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Tumor Necrosis Factor-α Stimulates the Maturation of Sterol Regulatory Element Binding Protein-1 in Human Hepatocytes through the Action of Neutral Sphingomyelinase*

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The mechanism by which genes involved in cholesterol biosynthesis and import are preferentially up-regulated in response to sterol depletion was elucidated with the cloning of sterol regulatory element binding protein-1 (SREBP-1). SREBP-1 is a transcription factor whose entry into the nucleus is gated by sterol-regulated proteolysis. We have investigated the role of tumor necrosis factor-α (TNF-α) as a mediator of SREBP-1 maturation in human hepatocytes. TNF-α is capable of inducing SREBP-1 maturation in a time- and dose-dependent manner that is consistent with the kinetics of TNF-α-mediated activation of neutral sphingomyelinase (N-SMase). Antibodies to N-SMase inhibit TNF-α-induced SREBP-1 maturation suggesting that N-SMase is a necessary component of this signal transduction pathway. Ceramide, a product of sphingomyelin hydrolysis, is also capable of inducing SREBP-1 maturation. The mature form of SREBP-1 generated by TNF-α, sphingomyelinase or ceramide treatment translocates to the nucleus and binds the sterol regulatory element. This promotes transcription of the gene upstream of the sterol regulatory element.

A unique finding of our studies is that ceramide stimulated SREBP-1 maturation even in the presence of cholesterol and 25-hydroxycholesterol both of which are known suppressors of SREBP-1 maturation. Our findings indicate that ceramide-mediated maturation of SREBP-1 maturation is a novel sterol-independent mechanism by which cholesterol homeostasis may be regulated.

The cytokine tumor necrosis factor (TNF[1]-α) elicits a wide range of biological effects including inflammatory, cytotoxic, antiviral, and proliferative processes (1). Despite significant progress in our understanding of the signal transducing mechanisms employed by TNF-α (2), they remain incompletely characterized. Elucidation of these pathways is complicated by the existence of at least two TNF receptors. These receptors share some common downstream effectors but also signal via receptor specific pathways.

One of the earliest events in TNF signaling is the activation of neutral sphingomyelinase (N-SMase). Neutral sphingomyelinase is a membrane bound enzyme that catalyzes the hydrolysis of sphingomyelin to ceramide and phosphocholine at a pH optima of 7.4 (3). The role of neutral sphingomyelinase in signal transduction has primarily been ascribed to its ability to generate the lipid second messenger ceramide. In addition to TNF-α, Fas receptor ligand (4, 5), vitamin D_3 (6), interleukin-1β (7), nerve growth factor (8), anti-CD28 antibodies (9), and γ-interferon (10) have all been shown to increase ceramide levels.

Sphingolipids, including ceramide, are increasingly appreciated as regulators of cell growth and differentiation (8, 11). Other lipids, such as cholesterol, have long been appreciated for their roles in cell physiology and pathophysiology. Cholesterol homeostasis in particular is a tightly regulated process. Dysregulation of cholesterol metabolism can lead to a variety of pathophysiological states including heart disease and stroke (12).

The focus of regulation for cholesterol homeostasis is the low density lipoprotein (LDL) receptor (12). The LDL receptor binds to cholesterol rich particles in the plasma and delivers them to cells. Transcription of the LDL receptor gene is suppressed when sterols accumulate and induced when sterols are depleted. Sterol sensitivity is conferred by a 10-base pair sequence upstream of the LDL receptor gene known as the sterol regulatory element (SRE) (13). The mature form of the sterol regulatory element binding protein-1 (SREBP-1) binds to the SRE and promotes transcription (14).

The activity of SREBP-1 is controlled by an extranuclear sequestration mechanism. Like nuclear factor-kB, SREBP-1 is synthesized as an inactive precursor that is proteolytically processed into a mature transcriptionally active form that translocates to the nucleus. Unlike nuclear factor-kB, however, SREBP-1 proteolysis is induced by sterol depletion and suppressed by sterol accumulation. Both molecules are proteolyzed in response to treatment with TNF-α.

Here we report that TNF-α is capable of inducing SREBP-1 proteolysis independent of the presence of sterols. Exogenously supplied sphingomyelinase and ceramide are also capable of inducing SREBP-1 proteolysis in a time- and dose-dependent manner. The kinetics of SREBP-1 maturation is consistent with those of neutral sphingomyelinase activation by TNF-α. Further, SREBP-1 maturation can be blocked with anti-N-SMase antibodies suggesting that neutral sphingomyelinase is necessary for TNF-α-induced sterol-independent SREBP-1 cleavage. The product of sterol-independent SREBP-1 proteolysis is capable of nuclear translocation and binds to the sterol regulatory element.

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[1] The abbreviations used are: TNF, tumor necrosis factor; N-SMase, neutral sphingomyelinase; LDL, low density lipoprotein; SRE, sterol regulatory element; SREBP-1, SRE binding protein-1; PBS, phosphate-buffered saline; C_2-ceramide, *N*-acetylsphingosine.

EXPERIMENTAL PROCEDURES

Materials—A continuous line of human hepatocytes designated HH-25 was prepared from normal human tissue (18). Alpha-modified minimal essential medium was purchased from Mediatech (Herndon, VA). Fetal bovine serum was purchased from Hyclone (Salt Lake City, UT). F10 medium and the insulin-transferrin-selenium supplement were purchased from Life Technologies, Inc. Human recombinant epidermal growth factor, platelet-derived growth factor, and TNF-α were from Upstate Biotechnology (Lake Placid, NY). C_2-ceramide (*N*-acetylsphingosine) was obtained from Matreya (Pleasant Gap, PA). [^{14}C]Sphingomyelin (specific activity 50 mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Anti-SREBP-1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sphingomyelinase from *Streptomyces* species and all other reagents were obtained from Sigma.

Cell Culture—HH-25 cells were grown in alpha-minimal essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml insulin, 0.1 μM selenium, 5.5 μg/ml transferrin, 0.5 μg/ml linoleic acid, and 10% fetal bovine serum (medium A). The cells were incubated in serum free F10 media for 30 to 60 min prior to initiating treatment with TNF-α, C_2-ceramide, or sphingomyelinase.

Cell Fractionation—Following treatment, the cells were washed with 5 ml of PBS and pelleted at 1500 $\times$ *g* for 10 min at 4 °C. The pellet was stored at −70 °C and lysed in 0.5 ml buffer A (10 mM HEPES, pH 7.4, 5 mM EDTA, 0.25 mM EGTA, 50 mM NaF, 7 mM β-mercaptoethanol, 0.35 M sucrose, 0.1% Nonidet P-40, and protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 μg/ml pepstatin). The lysate was centrifuged at 12,000 $\times$ *g* for 15 min at 4 °C to prepare a nuclear fraction.

Neutral Sphingomyelinase Assay—After stimulation with TNF-α for the indicated time intervals, the cells were washed once with 5 ml of PBS and harvested. The pellet was stored frozen at −70 °C and resuspended in 0.5 ml of buffer B (100 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 1 mM EDTA, and protease inhibitors). The cell suspension was sonicated 3 times (3-s bursts) using a probe sonicator and centrifuged at 500 $\times$ *g* at 4 °C for 5 min. The supernatant was used as the enzyme source.

100 μg of protein was assayed for neutral sphingomyelinase activity in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 0.1 mg of bovine serum albumin, 5 mM $MgCl_2$, and 50 μmol of [^{14}C]sphingomyelin (12,000 dpm). The assay was incubated at 37 °C for 1.5 h and terminated with the addition of 1 ml of 10% trichloroacetic acid. The precipitate was pelleted (1000 $\times$ *g* at 4 °C for 20 min) and 1 ml of the supernatant was extracted with 1 ml of anhydrous diethyl ether. 0.5 ml of the aqueous phase was removed for liquid scintillation counting.

Immunoblotting—50 μg of nuclear protein was separated by gel electrophoresis on a 7.5% polyacrylamide gel. Gels were calibrated by high range molecular weight markers (Bio-Rad) which were transferred to a polyvinylidene difluoride membrane and visualized with Coomassie staining. Rabbit polyclonal antibodies against SREBP-1 were used at 0.5 μg/ml according to the instructions of the manufacturer. The antibody was visualized with horseradish peroxidase-conjugated anti-rabbit IgG made in donkey (Amersham) using the enhanced chemiluminescence (ECL) Western blotting detection system kit (Amersham). Polyvinylidene difluoride membranes were exposed to hyperfilm ECL (Amersham) for the indicated time. Immunoblots were quantified via densitometry performed on a PDI densitometer scanner (model 20J7) coupled to a SPARC IRC workstation.

Indirect Immunofluorescence—Cultured HH-25 cells were grown on coverslips and treated as described. After treatment, the cells were washed 3 times with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (solution A). The cells were fixed with 3% paraformaldehyde in solution A for 10 min and permeabilized with 0.5% Triton X-100 in solution A for 6 min at room temperature. The coverslips were then washed 3 times for 5 min with solution A.

Primary antibody (anti-SREBP1) was used at a dilution of 0.5 μg/ml in PBS and applied for 1 h with gentle shaking. The cells were washed as above and a fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody was applied for 30 min according to the instructions of the manufacturer. The coverslips were washed, mounted on microscope slides, viewed, and photographed at the indicated magnification on a Zeiss Axiovert 25 fluorescence microscope.

DNA Laddering Assay—Cells were treated with either TNF-α, sphingomyelinase, or C_2-ceramide for 1 h at concentrations identical to those used in the SREBP-1 maturation studies. The cells were then washed twice with minimal essential medium and refed with medium A for 6 h. The cells were harvested and genomic DNA was prepared as described (22). Genomic DNA was electrophoresed and stained with ethidium bromide.

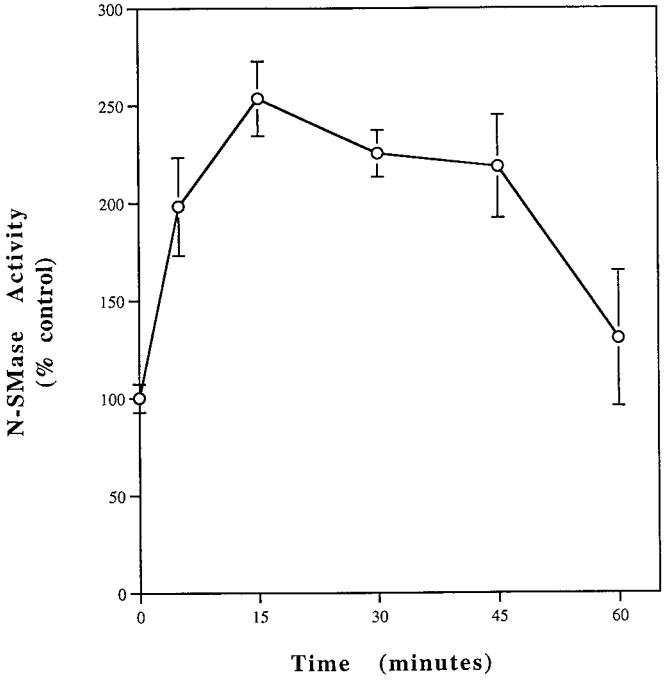

FIG. 1. **Effect of TNF-α on neutral sphingomyelinase activity.** Confluent cultures of HH-25 cells were washed once with PBS and incubated in serum free medium for 30 min prior to the addition of TNF-α (10 ng/ml). At the indicated time, cells were harvested in PBS, pelleted, and frozen. Cells were subsequently lysed as described under "Experimental Procedures." N-SMase assays were performed in duplicate as described. *Error bars* represent +1 S.D. from the mean.

Electrophoretic Mobility Shift Assays—Gel mobility shift assays were performed as follows. Each 20-μl reaction mixture contained 8–10 μg of nuclear protein plus a α-^{32}P-labeled 25-base pair oligonucleotide probe containing the SREBP-binding site (14) in binding buffer (10 mM HEPES, pH 7.5, 0.5 mM spermidine, 0.15 mM EDTA, 10 mM dithiothreitol, 0.35 mM sucrose). The reaction mixture was incubated at room temperature for 15 min and loaded directly onto a 6.5% polyacrylamide (49:0.6 acrylamide/bisacrylamide) gel in a buffer of 25 mM Tris borate (pH 8.0), 0.25 mM EDTA. In some experiments, antisera specific for SREBP or preimmune sera were added to reaction mixtures to determine the composition of protein-probe complexes. For these "supershift" assays, extracts were incubated with 1 μl of preimmune sera or an equal volume of anti-SREBP antisera at 4 °C for 30 min prior to addition of α-^{32}P-labeled probe. In all experiments, proteins were separated by electrophoresis at 200 V for 2 h at room temperature. Gels were dried and exposed to Kodak XAR film with intensifying screens. Assays were repeated with nuclear extracts obtained from three unique experiments and evaluated by phosphoimage analysis to ensure reproducibility of results.

RESULTS

The Effect of TNF-α on Neutral Sphingomyelinase Activity—Neutral sphingomyelinase activity increased rapidly with the addition of TNF-α (Fig. 1). A maximal 2.5-fold increase in activity was observed 15 min after TNF-α was added to the cells. The gradual return of N-SMase activity to control levels within 1 h contrasted the rapid onset of activation and is reflected in the asymmetric kinetic profile observed.

Kinetics of SREBP-1 Proteolysis—Sterol-independent SREBP-1 maturation in response to TNF-α closely paralleled the kinetics of TNF-α induced N-SMase activation. The mass of the mature form of SREBP-1 was found to increase 2-fold after 5 min and 3-fold after 15 min of incubation with TNF-α (Fig. 2). The amount of mature SREBP-1 returned to control levels within 1 h. This effect could not be recapitulated with epidermal growth factor or platelet-derived growth factor treatment

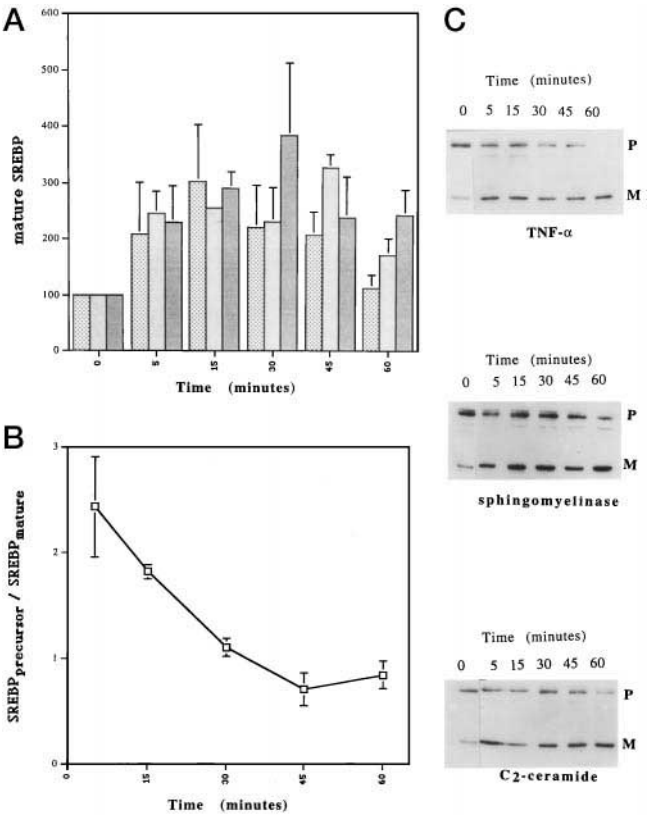

FIG. 2. **Effects of TNF-α, sphingomyelinase, and C_2-ceramide on the kinetics of SREBP-1 maturation.** Cells were maintained in medium supplemented with 1 mg/ml 25-hydroxycholesterol and 15 mg/ml cholesterol for 24 h before the experiment. The cells were treated for the indicated time as described under "Experimental Procedures." The cells were then harvested in PBS, pelleted, and frozen. Lysis and nuclear fractionation were performed as described. Nuclear fractions (50 mg of protein) were electrophoresed on a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Western blotting was performed as described. Band intensity was quantified via densitometry. *Error bars* represent +1 S.D. from the mean. *A*, the kinetics of SREBP maturation as measured by the increase in mature SREBP-1 are plotted. Fold increase was calculated by comparing each time point to the corresponding control value (TNF-α is represented by *stippled bars*, bacterial sphingomyelinase is represented by *light gray bars*, and C_2-ceramide by the *dark bars*). *B*, cells were treated with TNF-α (10 ng/ml) and prepared as described above. The bands corresponding to the precursor and mature forms of SREBP-1 were quantified and their ratio plotted. *C*, representative Western blots from which numerical data were derived. Incubation time is indicated above and applies to all conditions. The membranes were exposed to film for 15 s. *P* and *M* denote the precursor and mature forms of SREBP-1, respectively.

(data not shown). The increase in mature SREBP-1 levels was accompanied by a concomitant decrease in the intensity of the band corresponding to the precursor form of SREBP-1 see Fig. 2. After 60 min of treatment, significantly less precursor SREBP-1 is visible.

To incorporate the observed increase in mature SREBP-1 and the concomitant decrease in precursor SREBP-1 into a single variable, we plotted the ratio of precursor SREBP-1 to mature (Fig. 2). A maximal 1.5-fold decrease in the precursor to mature ratio occurred 45 min after TNF-α was added to the medium. The decrease in precursor to mature ratio was more pronounced in the initial 30 min of treatment. This is also consistent with the kinetics of TNF-α-induced N-SMase activation.

To explore the possibility that plasma membrane sphingomyelinase was involved in the signal transduction pathway leading to SREBP-1 proteolysis, we treated cells with exogenously supplied bacterial sphingomyelinase. Sphingomyelinase induced a dramatic change in the relative amounts of precursor and mature SREBP-1. As seen in Fig. 2, a 2.5-fold increase in mature SREBP-1 levels was observed after 15 min of treatment. Unlike TNF-α, the increase in mature SREBP-1 induced by sphingomyelinase persisted after 60 min. Sphingomyelinase was also capable of reducing the level of the precursor form of SREBP-1 (Fig. 2). Treatment with purified recombinant human sphingomyelinase produced similar results.

Since much of the signal-transducing ability of N-SMase has been ascribed to its ability to generate the lipid second messenger ceramide, we tested the ability of a cell-permeable ceramide analog C_2-ceramide (*N*-acetylsphingosine) to induce SREBP-1 maturation. C_2-ceramide also induces SREBP-1 maturation in a sterol-independent manner with greater magnitude than what was observed with either TNF-α or sphingomyelinase. C_2-ceramide increased the level of mature SREBP-1 4-fold after 30 min of treatment (Fig. 2). The persistent elevation of mature SREBP-1 levels observed with sphingomyelinase treatment also accompanied C_2-ceramide treatment. The increase in mature SREBP-1 is recapitulated with the addition of bovine brain ceramides but could not be induced with C_2-dihydroceramide, phospholipase A_2, or phospholipase D treatment (data not shown).

Effects of TNF-α, Sphingomyelinase, and C_2-ceramide on Apoptosis in Hepatocytes—To demonstrate that the observed maturation of SREBP-1 was not an artifact of the more general phenomenon of apoptosis-induced proteolysis, we performed DNA laddering assays. The 160-base pair DNA ladder characteristic of cells undergoing apoptosis was not observed in any of the samples (data not shown).

Effects of TNF-α, Sphingomyelinase, and C_2-ceramide Concentration on SREBP-1 Maturation—The extent of TNF-α induced SREBP-1 maturation did not vary appreciably with concentration. A maximal effect was observed with 10 ng/ml of TNF-α (Fig. 3). 250 milliunits of sphingomyelinase activity induced an 80% decrease in the precursor to mature ratio (Fig. 3). As little as 1 μM of C_2-ceramide was effective in producing an 81% maximal effect. The maximal effect, however, was obtained with a C_2-ceramide concentration of 50 μM (Fig. 3).

The Effect of Anti-N-SMase Antibodies on TNF-α-mediated SREBP-1 Maturation—The availability of anti-N-SMase antibodies allowed us to examine the effects of TNF-α on this pathway independent of N-SMase activation (10). Polyclonal anti-N-SMase antibodies at a dilution of 1:200 completely block TNF-α-induced SREBP-1 maturation (Fig. 4). The suppression of TNF-α-mediated SREBP-1 maturation was relieved with increasing antibody dilution. Preincubation with preimmune serum at the same dilution had no appreciable effect.

Effects of TNF-α, C_2-ceramide, and Sphingomyelinase on the Subcellular Localization of SREBP-1—To determine if the SREBP-1 fragment generated by TNF-α, C_2-ceramide, or sphingomyelinase treatment was capable of nuclear translocation, we pursued immunofluorescence studies. Previous immunofluorescence studies have relied on the overexpression of precursor and mature forms of SREBP-1 (14). We were able to visualize endogenous SREBP-1 in treated and untreated cells with polyclonal antibodies directed against the DNA binding domain of SREBP-1. Since the DNA binding domain is common to both the precursor and mature forms, we were able to examine the total distribution of endogenous SREBP-1.

TNF-α, C_2-ceramide, and sphingomyelinase are all capable of inducing changes in the subcellular localization of SREBP-1 (Fig. 5*A*). Untreated cells display an even staining pattern throughout their cell bodies. This is consistent with the localization of precursor SREBP-1 to intracellular membranes (14).

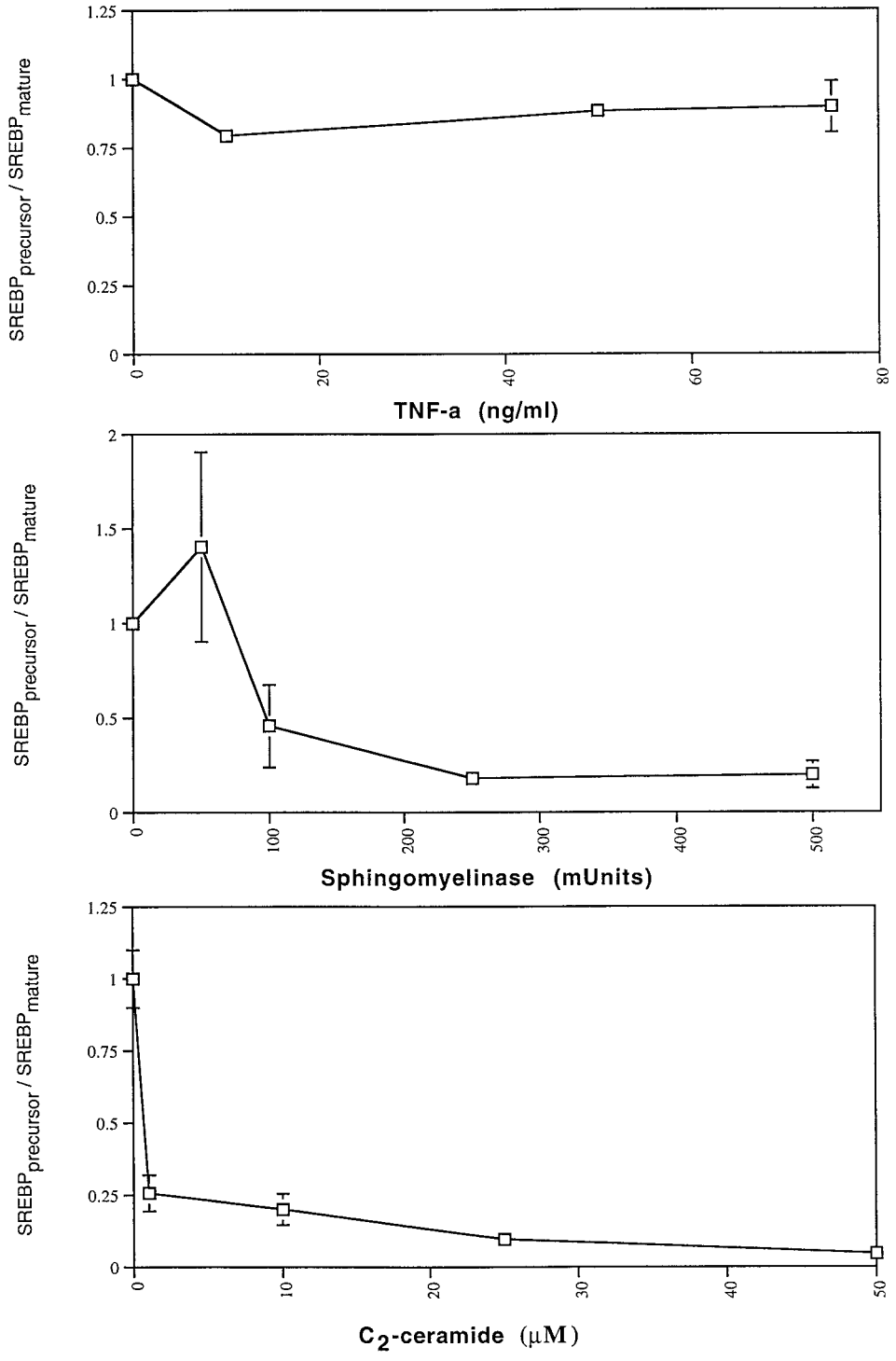

FIG. 3. **Effects of TNF-α, sphingomyelinase, and C_2-ceramide concentration on SREBP-1 maturation.** Cells were treated with either TNF-α, sphingomyelinase, or C_2-ceramide at the indicated concentrations. Nuclear pellets were prepared and electrophoresed (50 mg of protein). The bands corresponding to the precursor and mature forms of SREBP-1 were quantified. The precursor to mature ratios were normalized to a single control to facilitate comparison. The control ratio was arbitrarily assigned a value of 1. A unit of sphingomyelinase activity hydrolyzes 1.0 mmol of sphingomyelin per minute at 37 °C.

However, cells treated with TNF-α, C_2-ceramide, or sphingomyelinase exhibit intense nuclear staining and little extranuclear staining (Fig. 5, *B*, *C*, and *D*). The rapid change in the subcellular localization of SREBP-1 is consistent with a precursor/product relationship between the two forms and provides additional evidence that the mature SREBP-1 fragment generated by treatment is capable of nuclear translocation.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were pursued to demonstrate that the mature SREBP-1 fragment is additionally capable of binding to its consensus sequence. The amount of electrophoretically retarded probe increases with time following TNF-α treatment (Fig. 6*A*). The kinetics of this process is consistent with the activation of N-SMase. The amount of probe bound increases with sphingomyelinase and ceramide treatment. As expected, C_2-ceramide induces a more rapid accumulation of active nuclear SREBP-1 than either TNF-α or sphingomyelinase (Fig. 6, *A-C*). Antibodies directed toward the DNA binding domain of

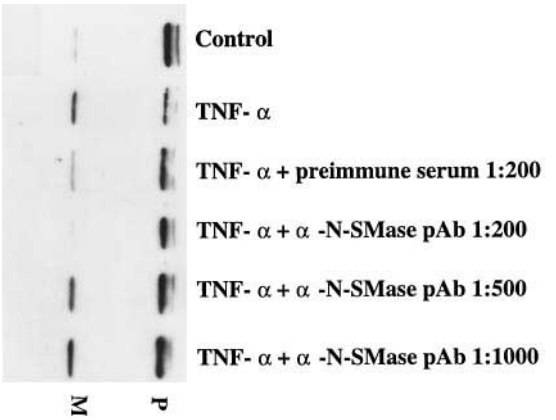

FIG. 4. **Effect of anti-N-SMase antibodies on TNF-α-induced SREBP-1 maturation.** Cells were maintained in medium supplemented with 1 mg/ml 25-hydroxycholesterol and 15 mg/ml cholesterol for 24 h before the experiment. The cells were switched to serum free media for 15 min and then incubated with anti-N-SMase antibodies or rabbit preimmune serum at the indicated dilution for 30 min prior to TNF-α addition (10 ng/ml). The cells were then harvested, pelleted, and lysed as described. The samples were electrophoresed on a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Bands were visualized as described. Film was exposed for 15 s.

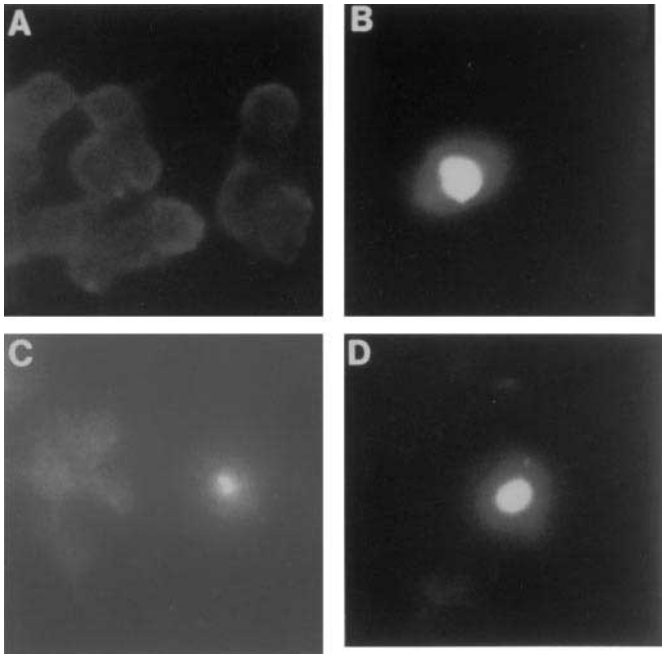

FIG. 5. **Indirect immunofluorescence of SREBP-1.** SREBP-1 was visualized with rabbit polyclonal antibodies directed toward the N-terminal DNA binding domain, which is common to both the precursor and mature forms. Cells were maintained in media supplemented with 1 mg/ml 25-hydroxycholesterol and 15 mg/ml cholesterol for 24 h before the experiment. Immunofluorescence was performed as described. All magnifications are × 40, and all photographs were taken of samples that were fixed 30 min after initiating treatment. *A*, control cells; *B*, cells treated with TNF-α (10 ng/ml); *C*, cells treated with sphingomyelinase (100 milliunits); *D*, cells treated with C_2-ceramide (10 mM).

SREBP successfully compete with the oligonucleotide probe for binding (Fig. 6*D*). Binding of the probe is not titrated by an unrelated oligonucleotide but is decreased with the addition of a nonradioactive competing probe (data not shown).

DISCUSSION

The importance of cholesterol homeostasis is underscored by the significant amount of redundancy that has been incorporated into its regulation. Cholesterol homeostasis is regulated at the transcriptional, translational, and enzymatic levels. The

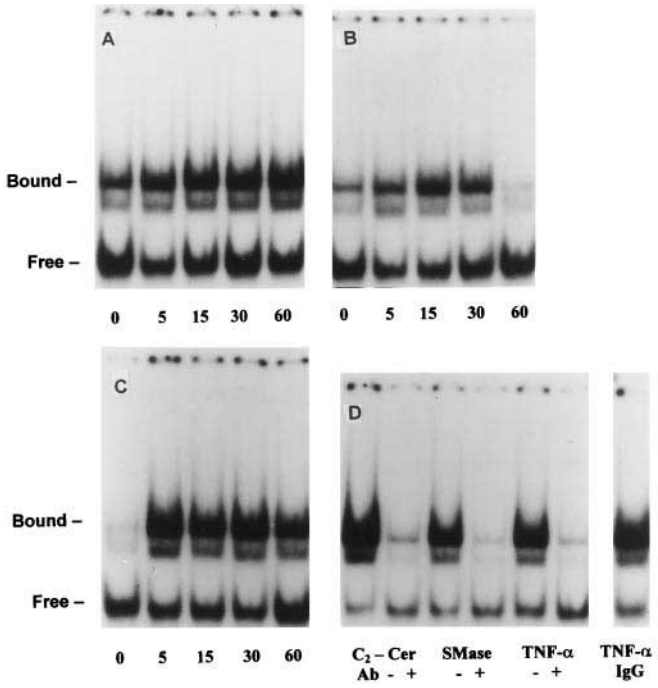

FIG. 6. **Electrophoretic mobility shift assays.** Cells were maintained in sterol-supplemented medium. Nuclear pellets were prepared and assayed as described under "Experimental Procedures." Probe that has been bound by mature SREBP-1 is indicated as "*Bound*". Unbound probe is indicated as "*Free*". The kinetics (in minutes) of SREBP-1 binding to the probe in response to treatment with TNF-α (10 ng/ml) (*A*), sphingomyelinase (100 milliunits) (*B*), and C2-ceramide (10 mM) (*C*). In *panel D*, the cells were treated with either TNF-α (10 ng/ml), sphingomyelinase (100 milliunits), or C_2-ceramide (10 mM) for 15 min. Supershift assays were then performed with antibodies raised against the DNA binding domain of SREBP-1. The presence or absence of antibody is indicated by (+) and (−), respectively. Preimmune IgG was used as a control.

observation that feedback inhibition plays an important role in cholesterol homeostasis has stimulated much work in elucidating the exact role of sterols in this process (12). The current data suggest a novel pathway by which SREBP-1 maturation could be effected in a sterol-independent manner.

TNF-α is capable of inducing SREBP-1 maturation in a sterol-independent manner in human hepatocytes. These findings are not a general response to growth factors, as they could not be recapitulated with epidermal growth factor or platelet-derived growth factor. The maturation, nuclear translocation, and SRE binding activity of SREBP-1 in response to TNF-α closely paralleled the kinetics of N-SMase activation. The effect of TNF-α on SREBP-1 maturation could be reconstituted with exogenously supplied bacterial or human sphingomyelinase (data not shown) or C_2-ceramide but could not be recapitulated with dihydroceramide, phospholipase A_2, or phospholipase D.

Preincubation with anti-N-SMase antibody effectively blocked TNF-α-induced SREBP-1 maturation. Inhibition was not observed with preimmune serum treatment and was relieved with increasing antibody dilution. Such findings are also confirmed by the ability of the antibody to inhibit TNF-α-induced increases in cholesterol ester synthesis and N-SMase-induced increases in ^{125}I-LDL binding, internalization, and degradation in human fibroblasts (15, 16).

The addition of C_2-ceramide, a water soluble ceramide analog, or bacterial sphingomyelinase mimicked the effect of TNF-α on SREBP-1 maturation. In fact, C_2-ceramide and sphingomyelinase induced more extensive SREBP-1 maturation than TNF-α. This may reflect the presence of a regulatory event upstream of ceramide generation that is effectively by-

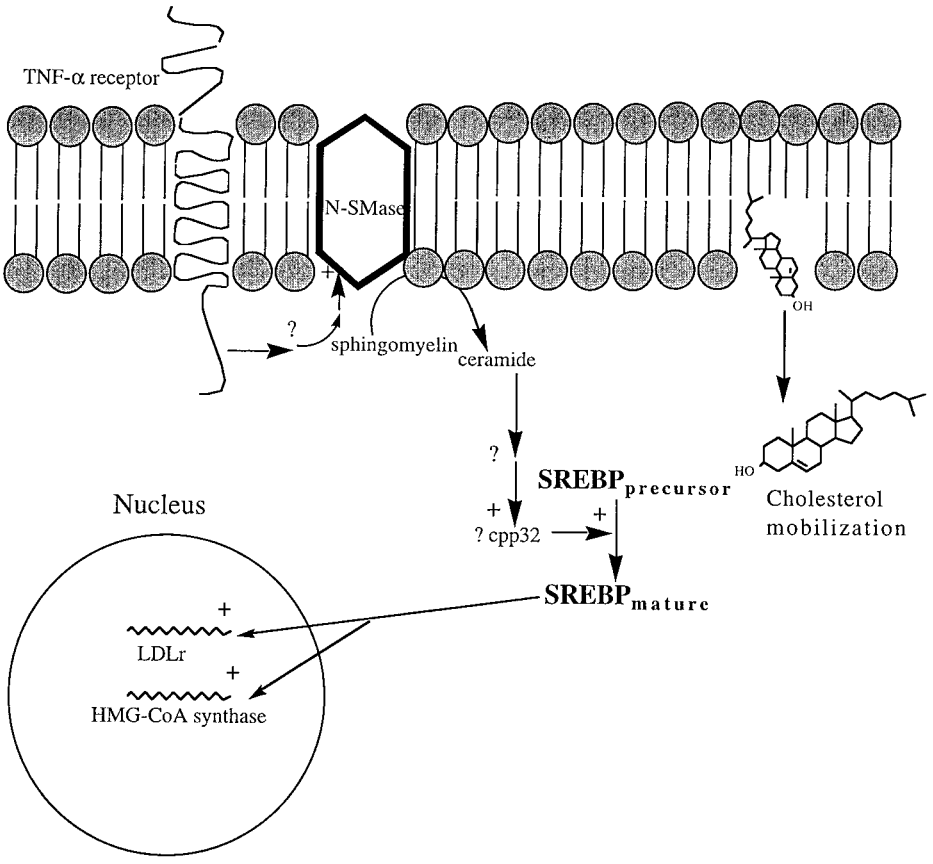

FIG. 7. **Hypothetical model depicting how TNF-α might induce SREBP-1 proteolysis and mobilize membrane cholesterol in human hepatocytes.**

passed with exogenous ceramide or sphingomyelinase. The lack of apparent dose dependence observed with TNF-α treatment might be attributable to saturable binding of the TNF-α receptors or an internal regulatory event that reduces the signaling capacity of the TNF-α receptors.

Previous studies have shown that an increase in SREBP-1 levels increases LDL receptor levels and sterol biogenesis (13). Our gel mobility shift experiments (Fig. 6) clearly indicate that TNF-α, N-SMase and C_2-ceramide all induce SREBP-1 levels in hepatocytes. In addition, we have previously shown that TNF-α induces sterol metabolism in cultured human fibroblasts (15) and LDL receptors (16, 17). Finally, our preliminary data indicate that indeed TNF-α induces LDL receptor mRNA levels in human hepatocytes. Collectively, these observations suggest that TNF-α-induced increase in mature SREBP-1 level is accompanied by increased LDL receptors and sterol metabolism.

TNF-α, C_2-ceramide, and sphingomyelinase did not induce apoptosis in our studies demonstrating that in hepatocytes, SREBP-1 maturation is not part of the more general phenomenon of apoptotic protein hydrolysis.

It has been speculated that cells undergoing apoptosis require cholesterol to maintain the integrity of their plasma membranes (18). The 160-base pair DNA ladder characteristic of cells undergoing apoptosis generally appears 4–8 h after an apoptosis-initiating stimuli is introduced. The kinetics of SREBP-1 maturation presented in this study would suggest that SREBP-1 proteolysis is a sufficiently early event to be involved in providing cholesterol to apoptotic cells. However, there was no evidence of apoptosis in the HH-25 human hepatocyte cell line used in this study. It is possible that the sterol-independent induction of SREBP-1 maturation in hepatocytes is a physiologic process that does not require that apoptosis be induced. Alternatively, the two pathways may diverge before the cell has been committed to apoptosis suggesting a manner in which sterol-independent SREBP-1 proteolysis could be employed independent of the induction of apoptosis. TNF-α is known to activate the apoptotic protease CPP32, and it has been shown that SREBP-1 is a physiological substrate (18). In a recent study, a cell-permeable ceramide was shown to induce the proteolytic cleavage of CPP32, a ced-3 interleukin-1β converting enzyme-like protease but not interleukin-1β converting enzyme (19). Thus, sterol-independent cleavage of SREBP-1 observed in our studies with human hepatocytes could also occur by ceramide generated by the TNF-α-induced N-SMase activation. This phenomenon may be reconstituted by the exogenous addition of N-SMase and/or C_2-ceramide to the hepatocytes. It is tempting to speculate that this pathway may represent a role for the apoptotic protease CPP32 in normal cellular homeostasis.

Previous studies have shown that cholesterol efflux from the membrane into the cytosol occurs with increased neutral sphingomyelinase activity (16). This effect is attributed to the depletion of plasma membrane sphingomyelin, which has been shown to exert a stabilizing effect on plasma membrane cholesterol stores through favorable thermodynamic interactions (20–22).

We propose a model (Fig. 7) that incorporates these data and suggests a mechanism by which TNF-α could initiate SREBP-1 proteolysis. According to this hypothesis, TNF-α binds to one or more of its cell surface receptors and in so doing promotes the activation of N-SMase. Recently, a candidate protein that mediates this activation has been cloned (23). N-SMase hydrolyzes membrane sphingomyelin into ceramide and phosphocholine. Ceramide, in turn, activates a protease perhaps CPP32 that mediates SREBP-1 maturation. The mature SREBP-1 then

migrates into the nucleus as shown and drives the transcription of genes with an upstream sterol regulatory element.

This model may be invoked to explain how sterol homeostasis can occur in the presence of increased cytosolic sterols, which would be predicted to suppress SREBP-1 maturation. The advantage conferred by the participation of neutral sphingomyelinase in cholesterol homeostasis is that it is capable of providing a short term solution to cholesterol starvation through mobilization of plasma membrane cholesterol and can facilitate long term compensatory mechanisms by promoting the maturation of SREBP-1.

Here, we report that TNF-α is capable of inducing SREBP-1 proteolysis independent of the presence of sterols. Exogenously supplied sphingomyelinase and ceramide are also capable of inducing SREBP-1 proteolysis in a time- and dose-dependent manner. The kinetics of SREBP-1 maturation is consistent with the activation of neutral sphingomyelinase by TNF-α. Furthermore, our preliminary data indicates that recombinant human N-SMase can also exert a time- and concentration-dependent induction of SREBP-1 maturation. In addition, anti-N-SMase antibodies block SREBP-1 maturation. Taken together, these findings suggest that neutral sphingomyelinase is necessary for TNF-α-induced sterol-independent SREBP-1 cleavage.

Our data implicate N-SMase in the TNF-α initiated signal transduction pathway leading to SREBP-1 maturation and provide evidence that ceramide is the second messenger employed. Further, they suggest a role for TNF-α in the regulation of cholesterol homeostasis. Additional studies are required to establish the role of cholesterol in apoptotic cells and the role of the different tumor necrosis factor receptors in SREBP-1 maturation. Better understanding of this pathway may yield novel targets for the pharmacological manipulation of serum cholesterol levels.

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