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The involvement of Cathepsin B and L in inflammation and cholesterol trafficking protein NPC2 secretion in macrophages

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Abstract

Obesity and its related chronic inflammation are the major risk factors for developing metabolic disturbances. The roles of cathepsin cysteine proteases have been tied to inflammation and atherosclerosis. Cathepsins are important functional links between inflammation, cholesterol metabolism, and atherosclerosis in obesity. NPC2, a lysosomal protein, plays an important role in cholesterol trafficking. The objective of this study was to examine the regulation of cathepsins and NPC2 in adipose tissue and macrophages in obesity, and the effect of modifying cathepsin activity in cholesterol metabolism and trafficking in macrophages. We found that high fat diet (HFD) feeding altered the mRNA and protein expression levels of cathepsin B and L and NPC2 in adipose tissue in mice; the differential regulation of these proteins was observed between adipose depots. In vitro studies showed that TNF-α reduces intracellular protein levels of CtB, CtL, and NPC2, but increases their secretion in 3T3-L1 adipocytes. Likewise, LPS stimulated the secretion of CtB and NPC2 in Raw264.7 macrophages. Using the inhibitors of cathepsin enzymatic activity, we found that cathepsin B and L regulate TNFα production, the expression and secretion of NPC2 protein, and the mRNA levels of the genes involved in cholesterol trafficking in macrophages. Our findings suggest that cathepsin B and L have a significant involvement in mediating the inflammatory response, in cholesterol trafficking, and in regulating NPC2 secretion.

Introduction

Chronic inflammation affiliated with obesity is known to alter metabolism which leads to the development of comorbidities such as atherosclerosis and heart disease. While this association is well established, many of the underlying changes that occur at the molecular level are not well understood. One class of proteins that has been found to have altered expression in obese subjects is cathepsins.

There are 11 isoforms of cathepsins known to mammals. Cathepsins are responsible for post-translational processing and the degradation of many proteins, which have essential

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Disclosure

There is no conflict of interest with all of the authors.

roles to maintain normal cellular and physiological functions. Due to their ubiquitous expression, they are generally considered housekeeping enzymes, however certain isoforms of cathepsins have been tied to adipogenesis (1), inflammation, and atherosclerosis. For example, it is known that cathepsin B (CtB) is necessary for TNF-α secretion, a mediator of inflammation (2). It has also been found that cathepsin L (CtL) is expressed at very high levels in atherosclerotic lesions (3, 4). Cathepsin D has been shown to play a crucial role in processing the cholesterol trafficking protein ATP-binding cassette transporter 1 (ABCA1); when cathepsin D is knocked out ABCA1 becomes trapped and cholesterol accumulates in the cell (5).

In addition to ABC-transporters, Niemann-Pick Type C (NPC) proteins, a class of lysosomal proteins, play an important role in cholesterol metabolism, particularly in cholesterol trafficking. Membrane-bound NPC1 and soluble NPC2 are both required for lipoprotein derived cholesterol to egress from endosomes and lysosmes (6). The function of NPC1 and NPC2 in the cholesterol trafficking has been reported to be through independent mechanisms (7). However, the evidence from another study supports that NPC2 and NPC1 function in concert to facilitate the export of unesterified cholesterol from lysosomes (8). Deficiency of NPC1 or NPC2 leads to the accumulation of LDL-derived cholesterol in the late endosomes and lysosomes. This causes the progressive neurodegeneration, hepatosplenomegaly, and premature death; the pathological state is called Niemann Pick C disease (9). There is very little data on the roles of cathepsins or NPC2 in adipose tissue, and none that currently links these two classes of proteins together.

We hypothesized that modulating cathepsin activity would influence NPC2 expression because both are lysosomal proteins and their alterations or deficiency are associated with an inflammatory state (10, 11). We also thought that exogenous cathepsin inhibitors would disrupt the production and secretion of pro-inflammatory cytokines in adipocytes and macrophages. The objectives of this study were to examine the regulation of cathepsins, specifically CtB, CtL, and NPC2 in adipose tissue and macrophages in genetic and diet-induced obesity. Additionally, we explored how the cathepsin activity regulates the expression of NPC2 and other genes involved in cholesterol metabolic pathways. We found that high fat diet feeding and inflammatory mediators regulate CtB, CtL, and NPC2 expression in adipose tissue and macrophages. This regulation displays a difference between fat depots. We also found that CtB and L regulated cytokine production, the expression of NPC2, and the genes involved in other cholesterol metabolic pathways in macrophages. Our findings indicate that CtB and L have important roles in regulating inflammation and cholesterol trafficking pathways in macrophages through interfering with the expression and secretion of NPC2 protein.

Methods and Procedures

Animal studies

Rats were Zucker lean (Fa/fa) and obese (fa/fa) on commercial rat diet (Rodent Laboratory Chow 5001, Purina Laboratories, St. Louis, MO). The rats were euthanized and epididymal fat pads were collected when the lean rats weighed 300g and the obese rats weighed 500–600g, at 8 to 10 weeks old.

Male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were housed in a specific pathogen-free facility. Animal handling followed the National Institutes of Health guidelines, and experimental procedures were approved by the University of Minnesota Animal Care and Use Committee. Animals were grouped into 3–4 mice/cage and at 3 weeks were started on a HFD (fat calories: 60%) obtained from Bio-Serv (F3282; New Brunswick, NJ) or a regular chow diet (RCD), with free access to water for all studies. For the Rosiglitazone (TZD) (GlaxoSmithKline, Philadelphia, PA) study, a group of the HFD mice were given an oral gavage of TZD (10mg/kg body weight/day) for 25 days after developing insulin resistance and obesity in response to 14 weeks of HFD. The epididymal and inguinal fat pads were collected and immediately frozen in liquid nitrogen and stored at −80°C.

Isolation of primary stramol vascular (SV) cells and adipocytes

Primary SV and adipocytes were prepared by collagenase digestion as described previously (12). Adipose tissue from epidydimal fat pad of male mice on RCD was placed in digestion vials containing KRBH buffer plus 0.1% albumin, minced and digested with collagenase to 20 mg/ml. After 2 hour digestion, SV cells and adipocytes were separated by centrifugation at 1200 rpm for 10 min. Isolated adipose cells were washed with KRBH buffer plus 0.1% albumin and subjected to RNA extraction.

Cell culture

3T3-L1 cells (Kindly provided by Dr. David Bernlohr in the Department of Biochemistry, Molecular Biology & Biophysics at the University of Minnesota) were cultured in 6-well plates using Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO) as described elsewhere (13, 14). Two days after 100% confluence was reached, the differentiation cocktail was applied as described previously. Cells were grown in DMEM with 10% fetal bovine serum (FBS) (JRH Biosciences, Inc. Lenexa, KS) and insulin for 8 days. At day 8, differentiated adipocytes were cultured in low glucose DMEM with 0.5% FBS for overnight. After serum starvation for overnight, cells were then treated with 0, 1.0 or 10 ng/ml of TNF-α (R & D systems, Inc., Minneapolis, MN) for 24 hours. At the end of the experiments, cells were prepared for protein collection using RIPA buffer (Sigma-Aldrich, St. Louis, MO) for western-blotting analysis. Culture medium was collected and concentrated by a Macrosep centrifugal device with molecular weight cutoff of 1 KDa (Pall Life Sciences, MI).

Raw 264.7 macrophages (Kindly provided by Dr. David Bernlohr in the Department of Biochemistry, Molecular Biology & Biophysics at the University of Minnesota) were cultured in 6 well plates using DMEM and 15% FBS. Cells were grown to approximately 70% confluence, followed by various treatments including LPS and LPS with cathepsin inhibitors. Lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, MO) was used at the concentration of 1Lg/mL for 24 hours. Cathepsin inhibitors were applied at concentrations of 10LM/well 3 hours prior to LPS treatment. The inhibitors used were: CtL inhibitor (NapSul-Ile-Trp-CHO, Enzo Life Sciences Farmingdale, NY), CtB inhibitor (CA-074Me, Enzo Life Science) and CtL and CtB combination inhibitor (E64) (Enzo Life Science). At the end of experiments, cells and medium were collected for RNA extraction and proteins for western-blotting.

Western blot analysis

Protein extracts were homogenized and solubilized using RIPA buffer with a proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The concentration was determined using Pierce bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA). Equal amounts of protein was loaded onto SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were immunoblotted according to the manufacturers using: CtL (Santa Cruz Biotechnology, Inc), CtB (R & D Systems, Minneapolis, MN), NPC2 (HEI) antibody (NPC2 antibody was kindly provided by Dr. Peter Lobel from Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School). After incubating with the primary antibody, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase. ECL Western Blotting detection systems (GE HealthCare BioSciences, Piscataway, NJ) were used to detect protein expression through antibody reactivity. Membranes were stripped using Restore Western stripping buffer (Thermo scientific Waltham, MA), and re-incubated with actin (Cell Signaling Technology, Danvers, MA) to test protein loading.

Cytokine detection

Cytokine/chemokine levels in the culture medium were evaluated by a multiplex method using the Bio-Rad, Bio-Plex multiplex cytokine secretion detection by the Cytokine Reference Laboratory, University of Minnesota.

RNA extraction and qPCR

RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) and protocol. RNA was purified using RQ treatment (Promega, Madison, WI), following their protocol. cDNA was synthesized using reverse transcript (RT) treatment (Promega, Madison, WI). Real-time qPCR was done using SYBR green SuperMix Universal kit (Invitrogen, Carlsbad, CA) and an ABI StepOnePlus real-time PCR Systems (Applied Biosystems, Foster City, CA, USA). Results are presented as levels of expression relative to that of controls after normalizing to β-actin as the internal control using the ΔCt method. Statistical significance was determined by 2-tailed Student's *t*-test.

Statistical analysis

Results were expressed as means ±SE. Differences in parameters between control and inhibitors or LPS and LPS with inhibitors were evaluated using a two-group *t* test with a 0.05 two-sided significance level. A P value <0.05 was considered significant.

RESULTS

Regulation of Cathepsins in adipose tissue of obese animals

In our unpublished microarray analysis, we noticed that in the Zucker obese rats, the mRNA levels of cathepsins (H, L, B, D and K), especially CtB and CtL were significantly upregulated selectively in the SV fraction of epididymal adipose tissue (data not shown). Herein, we examined the protein levels of CtL and CtB in adipose tissue of high-fat diet

(HFD)-induced obesity. Our results showed that CtL was differentially expressed and regulated between the two fat depots in the mice in response to the 14 weeks of HFD feeding. CtL protein was detected in the two forms: pro-CtL and mature CtL (Fig. 1A and 1B). HFD feeding selectively increased the protein levels of mature CtL in epididymal fat depot, but did not affect the expression of either form of CtL in inguinal fat depot (Fig. 1A and 1B). Similarly, the protein levels of CtB (Pro- and mature forms) were upregulated by HFD feeding in the epididymal depot of mice (Fig. 1A and 1C). However, in the inguinal tissue, the HFD reduced the protein levels of CtB, particularly pro-CtB form (Fig. 1A). The TZD (Rosiglitazone) treatment attenuated the expression of Pro- and mature forms of CtL in the epididymal fat depot, but did not alter the expression of CtB in either epididymal or inguinal depot (Fig. 1D and 1F). TZD did not appear to alter the protein levels of CtL and B in the inguinal fat depot (data not shown).

Adipose tissue is composed of heterogeneous cell populations which possess distinct roles in the biological processes. Therefore, it is necessary to dissect the cell populations that are responsible for the HFD-induced changes in CtL and B gene expression in adipose tissue. To this end, we isolated adipocyte and stromal-vascular (SV) fractions from adipose tissues of HFD-fed mice and RCD-fed mice as controls and examined the mRNA levels of CtL and B in these two fractions of cells. As shown in Fig 1F and 1G, the mRNA levels of CtL and B were increased by 2 fold in epididymal adipocytes isolated from HDF mice compared to RCD mice; this stimulatory effect of HFD was much more profound leading to 15–20 fold increase in CtL and B mRNA levels in epididymal SV cells. However, HFD had no significant effects on mRNA levels of CtL and B in inguinal adipocytes and SV cells (Fig. 1F and 1G).

Regulation of NPC2 in obese animals

Certain types of cathepsins have been found to be involved in the uptake of modified LDL and intracellular trafficking of cholesterol in macrophages, an important process for foam cell formation and atherogenesis (4). However, the molecular details, for instance, what molecules are involved in the regulation of this process have not been clearly elucidated. In addition to NPC1, NPC2 (another lysosomal protein) has been recently emphasized on its role as a cholesterol trafficking protein in intracellular cholesterol homeostasis. It is reasonable to raise the question of whether cathepsins and NPC1/NPC2 coordinately regulate cholesterol metabolism and inflammation. The regulation of NPC1 and NPC2 expression in adipose tissue and macrophages in obesity and inflammation has not been previously reported. We then determined how NPC1 and NPC2 expression in adipose tissue, adipocytes, and macrophages is regulated in obesity.

We showed that the mRNA expression levels of NPC2 were much (5 fold) higher than those of NPC1 in adipocytes (Fg. 2A), suggesting that NPC2 plays a dominant role in adipocytes. We therefore focused on the regulation of NPC2 in the subsequent studies. First, we found that the mRNA levels of NPC2 were markedly upregulated in epididymal adipose tissue of ob/ob mice when compared to lean controls (Fig. 2B). Second, we found that NPC2 protein levels also appeared to be differentially regulated by HFD between the two depots (Fig. 2C and 2D) in mice. In the epididymal depot, HFD induced an increase in the protein levels of

NPC2 compared to the RCD mice; where in the inguinal depot, HFD appeared to reduce the protein levels of NPC2. TZD treatment was not able to significantly reverse HFD-induced alteration in NPC2 protein levels in two depots (Fig. 2C and 2D).

Regulation of Cathepsin B and L and NPC2 by inflammatory mediators in adipocytes and macrophages

Inflammation plays a critical role in obesity, insulin resistance, and atherogenesis. Given CtB and L and NPC2 have roles in the regulation of inflammation and atherosclerosis; it is of importance to investigate whether inflammatory mediators regulate the expression of CtB and L and NPC2. In the first set of experiments, the effect of TNF- α on CtB and L and NPC2 protein expression and secretion was examined in 3T3-L1 adipocytes. As shown in Fig. 3A, TNF-α treatment for 24h reduced the intracellular levels of NPC2 protein, but enhanced NPC2 secretion at both doses (5 and 50 ng/ml) in adipocytes. Similar effects of TNFα on intracellular and secreted CtB and CtL were also observed in adipocytes (Fig. 3A).

Obesity induces a large infiltration of inflammatory macrophages in the SV fraction of adipose tissue, and the above data have demonstrated that SV cells are mainly responsible for HFD-induced upregualtion of CtL and B in adipose tissue. Therefore, it is of interest to examine inflammatory regulation of CtB and L and NPC2 in macrophages. Raw 264.7 macrophages were used in the following experiments. For both CtB and L, only mature forms were clearly detected in cultured adipocytes and Raw 264.7 macrophages. We found that lipopolysaccharides (LPS) treatment for 24 hours increased the intracellular and secreted levels of CtL protein in Raw macrophages (Fig. 3B). As illustrated in Fig 3C, LPS treatment reduced intracellular CtB protein, while it markedly increased the secretion of CtB protein in Raw 264.7 macrophages. It has been reported that NPC2 is present in multiple isoforms with molecular mass of 18.9 kDa, 16.3 kDa, and 14.5 kDa due to differences in glycosylation (12). In non-treated macrophages, three isoforms of NPC2 were detected; 14.5 kDa is the most abundant isoform of NPC2 (Fig. 3D). LPS treatment for 24h led to a significant increase in the intracellular protein levels of 14.5 kDa and 18.9 kDa isoforms, but caused the disappearance of the16.3 kDa isoform of NPC2 protein (Fig. 3D). Interestingly, in the culture medium, only 14.5 kDa isoform was detected in both non- and LPS-stimulated conditions; LPS significantly induced the secretion of NPC2 (Fig. 3D). These results have clearly shown that cathepsin B and L and NPC2 are regulated by inflammatory inducers in both adipocytes and macrophages.

Regulation of cytokine production by CathepsinB and L in macrophages

To further investigate the roles that CtB and CtL play in mediating inflammation, exogenous inhibitors of cathepsin B and L were added to cultured cells. Selective CtB inhibitor (CBI) and L inhibitor (CLI) and an inhibitor that target both cathepsins, E64 (11), were used. To determine the role that cathepsins have in the inflammatory response, inflammatory cytokines TNF-α, MCP-1, and IL-1β were detected in the conditioned medium of Raw macrophages treated without and with LPS (1μg/ml) in the presence or absence of cathepsin inhibitors. All macrophages treated with LPS, regardless of the use of cathepsin inhibitors, had a significantly higher secretion of cytokines compared to the control groups (Fig. 4A–4C).

Out of the three cytokines chosen TNF-α secretion was the most affected by CtL and CtB inhibition, especially CtB (Fig. 4A). TNF-α secretion was significantly muted when CtB inhibitors were used (Fig. 4A). This effect is supported by Ha, et al. (2) that found CtB necessary for the secretion of TNF-α. In addition, the cathepsin inhibitors significantly decrease the secretion of LPS-induced MCP-1 (Fig. 4B). This indicates the role that CtL and CtB have in mediating the secretion of MCP-1. In addition, we found that there was a trend toward decreased secretion of IL-1β with CBI alone; however E64 showed a significant increased secretion of IL-1β as compared to LPS alone. CtL appeared to have no effect on IL-1β secretion compared to LPS alone (Fig. 4C).

Regulation of Cathepsin B and L on NPC2 in macrophages

The results obtained from obese animals and in vitro studies suggest that inflammatory mediators regulate the expression of both CtB and L and NPC2 in a similar manner. To determine if CtB and L regulate NPC2 expression in macrophages, Raw macrophages were stimulated with LPS in the presence or absence of the cathepsin inhibitors (E64, CLI and CBI). After 24h treatment with E64, CLI, and CBI, the intracellular levels of 14.5 kDa NPC2 were increased, while the secreted levels of NPC2 were reduced in LPS non-treated macrophages (Fig. 5A). In the LPS-stimulated cells, the presence of three cathepsin inhibitors, significantly attenuated LPS-stimulated NPC2 secretion, but didn't much affect the intracellular NPC2 (Fig. 5A). To further elucidate the relationship between CtB and L and NPC2, a dose-dependent response of E64 effect on NPC2 was determined. As shown in Fig. 5B, E64 increased the intracellular NPC2 of non-treated cells at the concentration of 10LM, but this effect was not observed in LPS-treated cells (Fig. 5B). However, E64 significantly reduced the secretion of NPC2 in a dose-dependent manner in both non- and LPS-treated cells (Fig. 5B).

Effect of modulating cathepsin B and L activity on cholesterol metabolic pathways in macrophages

Our results have clearly associated the expression of CtL and CtB with NPC2 protein levels. To further determine if CtL and CtB have roles in the regulation of other cholesterol metabolic pathways in macrophages, we assessed the effect of cathepsin inhibitors on the mRNA levels of key transcription factors governing multiple pathways of cholesterol metabolism under LPS-stimulated and LPS non-stimulated conditions.

Our results showed a clear trend towards decreased SREBP2 expression with 24 hour LPS treatment and it was not cathepsin dependent (Fig. 6). This suggests that cathepsins may not be critical regulators of cholesterol synthesis. The oxidized LDL receptor (LDL-R ox) is a scavenger receptor that uptakes modified LDL. There is a clear link between oxidation and inflammation in the NFκB pathway activation (15, 16). We found that 24-hour LPS stimulation led to a significant upregulation of LDL-R ox mRNA levels as compared to the control group (Fig. 6). E64, CLI and CBI were all able to significantly reduce the LPS induction of the LDL-R ox, which clearly indicates that CtL and CtB have a role in regulating the expression of LDL-R ox gene (Fig. 6).

LXR-α is a transcription factor regulating the expression of cholesterol efflux genes, such as ABCA1 and ApoE. In the non-stimulated condition, 27 hour treatment of E64 let to a significant upregulation of LXR-α gene expression, while CLI significantly downregulated LXR-α gene expression (Fig. 6); the expression of LXRα target genes ApoE and ABCA1 were significantly increased by all of the three inhibitors. LPS treatment for 24 hours significantly reduced LXR-α gene expression (Fig. 6). Treatment with any of the three cathepsin inhibitors reversed the LPS-induced reduction in LXR-α (Fig. 6). Although the expression of ApoE and ABCA1 genes was affected by the inhibition of CtB and L activity, the magnitude of the effect is very small (Fig. 6). These results suggest that CtB and L modulate cholesterol homeostasis primarily through regulating the expression of genes involved in cholesterol uptake (LDL scavenger receptor and LDL-R ox) and cholesterol cellular trafficking (LXR and NPC2). However, modulating CtB and L activity seems not significantly affect the expression of cholesterol efflux genes (ApoE and ABCA1).

Discussion

Cathepsins are lysosomal proteases that are integral in protein turnover and cellular metabolism. The expression of cathepsins is tightly regulated, and an upregulation of cathepsins is correlated with pathologies including tumor growth, inflammation, and atherosclerosis. NPC2 is another lysosomal protein which has been recently emphasized on its potential role in cholesterol trafficking. However, the regulation of cathepsins and NPC2 in adipose tissue during obesity, particularly a regulatory relationship of these two types of proteins, has not been investigated. In this study, we examined the regulation of adipose CtL, CtB, and NPC2 expression in obesity and addressed the question of whether CtB and CtL are involved in the regulation of inflammation and cholesterol metabolism with focusing on cholesterol trafficking proteins, as both conditions are associated with the development of atherosclerosis, an obesity-related pathology.

We found increased expression of CtL and CtB in the epididymal adipose tissue in both Zucker obese rats (data not shown) and HFD-induced obese mice to be congruent with our hypothesis as well as the reports from other groups (1)(17). However, the adipose depot-different regulation of CtB and L was not investigated in the previous studies. Our results showed that chronic HFD feeding leads to increased expression of both CtL and CtB selectively in the epididymal adipose depot. We also found that the upregulation of CtL in epididymal adipose tissue was mitigated by TZD, where CtB did not appear to be affected by this treatment. This indicates that CtL is downregulated by a factor in the PPAR-γ pathway; a finding that is supported by CtL being a FOXO1 target, a transcription factor that directly inhibits PPAR-γ activation (18). Our results of dissecting the cell populations in adipose tissue that are responsive to HFD clearly demonstrated that macrophage-enriched SV cells are the predominant contributors to the increased expression of CtL and CtB in the epididymal adipose depot upon HFD feeding. Interestingly, HFD feeding did not alter the gene expression of CtL and CtB in neither adipocytes nor SV cells in inguinal adipose depot. This information implies that proinflammatory macrophages are the major source of CtB and L as epididymal (visceral) adipose depot, but not inguinal depot is known to be inflamed with increased accumulation of proinflammatory macrophages during obesity (19). These data suggest that CtL and CtB selectively mediate HFD-induced pathogenesis in visceral fat

depot, which is known to be closely associated with obesity-linked metabolic complications including inflammation and atherosclerosis.

Both NPC1 and NPC2 play important roles in trafficking LDL derived cholesterol around the cell from receptor mediated endocytosis into the endosomal system and maintaining cellular cholesterol homeostasis (20). NPC2 has been shown to be a ubiquitous and highly conserved secreted protein; our data showed that NPC2 is expressed at a higher level than NPC1 in macrophages. However, the expression and regulation of NPC2 in adipose tissue and its function in obesity is not clear. A previous study provided some evidence that NPC2 is important for the differentiation of adipocytes from skin fibroblasts (10). In this study, we showed that the NPC2 protein expression in adipose tissue is dysregulated in *ob/ob* and diet-induced obese (DIO) mice, and this dysregulation is depot-different. The influence of HFD on NPC2 protein expression in the different depots was opposite. In the inguinal adipose depot, HFD caused a decrease in NPC2 expression; where in the epididymal adipose depot, HFD led to an increase in NPC2 expression. The depot difference in the regulation of CtB and L expression suggests that different fat depots exert differential functions in inflammation and cholesterol metabolism. Previous studies have suggested that there is a relationship between inflammation and cathepsin expression and secretion. For example, there is evidence that TNF-α lead to increased secretion of CtL in adipose derived mesenchymal stem cells (21) and CtL expression is upregulated by LPS in dendritic cells (22). Consistently, our previous studies using quantitative proteomics technique showed that the administration of TZD to Zucker obese rats led to a reduction in the secretion of several cathepsins including CtB, D, G, H, and M from epididymal adipose tissue (23). Herein, we found that TNFα stimulates the secretion of CtL, CtB, and NPC2 proteins in 3T3-L1 adipocytes. Similarly, in Raw264.7 macrophages, LPS regulates the secretion of CtB, CtL, and NPC2, leading to the increased intracellular protein levels of CtL and NPC2 and increased secretion of CtB, CtL, and NPC2 into the culture medium. Since NPC2 expression has been correlated with adipogenesis and the development of insulin resistance (10), our findings that HFD induces the upregulation of NPC2 protein expression in adipose tissues and increased expression and secretion by inflammatory activation in macrophages and adipocytes suggest that NPC2 is involved in the inflammatory response in the obese state. The above results together suggest that CtL, CtB, and NPC2 expression can be modulated by inflammation in macrophages.

NPC2 is known to be involved in cholesterol metabolism. Both NPC2 and cathepsins are all primarily lysosomal proteins (4, 24); we showed that their expression levels are both modulated by inflammatory mediators LPS and TNFα. Therefore, we speculated that cathepsins and NPC2 may be functionally interregulated in inflammation and cholesterol metabolism. To test this relationship, we used cathepsin inhibitors to determine if blocking CtB and L activity would modulate NPC2 expression and secretion in macrophages. We were the first to demonstrate that LPS causes an increase in the intracellular concentration and secretion of NPC2 protein in macrophages. However, blocking CtB and L activity resulted in a differential consequence. For example, CtB and L inhibitors increased intracellular NPC2, but significantly reduced the secretion of NPC2 in the basal state (Fig. 3D). In the presence of LPS, CtB and L inhibitors also significantly attenuated LPS-stimulated NPC2 secretion, but only led to a slight increase in intracellular NPC2 (Fig. 5B).

These results clearly demonstrate that cathepsin inhibitors consistently block NPC2 secretion regardless of LPS presence, suggesting that CtB and L regulate NPC2 secretion. Additionally, our results showed that the magnitude of intracellular NPC2 increase induced by CtB and L inhibitors was much less than that induced by LPS. We also found that cathepsin inhibitors did not significantly change NPC2 mRNA expression, while LPS reduced NPC2 mRNA expression (data not shown). Thus, it is reasonable to speculate that CtB and L inhibition-caused intracellular NPC2 increase is primarily due to the block of NPC2 secretion. However, LPS-induced NPC2 increase may be attributive to the combined effect of stimulating NPC2 secretion via a cathepsin-dependent pathway and inhibiting NPC2 degradation via a cathepsin-independent pathway which cannot be completely overcome by inhibiting cathepsin activity (Fig. 7). Decreased NPC2 mRNA expression by LPS could be a negative feedback response to increased intracellular NPC2 protein levels. Therefore, we conclude that cathepsin activation pathway may be involved in the regulation of NPC2 activity primarily via modulating inflammation-induced NPC2 secretion.

The inhibition of CtB and L activity was able to significantly decrease the secretion of NPC2 in both the basal and LPS treated cells. However, the effects of cathepsin inhibitors on the gene expression of NPC2 and NPC1 were minimal (data not shown), supporting the speculation that CtL and CtB may primarily regulate the trafficking rather than the gene expression of NPC2 in macrophages. To provide more evidence supporting the conclusion that CtB and L are involved in the regulation of cholesterol trafficking, we examined the expression of transcription factors that control cholesterol metabolic homeostasis including SREBP2, a transcription factor that controls genes involved in biosynthesis of cholesterol, LXR-α, a transcription factor involved in cholesterol efflux, and oxLDL receptor, a scavenger receptor responsible for the uptake of oxidized LDL. LPS has also been shown to reduce reverse cholesterol transport (25) via the mechanisms involving a down-regulation of ABCA1 (26) and ApoE (27). Our results suggest that modulating the activity of CtB and CtL individually or together is able to largely or partially reverse LPS-induced changes in the expression of genes in cholesterol trafficking pathway, but not cholesterol biosynthesis, supporting the importance that Ct B and L play in regulating the expression of genes involved in cholesterol uptake and cellular trafficking.

In summary, we demonstrate that CtB and L are upregulated in obesity, especially in the visceral depot and SV fraction. NPC2 was up regulated in visceral obese adipose tissue. In macrophages, the inflammatory stimulation leads to an increase in intracellular protein levels and secretion CtL and NPC2 secretion. Inhibition of CtL and CtB enzymatic activity is shown to reduce NPC2 secretion and increase the expression of genes involved cholesterol trafficking. We conclude that CtB and L are important in cholesterol homeostasis through regulating the gene expression of LXR, oxLDL-R, ABCA1, ApoE, and modulating NPC2 secretion, however the mechanism of regulation needs to be further investigated.

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References

1. Yang M, Zhang Y, Pan J, et al. Cathepsin L activity controls adipogenesis and glucose tolerance. Nat Cell Biol. 2007; 9:970–977. [PubMed: 17643114]
2. Ha SD, Martins A, Khazaie K, et al. Cathepsin B is involved in the trafficking of TNF-alpha-containing vesicles to the plasma membrane in macrophages. J Immunol. 2008; 181:690–697. [PubMed: 18566436]
3. Li W, Kornmark L, Jonasson L, et al. Cathepsin L is significantly associated with apoptosis and plaque destabilization in human atherosclerosis. Atherosclerosis. 2009; 202:92–102. [PubMed: 18495127]
4. Lutgens SP, Cleutjens KB, Daemen MJ, et al. Cathepsin cysteine proteases in cardiovascular disease. FASEB J. 2007; 21:3029–3041. [PubMed: 17522380]
5. Haidar B, Kiss RS, Sarov-Blat L, et al. Cathepsin D, a lysosomal protease, regulates ABCA1-mediated lipid efflux. J Biol Chem. 2006; 281:39971–39981. [PubMed: 17032648]
6. Munkacsi AB, Pentchev PG, Sturley SL. Spreading the wealth: Niemann-Pick type C proteins bind and transport cholesterol. Cell Metab. 2009; 10:3–4. [PubMed: 19583946]
7. Goldman SD, Krise JP. Niemann-Pick C1 functions independently of Niemann-Pick C2 in the initial stage of retrograde transport of membrane-impermeable lysosomal cargo. J Biol Chem. 2010; 285:4983–4994. [PubMed: 20007703]
8. Infante RE, Wang ML, Radhakrishnan A, et al. NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes. Proc Natl Acad Sci U S A. 2008; 105:15287–15292. [PubMed: 18772377]
9. Peake KB, Vance JE. Defective cholesterol trafficking in Niemann-Pick C-deficient cells. FEBS Lett. 2010; 584:2731–2739. [PubMed: 20416299]
10. Csepeggi C, Jiang M, Kojima F, et al. Somatic cell plasticity and Niemann-Pick type C2 protein: fibroblast activation. J Biol Chem. 2011; 286:2078–2087. [PubMed: 21084287]
11. Rimkunas VM, Graham MJ, Crooke RM, et al. TNF-{alpha} plays a role in hepatocyte apoptosis in Niemann-Pick type C liver disease. J Lipid Res. 2009; 50:327–333. [PubMed: 18815434]
12. Chen X, Al-Hasani H, Olausson T, Wenthzel AM, Smith U, Cushman SW. Activity, phosphorylation state and subcellular distribution of GLUT4-targeted Akt2 in rat adipose cells. J Cell Sci. 2003; 116(Pt 17):3511–3518. [PubMed: 12876218]
13. Zhang Y, Chen X. Reducing selenoprotein P expression suppresses adipocyte differentiation as a result of increased preadipocyte inflammation. Am J Physiol Endocrinol Metab. 2011; 300:E77–E85. [PubMed: 20959537]
14. Zhang J, Wu Y, Zhang Y, et al. The role of lipocalin 2 in the regulation of inflammation in adipocytes and macrophages. Mol Endocrinol. 2008; 22:1416–1426. [PubMed: 18292240]
15. Miller DK, Calaycay JR, Chapman KT, et al. The IL-1 beta converting enzyme as a therapeutic target. Ann N Y Acad Sci. 1993; 696:133–148. [PubMed: 8109824]
16. Van Guilder GP, Hoetzer GL, Dengel DR, et al. Impaired endothelium-dependent vasodilation in normotensive and normoglycemic obese adult humans. J Cardiovasc Pharmacol. 2006; 47:310–313. [PubMed: 16495771]
17. Nadler ST, Stoehr JP, Schueler KL, et al. The expression of adipogenic genes is decreased in obesity and diabetes mellitus. Proc Natl Acad Sci U S A. 2000; 97:11371–11376. [PubMed: 11027337]
18. Yamazaki Y, Kamei Y, Sugita S, et al. The cathepsin L gene is a direct target of FOXO1 in skeletal muscle. Biochem J. 2010; 427:171–178. [PubMed: 20088826]
19. Wellen KE, Fucho R, Gregor MF, Furuhashi M, Morgan C, Lindstad T, Vaillancourt E, Gorgun CZ, Saatcioglu F, Hotamisligil GS. Coordinated regulation of nutrient and inflammatory responses by STAMP2 is essential for metabolic homeostasis. Cell. 2007; 129:537–548. [PubMed: 17482547]
20. Ikonen E. Cellular cholesterol trafficking and compartmentalization. Nat Rev Mol Cell Biol. 2008; 9:125–138. [PubMed: 18216769]

21. Lee MJ, Kim J, Kim MY, et al. Proteomic analysis of tumor necrosis factor-alpha-induced secretome of human adipose tissue-derived mesenchymal stem cells. J Proteome Res. 2010; 9:1754–1762. [PubMed: 20184379]

22. Menzel K, Hausmann M, Obermeier F, et al. Cathepsins B, L and D in inflammatory bowel disease macrophages and potential therapeutic effects of cathepsin inhibition in vivo. Clin Exp Immunol. 2006; 146:169–180. [PubMed: 16968411]

23. Chen X, Hunt D, Cushman SW, et al. Proteomic characterization of thiazolidinedione regulation of obese adipose secretome in Zucker obese rats. Proteomics Clin Appl. 2009; 3:1099–1111. [PubMed: 21137009]

24. Naureckiene S, Sleat DE, Lackland H, et al. Identification of HE1 as the second gene of Niemann-Pick C disease. Science. 2000; 290:2298–2301. [PubMed: 11125141]

25. McGillicuddy FC, de la Llera Moya M, Hinkle CC, et al. Inflammation impairs reverse cholesterol transport in vivo. Circulation. 2009; 119:1135–1145. [PubMed: 19221221]

26. Baranova I, Vishnyakova T, Bocharov A, et al. Lipopolysaccharide down regulates both scavenger receptor B1 and ATP binding cassette transporter A1 in RAW cells. Infect Immun. 2002; 70:2995–3003. [PubMed: 12010990]

27. Gafencu AV, Robciuc MR, Fuior E, et al. Inflammatory signaling pathways regulating ApoE gene expression in macrophages. J Biol Chem. 2007; 282:21776–21785. [PubMed: 17553793]

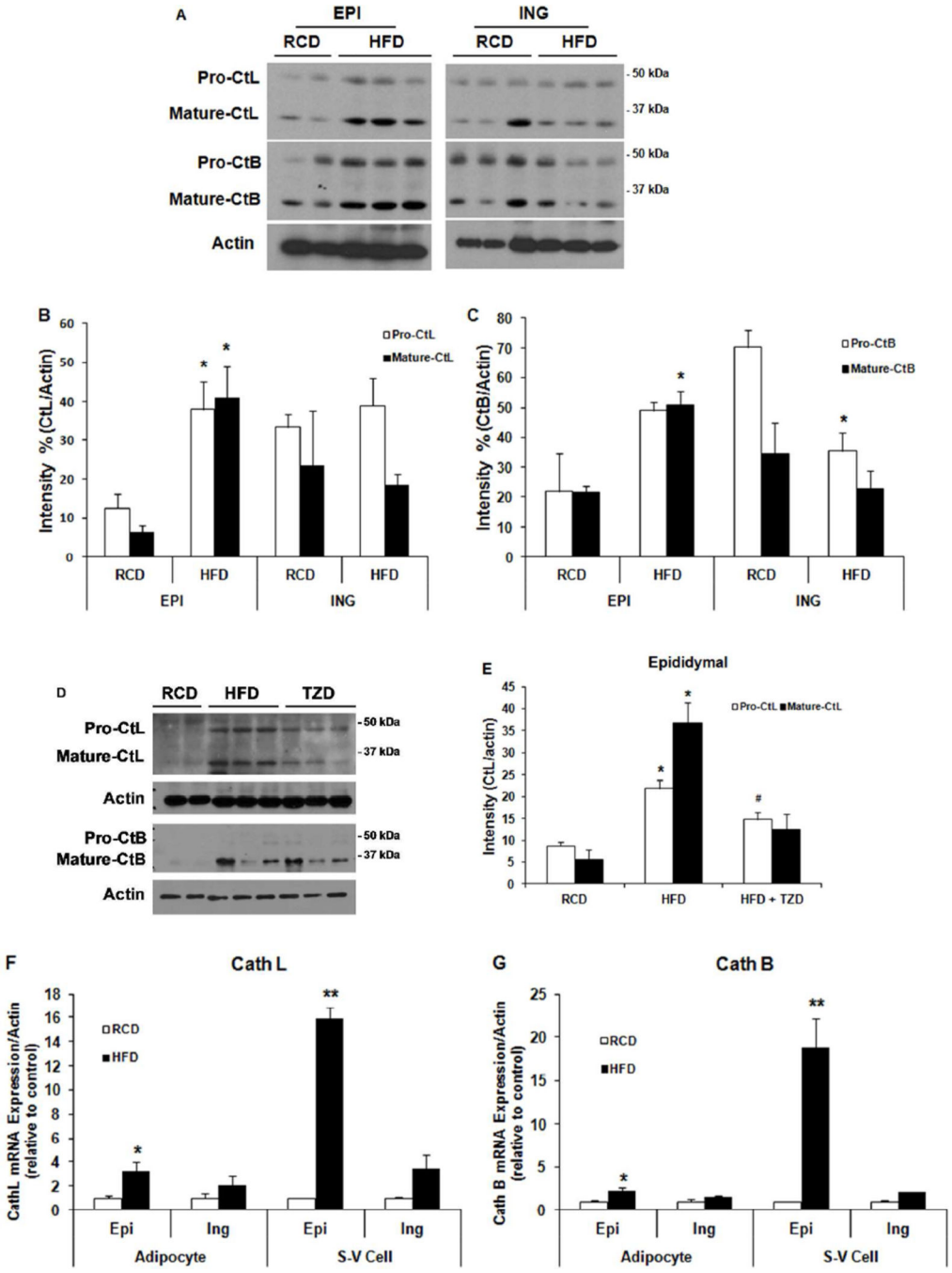

Figure 1.

Regulation of Cathepsin L and B in adipose tissue of HFD-induced obese mice. (A, B, and C) the protein expression and quantification of CtL and CtB in epididymal and inguinal adipose tissue of mice fed a RCD or HFD. (D and E) the effect of Rosi administration on CtL and CtB protein expression and quantification in epididymal adipose tissue of HFD-fed mice. Each lane represents the result from individual mouse. (F and G) mRNA expression of CtL and CtB in primary adipocytes and SV cells from 4–5 RCD- or HFD-fed mice. The data in the graphs represent Mean ± SE.

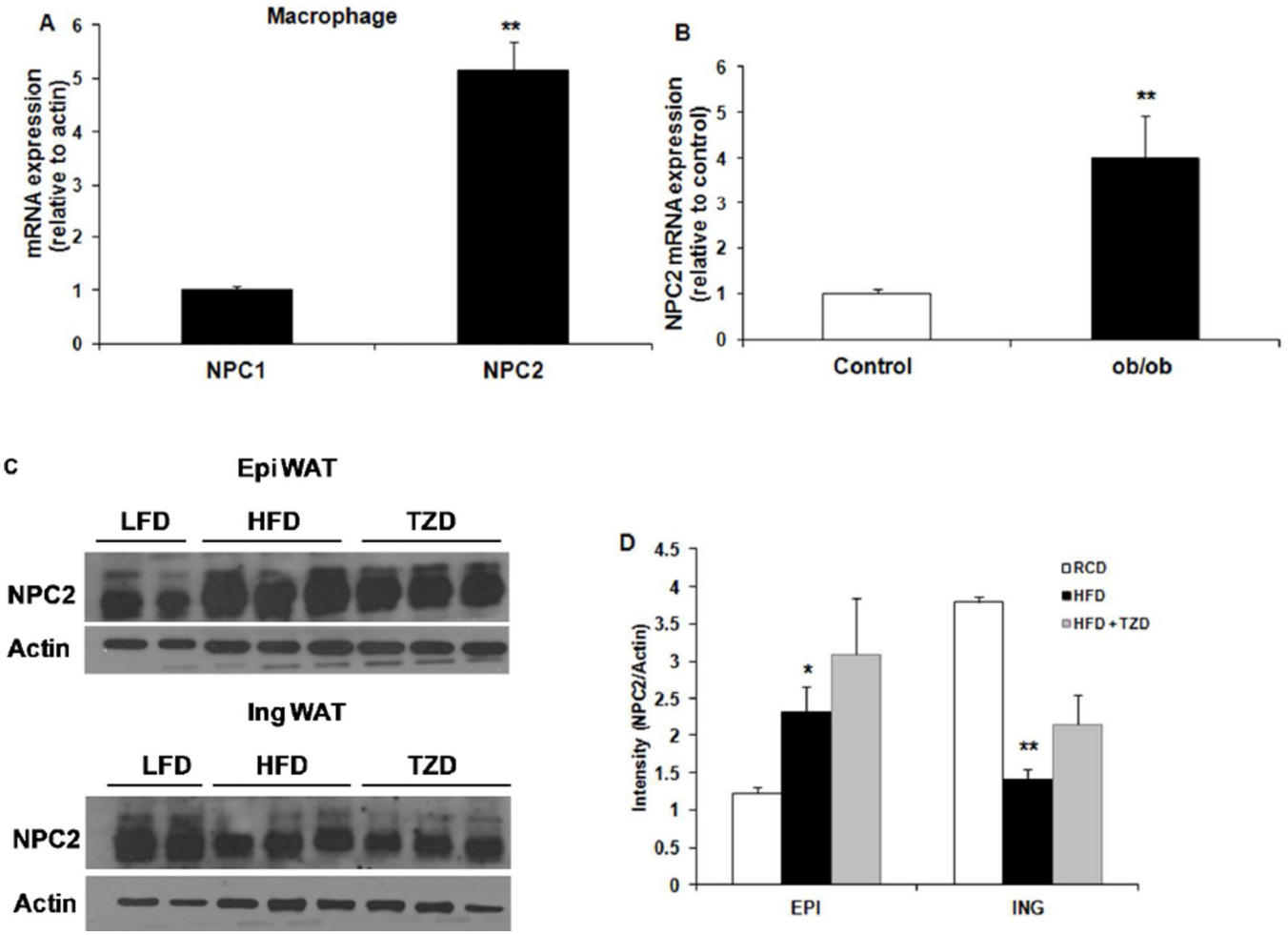

Figure 2.

Regulation of NPC2 in adipose tissue of ob/ob and HFD-induced obese mice. (A) NPC1 and NPC2 mRNA expression in primary adipocytes isolated from epididymal adipose depot of normal mice on RCD (n=4). (B) NPC2 mRNA expression in epididymal adipose tissue of lean control and ob/ob mice (n=6–8). (C and D) the protein expression and quantification of NPC2 and the effect of TZD administration on NPC2 expression in epididymal and inguinal adipose tissue in HFD mice. The data in the graphs represent Mean ± SE.

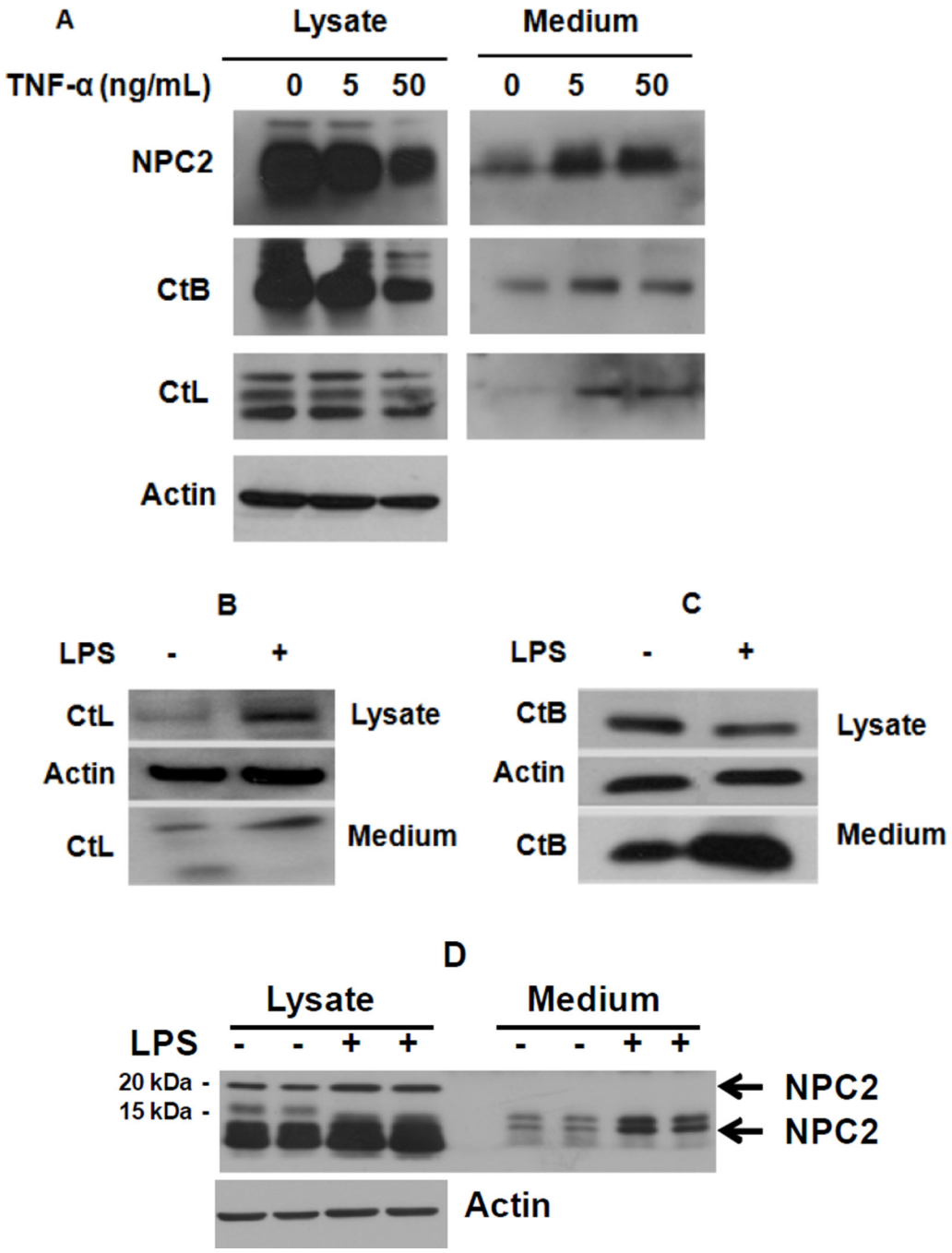

Figure 3.

Regulation of Cathepsin L and B and NPC2 by inflammatory mediators in adipocytes and macrophages. (A) Regulation of intracellular expression and secretion of Cathepsin L and B and NPC2 by TNFα in 3T3-L1 adipocytes. (B and C) Regulation of intracellular expression and secretion of Cathepsin L and B by LPS in Raw 264.7 macrophages. (D) Regulation of intracellular expression and secretion of NPC2 by LPS in Raw 264.7 macrophages.

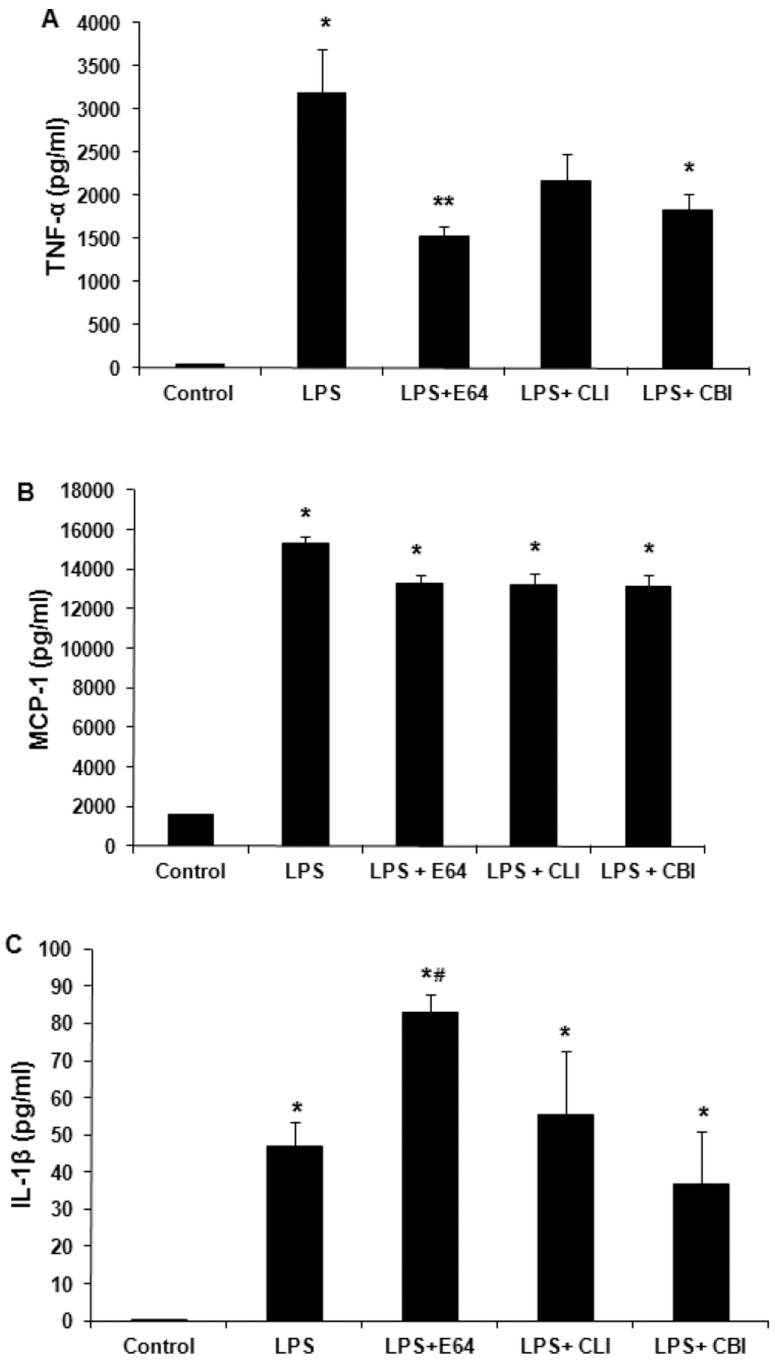

Figure 4.

The effect of Cathepsin activity on cytokine production in macrophages. (A) & (B) the effect of the inhibition of CtL and CtB enzymatic activity on TNFα and MCP-1 secretion in Raw 264.7 macrophages. The results represent mean ± SE of three independent experiments. * $P < 0.05$ vs control group; # $P < 0.05$ vs LPS-treated group.

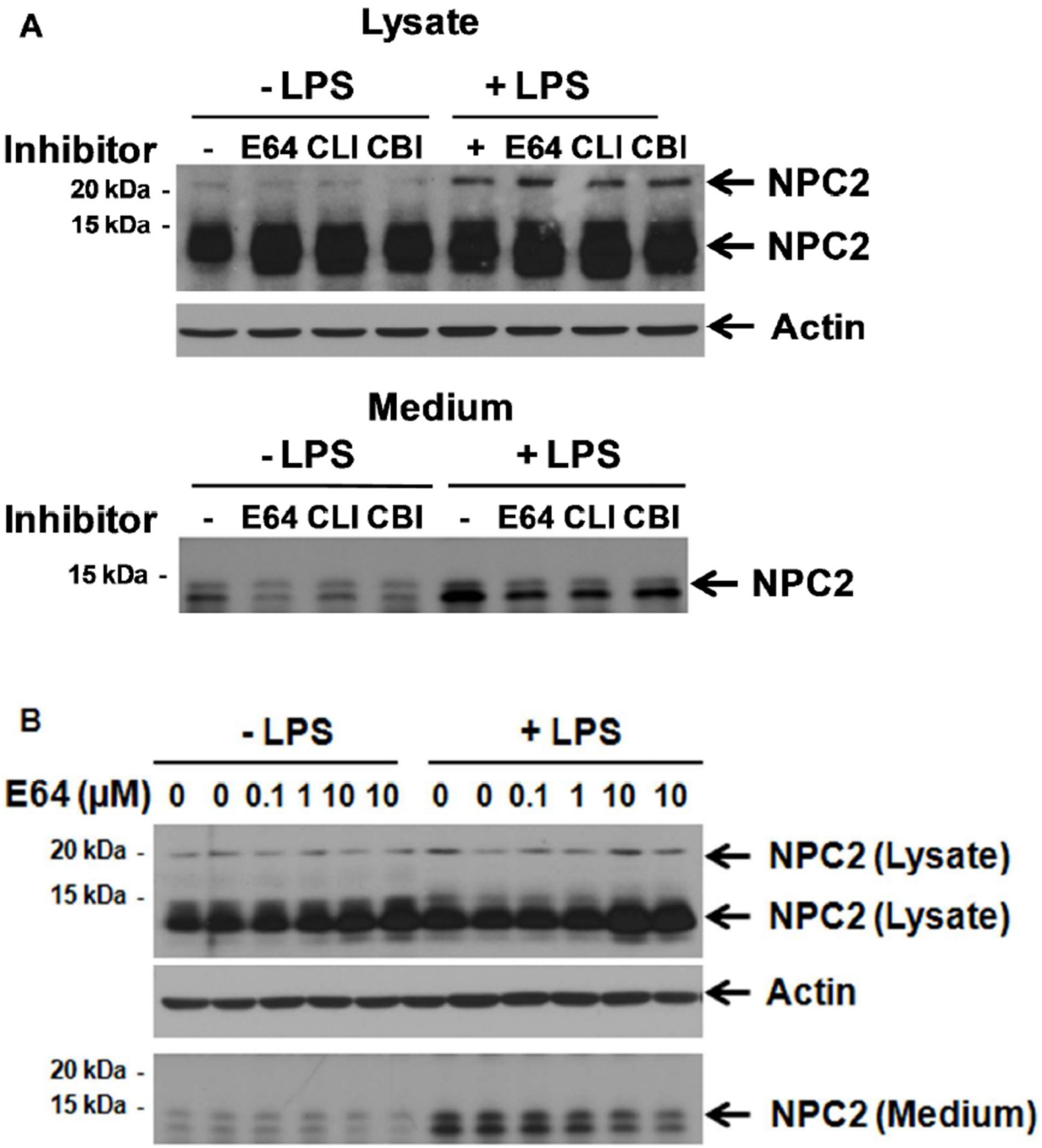

Figure 5.

The effect of cathepsin inhibition on NPC2 protein expression and secretion in macrophages. (A) the effects of CtL and CtB inhibitors on intracellular and secreted NPC2 protein in non-treated and LPS-treated conditions. (B) the dose-dependent effect of E64 on intracellular and secreted NPC2 protein in non-treated and LPS-treated conditions.

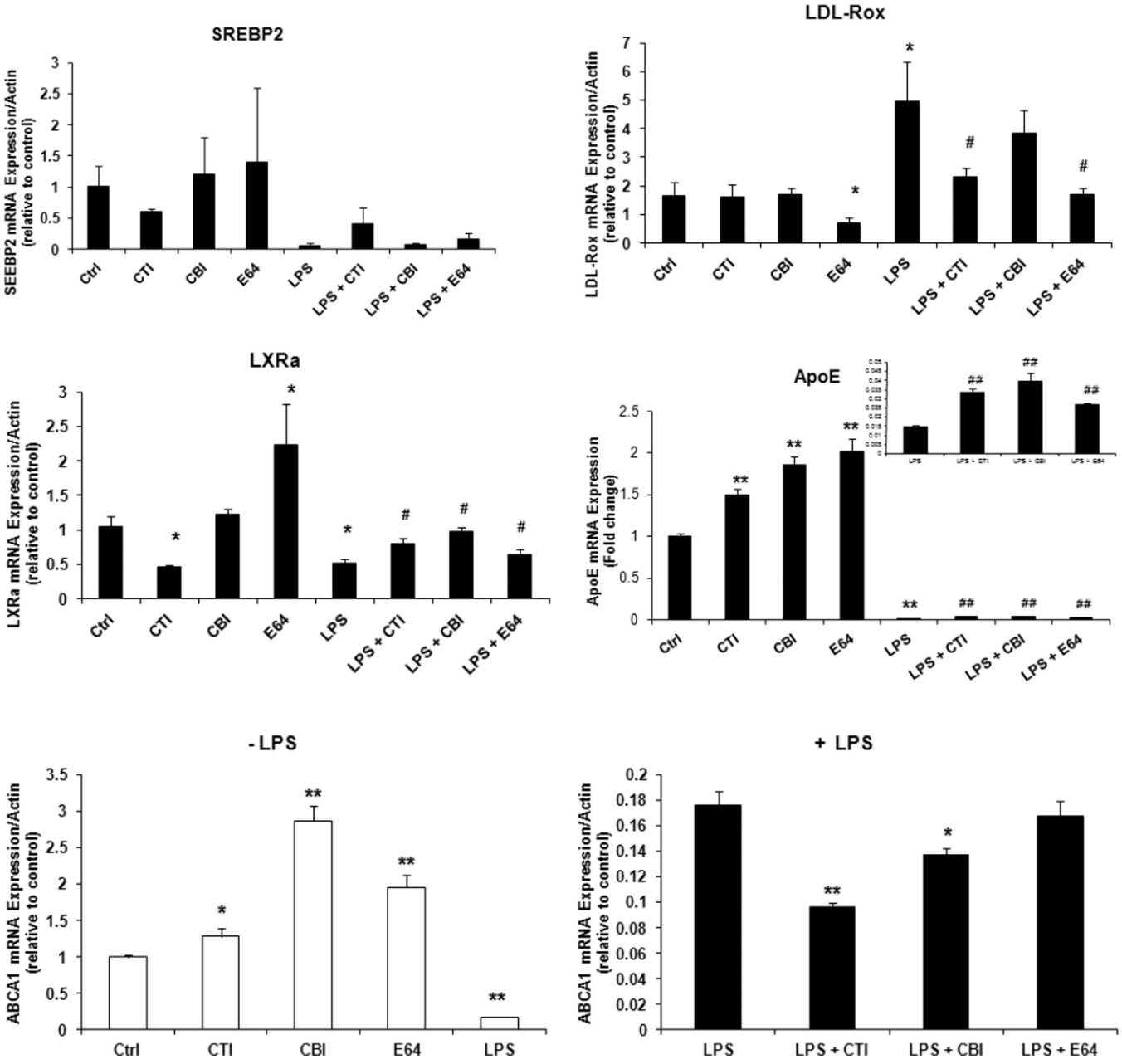

Figure 6.

The effect of Cathepsin activity on the mRNA expression of the genes involved in cholesterol metabolism in macrophages. The results represent mean ± SE of two independent experiments. * $P < 0.05$ vs control group; # $P < 0.05$ vs LPS-treated group.

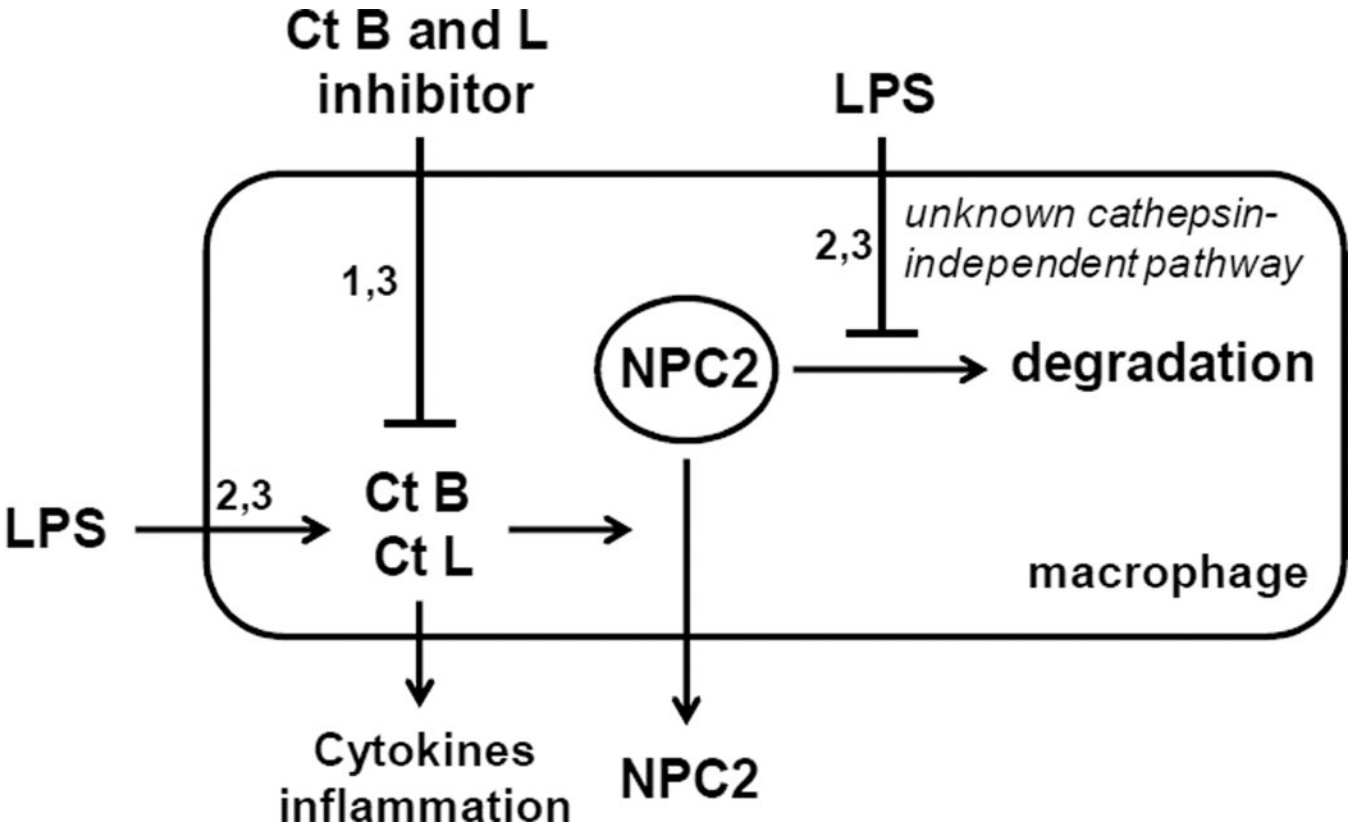

Figure 7.

Hypothetical model for the role of CtB and L in mediating LPS regulation of intracellular levels and secretion of NPC2 protein in macrophages. 1) Cathepsin inhibitors reduce NPC2 secretion and enhance intracellular NPC2; 2) LPS alone stimulates Ct B and L activity and inhibits NPC2 degradation via a cathepsin-independent pathway, leading to an increase in NPC2 secretion as well as intracellular NPC2 accumulation; 3) Cathpsin inhibitors block LPS-stimulated CtB and L activity leading to a decrease in NPC2 secretion, but a further increase in intracellular NPC2.