Among its diverse biologic effects, the cytokine tumor necrosis factor α causes the rapid nuclear translocation of the transcription factor, nuclear factor κB (NF-κB). The p55 tumor necrosis factor (TNF) receptor shares with the related APO-1/Fas antigen the ability to initiate apoptosis. We investigated the role of the sphingolipid mediator ceramide in the cytokine-induced signaling mechanisms leading to NF-κB activation and cell death. Several lines of evidence presented here suggest that ceramide generated in response to TNFα or Fas activation is not involved in NF-κB activation. (i) Cell-permeable ceramides and exogenous sphingomyelinase failed to induce either nuclear translocation of NF-κB or degradation of its cytosolic inhibitor, I-κB, in Jurkat T cells. (ii) Ceramide treatment of cells inhibited phorbol ester-induced activation of NF-κB. (iii) TNFα potently activated NF-κB in a cell line deficient in acid sphingomyelinase. (iv) TNFα activated NF-κB within minutes without altering ceramide levels. (v) Treatment of Jurkat cells with cross-linking antibodies to APO-1/Fas induced large scale increases in ceramide and apoptosis without affecting NF-κB. (vi) Ceramide generation in response to Fas activation was inhibited by N-acetylresinosilylvalinylalanlysaptaryl chloromethyl ketone, a peptide inhibitor of interleukin-1β-converting enzyme-like proteases, whereas TNFα-induced NF-κB activation was unaffected by the inhibitor. These results show that ceramide accumulation belongs selectively to the apoptotic pathway(s) induced by cytokines, and, if anything, ceramide may participate in negative feedback regulation of NF-κB.

Membrane glycerophospholipids, once thought to serve only as structural components of the cell, are now known to play central roles in a host of signal transduction pathways. Another class of lipids, the sphingolipids, have emerged recently as regulators of such diverse processes as cell growth and differentiation (1–5), cell cycle arrest (4, 5), cellular senescence (6), and programmed cell death (7–9). In particular, the sphingolipid ceramide, produced by hydrolysis of membrane sphingomyelin (for review, see Ref. 10), has received attention as an important bioeffector molecule, which may participate in mediating some of the actions of extracellular agents such as tumor necrosis factor α (TNFα) (11, 13), 1α,25-dihydroxyvitamin D3 (1, 2), γ-interferon (3), and APO-1/Fas (15, 14).

TNFα is a pleiotropic cytokine, which has a central role in mediating immune regulation and inflammatory response via binding to its 55- and 75-kDa membrane receptors, termed TNFR-1 and TNFR-2, respectively (for review, see Refs. 15 and 16). The APO-1/Fas antigen is a related member of the TNF receptor superfamily, which shares the ability to induce apoptosis in a number of hematopoietic cell lines (for review, see Ref. 17). Recent studies have shed some light on the upstream events that may mediate a common death signaling pathway for both TNF and Fas involving recruitment of the death domain-associated protein FADD (18) and the sequential activation of members of the interleukin-1β-converting enzyme (ICE)-like protease family (19, 20, 45). TNFα and Fas also both induce sphingomyelinase activation and the generation of ceramide, which can induce apoptosis and may play a role in apoptotic signaling by these cytokines (7–9).

TNFα is additionally known to activate the transcription factor NF-κB (21, 22) which is thought to mediate the TNFα-induced expression of a variety of genes including the IL-2 receptor. NF-κB belongs to the Rel family of transcription factors and in its inactive state exists in the cytosol as a heterodimer bound to the inhibitory complex I-κB (for review, see Refs. 23 and 24). Stimulation at the cell surface by cytokines such as TNFα and interleukin-1β (IL-1β) or by lipopolysaccharide initiates a poorly understood set of signaling events, which result in the phosphorylation and degradation of I-κB, thus allowing the free NF-κB dimer to translocate to the nucleus and initiate transcription of κB-responsive elements (25, 26). The TNF receptor-associated proteins TRADD and TRAF-2 have been implicated in signaling to NF-κB by TNFR-1 (27, 28). It is unclear whether ceramide generated in response to TNFα is involved in NF-κB activation. Some studies have suggested an essential role for this lipid second messenger in NF-κB activation and a dependence on ceramide generated by acid sphingomyelinase activity in particular (29–31). However, other studies have provided evidence against a role for ceramide in this signaling pathway (12, 32–35). Therefore, in the current study we sought to clarify the potential role of ceramide in the TNFα and Fas mechanisms of NF-κB activation.

In this study, we demonstrate that cell-permeable analogs of ceramide were unable to induce either I-κB degradation or nuclear translocation of NF-κB in intact Jurkat T cells. Likewise, treatment of cells with bacterial sphingomyelinase, which...
has been shown to increase intracellular ceramide levels via cleavage of membrane sphingomyelin (36), failed to activate NF-κB. TNFα remained a potent activator of NF-κB in cells from a patient with Niemann-Pick disease type A (NPA), which lack acid sphingomyelinase activity (37, 38).

Treatment of Jurkat T cells with cross-linking antibodies to APO-1/Fas caused both a marked increase in intracellular ceramide levels and apoptosis. However, Fas was unable to signal nuclear translocation of NF-κB at early or late time points. Pretreatment of Jurkat cells with YYAD.CMK, a site-specific inhibitor of ICE-like proteases (39), inhibited both Fas-induced ceramide generation and apoptosis, but not TNFα-induced NF-κB activation. Furthermore, in Jurkat cells treated with TNFα, we observed no increase in intracellular ceramide formation in the time course needed for activation of NF-κB (1–10 min). Thus, Fas induced intracellular ceramide increases in an ICE-like protease-dependent manner without activating NF-κB, whereas TNFα activated NF-κB within minutes independent of ceramide and ICE-like proteases. Finally, we show that ceramide inhibits PMA-induced activation of NF-κB. Taken together, these lines of evidence strongly suggest that ceramide is a specific component of apoptotic signaling pathways and not of the pathways leading to NF-κB activation.

EXPERIMENTAL PROCEDURES

Materials—Jurkat T cells were obtained from ATCC, Rockville, MD. Niemann-Pick A normal skin fibroblasts were obtained from the Coriell Institute (National Institute on Aging). TNFα was a kind gift from Dr. Phil Pekala (East Carolina University). Anti-Fas monoclonal antibody was purchased from Upstate Biotechnology, Inc. Staphylococcus aureus sphingomyelinase was purchased from Sigma. C2- and C6-ceramide were synthesized as described (2). γ-32P[ATP] was from NEN Life Science Products. Poly(dI:dC) and poly(dI:poly(dC)) were from Pharmacia Biotech Inc. Anti-NF-κB monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-IκBα monoclonal antibody was purchased from Rockland, Inc. YYAD.CMK (Bachem Bioscience, King of Prussia, PA) was dissolved in Me2SO before addition to medium (final MeSO concentration 0.2%, v/v), and appropriate solvent controls were used.

Cell Culture—Jurkat (human lymphocytic T cell leukemia) cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. Niemann-Pick type A skin fibroblasts and Jurkat cells were grown in minimal essential medium (Life Science Products). Poly(dI:dC) to attain a final concentration of 5 g/ml antipain, 20 μg/ml leupeptin, 20 μg/ml antipain, 20 μg/ml pepstatin, 1 mm phenylmethylsulfonyl fluoride (PMSF) to attain a final concentration of 5 × 105 cells/ml. Cells were washed and aliquots were stored at −80 °C for 4–12 h.

Oligonucleotide—The probe utilized was a synthetic NF-κB consensus oligonucleotide with the following sequence: 5′-AGTTGGAGGGACCTTCCCAAGCCG-3′. It was end-labeled using T4 kinase and [γ-32P]ATP. The mutant oligonucleotide used in competition experiments had the following sequence: 5′-AGTTGGAGGGACTTCCCAAGCCG-3′.

Western Blot Analysis—After treatments were carried out, cytosolic extracts from Jurkat cells were prepared by washing 107 cells in ice-cold PBS and resuspending pellet in ice-cold homogenizing buffer (20 mm Tris-HCl (pH 7.5), 250 mm sucrose, 10 mm EDTA (pH 7.4), 2 mm EDTA (pH 7.4), 1 mm phenylmethylsulfonyl fluoride, 0.02% leupeptin, and 0.1% Triton X-100). The cells were then lysed by sonication and ultra centrifuged at 40,000 rpm for 40 min at 4 °C to separate cytosolic from nuclear and membrane components. An aliquot of the supernatant was removed for protein determination, and the remainder of the supernatant was mixed gently for 20 min at 4 °C, and then microcentrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was mixed gently for 20 min at 4 °C, and then microcentrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was diluted with 40–70 ml of radiolabeled oligonucleotide probe (20,000–50,000 cpm) and then reacted with a variety of inducers of NF-κB, IκBα is phosphorylated and proteolyzed, thereby releasing NF-κB and allowing the free heterodimer to enter the nucleus and bind to target gene promoter regions (23, 24). IκBα proteolysis has been shown to be a necessary regulated step in the activation of NF-κB by TNFα (25, 26). Treatment of Jurkat T cells with TNFα resulted in proteolysis of IκBα within 10 min (data not was added to appropriate samples, which were then incubated for 1 h on ice prior to addition of Ficoll solution. Equal amounts of the reaction mixture were loaded on a 5% nonnondenaturing polyacrylamide gel in 1× TBE and were run at 200 V. Gels were transferred to Whatman filter paper, dried at 80 °C for 2 h, and exposed to film at −80 °C for 2–12 h.

RESULTS

Treatment of Jurkat Cells with Exogenous Ceramide Analogos and Bacterial Sphingomyelinase Does Not Induce Nuclear Translocation of NF-κB—To investigate whether TNFα-induced ceramide generation is a sufficient signal for NF-κB activation, Jurkat T cells were treated with varying concentrations of synthetic cell-permeable ceramide analogs and were then assayed for nuclear translocation of NF-κB (Fig. 1, A and B). These cell-permeable analogs have been shown to mimic the cytotoxic (apoptotic) effects of TNFα at micromolar concentrations (7). However, neither C2- nor C6-ceramide was able to induce nuclear translocation and activation of NF-κB as compared with untreated and TNFα-treated controls over both short and extended time courses. To evaluate the possibility that endogenously generated ceramide may provide a signal that the synthetic analogs lack, cells were treated with bacterial sphingomyelinase (Fig. 1C). Incubation of leukemic cell lines with bacterial sphingomyelinase has been shown to result in the hydrolysis of membrane sphingomyelin and the generation of intracellular ceramide in a dose- and time-dependent fashion (36). However, this treatment likewise failed to signal nuclear translocation of NF-κB in Jurkat T cells.

Activation of NF-κB was also studied by using Western blot analysis of its cytosolic inhibitor, IκBα. Upon treatment of cells with a variety of inducers of NF-κB, IκBα is phosphorylated and proteolyzed, thereby releasing NF-κB and allowing the free heterodimer to enter the nucleus and bind to target gene promoter regions (23, 24). IκBα proteolysis has been shown to be a necessary regulated step in the activation of NF-κB by TNFα (25, 26). Treatment of Jurkat T cells with TNFα resulted in proteolysis of IκBα within 10 min (data not
mediated pathways from the signaling events leading to NF-
lar (Fig. 2), further suggesting the divergence of ceramide-
particular is a necessary and sufficient signal for NF-
a ceramide signal generated by the acid sphingomyelinase in
neutral and acidic pH optima, and there has been evidence that
shown), and after 30 min near-complete proteolysis of the band
was observed (Fig. 2). Treatment with varying concentrations
of cell-permeable ceramide and bacterial sphingomyelinase did
not induce I-\kappa B proteolysis as compared with untreated con-
trol (Fig. 2), further suggesting the divergence of ceramide-
mediated pathways from the signaling events leading to NF-\kappa B
activation.

TNF\alpha Activates NF-\kappa B in the Absence of Acid Sphingomyelinase—TNF\alpha signaling through the 55-kDa receptor has been shown to result in activation of sphingomyelinase with both neutral and acidic pH optima, and there has been evidence that a ceramide signal generated by the acid sphingomyelinase in particular is a necessary and sufficient signal for NF-\kappa B activation (29). To more closely evaluate the possible role of acid sphingomyelinase in this pathway, we studied skin fibroblasts—

Neutral magnesium-dependent sphingomyelinase activity was measured as described under “Experimental Procedures.”

| Acid sphingomyelinase | Neutral magnesium-dependent sphingomyelinase |
|------------------------|---------------------------------------------|
| NPA                    | 0.25                                        |
| Normal skin fibroblasts| 2.50                                        |

* nmol/mg protein/h.
occur after treatment with Fas ligand and TNF activation and ceramide generation have also been shown to involve the ICE-like family of proteases (19, 20, 46). Sphingomyelinase associated protein FADD and activation of members of the death domain–death effector domain protein complex have been shown to involve recruitment of the death domain–death effector domain protein complex (39) (Fig. 5A).

We also sought to determine if inhibition of ICE-like protease activity and ceramide generation would have an effect on Fas-induced apoptosis. At the time of lipid extraction, cell viability was determined by trypan blue exclusion (Fig. 5B). In samples treated with anti-Fas antibody alone, the increase in ceramide levels observed was accompanied by the induction of 70% cell death after 12 h. In samples pretreated with YVAD.CMK, Fas-induced apoptosis was reduced to 10% after 12 h, a 7-fold reduction in cell death.

TNFα and Fas Signaling via Ceramide

FIG. 3. Effect of TNFα on NF-κB activation in NPA and normal skin fibroblasts. A, NPA and normal skin fibroblasts were left untreated or were treated with 2 nM TNFα for 15 min prior to extraction of nuclear proteins. EMSA was performed as described under “Experimental Procedures.” B, specificity of gel shift complexes in NPA fibroblasts. EMSA was performed using nuclear extracts of untreated (lane 1) and 2 nM TNFα-treated NPA cells (lanes 2–6). Protein-DNA binding reactions were carried out in the presence of 32P-labeled NF-κB consensus oligonucleotide alone (lanes 1 and 2) or with the following additions: monoclonal antibody to NF-κB p65 subunit (lane 3), monoclonal antibody to c-Rel protein (lane 4), excess unlabeled consensus (WT) oligonucleotide (lane 5), or excess unlabeled mutant NF-κB oligonucleotide (lane 6).

FIG. 4. Effect of anti-Fas antibody on ceramide levels and NF-κB in Jurkat T cells. Cells in each experiment were treated with anti-Fas cross-linking antibody at a concentration of 100 ng/ml. A, 5 × 106 cells were either left untreated (open squares) or were treated with anti-Fas antibody (closed squares) for the indicated times prior to harvesting. Lipids were extracted and ceramide levels determined as described under “Experimental Procedures.” B, cells were treated with either 2 nM TNFα for 15 min or with anti-Fas antibody (Fas Ab) for the indicated times prior to extraction of nuclear proteins. EMSA was performed as described under “Experimental Procedures.” n.s., nonspecific bands.
pathway leading to NF-κB activation.

Members of the ICE-like protease family have been shown to be involved in APO-1/Fas-induced apoptosis (19, 20), and appear to be upstream of sphingomyelinase activation and ceramide generation induced by this cytokine as described above. To clarify whether ICE-like protease activity was additionally an upstream modulator of TNF-α-induced activation of NF-κB, we treated Jurkat T cells with TNF-α after 30 min of preincubation with YVAD.CMK (Fig. 6B). Pretreatment with 20 and 100 μM YVAD.CMK had no effect on the ability of TNF-α to potently induce nuclear translocation of NF-κB within 10 min, suggesting that ICE-like protease activity is not involved in NF-κB activation by TNF-α.

Ceramide Inhibits PMA Activation of NF-κB—Additional studies examining the interactions of ceramide with other inducers of NF-κB led to an investigation of the effects of C2- and C6-ceramide on activation of NF-κB by PMA, an activator of protein kinase C that is known to activate NF-κB. In these studies, cells were treated with 50 nM PMA alone or in the presence of 10 μM C2- or C6-ceramide. PMA alone caused significant activation of NF-κB as shown in Fig. 7A. Both C2- and C6-ceramide inhibited activation of NF-κB in response to PMA (Fig. 7A). Interestingly, C2- and C6-ceramide at 5–20 μM did not inhibit TNF-α-induced activation of NF-κB (Fig. 7B), concentrations that were sufficient to inhibit PMA-induced activation, thus demonstrating that the effects of ceramide are not a result

FIG. 5. Effect of the protease inhibitor YVAD.CMK on Fas-induced ceramide generation and cell death in Jurkat cells. A, 5 × 10⁶ cells were treated with anti-Fas cross-linking antibody at a concentration of 100 ng/ml for the indicated times prior to harvesting. 30 min prior to the addition of Fas, cells were pretreated with either Me₂SO vehicle (open squares) or 100 μM YVAD.CMK in Me₂SO (closed squares). At the indicated times, lipids were extracted and ceramide levels determined as described under “Experimental Procedures.” Results are expressed as fold change in ceramide levels over untreated vehicle control. B, 3 × 10⁵ cells were treated with either Me₂SO vehicle alone (DMSO, black bars), vehicle + 100 ng/ml anti-Fas antibody (hatched bars), or 100 μM YVAD.CMK + 100 ng/ml anti-Fas antibody (white bars). Me₂SO vehicle or YVAD.CMK was added to cells 30 min prior to the addition of anti-Fas antibody. Viability was assessed by trypan blue exclusion at the indicated times after the addition of anti-Fas antibody. Results are representative of two separate experiments.

FIG. 6. Role of ceramide generation and ICE-like protease activity in TNF-α-induced NF-κB activation. A, 5 × 10⁶ cells in 1-ml volumes were either left untreated (open squares) or were treated with 2 nM TNF-α (closed squares) for the indicated times before reactions were stopped by the addition of 15 ml of ice-cold PBS. Lipids were extracted and ceramide levels measured as described under “Experimental Procedures.” B, cells were either left untreated or were treated with 2 nM TNF-α 30 min prior to the addition of TNF-α, cells were incubated with the indicated concentrations of YVAD.CMK. 15 min after the addition of TNF-α, nuclear proteins were extracted and EMSA performed as described under “Experimental Procedures.” n.s., nonspecific bands.
of nonspecific interruption of the NF-κB complex. Importantly, these results demonstrate that ceramide is capable of inhibiting NF-κB, probably through inhibition of the PKC pathway (which does not appear to participate in TNFα action; Ref. 24).

**DISCUSSION**

TNFα is known to induce a number of diverse biologic effects (the nature of which vary depending on target cell type) including cytotoxicity, cell differentiation, and antiviral activity (47). TNFα is one of a group of ligands, including certain cytokines, hormones, and growth factors, which cause the activation of sphingomyelinases resulting in the generation of the lipid mediator ceramide (for review, see Ref. 10). With the discoveries that TNFα could both induce sphingomyelin hydrolysis and ceramide generation (3) and cause nuclear translocation of NF-κB through an undefined signaling mechanism, it was logical to pursue the hypothesis that ceramide may be the second messenger responsible for the TNFα-induced activation of NF-κB. However, despite the recent attention this hypothesis has received and the high degree of interest in delineating the exact mechanisms by which ligand binding at the cell surface results in nuclear translocation of NF-κB, the potential role of ceramide in this process has remained unresolved.

Some evidence has suggested an essential role for SMase activity and ceramide generation in NF-κB activation by TNFα, the strongest of which has come from studies of permeabilized cells (29, 31). Schütze et al. showed that in permeabilized Jurkat T cells, treatment with exogenous SMase and with low (2.5–50 nM) concentrations of ceramide caused enhanced NF-κB binding activity as assessed by EMSA (31). A subsequent study by this group using nuclei-free lysates of Jurkat cells showed in vitro induction of IκB proteolysis by SMase and ceramide within 5 and 1 min, respectively (48). In addition to implicating ceramide in this pathway, these studies have also suggested that the TNFα-induced activation of NF-κB depends specifically on activation of acidic (endosomal) SMase rather than neutral, Mg2+-dependent (membrane-associated) SMase (29, 31). A truncated form of the p55 TNF receptor lacking the ability to activate acid sphingomyelinase was unable to signal NF-κB activation in response to TNFα, suggesting an essential role for sphingomyelinase with acid pH optima (29). Two studies utilizing intact cell systems have suggested a role for ceramide in this process. Yang et al. (30) described enhanced NF-κB binding activity on EMSA in response to direct treatment of HL-60 cells with exogenous bacterial SMase and N-octanoylsphingosine (C8-ceramide), although to a significantly lesser degree than that observed with TNFα treatment. Johns et al. (34) observed minimal activation of NF-κB by exogenous ceramide on EMSA, and activation of an NF-κB-dependent reporter to an extent comparable to TNFα only at very high ceramide concentrations (500 μM).

Additional work, however, has provided conflicting evidence and has suggested that the sphingomyelin cycle and ceramide generation are not involved in the TNFα-induced activation of NF-κB (12, 32, 33, 35, 49). Betts et al. (32) observed potent NF-κB activation in response to TNFα treatment of HL-60 cells, despite observing no appreciable changes in intracellular ceramide levels. Several studies demonstrated that cell-permeable analogs of ceramide could mimic the growth-inhibitory and apoptotic effects of endogenous ceramide but could not induce NF-κB activation (12, 32, 49). Likewise, a study by our group and others recently demonstrated that, while C2-ceramide and bacterial SMase activated Jun kinase (JNK, also known as stress-activated protein kinase), these treatments failed to cause nuclear translocation of NF-κB in HL-60 cells (49). A recent study by Higuchi et al. (35) using the myelogenous leukemia line ML-1a found that the addition of exogenous cell-permeable ceramide analogs was not sufficient to induce either NF-κB activation or DNA fragmentation (in contrast to TNFα, which was shown to induce both in this cell line). Thus there exists contradictory evidence as to whether or not a ceramide second messenger comprises an essential component of the signaling machinery that allows NF-κB to dissociate from IκB and to translocate to the nucleus of target cells.

In contrast to the studies with TNFα, little is known concerning the relationship of Fas activators, ceramide, and NF-κB activation. Activation of Fas in several cell types results in ceramide accumulation, which has been associated with the apoptotic response of these cells (13, 14). On the other hand, the relationship between Fas and NF-κB appears to be more complex. Activation of Fas in U937 cells did not result in activation of NF-κB (50), whereas activation of Fas in SV80 fibroblasts (transfected with Fas) and in T24 cells caused activation of NF-κB (51, 52). Since Fas shares with TNFα the apoptotic and ceramide responses, it potentially provides for a useful model system to investigate the relationship of ceramide to activation of NF-κB.

Several observations in the present study argue against a role for ceramide in the TNFα-induced signaling cascade leading to NF-κB activation. First, cell-permeable C2- and C6-ceramide analogs, which mimic the apoptotic effects of endoge-
A distinct member of the ICE family that acts on poly(ADP-ribose) kinase (SMase) activity is not necessary for NF-κB activation (Fig. 4).

Examined whether acid SMase activation in particular was a clear-cut dichotomy in the activation of NF-κB (Fig. 6). Jurkat cells within minutes without affecting ceramide levels. We demonstrate here that TNFα is able to activate NF-κB, IκB. Likewise, direct addition of exogenous bacterial SMase did not cause IκB proteolysis or NF-κB nuclear translocation (Figs. 1 and 2). We also examined whether acid SMase activation in particular was a required component of TNFα-induced NF-κB activation (as has been proposed previously; Ref. 29) using Niemann-Pick A fibroblasts. We have shown here that cells completely deficient in acid sphingomyelinase retained the ability to normally activate NF-κB in response to TNFα (Fig. 3). While this work was in progress, a study by Kuno et al. (33) likewise described the ability of Niemann-Pick fibroblasts to activate NF-κB in response to TNFα and IL-1 despite lacking acid sphingomyelinase. Additional evidence against a role for acid SMase in cytokine activation of NF-κB comes from work using the acid SMase inhibitor SR33557 (35). Inhibition of acid SMase prevented TNFα-induced DNA fragmentation, but had no effect on TNFα-induced NF-κB activation. These results are in contrast to findings by Wiegmann et al. (29) and suggest that acid SMase activity is not necessary for NF-κB activation. Also, we demonstrate here that TNFα is able to activate NF-κB in Jurkat cells within minutes without affecting ceramide levels (Fig. 6A). The studies in the Fas-activated system also provide a clear-cut dichotomy in the activation of NF-κB and ceramide accumulation. Whereas APO-1/Fas induced significant cell death and ceramide accumulation in the Jurkat cells, it was unable to induce NF-κB activation at early or late time points (Fig. 4).

Taken together, these results suggest that ceramide is neither a sufficient nor necessary signal to induce nuclear translocation of NF-κB in intact cells. Indeed, the studies with PMA-induced activation of NF-κB (Fig. 7) show that ceramide has inhibitory rather than stimulatory effects on activation of NF-κB.

The question remains as to why such discrepancies exist in the evidence put forth for the potential role of acid sphingomyelinase and ceramide in this signaling pathway. One possibility is that an accurate comparison of studies performed using permeabilized cells with those performed on membrane-intact cells is not possible with respect to this signaling cascade. Treatment with permeabilizing agents may fundamentally alter the intracellular environment in ways not easily quantified, for example, by inducing higher levels of proteolysis, such that the signaling events observed in this system may not be physiologically relevant. Moreover, those studies relied heavily on D609 as an inhibitor of phosphatidylcholine-specific phospholipase C, which has been proposed as an upstream activator of the acid sphingomyelinase. This putative phosphatidylcholine-specific phospholipase C is a poorly characterized enzyme, the specificity of the inhibitor has not been determined, and, at best, the studies with the inhibitor may implicate the phospholipase C and not the sphingomyelinase. The studies with the Niemann-Pick fibroblasts are more persuasive in ruling out a role for the acid sphingomyelinase in activation of NF-κB.

Our evidence suggests a model in which TNFα and APO-1/Fas are each able to initiate an apoptotic signaling pathway, which involves downstream activation of ICE-like proteases and ceramide generation, while TNFα additionally induces a distinct set of events, independent of ICE-like proteases and ceramide, resulting in immune modulation via NF-κB activation (Fig. 8).

Recent studies have shown that trimerization of the TNFR-1 upon binding its ligand results in recruitment of the TNFR-1 death domain-associated protein TRADD (27). This complex, in turn, has been shown to directly interact with FADD. Thus, it appears that FADD is a point of convergence between the signaling cascades of TNF and APO-1/Fas (53). The common result is the FADD-dependent activation of a series of ICE-like proteases (including MACH/FLICE), which are beginning to be elucidated and which are thought to play a crucial role in the induction of apoptosis (19, 20). Here we have shown that treatment with antibodies to APO-1/Fas causes up to a 6-fold elevation in ceramide levels and that ceramide generation precedes the induction of cell death in this line of Jurkat cells (Figs. 4 and 5). Additionally, using the ICE-like protease inhibitor YVAD.CMK, we show that ceramide generation appears to be downstream of the activity of ICE-like proteases in this pathway; pretreatment with YVAD.CMK inhibited ceramide generation and subsequent cell death (Fig. 5). These results concur with a recent study of the Drosophila Reaper protein Reaper, which showed that Reaper-induced ceramide generation and apoptosis were largely inhibited in a Drosophila cell line by a peptide inhibitor of ICE-like protease activity (54). Finally, we show here that TNFα is able to potently activate NF-κB despite inhibition of ICE-like proteases by YVAD.CMK (Fig. 6B). These lines of evidence strongly support the hypothesis that ceramide generation is a downstream component of an apoptotic pathway which involves ICE-like protease activation and which is distinct from mechanisms leading to NF-κB activation (Fig. 8).

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