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Tumor Necrosis Factor α Activates NF-κB in Acid Sphingomyelinase-deficient Mouse Embryonic Fibroblasts*

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Tumor necrosis factor α (TNF-α) is one of the most potent inducer of the nuclear transcription factor κB (NF-κB). Activation of NF-κB is initiated by phosphorylation of the inhibitory subunit of the IκB-α-NF-κB complex. This leads to the dissociation of the complex and degradation of IκB-α. NF-κB is translocated into the nucleus. The sphingomyelin pathway is thought to mediate the TNF-α-induced activation of NF-κB by its second messenger ceramide. We have used the recently established acid sphingomyelinase-deficient mouse line (*asmase*$^{-/-}$ mice) to evaluate the role of acid sphingomyelinase in the TNF-α-induced signal transduction pathway. Here we present experimental evidence that acid sphingomyelinase is not involved in the TNF-α-induced activation of NF-κB. TNF-α treatment induced the dissociation and degradation of IκB-α and the nuclear translocation of NF-κB in embryonic fibroblasts derived from *asmase*$^{-/-}$ and wild type mice indiscriminately.

A ubiquitous, evolutionarily conserved signaling pathway with sphingomyelin as precursor lipid for the second messenger ceramide has been postulated. It is comparable with the other well known signal transduction systems (for reviews, see Refs. 1–3).

The second messenger ceramide is generated by hydrolysis of sphingomyelin by sphingomyelinases. At least two sphingomyelinases are reported to participate in signal transduction, acid (ASM)[1] and neutral sphingomyelinase (NSM). Acid sphingomyelinase is a lysosomal hydrolase with a pH optimum of 4.5–5.5 required for membrane turnover (4). The neutral, Mg^{2+}-dependent isoform (pH optimum 7.4) is localized on the outer leaflet of the plasma membrane (5). While the ASM is well characterized at the molecular level (6, 7) and an ASM null-allelic mouse model is available (8, 9), the NSM has only partially been purified (10, 11).

Sphingomyelinase activation has been linked to several cell surface receptors (*e.g.* the 55-kDa TNF-α receptor (TR55) or the 80-kDa interleukin 1 receptor (2)). Activation of ASM has been described for TNF-α, Fas, and CD28 (12–14), and of NSM for TNF-α, vitamin D_3, interleukin 1–1β, and ionizing radiation (12, 15–17).

As direct targets for ceramide action ceramide-activated protein kinase (18), ceramide-activated protein phosphatase (19), protein kinase C-ζ (20, 21) and the putative guanine nucleotide exchange factor Vav (22) have been identified.

Ceramide is thought to mediate several physiological effects in different cell types, including the activation of the transcription factor NF-κB (23), induction of apoptosis (24, 25), and mitogenic signaling (26).

NF-κB, first described as a B-cell-specific factor responsible for expression of the immunoglobulin-κ gene (27), participates in the regulation of several genes, most of them being involved in the early events of immune, acute phase, or inflammatory responses (for review, see Ref. 28). NF-κB is also responsible for the transcriptional activation of many viral genes, *e.g.* of the human immunodeficiency virus-1 (29). Inactive NF-κB is a heterodimer consisting of a 50-kDa and a 65-kDa subunit retained in the cytoplasm by association with a 36-kDa inhibitory subunit IκB-α (30–32). This complex can be activated by several effector molecules like TNF-α or interleukin 1 (33), starting with serine phosphorylation and subsequent ubiquitination and proteolytic degradation of IκB-α (34–37). The free NF-κB dimer translocates into the nucleus and activates the transcription of several genes (28).

Recently it has been reported that different receptor domains of the TR55 activate the different sphingomyelinases (12). Truncated TR55 mutants, which lack the so-called death domain, an 80-amino acid residue C-terminal sequence, neither showed NF-κB activation nor an increase of ASM activity, while the NSM activation was not affected when expressed in 70Z/3 cells, suggesting that activation of NF-κB is mediated exclusively by the acid sphingomyelinase (12, 23). Following these results activation of NF-κB by TNF-α should be precluded in ASM-deficient cells.

In the present study we used primary embryonic fibroblasts (EMFIs) from the recently established ASM-deficient mouse line (8) (*asmase*$^{-/-}$ EMFIs) to elucidate the role of the acid sphingomyelinase in the TNF-α-induced activation of NF-κB.

In contrast to previous reports (12, 23), we found no moderation of the NF-κB activation pathway in the response to TNF-α in cells derived from *asmase*$^{-/-}$ mice in comparison with wild type mice.

Neither changes in ASM or NSM activitiy nor alterations in sphingomyelin or ceramide concentration have been determined after TNF-α treatment.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—EMFIs (fourth to sixth passage) derived from wild type (C57/Bl6) and *asmase*$^{-/-}$ (C57/Bl6 × 129/01a *asmase*$^{-/-}$)

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[1] The abbreviations used are: ASM, acid sphingomyelinase; NSM, neutral sphingomyelinase; TNF-α, tumor necrosis factor α; NF-κB, nuclear transcription factor κB; IκB-α, inhibitory subunit; TR55, 55-kDa TNF receptor; DTT, dithiothreitol; BSA, bovine serum albumin; PBS, phosphate-buffered saline: BCA, bicinchoninic acid; TLC, thin layer chromatography; EMFIs, embryonic fibroblasts; HEK, human embryonic kidney; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s).

e 14 embryos were grown in Dulbecco's modified Eagle's medium (Seromed) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator containing 5% CO_2.

Rabbit polyclonal anti-NF-κB p65, rabbit polyclonal anti-NF-κB p50, and rabbit polyclonal anti-IκB-α antibodies were purchased from Santa Cruz Biotechnology Inc.

Alkaline phosphatase-labeled goat anti-rabbit IgG second antibody, phenylmethylsulfonyl fluoride, benzamidine, pepstatin A, and DTT were purchased from Sigma and [γ-^{32}P]ATP and [α-^{32}P]dATP from Amersham Corp.

Recombinant human TNF-α (specific activity, 6.6×10^6 units/mg) was kindly provided by BASF/Knoll, Germany.

Cell Stimulation and Preparation of Nuclear and Cytoplasmic Extracts—EMFIs were grown in serum-free medium for 4 h prior to stimulation, and 100 ng/ml medium TNF-α was added for the indicated times. Stimulation was stopped by removing the medium and washing the cell layer with cold PBS.

Proteins were fractionated as described previously (33) with some modifications. Briefly, cells were scraped with a rubber policeman into fresh, cold PBS and washed twice. After low-speed centrifugation ($200 \times g$) the cell pellet was resuspended in lysis buffer (10 mM Tris/HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine) and placed for 10 min on ice. Nuclei were separated from the cytoplasm by centrifugation at 2500 rpm in a microcentrifuge (Eppendorf) at 4 °C for 5 min, washed briefly with lysis buffer without Nonidet P-40, resuspended in nuclear extraction buffer (20 mM Tris/HCl, pH 8.0, 20 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol), and lysed by adding NaCl to a final concentration of 400 mM. The suspension was kept for 10 min on ice, vortexed, and centrifugated for 10 min (14.000 rpm). The supernatant contained the nuclear protein extract.

The supernatant of the first centrifugation step, which contained the cytoplasmic fraction, was centrifugated at 14,000 rpm and the resulting supernatant used for Western blot analysis.

Western Blot Analysis—50 μg of protein of cytoplasmic extract was subjected to SDS-polyacrylamide gel electrophoresis on 15% polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell) for 3 h with 1 mA/cm^2. The membrane was blocked for 2 h with 3% BSA in Tris-buffered saline and incubated overnight at 4 °C with 300 ng of anti-IκB-α polyclonal antibody/ml of Tris-buffered saline, 1% BSA. After incubation with the second antibody for 1 h at room temperature in Tris-buffered saline, 1% BSA, the bands were stained using 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitro blue tetrazolium (Boehringer Mannheim).

Electrophoretic Mobility Shift Assay—5–10 μg of nuclear proteins (determined by the BCA assay (Pierce)) were incubated with 3 μg of poly(dI-dC) (Boehringer Mannheim), 0.4 pmol of double-stranded ^{32}P-labeled oligonucleotide containing the NF-κB binding site of the human immunodeficiency virus long terminal repeat (5′-ATCAGGGACTTTCGCTGGGGACTTTCCG-3′) in a total volume of 20 μl in a buffer containing 20 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 40 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, and 10% glycerol. After 30 min at room temperature samples were separated on native 4% polyacrylamide gels using 0.25 × TBE (0.0225 M Tris borate, 0.0005 M EDTA, pH 8.0) as running buffer. Competition studies were carried out with two unrelated double-stranded oligonucleotides A (5′-ATCAAGATATGAAAGAGTCTGAACATAGCACCA-3′) and B (5′-GTCTTATTCACTGCTAGCAACCATGGGCTGAAG-3′). For supershift analysis the NF-κB-double-stranded oligodeoxynucleotide complexes were further exposed to a polyclonal antibody recognizing the p50 subunit of NF-κB. Gels were dried, and radioactive signals were analyzed with a PhosphorImager (Molecular Dynamics).

Immunofluorescence Microscopy—EMFIs from wild type and *asmase*$^{-/-}$ mice were grown in serum-free medium for 4 h and stimulated for 20 min with 100 ng/ml TNF-α. After discarding the medium, cells were fixed with a mixture of acetone/methanol (1:1) and incubated with anti-NF-κB p65 antibody (300 ng/ml in PBS, 3% BSA) for 1 h. Cells were washed three times with PBS, 0.5% Triton X-100, incubated with a Cy3-coupled anti-rabbit IgG antibody (Jackson ImmunoResearch) for 1 h and after further washings analyzed under a fluorescence microscope (Zeiss).

Northern Blot Hybridization Analysis—40 μg of total RNA prepared by the guanidine thiocyanate-phenol method (38) was separated on a 2% formaldehyde-agarose gel and blotted onto a GeneScreen Plus nylon-membrane (DuPont) following the manufacturer's protocol.

The blot was probed with a 125-bp fragment derived from the 5′ end of the IκB-α cDNA sequence and a 348-bp fragment derived from the β-actin cDNA.

RT-PCR Analysis—For first strand cDNA synthesis RNA was transcribed in a reverse transcriptase reaction. The reaction mixture contained in a total volume of 10 μl: 0.5 mM of each deoxynucleotide (Boehringer Mannheim), 5 μM random hexanucleotide primers (Boehringer Mannheim), 10 mM DTT (Life Technologies, Inc.), 20 units of RNase inhibitor (Promega), 100 units of Superscript II (Life Technologies, Inc.), and 100 ng of total RNA in a reverse transcriptase buffer (Life Technologies, Inc.). Samples were incubated for 1 h at 37 °C.

One-half of the RT reaction mixture was used for the PCR reaction with the primers mIκB-s (5′-GCCCCGCACAGCCATGTTTCAG-3′) and mIκB-as (5′-CATGGAGTCCAGGCCGCTGTCGTG-3′) for IκB-α. The other half was used for the β-actin control with the oligonucleotides mβ-actin-s (5′-TGGAATCCTGTGGCATCCATGAA-3′) and mβ-actin-as (5′-TAAAACGCAGCTCAGTAACAGTC-3′).

Fragments were amplified in a thermal cycler (Perkin-Elmer) with the following program: 5 min 94 °C (1 ×); 45 s 94 °C, 60 s 60 °C, 90 s 72 °C (25 ×); 10 min 72 °C (1 ×).

PCR products were separated on a 2% TBE-agarose gel and analyzed under UV light.

Sphingomyelinase Assays—EMFIs were incubated with 100 ng/ml TNF-α, and ASM activities were measured following a modified method described previously (39). Briefly, 4×10^6 cells were grown in serum-free medium for 4 h and incubated with TNF-α for 2 and 4 min, respectively (12). The medium was discarded, cells were scraped into cold PBS, washed once, resuspended in 200 μl of 0.2% Triton X-100, and placed on ice for 10 min. After centrifugation at $800 \times g$ for 5 min, 10 μg of protein of the supernatant was diluted to a final volume of 90 μl with buffer (250 mM sodium acetate, pH 4.5, 1 mM EDTA). 10 μl of substrate (1 nmol of [*N*-$^{14}CH_3$]sphingomyelin/μl of buffer (specific activity, 8000 dpm/nmol) (40)) was added and the mixture incubated for 60 min at 37 °C.

For detection of NSM activity (12, 41), cells were stimulated with TNF-α for 1 and 2 min, transferred into cold PBS, washed, resuspended in 200 μl of buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 10 mM $MgCl_2$, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 750 μM ATP, 0.2% Triton X-100), and placed for 10 min on ice. Conditions of incubation and enzyme assay were as described for ASM except that 10 μg of protein was resolved in 20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, and incubation time was extended to 120 min. [^{14}C]Phosphorylcholine was extracted by adding 200 μl of H_2O and 800 μl of chloroform/methanol (2:1) mixture, and the radioactivity of the aqueous phase was determined by scintillation counting.

Radioactive Labeling of Cells—Fibroblasts were metabolically labeled with [1-^{14}C]palmitic acid (specific activity, 0.81×10^7 dpm/mg, 20 μg/ml culture medium) for 36 h. Before TNF-α stimulation the medium was removed, and cells were grown in serum-free medium for 4 h.

Lipid Extraction and Thin Layer Chromatography—Stimulation of cells was stopped by removing the medium and immediate washing with cold PBS. Cells were scraped from the culture dishes, centrifugated, and the cell pellet suspended in chloroform/methanol (2:1) for lipid extraction. Phases were separated by centrifugation for 10 min at $4000 \times g$. The organic phase was dried under nitrogen, and lipids were dissolved in chloroform/methanol (2:1). Radioactive lipids were measured in a liquid scintillation counter (Wallac 1409), and aliquots were separated on TLC plates (Merck) in chloroform/methanol/water (65:25:4). Radioactivity was detected and quantified with a PhosphorImager (Molecular Dynamics) using internal standards.

RESULTS

We studied the role of sphingomyelin and ASM within the postulated signal transduction chain initiated by TNF-α and leading to the NF-κB activation in EMFIs derived from wild type and *asmase*$^{-/-}$ mice. The genotype of *asmase*$^{-/-}$ EMFIs was confirmed by Southern blot hybridization analysis of the *Eco*RI-restricted DNA using the previously described probe (8). In addition the ASM activity of wild type and the null mutant fibroblasts was assayed using [*N*-$^{14}CH_3$]sphingomyelin as substrate. The *asmase*$^{-/-}$ fibroblasts showed no ASM activity.

Southern blot analysis and enzyme assay therefore clearly proved the complete ASM deficiency of the mutant EMFIs.

Degradation and Resynthesis of IκB-α after TNF-α Stimulation—TNF-α rapidly activates NF-κB (28) and leads to degradation of IκB-α (33, 37), which is rapidly resynthesized. We studied first the kinetics of IκB-α degradation of wild type and

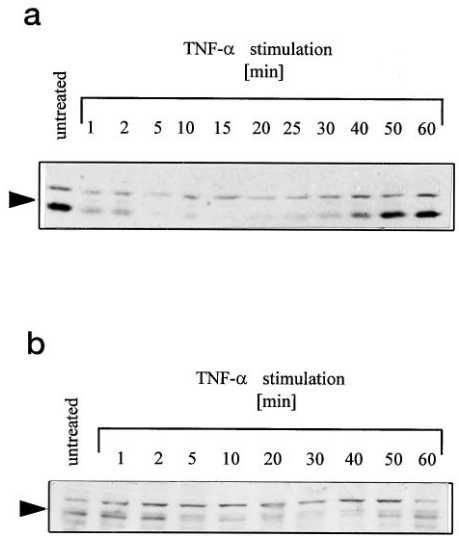

FIG. 1. **TNF-α induces IκB-α degradation in wild type and *asmase*$^{-/-}$ EMFIs.** Western blot analysis of cytoplasmic extracts of wild type (*a*) and *asmase*$^{-/-}$ EMFIs (*b*). Cells were stimulated for the indicated times with TNF-α. Cytoplasmic extracts were prepared and analyzed for IκB-α. The *arrows* indicate the specific 36-kDa signal.

asmase$^{-/-}$ EMFIs after TNF-α stimulation over the time period indicated in Fig. 1. Western blot hybridization analysis showed the disappearance of the IκB-α signal within 10–20 min identically in cells with both genotypes. Resynthesis restored the IκB-α level within 60 min to the level of the unstimulated cells. IκB-α was recognized by the polyclonal antibody as a 36-kDa protein.

We also monitored the NF-κB-induced resynthesis of IκB-α following its degradation (28, 42, 43) by measuring the increase of IκB-α transcription in Northern blot hybridization analysis (Fig. 2*a*). IκB-α mRNA was hardly measured in unstimulated wild type and *asmase*$^{-/-}$ cells. However within 60 min after TNF-α stimulation a strong IκB-α mRNA signal appeared in wild type and in the mutant fibroblasts. Relating the IκB-α mRNA signals with the respective constitutively expressed β-actin signal and quantification of the signals by phosphorimaging clearly indicated that *asmase*$^{-/-}$ and wild type EMFIs showed an identical expression rate.

IκB-α is constitutively expressed at a very low level. The low concentration of mRNA was detected by amplification of a specific 125-bp fragment by RT-PCR. Fig. 2*b* shows the RT-PCR pattern of EMFIs of the wild type and *asmase*$^{-/-}$ genotype, quiescent and after TNF-α stimulation for 30 and 60 min, respectively. Identical patterns of the expected 125-bp PCR fragment were observed in wild type and *asmase*$^{-/-}$ EMFIs, the intensity of the 348-bp β-actin PCR fragment in all lanes proved the Northern blot hybridization analysis.

Nuclear Translocation of NF-κB—TNF-α stimulation induces the dissociation of the IκB-NF-κB complex liberating NF-κB for nuclear translocation and DNA binding (28). We followed the kinetics of NF-κB nuclear translocation and DNA binding in the electrophoretic mobility shift assay. Double-stranded, ^{32}P-labeled oligodeoxynucleotides resembling the NF-κB binding site of the human immunodeficiency virus long terminal repeat were incubated with nuclear protein extracts of wild type and *asmase*$^{-/-}$ EMFIs stimulated with TNF-α for the time periods indicated in Fig. 3*a*.

Within 5 min of TNF-α stimulation the strong nuclear import reached a plateau, which was stable during prolonged TNF-α stimulation. The intensities of NF-κB oligonucleotide complexes formed in nuclear extracts of wild type and *asmase*$^{-/-}$ EMFIs were identical.

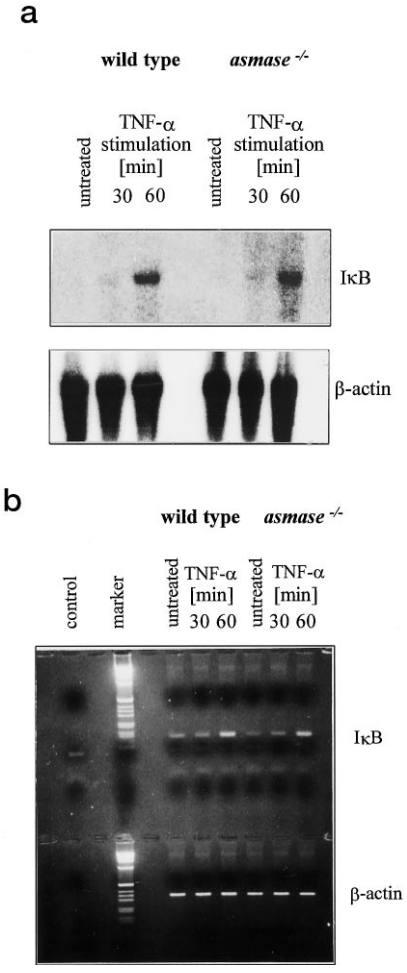

FIG. 2. **IκB-α is resynthesized after prolonged TNF-α stimulation.** Northern blot hybridization analysis of total RNA of wild type and *asmase*$^{-/-}$ EMFIs (*a*). Cells were treated for the indicated times with TNF-α. RNA was prepared, and the blot was probed with specific fragments for detection of IκB-α transcripts and the constitutively expressed β-actin. While the IκB-α signals were absent in unstimulated cells, transcription increased during prolonged TNF-α stimulation. Low constitutive levels of IκB-α transcription were detected by RT-PCR (*b*). After TNF-α stimulation the intensities of the signals increased. This is in agreement with the data documented by Northern blot hybridization. The specific 125- and 348-bp bands were indicative for IκB-α and β-actin, respectively. The control lane shows a PCR without template.

The specificity of the signals was proven in the control experiments (Fig. 3*b*). For an *in vivo* proof the nuclear import of NF-κB after TNF-α stimulation in wild type and *asmase*$^{-/-}$ EMFIs was followed by immunofluorescence microscopy. After TNF-α stimulation cells were permeabilized and incubated with the anti-NF-κB p65 antibody and subsequently with a Cy3-conjugated anti-rabbit IgG second antibody.

The cellular distribution of the NF-κB-α signal in untreated and TNF-α-stimulated wild type and *asmase*$^{-/-}$ EMFIs is compared in Fig. 4. The nuclear staining of both TNF-α-treated cells (Fig. 4, *b* and *d*) was equal, whereas the untreated cells (Fig. 4, *a* and *c*) showed no nuclear immunofluorescence.

The immunocytochemical data fully support the results of our biochemical analysis. After TNF-α stimulation, wild type as well as mutant EMFIs indiscriminately translocated NF-κB within a short time into the nucleus for DNA binding.

Sphingomyelinase Activity after TNF-α Stimulation in Wild Type and asmase$^{-/-}$ *EMFIs*—Previous reports indicate that TNF-α stimulation of U937 cells increases NSM activity 2–3-fold within 90 s and ASM activity within 4 min (12). A rapid 70% increase of ceramide concentration within 2–3 min and a

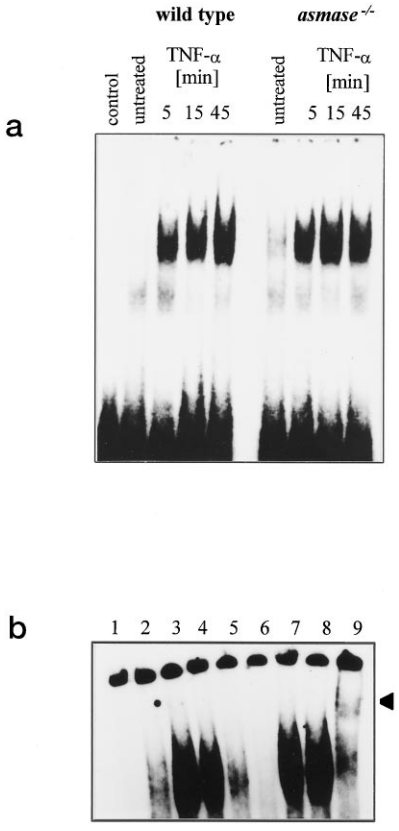

FIG. 3. **NF-κB is translocated into the nucleus shortly after TNF-α stimulation.** Electrophoretic mobility shift assay of wild type and *asmase*$^{-/-}$ EMFIs (*a*). Cells were stimulated for the indicated times with TNF-α, and NF-κB binding activity in nuclear extracts was tested. The control lane contained no protein. For proving the specificity of the NF-κB signal (*b*), wild type EMFIs were left untreated or stimulated with TNF-α for 10 min and nuclear extracts were prepared and 10 μg subjected to electrophoretic mobility shift assay. *Lane 1*, control without protein. *Lane 2*, extracts from untreated cells. *Lane 3*, extracts from TNF-α-treated cells. *Lane 4*, same exctracts as in *lane 3* but with addition of equal amount of unlabeled oligonucleotide. *Lane 5*, same extracts as in *lane 3* with 10-fold molar excess of unlabeled oligonucleotide. *Lane 6*, same extracts as in *lane 3* with 100-fold molar excess of unlabeled oligonucleotide. *Lane 7*, same extracts as in *lane 3* with 100-fold molar excess of unlabeled unrelated oligonucleotide A. *Lane 8*, same extracts as in *lane 3* with 100-fold molar excess of unlabeled unrelated oligonucleotide B. *Lane 9*, supershift using anti-p50 antibody. The *arrow* indicates the supershifted band.

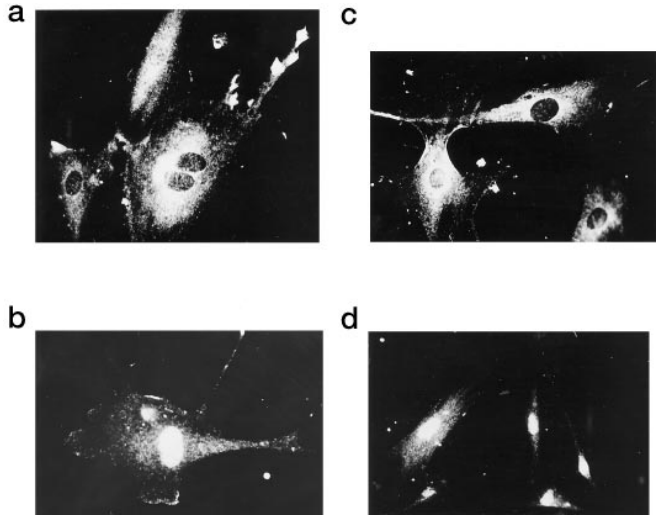

FIG. 4. **Immunocytochemical proof of nuclear translocation of NF-κB.** Wild type and *asmase*$^{-/-}$ EMFIs were left untreated (*a, c*) or stimulated with TNF-α for 20 min (*b, d*), and cellular localization of NF-κB was detected by immunofluorescence microscopy. Untreated wild type (*a*) as well as *asmase*$^{-/-}$ (*c*) EMFIs showed cytoplasmic, but no nuclear staining, while TNF-α-treated wild type (*b*) and *asmase*$^{-/-}$ (*d*) cells showed strong nuclear immunofluorescence.

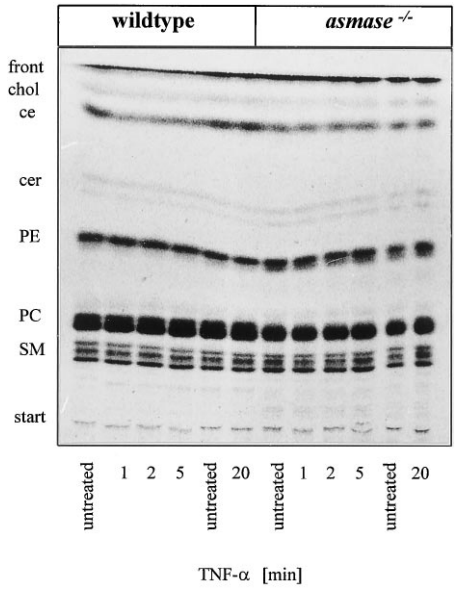

FIG. 5. **Lipid analysis of wild type and *asmase*$^{-/-}$ EMFIs.** Wild type and *asmase*$^{-/-}$ EMFIs were labeled 36 h with [1-^{14}C]palmitate and stimulated for the indicated times with TNF-α. Lipids were extracted and separated by TLC. Bands were assigned using standard lipids. No changes in sphingomyelin or ceramide levels could be determined by measuring the radioactivity with a PhosphorImager. Abbreviations are: *chol*, cholesterol; *ce*, ceramide; *cer*, cerebroside; PE, phosphatidylethanolamine; *PC*, phosphatidylcholine; *SM*, sphingomyelin.

decrease of sphingomyelin concentration reaching 20% of control values of untreated cells after 5 min has been reported for Jurkat cells (23).

When mouse embryonic fibroblasts with the wild type and ASM deficiency genotype were assayed after 0, 2, and 4 min of TNF-α stimulation, no significant increase of ASM activity of wild type was observed. The NSM assay (41) at 0, 1, and 2 min after TNF-α stimulation indicated neither a measurable NSM activity nor an increase of the enzyme activity in wild type and *asmase*$^{-/-}$ EMFIs. Neutral sphingomyelinase is present only at a low level in nonneuronal tissue (8, 44).

These results are supported by the measurement of the intracellular ceramide concentrations in wild type and *asmase*$^{-/-}$ EMFIs after TNF-α stimulation. We prelabeled EMFIs with [1-^{14}C]palmitate for 36 h. Cells were thoroughly washed and stimulated with TNF-α for 1, 2, 5, and 20 min. Total lipids of EMFIs were isolated and separated by thin layer chromatography. The intensity of the radioactive ceramide and sphingomyelin bands were calculated and correlated with the individual phospholipid classes using the Image Quant software of the PhosphorImager (Fig. 5). Neither EMFIs from wild type nor ASM-deficient mice showed significant alterations of the sphingomyelin and ceramide concentrations after TNF-α stimulation.

NF-κB Activation by TNF-α Did Not Relate to ASM Overexpression in HEK Cells—Jurkat T-cells, which overexpress recombinant ASM, constitutively show a dose-dependent NF-κB-specific transcription of a reporter gene without an external stimulus (12).

We generated a stable, murine ASM-expressing human embryonic kidney (HEK 293) cell line with an ASM activity 20-fold above basis level. They were stimulated with TNF-α for the periods indicated in Fig. 6 and compared with wild type HEK

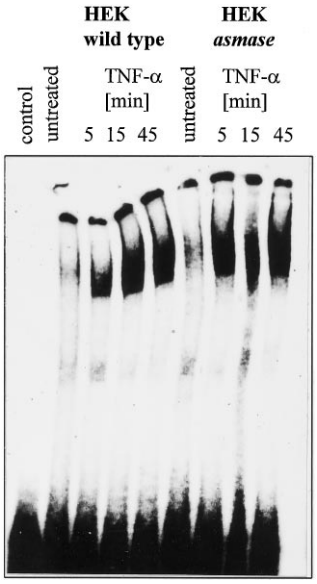

FIG. 6. **ASM-overexpressing HEK cells activate NF-κB indiscriminately from wild type cells.** Wild type and ASM-overexpressing HEK cells were stimulated for the indicated times with TNF-α. Nuclear extracts were submitted to electrophoretic mobility shift assay. No difference could be determined in the kinetics or the intensity of the signals.

cells.

The time course of the NF-κB nuclear translocation followed by the electrophoretic mobility shift assay was indistinguishable in wild type and ASM-overexpressing HEK cells and showed the same kinetics as in the EMFIs of wild type and *asmase*$^{-/-}$ mice.

DISCUSSION

The *asmase*$^{-/-}$ mouse line offers a unique opportunity for studies on the function of ASM and NSM in the recently postulated signal transduction pathways triggered by TNF-α and interleukin 1 (2) or other effector molecules, *e.g.* CD28, Fas, or vitamin D_3 (1). TNF-α is supposed to activate both acid and neutral sphingomyelinase. Initiation of NF-κB nuclear translocation is supposed to be transmitted exclusively by acid (lysosomal) sphingomyelinase generating the proposed second messenger ceramide. Diacylglycerol, which is released by a phosphatidylcholine-specific phospholipase C, has been postulated as a link between TNF-α receptor 55 and lysosomal sphingomyelinase (23). However, the existence of the enzyme remains uncertain.

Taking these reports for granted, NF-κB activation should be precluded in ASM-deficient cells.

The results described here indicate that NF-κB is activated in embryonic fibroblasts from wild type and *asmase*$^{-/-}$ mice when stimulated with TNF-α. The IκB-α-NF-κB complex is dissociated as demonstrated by the rapid transient proteolysis and resynthesis of IκB-α. Free cytosolic NF-κB is translocated into the nucleus shortly after TNF-α stimulation in EMFIs of both genotypes, which was proven by the electrophoretic mobility shift assay with nuclear protein extracts and by immunocytochemistry.

Our results clearly exclude acid sphingomyelinase from the signaling pathway leading to the TNF-α-induced activation of NF-κB. Additionally no increase in ASM enzyme activity after short TNF-α incubation in wild type EMFIs was observed.

Similar observations were made in studies with ASM-deficient Niemann-Pick disease A human fibroblasts (45, 46).

NSM activity of wild type and *asmase*$^{-/-}$ EMFIs is apparently also not stimulated by TNF-α. The enzyme activity in extraneural cells so far analyzed is very low, and high activities were only measured in the central nervous system (8). Embryonic fibroblasts seem to be almost devoid of NSM activity, as shown by the enzyme assay, which is in agreement with a previous report (47). If NSM of EMFIs is participating in the ceramide-activated pathway, then the activity is below the detection level of the assay used. Further studies will address the analysis of the function of NSM.

It should be mentioned that most data for the function of ASM and NSM in signal transduction have been derived mainly from myeloid-lymphoid cell lines.

Recent reports showed that NF-κB activation protects mouse embryonic fibroblasts from induction of apoptosis, suggesting a dissection of the TNF-α-induced signaling pathways (48–51). In our analysis we showed that neither acid nor neutral sphingomyelinase were activated, and no ceramide was generated in the TNF-α-induced activation of NF-κB. Therefore we conclude that sphingomyelinases are not of general importance in TNF-α responses at all.

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REFERENCES

1. Spiegel, S., Foster, D., and Kolesnick, R. (1996) *Curr. Opin. Cell Biol.* **8,** 159–167
2. Kolesnick, R., and Golde, D. W. (1994) *Cell* **77,** 325–328
3. Hannun, Y. A. (1994) *J. Biol. Chem.* **269,** 3125–3128
4. Quintern, L. E., Weitz, G., Nehrkorn, H., Tager, J. M., Schram, A. W., and Sandhoff, K. (1987) *Biochim. Biophys. Acta* **922,** 323–336
5. Das, D. V., Cook, H. W., and Spence, M. W. (1984) *Biochim. Biophys. Acta* **777,** 339–342
6. Newrzella, D., and Stoffel, W. (1996) *J. Biol. Chem.* **271,** 32089–32095
7. Hurwitz, R., Ferlinz, K., Vielhaber, G., Moczall, H., and Sandhoff, K. (1994) *J. Biol. Chem.* **269,** 5440–5445
8. Otterbach, B., and Stoffel, W. (1995) *Cell* **81,** 1053–1061
9. Horinouchi, K., Ehrlich, S., Perl, D. P., Ferlinz, K., Bisgaier, C. L., Sandhoff, K., Desnick, R. J., Steward, C. L., and Schuchman, E. H. (1995) *Nat. Genet.* **10,** 288–293
10. Chatterjee, S., and Gosh, N. (1989) *J. Biol. Chem.* **264,** 12554–12561
11. Maruyama, E. N., and Arima, M. (1989) *J. Neurochem.* **52,** 611–618
12. Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., and Krönke, M. (1994) *Cell* **78,** 1005–1015
13. Cifone, M. G., Roncaioli, P., De Maria, R., Camarda, G., Santoni, A., Ruberti, G., and Testi, R. (1995) *EMBO J.* **14,** 5859–5868
14. Boucher, L.-M., Wiegmann, K., Futterer, A., Pfeffer, K., Machleidt, T., Schütze, S., Mak, T. W., and Krönke, M. (1995) *J. Exp. Med.* **181,** 2059–2068
15. Okazaki, T., Bell, R. M., and Hannun, Y. A. (1989) *J. Biol. Chem.* **264,** 19076–19080
16. Mathias, S., Younes, A., Kan, C.-C., Orlow, I., Joseph, C., and Kolesnick, R. N. (1993) *Science* **259,** 1769–1771
17. Haimovitz-Friedman, A., Kan, C., Ehleiter, D., Persaud, R., McLoughlin, M., Fuks, Z., and Kolesnick, R. N. (1994) *J. Exp. Med.* **180,** 525–535
18. Liu, J., Mathias, S., Yang, Z., and Kolesnick, R. N. (1994) *J. Biol. Chem.* **269,** 3047–3052
19. Dobrowsky, R. T., and Hannun, Y. A. (1992) *J. Biol. Chem.* **267,** 5048–5051
20. Lozano, B., Berra, E., Municio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L., and Moscat, J. (1994) *J. Biol. Chem.* **269,** 19200–19202
21. Müller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D., and Pfizenmaier, K. (1995) *EMBO J.* **14,** 1961–1969
22. Gulbins, E., Coggeshall, K. M., Langlet, C., Baier, G., Bonnefoy-Berad, N., Burn, P., Wittinghofer, A., Katzav, S., and Altman, A. (1994) *Mol. Cell. Biol.* **14,** 906–913
23. Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Krönke, M. (1992) *Cell* **71,** 765–776
24. Obeid, L., Linardic, C. W., Karolak, L. A., and Hannun, Y. A. (1993) *Science* **259,** 1769–1771
25. Jarvis, W. D., Kolesnick, R. N., Fornari, F. A., Traylor, R. S., Gewirtz, D. A, and Grant, S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 73–77
26. Okazaki, T., Bielawska, A., Bell, R. M., and Hannun, Y. A. (1990) *J. Biol. Chem.* **265,** 15823–15831
27. Sen, R., and Baltimore, D. (1986) *Cell* **47,** 921–928
28. Baeurle, P. A., and Henkel, T. (1994) *Annu. Rev. Immunol.* **12,** 141–179
29. Nabel, G., and Baltimore, D. (1987) *Nature* **326,** 711–713
30. Baeuerle, P. A., and Baltimore, D. (1988) *Cell* **53,** 211–217
31. Baeuerle, P. A., and Baltimore, D. (1989) *Genes Dev.* **3,** 1689–1698
32. Beg, A. A., Ruben, S. M., Scheinmann, R. I., Haskill, S., Rosen, C. A., and Baldwin, A. S., Jr. (1992) *Genes Dev.* **6,** 1899–1913
33. Beg, A. A., Finco, T. S., Nantermet, P. V., and Baldwin, A. S., Jr. (1993) *Mol. Cell. Biol.* **13,** 3301–3310
34. Gosh, S., and Baltimore, D. (1990) *Nature* **344,** 678–682
35. Palombella, V. J., Rando, O. R., Goldberg, A. L., and Maniatis, T. (1994) *Cell* **78,** 773–785
36. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D.,

and Maniatis, T. (1995) *Genes Dev.* **9,** 1586–1597
37. Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neria, Y., and Baeuerle, P. A. (1993) *Nature* **365,** 182–185
38. Chomczynski, P., and Sacci, N. (1987) *Anal. Biochem.* **162,** 156–159
39. Quintern, L. E., and Sandhoff, K. (1991) *Methods Enzymol.* **197,** 536–540
40. Stoffel, W., LeKim, D., and Tschung, T. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352,** 1058–1064
41. Schütze, S., and Krönke, M. (1995) *Cytokines: A Practical Approach* (Balkwill, F. R., ed) pp. 93–109, 2nd Ed., Oxford University Press
42. de Martin, R., Vanhove, B., Cheng, Q., Hofer, E., Csizmadia, V., Winkler, H., and Bach, F. H. (1993) *EMBO J.* **12,** 2773–2779
43. Sun, S. C., Ganchi, P. A., Ballard, D. W., and Greene, W. C. (1993) *Science* **259,** 1912–1915
44. Spence, M. W., Burgess, J. K., and Sperker, E. R. (1979) *Brain Res.* **168,** 543–551
45. Andrieu, N., Salvayre, R., and Levade, T. (1994) *Biochem. J.* **303,** 341–345
46. Kuno, K., Sukegawa, K., Ishikawa, Orii, T., and Matsushima, K. (1994) *Int. Immunol.* **6,** 1269–1272
47. Levade, T., Gatt, S., and Salvayre, R. (1991) *Biochem. J.* **275,** 211–217
48. Beg, A. A., and Baltimore, D. (1996) *Science* **274,** 782–784
49. Wang, C.-Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) *Science* **274,** 784–787
50. van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) *Science* **274,** 287–289
51. Liu, Z., Hsu, H., Goeddel, D. V., and Karin, M. (1996) *Cell* **87,** 565–576