Involvement of Cathepsins B and L in Inflammation and Cholesterol Trafficking Protein NPC2 Secretion in Macrophages

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Objective: 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.

Design and Methods: Cathepsins and NPC2 mRNA expression and protein secretion were detected in obese adipose tissue as well as 3T3-L1 adipocytes and Raw 264.7 macrophages in response to inflammatory stimuli and cathepsin inhibitors.

Results: It was found that high-fat diet feeding altered the mRNA and protein expression levels of cathepsins B and L (CtB and CtL) 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 Raw 264.7 macrophages. Using the inhibitors of cathepsin enzymatic activity, it was found that CtB and CtL regulate TNF-α production, the expression and secretion of NPC2 protein, and the mRNA levels of the genes involved in cholesterol trafficking in macrophages.

Conclusion: These findings suggest that CtB and CtL 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 that leads to the development of comorbidities such as atherosclerosis and heart disease. Although 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 roles to maintain normal cellular and physiological functions. Because of 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 lysosomes (6). The function of NPC1 and NPC2 in the cholesterol trafficking has

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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 proinflammatory 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 (HFD) 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 CtL regulated cytokine production, the expression of NPC2, and the genes involved in other cholesterol metabolic pathways in macrophages. Our findings indicate that CtB and CtL 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 300 g and the obese rats weighed 500-600 g 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 three classes of proteins together.

Isolation of primary stromal vascular cells and adipocytes
Primary stromal vascular (SV) and adipocytes were prepared by collagenase digestion as described previously (12). Adipose tissue from epididymal fat pad of male mice on RCD was placed in digestion vials containing KRBH buffer [Kreb's-Ringer buffered with HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid)] plus 0.1% albumin, minced, and digested with collagenase to 20 mg/mL. After 2-h digestion, SV cells and adipocytes were separated by centrifugation at 1200 rpm for 10 min. The isolated adipose cells were washed with KRHB 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 and Biophysics at the University of Minnesota) were cultured in six-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% FBS (JRH Biosciences, 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, Minneapolis, MN) for 24 h. At the end of the experiments, cells were prepared for protein collection using RIPA buffer (Sigma-Aldrich) for Western blotting analysis. Culture medium was collected and concentrated by a Macrostep centrifugal device with molecular weight cutoff of 1 kDa (Pall Life Sciences, MI).

Raw 264.7 macrophages (kindly provided by Dr. David Bernlohr) were cultured in six-well plates using DMEM and 15% FBS. Cells were grown to ~70% confluence, followed by various treatments including LPS and LPS with cathepsin inhibitors. Lipopolysaccharide (LPS; Sigma-Aldrich) was used at the concentration of 1 μg/mL for 24 h. Cathepsin inhibitors were applied at concentrations of 10 μM per well 3 h prior to LPS treatment. The following inhibitors were used: CtL inhibitor (CLI; NapSul-Ile-Trp-CHO; Enzo Life Sciences, Farmingdale, NY), CtB inhibitor (CBL, CA-074Me; Enzo Life Sciences), 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 Radio-Immunoprecipitation Assay (RIPA) buffer with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The concentration was determined using Pierce bicinchoninic acid method (Pierce Chemical, Rockford, IL). 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), CtB (R&D Systems), and 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 Health Care 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 reincubated 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) and protocol. RNA was purified using RQ treatment (Promega, Madison, WI), following their protocol. cDNA was synthesized using reverse transcript treatment (Promega). Real-time qPCR was done using SYBR green SuperMix Universal kit (Invitrogen) and an ABI StepOnePlus real-time PCR Systems (Applied Biosystems, Foster City, CA). The 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 two-tailed Student’s t-test.

**Statistical analysis**

The results were expressed as mean ± 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 of <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 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 (Figure 1A,B). HFD feeding selectively increased the protein levels of mature CtL in epididymal fat depot, but not able to significantly reverse HFD-induced alteration in NPC2 protein levels in the two depots (Figure 2C,D).

**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 atherosclerosis (4). However, the molecular details, for instance, what molecules are involved in the regulation of this process, have not been clearly elucidated. We then determined how NPC1 and NPC2 expression in adipose tissue and macrophages in obesity and inflammation has not been previously reported. 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 with lean controls (Figure 2B). Second, we found that NPC2 protein levels also appeared to be differentially regulated by HFD between the two depots (Figure 2C,D) in mice. In the epididymal depot, HFD induced an increase in the protein levels of NPC2 when compared with the RCD mice, whereas 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 the two depots (Figure 2C,D).

**Regulation of CtB and CtL and NPC2 by inflammatory mediators in adipocytes and macrophages**

Inflammation plays a critical role in obesity, insulin resistance, and atherogenesis. Given that CtB and CtL 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 CtL and NPC2. In the first set of experiments, the effect of TNF-α on CtB and CtL and NPC2 protein expression and secretion was examined in 3T3-L1 adipocytes. As shown in Figure 3A, TNF-α treatment for 24 h reduced the intracellular levels of NPC2 protein, but enhanced NPC2 secretion at both doses (5 and 50 ng/ml) in adipocytes. Similar effects of TNF-α on intracellular and secreted CtB and CtL were also observed in adipocytes (Figure 3A).

Obesity induces a large infiltration of inflammatory macrophages in the SV fraction of adipose tissue, and the above data have
FIGURE 1 Regulation of cathepsin L and B in adipose tissue of HFD-induced obese mice. A–C: The protein expression and quantification of CtL and CtB in epididymal and inguinal adipose tissue of mice fed with RCD or HFD. D and E: The effect of TZD 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.
demonstrated that SV cells are mainly responsible for HFD-induced upregulation of CtL and CtB in adipose tissue. Therefore, it is of interest to examine the inflammatory regulation of CtB and CtL and NPC2 in macrophages. Raw 264.7 macrophages were used in the following experiments. For both CtB and CtL, only mature forms were clearly detected in cultured adipocytes and Raw 264.7 macrophages. We found that LPS treatment for 24 h increased the intracellular and secreted levels of CtL protein in Raw macrophages (Figure 3B). As illustrated in Figure 3C, LPS treatment reduced intracellular CtB protein, whereas 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, 16.3, and 14.5 kDa due to differences in glycosylation (12). In nontreated macrophages, three isoforms of NPC2 were detected; 14.5 kDa is the most abundant isoform of NPC2 (Figure 3D). LPS treatment for 24 h led to a significant increase in the intracellular protein levels of 14.5 and 18.9 kDa isoforms, but caused the disappearance of the 16.3-kDa isoform of NPC2 protein (Figure 3D). Interestingly, in the culture medium, only the 14.5-kDa isoform was detected in both nonstimulated and LPS-stimulated conditions; LPS significantly induced the secretion of NPC2 (Figure 3D). These results have clearly shown that CtB and CtL and NPC2 are regulated by inflammatory inducers in both adipocytes and macrophages.

Regulation of cytokine production by CtB and CtL in macrophages

To further investigate the roles that CtB and CtL play in mediating inflammation, exogenous inhibitors of CtB and CtL were added to cultured cells. Selective CBI and 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 (1 μg/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 when compared with the control groups (Figure 4A-C).

Of the three cytokines chosen, TNF-α secretion was the most affected by CtL and CtB inhibition, especially CtB (Figure 4A). TNF-α secretion was significantly muted when CBIs were used (Figure 4A). This effect is supported by Ha et al. (2) who found that CtB is necessary for the secretion of TNF-α. In addition, the cathepsin inhibitors significantly decrease the secretion of LPS-induced MCP-1 (Figure 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 when compared with LPS alone. Ctl appeared to have no effect on IL-1β secretion when compared with LPS alone (Figure 4C).

Regulation of CtB andCtl 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 Ctl and NPC2 in a similar manner. To determine if CtB and Ctl regulate NPC2 expression in macrophages, Raw macrophages were stimulated with LPS in the presence or absence of the cathepsin inhibitors (E64, Cli, and Cbl). After 24-h treatment with E64, Cli, and Cbl, the intracellular levels of 14.5-kDa NPC2 were increased, whereas the secreted levels of NPC2 were reduced in LPS non-treated macrophages (Figure 5A). In the LPS-stimulated cells, the presence of three cathepsin inhibitors significantly attenuated LPS-stimulated NPC2 secretion, but did not much affect the intracellular NPC2 (Figure 5A). To further elucidate the relationship between CtB and Ctl and NPC2, a dose-dependent response of E64 effect on NPC2 was determined. As shown in Figure 5B, E64 increased the intracellular NPC2 of nontreated cells at the concentration of 10 μM; however, this effect was not observed in LPS-treated cells (Figure 5B).

Effect of modulating CtB and Ctl 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 toward decreased SREBP2 expression with 24-h LPS treatment and it was not cathepsin dependent (Figure 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 the 24-h LPS stimulation led to a significant upregulation of LDL-R ox mRNA levels when compared with the control group (Figure 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 (Figure 6).

LXR-α is a transcription factor regulating the expression of cholesterol efflux genes, such as ABCAI and ApoE. In the nonstimulated condition, 27-h treatment of E64 led to a significant upregulation of LXR-α gene expression, whereas CLI significantly downregulated LXR-α gene expression (Figure 6); the expression of LXRα target genes ApoE and ABCAI were significantly increased by all of the three inhibitors. LPS treatment for 24 h significantly reduced LXR-α gene expression (Figure 6). Treatment with any of the three cathepsin inhibitors reversed the LPS-induced reduction in LXR-α (Figure 6). Although the expression of ApoE and ABCAI genes was affected by the inhibition of CtB and CtL activity, the magnitude of the effect is very small (Figure 6). These results suggest that CtB and CtL 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 CtL activity seems to not significantly affect the expression of cholesterol efflux genes (ApoE and ABCAI).

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 that 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 CtL 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 peroxisome proliferator-activated receptor gamma (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 on 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 CtL and CtB as epididymal (visceral) adipose depot; however, inguinal depot is not 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 are 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 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, whereas in the epididymal adipose depot, HFD led to an increase in NPC2 expression. The depot difference in the regulation of CtB and CtL 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 stimulates the secretion of CtL, CtB, and NPC2 proteins in 3T3-L1 adipocytes. Similarly, in Raw 264.7 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. As 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.

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.
NPC2 is known to be involved in cholesterol metabolism. Both NPC2 and cathepsins are all primarily lysosomal proteins (4,24), and we showed that their expression levels are both modulated by inflammatory mediators LPS and TNF-α. 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 CtL 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 CtL activity resulted in a differential consequence. For example, CtB and CtL inhibitors increased intracellular NPC2, but significantly reduced the secretion of NPC2 in the basal state (Figure 3D). In the presence of LPS, CtB and CtL inhibitors also significantly attenuated LPS-stimulated NPC2 secretion, but only led to a slight increase in intracellular NPC2 (Figure 5B). These results clearly demonstrate that cathepsin inhibitors consistently block NPC2 secretion regardless of LPS presence, suggesting that CtB and CtL regulate NPC2 secretion. Additionally, our results showed that the magnitude of intracellular NPC2 increase induced by CtB and CtL inhibitors was much less than that induced by LPS. We also found that cathepsin inhibitors did not significantly change NPC2 mRNA expression, whereas LPS reduced NPC2 mRNA expression (data not shown). Thus, it is reasonable to speculate that CtB and CtL inhibition-induced intracellular NPC2 increase is primarily due to the block of NPC2 secretion. However, LPS-induced NPC2 secretion may be attributable to the combined effect of stimulating NPC2 secretion via a cathepsin-dependent pathway and inhibiting NPC2 degradation via a cathepsin-independent pathway that cannot be completely overcome by inhibiting cathepsin activity (Figure 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 CtL 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 CtL 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 LDL-R ox, 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 downregulation 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 CtB and CtL play in regulating the expression of genes involved in cholesterol uptake and cellular trafficking.

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

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