Roles for C_{16}-ceramide and Sphingosine 1-Phosphate in Regulating Hepatocyte Apoptosis in Response to Tumor Necrosis Factor-α*

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Tumor necrosis factor (TNF-α) signals cell death and simultaneously induces the generation of ceramide, which is metabolized to sphingosine and sphingosine 1-phosphate (SIP) by ceramidase (CDase) and sphingosine kinase. Because the dynamic balance between the intracellular levels of ceramide and SIP (the “ceramide/SIP rheostat”) may determine cell survival, we investigated these sphingolipid signaling pathways in TNF-α-induced apoptosis of primary hepatocytes. Endogenous C_{16}-ceramide was elevated during TNF-α-induced apoptosis in both rat and mouse primary hepatocytes. The putative acid sphingomyelinase (ASMase) inhibitor imipramine inhibited TNF-α-induced apoptosis and C_{16}-ceramide increase as did the knock out of ASMase. Overexpression of neutral CDase (NCDase) inhibited the TNF-α-induced increase of C_{16}-ceramide and apoptosis in rat primary hepatocytes. Moreover, NCDase inhibited liver injury and hepatocyte apoptosis in mice treated with β-galactosamine plus TNF-α. This protective effect was abrogated by the sphingosine kinase inhibitor N,N-dimethylsphingosine, suggesting that the survival effect of NCDase is due to not only C_{16}-ceramide reduction but also SIP formation. Administration of SIP or overexpression of NCDase activated the pro-survival kinase AKT, and overexpression of dominant negative AKT blocked the survival effect of NCDase. In conclusion, activation of ASMase and generation of C_{16}-ceramide contributed to TNF-α-induced hepatocyte apoptosis. NCDase prevented apoptosis both by reducing C_{16}-ceramide and by activation of AKT through SIP formation. Therefore, the cross-talk between sphingolipids and AKT pathway may determine hepatocyte apoptosis by TNF-α.

Tumor necrosis factor (TNF)α is a multifunctional cytokine that plays a role in inflammation, immunity, antiviral responses, and a variety of diseases. TNF-α is particularly important in the pathophysiology of hepatocytes, mediating viral hepatitis, alcoholic liver disease, and fulminant hepatitis (1). TNF-α activates a variety of cellular signal transduction pathways, some of which result in apoptosis (2, 3). Ceramides are sphingolipid-derived signaling and regulatory intermediates that play a role in the stress response and cell death (4). Stress stimuli such as TNF-α, Fas ligand, oxidative stress, growth factor withdrawal, anticancer drugs, ionizing radiation, heat shock, or ultraviolet light induce an elevation in the endogenous cellular levels of ceramides (4, 5). Ceramides are generated from the major membrane sphingolipid sphingomyelin by acid or neutral sphingomyelinas (ASMase), enzymes that are activated in response to TNF-α and other cytokines (6). ASMase knock-out mice are resistant against TNF-α-induced liver injury (7). Thus, ceramides generated by ASMase have an important role in TNF-α-induced hepatitis cell death.

Besides apoptotic signals, TNF-α also activates molecules that protect cells from apoptosis. For example, TNF-α transmits anti-apoptotic signals via nuclear factor (NF)-κB and phosphatidylinositol 3-kinase (PI3K)/AKT. Blocking these signaling pathways results in sensitization of hepatocytes to apoptosis induced by TNF-α (8, 9). In addition TNF-α activates sphingosine kinase (SphK), which converts sphingosine to sphingosine 1-phosphate (SIP). This lipid-derived mediator prevents the cytotoxic action of TNF-α (8, 10). Moreover, a model has been proposed in which the dynamic balance between the intracellular levels of ceramide and SIP (the “ceramide/SIP rheostat”) is an important factor that determines whether a cell survives or dies (11).

Ceramide is deacylated by either acid or neutral ceramidase (ACDase or NCDase) and converted to sphingosine. Overexpression of ACDase, which is localized in the lysosomes (12), protects from TNF-α-induced apoptosis in murine fibrosarcoma L929 cells by reduction of intracellular ceramide and therefore in a shift of the ceramide/SIP rheostat in the direction of cell survival (13). Besides an acidic form, a neutral ceramidase has been identified and cloned (14–16). NCDase is a membrane-bound enzyme that catalyzes the hydrolysis of N-acyl linkage in ceramide (10). In rat hepatocytes, NCDase is localized in late endosomes/lysosomes because of the presence of a functional di-Leu motif in the putative amino acid sequences in NCDases, which is a sorting signal for vesicular transport from plasma membranes.
was performed with anti-cleaved caspase (−3) and caspase 3

TNF-α generating its clearance by overexpression of NCDase protected from important role of AKT activation in this protective response.

periods of time. Cell death was determined by double staining with Hoechst 33258 (purchased from Nacalai Tesque (Tokyo, Japan). All other reagents used benzimide), propidium iodide, and anti-

ride, LY 294002, ASMase from human placenta, Hoechst 33258 (bis-
sphingosine (DMS), S1P, pertussis toxin (PTX), imipramine hydrochlo-

N, N,

was obtained from R&D Systems (Minneapolis MN).

membrane to endosomes/lysosomes, (10). More importantly, the roles of NCDases in TNF-α-induced hepatocyte apoptosis have not been addressed.

In the present study, we evaluated the role of endogenous ceramide in apoptosis of rat primary hepatocytes induced by TNF-α. Preventing formation of endogenous ceramide or accelerating its clearance by overexpression of NCDase protected from TNF-α induced hepatocyte apoptosis. We provide evidence for an important role of AKT activation in this protective response.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media for rat and mouse primary hepatocytes, Waymouth’s MB 752/1 and RPMI 1640, and recombinant human insulin were purchased from Invitrogen. Recombinant mouse TNF-α was obtained from R & D Systems (Minneapolis MN). N, N-Dimethyl-

phosphoglycine (DMS), S1P, pertussis toxin (PTX), imipramine hydrochloride, L-arginine, and anti-caspase-3 (clone AC-15) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 

were of the highest analytical grade available.

Primary Hepatocyte Cultures and Treatments—Sprague-Dawley male rats (200–250 g) or ASMase knock-out mice (ASMase−/−), maintained in C57BL/6 background, were anesthetized with ketamine and xylazine administered by intraperitoneal injection. Hepatocytes were then isolated by a nonrecirculating in situ collagenase perfusion of livers cannulating through the portal vein by a procedure modified from Moldeus et al. (17). Livers were first perfused in situ with 0.5 mM EGTA containing calcium-free salt solution, followed by perfusion with solution containing 0.02% collagenase D (Roche Applied Science). The liver was then gently minced on a Petri dish and filtered with polycarbonate mesh (3-60/42, Sefar America, Kansas City, MO). Hepatocytes were washed three times and centrifuged at 50 × g for 1 min. Cell viability was consistently 85% as determined by trypan blue exclusion. Cells (1 × 10^7 and 1 × 10^5) were plated on 12-well and 60-mm dishes coated with rat collagen type I in Waymouth’s medium containing 10% fetal bovine serum containing antibiotics (plating medium). After 4 h, the culture was washed with PBS and changed to serum-free RPMI 1640 containing antibiotics in the presence or absence of recombinant adenoviruses (Ad5GFP, Ad5IκB, Ad5NCDase, Ad5CA-AKT, Ad5DN-AKT, and Ad5NFB-Luc). After 2 h of incubation, the culture media were changed to serum-free RPMI 1640 containing antibiotics, and the cells were incubated for another 16 h. Before stimulation with 30 ng/ml mouse TNF-α or 1 μM S1P, the cells were washed with PBS and, if necessary, incubated for 1 or 2 h in serum-free RPMI 1640 containing the indicated agent(s): 50 μM imipramine in H_2O, 10 μM DMS in Me_3SO, ASMase (1

FIG. 1. The IκB super repressor sensitizes rat hepatocytes to TNF-α-mediated cell death. A. NF-κB activation of primary rat hepatocytes infected with or without Ad5IκB by TNF-α (30 ng/ml, for 8 h) was assessed by reporter gene assays using 3× NFκB luciferase (κB3-Luc) expressing adenovirus (10 μL/NI). B. primary rat hepatocytes infected with Ad5GFP or Ad5IκB (10 μL/NI) were treated with TNF-α for various periods of time. Cell death was determined by double staining with Hoechst 33258 (left panel) and propidium iodide (right panel) to detect apoptotic and necrotic cells, respectively. At least 500 cells were counted, and cell death was expressed as a percentage of total cells. Results are expressed as means ± S.D. from at least three independent experiments. *, p < 0.01 using Student’s t test. C. primary rat hepatocytes infected with Ad5GFP or Ad5IκB were treated with TNF-α (30 ng/ml) for various periods of time. Extracted proteins were subjected to SDS-PAGE, and immunoblotting was performed with anti-cleaved caspase (casp)-3 and β-actin antibodies. The results shown are representative of at least two independent experiments. D. primary rat hepatocytes infected with Ad5GFP or Ad5IκB were incubated with TNF-α for the indicated times. The levels of individual ceramide molecular species were measured by using MS analysis. The results are expressed as picomoles of lipid/μg of protein, and they represent the mean from two independent experiments.
NCDase Protects Hepatocyte Apoptosis Dependence on AKT

In some experiments, hepatocytes were pretreated with PTX for 12 h before adenovirus infection or S1P treatment.

Adenoviruses—The adenovirus five variants Ad5IxB, Ad5GFP, Ad5CA-ART, Ad5DN-ART, and Ad5NF-b-Luc expressing hemagglutinin (HA)-IxB (S32A, S36A), green fluorescent protein, HA constitutively active (CA)-AKT encoding an amino-terminal myristoylation signal, HA-dominant negative (DN)-AKT, and an NF-κB-driven luciferase reporter, respectively, have been described previously (9, 18–20). The recombinant replication-deficient adenovirus Ad5NCDase was constructed by AdEasy™ adeno viral vector system (Stratagene, La Jolla, CA). Briefly, the full-length of human NCDase DNA (GenBank™ accession number AF449759) was subcloned into gTrack adenoviral vector. The plasmid DNA was prepared by the alkaline lysis method and transfected into BJ5183-AD-1 electroporation-competent cells. The virus was grown in 293 cells and purified by banding twice on CsCl gradients and then stored in 10% (v/v) glycerol at −20 °C.

Assessment of Cell Death—To assess cell death, cell cultures were double-stained with propidium iodide and Hoechst 33258. Propidium iodide is a vital nucleic acid-staining dye that penetrates cells with compromised plasma membrane (necrotic cells). Morphological changes in the nuclei of cells undergoing apoptotic cell death were determined by staining with the DNA-binding fluorochrome Hoechst 33258. Apoptotic nuclear changes include condensation, margination, and segmentation of the nuclei into several fragments. Briefly, cells were stained with both dyes (100 μM) for 20 min and examined under a fluorescent microscope (Olympus IX72, Tokyo, Japan). Quantitation of apoptotic and necrotic cells was performed by counting at least 500 cells and was expressed as a percentage of total cells counted.

Animal Treatment—The experiments were conducted in accordance with the institutional guidelines by Columbia University. Eight-week-old male BALB/c mice were infected with Ad5GFP or Ad5NCDase (1 × 109 plaque-forming units) by intravenously injecting the tail vein. GalN was dissolved in sterile, nonpyrogenic saline solution and was administered to the mice intraperitoneally (20 mg/mouse), 2 days after adenovirus administration. Recombinant mouse TNF-α was diluted with pyrogen-free saline and injected to mice intravenously (0.5 μg/mouse) 30 min after GalN treatment. Treated animals were anesthetized and killed by withdrawal of blood from the inferior vena cava. Hepatocellular injury was monitored biochemically by measuring serum alanine aminotransferase (ALT) activity. The liver was excised, fixed with 10% buffered formalin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin for light microscopic examination. Apoptotic cells were estimated by the terminal deoxynucleotidyltransferase nick-end labeling (TUNEL) assay, which relies on incorporation of labeled dUTP at sites of DNA breaks. For the TUNEL procedure, all reagents, including buffers, were parts of a kit (ApopTag, Chemicon, Temecula, CA). Procedures were carried out according to the manufacturer’s instructions.

Western Blot Analysis—For the preparation of total cell proteins, cells or frozen liver were sonicated in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.8, 150 mM NaCl, 10 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 0.3 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 0.1 mM sodium molybdate, 0.5 mM 4-deoxyxypiridoxine). The proteins were separated by SDS-PAGE and were electrophoretically transferred onto nitrocellulose membrane. The membranes were first incubated with the primary antibodies and then incubated with the anti-mouse or -rabbit horseradish peroxidase-coupled secondary antibodies. Detection was performed with an ECL system.

Reverse Transcription-PCR Analysis—Total RNAs isolated from hepatocytes infected with Ad5NCDase or Ad5GFP were reverse-transcribed using random hexamer mixed primers. cDNAs were amplified with the following primer sets: human NCDase sense, 5′-AGAAACTC-GATGGGTCCAATCG-3′; and human NCDase antisense, 5′-GGGA-

FIG. 2. ASMase inhibitor treatment, imipramine, or ASMase(−/−) hepatocytes are resistant to cell death induced by TNF-α. A, rat primary hepatocytes infected with Ad5IxB were pretreated with or without imipramine (50 μM) for 1 h and then treated with TNF-α (30 ng/ml) for the indicated times (left panel). Mouse primary hepatocytes from wild type or ASMase(−/−) mice were infected with Ad5IxB and then exposed to TNF-α for the indicated times (right panel). Apoptotic nuclei cells stained with Hoechst 33258 (upper panel) and necrotic cells with propidium iodide (lower panel) were counted, and the percentage of cell apoptosis and necrosis out of 500 cells was determined. Data are means ± S.D. from at least three independent experiments. *, p < 0.01 using Student’s t test. B, the C16′′ and total ceramide (Cer) contents of rat primary hepatocytes (left panel) or mouse primary hepatocytes (right panel) at 12 h after TNF-α treatment were measured by using MS analysis. Data are means from two independent experiments. C, mouse primary hepatocytes from wild type or ASMase(−/−) mice were infected with Ad5IxB or Ad5GFP and then exposed to TNF-α with or without ASMase (1 IU/ml) for 8 h. Apoptotic nuclei cells stained with Hoechst 33258. N. S., not significant.
CAAGTGCTATTGGCGTTA-3' rat NCDase sense, 5'-TGAGAGACGACGTGAAAGCCGC-3', and rat NCDase antisense, 5'-TGCGATAACGA-CAGTCATATCC, at an annealing temperature of 58 °C. After amplification, PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

**NF-κB Reporter Gene Assay**—Hepatocytes were infected with Ad5NF-κB Bluc (10 m.o.i.) and Ad5IκB or Ad5GFP for 18 h. NF-κB-dependent gene transcription was analyzed 8 h later on a luminometer (FLUOstar OPTIMA, BMG LABTECH, Inc., Durham, NC) using an enhanced luciferase assay kit (Pharmingen) and adjusted for protein content.

**Mass Spectrometric Analysis of Lipids**—These were performed using electrospray ionization MS/MS analysis on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in multiple reaction monitoring positive ionization mode as described previously (21).

**Measurement of S1P Formation**—S1P formation was measured as described previously (8) with slight modifications. For the radiolabeling of sphingolipids, the primary rat hepatocytes were incubated with the plating medium containing [3H]serine (2 μCi/ml) for 12 h. The medium was then changed to serum-free RPMI 1640 medium containing [3H]serine, and the cells were incubated for another 18 h with or without adenovirus infection. The radiolabeled cells were stimulated with or without S1P, and the cellular lipids were extracted by the method of Bligh and Dyer and separated on TLC plates in the solvent.

**Fig. 3.** Overexpression of NCDase reduces hepatocyte apoptosis induced by TNF-α. A. cDNAs derived from Ad5GFP- or Ad5NCDase-infected rat primary hepatocytes RNA were analyzed by reverse transcription-PCR using specific oligonucleotide primers of human NCDase, rat NCDase, and β-actin. PCR with RNA was performed as negative control. B, primary rat hepatocytes infected with Ad5NCDase plus Ad5GFP or Ad5IκB was treated with TNF-α (30 ng/ml) for 8 h. Apoptotic nuclei cells stained with Hoechst 33342 were counted among 500 cells, and the percentage of cell apoptosis was determined (upper panel). Data are means ± S.D. from at least three independent experiments. *, p < 0.01 using Student’s t test. Cellular protein was extracted 12 h after TNF-α administration and subjected to SDS-PAGE. Immunoblotting was performed with anti-cleaved caspase-3 and β-actin antibodies. A densitometric scan of the Western blot analysis was performed (lower panel). Cleaved caspase-3/β-actin ratio is indicated. Data are the means from two independent experiments. C, hepatocytes infected with Ad5IκB and Ad5NCDase, or Ad5GFP were treated with TNF-α for 12 h. The C16- and total ceramide contents were measured using MS analysis. Data are means from two independent experiments.
system of 1-butanol/acetic acid/water (60:20:20, v/v). The radiolabeled S1P spot, identified by comigration with an authentic standard, was scraped off from the plate, and the radioactivity was measured in a liquid scintillation counter.

RESULTS
Increase of C16-ceramide Levels during TNF-α/H9251-induced Hepatocyte Apoptosis—TNF-α induced NF-κB activation in rat primary hepatocytes as determined by luciferase assay. The activation was almost abolished when hepatocytes were infected with Ad5IκB but not with the control adenovirus Ad5GFP (Fig. 1A). Normal hepatocytes are usually resistant to the cytotoxicity of TNF-α. However, infection with Ad5IκB sensitizes hepatocytes to TNF-α-mediated apoptosis (8, 19, 22, 23). To determine the extent of cell death induced by TNF-α under these conditions, hepatocytes were labeled with propidium iodide, a vital stain, and the permeable DNA-binding fluorochrome Hoechst 33258. Ad5IκB infection sensitized hepatocytes to both apoptosis and necrosis induced by TNF-α (Fig. 1B), as reported previously. Previous studies have demonstrated that TNF-α causes activation of caspases in hepatocytes infected with Ad5IκB (8, 22). Cleavage of caspase-3 occurred within 12 h after TNF-α treatment (Fig. 1C). Moreover, Ad5CrmA infection, which expresses crmA, a serpin inhibitor of a subset of caspases including caspases-1 and -8 (24, 25), completely prevented cell death (data not shown).

Previous studies demonstrated that TNF-α induces ceramide formation in hepatocytes by activation of ASMase (7, 26), and ceramide is thought to be a lipid mediator involved in the apoptotic process (2, 27). Although total ceramide levels in response to TNF-α did not show remarkable change in both Ad5GFP- and Ad5IκB-infected hepatocytes (Fig. 1D), C16-ceramide increased 1.7-fold in Ad5IκB-infected hepatocytes after TNF-α treatment (Fig. 1D). Thus, TNF-α-induced cell death was accompanied with selective increase in the levels of only one ceramide species.

![Fig. 4](http://www.jbc.org/)

**FIG. 4.** Overexpression of NCDase reduces liver damage induced by TNF-α. BALB/c mice were infected with Ad5GFP or Ad5NCDase (1 × 10⁶ plaque-forming units/mouse) by intravenous administration. 48 h later, the mice were treated with (per mouse) 20 mg of GalN intraperitoneally 30 min before TNF-α treatment. Recombinant mouse TNF-α was injected intravenously (0.5 μg/mouse). A, serum ALT levels were determined 6 h after TNF-α injection. Data are means ± S.D. from at least three independent experiments. *, p < 0.05 using Student’s t test. B, liver sections were stained with hematoxylin and eosin for light microscopic examination (upper panel). Apoptotic nuclei in mouse liver were identified using TUNEL (lower panel). C, protein extracts from liver tissue were subjected to SDS-PAGE and immunoblotting was performed with anti-cleaved caspase-3 (casp-3) and β-actin antibodies. The results shown are representative of three independent experiments.

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<th>TABLE I</th>
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<td>Changes in the sphingolipid profile in Ad5NCDase-infected hepatocytes</td>
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<td>Rat primary hepatocytes were infected with Ad5GFP or Ad5NCDase. Sphingosine and the different ceramide species were examined by MS analysis. The results are expressed as picomoles of lipid/μg of protein, and they represent the mean from two independent experiments. The abbreviations used are as follows: Cer, ceramide; dh-Cer, dihydroceramide.</td>
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![Casp-3 Blot](http://www.jbc.org/)

**Casp-3 Blot**

Ad5GFP + GalN Ad5GFP + GalN + TNF Ad5NCD + GalN + TNF

**NCDase Protects Hepatocyte Apoptosis Dependence on AKT**

![AKT Blot](http://www.jbc.org/)
Protective Effect of ASMase Inhibition against Hepatocyte Apoptosis by TNF-α—We next aimed to determine the role of C16-ceramide in cell death. We initially attempted to examine the effects of exogenous C16-ceramide. However, rat primary hepatocytes did not take up C16-ceramide even when dissolved in ethanol/dodecane (98:2, v/v) (data not shown). Therefore, we resorted to a combination of pharmacologic, molecular, and genetic approaches to investigate the role of endogenous ceramide.

It was reported previously (7) that ASMase knock-out mice are resistant to TNF-α-induced liver injury. Imipramine, a tricyclic antidepressant, induces the proteolysis of the active 72-kDa ASMase form and hence inhibits ASMase activity (28). Pretreatment with imipramine protected rat primary hepatocytes from apoptosis and necrosis induced by Ad5IκB plus TNF-α (Fig. 2A, left panel). However, because imipramine may have pleiotropic effects, we also used ASMase knock-out mice. Indeed, primary hepatocytes from ASMase knock-out mice showed less apoptosis and necrosis than wild type hepatocytes (Fig. 2A, right panel). Furthermore, imipramine treatment or ASMase knock-out cells prevented the increase of C16-ceramide by Ad5IκB plus TNF-α (Fig. 2B), and exogenous ASMase administration reversed susceptibility of apoptosis in ASMase knock-out hepatocytes (Fig. 2C). These results suggest that ASMase activation and C16-ceramide accumulation contribute to hepatocyte apoptosis.

Protective Effects of NCDase Overexpression in Hepatocytes—Ceramide levels are regulated not only through formation (e.g., by SMase) but also by degradation through the action of CDases. Besides an acidic form, which is localized in the lysosomes (12), a neutral ceramidase has been identified and cloned (14, 16). We hypothesized that this neutral ceramidase may play a role in countervailing ceramide generation by the sphingomyelinases. Thus, we constructed a human NCDase expressing adenovirus, and we investigated its effects on TNF-α-induced apoptosis. PCR analysis revealed that human NCDase was expressed in Ad5NCDase-infected rat primary hepatocytes and that endogenous rat NCDase levels were not affected by Ad5NCDase infection (Fig. 3A). Ad5NCDase infection increased the survival of rat primary hepatocyte apoptosis after TNF-α and reduced caspase-3 cleavage (Fig. 3B), but Ad5NCDase did not affect the expression of IκB mutant form by Ad5IκB (supplemental Fig. 1). Ad5NCDase induced the largest decrease in C16-ceramide (40% reduction) (Table I) followed by C18:1- and C18-ceramide (20% reduction). On the other hand, C24- and C24:1-ceramide levels were not significantly affected. The levels of C14-, dihydro-C16-, and C20-ceramide were extremely low in hepatocytes. Ad5NCDase infection inhibited the increase of C16-ceramide by Ad5IκB plus TNF-α (Fig. 3C). To assess further the protective role of NCDase during hepatocyte apoptosis, its effect on TNF-α-mediated liver injury was examined in vivo. Administration of Ad5NCDase via tail vein, which expresses both NCDase and GFP, resulted in GFP expression and NCDase mRNA expression in liver of BALB/c mice (supplemental Fig. 2). In BALB/c mice infected
with Ad5GFP, GalN plus TNF-α elicited liver damage, and the serum ALT level increased to about 7000 IU/liter at 6 h (Fig. 4A). Histological analysis revealed that GalN plus TNF-α resulted in pronounced hepatocyte destruction in large areas of the liver (Fig. 4B, upper panel), with many TUNEL-positive hepatocytes (Fig. 4B, lower panel), and an increase in caspase-3 cleavage (Fig. 4C). Overexpression of NCDase reduced serum ALT levels by 70% (Fig. 4A). Moreover, overexpression of NCDase reversed morphological changes induced by GalN plus TNF-α and strongly reduced the number of TUNEL-positive hepatocytes (Fig. 4B) and caspase-3 cleavage (Fig. 4C). Thus, overexpression of neutral ceramidase protects from TNF-α-induced hepatotoxicity.

Effects of SphK Inhibitor, DMS, on Hepatocyte Apoptosis—Ceramide is hydrolyzed to sphingosine, which is subsequently converted to S1P by SphK. Previous studies demonstrated that SphK activation can mediate anti-apoptotic actions via S1P formation (8, 29). NCDase reduced sphingosine in rat hepatocytes (Table I), suggesting that sphingosine was probably metabolized to another sphingolipid like S1P. Indeed, pretreatment with the SphK inhibitor DMS blocked the anti-apoptotic effect of NCDase overexpression (Fig. 5A, upper panel), and it prevented the cleavage of caspase-3 (Fig. 5A, lower panel). Moreover, NCDase overexpression increased S1P level, and DMS reversed this effect (Fig. 5B). Thus, the anti-apoptotic effect of NCDase overexpression was not due to ceramide degradation alone, but appears to be contributed to by the formation and phosphorylation of sphingosine and a change in the balance of C16-ceramide and S1P.

To begin to elucidate the cytoprotective mechanisms of NCDase, we characterized the effects of NCDase on TNF-α-induced cytoprotective pathways. Because NF-κB activation was already completely blocked by Ad5I and not further modified by NCDase under the conditions of our study, we focused on potential effects of NCDase on the anti-apoptotic mediator AKT (8, 23, 30, 31). Infection of Ad5NCDase induced phosphorylation of AKT, and DMS inhibited this effect in a concentration-dependent manner (Fig. 5C). DMS could not inhibit AKT phosphorylation by insulin (data not shown), suggesting that this effect of DMS was specifically due to SphK inhibition. Moreover, exogenous S1P administration induced phosphorylation of AKT (Fig. 5D). Thus, NCDase overexpression resulted in AKT phosphorylation through S1P formation.

Accumulating evidence indicates that S1P formed by SphK acts not only as an autocrine and/or paracrine ligand via the Edg receptor but also as an intracellular second messenger (32). To examine the involvement of Edg receptor(s) in the activation of AKT, hepatocytes were pretreated with PTX for...
DISCUSSION

TNF-α is a potent mediator of hepatotoxicity in vivo and in cultured hepatocytes. However, TNF-α simultaneously activates cytotoxic and cytoprotective signaling pathways, and TNF-α alone does not induce cell death in normal hepatocytes (22, 23, 26). Although several TNF-α-induced pro- and anti-apoptotic pathway have been characterized in hepatocytes, including NF-κB, c-Jun NH2-terminal kinase/AP-1, and AKT, the role of sphingolipids in TNF-α-induced cell death is not completely understood (8, 22, 23, 33, 34). The present study was undertaken to address specifically the role of sphingolipids in the apoptotic signaling of TNF-α in hepatocytes using Ad5IxB-infected rat and mouse primary hepatocytes. Our results demonstrate that overexpression of NCDase inhibits TNF-α-induced hepatocyte apoptosis via AKT activation. The results raise novel therapeutic possibilities for ameliorating acute liver injury.

TNF-α has been reported to induce ceramide formation, at least in part, by activation of ASMase (26). Ceramide generation by ASMase is involved in TNF receptor or Fas-induced signaling. Deoxycholic acid activates the c-Jun NH2-terminal kinase pathway via Fas translocation to the plasma membrane, which is induced by ceramide generation by ASMase activation in primary hepatocytes (35). ASMase knock-out mice are resistant to TNF-α-induced liver injury (7). Indeed, administration of TNF-α increased C16-ceramide content in both rat and mouse primary hepatocytes, and the ASMase inhibitor imipramine or ASMase knock out blocked increase of C16-ceramide and apoptosis induced by TNF-α. Moreover, the de novo ceramide synthase inhibitor fumonisin B1 or the neutral sphingomyelinase inhibitor N-acetylcysteine (36) did not inhibit this apoptosis (data not shown). These results suggest that activation of ASMase contributes to TNF-α-induced hepatocyte apoptosis. These results and previous reports lead us to speculate that C16-ceramide formation is specifically required for apoptosis induced by TNF-α.

TNF receptor I and Fas are associated with lipid rafts, and it is believed that the generation of ceramide by ASMase within these cholesterol- and sphingolipid-rich domains is required for TNF receptor- and Fas-mediated apoptosis (37–40). However, pretreatment of methyl-β-cyclodextrin, a cholesterol-depleting agent that is commonly used to disrupt lipid rafts, did not prevent hepatocyte apoptosis in our model (data not shown).

Another pathway by which ceramides may contribute to apoptosis is the generation of glucosylceramide and glycosphingolipids (such as GD3). It has been reported that GD3 has apoptotic effects by affecting mitochondria (41, 42). In hepatocytes, GD3 is targeted to mitochondria after TNF-α treatment, and administration of exogenous GD3 induces apoptosis in hepatocytes sensitized by depletion of mitochondrial glutathione (7). However, exogenous GD3 (50 μM) did not induce cell death in Ad5IxB-infected hepatocytes (data not shown). Thus, both GD3 formation and ceramide generation in lipid rafts do not appear to exert major effects on hepatocyte apoptosis in this model.

Ceramides are also converted into sphingosine by CDase, and sphingosine is phosphorylated by SphK to S1P. Although ceramide has been regarded as a proapoptotic factor, S1P has been implicated as a survival factor (43). Ceramide and its metabolite S1P often exert opposing effects, thus the balance between these two sphingolipids may be important for cell fate (11, 43, 44). CDase is classified into the following three groups based on optimum catalytic pH: ACDase, alkaline, and NC-Dase. We now report that overexpression of NCDase reduced hepatocyte apoptosis by TNF-α both in tissue culture as well as in vivo. Most interestingly, in NCDase overexpressing hepatocytes, total ceramide levels were not modulated/changed because C24-ceramide and C24:1-ceramide, the major contents of ceramides in hepatocytes, were not affected; however, C16-ceramide was significantly reduced, again implicating this specific ceramide in mediating apoptosis. In addition to changes in ceramide, overexpression of NCDase also decreased sphingosine and increased S1P levels, suggesting that the metabolite was further converted to S1P. The SphK inhibitor DMS eliminated the anti-apoptotic effect of NCDase. Thus, the anti-apoptotic effect of NCDase is due to both C16-ceramide reduction and S1P formation. S1P stimulates many signaling pathways, such as cAMP-dependent kinase, focal adhesion kinase, extracellular signal-regulated kinase, AP-1, and NF-κB (10, 32). S1P also activates the PI3K/AKT pathway in endothelial differentiation gene (EDG3 receptor overexpressing Chinese hamster ovary cells (45) and human hepatocytes (8). The PI3K product, phosphatidylinositol 3,4,5-trisphosphate activates phosphatidylinositol-dependent kinase, which activates AKT. Activation of AKT protects cells from apoptosis induced by TNF-α (8, 23, 30, 31) by phosphorylating MDM2, AFX, FKHR, mTOR, and BAD (46, 47). Reciprocally, ceramide has been shown to
that ACDase also protects from TNF-α-receptor(s) in primary hepatocytes. Although it has been shown that ACDase also protects from TNF-α-induced apoptosis (13), it is not clear whether this protective effect is mediated through TNF receptor activation. Further studies need to examine the effects of several kinds of CDases on apoptosis signals.

In summary, we have shown that overexpression of NCDase protects from TNF-α-induced hepatocyte apoptosis via AKT activation by S1P (Fig. 7). Our results suggest that the regulation of NCDase may present a new therapeutic approach to block apoptosis in liver disease.

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Roles for $\text{C}_{16}\text{-ceramide}$ and Sphingosine 1-Phosphate in Regulating Hepatocyte Apoptosis in Response to Tumor Necrosis Factor- $\alpha$
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