Tumor Necrosis Factor-α Inhibits Expression of CTP:Phosphocholine Cytidylyltransferase*

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We investigated the effects of tumor necrosis factor α (TNFα), a key cytokine involved in inflammatory lung disease, on phosphatidylcholine (PtdCho) biosynthesis in a murine alveolar type II epithelial cell line (MLE-12). TNFα significantly inhibited [3H]choline incorporation into PtdCho after 24 h of exposure. TNFα reduced the activity of CTP:phosphocholine cytidylyltransferase (CCT), the rate-regulatory enzyme within the CDP-choline pathway, by 40% compared with control, but it did not alter activities of choline kinase or cholinephosphotransferase. Immuno blotting revealed that TNFα inhibition of CCT activity was associated with a uniform decrease in the mass of CCTα in total cell lysates, cytosolic, microsomal, and nuclear subfractions of MLE cells. Northern blotting revealed no effects of the cytokine on steady-state levels of CCTα mRNA, and CCTβ mRNA was not detected. Incorporation of [32P]methionine into immunoprecipitable CCTα protein in pulse and pulse-chase studies revealed that TNFα did not alter de novo synthesis of enzyme, but it substantially accelerated turnover of CCTα. Addition of N-acetyl-Leu-Leu-Nle-CHO (ALLN), the calpain I inhibitor, or lactacystin, the 20 S proteasome inhibitor, blocked the inhibition of PtdCho biosynthesis mediated by TNFα. TNFα-induced degradation of CCTα protein was partially blocked by ALLN or lactacystin. CCT was ubiquitinated, and ubiquitination increased after TNFα exposure. m-Calpain degraded both purified CCT and CCT in cellular extracts. Thus, TNFα inhibits PtdCho synthesis by modulating CCT protein stability via the ubiquitin-proteasome and calpain-mediated proteolytic pathways.

Phosphatidylcholine (PtdCho (1),1 the most abundant mammalian phospholipid, has several well recognized roles, including serving as an integral component of pulmonary alveolar surfactant and providing the cell with a reservoir for the generation of bioactive lipid second messengers. The biosynthesis of PtdCho is tightly regulated within the CDP-choline pathway (1). A key step in this pathway is the conversion of choline phosphate to CDP-choline, which is catalyzed by the rate-limiting enzyme CTP-phosphocholine cytidylyltransferase (CCT, EC 2.7.7.15 (1)).

CCT localizes primarily to the endoplasmic reticulum and nucleus (2), but it is also found associated with Golgi and transport vesicles (1, 3). CCT activity in cells is controlled primarily by association with membrane lipids and by gene expression (1). Positive and negative regulatory lipids govern CCT activity when presented within the context of a PtdCho lamellar structure. Activating lipids include unsaturated fatty acids, anionic phospholipids, and diacylglycerol, whereas negative regulatory lipids include sphingosine and lysophosphatidylcholine (4–7). Phosphorylation of CCT modulates activity by attenuating lipid activation, and an inverse correlation exists between CCT phosphorylation and PtdCho synthesis in some systems (8). Protein kinases that phosphorylate purified CCT in vitro include p42/44 mitogen-activated protein kinase, protein kinase Ca, and casein kinase II (9, 10). Although there is substantial evidence supporting regulation of CCT by biochemical and protein modification mechanisms, changes in the level of mRNA and protein have also been identified (11–14). In this regard, studies have shown that the developmental increase in CCT mRNA in alveolar epithelium is due to an increase in the stability of the CCT transcript (15).

Three isoforms of CCT exist in cells as follows: CCTα, CCTβ1, and CCTβ2 (2, 16). The 367-amino acid CCTα isoform contains four distinct functional domains including an aminoterminal nuclear targeting domain, a catalytic sequence, a regulatory domain consisting of amphipathic α-helices that interact with lipids, and a carboxyl-terminal phosphorylation domain. Unlike CCTα, the CCTβ isoforms lack a nuclear localization motif and differ in their carboxyl-terminal phosphorylation sites (2, 16). However, the CCTβ isoforms exhibit full catalytic activity and, like CCTα, require the presence of lipid regulators for optimal activation. The control mechanisms underlying the differential expression of CCTα and CCTβ are not known.

Several factors such as phorbol ester (17) colony-stimulating factor 1 (11), cholecystokinin (13), and exogenous lipoproteins (18, 19) have been used to modulate CCT activity to investigate the physiological mechanisms that govern PtdCho synthesis. In the lung, TNFα is a key cytokine released by alveolar macrophages that has been implicated as a major factor for inducing acute lung injury associated with sepsis (20). Many of the manifestations of sepsis can be reproduced by the in vivo administration of this cytokine, and these deleterious effects can be attenuated by treatment with TNFα antibodies (21).

Sepsis-associated injury is characterized by elevated serum...
levels of TNFα and with reduced levels of PtdCho within critical organs (22, 23). Specifically, one important feature of TNFα-induced acute lung injury is depletion of alveolar surfactant PtdCho, which may occur as a result of accelerated PtdCho hydrolysis (22, 24, 25). TNFα stimulates PtdCho hydrolysis rapidly, often within minutes after cytokine exposure (26). However, there is limited information about long term effects of TNFα on phospholipid metabolism and whether the cytokine might regulate PtdCho biosynthesis. Thus, the primary objectives of this study were to investigate whether exposure to TNFα down-regulates PtdCho biosynthesis in alveolar epithelial cells and to characterize the mechanisms responsible for reduced cellular PtdCho levels after long term cytokine exposure. Recent studies demonstrating that TNFα suppresses [14C]glucose incorporation into disaturated phosphatidylcholine (DSPtdCho (27)), a marker of surfactant phospholipid, led us to hypothesize that the inhibitory actions of TNFα on surfactant phospholipid metabolism might be mediated by inhibition of PtdCho synthesis at the CTP:phosphocholine cytidylyltransferase step.

EXPERIMENTAL PROCEDURES

Materials—Human tumor necrosis factor α (1 μg = 1.1 × 10⁵ activity units) was obtained from Endogen (Minneapolis, MN). Silica LK5D (250 mm × 20 × 20 cm) TLC plates were purchased from Whatman. Hite's medium was obtained from the University of Iowa Tissue Culture and Hybridoma Facility (Iowa City, IA). All radiochemicals were purchased from NEN Life Science Products. The MLE-12 cell line was kindly provided by Dr. Jeffrey Whitsett (Cincinnati (28)). Lactacystin, N-Acetyl-Leu-Leu-Val-Try (ALLN), and calpeptin were purchased from Calbiochem. Immunoblotting membranes were obtained from Millipore (Bedford, MA), and Sepharose CL-4B was from Sigma. The ECL Western blotting detection system and GammaBind® Plus SuperSephose® were from Amersham Pharmacia Biotech. Protein A was purchased from Repligen (Cambridge, MA). Anti-ubiquitin rabbit polyclonal antibody was purchased from StressGen Biotechnologies Corp. (Victoria, Canada). Anti-CCTα and anti-CCTβ rabbit polyclonal antisera were prepared against synthetic peptides as described previously (2, 16). The anti-CCTα rabbit polyclonal antisera was raised against a synthetic peptide (MDAQQASSYVRSSKRRKE) corresponding to the first 17 amino acids of CCTβ. The anti-CCTβ rabbit polyclonal antisera was raised against a synthetic peptide (MEEITEHTCQPORL) corresponding to amino acids 27–39 of CCTβ. A rabbit polyclonal antibody to a synthetic peptide corresponding to residues 164–176 of CCTα and cross-reactive with CCTβ (29) was generated by Covance Research Products Inc. (Richmond, CA) and is termed pan-reactive. The CCTα and CCTβ cDNAs were described (32). The random primed labeling kit used was Redprime II™ (Buckinghamshire, UK).

Cell Culture—Cells were cultured in Hite's medium with the inclusion of 2% fetal bovine serum for 48 h and then cultured in Hite's medium alone for 2–24 h in the presence or absence of TNFα (500 ng/ml) (27). In some studies, cells were preincubated with lactacystin (1–5 μM) or ALLN (10–40 μg/ml) alone, or with both protease inhibitors for 1 h with or without subsequent exposure of cells to TNFα. Cells were isolated after brief sonication in Buffer A (150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2 mM dithiothreitol, 0.025% sodium azide, 1 mM PMSF, pH 7.4) at 4 °C prior to analysis. Cell lysates were prepared using a sonicator (Heat Systems-Ultrasonics Inc., Plainview, NY). Cell nuclei were prepared by nuclease treatment of cellular lysates as described (31).

Cell Viability—Efforts of TNFα on cell viability were measured by a standard chromium-51 release assay. Cells were pulsed with 1 μCi of [55Cr]-labeled sodium chromate overnight. After labeling, cells were rinsed 4 times with Hite's medium and subsequently incubated for an additional 24 h with or without TNFα (500 ng/ml). An aliquot of medium was collected for gamma counting. Cell-specific activity was calculated as a ratio of medium cpm/total cpm as described (32). Cell cultures were also examined morphologically using an Olympus IX 70 inverted microscope (Leeds Precision Instruments, Inc., Minneapolis, MN).

Phosphatidylcholine (PtdCho) and Disaturated Phosphatidylcholine (DSPtdCho) Analysis—Lipids were extracted from equal amounts of cellular protein using the method of Bligh and Dyer (33). Lipids were dried under nitrogen gas, spotted on silica LK5D plates, and resolved in chloroform:methanol:petroleum ether:acetic acid:boric acid (40:20:30:10:1.8, v/v/v/v/w) (34). Samples that comigrated with PtdCho standard as detected by exposure to iodine vapor were scraped from the silica gel and quantitatively assayed for phosphorus content (35). In other studies, cells were pulsed with 1 μCi of [methyl-3H]-choline chloride during the final 2 h of incubation. Cellular lipids were extracted; PtdCho was isolated using TLC and then used in the standard chromium-51 assay as described (36). Incorporation of label into PtdCho and DSPtdCho were then quantitated using scintillation counting.

Enzyme Assays—The activity of choline kinase was assayed as described (37). The reaction mixture (0.1-ml volume) contained 100 mM Tris-HCl buffer, pH 8.0, 10 mM magnesium acetate, 0.016 mM [3H]choline phosphate (5000 cpm/mmol), 10 μM ATP, and 50–100 μg of cell sample. After a 1-h incubation at 37 °C, the reaction was terminated with 0.02 ml of cold 50% trichloroacetic acid. Aliquots (20 μl) of the mixture were spotted on Whatman 3MM paper and choline metabolites resolved using paper chromatography as described (37). Spots that comigrated with the radiolabeled standard, choline phosphate, were cut and used for scintillation counting.

CCT activity was determined by measuring the rate of incorporation of [methyl-3H]phosphocholine into CDP-choline using a charcoal extraction method (30). All assays were performed without the inclusion of a lipid activator in the reaction mixture unless otherwise stated.

The activity of cholinephosphotransferase was assayed as described (38). Each reaction mixture contained 50 mM Tris-HCl buffer, pH 8.0, 1 mM ATP, 0.5 mM 2,3-diacylglycerol, 0.5 mM [14C]CDP-choline (specific activity 1110 dpm/nmol), 5 mM dithiothreitol, 5 mM EDTA, 10 mM MgCl₂, and 30–40 μg of sample. The lipid substrate was prepared by combining appropriate amounts of 1,2-diacylglycerol (1 mM) and phosphatidylglycerol (0.8 mM) in a test tube, drying under nitrogen gas, and brief sonication prior to addition to the assay mixture to achieve the final desired concentration. The reaction proceeded for 1 h at 37 °C and was terminated with 4 ml of methanol:chloroform:water (2:1.7:ν, ν). The remainder of the assay was performed exactly as described (38).

Immunoblot Analysis—For immunoblot analysis, equal amounts of protein from cell homogenates, cytosolic, microsomal, and nuclear extracts were assessed in control and TNFα-treated MLE cells. Each sample was adjusted to give a final concentration of 60 mM Tris-HCl, pH 8.0, 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 5% β-mercaptoethanol and heated at 100 °F for 5 min. Samples were then electrophoresed through a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. CCTα and CCTβ isoforms were detected by using the ECL Western blotting detection system as instructed by the manufacturer. The dilution factor for anti-CCT isoform-specific antibodies was 1:1000. The pan-reactive CCTα and CCTβ rabbit polyclonal antibody was used for immunoprecipitation studies (29).

Detection of CCTα mRNA—Total cellular RNA was isolated from cells by cesium chloride gradient centrifugation following lysis with guanidine thiocyanate (39). Total RNA (30 μg) containing ethidium bromide was separated electrophoretically using a 1% agarose gel containing 2.2 M formaldehyde with a circulating running buffer of 20 mM Tris, 1 mM EDTA, 0.5% sodium acetate, and 10% glycerol. The RNA was capillary-blotted to a nitrocellulose membrane using a Turboblot apparatus and hybridized at 42 °C with 7.5 × 10⁵ cpm/15 ml of a 32P-labeled probe using standard Northern blot hybridization protocols (16). Probes were prepared by random primed labeling of cDNA fragments isolated from agarose gels using a Genease kit. The probes were prepared for the following cDNAs inserted in pcDNA3: a 1.3-kb HindIII-BamHI fragment of the rodent CCTα, a 1.3-kb BamHI-XhoI fragment from the human CCTβ. The blot was washed four times for 1 min at 22 °C and then 42 °C for 10 min, following hybridization with the CCTα probe, or four times for 1 min at 22 °C only following hybridization with the CCTβ probe.

Single Immunoprecipitation and Sequential Immunoprecipitation with Anti-CCT and Anti-ubiquitin Antibodies—Single immunoprecipitations were used to evaluate CCTα synthesis and degradation, whereas sequential immunoprecipitations were performed to determine if CCTα becomes ubiquitinated in MLE cells. CCTα synthesis was assessed in control and TNFα-treated cells by labeling cells with [35S]methionine (60 μCi/ml in methionine-deficient medium) during the last 4 h of culture at 37 °C. Cells were pulse-labeled with [35S]methionine, washed once with buffer (10 mM Na₂HPO₄, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 20 μM leupeptin, 1% Triton X-100, 0.1% SDS, 20 mM l-cysteine, and 2 mM l-cysteine; pH 7.4), sonicated briefly, centrifuged at 15,000 × g for 30 s, and then preclarified for 2 h at 4 °C using Sepharose CL-4B and preimmune rabbit serum. Cleared supernatants, containing equal amounts of protein (500 μg), were incubated overnight at 4 °C with 1 μg of rabbit anti-CCT antibody, which
was previously bound to GammaBind® Plus Sepharose®. The following morning, the immunoprecipitates were washed with lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5 mM EGTA, 1 mM PMSF, 50 mM NaF, 1 mM NaVO4, 1 mM PMSF, and 1 mM aprotinin; pH 7.6), and the pellets were placed in SDS protein sample buffer and heated to 95 °C for 5 min. Soluble proteins were separated using 10% SDS-PAGE, and the gels were stained with Coomassie Blue, destained, and then dried for both autoradiography and liquid scintillation counting of excised 42-kDa protein bands.

Turnover of CCTα was determined using pulse-chase procedures. MLE-12 cells were preincubated for 1 h in methionine-deficient medium and then pulsed with [35S]methionine (60 Ci/ml) for 4 h at 37 °C. Cells were rinsed twice in similar medium and chased with serum-free Hite’s medium containing 10 mM methionine and 3 mM cysteine for 0 to 24 h in the presence or absence of TNFα. Cells were scraped and lysed in RIPA buffer, and CCTα was immunoprecipitated prior to separation using SDS-PAGE as described above.

For detection of ubiquitinated CCTα, MLE-12 cells were labeled with [35S]methionine as described above. The radiolabeled cells were washed with ice-cold phosphate-buffered saline and solubilized in RIPA buffer. CCTα was immunoprecipitated using 1 μg of rabbit anti-CCT antibody as described above. The CCT immunoprecipitates were washed twice with lysis buffer, resuspended in 400 μl of the same buffer, and boiled for 4 min to release proteins from the GammaBind® Plus Sepharose® beads. Samples were placed on ice for at least 10 min and allowed to cool. These samples were then used for a second round of immunoprecipitation, which was conducted overnight at 4 °C using rabbit anti-ubiquitin antibody at a dilution of 1:100. The ubiquitin immunocomplexes were captured with protein A and Sepharose CL-B4 and subsequently analyzed by SDS-PAGE and autoradiography of dried gels.

Assay of Calpain and 20 S Proteasome Proteolytic Activity—Incubations for calpain activity were conducted for 16 h at 30 °C in a reaction mixture (total volume, 150 μl) containing calpain (0–500 μg; 0.00–0.90 units), 20 mM Tris, pH 7.5, 50 mM NaCl, 1 mM diithiothreitol, 0.5 mM EGTA, 0.5 mM EDTA, and 20 mM glycerol. Calpastatin (50 μg), a calpain protease inhibitor, was added to selected samples. Proteins from cellular lysates (300 μg) and a 20 S proteasome preparation (30 μg) purified from rat liver cytosol using octylglucoside were used as substrates (40). The reaction was started by adding 1 μl of CaCl2 to a final concentration of 3 mM and was terminated by adding SDS protein sample buffer and heating samples to 95 °C. Degradation of CCTα protein was evaluated by immunoblot analysis.

For assay of 20 S proteasome, the reaction mixture (total volume, 100 μl) contained 0–40 μg of 20 S proteasome, 40 mM Tris-HCl, pH 8.0, 0.04% w/v SDS, and 2 mM β-mercaptoethanol. Rat liver CCTα protein (30 μg) served as substrate. Reactions were carried out for 24 h at 37 °C and then stopped by adding SDS protein sample buffer and heating samples to 95 °C. In selected assays, proteasomes were pretreated with an inhibitor, 50 μM lactacystin, for 10 min at 37 °C before addition of protein substrate. Immunoblotting was then conducted to evaluate CCTα degradation in these samples.

RESULTS

Cell Viability—Cells cultured in the presence or absence of TNFα exhibited no significant differences in cell cytotoxicity as determined by 51Cr release (0.109 ± 0.014 [control] versus 0.132 ± 0.008 [TNFα]). No morphological evidence of cell death after 24 h of TNFα exposure was observed (500 ng/ml, data not shown). Cells analyzed by trypan blue exclusion were approximately 90% viable under control and TNFα-treated conditions.

PtdCho and DSPtdCho Analysis—TNFα significantly decreased the mass of PtdCho by 27% after 24 h of exposure (Fig. 4A). The magnitude of these effects are remarkable because in primary alveolar type II epithelial cells, PtdCho accounts for nearly 70% of the total phospholipid in MLE cells (41). To determine if the reduction in PtdCho mass was due to a decrease in phospholipid synthesis, we examined the incorporation of [methyl-3H]choline into PtdCho after pulping cells with 1 μCi of the label. TNFα significantly decreased choline incorporation into PtdCho by approximately 40% after 24 h of exposure (Fig. 1B). An even greater effect of TNFα was observed on DSPtdCho, which accounts for approximately 50% of the total PtdCho in MLE cells and is the major surface-active component of surfactant synthesized by alveolar type II epithelial cells. TNFα effects were primarily directed toward the DSPtdCho pool, as it substantially reduced DSPtdCho mass by 38% (Fig. 1A (inset)) and choline incorporation into DSPtdCho by 76% (p = 0.001, Fig. 1B (inset)). These results show that TNFα substantially reduces the biosynthesis of PtdCho, an essential component of surfactant, in alveolar epithelial cells.

Enzymes Assays—To confirm whether TNFα inhibits PtdCho synthesis, we assayed the activities of enzymes within the CDP-choline pathway, the principal pathway for PtdCho synthesis. There were no significant effects of the cytokine on choline kinase activity, the first committed step in the pathway, or on cholinephosphotransferase, the terminal enzyme involved in PtdCho synthesis (Table I). However, TNFα significantly decreased CCT activity to approximately 40% of control values after 24 h of treatment (p < 0.001, Table I) and 50% of control after 30 h (data not shown). These negative effects of TNFα were not only observed in cellular homogenates but also in the soluble, microsomal, and nuclear fractions (data not shown). The presence of the lipid activator (PtdCho:sphingoid acid) in the reaction mixture had little effect, as enzyme activity decreased 47% after 24 h of TNFα treatment. These results indicate that the primary effect of TNFα is to down-regulate the conversion of choline phosphate to CDP-choline at the rate-limiting step within the PtdCho biosynthetic pathway.

Immunoblot Analysis—We performed immunoblot analysis to determine if TNFα inhibited CCT activity by decreasing the amount of enzyme. Although two distinct CCT isoforms have been described in mammalian tissues, CCTα appears to be the only isoform detectable in MLE-12 cells. TNFα significantly decreased the amount of steady-state CCTα after long-term exposure to the cytokine. No effects on immunoreactive levels were seen after 4 h of TNFα treatment, and modest inhibitory effects of the cytokine were observed after 12 h of exposure (Fig. 2A). However, densitometric analysis showed that 24 h of TNFα exposure overall produced a 50–70% decrease in the amount of immunoreactive enzyme in these cells. Following TNFα treatment, the levels of immunoreactive CCTα uniformly decreased in total cell lysates and in the cytosolic and microsomal fractions compared with control (Fig. 2B). The most significant effect of TNFα was observed in the soluble fraction, where control cells contained 3-fold greater amounts of CCTα.
were pulsed with 1 inset) were quantitated using scintillation counting. The results represent the mean ± S.E. of three independent experiments. *, p < 0.05 versus control; **, p < 0.005 versus control.

![Image](65x161 to 281x729)

FIG. 1. Effect of TNFα on PtdCho content and choline incorporation into PtdCho in MLE cells. Cells were incubated in Hite's medium with the inclusion of 2% fetal bovine serum for 48 h prior to changing the medium to Hite's serum-free medium with or without the addition of TNFα (500 ng/ml) for the indicated times. A, lipids were extracted using a Bligh-Dyer method, PtdCho resolved using TLC, and the phosphatidylcholine content (nmol phosphorus/mg protein) determined by densitometric analysis of immunoblots (Fig. 5, A). The cytokine, however, substantially enhanced CTA degradation (Fig. 4B). The turnover of newly synthesized CTA was examined in pulse-chase experiments in which the amount of [3H]choline incorporated into immunoprecipitable CTA was determined after a 4-h pulse, followed by a chase (0–24 h) with unlabeled methionine conducted in the presence or absence of TNFα. At 0 h (start of chase), equal amounts of CTA were present in control and TNFα-treated cells (Fig. 4B).

However, after only 4 h of TNFα treatment, CTA degradation was detectable, and by 16 h, substantially less CTA was observed in TNFα samples compared with control. TNFα treatment resulted in further CTA degradation at 24 h in control MLE cells and reduced by approximately 50% by TNFα (Fig. 4D). These results indicate that TNFα decreases PtdCho synthesis by selectively enhancing the degradation of CTA.

Effect of TNFα on PtdCho content and choline incorporation into PtdCho in MLE cells. Cells were incubated in Hite's medium with the inclusion of 2% fetal bovine serum for 48 h prior to changing the medium to Hite's serum-free medium with or without the addition of TNFα (500 ng/ml) for the indicated times. A, lipids were extracted using a Bligh-Dyer method, PtdCho resolved using TLC, and the lipid reacted with osmium tetroxide prior to a run in the second dimension. The levels of PtdCho and DSPtdCho (inset) were quantitatively assayed for using the phosphorus assay. B, in other studies, cells were pulsed with 1 μCi of [methyl-3H]choline chloride during the final 2 h of incubation. Cellular lipids were extracted, and the lipids were processed as described above. Incorporation of label into PtdCho and DSPtdCho (inset) were then quantitated using scintillation counting. The results represent the mean ± S.E. of three independent experiments. *, p < 0.05 versus control; **, p < 0.005 versus control.
bition of surfactant PtdCho synthesis.

Effect of m-Calpain on CCT Proteolysis—To determine if CCT could be a potential substrate for TNFα-activated proteases, in vitro assays were conducted using purified CCTα in the presence of catalytically active m-calpain. The products of the reaction were then analyzed by immunoblotting using the pan-reactive anti-CCT antibody. Incubation of purified CCTα with calpain produced a dose-dependent decrease in the levels of the 42-kDa band at each time point are shown after performing densitometry of the immunoblots. Values are expressed as densitometric ratios after normalizing CCTα to control. The results represent the mean ± S.E. of three independent experiments (24 h, n = 9). B, the amount of CCTα was assayed in cell homogenates (H), and in cytosolic (C), microsomal (M), and nuclear (N) preparations after 24 h of TNFα exposure. Lanes were loaded with equal amounts of protein (400 μg). Right, shown are the levels of β-actin using similar experimental conditions.

Fig. 3. Effect of TNFα on CCT mRNA. MLE cells were cultured under conditions as described in Fig. 1 with or without TNFα (500 ng/ml) for the indicated times. Total cellular RNA was separated (30 μg) on 1% agarose gels, transferred to nitrocellulose membranes, and probed with CCTα or CCTβ cDNAs. Below, ethidium bromide staining of RNA gels was visualized to confirm equal RNA loading.
20 S proteasome which functions as the proteolytic core of the 26 S proteasome complex. The 20 S proteasome induced only partial degradation of the enzyme up to a concentration of 20 nmol in the reaction mixture (data not shown). The results may be attributed to the fact that unlike the 26 S proteasome, the 20 S proteasome lacks recognition sites for ubiquitinated proteins. Collectively, these results indicate that CCT serves as a substrate for calpain and possibly the proteasomal complex.

Sequential Immunoprecipitation with Anti-CCT and Anti-ubiquitin Antibodies—The data above support a role for the proteasome pathway in partially mediating TNFα-induced degradation of CCT. To identify whether TNFα-induced proteolysis of CCT involved a ubiquitin-dependent or ubiquitin-independent proteasome pathway, cells were first exposed to TNFα, lactacystin, or a combination of lactacystin with TNFα. Immunoblotting studies were conducted probing with an anti-ubiquitin antibody to determine if TNFα induces protein ubiquitination in MLE cells. As shown in Fig. 8A, in the presence of TNFα, accumulation of several high molecular weight bands became discernible. Similar high molecular weight poly-ubiquitinated proteins were observed after cells were treated with lactacystin. Cells exposed to a combination of TNFα and the proteasome inhibitor expressed the highest levels of ubiquitinated proteins (Fig. 8A). Thus, these results indicate that TNFα stimulates protein ubiquitination within the alveolar epithelial cell line.

To determine if CCT is a direct substrate for ubiquitination, separate studies were conducted where unlabeled cells were lysed, and the enzyme was immunoprecipitated using the pan-reactive CCT antibody. The samples were then resolved by SDS-PAGE, immunoblotted using a rabbit polyclonal ubiquitin antibody, and ubiquitinated CCT detected by using the ECL detection system. By using these methods, under steady-state conditions low levels of the enzyme are targeted for ubiquitin-dependent proteolysis. These levels are increased in the setting of TNFα exposure.
DISCUSSION

Our results show for the first time that TNFα modulates PtdCho synthesis by inhibiting CCT activity via proteolytic degradation of the enzyme. Based on the current results, it appears that specific degradative pathways exist in cells to control expression of CCT. These pathways include a constitutively active ubiquitin-proteasome pathway that may be important in regulating enzyme turnover under steady-state conditions. This system, together with an inducible calpain pathway, likely mediates TNFα effects on CCT proteolysis. The results should prompt investigations into the molecular mechanisms by which CCT is selected for degradation under normal or pro-inflammatory conditions. Results from such work could lead to maneuvers directed at modulation of these degradative pathways which in turn could serve as a means to control CCT lifespan within cells.

TNFα decreased the activity of CCT selectively. In contrast to effects of interleukin-1β, there were no appreciable effects of TNFα on other enzymatic steps within the biosynthetic pathway (44). Immunoblotting of MLE-12 cells and primary alveolar type II cells in both control and TNFα-treated extracts with antisera specific for the CCTα and CCTβ isoforms indicated that CCTα was the only isoform detected in these cells. Thus, from these and other recent results (2), CCTα appears to be the predominant isoform involved in surfactant phospholipid synthesis in mature alveolar type II cell epithelium. Further analysis confirmed that inhibition of CCT activity was due to a reduction of total cellular enzyme mass. A global decrease in CCT protein was observed in all subcellular fractions that were analyzed (Fig. 2), arguing against the likelihood that a shift in

FIG. 5. Effect of proteasome and calpain inhibitors on TNFα regulation of CCTα expression. A, MLE cells cultured in serum-free Hite’s medium (CON, control) were pretreated with either lactacytin (LAC, 5 μM) or ALLN (40 μg/ml) for 1 h prior to subsequent exposure to Hite’s medium or Hite’s medium containing TNFα (500 ng/ml) for 24 h. Inhibitors were removed immediately prior to TNFα treatments by washing cells with serum-free Hite’s medium. The amount of CCTα was assayed in cell lysates using immunoblotting. A pan-reactive antibody raised against CCT was used for immunoblotting. The far left lane shows purified CCT standard. B, densitometric analysis of the immunoreactive 42-kDa bands from each condition in A was performed. The mean ± S.E. from three separate experiments is shown using arbitrary OD units. C, cells were pretreated with the combination of lactacytin (LAC, 2 μM) plus ALLN (20 μg/ml) for 1 h prior to subsequent exposure to Hite’s medium with or without TNFα (500 ng/ml).

FIG. 6. Effect of proteasome and calpain inhibitors on TNFα-induced decrease in DSPtdCho content in MLE cells. Cells cultured in serum-free Hite’s medium (Control) were pretreated with either lactacytin (Lac, 5 μM) or ALLN (40 μg/ml) for 1 h prior to subsequent exposure to Hite’s medium or Hite’s medium containing TNFα (500 ng/ml) for 24 h. Cells were harvested, and levels of DSPtdCho mass were determined as described in Fig. 1A. Values are expressed as nanomoles of phospholipid phosphorus/mg protein. The results represent the mean ± S.E. from three separate experiments. *, p < 0.05 versus control.
cellular CCT content from a membrane-bound form to a cytosolic form accompanied the down-regulation of activity. It is unlikely that TNF\(_\text{a}\) altered translational efficiency because metabolic labeling studies showed no changes in newly synthesized CCT\(_\text{a}\) but clearly showed that the cytokine accelerated enzyme turnover. The native enzyme had a half-life (\(t_{1/2} = 5.8\) h) consistent with half-lives of other regulatory enzymes (45), but it was much more labile after cytokine stimulation (Fig. 4).

There have been no prior studies that have investigated which proteolytic pathways control CCT turnover. Groblewski et al. (13) showed that CCT can be degraded in response to cholecystokinin and these effects could be influenced by en-

![Fig. 7. CCT proteolysis by purified m-calpain.](image)

![Fig. 8. TNF\(_\text{a}\) stimulates ubiquitination of CCT.](image)
zyme phosphorylation. The primary mechanisms for protein catabolism in viable cells include lysosomal proteolysis, calcium-activated neutral proteolysis (calpains), and the ATP-dependent ubiquitin-proteasome pathway. Of these, the calpain and ubiquitin-proteasome systems have been most prominently identified in mediating TNFα proteolysis (42, 43, 46). For example, TNFα-induced IκBα proteolysis is mediated by both the calpain and ubiquitin-proteasome pathways (42), and IκBα degradation in response to the cytokine was also observed in our cells (data not shown). However, the kinetics of CCT degradation in alveolar epithelial cells and IκBα degradation in HepG2 cells differ significantly (42). IκBα is rapidly degraded within a few minutes with an 50% reduction in the half-life of the protein and is effectively eliminated during the acute phase response to TNFα. On the other hand, CCTα, a metabolic regulatory protein with an estimated half-life of 8 h, is degraded at a slower rate, and its half-life is reduced 56% in response to the cytokine (Fig. 4D). The differences between the kinetics of CCTα degradation and IκBα degradation are probably due to multiple factors. First, the various cellular responses to TNFα are dose- and time-dependent (47) and also cell type-dependent. For example, unlike proteolysis of IκBα, p21WAF1 proteolysis in ME-180 cells is not detectable until 4 h after TNF addition (42, 48). The kinetics of calpain-mediated proteolysis also differ with respect to different protein substrates due to the fact that there are multiple active forms of μ-calpain within cells that have unique substrate specificities and distinct functional roles (49).

Second, the molecular context of CCT can influence the rate of proteolysis. For example, the presence of DNA decreases the Ca²⁺ requirement for degradation of nuclear matrix proteins (50), phosphorylation of connexin-32 prevents its proteolysis (51), interaction of calmodulin with brain spectrin results in a marked acceleration of the rate of spectrin degradation by calpains (52), and binding of polyamines to spermidine/spermine N⁴-acytetyltransferase prevents proteasomal degradation (53). CCTα resistance to proteolytic degradation correlates with binding to membrane lipid (54), suggesting the possibility that nucleoplasmic CCTa might be more susceptible to digestion by calpain or the proteasome complex compared with CCTα located at membranous sites. In addition, the rate of CCTα modification by ubiquitination (Fig. 8) or phosphorylation in response to TNFα likely plays a role in determining the kinetics of proteolysis. CCTα phosphorylation stabilizes the protein from degradation in response to cholecytokinin (13), suggesting by analogy that dephosphorylation could reduce its half-life in response to TNFα.

These effects of TNFα-inducible CCT proteolysis are also partially blocked by inhibitors of either calpain or the 20 S proteasome. Interestingly, CCTα degradation was nearly completely blocked by the combination of both inhibitors. Perhaps more importantly, inhibition of the ubiquitin and calpain pathways totally abrogated the TNFα-induced decrease in DSPtdCho mass (Fig. 6). Lactacystin is a selective, potent, inhibitor of the proteasome without effects on either serine or cysteine proteases, whereas ALLN is less specific for cysteine proteases, such as calpain, especially at higher doses (55). Thus, we performed direct in vitro studies examining whether purified CCT was a substrate for catalytically active m-calpain, and it degraded purified CCT in a dose-dependent manner resulting in the generation of low molecular weight degradation products (Fig. 7). Our inability to detect similar proteolytic fragments in cells following TNFα treatment is probably due to several factors. The data indicate that CCTα is targeted by two proteolytic systems, as is IκBα where fragments also were not easily observed and Western blots required long gel exposures (42).

Whereas two major fragments of about 40 and 20 kDa resulted from calpain digestion of purified CCTα in vitro (Fig. 7), recent studies indicate that the sizes of products of the 20 S and 26 S proteasome are only 500 Da (56). Such small products are only detected in vitro by more sensitive methods and would run near the dye front of the PAGE. Thus, the proteolytic fragments resulting from digestion by the combination of calpain and/or the proteasome are probably very small, on the order of 5–6 residues. Another factor contributing to this result is the fact that the analyses were performed at 4- to 8-h intervals following cytokine addition (Figs. 2 and 4), and it has been shown that proteasome fragments are subject to further rapid hydrolysis by endopeptidases (57), thus probably eliminating the products of calpain/proteasome degradation.

Because both ubiquitin-dependent and ubiquitin-independent proteasome pathways have been described (58), we performed additional studies to determine if CCT and ubiquitin coimmunoprecipitated. Our pulse-chase and sequential immunoprecipitation studies indicate that radiolabeled CCT, which represents a relatively small pool of total cellular enzyme, apparently is not ubiquitinated in the absence of TNFα (Fig. 8D). However, it is likely that some CCT is targeted for ubiquitination under steady-state conditions perhaps as the enzyme becomes misfolded or modified post-synthetically (Fig. 8C). Thus, low levels of a constitutively active ubiquitin-dependent proteolytic pathway appear to regulate enzyme turnover in the native state. The activity of this pathway is stimulated after TNFα exposure, and results in a greater amount of CCT that becomes ubiquitinated (Fig. 8). We suspect that both the ubiquitin-proteasome and calpain systems may be relevant to CCT stability because purified CCT was a substrate for m-calpain in vitro, and these proteolytic systems localize both in the cytoplasm and nucleus (59, 60).

The mechanisms by which calpain and the proteasome are activated by TNFα and how CCT is targeted for degradation require further study. It appears that lipid mediators implicated in TNFα signaling can stimulate calpain activity (61). On the other hand, proteins that are abnormally synthesized or post-translationally are often targeted for ubiquitination (42, 60). Since TNFα alters oxidant tone within cells (62), it is possible that oxidation of CCT methionine residues, or other post-synthetic modifications such as serine phosphorylation, or alterations in NH₂-terminal protein folding could destabilize the enzyme facilitating incorporation into the ubiquitin pathway.

Unlike the present results showing alterations in CCT protein stability, most prior studies have shown that enzyme activity in cells is regulated by other post-translational events involving lipid regulation or changes in the degree of enzyme phosphorylation (1). These mechanisms might occur independently or act in concert with TNFα-induced CCT proteolysis. For example, the cytokine stimulates phospholipase A₂ and neutral sphingomyelinase activities (24, 25, 63, 64). One potential consequence of activation of these hydrolases is the generation of bioactive lipid intermediates such as lysophosphatidylcholine, ceramide, or potentially sphingosine which have been linked to inhibition of either CCT activity or PtdCho synthesis (4, 6, 30, 65). Ceramide, resulting from TNFα activation of sphingomyelinase, has also been shown to activate calpain (61). Furthermore, TNFα has been shown to stimulate mitogen-activated protein kinase (9, 66). Preliminary studies in our laboratory reveal that the cytokine activates p42/p44 extracellularly regulated kinase and protein kinase Cα, which may modulate CCT activity by stimulating enzyme phosphorylation. However, these studies indicate that the sizes of products of the 20 S and 26 S proteasome are only 500 Da (56). Such small products are only detected in vitro by more sensitive methods and would run near the dye front of the PAGE. Thus, the proteolytic fragments resulting from digestion by the combination of calpain and/or the proteasome are probably very small, on the order of 5–6 residues. Another factor contributing to this result is the fact that the analyses were performed at 4- to 8-h intervals following cytokine addition (Figs. 2 and 4), and it has been shown that proteasome fragments are subject to further rapid hydrolysis by endopeptidases (57), thus probably eliminating the products of calpain/proteasome degradation.
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lum (64) simultaneously with an increase in CCT phosphorylation suggesting that these pathways are initial upstream events in TNFα signaling. Whether these changes in enzyme phosphorylation or alterations in lipid association of CCT affect its vulnerability to proteolysis in the setting of TNFα exposure require further investigation. Results of such work might lead to novel therapies targeted at protease inhibition in the setting of sepsis-associated lung injury.

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