TNF-α decreases ABCA1 expression and attenuates HDL cholesterol efflux in the human intestinal cell line Caco-2

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Abstract HDL cholesterol levels are decreased in Crohn's disease, a tumor necrosis factor-α (TNF-α)–driven chronic inflammatory condition involving the gastrointestinal tract. ATP-binding cassette transporter A1 (ABCA1), one of several liver X receptor (LXR) target genes, is a cell surface transporter that mediates the rate-controlling step in HDL synthesis. The regulation of ABCA1 and HDL cholesterol efflux by TNF-α was investigated in the human intestinal cell line Caco-2. In response to cholesterol micelles or T0901317, an LXR nonsterol agonist, TNF-α decreased the basolateral efflux of cholesterol to apolipoprotein A1 (apoA1). TNF-α, by attenuating ABCA1 promoter activity, markedly decreased ABCA1 gene expression without attenuating the expression of LXR-α, LXR-β, and most other LXR target genes, such as ABCG1, FAS, ABCG8, scavenger receptor-B1 (SR-B1), and apoC1. TNF-α also decreased ABCA1 mass by markedly enhancing the rate of ABCA1 degradation and modestly inhibiting its rate of synthesis. Inhibitors of the nuclear factor-κB (NF-κB) pathway, which is activated by TNF-α, partially reverse the effect of TNF-α on ABCA1 protein expression. The results suggest that TNF-α, the major cytokine implicated in the inflammation of Crohn's disease, decreases HDL cholesterol levels by attenuating the expression of intestinal ABCA1 and cholesterol efflux to apoA1.—Field, F. J., K. Watt, and S. N. Mathur. **TNF-α decreases ABCA1 expression and attenuates HDL cholesterol efflux in the human intestinal cell line Caco-2.** ***J. Lipid Res.*** **2010.** 51: **1407–1415.**

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There is a reciprocal relationship between atherosclerotic heart disease and HDL levels (1–3). It is believed that HDL plays a pivotal role in reverse cholesterol transport, a process that delivers cholesterol from the arterial wall back to the liver for disposal (4, 5). Reverse cholesterol transport is facilitated by a cell-surface transporter called ATP-binding cassette transporter A1 (ABCA1). This transporter mediates the rate-controlling step in HDL formation by promoting the efflux of cholesterol and phospholipids to apolipoprotein A1 (apoA1) (4, 6–8). Mutations in ABCA1 are responsible for the phenotype found in Tangier disease. This rare disorder is characterized by severe HDL deficiency, accumulation of cholesteryl esters in macrophages in various tissues, and premature atherosclerosis (9–12). Thus, it is clear that ABCA1 is critical for the synthesis of HDL particles and plays a protective role in preventing atherosclerosis.

ABCA1 is a target gene for liver X receptor (LXR), a nuclear hormone transcription factor (13–15). LXR is believed to act as a cellular sterol sensor. By responding to an influx of cholesterol, LXR activates genes that transcribe proteins that enhance the elimination, or limit the accumulation, of cellular cholesterol (14, 16–20). Besides ABCA1, cholesterol 7α-hydroxylase, lipoprotein lipase, cholesterol ester transfer protein, fatty acid synthase, apo C1, SREBP-1c, and other ABC sterol transporters, such as ABCG1, ABCG5, and ABCG8 are gene targets for LXR (13–16, 18, 20). It has been proposed that targeting LXR may be a mechanism for enhancing the removal of cholesterol from the body.

It is now clear that the liver and intestine are the major sources of circulating plasma HDL (21–23). Dysfunction of either organ, therefore, could lead to lower HDL levels and increased risk of atherosclerosis. Indeed, patients with active Crohn's disease, a chronic inflammatory disease of the gut, do have low HDL levels and evidence for accelerated atherogenesis (24). The reasons for this are not clear. There is a suggestion, however, that the proinflammatory

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Abbreviations: ABC, ATP-binding cassette transporter; apo, apolipoprotein; CYP7A1, cytochrome P450 7A1; LXR, liver X receptor; NF, nuclear factor; NPC1, Niemann Pick C1; SR-B1, scavenger receptor-B1; SREBP, sterol-regulatory element binding protein; TNF-α, tumor necrosis factor-α; TC, taurocholate.

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cytokine, TNF-α, decreases HDL levels (see ref. 25 for a review). Because TNF-α is recognized to play a major role in the inflammatory process in Crohn's disease (26–28) it raises the possibility that TNF-α might alter intestinal ABCA1 expression/function resulting in the low HDL levels observed in this disease. The present study was undertaken to investigate the possible regulation of intestinal ABCA1 by TNF-α. The results clearly demonstrate that TNF-α decreases the expression of ABCA1 in cultured intestinal cells, which leads to attenuation of cholesterol efflux in response to LXR activation. This effect can be partially reversed by preventing activation of the nuclear factor NF- kappa B pathway by TNF-α (29).

EXPERIMENTAL PROCEDURES

Materials

[^{3}H] cholesterol (48.3 Ci/mmol) and easytag express protein labeling mix (L-[^{35}S] methionine and L-[^{35}S] cysteine) were obtained from Perkin Elmer Life Sciences (Boston, MA). Metformin (1,1-dimethylbiguanide, hydrochloride) and 5-aminoimidazole-4-carboxamide riboside (AICAR) were from Calbiochem. Protease inhibitor cocktail, 5,6-dichloro-1-b-ribofuranosyl-benzimidazole (DRB), sodium taurocholate, Tri Reagent, monoclonal anti-β-actin antibody (clone AC-15) were from Sigma Chemicals (St. Louis, MO). Rabbit polyclonal anti-human ABCA1 was from Cayman Chemicals (Ann Arbor, MI). Polyclonal anti-human ABCG1 antibody, mouse monoclonal anti-human ABCA1 antibody, and mouse monoclonal anti-NPC1 were from Abcam Inc. (Cambridge, MA). Anti-human ABCG1 rabbit monoclonal antibody and ABCG1 blocking peptide were purchased from Epitomics (Burlingame, CA). The bicinchoninic protein assay kit, stabilized goat anti-rabbit HRP conjugated antibody, goat anti-mouse HRP conjugated antibody, and superSignal west femto maximum sensitivity substrate chemiluminescent detection kit were from Pierce Biotechnology, Inc. (Rockford, IL). T0901317 was a gift from Tularik, Inc. (San Francisco, CA). Dynabeads Protein G was purchased from Invitrogen. Human apolipoprotein A1 was purchased from Meridian Life Science, Inc. (Saco, ME). MTP inhibitor BMS-201038 was a gift from Bristol Myers Squibb (New Brunswick, NJ). Dual-Glo Luciferase Assay System was obtained from Promega. TNF-α was obtained from Cell Sciences (Canton, MA).

Cell culture

Caco-2 cells were cultured in T-75 flasks (Corning Glassworks, Corning, NY) in DMEM (GIBCO, Grand Island, NY) with 4.5 g/l glucose, and supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Once the flasks reached 80% confluence, the cells were split and seeded at a density of 0.2×10^5 cells/well onto polyester membranes (0.4 μm pore size, 24 mm diameter) inserted into transwells (Costar, Cambridge, MA). Cells were fed every other day and were used 14 days after seeding.

Micelle preparation

Appropriate volumes of ethanol stock solutions containing taurocholate and cholesterol were evaporated under nitrogen, and the dried lipids were dissolved in DMEM. The resulting solution was stirred vigorously at 37°C until clear.

Estimation of efflux of [^{3}H] cholesterol into the basolateral medium

Caco-2 cells were incubated overnight with 1.5 ml of 10% fetal calf serum (FCS)-DMEM containing 2.5 μCi [^{3}H] cholesterol to uniformly label cellular cholesterol. Radiolabeled cholesterol was added to the apical medium in ethanol (<0.5%, final concentration). To remove unincorporated labeled cholesterol, the filter insert was washed twice with DMEM and transferred to a new 6-well plate. The basolateral medium was replaced with 2.5 ml of DMEM containing 3 μg/ml apoA1. In dishes that would contain TNF-α, the cytokine was added to both the apical and basolateral medium at a concentration of 100 ng/ml. The cells were then incubated at 37°C in 1.5 ml of DMEM, on the apical side, containing 5 mM taurocholate and 250 μM cholesterol (cholesterol micelles) or 0 or 2 μM T0901317, 0.05 μM MTP inhibitor, BMS-201038, and/or other treatments as described in the figure legends. At the end of the incubation, the basolateral medium was collected, and a sample was taken to estimate the percent of [^{3}H] cholesterol effluxed into the medium. The apical medium was removed, and the cells were washed two times with 1.5 ml of cold DMEM. Cell lipids were extracted with 1.5 ml of hexane/isopropyl alcohol/water (3/2/0.1, v/v/v). A portion of the lipid extract was taken and counted in a Packard liquid scintillation counter to determine cellular cholesterol label. The radioactivity recovered in the basolateral medium, and the total [^{3}H] cholesterol radioactivity recovered (cells, apical, and basolateral medium) was used to calculate the percent of total [^{3}H] cholesterol effluxed into the basolateral medium.

RNA estimation by real time quantitative RT-PCR

mRNA levels were estimated by quantitative RT-PCR as described previously (30). The following primers were used to estimate human gene expression: fatty acid synthase, forward, 5′- ACAGGGACAACCTGGAGTTCT -3′, reverse, 5′- CTGTGGTCCCACTTGATGAGT -3′; ApoC1, forward, 5′- TGAACTTTCTGCCAAGATGC -3′, reverse, 5′- GGTGTGGGAAATTTCAGAGG -3′; LXR-α, forward, 5′- GGTACAACCCTGGGAGTGAG -3′, reverse, 5′- TGGGGTTGATGAATTCCACT -3′; NPC1, forward, 5′- TGAGCTCTGGTCTGCAGTCATCAT -3′, reverse, 5′- TCACCGTGAACGCTCTGGTTATGT -3′; LXR-β, forward, 5′- CGCTACAACCACGAGACAGA -3′, reverse, 5′- GGTTGATGAACTCCACCTGC -3′; CYP7A1, forward, 5′- CAGAACTGAATGACCTGCCA -3′, reverse, 5′- GGTGCAAAGTGAAATCCTCC -3′. These primers were designed using qPrimerDepot database (http://primerdepot.nci.nih.gov/). The sequences for the other primers have been described previously (30, 31). The values were normalized using 18S rRNA as endogenous internal standard. The relative expression of the gene was calculated using comparative C_T method (32). The following are the qPCR Ct values for the respective genes: 18S, 10; ABCA1, 20; ABCG1, 27; SREBP-1c, 25; FAS, 22; ABCG8, 32; NPC1, 24; Apo C1, 26; CYP7A1, 26; LXR-α, 23; LXR-β, 18.

To estimate stability of ABCA1 mRNA, cells were incubated for 18 h with cholesterol micelles in the presence or absence of TNF-α. Six wells for each treatment received 0 or 157 μM DRB apically for 6 h to inhibit new mRNA synthesis. The amount of mRNA remaining in the cells was estimated by real-time quantitative RT-PCR.

Immunoblot analysis of ABCA1, NPC1, and ABCG1

Cells were incubated at 37°C for 18 h in 1.5 ml of apical medium containing 5 mM taurocholate and 250 μM cholesterol (cholesterol micelles) or 0 or 2 μM of T0901317 and the indicated amounts of other compounds. The cells were washed and harvested in 1 ml of DMEM buffer. Immunoblot analysis

of ABCA1, NPC1, and ABCG1 was performed as previously described (30). Equal loading of the samples was assured by using equal amount of protein per sample and by density of the actin band on the blot. To validate that the ABCG1 antibody detected ABCG1 protein, an ABCG1 blocking peptide resulted in the complete elimination of the ABCG1 band observed on immunoblot.

Degradation of ABCA1 protein

Cells were incubated for 18 h with micelles containing cholesterol in the presence or absence of TNF-α. At the end of the incubation, fresh medium containing original treatments was added on apical and basal sides. To inhibit new protein synthesis, 100 μM cycloheximide was added to the apical medium (33, 34). The cells were harvested at various time intervals indicated in Fig. 6. The amount of ABCA1 remaining in the cells was estimated by immunoblotting.

Synthesis of ^{35}S-methionine labeled ABCA1 protein

Cells were incubated for 18 h with micelles containing cholesterol in the presence or absence of TNF-α. The medium was removed, and cells were incubated with methionine-deficient medium for 30 min. Fresh methionine-deficient medium containing 290 μC of ^{35}S-methionine apically and all the treatments used in the first incubation were added, and the incubation was continued for 2, 4, and 6 h. The radioactivity in newly synthesized ABCA1 was determined by immunoprecipitation with rabbit polyclonal anti-human ABCA1 antibody. Briefly, the cells were washed and harvested in 0.5 ml of RIPA buffer containing protease inhibitors. The cell suspension was sonicated for 10 s, followed by centrifugation at 16000 *g* for 10 min. The supernatant was collected, and an equal amount of protein (200 μg) was taken for immunoprecipitation with 1 μg rabbit polyclonal anti-human ABCA1 antibody. This mixture was incubated overnight at 4°C with gentle mixing. Ten microliters of Dynabeads Protein G slurry was added to the immunocomplexes and incubated at 37°C for 4 h. The immunoprecipitate was washed 4 times according the manufacturer's protocol. The antigen-antibody complex was eluted from the beads and dissociated with 5 μl of 0.1 M glycine-HCl, pH 3.5, followed by 15 μl of 5× Laemmli sample buffer. This mixture was applied quantitatively to the SDS-acrylamide gel and transferred to an Immobilon-P PVDF membrane (Millipore, Bedford, MA). The radioactivity in ABCA1 protein was detected by exposing the blot to BIO-MAX X-ray film. The amount of radiolabeled, newly synthesized ABCA1 protein was quantitated by measuring density by NIH-image.

ABCA1 promoter activity

The quantity 0.045 ml of a transfection complex solution containing 3 μg of ABCA1 promoter plasmid (pGL3-Basic firefly luciferase vector, −949/+274, GeneBank accession number AF287262) (35), 10 ng of Renilla luciferase plasmid (phRL-TK vector, Catalog # E6241, Promega), 1 μl PLUS reagent, and 3 μl lipofectamine LTX reagent (prepared in Opti-MEM reduced serum medium according to the manufacturer's instructions) was added to each well of a 24-well plate. The transfection solution was mixed with 0.24×10^6 Caco-2 cells suspended in 0.05 ml of Opti-MEM reduced serum medium and incubated under sterile conditions at room temperature for 10 min, followed by addition of 0.5 ml Opti-MEM, and transferred to a 5% CO-2 incubator at 37°C. After incubation, 1 ml of complete medium containing 0.5 ml of 10% FCS with antibiotics and 0.5 ml of conditioned medium was added, and the incubation continued for 24 h. On the next day, the medium was replaced with 10% FCS-DMEM containing antibiotics, and the cells incubated for another 6 h. At the end of this incubation, cells were washed with DMEM and 1 ml of fresh DMEM containing 200 ng TNF-α/ml was added to each well for 18 h. The cells were washed and harvested. Firefly and Renilla luciferase activity was estimated using Dual-Glo Luciferase Assay System from Promega.

Other analyses

Protein content was estimated using the bicinchoninic acid assay kit (Pierce, Rockford, IL). Unpaired *t*-test or one-way ANOVA was performed to compare the treatments using SigmaPlot version 11 for Windows.

RESULTS

TNF-α attenuates ABCA1-mediated cholesterol efflux

To address whether TNF-α alters cholesterol efflux to apoA1, cells grown on micropore filters separating an upper and lower well were labeled with cholesterol overnight. They were then incubated apically with taurocholate micelles containing 250 μM cholesterol to enhance ABCA1 expression and cholesterol efflux (30, 36). In dishes that would contain TNF-α, the cytokine was added to both the apical and basolateral medium at a concentration of 100 ng/ml. ApoA1 was also added to the basolateral medium. After 18 h incubation, the percentage of labeled cholesterol recovered in the basolateral medium was estimated. **Fig. 1** shows these results. Compared with control cells incubated without TNF-α, the amount of labeled cholesterol recovered from the lower well of cells incubated with the cytokine was modestly, but significantly, decreased (left two bars). In another set of cells, ABCA1-mediated cholesterol efflux was induced by a nonsterol LXR agonist, T0901317, in the presence or absence of TNF-α (right two

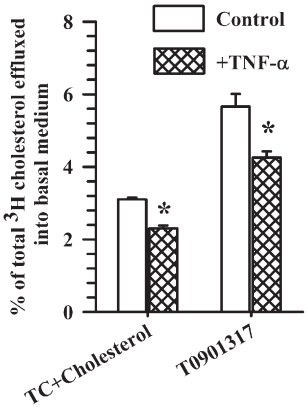

Fig. 1. Effect of TNF-α on cholesterol efflux. Cells were prelabeled for 24 h with [^{3}H] cholesterol as described in "Experimental Procedures." After thorough washing to remove unincorporated label, they were incubated with either taurocholate micelles containing 250 μM cholesterol or an LXR agonist T0901317 with or without 100 ng/ml of TNF-α added to both the apical and basolateral medium. ApoA1 was also added to the basolateral medium. Following 18-h incubation, the amount of labeled cholesterol recovered in the basolateral medium was estimated as described in "Experimental Procedures." Values for each treatment represent a mean ± SE of 6 individual wells of a representative experiment from three separate experiments all showing similar results. *$P < 0.05$ versus control.

bars). The results were similar. TNF-α decreased the basolateral efflux of cholesterol to apoA1. Thus, whether ABCA1-mediated cholesterol efflux was enhanced by cholesterol or a synthetic nonsterol LXR agonist, the addition of TNF-α significantly attenuated cholesterol efflux into the basolateral medium.

TNF-α decreases the expression of ABCA1

To investigate if TNF-α alters the expression of ABCA1, cells were incubated for 18 h with micelles containing cholesterol or the LXR agonist T0901317 in the presence or absence of TNF-α. Following incubation, ABCA1 mRNA levels were estimated by quantitative RT-PCR. **Fig. 2A** shows these results. It is clear from these data that TNF-α caused a decrease in ABCA1 mRNA levels. This decrease in ABCA1 gene expression by TNF-α was not due to a change in ABCA1 mRNA stability because the percentage of ABCA1 mRNA remaining following incubation with TNF-α was similar whether in the presence or absence of a potent inhibitor of mRNA synthesis, 5,6-dichloro-1-b-ribofuranosyl-benzimidazole (data not shown).

To further support the notion that TNF-α attenuates transcription of ABCA1, the effect of TNF-α on the activity of the ABCA1 promoter was estimated. Cells were transfected with a luciferase-expressing plasmid containing the ABCA1 promoter. Promoter activity was then estimated following incubation with or without TNF-α. As shown in Fig. 2B, promoter activity was markedly decreased in cells incubated with TNF-α, which suggests that the decrease in ABCA1 gene expression by TNF-α was due to a decrease in its transcription.

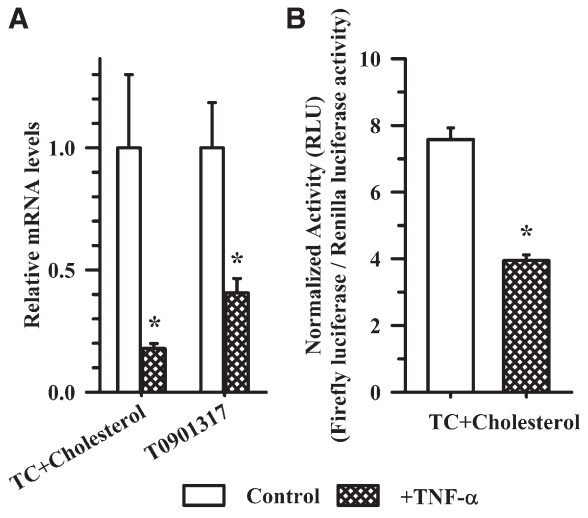

Fig. 2. Effect of TNF-α on ABCA1 gene expression and promoter activity. (A) Cells were incubated for 18 h with micelles containing 250 μM cholesterol or the LXR agonist T0901317 with or without 100 ng/ml of TNF-α. Following incubation, ABCA1 mRNA levels were estimated by quantitative RT-PCR. (B) Cells were transfected with a luciferase-expressing plasmid containing the ABCA1 promoter. The cells were then incubated for 18 h with micelles containing 250 μM cholesterol in the presence or absence of 100 ng/ml of TNF-α, and promoter activity was estimated as described in "Experimental Procedures." Values for each treatment in (A) represent a mean ± SE of six individual wells of a representative experiment of four separate experiments all showing similar results. Values for each treatment in (B) represent a mean ± SE of six individual wells. *$P < 0.05$ versus control.

To address whether the effect of TNF-α on ABCA1 gene expression was specific for this LXR-responsive gene, the effect of TNF-α on other gene targets of LXR was also examined (**Fig. 3**). Cells were again incubated for 18 h with micelles containing 250 μM of cholesterol or the LXR agonist T0901317 in the presence or absence of 100 ng/ml TNF-α. Following incubation, mRNA levels for the respective genes were estimated by RT-PCR. In contrast to the marked decrease in ABCA1 gene expression by TNF-α, five other LXR target genes—ABCG1, FAS, ABCG8, SR-B1, and apoC1—were not attenuated by TNF-α. Two other LXR target genes, SREBP-1c and CYP7A1, however, were significantly decreased by TNF-α. Moreover, gene expression for LXR-α and -β were not affected by the cytokine. Gene expression of NPC1, a protein felt to play a role in cholesterol efflux (30, 37), was not altered by TNF-α, nor was NPC1L1, a protein critical in the absorption of cholesterol from the lumen (data not shown).

Fig. 4 shows the effect of TNF-α on ABCA1, NPC1, and ABCG1 mass. The left panels show results using cholesterol micelles to enhance ABCA1-mediated cholesterol efflux and ABCA1 expression, and the right panels show results using the LXR agonist. The right and left panels are from two separate experiments/blots performed on different days, so the blots cannot be compared. Similar to what was observed above, however, TNF-α significantly decreased ABCA1 mass without altering the amount of NPC1 or ABCG1 mass.

To investigate a possible mechanism by which TNF-α caused a decrease in ABCA1 mass, the effect of TNF-α on ABCA1 protein synthesis and degradation was studied. Cells were incubated for 18 h with micelles containing cholesterol in the presence or absence of TNF-α. Following incubation, cells were pulsed for 2, 4, and 6 h with ^{35}S-methionine, and the incorporation of methionine into ABCA1 protein was estimated. The results demonstrate that TNF-α only modestly attenuated the rate of synthesis of ABCA1 (**Fig. 5**).

Then the effect of TNF-α on the rate of ABCA1 protein degradation was estimated. Following incubation as described above, cycloheximide was added to prevent new protein synthesis. Cells were harvested over a 10-h period, and the ABCA1 mass was estimated by immunoblotting. The results are shown in **Fig. 6**. The half-life of ABCA1 protein in control cells was approximately 10 h. In contrast, the half-life of ABCA1 in cells incubated with TNF-α was 5 h, half that of control cells. The combined results suggest that TNF-α decreases ABCA1 mass by modestly attenuating its synthesis and by markedly enhancing its rate of degradation.

Inhibitors of the NF-κB pathway attenuate the effect of TNF-α on ABCA1 expression

To address whether the downregulation of ABCA1 expression by TNF-α was acting through the NF-κB pathway, inhibitors of this pathway were used to prevent the effect

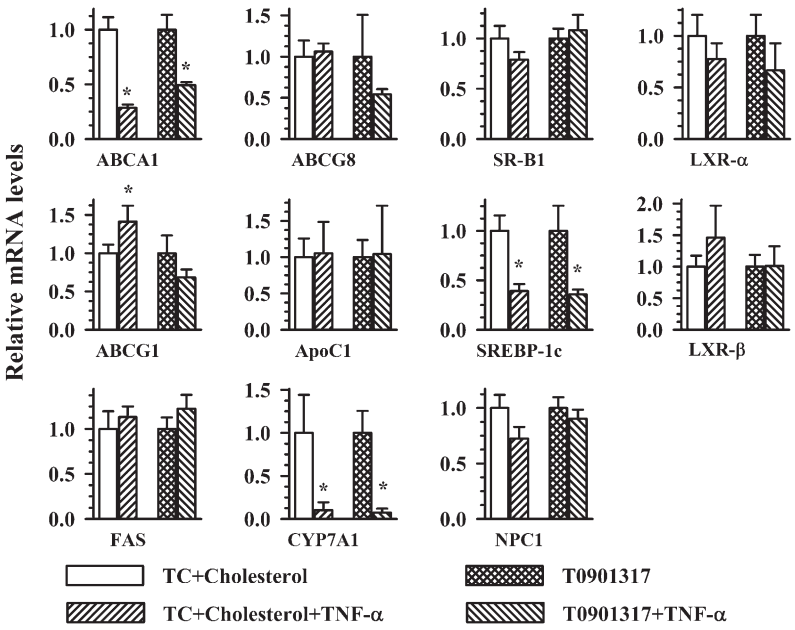

Fig. 3. Effect of TNF-α on LXR target genes. Cells were incubated for 18 h with micelles containing 250 μM cholesterol or the LXR agonist T0901317 in the presence or absence of 100 ng/ml of TNF-α. Following the incubation, mRNA levels for the respective genes were estimated by quantitative RT-PCR. Values for each treatment represent a mean ± SE of 6–8 individual wells. Similar results were observed for ABCA1, ABCG1, SREBP-1c, FAS, ABCG8, and NPC1 in five other separate experiments. *$P < 0.05$ versus control.

of TNF-α. Cells were incubated with micelles containing cholesterol in the presence or absence of TNF-α, as well as two individual inhibitors of the NF-κB pathway, AICAR and metformin (29). Following incubation, ABCA1 mRNA levels and mass were estimated. **Fig. 7** shows these results. It is clear from these data that known inhibitors of the NF-κB pathway attenuate, but do not completely ameliorate, the inhibitory effect of TNF-α on ABCA1 gene and protein expression. This suggests that the NF-κB pathway plays a role in the inhibitory effect of TNF-α on ABCA1 expression.

DISCUSSION

The results of the present study clearly demonstrate that the proinflammatory cytokine TNF-α attenuates the expression of intestinal ABCA1, which results in a decrease in cholesterol efflux to apoA1. TNF-α decreases ABCA1 transcription by attenuating its promoter activity and decreases ABCA1 mass by decreasing synthesis and enhancing degradation of the protein. Furthermore, an intact NF-κB pathway is necessary for TNF-α to have its full inhibitory effect on ABCA1 expression.

In the macrophage, a cell in which ABCA1 and cholesterol efflux have been extensively studied, it is evident that the ABC transporter can be regulated at several levels (see refs. 38, 39 for reviews). Our prior results in intestinal cells also support that notion (36, 40). In the present study, we show evidence for transcriptional, translational, and post-translational regulation of ABCA1 by TNF-α. The cytokine caused a consistent and reproducible decrease in ABCA1 mRNA levels, which suggests that TNF-α attenuates intestinal ABCA1 gene transcription. Indeed, we found that TNF-α caused a marked decrease in ABCA1 promoter activity. In macrophages, however, the effect of TNF-α on ABCA1 gene expression is not at all clear. For example, in J774 cells, Khovidhunkit et al. (41) found that TNF-α decreased ABCA1 mRNA levels; whereas, in mouse peritoneal macrophages, TNF-α had either no effect or actually enhanced ABCA1 gene expression (42, 43). Obviously, intestinal cells and macrophages differ significantly in their function and structure. This could explain why the regulation of ABCA1 gene expression by TNF-α may be different in these two cells. The cause for the observed differences noted above within macrophages, however, remains unclear.

There are data to suggest that the levels of ABCA1 mRNA do not accurately reflect the amount of ABCA1

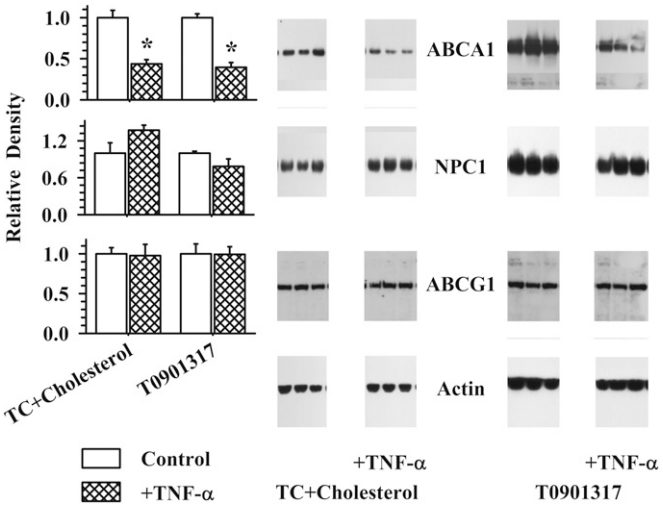

Fig. 4. Effect of TNF-α on ABCA1, ABCG1, and NPC1 mass. Cells were incubated for 18 h with micelles containing 250 μM cholesterol or the LXR agonist T0901317 in the presence or absence of 100 ng/ml of TNF-α. Following incubation, ABCA1, ABCG1, and NPC1 mass was estimated by Western immunoblotting as described in "Experimental Procedures." Values for each treatment represent a mean ± SE of six individual wells of a representative experiment from three separate experiments all showing similar results. *$P < 0.05$ versus control.

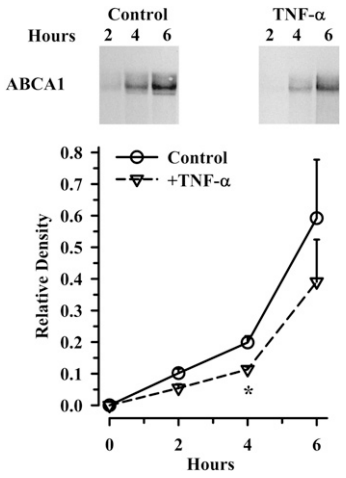

Fig. 5. Effect of TNF-α on ABCA1 synthesis. Cells were incubated for 18 h with micelles containing 250 μM cholesterol in the presence or absence of 100 ng/ml of TNF-α. Following incubation, cells were pulsed with ^{35}S-methionine for 2, 4, or 6 h. The incorporation of labeled methionine into ABCA1 was estimated as described in "Experimental Procedures." Values for each treatment represent a mean ± SE of three individual wells of a representative experiment from two separate experiments with a total of 7 individual wells all showing a decrease in the incorporation of label into ABCA1 in cells incubated with TNF-α. *P < 0.05 versus control.

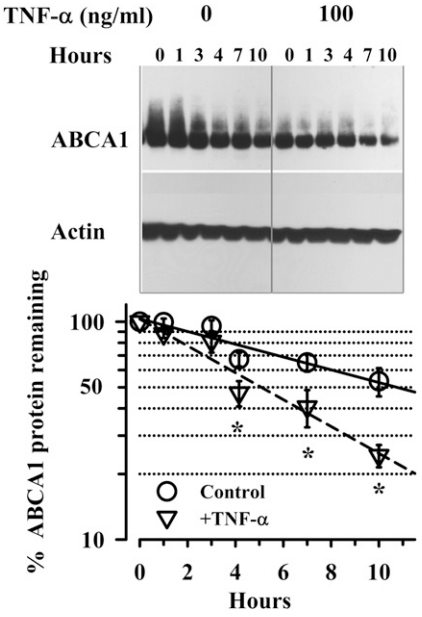

Fig. 6. Effect of TNF-α on ABCA1 degradation. Cells were incubated for 18 h with micelles containing 250 μM cholesterol in the presence or absence of 100 ng/ml of TNF-α. Following incubation, 100 μM of cycloheximide was added, and the cells were harvested over a 10 h period. ABCA1 mass was estimated by Western immunoblotting. Values for each treatment represent a mean ± SE of four individual wells of a representative experiment from two separate experiments showing similar results. *P < 0.05 versus control.

protein within cells (44). This indicates that posttranscriptional regulation of ABCA1 protein occurs and is likely important for regulating its activity within cells. Our results are in agreement. The rate of synthesis of ABCA1 protein was only modestly decreased by TNF-α, but TNF-α caused a marked increase in the rate of ABCA1 degradation. Degradation of ABCA1 is felt to play an important role in modulating the amount of this cellular transporter. In the macrophage, ABCA1 turns over relatively rapidly with an observed half-life between one and 2 h (45–47). In contrast, in Caco-2 cells, ABCA1 turns over much more slowly with an estimated half-life between 8 and 10 h. ApoA1 is thought to stabilize or protect the ABCA1 protein from degradation (46, 48). In the macrophage, this protection from degradation occurs at the cell surface when apoA1 interacts directly with the transporter. In the intestinal cell, however, apoA1 is continually synthesized within the cell and eventually secreted at the basolateral membrane, the cell surface site of ABCA1 (36, 49). Perhaps in an intestinal cell, apoA1 interacts with ABCA1 intracellularly as well as at the cell surface. This interaction could protect the ABC transporter from degradation and would explain the prolonged half-life observed in intestinal cells compared with that observed in the macrophage. Moreover, it would make sense for the macrophage to be able to rapidly regulate the amount of ABCA1 at the cell surface by regulating its rate of degradation. In times of cholesterol excess, it would be important for the cell to rapidly enhance the amount of ABCA1 on the cell surface to facilitate efflux of cholesterol to apoA1. In times of cholesterol deficit or in the absence of apoA1, ABCA1 would be more rapidly degraded, and HDL cholesterol efflux would be minimal. In the intestine, a major site of HDL production, the absorptive cell is constitutively synthesizing and secreting apoA1 and is continually transporting biliary or dietary cholesterol. It would seem important that intestinal ABCA1 not be limiting so as to accommodate large amounts of, or rapid changes in, cholesterol flux to maintain HDL levels.

Despite the fact that TNF-α reduced ABCA1 protein by 50% or more, the decrease observed in cholesterol efflux was much less. In the intestine, there is another abundant ABC transporter, ABCG1 (36). ABCG1 also facilitates the efflux of cholesterol to apoA1. In peripheral cells, ABCG1 can substitute for ABCA1 or even be enhanced when ABCA1 is absent or attenuated (see ref. 50 for review). Indeed, in animals that are made deficient in ABCA1, gene expression for ABCG1 is augmented. In the present study, TNF-α did not significantly alter the gene or protein expression of ABCG1. It is possible, therefore, that ABCG1 partially compensates for the loss of ABCA1 due to TNF-α. Moreover, the observation that TNF-α decreased ABCA1 expression, but not that of ABCG1, strongly suggests that TNF-α does not alter LXR or the interaction of LXR with its target gene. This is further supported by the observation that TNF-α did not alter LXR-α or -β expression. When other LXR target genes were examined (FAS, SR-BI, ApoC1, and ABCG8), none of these were attenuated by TNF-α; the only exceptions were SREBP-1c and CYP7A1. It is also possible that TNF-α attenuates ABCA1 within intracellular compartments but does not alter (or alters only

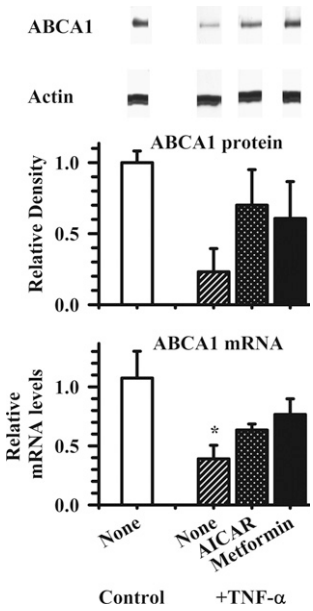

Fig. 7. NF- kappa B pathway inhibitors attenuate the inhibitory effect of TNF-α on ABCA1 expression. Cells were incubated for 18 h with micelles containing 250 μM cholesterol in the presence or absence of 100 ng/ml of TNF-α and inhibitors of the NF- kappa B pathway AICAR (0.5 mM) or metformin (1 mM). Following the incubation, ABCA1 mRNA levels and mass were estimated. Values for each treatment represent a mean ± SE of three individual wells. The data were analyzed using one-way ANOVA. $*P < 0.05$ versus control.

modestly), the amount of cell surface ABCA1. If true, and if ABCA1 does not facilitate the trafficking of cholesterol from intracellular pools to the cell surface for export, this could explain why cholesterol efflux is partially maintained in cells exposed to TNF-α. Data about intestine and other cell types support an intracellular and plasma membrane location for ABCA1 (23, 51, 52). To our knowledge, however, there are no data that support a specific regulation of ABCA1 located intracellularly versus that which is located on the plasma membrane. There are also other cholesterol efflux pathways that exist, such as passive or diffusional efflux or SR-B1–mediated efflux (53). With the attenuation of ABCA1, these other pathways could be playing a more major role in intestinal HDL production independent of ABC-mediated cholesterol efflux (53).

The present results would indicate that in Crohn's disease, a disease known to have elevated levels of serum and intestinal TNF-α (26–28), intestinal HDL production and, thus, plasma HDL levels, would be decreased. In human clinical studies, this appears to be the case (24). One could argue, however, that independently of the effects of TNF-α on ABCA1 expression, the marked intestinal inflammation and underlying histopathology seen in Crohn's disease, in and of itself could cause disruption of HDL production. Indeed, in hamster intestine, we found that compared with duodenum and jejunum, ABCA1 mRNA levels were highest in ileum (54), the location most affected in patients with small intestinal Crohn's disease. Although one should be cautious in equating intestinal ABCA1 mRNA levels with ABCA1 protein levels (44), it is possible that, independent of TNF-α, the severely diseased segment could impact intestinal HDL production and plasma HDL levels. The logical argument against this is that not all Crohn's disease involves the terminal ileum. In rheumatoid arthritis, another disease state with decreased HDL levels and high circulating levels of TNF-α, the intestine is not inflamed. Moreover, inhibiting TNF-α with an intravenous infusion of an antibody directed against the cytokine results in increased HDL levels in both Crohn's disease and rheumatoid arthritis (55, 56).

There is good evidence that NF-κB activity is upregulated in patients with Crohn's disease. Numerous NF-κB–dependent proinflammatory mediators, including interleukin (IL)-1β, TNF-α, IL12p40, and IL-23p19, are elevated in patients with inflammatory bowel disease (see ref. 57 for review). Importantly and germane to the present study, TNF-α is a major activator of the NF-κB pathway. In addition, the promoter of ABCA1 contains an NF-κB binding site (58). Because TNF-α attenuates the activity of the ABCA1 promoter, we postulated that the regulation of ABCA1 expression by TNF-α was being mediated through the NF-κB pathway. Although potent inhibitors of NF-κB, AICAR and metformin (29), modestly reversed the effects of the cytokine on ABCA1 expression, they did not completely prevent it. This would suggest that other TNF-α pathways, such as those acting through AP1 transcription factors or mitogen-activated protein kinase (MAPK) may be involved (see ref. 57 for review). Further studies would be required to more specifically address these possibilities.

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