Activation of Acid Sphingomyelinase by Interleukin-1 (IL-1)
Requires the IL-1 Receptor Accessory Protein*

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The cytokine interleukin-1 (IL-1) plays an important role in inflammation and regulation of immune responses, but the mechanisms of its signal transduction and cell activation processes are incompletely understood. Ceramide generated by sphingomyelinases (SMases) is known to function as an important second messenger molecule in the signaling pathway of IL-1 and tumor necrosis factor. To investigate the activation of SMases by IL-1, we used an IL-1 receptor type I (IL-1RI)-positive EL4 thymoma cell line, which is defective in IL-1R accessory protein (IL-1RACP) expression. In this cell line (EL4D6/76), tumor necrosis factor induced ligand/receptor internalization, NFκB nuclear translocation, IL-2 production, and the activation of neutral (N)-SMase and acid (A)-SMase. In contrast, stimulation with IL-1 resulted only in the activation of N-SMase whereas ligand/receptor internalization, NFκB translocation, IL-2 production, and activation of A-SMase were not detected. Transfection of this functionally defective EL4D6/76 with IL-1RACP cDNA restored these functions. These data suggest that A-SMase activity is strongly linked with the internalization of IL-1RI mediated by IL-1RACP and that A-SMase and N-SMase are activated by different pathways.

Interleukin-1 (IL-1) and tumor necrosis factor (TNF) belong to a group of pro-inflammatory cytokines with overlapping biological activities, which might be brought about by common signaling mechanisms (1–3). In the past few years, several groups have reported the involvement of sphingomyelin breakdown in the signaling of IL-1 and TNF (4–6). Ceramide generated by sphingomyelinases (SMases) is an important second messenger molecule in signal transduction pathways of IL-1 (7, 8), TNF (9), and CD28 (10). Ceramides appear to be involved in cell differentiation, apoptosis, and cell cycle arrest (11–13), e.g. ceramide was able to mimic interferon- γ and TNF effects in the differentiation of the monocytic cell line HL60 (14). Different types of cell-permeable ceramides induced apoptosis in various cell systems (15, 16). In cell cycle studies, C2-ceramides have been demonstrated to induce growth suppression by dephosphorylation of Rb (17, 18). For IL-1β, the involvement of sphingomyelin hydrolysis to ceramide and stimulation of a ceramide-activated protein kinase has been reported (19, 20). Synthetic cell-permeable ceramides or exogenous SMase have been shown to bypass IL-1 receptor activation in EL4 cells and mimic biologic activities of this cytokine (8). The activity of ceramide-activated protein kinase is directed to c-Raf-1 and appears to be activated by TNF and IL-1. Other targets of downstream signaling processes of ceramides are ceramide-activated protein phosphatase (21, 22) and protein kinase C (23). Additional events in the signaling cascade of ceramides are the phosphorylation of mitogen-activated protein kinase and activation of the c-Raf-1 kinase (24, 25).

Binding of TNF to the 55-kDa TNF receptor activated two different types of SMases, a membrane-associated neutral (N)-SMase and an endosomally located acid (A)-SMase (9). Structure-function analyses of the p55 TNF receptor revealed that the SMases are activated independently through different cytoplasmic domains of the receptor (26). Diacylglycerol (DAG) generated by a phosphatidylinositol-specific phospholipase C (PC-PLC) has been reported to serve as important factor of activation of A-SMase, which, through the generation of ceramide, is a co-factor for the activation of NFκB (27). A key event in NFκB activation is the rapid degradation of the inhibitory protein IκB. In a cell-free system, SMase and synthetic ceramide could directly induce IκB degradation, strongly indicating the involvement of SMase in NFκB activation (28). On the other hand, N-SMase seems to exert its signaling capacity via proline-directed protein kinases, like ceramide-activated protein kinase and mitogen-activated protein kinase, which acts in turn on phospholipase A2 (6).

IL-1 activity is represented by three structurally related molecules (3, 29, 30). IL-1α and IL-1β act in an agonistic manner and are internalized after binding to the receptor. The IL-1 receptor antagonist (IL-1Ra) blocks the binding of the agonist and inhibits the internalization of the receptor (31). Two types of receptors have been described with molecular masses of 85 kDa for the type I receptor (IL-1RI) and 65 kDa for the IL-1 type II receptor (IL-1 RII) (32), but binding to only IL-1RI has been shown to induce cell activation. IL-1RII does not trigger a signaling cascade and presumably inhibits IL-1 activity by acting as a decoy target for IL-1 (33, 34). Binding of IL-1 to the IL-1RI leads to the association of serine/threonine kinases (35, 36).

Recently an IL-1RI accessory protein (IL-1RACP) was described which does not bind IL-1 but associates with and increases the affinity of IL-1RI (37). We have previously described an IL-1RI-positive subclone of EL4 cells, EL4D6/76,
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FIG. 1. A, IL-2 production by EL4 5D3 and EL4D6/76 cells. Cells were incubated in culture medium or stimulated with 10 ng/ml PMA, 10 ng/ml PMA + 150 pg/ml rhIL-1α, or 10 ng/ml PMA + 100 ng/ml rmTNF. After 18 h, supernatants were collected and IL-2 production quantified by enzyme-linked immunosorbent assay. No IL-2 was detected after stimulation with 150 pg/ml rhIL-1α or 100 ng/ml rmTNF alone. Specific binding = PMA + IL-1/PMA or PMA + TNF/PMA. B, internalization of 125I-IL-1 (37°C) by EL4 5D3 and EL4D6/76 cells. Cells were incubated with 500 pCi 125I-IL-1α for 4 h at 37°C or 4°C. For determination of total cell-associated radioactivity, cells were centrifuged through an oil mixture (see "Experimental Procedures"). In a parallel reaction, surface-bound 125I-IL-1α was removed by a pH 3 washing step and the internalized radioactivity was measured in the cell pellet after centrifugation. In the present study, therefore, we investigated the activation of SMase by different components of the IL-1R complex. Evidence is provided that IL-1R internalization is required for the activation of the endosomal A-SMase. Ceramide produced by A-SMase provides an important signal for further downstream events like NFκB activation or IL-2 production. The lack of A-SMase activation may thus explain the unresponsiveness of the IL-1RI internalization-defective cells.

Cell Culture and Biological Reagents—EL4 cells and corresponding transfectants were cultured in RPMI 1640 containing 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in air with 5% CO2. For stimulation, 2.5 × 106 cells were seeded in 48-well plates at a density of 1 × 106 cells/ml. Human (h) recombinant (r) IL-1α (rhIL-1α) was kindly provided by Drs. A. Stern and P. Lomedico (Hoffmann-La Roche, Nutley, NJ). The specific activity was 5 × 1012 units/mg as determined by the lymphocyte activating factor assay and used at a concentration of 10 units/ml, representing a concentration of 150 pg/ml. Recombinant mouse (m) and human (h) TNF was a kind gift of Knoll AG (Ludwigshafen, Germany).

Cytokine Assay—IL-2 activity of the cell supernatants was quantified by enzyme-linked immunosorbent assay with the IL-2 Mini Kit (Biozol, Eching, Germany). The assay was performed according to the manufacturer’s instructions. The IL-2 detection limit was 50 pg/ml.

Internalization Assay—To measure the internalization of 125I-IL-1α, 2 × 106 cells were incubated for 4 h at 37°C or 4°C in 200 μl of medium, pH 7.4, containing 500 pCi 125I-IL-1α (Amersham-Buchler, Braunschweig, Germany). Nonspecific binding was determined by adding a 100-fold excess of unlabeled rhIL-1α. Cell surface-bound radioactivity was removed by washing the cells in medium, pH 3.0, for 2 min. Subsequently, the cells were centrifuged through a mixture of dibutyl phthalate and bis(2-ethylhexyl) phthalate (3:2) (Merck, Darmstadt, Germany). To determine the total cell-associated 125I-IL-1α, the cells were passed through the mixture of dibutyl phthalate and bis(2-ethylhexyl) phthalate without washing. Radioactivity in the cell pellets was measured using a γ-counter.

For detection of internalized 125I-TNF, 2 × 106 cells were incubated for 1 h at 4°C with 1 ng/ml 125I-TNF (recombinant TNF, NEN Life Science Products, specific activity 2160 kBq/μg) to saturate cell surface receptors. Nonspecific binding was determined by adding an 200-fold excess of unlabeled TNF together with 125I-TNF. After washing the cells three times in cold phosphate-buffered saline, temperature was shifted to 37°C to allow receptor internalization or kept at 4°C. To determine the amount of internalized 125I-TNF receptor complexes, noninternalized ligand was removed by centrifuging (50 × g) the cells through serial pH 3.0 gradients consisting of (a) 0.5 ml of culture medium supplemented with 20% Ficoll; (b) a second layer of 0.5 ml of 50 μM glycine-HCl, pH 3.0, 100 mM NaCl supplemented with 10% Ficoll; and (c) a third layer of 0.5 ml of culture medium containing 5% Ficoll. To determine the total amount of cell-associated 125I-TNF, a second aliquot of cells was passed through a gradient, in which the second layer was replaced by phosphate-buffered saline, pH 7.3, containing 10% Ficoll. Radioactivity of the cell pellets was determined by counting in a γ-counter.

Specific binding was calculated by subtracting nonspecific from total binding, and the amount of internalized 125I-ligands was calculated as percent of specific binding determined at neutral pH.

Electrophoretic Mobility Shift Assay—Following stimulation of cells (5 × 106 at 106 cells/ml density) for the times indicated in the figures, nuclear extracts were prepared according to Schreiber et al. (43). The protein concentration of the nuclear extracts was measured using a BCA assay (Pierce, Hamburg, Germany) with bovine serum albumin (Sigma, Deisenhofen, Germany) as standard protein. The double-stranded NFκB-specific oligonucleotide, containing two tandemly arranged NFκB binding sites of HIV long terminal repeat enhancer (5'-ATCCAGGGACTTTCCGGGAATTC-3') was end-labeled with [γ-32P]ATP (Amersham-Buchler, Braunschweig, Germany) using T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany) and purified with Nick columns (Pharmacia, Freiburg, Germany). Nuclear extracts (10 μg) were incubated for 15 min at room temperature in

which binds IL-1 with high affinity but does not respond with IL-1RI internalization or IL-2 production (38, 39). This defect can be overcome by intracellular delivery of IL-1 (40) or by transfection with IL-1RαcP, which reconstituted the IL-1RI internalization and functional defects (41, 42).

In the present study, therefore, we investigated the activation of SMase by different components of the IL-1R complex.
binding buffer (5 mM HEPES, pH 7.8, 5 mM MgCl₂, 50 mM KCl, 5 mM dithiothreitol, 10% glyceral, 50 mM poly(dI-dC), final volume 20 μL). The 32P-labeled double-stranded oligonucleotide was then added, and the reaction mixture was incubated for another 15 min. In competition experiments, an 200-fold excess of the unlabeled oligonucleotide and Oct2A site (5′-CTACGGAGATCTAGGCAATCTCCCTCTCCCTG-3′) was added. For supershift experiments, the binding reaction mix was incubated with the indicated amounts of antibodies for an additional 1 h. The samples were fractionated on a low ionic strength sucrose gradient and the proteins were detected by autoradiography.

FIG. 2. Activation of NFκB in EL4 5D3 and EL4D6/76 induced by IL-1α (A) and TNF (B). Cells were left untreated or incubated with 150 pg/ml IL-1α and 100 pg/ml TNF for the indicated times. Nuclear extracts were prepared, and NFκB binding was analyzed by electrophoretic mobility shift assay using a 32P-labeled NFκB binding site from HIV long terminal repeat.

This defect in IL-1 responsiveness was shown to correlate with a defect in internalization of receptor-bound IL-1. Only EL4 5D3 cells were able to internalize IL-1, whereas EL4D6/76 cells were deficient (Fig. 1B). To investigate whether this defect of EL4D6/76 cells is also specific for IL-1, we tested both cell lines for their capability to internalize TNF. Fig. 1C shows that TNF was internalized in both cell lines to the same extent. Activation of the IL-1RI by IL-1 leads to the rapid activation of the transcription factor NFκB. To examine whether the functional defects in IL-1 responsiveness correlated with defective NFκB activation, cells were stimulated with saturating concentrations of IL-1 and TNF. As shown in Fig. 2, TNF but not IL-1 was able to trigger the rapid activation of NFκB in EL4D6/76 cells, whereas the IL-1-responder EL4 5D3 responded to both stimuli equally well. Taken together, these data show the defect of internalization and function is specific for IL-1RI. Furthermore, the binding of IL-1 to the IL-1RI is not sufficient for triggering the nuclear translocation of NFκB.

Induction of SMase activity was shown to be a very early event after TNF receptor or IL-1R triggering. To address the question whether the activity of the N- and A-SMase is coupled to a functional receptor, cells were stimulated with TNF or IL-1 and the activities of the N- and A-SMase were measured. In IL-1-stimulated IL-1-responsive EL4 5D3 cells, the activity of the N-SMase peaked after 90 s (Fig. 3A), whereas the maximum of A-SMase activity was detected after 3 min (Fig. 3B). Interestingly, IL-1-stimulated N-SMase activity in IL-1-nonresponsive EL4D6/76 cells (Fig. 3C), whereas no increase of A-SMase activity was detected (Fig. 3D). Again, stimulation with TNF led to activation of both N-SMase and A-SMase in both sublines (Fig. 3, A–D). As we have shown before, both EL4 5D3 and EL4D6/76 cells were able to internalize and respond to TNF. These data suggested that IL-1R internalization and activation of A-SMase but not N-SMase are functionally coupled. The enzymatic activities of the crude preparations of A- and N-SMase were analyzed. The enzymes showed classical Michaelis-Menten kinetics with IL-1 not significantly affecting K_m, but increasing V_max of both SMases (Fig. 4).

Recently, we found that expression of IL-1RAcP in EL4D6/76 reconstituted IL-1 responsiveness with respect to internalization.
tion of IL-1 and IL-2 secretion (41). To investigate whether activation of A-SMase is linked to a functionally competent IL-1 receptor that is capable of receptor internalization, we used transfectants of the IL-1-nonresponding line EL4 D6/76, which stably expressed the IL-1RAcP. The ability to activate A-SMase in EL4D6/76 cells was also reconstituted in IL-1RAcP transfectants. As shown in Fig. 5, IL-1 did not stimulate A-SMase in EL4D6/76 cells. The corresponding IL-1RAcP transfectants, however, showed the typical A-SMase activation pattern with maximum activity at 3 min after IL-1 stimulation. A-SMase through production of ceramide provides an important cofactor for NFκB activation. When the IL-1-responsive EL4 5D3 and IL-1-nonresponsive EL4D6/76 were stimulated with IL-1, NFκB was activated in EL4 5D3 but not in the nontransfected EL4D6/76 (Fig. 6A). Four corresponding IL-1RAcP transfectants, however, clearly showed activated NFκB after stimulation with IL-1 (Fig. 6A). The identity of NFκB was confirmed in competition experiments with a 200-fold excess of unlabeled NFκB oligonucleotides in two representative IL-1RAcP transfectants (Fig. 6B). In contrast, a 200-fold excess of cold Oct2A oligonucleotide had no inhibitory effect on the formation of the NFκB complex. In supershift experiments, an anti-RelA antibody specifically inhibited the formation of the NFκB complex. An anti-RelB antibody was not able to replace the complex, indicating the involvement of the p65 rather than p68 subunit in the formation of the NFκB complex (Fig. 6B).


During the last few years, the importance of ceramides as second messenger molecules generated by the breakdown of sphingomyelin has become evident (5, 6). Previous studies indicated that the TNF signal activates two forms of sphingomyelinases, a membrane-bound N-SMase and DAG-dependent endo/lysosomal A-SMase (27). These two forms are triggered independently from each other and lead into different signaling pathways (9). A-SMase has been identified as a candidate for activation of NFκB and IL-2 production. In contrast, A-SMase activation selectively; neither N-SMase activity nor PC-PLC was affected (27).

As IL-1 is another potent activator of SMases and NFκB, we, therefore, investigated the activation of A- and N-SMase by IL-1 and the relation to different components of the IL-1RI in two sublines of the EL4 thymoma cell line. The subline EL4 5D3 can be activated by IL-1, whereas EL4D6/76 cannot be activated although high affinity IL-1 binding sites are present (39). The defects in internalization of the IL-1R complex, in activation of A-SMase, and in nuclear translocation of NFκB are not essential for NFκB activation. Thus, ceramide per se might not be an activator of NFκB but only ceramide generated by A-SMase in a distinct cellular compartment. The importance of compartmentalization is underlined by investigations of Liu et al. (44). They have shown that IL-1β stimulated DAG and ceramide production only in caveolae fractions of fibroblasts. DAG induced by IL-1 in other cellular fractions was not coupled to ceramide production. In our experiments which are not shown in this paper, incubation with C2- and C8-ceramides did not activate NFκB in both cell lines. Therefore, since A-SMase appears to be required for IL-1-induced NFκB activation and ceramide analogs do not induce this event, ceramide might be a necessary but not sufficient co-signal for NFκB activation. We also found previously that exogenous sphingomyelinases or sphingosine were not able to co-stimulate IL-2 production in EL4 cells (45). Therefore, it might be possible that small amounts of A-SMase-derived ceramide in specialized compartments contribute to activation of NFκB or IL-2 production. In A-SMase-deficient Niemann-Pick fibroblasts, however, NFκB activation is induced by IL-1, indicating that A-SMase activity is not essential for NFκB activation (46).

In addition to internalization of IL-1 and IL-2 production, we found that IL-1-stimulated A-SMase activity was also reconstituted by transfection in four independent stable transfectants of EL4D6/76 cells (Fig. 5). Simultaneously, the activation of IL-1 and IL-2 production, we found that IL-1-stimulated A-SMase activity was also reconstituted by transfection in four independent stable transfectants of EL4D6/76 cells (Fig. 5). Simultaneously, the activity of NFκB was restored, strongly supporting the existence of a link between A-SMase activity and NFκB activation. Thus, A-SMase activity, in contrast to N-SMase activity, correlated with internalization of a functional IL-1RI complex. The data suggest that A-SMase activation requires a functional receptor complex that is capable of receptor internalization.

**DISCUSSION**

**FIG. 4. IL-1α stimulates the increase of activities of N- and A-SMase in EL4 5D3 cells.** A, cells were left untreated or stimulated with IL-1α for 1.5 min. Membrane fractions were prepared as described under "Experimental Procedures." Protein (15 μg) was incubated in triplicate for 1 h at 37 °C with varying concentrations of [N-methyl-14C]sphingomyelin (0.15–90 μM). Lipids were extracted, and radioactive phosphorylcholine was quantified in the aqueous phase by lipid scintillation counting. The rate of sphingomyelin breakdown was calculated and the results were plotted double reciprocally. Shown are values of representative experiment (n = 3). B, cells were left untreated or stimulated with IL-1α for 3 min. Cell lysates were prepared for A-SMase assays. Protein (10 μg) was incubated for 1 h at 37 °C with varying concentrations of [N-methyl-14C]sphingomyelin (0.15–90 μM). Enzymatic activities were determined and plotted double reciprocally. Shown are values of representative experiments (n = 3). C, Kₘ and Vₘₐₓ were calculated from A and B according to Lineweaver and Burk (52).
The need of internalization for cytokine action is controversially discussed in the literature. Endocytosis is reported to play a critical role in TNF-induced gene expression and induction of cytolysis (47–49). In EL4 cells, IL-1 signaling and internalization correlate (38, 39) and an intracellular activation loop of IL-1 seems to be operative in EL4 (40). On the other hand, in Jurkat cells, internalization and nuclear localization of IL-1 was not sufficient for activation of the IL-2 promoter (50). Andrieu et al. (51) suggest that cytokine-receptor internalization is not required for activation of the sphingomyelin pathway because they found similar degradation of sphingomyelin and generation of ceramide in cells when endocytosis was blocked by low temperature and hypertonicity. These data, however, do not exclude the requirement for the internalization of the receptor complex to activate A-SMase, as there was no distinction made between ceramide produced by A- or N-SMase. It is therefore possible that the increased ceramide level results from N-SMase activity, which in our experiments did not require IL-1R internalization.

A possible mechanism for the activation of A-SMase is indicated by data obtained from studies with caveolae fractions. The caveola is a membrane domain that can undergo an internalization cycle. Invagination of the membrane is followed by the formation of plasmalemmal vesicles, which provide an optimal microenvironment for activation of A-SMase. IL-1b-stimulated production of DAG in a caveola-rich membrane fraction of whole fibroblasts. This was followed by a degradation of sphingomyelin and a concomitant increase of ceramide. Additionally, A-SMase activity could be detected in the caveolae fractions (44). In TNF signaling, the activation of A-SMase by 1,2-DAG generated by membrane-located PC-PLC has been reported (27). Thus, activation of A-SMase by IL-1 may occur via co-internalization of 1,2-DAG with the caveolae-associated IL-1/IL-1RI complex, if PC-PLC activation occurs in close vicinity to the membrane receptors.

In conclusion, the present study shows that the IL-1-induced increase in A-SMase activity and concomitant activation of NFκB are dependent on the presence of IL-1RAcP. Ceramide

![Figure 5](image5.png) **FIG. 5.** Reconstitution of IL-1 induced A-SMase activity in EL4D6/76 by transfection with IL-1RAcP. EL4D6/76 cells were transfected with IL-1RAcP cDNA and stable transfectants selected. EL4 D6/76 parental cells and four independent clones (EL4 1F4, EL4 1G6, EL4 10B5, and EL4 10G12) expressing IL-1RAcP mRNA were stimulated for the indicated periods of time with 1.5 ng/ml IL-1α or left untreated for control and A-SMase activity was assayed as described under “Experimental Procedures.” A-SMase activity is expressed in percentage of control. The standard errors were always lower than 4% of the mean.

![Figure 6](image6.png) **FIG. 6.** Reconstitution of IL-1-mediated NFκB activation in EL4D6/76 when transfected with IL-1RAcP. The NFκB complex was confirmed in competition experiments and supershift analyses. A, EL4 5D3, EL4 D6/76, and stably IL-1RAcP transfected EL4D6/76 cells (EL4 1F4, EL4 1G6, EL4 10B5, and EL4 10G12) were either left untreated or stimulated for 1 h with 150 pg/ml IL-1. After the indicated periods of time, nuclear extracts were prepared and NFκB binding activity was detected by EMSA using the 32P-labeled NFκB binding site from HIV long terminal repeat. B, two transfectants (EL4 10B5 and EL4 10G12) were left untreated or stimulated with 150 pg/ml IL-1 for 1 h before nuclear extracts were prepared. Again, NFκB binding activity was detected by EMSA. Additionally, competition experiments with unlabeled NFκB and Oct2A oligonucleotides, respectively, were performed. The nuclear extracts were incubated with the radiolabeled NFκB binding oligonucleotide, and either a 200-fold excess of unlabeled NFκB or Oct2A site before the reaction mix was separated by gel electrophoresis. In supershift experiments, 1 μg of anti-RelA or anti-RelB antibody was added to the reaction mix for an additional 1 h before separation by gel electrophoresis.
produced by A-SMase, therefore, might represent the functional link between IL-1RI internalization and activation of NFκB and IL-2 production.

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