_2-ceramide applied at 3 μM (16, 20) results in an intracellular C_2-ceramide concentration that is equimolar to the concentration of ceramides generated endogenously following cell treatment with TNFα (15).

Because C_2-ceramide is an amphiphilic lipid analog that may have nonspecific activities, it was important to establish the specificity of its activation of JNK/SAPK, a family of kinases activated by a wide variety of stress stimuli (7). DL-*erythro*-dihydro-C_2-ceramide is a close structural analog of C_2-ceramide lacking the *trans*-unsaturated bond in the sphingosine moiety. Previous studies (20) demonstrated that the uptake and metabolism of radiolabeled C_2-ceramide and dihydro-C_2-ceramide are similar. DL-*erythro*-dihydro-C_2-ceramide fails to activate JNK/SAPK activity (Fig. 1*C*, *lane 8*). Therefore, the effects of C_2-ceramide on JNK/SAPK are specific and suggest a specific interaction of ceramide with a component of the JNK/SAPK pathway.

The effect of ceramide on the induction of NFκB activity is unresolved, in that ceramide treatment of intact cells induces binding activity in one report (31) but not others (3, 33, 34). TNFα potently stimulated NF-κB DNA binding activity (Fig. 2*A*, *lane 7*), with the predominant form of NFκB consisting of p65/p50 heterodimers (Fig. 2*B*, *lane 3*). However, treatment with bacterial sphingomyelinase (Fig. 2*A*, *lanes 5* and *6*) or C_2-ceramide (Fig. 2*A*, *lanes 2–4*) failed to induce NFκB binding activity.

TNFα is a weak inducer of MAP kinase (ERK1 and ERK2) activity relative to its induction of JNK/SAPK or relative to the induction of MAP kinases by growth factors such as epidermal growth factor (7, 8). In HL-60 cells, high concentrations of TNFα weakly activate MAP kinase activity using recombinant GST-Elk activation domain as a substrate in an immune complex kinase assay (1.5-fold over unstimulated levels) (Fig. 3, *lane 5*). As reported elsewhere (35), high concentrations of exogenous sphingomyelinase activate MAP kinase activity (Fig. 3, *lane 7*). However, treatment of cells with C_2-ceramide almost completely attenuates basal MAP kinase activity in a concentration-dependent manner (Fig. 3, *lanes 2*, *3*, and *8*), and

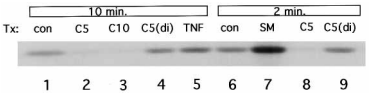

FIG. 3. **MAP kinase activity is stimulated by sphingomyelinase but not ceramide.** An immune complex MAP kinase assay was performed with HL-60 cell extracts prepared following cell treatment (*Tx*) with the indicated agonists for 10 min (*lanes 1–5*) or 2 min (*lanes 6–9*). *C5* and *C10*, 5 and 10 μM D-e-C_2-ceramide; *C5(di)*, 5 μM DL-*erythro*-dihydro-C_2-ceramide; *SM*, 100 milliunits/ml *Staphylococcus aureus* sphingomyelinase; *TNF*, 50 ng/ml recombinant human TNFα; *con*, control. GST-ElkC was used as the substrate. Equal substrate loading was confirmed in all cases by Coomassie Blue staining of the gels prior to autoradiography.

this effect was not observed with dihydro-C_2-ceramide-treated cells (Fig. 3, *lanes 4* and *9*). Similar results are obtained using myelin basic protein as a substrate for MAP kinase in an immune complex kinase assay or using an in-gel MAP kinase assay (36) with MBP incorporated into the gel (data not shown).

The ability of ceramide to activate JNK/SAPK predicts that ceramide would activate downstream targets of this kinase cascade. The c-*jun* gene contains two non-consensus AP-1 binding sites in its promoter, which are recognized with high affinity by protein complexes containing c-Jun and ATF-2 (37, 38). The transcriptional activation domains of both c-Jun (11) and ATF-2 (12) are phosphorylated and activated by JNK/SAPK. TNFα, C_2-ceramide, and sphingomyelinase stimulate c-*jun* mRNA levels in HL-60 cells (Fig. 4*A*). Thus, all three agents that stimulate JNK/SAPK activity also lead to enhanced expression of the c-*jun* gene. The heightened ability of sphingomyelinase to activate c-*jun* transcription may be due to its activation of Elk-1 (Fig. 3), which can stimulate c-*fos* transcription and hence increase total AP-1 activity.

To further assess the functional sequela of stimulation by TNFα or bacterial sphingomyelinase, we measured transcriptional activities with reporter gene assays in L929 fibrosarcoma cells, which are efficiently transfected and are responsive to TNFα (21). TNFα and sphingomyelinase are equally effective in stimulating the reporter gene driven by the c-*jun* promoter (Jun-Luc, Fig. 4*B*, *top panel*), consistent with their stimulation of JNK/SAPK activity (Fig. 1) and c-*jun* mRNA level (Fig. 4*A*). The transcriptional activity of a fusion protein consisting of the Gal4 DNA binding domain and the Elk-1 transactivation domain (Gal-ElkC (14); a MAP kinase substrate), measured by a reporter plasmid containing a luciferase gene with Gal4 binding sites (5XGal-luciferase), is activated by sphingomyelinase and to a lesser degree by TNFα (Fig. 4*B*, *middle panel*), in accord with their stimulation of MAP kinase activity *in vitro* (Fig. 3). TNFα but not sphingomyelinase markedly stimulated an NF-κB-responsive reporter gene ((KB)3-luciferase, Fig. 4*B*, *bottom panel*), consistent with the effect of these agents on NF-κB DNA binding activity (Fig. 2).

TNFα signals through two cell surface receptors, TNF-R1 (p55) and TNF-R2 (p75), which contain no apparent catalytic activity and whose intracellular domains are not homologous to characterized signaling proteins. Two proteins, TRAF1 and TRAF2, bind to the cytoplasmic domain of TNF-R2 (39). An unrelated protein, TRADD, binds to the TNF-R1-associated death domain and might be involved in TNF-induced apoptosis and NF-κB activation (40). Most of TNFα's biological effects are mediated by TNF-R1 (41–43), but the mechanisms by which second messengers are recruited are unknown. Several of TNFα's second messengers are activated via TNF-R1, including the activation of a sphingomyelinase, protein kinase C, and phospholipase A_2 (41). By using differences in the species specificity of TNFα (44, 45), we have found that TNFα also activates JNK/SAPK through TNF-R1 (data not shown).

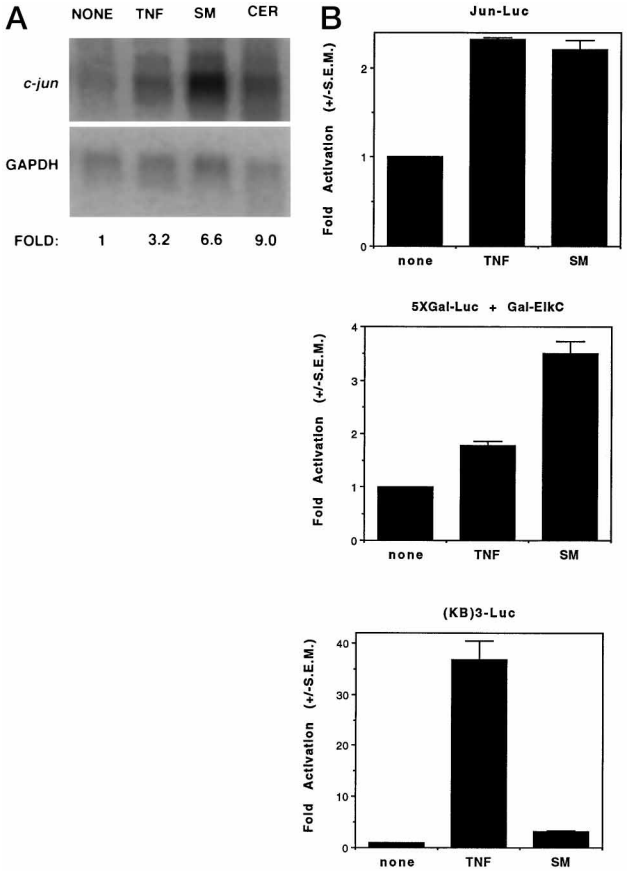

FIG. 4. **Stimulation of c-*jun* transcriptional activity.** *A*, c-*jun* mRNA levels are increased following TNFα, sphingomyelinase, and C_2-ceramide treatment. HL-60 cells were incubated with TNFα (*TNF*, 20 ng/ml), sphingomyelinase (*SM*, 100 milliunits/ml), C_2-ceramide (*CER*, 10 μM), or untreated (*NONE*) for 1 h, and total RNA was prepared followed by electrophoresis and Northern blotting. The blot was hybridized first with a radioactively labeled human c-*jun* probe (*upper panel*) and then with a labeled human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe. Blots were quantitated by phosphor image analysis, and -fold induction of c-*jun* mRNA normalized to GAPDH mRNA (*FOLD*) is indicated. *B*, activity of luciferase reporter genes in the L929 fibrosarcoma cell line. Jun-luciferase (*Luc*), or 5XGal-luciferase plus Gal-ElkC, or (KB)3-luciferase (three copies of the consensus NF-κB response element linked to luciferase) was transfected into L929 cells. Cells were then treated with TNFα (20 ng/ml) or sphingomyelinase (100 milliunits/ml) for 5 h, and extracts were prepared for determination of luciferase activity. -Fold activation represents reporter activity relative to cells treated with vehicle only. Results from one representative experiment performed in duplicate are shown ($\pm$S.E.). Results were consistent over at least three experiments.

Recent studies have delineated the steps immediately upstream of JNK/SAPK activation (46–48), but connections between these cytoplasmic and nuclear kinases and upstream messengers leading to their activation are unresolved. This study demonstrates that ceramide is a link between binding of TNFα to TNFR1 and activation of a cytoplasmic kinase cascade that results in stimulation of JNK/SAPK activity and c-*jun* expression. TNFα and C_2-ceramide activate JNK/SAPK to the same degree (Fig. 1) and stimulate activity with similar kinetics in HL-60 cells. We have previously demonstrated that JNK1 (also known as SAPKγ) is a major component of TNFα-activated JNK/SAPK (8). By activating JNK/SAPK, TNFα and ceramide activate a subset of AP-1 transcription factors, such as c-Jun and ATF-2, which in turn will preferentially induce genes with specific non-consensus AP-1 binding sites, such as the c-*jun* gene itself (Fig. 4). Therefore, this study provides strong evidence that ceramide functions as the second messenger in TNFα signaling resulting in the activation of

JNK/SAPK and c-*jun*.

TNFα is not a potent activator of MAP kinases (7, 8), and we demonstrate here that C_2-ceramide actually decreases MAP kinase activity in HL-60 cells. Inhibition of MAP kinase activity may result from the activity of a ceramide-activated protein phosphatase (17, 18) acting on a component of the MAP kinase cascade. Ceramide-activated protein phosphatase is a phosphatase of the PP2A class, and PP2A activity on both MAP kinases and MEKs has been described (reviewed in Ref. 49). The weak activation of MAP kinase activity by TNFα may be the result of ceramide-independent activation of a component (MEK or MAP kinase itself) downstream of Raf-1 and/or by activation of protein kinase C (50).

TNFα is a potent inducer of NFκB (5, 51) (Figs. 2 and 4), perhaps through the activation of protein kinase C ζ (52), and ceramide potentiates the stimulation of NFκB by TNFα (3). Ceramide stimulates NF-κB binding activity in permeabilized cells, which has been attributed to the activation of an acidic sphingomyelinase (32). However, extracts derived from intact HL-60 cells treated with exogenous sphingomyelinase or C_2-ceramide have elevated JNK/SAPK activity but not NF-κB DNA binding activity. Perhaps these different results reflect the existence of separate ceramide pools, such that C_2-ceramide and the ceramide generated by exogenous bacterial sphingomyelinase are in a different cellular compartment than the ceramide generated by acidic sphingomyelinase.

The evidence is pointing to the existence of a novel signal transduction pathway that may be specifically involved in the stress response. A number of agents of systemic stress (*e.g.* TNFα) and tissue injury (hypoxia, UV irradiation, and chemotherapeutic agents) activate both a neutral sphingomyelinase and JNK/SAPK. This study provides a link between the sphingomyelinase/ceramide pathway and the JNK/SAPK pathway by demonstrating that ceramide functions upstream of JNK/SAPK, resulting in activation of nuclear downstream effects.

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