Low Temperatures and Hypertonicity Do Not Block Cytokine-induced Stimulation of the Sphingomyelin Pathway but Inhibit Nuclear Factor-κB Activation*

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Nathalie Andrieu, Robert Salvayre, Jean-Pierre Jaffrezou, and Thierry Levade

From the Laboratoire de Biochimie, "Maladies Métaboliques," CJF INSERM 9206, Institut Louis Bugnard, C.H.U. Rangueil, Toulouse, France and CNRS, Centre Claudius Regaud, Toulouse, France

In order to better understand the significance of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)-receptor internalization in the sphingomyelin pathway, we investigated receptor signaling under conditions in which receptor internalization is blocked. We demonstrate that human recombinant TNF-α and IL-1β both induced sphingomyelin and phosphatidylcholine hydrolysis at either 4, 14, or 37°C in human skin fibroblasts and U937 monocytic cells. Cytokine-induced sphingomyelin degradation also occurred when endocytosis was inhibited by incubating the cells in hypertonic medium. While internalization was not required for the production of ceramide, activation of the transcription factor NF-κB was strongly reduced when cells were stimulated with TNF at low temperature or in hypertonic medium. Under these conditions, activation of NF-κB by the cell-permeant C2-ceramide (N-acetylphospho sphingosine), by exogenous sphingomyelinase or by phorbol myristate acetate was also inhibited. These results suggest that low temperature and hypertonicity, two inhibitors of receptor internalization, (i) do not affect the TNF-α or IL-1β-induced sphingomyelin hydrolysis, but (ii) do inhibit a step distal to ceramide of the intracellular signaling pathway leading to NF-κB activation.

Accumulating evidence suggests an important role for sphingomyelin (ceramide phosphocholine, SPM) hydrolysis and ceramide generation in cytokine signaling (1-4). Indeed, the action of tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β), i.e. two unrelated cytokines which share many biological activities (5), seems to be mediated by this recently identified SPM pathway (6-15). This pathway also appears to transduce the cellular responses to other extracellular agents, such as dihydroxyvitamin D3 (16), γ-interferon (6), nerve growth factor (17), ionizing radiation (18), and CD95 (Fas antigen) engagement (19). Thus, through the generation of ceramide, the SPM hydrolysis has emerged as an initial key signal in the regulation of diverse cell functions, including differentiation (6, 16), inhibition (20), or stimulation (21) of cell growth and apoptosis (11, 18, 19, 22). Current arguments for the importance of the SPM pathway in TNF-α or IL-1β cell signaling are based on the observations that, like the cytokine, ceramide could trigger membrane-bound serine/threonine kinase (7), a cytosolic protein phosphatase (23), a mitogen-activated protein kinase (24) and promote down-regulation of c-myc proto-oncogene RNA levels (6), enhancement of cyclooxygenase gene expression (14), and activation of transcription factors such as nuclear factor κB (NF-κB) (9, 13, 25, 26) and AP-1 (27).

The specific binding of TNF-α and IL-1β to cell surface receptors appears to be the first step for mediating most of the biological responses to these cytokines (5, 28, 29). Binding of TNF-α and IL-1β is then followed by internalization of the cytokine-receptor complex by receptor-mediated endocytosis (30, 31). However, whether internalization of these two ligands is a necessary step for induction of cellular responses in general is still under debate (28, 29). For instance, internalization of TNF-α seems to be required for mediating its cytotoxic effects (32), but not for inducing phosphorylation of a 26-kDa cytosolic protein involved in TNF signaling (33). Internalization of IL-1β is also believed to be important for its physiological responses, but it has been reported that IL-1 can stimulate rapid diacylglycerol production without being internalized (34). Elsewhere, the role of internalization in the triggering of SPM pathway has never been investigated.

The present study was undertaken to determine the importance of cytokine and cytokine receptor internalization in the stimulation of the SPM pathway. We showed that, under a variety of conditions that rule out receptor-mediated endocytosis, both TNF-α and IL-1β can trigger SPM hydrolysis but not the ceramide-stimulated activation of NF-κB.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant TNF-α and IL-1β were supplied from PeproTech-Tebu (Le Perray en Yvelines, France). Choline chloride, [methyl-3H] (86 Ci/mmol)- and [9,10-3H]palmitic acid (53 Ci/mmol) were obtained from Amersham (Les Ulis, France); [γ-32P]-adenosine (7000 Ci/mmol) was from ICN Biomedicals (Orsay, France). N-Acetylphosphosphinosine (C2-ceramide) was purchased from Matreya (Pleasant Gap, PA) or Sigma; DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) was from Molecular Probes (Eugene, OR). Sphingomyelinase (Bacillus cereus), poly(dl-dc), dithiothreitol, Nonidet P-40, phorbol myristate acetate, and sucrose were from Sigma. All solvents and other reagents obtained from Merck or SDS (Payrin, France) were of analytical grade. RPMI 1640 medium, penicillin, streptomycin, l-glutamine, trypsin-EDTA, and fetal calf serum were from Life Technologies, Inc. (Cergy-Pontoise, France).

Cell Cultures—The human monocytcuc cell line U937 (purchased from the ATCC) and human normal skin fibroblasts (obtained from our laboratory or from the Laboratoire de Culture de Peau, CHU Rangueil, Toulouse, France) were routinely grown in RPMI 1640 medium supple-
Receptor-mediated Endocytosis of LDL—Human plasma LDL were fluorescently labeled with Dil (35), according to a previously described procedure to prepare radioactive SPM-labeled LDL (36). The fluorescent LDL preparation contained about 2.5 ng of DiI/apoprotein B. After a 24-h preincubation in the presence of RPMI 1640 medium containing 2% Ulsretor HY, cells were incubated for 30 min at 4, 14, or 37 °C in the presence or absence of 0.31 M NaCl or 0.4 M sucrose. Then, Dil-labeled LDL (about 40 μg/ml) were added to the cells and the incubation continued under the same conditions for an additional 60 min. The cells were washed with PBS three times and fixed with ice-cold phosphate-buffered saline containing 2 mg/ml bovine serum albumin and twice with phosphate-buffered saline alone. Non specific uptake of LDL was evaluated by incubation in the presence of a 1250 μg/ml of native, unlabeled LDL. The fluorescence intensity of the cellular lipid extract was determined using a Jobin-Yvon three-dimensional spectrofluorometer (at 520 and 568 nm for the excitation and emission wavelengths, respectively). Alternatively, the fluorescence of fibroblast monolayers grown on glass coverslips and treated as described above was examined using a Leitz Diaplan microscope using the appropriate filter package.

Metabolic Labeling of Cellular Choline Phospholipids—Cells were incubated in RPMI medium containing 1% fetal calf serum and [methyl-3H]choline (1 μCi/ml). After 48-h incubation, the radioactive medium was removed, and cells were chased for 2 h in 1% fetal calf serum enriched medium. Then, 30 min prior to addition of TNF-α (2 μM) or IL-1β (6 μM), cells were preincubated at the indicated temperature (4, 14, or 37 °C) and in the absence or presence of NaCl (final concentration 0.31 M or sucrose (0.4 M). After various times of incubation with the cytokine under the above-mentioned conditions, the cells were harvested (using a rubber policeman for fibroblasts) and sedimented by slow speed centrifugation. Cell pellets were immediately frozen at −20 °C.

Metabolic Labeling with [3H]Palmitic Acid—For estimation of ceramide, cells were incubated in RPMI medium containing 1% fetal calf serum and [3H]palmitic acid (1 μCi/ml). After 12-h incubation, the radioactive medium was removed, and cells were chased for 2 h in RPMI medium supplemented with 10% serum. After centrifugation at 15,000 g for 15 min (38), the supernatant was removed, and the cell pellets were washed once with PBS and then in a buffer containing 100 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 0.5 μM leupeptin. The cells were solubilized by lysing in 0.5% Triton X-100 and 0.5% sodium deoxycholate. The cell homogenates were assayed for protein content (40).

Lipid Extraction and Analyses—Cell pellets were suspended in 0.6 ml of distilled water and cells disrupted by brief sonication (10 s) using a Soniprep MSE probe sonicator. An aliquot was taken for protein content (40) determination (37). The remaining cell homogenate was extracted with a synthetic upper phase consisting of chloroform/methanol/water (2:1:0.8, by volume) and then petroleum ether/diethyl ether/acetic acid (80:20:2, by volume) as developing solvent systems. Radioactive lipids were localized on the plate using a Berthold LB radiomachromatic scanner. Lipid spots were scraped and counted by liquid scintillation (Packard Tricarb 4530 spectrometer) using 5 ml of Aquasafe 300 (Zinsser BAI). In some experiments, the mass of total phospholipids and SPM was quantitated by measuring their phosphate content (40).

Nuclear Extract Preparations—Nuclear extracts were prepared essentially as described (41). Briefly, cell pellets were homogenized at 4 °C in 400 μl of buffer A (10 mM Hepes, pH 7.8, 10 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). The cells were allowed to swell for 15 min on ice, after which Nonidet P-40 (0.6% final concentration) was added, and the tubes were vortexed. Nuclei were pelleted by centrifugation at 1200 × g for 20 min at 4 °C. The supernatant was discarded, and the nuclei were suspended in buffer A. After centrifuging at 2000 × g for 20 min, the nuclei were suspended in 25 μl of buffer C (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 10 μg/ml leupeptin) and incubated for 30 min on ice. After centrifuging at 15,000 × g for 20 min, the supernatant was collected and stored at −80 °C. Protein concentrations were determined using a bicinchoninic acid method (33).

Electrophoretic Mobility Shift Assays—Double-stranded NF-κB consensus oligonucleotide (Promega, Charbonnieres, France) was end-labeled using T4 polynucleotide kinase and [γ-32P]ATP according to the manufacturer’s instructions. Binding reactions were performed in a total volume of 20 μl containing radiolabeled oligonucleotide (200,000 cpm), binding buffer (with the following final concentrations: 2 mM Hepes, pH 7.8, 50 mM NaCl, 1 mM MgCl2, 0.3 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, 1.2 μg poly(dI-dC), and 0.4 μg of bovine serum albumin) and 5 μg of nuclear extract. After incubation for 20 min at room temperature, the samples were subjected to electrophoresis on a 4% acrylamide (acylamide/bisacrylamide, 30:1) gel for 3–4 h at 100 V/17 mA (42). Prior to sample loading, the gel was run for 1 h at 100 V/17 mA. After electrophoresis, the gel was dried by heating under vacuum and exposed to an x-ray film (Hyperfilm, Amersham) at −80 °C. Quantitation of the radiolabeled probe-NF-κB complexes was carried out by liquid scintillation counting of the gel bands.

RESULTS

Low Temperatures and Hypertonic Media Inhibit Receptor-mediated Endocytosis on Skin Fibroblasts and U937 Cells—Various treatments of cultured cells are known to severely reduce receptor-mediated endocytosis; these include incubation at temperatures below 15–18 °C (43) and treatment with hypertonic medium (44, 45). To document the effect of such perturbations on the receptor-mediated endocytosis by the cells we studied here, i.e., human skin fibroblasts and U937 cells, we monitored the uptake of fluorescently labeled LDL. As illustrated in Fig. IA, fibroblasts exposed to Dil-labeled LDL for 60 min at 37 °C displayed a strong intracellular fluorescence that was distributed into numerous perinuclear vacuoles, most probably corresponding to large endosomes (45). In contrast, fibroblasts incubated at 14 °C or at 37 °C in the presence of 0.31 M NaCl or 0.4 M sucrose showed a much lower and diffuse fluorescence that appeared to localize on the cell surfaces (Fig. 1, B–D). No fluorescent fibroblasts could be seen after incubation at 4 °C (data not shown). Quantitation of the fluorescence intensity using a spectrofluorometer (after subtracting the fluorescence due to nonspecific uptake) indicated that receptor-mediated endocytosis of Dil-labeled LDL by skin fibroblasts was inhibited by 99% at 4 °C, 96% at 14 °C, 98% in 0.31 M NaCl, and 91% in 0.4 M sucrose (mean of three to five separate experiments). Very similar results were obtained on U937 cells. These data corroborate previous observations (43–45) and demonstrate that in these particular two cell types low temperatures and hyperosmolarity strongly inhibit receptor-mediated endocytosis.

Low Temperatures Do Not Affect TNF-α-induced SPM Hydrol-
ysis—Initial studies examined the effect of low temperature on the triggering of SPM pathway by TNF-α or IL-1β in human skin fibroblasts. Our first experiments were performed at
In U937 cells, TNF-α action on a different cell type, the human U937 monocytic cell fibroblasts, subsequent experiments investigated the cytokine (data not shown). Comparable results were obtained after stimulation at 14°C and 4°C. Quite unexpectedly, at this temperature, both TNF-α and IL-1β were able to promote SPM (and phosphatidylcholine) degradation on skin fibroblasts (Fig. 2, C and D). Of note, the time course and extent of response were very similar to those obtained at 37°C.

Further evidence that the SPM pathway could be activated at low temperature was provided by measurement of ceramide production. Skin fibroblasts were metabolically labeled with [3H]palmitic acid as described under “Experimental Procedures.” After 48-h labeling, the medium was removed and cells chased in medium containing 1% fetal calf serum. Prior to addition of 2 nM TNF-α or 60 nM IL-1β cells were preincubated for 30 min at the indicated temperature. A control experiment was simultaneously carried out in the absence of cytokine. At the indicated time points, incubations were stopped and phosphatidylcholine (A and C) and SPM (B and D) levels (dpm/mg of cell protein) determined as described under “Experimental Procedures.” Total cell-associated radioactivity averaged 2,157,500 ± 174,600 dpm/mg protein. The phospholipid levels are expressed as percentage of the value observed at time 0. In the presence of TNF, the values correspond to the mean ± S.E. of four independent experiments (one representative experiment is shown for IL-1).

14°C. As illustrated in Fig. 2, TNF induced a SPM cycle at 14°C as well as at 37°C. At both temperatures, maximal hydrolysis (about 20–25%) was detected at 30 min, and cells chased in medium containing 1% fetal calf serum. Prior to addition of 2 nM TNF-α or 60 nM IL-1β cells were preincubated for 30 min at the indicated temperature. A control experiment was simultaneously carried out in the absence of cytokine. At the indicated time points, incubations were stopped and lipids were extracted, separated by thin-layer chromatography, and ceramide content (dpm/mg of cell protein) determined as described under “Experimental Procedures.” Values are expressed as percentage of radioactivity at time 0 (basal levels of ceramide averaged 89,690 ± 9870 dpm/mg of protein). The data are from one representative experiment out of two or three independent experiments.

To further document the role of receptor internalization in triggering of the SPM pathway, the effect of other treatments which inhibit endocytosis was studied. In particular, incubation of cells in hypertonic medium strongly reduces receptor-mediated endocytosis (Refs. 44 and 45; our data). We therefore investigated the effects of addition of 0.31 M NaCl or 0.4 M sucrose in the incubation medium of skin fibroblasts on the cytokine response. As illustrated in Fig. 5, even in the presence of hypertonic medium, both TNF-α and IL-1β could normally stimulate the hydrolysis of SPM (and phosphatidylcholine).

The results of Fig. 4, B and C, clearly demonstrate that activation of SPM (and phosphatidylcholine) degradation in response to TNF-α at 14 or 4°C was as effective as at 37°C. Such effects were further confirmed by determining the mass of cellular phospholipids and SPM. Lipid phosphorus assays indicated that at 4°C both total phospholipids and SPM contents similarly decreased by about 15 and 30% at the early and late cycles, respectively (data not shown).

Experimental Procedures. After 48-h labeling, the medium was removed and cells chased in medium containing 1% fetal calf serum. Prior to addition of 2 nM TNF-α, cells were preincubated for 30 min at the indicated temperature. At the indicated time points, incubations were stopped, total lipids were extracted, separated by thin-layer chromatography, and ceramide content (dpm/mg of cell protein) determined as described under “Experimental Procedures.” Values are expressed as percentage of radioactivity at time 0 (basal levels of ceramide averaged 89,690 ± 9870 dpm/mg of protein). The data are from one representative experiment out of two or three independent experiments.

Further evidence that the SPM pathway could be activated at low temperature was provided by measurement of ceramide production. Skin fibroblasts were metabolically labeled to equilibrium with [3H]palmitic acid and then stimulated with TNF-α. As shown in Fig. 3, addition of TNF at both 37 and 4°C resulted in ceramide generation. Peak effect was observed at 30 min, i.e. concomitant to maximal hydrolysis of SPM (see Fig. 2). Comparable results were obtained after stimulation at 14°C (data not shown).

While the above studies were performed on human skin fibroblasts, subsequent experiments investigated the cytokine action on a different cell type, the human U937 monocytic cell line, in which previous works have studied the SPM pathway (9, 11, 13, 19). In U937 cells, TNF-α activates two successive cycles of choline-phospholipid hydrolysis. As already reported (9, 10, 13, 46), stimulation of phosphatidylcholine degradation in U937 cells occurred at about 2 min and then at about 20 min after addition of TNF (Fig. 4A), maximal degradation being usually observed in the second cycle. TNF-α also stimulated two cycles of SPM hydrolysis which closely paralleled those of phosphatidylcholine (Fig. 4A).

We also observed two cycles of phosphatidylcholine and SPM hydrolysis in U937 cells after treatment with a chemotherapeutic agent (jaffrézou, j. p., levade, t., bettaieb, a., maestre, n., rousse, a., and laurent, g., submitted for publication).
dium simply retarded the activation of NF-κB. As shown in Fig. 6B, TNF-induced nuclear translocation of NF-κB in control cells was detected at 5 min, and a maximal effect was achieved after 30 min of stimulation (i.e., with comparable or slightly faster kinetics than for SPM degradation). Coincubation in hypertonic medium resulted in strong inhibition of NF-κB activation at all time points examined (Fig. 6B). This result raised the possibility that low temperature and hypertonicity may inhibit the steps necessary for ceramide to activate NF-κB translocation.

To further elucidate the absence of NF-κB activation under conditions which block endocytosis, cells were treated directly with C2-ceramide, a cell-permeant ceramide analog (6, 11, 14, 18, 20, 21) or with bacterial sphingomyelinase to generate ceramide in cell membranes (6, 15, 25). C2-ceramide activated NF-κB nuclear translocation in skin fibroblasts or U937 cells (Fig. 6C, lanes 3 and 4). Incubation of the cells with C2-ceramide at 4 °C or in hypertonic media resulted in significant inhibition of NF-κB activation (Fig. 6C, lanes 5 and 6); similar data were obtained when treating the cells by exogenous sphingomyelinase (Fig. 6D). Interestingly, NF-κB nuclear translocation by phorbol myristate acetate, a well-known activator of NF-κB (see, for instance, Ref. 41), was also inhibited by low temperature and hypertonicity (Fig. 6E, lanes 4–6). Thus, it can be concluded from these studies that these inhibitors also affect a step distal to both TNF and ceramide, which is required for NF-κB activation.

**DISCUSSION**

A large number of biochemical events induced by TNF-α or IL-1β have been reported to be mimicked by ceramide, supporting the assertion that the so-called SPM pathway could mediate the action of these two cytokines (1–4). The binding of TNF-α and IL-1β to their respective receptors is known to be rapidly followed by endocytosis (30, 31). However, the role of receptor internalization in TNF-α or IL-1β signal transduction in general, and in stimulation of the SPM pathway in particular, remains to be clearly established. We report here that the SPM pathway can be triggered under experimental conditions that rule out receptor internalization.

Previous reports have clearly demonstrated that receptor-mediated endocytosis is temperature-dependent (43) and inhibited by hypertonicity (44, 45). Use of these treatments was selected in our study because they are effective (see our data) and noninvasive methods to inhibit endocytosis, whereas experiments using metabolic poisons may be limited in their interpretation due to nonspecific effects unrelated to endocytosis. In addition, lowering the temperature below 17 °C or incubation in hypertonic medium have been reported to severely reduce the cellular internalization of TNF-α (30, 47, 48) and IL-1β (31, 49). In contrast, we demonstrate that under these conditions TNF-α and IL-1β activate SPM hydrolysis as well in two different cell types. At low temperature or in hypertonic medium, the kinetics and extent of SPM (and phosphatidylycerino
Effect of Inhibition of Endocytosis on Sphingomyelin Pathway and NF-κB

Fig. 6. Effect of low temperature and hypertonic media on the activation of NF-κB. In A, normal skin fibroblasts were left untreated (lane 1) or treated for the indicated times with 2 nM TNF-α at 37 °C (lanes 2–7), 4 °C (lane 8), 14 °C (lane 10) or in the presence of 0.4 M sucrose (lane 9). Nuclear extracts were prepared as described under “Experimental Procedures,” and equal amounts were incubated with [γ-32P]ATP-labeled NF-κB oligonucleotide probe. Specificity was determined by competitive experiments in the presence of 25- or 50-fold excess unlabelled NF-κB (lanes 3 and 4) or AP1 (lanes 5 and 6) consensus oligonucleotides. Similar results were obtained on U937 cells. In B, fibroblasts were left untreated (lane 1) or treated for the indicated times with 2 nM TNF-α in the absence (lanes 2, 4, 6, 8, and 10) or presence of 0.31 M NaCl (lanes 3, 5, 7, 9, and 11). In C, U937 cells were left untreated (lane 1) or incubated for 30 min with 3 nM TNF-α at 37 °C (lane 2) or treated for 60 min with 10 μM C2-ceramide at 37 °C (lane 3) or with 30 μM C2-ceramide at 37 °C (lane 4), at 4 °C (lane 5), or in the presence of 0.4 M sucrose (lane 6). In D, fibroblasts were left untreated (lane 2) or incubated for 30 min with 2 nM TNF-α at 37 °C (lane 1) or treated with 100 milliunits/ml sphingomyelinase from B. cereus (SPMase) for 30 min at 37 °C (lane 3), at 4 °C (lane 4), or in the presence of 0.4 M sucrose (lane 5) or 0.31 M NaCl (lane 6). In E, U937 cells were left untreated (lane 1) or incubated for 30 min with 2 nM TNF-α at 37 °C (lane 2) or treated with 0.2 μM phorbol myristate acetate (PMA) for 3 h at 37 °C (lane 3), at 4 °C (lane 4), or in the presence of 0.31 M NaCl (lane 5) or 0.4 M sucrose (lane 6). The gels are representative of two to six separate experiments.

Thus, the present data strongly suggest that cytokine-receptor internalization is not required for activation of the SPM pathway. This contrasts with the general belief that internalization of TNF is important for induction of cytolysis (28, 32, 50). TNF-induced gene expression in cultured human endothelial cells has also been reported to be dependent on endocytosis (48). However, there are reports describing biologic effects of cytokines under conditions where receptor internalization is excluded. For instance, microinjected TNF seems to exhibit cytotoxic activity (51). At low temperature, TNF can also stimulate the phosphorylation of a p26 cytokolic protein (33). The role of internalization in IL-1β signaling also remains unclear (49). A subclone of EL4 cells has been described that binds but does not respond to IL-1 possibly due to its inability to internalize the cytokine (52). However, Rosoff et al. (34) have reported that IL-1β induces a very rapid increase in diacylglycerol in Jurkat cells in the absence of detectable specific binding of the cytokine. In addition, internalization of IL-1β does not correlate with its ability to transduce the IL-1 signal for the induction of IL-2 gene expression, an important physiological effect of the hormone, since mutant receptors lacking most of the cytoplasmic domain still can bind and internalize IL-1, but they do not allow activation of the IL-2 promoter (53). Other instances where cytokine receptor internalization is not required for signal transduction include protein tyrosine phosphorylation induced by macrophage-colony-stimulating factor (54) or granulocyte-macrophage-colony-stimulating factor (55).

Our findings on activation of the SPM pathway in the absence of endocytosis have important biological implications. First, they suggest that SPM (and phosphatidylcholine) hydrolysis is a proximal event in TNF-α or IL-1β signal transduction, which likely occurs in close vicinity of the cytokine receptor, i.e. at the plasma membrane. Such a subcellular topology for cytokine-induced ceramide generation is perfectly consistent with previous suggestions for a tight coupling of the SPM pathway to TNF-α and IL-1 receptors (8, 15) and with recent observations that the signaling pool of SPM localizes to the plasma membrane (56).3 The second implication of our results is that the sphingomyelinase which is activated upon cytokine binding should be located at or near the plasma membrane. Two enzymes could potentially account for SPM hydrolysis at this subcellular site: the plasma membrane bound, neutral, magnesium-dependent sphingomyelinase (57), which has been re-

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3 N. Andrieu, R. Salvayre, and T. Levade, submitted for publication.
ported to be stimulated by TNF and vitamin D3 (2, 3, 13, 16, 58), and the (possibly translocated) cytosolic, neutral, magne
sium-dependent sphingomyelinase (4, 59). Whereas the question of which of these (or another, yet uncharacterized) neutral sphingomyelinases is actually activated still remains to be elucidated, our data make very unlikely the hypothesis (9, 13) for the involvement of an endolysosomal sphingomyelinase in cytokine signaling. This conclusion that add sphingomyelinase is probably not implicated in TNF-κB or IL-1β signal transduction is corroborated by two independent observations which examined different actions of the cytokines (26, 39). Finally, the evidence that at 4 °C, or in hypertonic medium, the SPM decrease induced by addition of TNF-α or IL-1β is followed by a recovery to the starting levels indicates that such a recovery is probably due to the reversibility of the SPM cycle rather than to the loss of cytokine activity (desensitization) by down-regulation of the cytokine receptors. Indeed, down-modulation of the TNF receptors is a result of the internalization of p60 receptor (60). Since the p60 receptor triggers the SPM pathway (10, 13, 61), it is therefore likely that, under conditions where internalization is blocked, the reversibility of SPM degradation is not due to down-regulation of the TNF receptor.

Another interesting feature of the present study is the surprising observation that SPM (and phosphatidylcholine) hydrolisis occurred at 4 °C (or 14 °C) as well as at 37 °C. Although many types of enzyme activity are reduced at 4 °C, there are some well documented examples of enzymes fully active at low temperature, e.g. tyrosine kinases (see Ref. 55 and references therein). However, to our knowledge, there has been no report of mammalian phospholipases in intact cells retaining their activity at 4 °C. Of note, some enzymes of lipid metabolism are active at low temperature, either in vitro or in vivo, such as snake venom (62) or Escherichia coli sphingomyelinase A2, and rat hormone-sensitive lipase (63). The fact that the phospholipid-degrading enzymes stimulated by cytokines are insensitive to low temperatures suggests that the SPM pathway might represent a crucial signal transduction pathway, possibly present in polymorphonuclear cells or at least able to support a cold adaptability.

Cytokine-dependent activation of transcription factors such as NF-κB is a central mechanism involved in transduction of the multiple biological actions of TNF-α and IL-1β (28, 29, 41). SPM hydrolysis and ceramide generation have recently been proposed as mediators of NF-κB signaling (9, 25), although ceramide might not be required nor sufficient for NF-κB activation (4, 20, 27, 64, 65). Under the conditions we used to inhibit receptor internalization, although cytokines were fully capable of inducing the degradation of SPM and the production of ceramide, NF-κB translocation to the nucleus was inhibited.4 This result can be in agreement with the observation that TNF-induced expression of various genes was decreased by inhibiting cytokine endocytosis (48).

Several hypotheses may account for the strong inhibition of NF-κB activation while the SPM pathway is activated. The first possibility is that the steps between ceramide and NF-κB activation are inhibited by cold and hypertonicity. Indeed, NF-κB activation triggered by exogenous sphingomyelinase or by C2-ceramide is also quite limited under these conditions. One could argue that low temperatures might inhibit the internalization of ceramide; however, hypertonic medium does not affect fluid-phase endocytosis (44). The observation that phorbol ester stimulation of NF-κB was also impaired under-

4 H. Chap, personal communication.

5 This result might explain why, in all the conditions we tested for blocking endocytosis, TNF-α was unable to induce apoptosis of U937 cells as followed by DNA fragmentation (data not shown).

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