

Lysosomal Cathepsin D contributes to cell death during adipocyte hypertrophy

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Abbreviations: AT, adipose tissue; EAT, epididymal adipose tissue; SVF, stromal vascular fraction; CTSD, Cathepsin D; CTSB, Cathepsin B; ROS, reactive oxygen species

Obesity has reached epidemic proportions in most of the western world. With obesity comes a variety of adverse health effects such as insulin resistance, dyslipidemia, hypertension, glucose intolerance, and hepatic steatosis. It has become clear that a state of low grade chronic inflammation, typically associated with obesity, and characterized by macrophage infiltration of adipose tissue (AT) and increased production of pro-inflammatory cytokines, plays a crucial role in the development of insulin resistance. The pathogenic mechanisms resulting in AT macrophage recruitment are under intense investigation and remain incompletely understood. We recently demonstrated that lysosomal permeabilization, and subsequent Cathepsin B (CTSB) activation, occurs at the early stages of high fat diet induced weight gain and is preceded by macrophage infiltration into hypertrophied AT resulting in adipocyte cell death through mitochondrial dysfunction. In this report, we demonstrated that another key Cathepsin, Cathepsin D (CTSD), is also activated at the early stages of weight gain. In addition, activated CTSD induced proapoptotic protein activation. In conclusion, our data identify lysosomal CTSD as a potential key mediator of adipocyte cell death during weight gain and obesity.

Introduction

Obesity rates have been growing dramatically in most of the world. Obesity has become a serious public health problem as it is closely associated with a number of adverse health outcomes—including the development of a cluster of disorders grouped into the so-called metabolic syndrome.^{1–6} Insulin resistance is the common denominator in metabolic syndrome, and a state of low grade chronic inflammation—characterized by macrophage infiltration into AT with production of pro-inflammatory cytokines such as TNF- α and IL-6—has been identified as a central mechanism in the development of insulin resistance.^{7–9} Recently, increased adipocyte cell death has been proposed as an initial event that contributes to macrophage infiltration.¹⁰ The infiltrating macrophages in the AT are typically found surrounding a single dead adipocyte forming crown-like structures in both obese mice and obese humans.^{11–13} However, very limited data are currently available regarding the biochemical pathway(s) that may initiate adipocyte cell death and subsequent macrophage infiltration during weight gain. We recently focused on the potential role of the lysosomal pathway of cell death in these events. Accumulating evidence suggests a central role of lysosomes in both necrotic and apoptotic cell death, and in both circumstances it appears to be an early event that precedes other features characteristic of the cell death process.¹⁴ Initiation of cell death in response to

different stimuli such as lipid mediators, lysosomotropic agents, and pro-inflammatory cytokines, including TNF- α , may result in lysosomal permeabilization. During this process, lysosomal proteases are activated and released from the lysosomes into the cytosol where they can cleave and activate various pro-apoptotic Bcl-2 family proteins resulting in mitochondrial dysfunction and cell death. Among the 11 human Cathepsins known (B, H, L, S, F, K, C, W, X, V, and O), Cathepsin B (lysosomal cysteine protease) and Cathepsin D (lysosomal aspartic protease) are the most abundantly expressed in the lysosome, as well as the most stable at the neutral pH of the cytosol.¹⁴

We recently reported that lysosomal destabilization associated with activation of lysosomal cysteine protease, Cathepsin B (CTSB), occurs in both cultured adipocytes exposed to saturated fatty acids *in vitro*, as well as *in vivo* in adipocytes from obese mice fed a high fat (HFAT) diet.¹⁵ After 2 weeks of HFAT diet, analysis of mouse AT showed slight CTSB activation, F4/80⁺-CD11b⁺ macrophage infiltration, and adipocyte cell death were detected. After 6 weeks of HFAT diet, CTSB activation was increased, associated with a significant amount of F4/80⁺-CD11b⁺ macrophage infiltration, the production of a variety of proinflammatory cytokines, and induction of adipocyte cell death. We also revealed that mitochondrial dysfunction occurred with a significant increase in reactive oxygen species (ROS) production and the release of cytochrome c from the mitochondria

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into the cytosol as a downstream effect of CTSB activation associated with adipocyte cell death. Taken together, our data demonstrate that lysosomal permeabilization is important event that initiates adipocyte cell death, macrophage infiltration and AT inflammation. In this study, we focus on Cathepsin D (CTSD) as another key Cathepsin in lysosomal permeabilization and proapoptotic protein to mediating mitochondrial dysfunction in hypertrophied AT of obese mice.

Results

Cathepsin D is activated in hypertrophied adipose tissue, or adipocytes, and induced upregulation of proapoptotic proteins. Since CTSB has been shown to be an important initiator of cell death, we hypothesized that CTSD may also have a significant role during adipocyte hypertrophy associated with weight gain. To investigate whether CTSD is activated in the hypertrophied adipocytes, we placed wild-type mice on a HFAT, or control chow (CTL), diet for 12 weeks and examined CTSD activation in the epididymal adipose tissue (EAT) by immunoblotting. CTSD is synthesized as an inactive preprocathepsin D (43 kDa), which then gets cleaved and glycosylated to form a procathepsin D (46 kDa), which is then further cleaved to CTSD heavy chain (28 kDa) and light chain (15 kDa). As a result, the active form of CTSD (heavy chain) is clearly separated from CTSD precursor (prepro and pro) on the blot. The CTSD active form, as well as CTSD precursor, was significantly increased in the HFAT diet mice compared with the mouse fed a CTL diet (Fig. 1A, the upper column), showing a positive correlation of CTSD activation (ratio of active form to precursors) with epididymal fat weights (Fig. 1B; $r = 0.77$, $P < 0.02$) and total body weights (Fig. 1C; $r = 0.9$, $P < 0.001$). Furthermore, we examined the expression of Bax and Bid, key proapoptotic proteins that CTSD has been shown to cleave and activate, in the AT. The Bax and Bid expressions were significantly upregulated in the HFAT diet fed mice via immunoblotting (Fig. 1A, middle and lower columns). Notably, there is a significant positive correlation between CTSD activation and Bax expression levels (Fig. 1D; $r = 0.9$, $P < 0.001$) as well as Bid expression levels (Fig. 1E; $r = 0.76$, $P < 0.02$). Furthermore, the increase of CTSD expression and Bax activation were detected in the adipocytes of HFAT diet fed mice via immunofluorescence with anti-CTSD and anti-Bax (6A7) (Fig. 1F). To investigate the association of these events with macrophage infiltration, we isolated stromal vascular fraction (SVF) from EAT and analyzed the number of F4/80⁺-CD11b⁺ macrophages by flow cytometry. A significant amount of F4/80⁺-CD11b⁺ macrophages were infiltrated into the adipose tissue of the mice fed a HFAT diet (Fig. 1G).

Next, we investigated CTSD activation in a genetically obese mouse model (leptin-deficient ob/ob mouse) at age 11 weeks. We isolated epididymal adipocytes to eliminate CTSD expression in the infiltrated macrophages because inflammatory macrophages are the dominant source of CTSD expression.¹⁶ The CTSD active form, as well as CTSD precursor, was significantly increased in the epididymal adipocytes of the ob/ob mouse compared with the ob control (ob CTL) mouse (Fig. 2A, the upper column),

showing a significant positive correlation between CTSD activation and epididymal fat weights (Fig. 2B; $r = 0.96$, $P < 0.001$) and total body weights (Fig. 2C; $r = 0.83$, $P < 0.04$). In addition, we detected upregulation of Bax expression in the adipocytes from ob/ob EAT (Fig. 2A, lower column). To investigate the association of these events with macrophage infiltration, we isolated SVF from EAT and analyzed the number of F4/80⁺-CD11b⁺ macrophages by flow cytometry. The number of infiltrated F4/80⁺-CD11b⁺ macrophages was increased 2.3 fold in the ob/ob mice compare with the ob CTL mice (Fig. 2D), exactly the same as the ratio of infiltrated F4/80⁺-CD11b⁺ macrophages in the mice fed a HFAT diet for 12 weeks (Fig. 1F). These results suggest that a lysosomal-mitochondrial axis involving CTSD and Bax as a potential mechanism involved in adipocyte cell death and macrophage infiltration during obesity.

Cathepsin D is activated from an early stage in hypertrophied adipose tissue. The last question is whether CTSD activation occurs at an early stage in hypertrophied AT. We checked the CTSD activation in the AT of mice on the HFAT or CTL diet at the both 2 week and 6 week timepoints. The CTSD activation was slightly increased in the mice fed a HFAT diet for 2 weeks, and it was markedly increased after 6 weeks on this diet as detected by immunoblot (Fig. 3A) and CTSD activity assays (Fig. 3B). In proportion to CTSD activation, the epididymal adipocytes were slightly hypertrophied at 2 weeks and it was markedly hypertrophied at 6 weeks on HFAT diet (Fig. 3C). These results indicate that CTSD activation occurs early in the process of adipocyte hypertrophy induced by weight gain.

Discussion

It is well known that infiltrated macrophages make crown-like structures around adipocytes and cause adipocyte cell death. However, the pathogenic mechanisms resulting in AT macrophage recruitment are incompletely understood. In our previous work, we focused on the potential role of lysosomal destabilization and mitochondrial dysfunction in the hypertrophied adipocytes during weight gain and reported that lysosomal destabilization associated with activation of lysosomal cysteine protease, Cathepsin B (CTSB), occurs in hypertrophied adipocytes in mice fed a HFAT diet.¹⁵ Notably, CTSB activation was remarkably increased at 2 weeks after initiation of the HFAT diet, whereas there is a slight increase of infiltrated F4/80⁺-CD11b⁺ macrophages into the AT and adipocyte cell death. After 6 weeks on the HFAT diet, we observed a further increase in CTSB activation, a significant amount of infiltrated F4/80⁺-CD11b⁺ macrophages into the AT, and adipocyte cell death resulting in the production of a variety of proinflammatory cytokines, as well as an increase in the iNOS to arginase 1 ratio. These results strongly suggest that lysosomal permeabilization and CTSB activation in adipocytes precedes the infiltration of pro-inflammatory M1 type macrophages into AT. We also found that mitochondrial dysfunction occurred with a significant increase in reactive oxygen species (ROS) production and the subsequent release of cytochrome c from the mitochondria into the cytosol, key indicators of mitochondrial dysfunction. ROS generated following

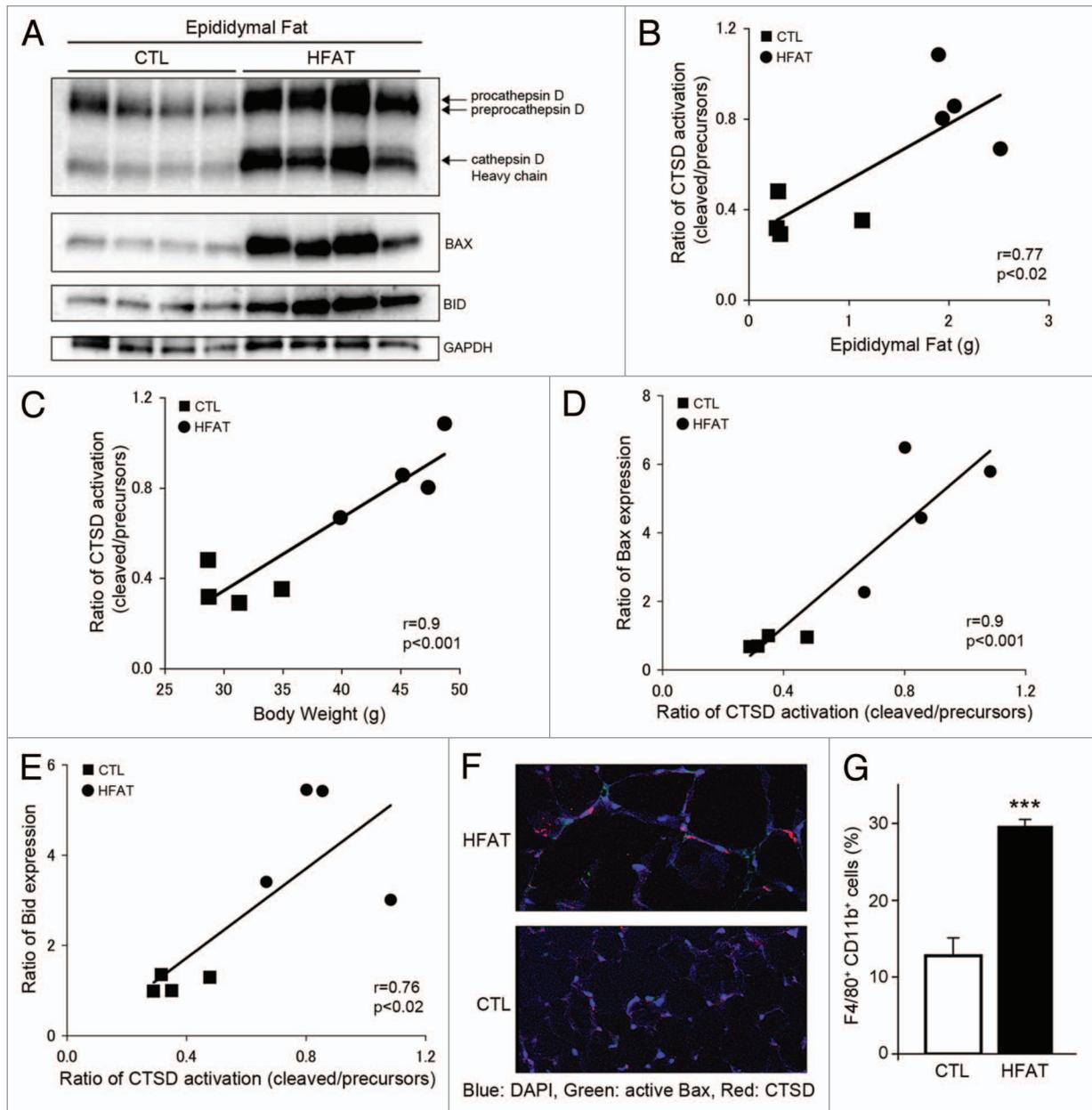


Figure 1. CTSD activation associated with macrophage infiltration occurs in hypertrophied adipose tissue of diet-induced obese mice, and induces upregulation of pro-apoptotic proteins. C57BL/6 male mice were fed high fat (HFAT) or standard low fat control (CTL) diet for 12 weeks. (A) Immunoblot analysis of CTSD, Bax, and Bid in epididymal adipose tissue. (B) Correlation graph between ratio of CTSD activation and epididymal adipose tissue weight. (C) Correlation graph between ratio of CTSD activation and mouse body weight. (D) Correlation graph between ratio of CTSD activation and Bax expression. (E) Correlation graph between ratio of CTSD activation and Bid expression. (F) Representative microphotograph of double immunofluorescence for expression of CTSD and active Bax in epididymal adipose section. (G) Quantification of F4/80⁺ CD11b⁺ cells by flow cytometry analysis of epididymal adipose tissue. Data are presented as mean \pm SEM, *** $P < 0.001$.

mitochondrial damage, and possible other factors of mitochondrial origin, could also feed back to the lysosome, resulting in further lysosomal breakdown and cell damage. Moreover, CTSD-deficient mice showed a decrease in lysosomal permeabilization resulting in protection against adipocyte cell death and less F4/80⁺-CD11b⁺ macrophage infiltration typically associated with obesity. Taken together, our previous data strongly suggest that lysosomal permeabilization and CTSD activation occur at an

early stage of weight gain resulting in adipocyte death through mitochondrial dysfunction.

However, there were still missing pieces of the puzzle: (1) The function of Cathepsin D (CTSD), another abundant key Cathepsin, in the hypertrophied AT and (2) the direct evidence connecting lysosomal permeabilization with mitochondrial dysfunction. Indeed, there are reports that CTSD activity is increased in mice fed a HFAT diet and CTSD mRNA levels are

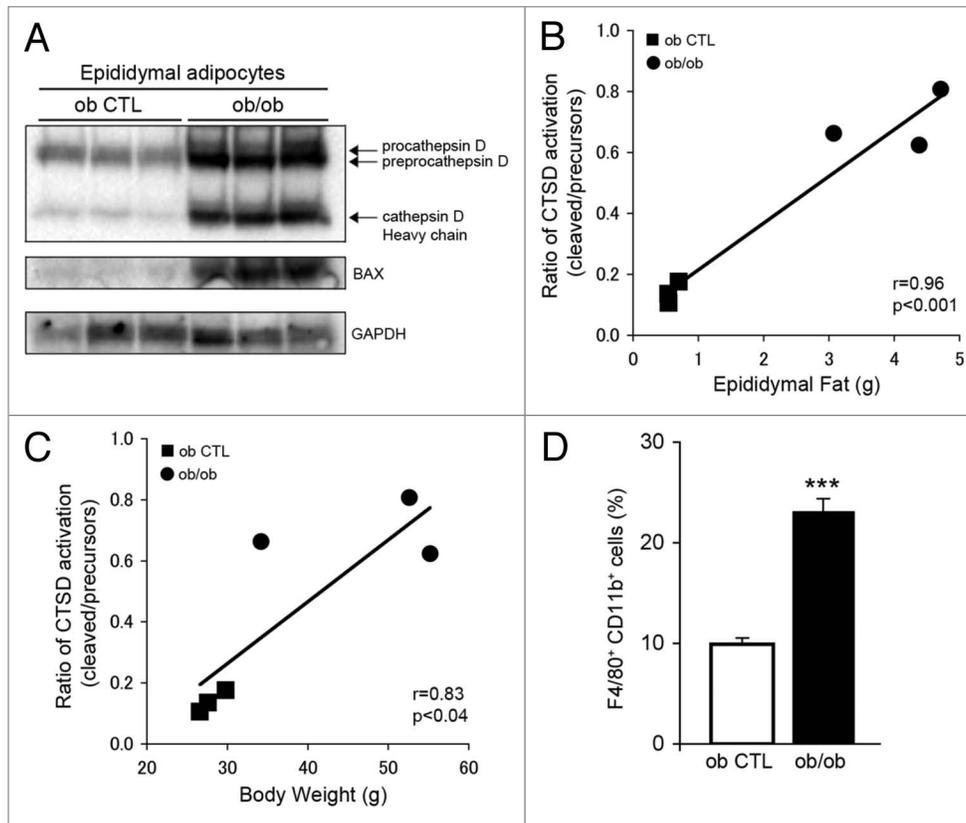


Figure 2. CTSD activation associated with macrophage infiltration occurs in hypertrophied adipocytes of genetically obese mice, and induces upregulation of pro-apoptotic proteins. Leptin-deficient ob/ob male mice, or ob control (ob CTL) male mice, were used. (A) Immunoblot analysis of CTSD, and Bax in epididymal adipocytes. (B) Correlation graph between ratio of CTSD activation and epididymal adipose tissue weight. (C) Correlation graph between ratio of CTSD activation and mouse body weight. (D) Quantification of F4/80-CD11b positive cells by flow cytometry analysis of epididymal adipose tissue. Data are presented as mean \pm SEM, *** P < 0.001.

upregulated in AT of obese mice and humans,^{17,18} but no evidence to connect lysosomal permeabilization and mitochondrial dysfunction. In this study, we demonstrated that CTSD activation also occurs at an early stage during weight gain resulting in the upregulation of proapoptotic proteins, Bax and Bid. Taken together, we propose the following model for the role of lysosome permeabilization in adipocytes during weight gain and obesity (Fig. 4): (1) Lysosomal permeabilization occurs at an early stage during adipocyte hypertrophy and results in the release and activation of Cathepsins including CTSD into the cytosol, (2) CTSD induce activation of proapoptotic Bcl-2 family proteins resulting in mitochondrial dysfunction with the resultant increase of ROS and release of cytochrome c into the cytosol, and (3) increased adipocyte cell death results in the recruitment of macrophages into the hypertrophied adipocyte thus releasing cytokines as pro-inflammatory factors. Therapy targeted at inhibiting the lysosomal pathway, such as CTSD or CTSD inhibitors, may be attractive new therapeutic strategies for treatment of obesity-associated metabolic complications. Future studies are necessary to better dissect the role of another cell death pathway, such as endoplasmic reticulum stress,¹⁹ and how it interacts with lysosomal permeabilization in hypertrophied adipocyte. In addition, Cathepsin null mice, such as CTSD, are

warranted to further elucidate the role of lysosomal initiated cell death in AT pathobiology associated with obesity and metabolic syndrome.

Materials and Methods

Animal studies. UCSD Animal Care approved of the experimental protocol. Male C57BL/6 mice, 20–25 g of body weight (Harlan Laboratory) were placed on either a high fat (HFAT) diet consisting of 42% Kcal from fat, 42.7% carbohydrate, 15.2% protein, 4% mineral mixture (TD 88137, Teklad Mills), or regular chow diet consisting of 6% fat (CTL) (TD 2918, Teklad Mills), for 2, 6, and 12 weeks. Total body weight was measured weekly. Leptin-deficient ob/ob and ob-control mice, (age 11 weeks) were purchased from Jackson Laboratory.

Immunoblot analysis. Adipose tissue lysates were prepared using lysis buffer (50 mM Tris, 6.4 mM NaCl, 1 mM EDTA, and 1% Triton X100). Cell lysates were run on SDS-PAGE, and transferred on Nitrocellulose membrane. Adipocyte lysates were prepared using NativePAGE sample buffer with 1% DDM (Lifetechnologies). The bands were detected with anti-Cathepsin D antibody (GeneTex), anti-Bax (Cell Signaling), anti-Bax (6A7) (Abcam), anti-Bid (Cell Signaling), or anti-GAPDH (GeneTex).

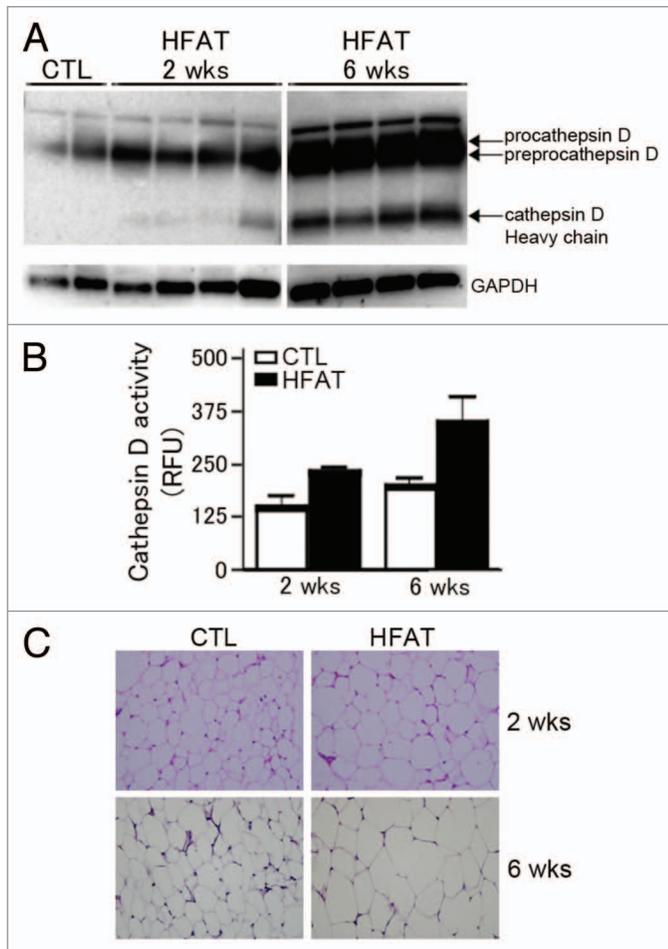


Figure 3. CTSD activation in adipose tissue occurs early during weight gain. C57BL/6 male mice were fed high fat (HFAT) or standard low fat control (CTL) diet for 2 or 6 weeks. (A) Immunoblot analysis of CTSD in epididymal adipose tissue. (B) CTSD activity assay in epididymal adipose tissue. (C) Representative microphotograph of H&E staining of epididymal adipose section.

Histopathology and immunohistochemistry. EAT was fixed in 4% paraformaldehyde and embedded in Tissue Path (Fisher Scientific). Tissue sections (4 μ m) were prepared and hematoxylin and eosin-stained adipose specimens were evaluated by light microscopy. The expression of CTSD and active Bax

was assessed by immunofluorescence with primary antibodies, anti-CTSD and anti-Bax (6A7), followed by secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 594 (Lifetechnologies) and examined using a laser confocal microscopy (Leica SP5).

Measurement of Cathepsin D (CTSD) activity. CTSD activity was determined fluorometrically in whole-cell lysates using the InnoZyme Cathepsin D activity assay (Calbiochem) according to the manufacturer's instructions. Fluorescence was measured with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The Cathepsin D substrate was co-incubated with Cathepsin D inhibitor CA-074 (50 μ M) as a negative control.

Isolation of adipocytes and stromal vascular fraction. EAT was minced and centrifuged at 500 rpm for 5 min. Floating pieces of AT were incubated with 1 mg/ml of collagenase type II (Worthington Biochemical) in Tyrode buffer (containing 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 0.33 mM NaH₂PO₄, 5 mM HEPES, and 5 mM glucose) with gentle stirring for 20 min at 37 °C. After being filtered through a 250 μ m mesh and centrifuged at 500 rpm for 5 min, adipocytes were collected in the upper white layer and stromal cells were collected from the remaining supernatant. Adipocytes were washed twice by centrifugation at 500 rpm for 5 min. The stromal vascular fraction (SVF) cells were pelleted by centrifugation at 1000 rpm for 10 min, incubated with erythrocyte-lysing buffer (eBioscience), and washed with PBS twice. SVF cells were suspended with 3% FBS-PBS and incubated with labeled antibodies, F4/80 (AbD Serotec) and CD11b (eBioscience). Macrophage infiltration was analyzed by Flow Cytometer (Becton Dickinson, LSR II).

Statistical analysis. All data are expressed as the mean \pm SEM. Differences were considered to be statistically significant at $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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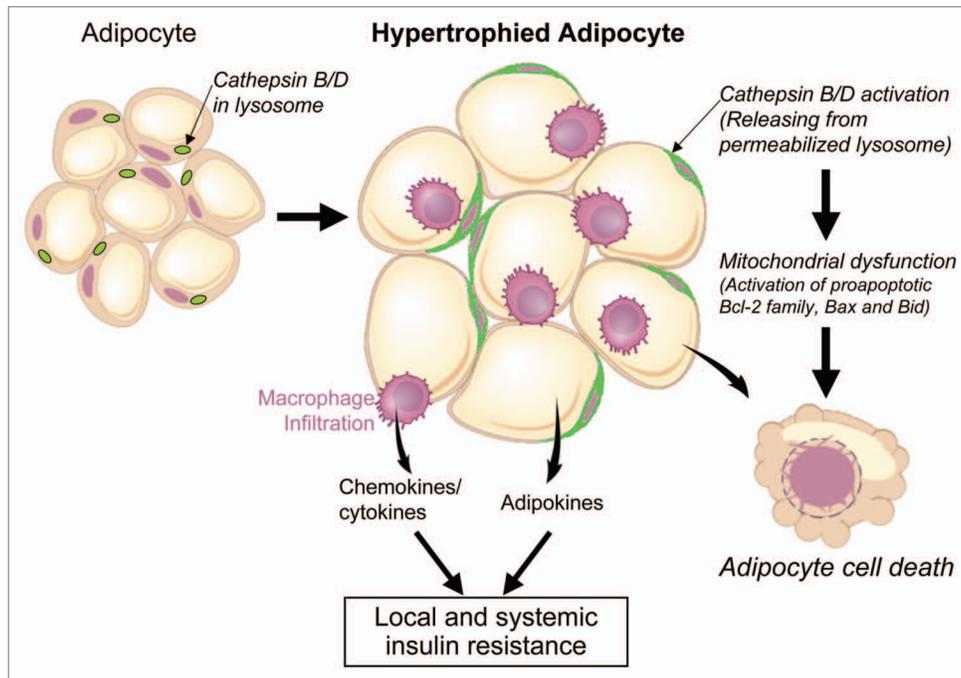


Figure 4. Our model for the role of lysosome permeabilization during weight gain and obesity. Cathepsins CTSB and CTSD stay in the lysosomes in the normal adipocyte. During hypertrophic adipocytes associated with weight gain and obesity, activated CTSB and CTSD are released from permeabilized lysosomes into the cytosol and trigger activation of pro-apoptotic Bcl-2 family proteins (Bax and Bid) followed by an increase in adipocyte cell death through mitochondrial dysfunction. Increased adipocyte cell death results in the recruitment of macrophages into the hypertrophied adipocyte and subsequent release of cytokines as proinflammatory factors.

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