Divergent role of ceramide generated by exogenous sphingomyelinases on NF-κB activation and apoptosis in human colon HT-29 cells

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Received 4 June 2002; revised 13 July 2002; accepted 15 July 2002

First published online 30 July 2002

Edited by Guido Tettamanti

Abstract This study examined the role of ceramide generated by exogenous sphingomyelinases (SMases) on transcription nuclear factor-κB (NF-κB) activation and apoptosis in human colon epithelial HT-29 cells. Exogenous neutral (N) and acidic (A) SMase activated NF-κB with different kinetics, accounting for the diverse pattern of DNA binding of NF-κB complexes activated by tumor necrosis factor-α (TNF). NSMase activated predominantly RelA/p52 and RelA/p50 dimers within 30 min, while ASMase activated the p50/p50 homodimer by 20 h. The predominant activation of RelA-containing κB complexes by TNF or NSMase paralleled the induction of interleukin-8. HT-29 cells were sensitive to ASMase and TNF but resistant to NSMase. However, the apoptotic potential of NSMase was masked by NF-κB, as its prior inactivation sensitized HT-29 cells to NSMase. Thus, the generation of ceramide by exogenous SMases participates differentially in inflammation and apoptosis.

Key words: Ceramide; Sphingomyelinase; Apoptosis; Transcription nuclear factor-κB

1. Introduction

Tumor necrosis factor-α (TNF) triggers diverse signaling pathways that account for divergent cellular responses such as inflammation or apoptosis. TNF-induced apoptosis is mediated by its binding to TNF plasma membrane receptor-1 (TNFR-1), causing receptor oligomerization and subsequent recruitment of the TNFR-associated adapter protein with death domain (TRADD) [1–4]. The binding of TRADD to Fas-associated protein with death domain (FADD) [5,6] activates caspase-8, which, in turn, cuts specific target proteins including pro-caspase-3 [7–9]. Furthermore, the binding of TRADD to the downstream transducer protein TNFR-associated factor-2 and the transcription nuclear factor-κB (NF-κB)-inducing kinase results in the activation of NF-κB, which activates the transcription of antiapoptotic genes that antagonize the FADD-mediated death signals [10–12]. NF-κB is usually kept inactive in the cytoplasm through association with an endogenous inhibitor protein of the NF-κB (IκB) family. The activation of NF-κB by TNF involves the phosphorylation of IκB targeting its subsequent degradation by the proteasome. The released subunits of NF-κB translocate to the nuclei where they bind to consensus binding sites in the promoter/enhancer region of target genes.

Ceramide is a sphingolipid increasingly recognized as an important mediator of signal transduction processes leading to a variety of cellular responses, including apoptosis [13,14]. Ceramide in cells can arise by several mechanisms [15]. In addition to the de novo ceramide synthesis through serine-palmitoyl transferase and ceramide synthase, the levels of this lipid can increase by the action of sphingomyelinase (SMase). SMase is a principal enzyme catalyzing the hydrolysis of sphingomyelin to ceramide and phosphocholine. Out of the various SMases described, two types of SMases in mammalian cells are of particular relevance in signaling pathways; neutral SMase with an optimum pH at around 7.5, membrane bound and Mg^{2+}-dependent, and acidic SMase with an optimum pH at around 4.8, further subclassified to two isoforms, an endosomal/lysosomal acidic SMase and a secretory Zn^{2+}-dependent SMase [16–18]. Indeed, TNF activates neutral and acidic SMase activities [13–16], indicating the ability of TNF to generate ceramide at different intracellular locations, suggesting the potential involvement of ceramide in distinct signaling pathways.

A common strategy used to assess the role of endogenous ceramide on cellular responses has been the use of exogenous SMases. The Mg^{2+}-dependent, neutral pH optimum bacterial SMase from *Bacillus cereus* (NSMase) has been widely used to examine the role of ceramide on gene regulation, inflammation and apoptosis in a variety of cell types [19–25]. The acidic human placenta SMase (ASMase) is an acidic pH optimum SMase, which has been shown to enter cells via endocytosis becoming active in acidic compartments [22,26,27]. Consistent with these features, mannose 6-phosphate and pH gradient-collapsing agents, e.g. monensin, have been reported to prevent the effects of this enzyme on reactive oxygen species generation, caspase activation and apoptosis [26,27].

Thus, the purpose of our work was to examine the role of ceramide generated by exogenous SMases on NF-κB activation and apoptosis in human HT-29 cells. We provide evi-

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Abbreviations: ASMase, acidic human placenta sphingomyelinase; FADD, Fas-associated protein with death domain; IL-8, interleukin-8; NF-κB, transcription nuclear factor-κB; NSMase, neutral *Bacillus cereus* sphingomyelinase; SMase, sphingomyelinase; TNF, tumor necrosis factor-α; TNFR-1, TNF plasma membrane receptor-1; TRADD, TNFR-associated protein with death domain

0014-5793/02/$22.00
PII: S0014-5793(02)03106-X

dence that NSMase induces interleukin-8 (IL-8) expression and that its apoptotic potential is counteracted by RelA-containing κB members activated by NSMase. In contrast, HT-29 cells exhibit intrinsic sensitivity to ASMase accompanied by the predominant activation of p50/p50.

2. Materials and methods

2.1. Cell culture and experimental conditions

The human colon cancer cell line HT29 was purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). HT-29 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose in the presence of 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum. Cells (5×10^5 cells/ml) were seeded into 6-well plates and confluent cell monolayers were then incubated for various periods of time with human recombinant TNF (Promega, 14–280 ng/ml), *B. cereus* NSMase or human placenta ASMase (0.1–0.5 U/ml) or appropriate vehicle as described previously [22,27]. In some cases, cells were pretreated with Bay 11-7085 before exposure to TNF or exogenous SMases. To examine whether the various treatments induced HT-29 differentiation, we analyzed the release of carcinoembryogenic antigen (CEA). After cell treatment with TNF, NSMase or ASMase, culture media was collected and centrifuged (10 min, $2000\times g$). The level of CEA in the supernatants was determined by the Abbott CEA-EAI monoclonal One-Step kit and normalized to the number of attached cells. Under the experimental conditions used the level of CEA varied from 5 to 7 ng/mg protein and did not change by the various treatments.

2.2. Determination of NSMase and ASMase activities

Mg^{2+}-dependent NSMase as well as ASMase activity were determined by monitoring [*N*-methyl-^{14}C]sphingomyelin (56.6 mCi/mmol) hydrolysis as described before [16,27]. SMase activities were determined from cell extracts prepared after TNF treatment as described [16]. Briefly, following TNF treatment HT-29, cells were immersed in a methanol–dry ice bath. Cells were scraped, spun ($800\times g$) for 5 min at 4°C and washed two times with phosphate-buffered saline. To measure NSMase, cell pellets were dissolved in a buffer containing 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, 5 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 30 mM *p*-nitrophenylphosphate, 10 mM β-glycerophosphate, 750 μM ATP, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM leupeptin, 10 μM pepstatin and 0.2% Triton X-100. After incubation for 5 min at 4°C, cells were homogenized by repeatedly squeezing the cells through an 18-gauge needle. Nuclei and cell debris were removed by low speed centrifugation ($800\times g$). Supernatants (30–50 μg protein) were incubated for 2 h at 37°C in a buffer containing 20 mM HEPES, 1 mM $MgCl_2$ (pH 7.4) and 2.25 μl [*N*-methyl-^{14}C]sphingomyelin. To measure ASMase, cell pellets were resuspended in 200 μl of 0.2% Triton X-100 and incubated for 15 min at 4°C followed by centrifugation in a microfuge at 14 000 rpm for 10 min at 4°C. Supernatants (30–50 μg protein) were incubated for 2 h at 37°C in a buffer (50 μl final volume) containing 250 mM sodium acetate, 1 mM EDTA (pH 5.0) and 2.25 μl of [*N*-methyl-^{14}C]sphingomyelin. Phosphorylcholine was extracted with 800 μl chloroform:methanol (2:1, v/v) and 250 μl water and identified by thin-layer chromatography and routinely determined by scintillation counting.

2.3. Measurement of ceramide

Cellular lipids after treatment with TNF, NSMase and ASMase were extracted in chloroform:methanol (1:1, v/v). Ceramide levels were determined by the diacylglycerol kinase assay in TLC plates developed in chloroform:acetic acid (9:1, v/v) or high-performance liquid chromatography (HPLC) after derivatization of the sphingoid base with *o*-phthalaldehyde following deacylation of ceramide as characterized previously [27].

2.4. Electrophoretic mobility shift assays

Activation of NF-κB was examined using consensus oligonucleotide κB probe labeled by T4 polynucleotide kinase using nuclear extracts as described before [28]. Cells were washed twice with ice-cold phosphate-buffered saline and collected with a rubber policeman after the various treatments. Cells were resuspended in 10 mM HEPES, pH 7.8, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml TLCK and kept on ice for 15 min. Cells were then lysed with Nonidet P-40 (10%), and the nuclear pellet was recovered after centrifugation at $13\,000\times g$ at 4°C for 30 s. The nuclear pellet was resuspended in ice-cold 20 mM HEPES, pH 7.4, containing 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml TLCK and stored at 80°C. Supershift assays were done using human antibodies anti-RelA (p65), p50, p52, c-Rel and RelB as described previously [28].

2.5. IL-8 expression

Total cellular RNA was isolated using the Trizol reagent (Life Technologies, Barcelona, Spain). A cDNA probe for IL-8 was generated by polymerase chain reaction using the following primers 5′(+1584–1603)-ATGATCTCCAAGCTGGCCG-3′ and 5′(−2877–2896)-GTCTCTCTTCCATCATCAGAAAGC-5′, designed from the genomic sequence of IL-8 (GenBank accession number M28130). The 226-bp cDNA fragment was cloned into pGemT (Promega, Madison, WI, USA) and sequenced. Twenty μg total RNA was fractionated in a 1% agarose gel under denaturing conditions and transferred on nylon membrane, fixed by baking at 80°C for 2 h. The membranes were prehybridized at 65°C and hybridizations were performed using the Random Prime II labeling system (Amersham). RNA levels were calculated relative to the 18S used as internal reference control and expressed as percentage of control. Densitometric quantitation of autoradiographs was performed with a densitometer Preference (Seba, France).

2.6. Determination of cell death and apoptosis

Cell survival was quantitated by measurement of lactate dehydrogenase (LDH) in culture supernatants and in the remaining cell monolayer after lysis with 0.1% Triton X-100, expressing the percentage of LDH release in the medium as the fraction of total LDH (medium plus cells) as described [29]. Alternatively, cell viability was ascertained by the MTT assay adding MTT (20 μl of 5 mg/ml MTT in phosphate-buffered saline) and the absorbance was measured at 570 nm [30]. Apoptotic features of cell death were determined by staining of the cells with the DNA binding fluorochrome H33258 assessing chromatin condensation by fluorescence microscope analyses or nuclear DNA fragmentation by gel electrophoresis as described previously [29,31].

2.7. Statistics analyses

Results are expressed as the mean ± S.D. and are averages of three to five values per experiment. Differences between treatments were compared using an analysis of variance for repeated measures and statistical analyses of mean values for multiple comparisons were made by one-way analysis of variance.

3. Results

3.1. Activation of NF-κB by exogenous SMases

Since SMases can generate ceramide from the hydrolysis of sphingomyelin, we first determined the level of ceramide generated upon treatment of HT-29 cells with exogenous NSMase and ASMase. Exogenous NSMase and ASMase increased ceramide levels in a time-dependent manner, peaking at 1–2 h and declining thereafter to basal levels (Fig. 1). The magnitude of ceramide generation increased with increasing dose of exogenous SMases. Likewise, TNF stimulated an early ceramide generation between 1 and 2 h, the magnitude of which was dependent on the dose of TNF; however, unlike exogenous SMases, the stimulated ceramide generation by TNF was more sustained remaining above control levels by 10 h post incubation (Fig. 1).

Moreover, since activation of NF-κB is a prominent effect of TNF, we next analyzed the activation of NF-κB by exogenous SMases. The DNA binding activity of NF-κB revealed the presence of several complexes, denoted by the arrows, that

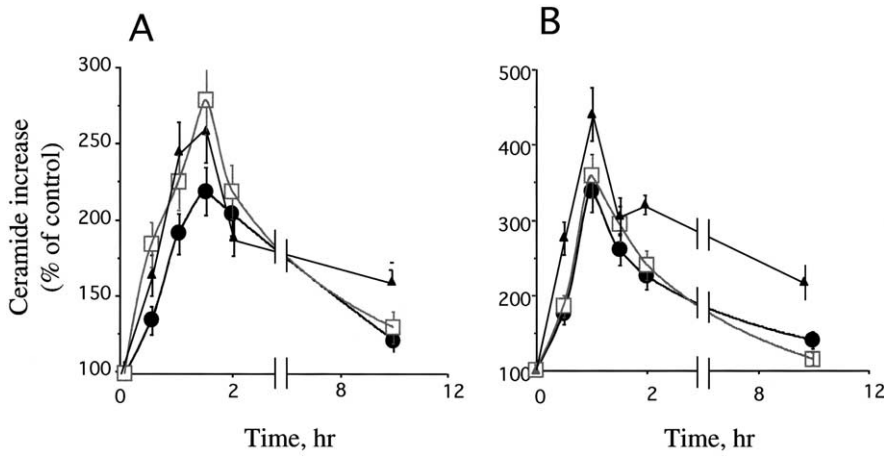

Fig. 1. Ceramide levels generated by TNF or exogenous SMases. A: HT-29 cells were exposed to TNF (14 ng/ml), NSMase or ASMase (0.1 U/ml) for the indicated periods of time. B: As A except that cells were incubated with 280 ng/ml of TNF or 0.5 U/ml of NSMase and ASMase. Lipid extracts were isolated and ceramide determined by HPLC. Control ceramide levels were 1.8 ± 0.3 nmol/mg protein. Triangles, TNF; squares, NSMase; circles, ASMase. Results are the mean ± S.D. of five independent experiments.

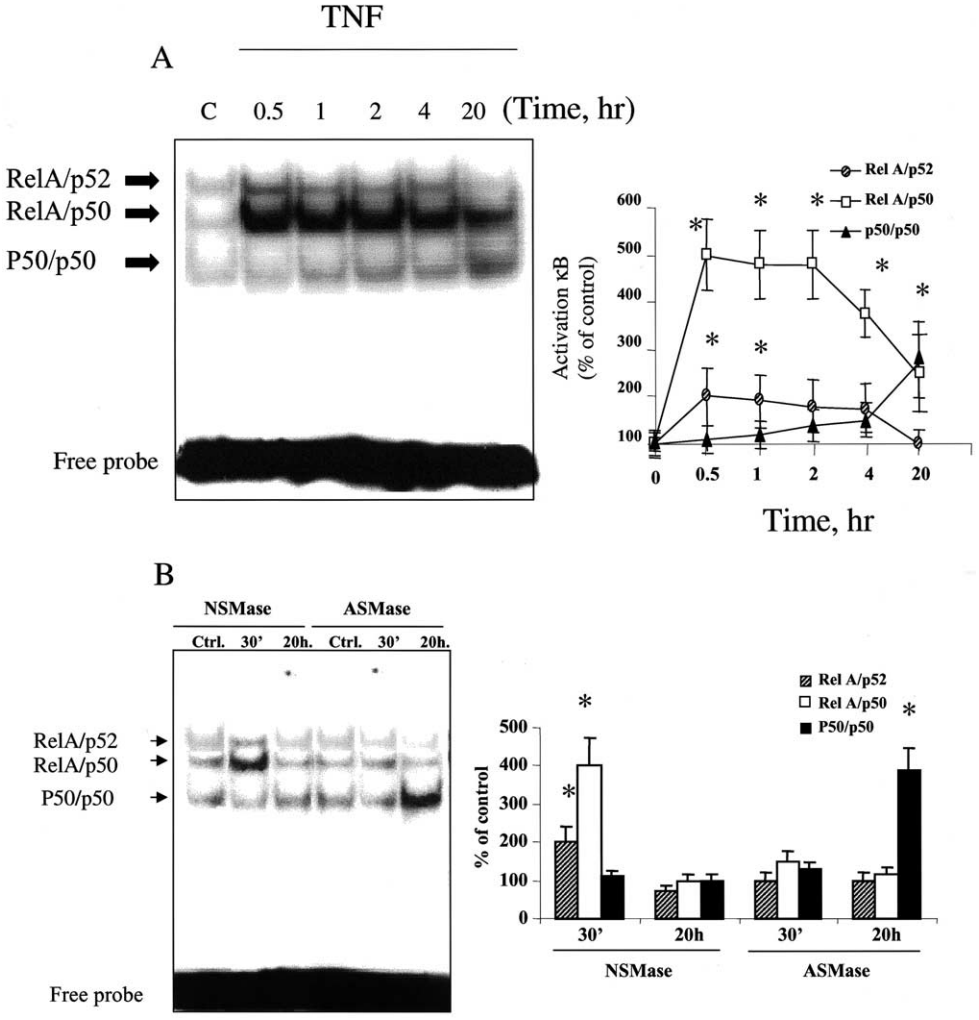

Fig. 2. Activation of NF-κB by TNF and exogenous SMases. HT-29 cells were incubated with either TNF (280 ng/ml) (A) or exogenous NSMase or ASMase (0.5 U/ml, each) (B) for the indicated periods of time and nuclear extracts were assayed for κB DNA binding. Representative experiment out of five independent ones giving similar results is shown. The magnitude of activation of the individual κB complexes was estimated by densitometry. The quantitation of κB complexes is expressed as the mean ± S.D. of five independent experiments. The variability between individual experiments was below 13%; $*P < 0.05$ vs. control.

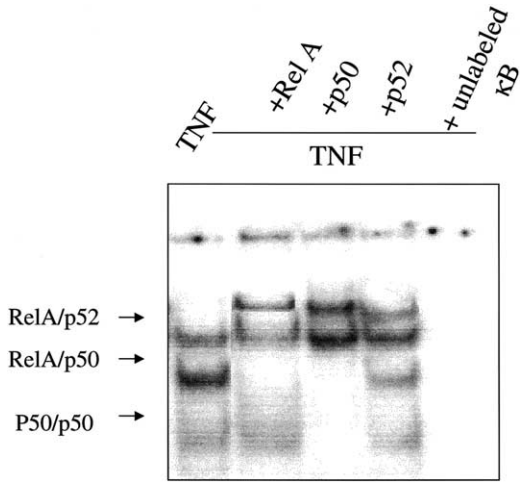

Fig. 3. Supershift assay of active κB complexes. Nuclear extracts from HT-29 cells after TNF exposure (280 ng/ml for 2 h) were incubated with different antibodies against different members of κB as indicated. In addition, nuclear extracts were exposed to a molar excess of unlabeled κB oligonucleotide to check for specificity of the different κB complexes. Representative experiment out of five independent ones giving similar results.

were activated by TNF at different time points (Fig. 2A). As revealed by the supershift assays, there were three complexes, RelA/p52, RelA/p50 and p50/p50, all of which were displaced by unlabeled oligonucleotide (Fig. 3). The DNA binding activity of the two upper bands, corresponding to RelA/p52 and RelA/p50 heterodimers, increased by 30 min post treatment and declined over time; however, the lower band, corresponding to p50/p50 homodimer, increased gradually over time from 4 to 20 h (Fig. 2A).

NSMase enhanced the DNA binding of RelA/p52 and RelA/p50 at 30 min post incubation (Fig. 2B). The activation of these complexes were maintained for up to 4 h (not shown) and declined at 20 h post treatment (Fig. 2B). However, in contrast to TNF, there was no evidence for the activation of p50/p50 homodimer by NSMase (Fig. 2B). In contrast to this pattern, the predominant κB complex activated by ASMase was the p50/p50 homodimer whose activation was detectable by 12 h post treatment (not shown); at 20 h after TNF treatment the p50 homodimer was the only active complex detected (Fig. 2B). Unlike NSMase, there was no evidence for RelA-containing κB members by ASMase (Fig. 2B). Thus, these data show that exogenous NSMase and ASMase activated distinct κB complexes.

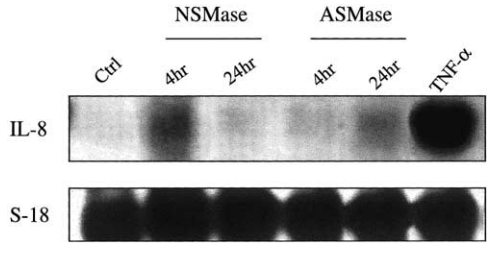

Fig. 4. IL-8 mRNA level in response to exogenous SMases. HT-29 cells were treated with either NSMase or ASMase (0.5 U/ml) for the indicated periods of time or TNF (280 ng/ml, for 12 h) and the IL-8 mRNA expression was quantitated relative to the expression of 18S as reference control. Northern blot representative of four different experiments yielding similar results.

3.2. IL-8 induction by exogenous SMases

Since NF-κB plays a key role as a pro-inflammatory transcription factor by stimulating the synthesis of inflammatory cytokines [32,33], we next examined the expression of IL-8 in HT-29 cells following NSMase and ASMase treatment. Northern blot analyses indicated that TNF induced a strong expression of IL-8 ($640 \pm 42\%$ over control, $n = 4$, $P < 0.005$) (Fig. 4). NSMase induced the levels of IL-8 mRNA by 4 h ($356 \pm 44\%$ over control, $n = 4$, $P < 0.005$), and ASMase induced a weaker IL-8 expression ($152 \pm 34\%$ over control at 24 h post incubation, $n = 4$, $P < 0.05$) (Fig. 4).

3.3. Divergence of NSMase and ASMase in the induction of apoptosis

To determine whether ceramide generated by exogenous SMases participates in the cytotoxic effects of TNF, we next examined the apoptotic potential of either SMase compared to that of TNF. TNF induced a dose-dependent loss of viability, which was significant at 24 or 12 h post incubation at 14 ng/ml or 280 ng/ml, respectively ($P < 0.05$) (Fig. 5A). This form of cell death was accompanied by disruption of chromatin integrity, indicative of apoptosis (not shown). In contrast, NSMase failed to kill HT-29 cells at either of the doses examined (Fig. 5). On the other hand, ASMase mimicked the cytotoxic effect of TNF, being significant at 48 or 12 h of incubation with 0.1 U/ml or 0.5 U/ml, respectively ($P < 0.05$) (Fig. 5). The apoptotic potential of ASMase was prevented by mannose 6-phosphate and monensin through competition with the mannose 6-phosphate receptor [34] and pH gradient collapse in acidic compartments [26,27] (not shown).

3.4. NF-κB activation unmasks the apoptotic potential of NSMase

To examine if the inability of NSMase to induce apoptosis was masked by the activation of survival pathways dependent on NF-κB, we pretreated cells with agents that block NF-κB activation before incubation with NSMase. Bay 11-7085 has been shown to suppress NF-κB activation by interfering with

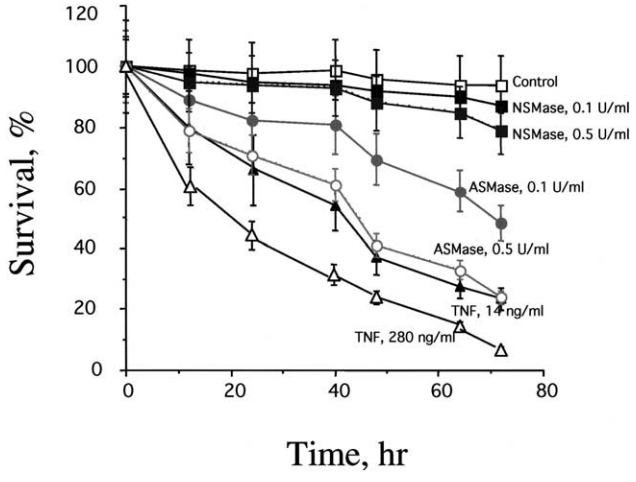

Fig. 5. Time-dependent viability of HT-29 cells following TNF or exogenous SMases incubation. HT-29 cells were exposed to either TNF (triangles), NSMase (squares) or ASMase (circles) at the indicated doses for different periods of time and cell viability was ascertained by LDH release into the medium and expressed as percentage of total LDH found in cells plus medium. Results are the mean ± S.D. of five independent experiments.

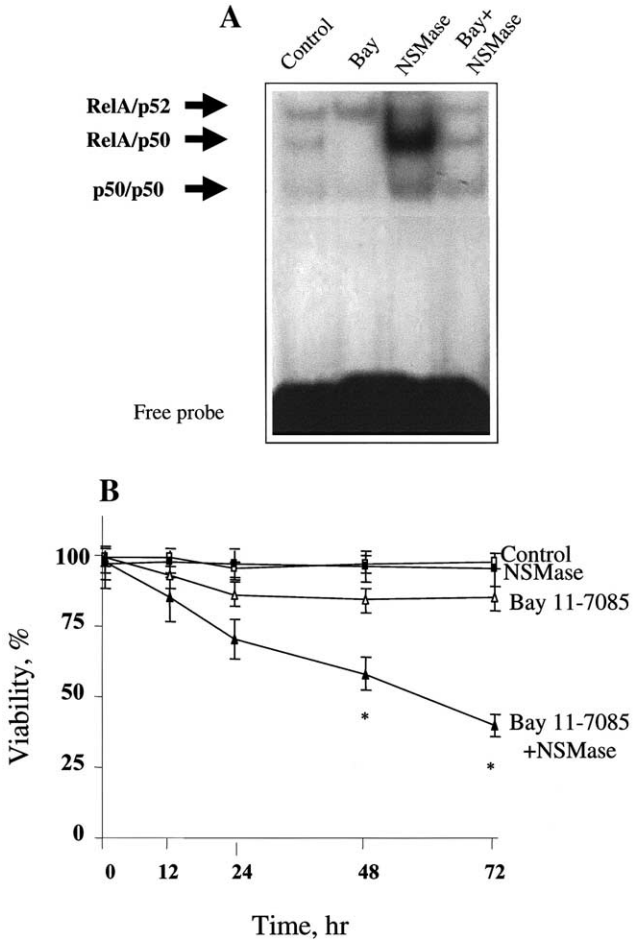

Fig. 6. Inactivation of NF-κB and apoptosis induced by NSMase. HT-29 cells were pretreated with Bay 11-7085 (20 μM) before incubation with NSMase (0.5 U/ml) for 2 h, and nuclear extracts were used for NF-κB activation (A). Parallel culture plates were treated with NSMase for various periods of time in the absence or presence of Bay 11-7083 and cell survival was determined by LDH release into the medium (B). Results are the mean ± S.D. of four individual experiments. $*P < 0.05$ vs. control.

the IκBα phosphorylation [35]. Consistent with this mechanism of action, pretreatment of HT-29 cells with Bay 11-7085 suppressed NF-κB activation induced by NSMase (Fig. 6A). To examine whether NF-κB inactivation sensitized HT-29 cells to NSMase, we next determined the effect of Bay 11-7085 on cell survival. This strategy unmasked the apoptotic potential of NSMase as shown by the progressive loss of cell viability (Fig. 6B). This form of cell death was apoptotic as verified by chromatin disruption (not shown). Taken together these findings reveal the importance of NF-κB activation in controlling the survival of HT-29 cells to NSMase exposure.

4. Discussion

This study examined the role of ceramide generated by exogenous SMases on NF-κB activation and apoptosis in human colon epithelial HT-29 cells. Our data reveal that exogenous NSMase mimicked the ability of TNF to activate NF-κB. Both stimuli enhanced the DNA binding activity of RelA/p52 and RelA/p50 heterodimers, confirming previous results in human gastric epithelial cells [21]. Consistent with the role of NF-κB activation in gene regulation, we show the inducible expression of pro-inflammatory chemokine IL-8 in response to TNF and NSMase. The transcriptional expression of IL-8 has been shown to require the combination of several transcription factors, including NF-κB and AP-1 or NF-κB and NF-IL6, as reported for different cell types [36–38]. It is conceivable that the recruitment and activation of these combinations may account for the greater transcriptional expression of IL-8 by TNF compared to NSMase.

An intriguing finding of our study is that exogenous NSMase did not signal cell death. Indeed, this resistance of HT-29 cells to exogenous bacterial NSMase confirms previous reports [19,21], although these studies did not address the activation of NF-κB by NSMase. In pursuing this issue further, we examined whether NF-κB activation may have protected HT-29 cells against NSMase-induced apoptosis, as it is known that NF-κB induces the expression of antiapoptotic genes. To this end, cells were pretreated with Bay 11-7085 that interferes with IκBα phosphorylation, thus preventing the proteasome-stimulated hydrolysis of IκB. Since IκB sequesters NF-κB inactive in the cytosol, this agent abolishes the nuclear translocation of active κB members. As confirmed by electrophoretic mobility shift assays, Bay 11-7085 blocked the activation of NF-κB induced by NSMase. Indeed, using this approach we show that Bay 11-7085 renders HT-29 cells sensitive to exogenous NSMase treatment, thus unmasking the apoptotic potential of exogenous NSMase. Previous studies indicated a differential apoptotic outcome of treating Molt-4 cells with exogenous NSMase vs. its transfection, despite that both strategies generated similar ceramide levels [20]. These findings promoted the concept of the existence of an apoptotic ceramide pool whose ability to signal cell death appears to depend on the cellular location within the plasma membrane where ceramide is generated by bacterial NSMase [20]. In contrast to this view, we provide evidence that the exogenous bacterial NSMase-induced ceramide generation has the potential to signal apoptotic cell death in HT-29 cells. However, the impact of this process is counteracted by the induction of survival genes dependent on NF-κB activation, and, hence our data show that the pool of ceramide generated by NSMase is linked to both NF-κB and apoptotic pathways.

Our data indicate the activation of NF-κB in response to exogenous ASMase. Compared to exogenous NSMase, this process was a late event resulting in the predominant activation of the homodimer p50/p50. Although we have not addressed the signaling events leading to the selective activation of p50/p50 by ASMase, inhibitors of IκBα phosphorylation prevented the activation of p50/p50 by ASMase. Hence, it is conceivable that ASMase may activate the IκB kinase complex, which in turn leads to phosphorylation and proteasome-limited proteolysis of p105, the precursor form of p50 [39]. Further work will be required to discern whether ASMase activates the kinase complex or inhibits a cellular phosphatase.

The pattern of gene induction by p50/p50 homodimer may differ from that induced by RelA-containing heterodimers. While RelA/p52 can induce the expression of survival genes [10–12,21], p50/p50 homodimer has been reported to signal the repression of pro-inflammatory gene transcription, which may play a role in the resolution of inflammation [40]. In addition, a role has been suggested of p50/p50 in the expres-

sion of pro-apoptotic p53 and Bax involved in the apoptosis of leukocytes [40]. However, the sensitivity of HT-29 cells to exogenous ASMase was unaltered when the activation of this predominant κB complex activated by ASMase (p50/p50 homodimer) was prevented. These findings suggest that the p50/p50 homodimer is not directly involved in the generation of death signals.

Overall, to the best of our knowledge this is the first report analyzing the effects of diverse exogenous SMases in the regulation of NF-κB activation and apoptosis in human colon epithelial cells. Our findings suggest the intriguing possibility that the fate of endogenous ceramide generated by the action of exogenous NSMase or ASMase in activating distinct NF-κB complexes resulting or not in apoptosis seems to depend on the cellular site where ceramide is released.

Acknowledgements: This work has been performed with excellent technical assistance of Susana Núñez. The work presented was supported by the Research Center for Liver and Pancreatic Diseases, P50 AA11999, funded by the U.S. National Institute on Alcohol Abuse and Alcoholism, Plan Nacional de I+D, Grants SAF 99-0138, 2FD97-0988, SAF01-2118 and Fondo de Investigaciones Sanitarias FIS 00-907. Dr. García-Ruiz is a Sistema Nacional de Salud (SNS) investigator.

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