Adipocyte hypertrophy is associated with lysosomal permeability both in vivo and in vitro: role in adipose tissue inflammation

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Gornicka A, Fettig J, Eguchi A, Berk MP, Thapaliya S, Dixon LJ, Feldstein AE. Adipocyte hypertrophy is associated with lysosomal permeability both in vivo and in vitro: role in adipose tissue inflammation. Am J Physiol Endocrinol Metab 303: E597-E606, 2012. First published June 26, 2012; doi:10.1152/ajpendo.00022.2012.—Obesity in both humans and rodents is characterized by adipocyte hypertrophy and the presence of death adipocytes surrounded by macrophages forming “crown-like structures.” However, the biochemical pathways involved in triggering adipocyte death as well as the role of death adipocytes in adipose tissue remodeling and macrophage infiltration remain poorly understood. We now show that induction of adipocyte hypertrophy by incubation of mature adipocytes with saturated fatty acids results in lysosomal destabilization and cathepsin B (ctsb), a key lysosomal cysteine protease, activation and redistribution into the cytosol. ctsb activation was required for the lysosomal permeabilization, and its inhibition protected cells against mitochondrial dysfunction. With the use of a dietary murine model of obesity, ctsb activation was detected in adipose tissue of these mice. This is an early event during weight gain that correlates with the presence of death adipocytes, and precedes macrophage infiltration of adipose tissue. Moreover, ctsb-deficient mice showed decreased lysosomal permeabilization in adipocytes and were protected against adipocyte cell death and macrophage infiltration to adipose tissue independent of body weight. These data strongly suggest that ctsb activation and lysosomal permeabilization in adipocytes are key initial events that contribute to the adipocyte cell death and macrophage infiltration into adipose tissue associated with obesity. Inhibition of ctsb activation may be a new therapeutic strategy for the treatment of obesity-associated metabolic complications.

MATERIALS AND METHODS

Cell culture and treatment with free fatty acids. Mouse 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were grown and maintained at no higher than 70% confluence in Dulbecco’s modified Eagle medium (GIBCO, Camarillo, CA) supplemented with 10% FBS (Cellgro, Manassas, VA), penicillin, and streptomycin (growth medium) at 37°C in a 10% CO2 incubator. Medium was replaced every other day until cells reached confluence. To induce differentiation into mature 3T3-L1 adipocytes, cells were grown 2 days postconfluence in growth medium, and then the cells were induced to differentiate in growth medium supplemented with insulin, 3-isobutyl-1-methylxanthine, and dexamethasone (Cayman Chemical, Ann Arbor, MI), as described previously (30). Three days postinduction, medium was replaced with insulin-only medium (growth medium supplemented with only insulin) for an additional 5–7 days. Insulin-only medium was replaced every other day during this period, and accumulation of lipid droplet was monitored microscopically. At least 95% of the cells showed an adipocyte phenotype at the end of the differentiation period. Differentiated mature 3T3-L1 adipocytes were exposed to the free fatty acid (FFA) palmitic acid (500 μM), or stearic acid (500 μM), in media containing 1% BSA for up to 18 h. In selected experiments, cells were incubated with the fatty acid mixture in the presence or absence of the cathepsin B (ctsb)-selective inhibitor (100 μM Ac-LVK-CHO; Calbiochem, Gibbstown, NJ).

Lysotracker Red loading. Lysosomal permeabilization was determined by staining with Lysotracker Red (577/590 nm), an acidophilic fluorescent dye that loads predominantly into lysosomes (Molecular Probes, Carlsbad, CA) in unfixed live cells and visualized using fluorescent microscopy. The percentage of cells displaying positive the obesity-associated inflammatory state and inhibits subsequent development of insulin resistance (12, 15, 25, 26).

The pathogenic mechanisms resulting in ATM recruitment are under intense investigation and remain incompletely understood. Increased production and release of certain chemokines by adipocytes, potentially as a result of local hypoxia in an expanding AT bed, has been implicated as a possible mechanism resulting in ATM recruitment (18, 29). More recently, adipocyte cell death with formation of “crown-like structures” characterized by isolated dead adipocytes surrounded by macrophages has been described in both AT from obese mice and obese humans (4, 16, 22). Indeed, adipocyte cell death appears to be an important contributor to ATM recruitment in experimental models of obesity and insulin resistance. However, very limited information is currently available regarding the biochemical pathways that initiate adipocyte cell death during weight gain. Thus, the overall objective of this study was to characterize the biochemical and molecular mechanisms involved in triggering cell death during adipocyte hypertrophy and their role in AT remodeling and macrophage infiltration.

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staining was counted in 30 random fields as we have described previously in detail (7, 14).

Measurement of cathepsin activity. Ctsb activity was determined fluorometrically in whole cell lysates using the ImmoZyme ctsb 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 ctsb substrate was covalently attached to ctsb inhibitor CA-074 (50 μM) as a negative control.

Measurements of cellular reactive oxygen species. The effect of FFA overaccumulation on adipocyte reactive oxygen species (ROS) production was monitored using the nonfluorescent cell-permeant compound 2′,7′-dichlorofluorescin diacetate (DCFH-DA). This technique is based on the principle that, on oxidation by ROS, the deesterified form of this compound becomes the fluorescent compound dichlorofluorescein (DCF). Adipocytes were loaded with 10 μM DCFH-DA (Molecular Probes) and treated with FFAs in Hanks’ balanced salt solution for 6 h at 37°C. Cells were lysed in Reporter lysis buffer (Promega, Madison, WI), and the fluorescence was measured with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Generation of ROS in AT was measured in fresh tissue homogenates by using DCFH-DA (13). The assay was performed within 1 h after tissue harvest in buffer containing 130 mM KCl, 5 mM MgCl2, 20 mM NaH2PO4, 20 mM Tris-HCl, and 30 mM glucose (pH 7.4), plus 2 mM malate and 2 mM pyruvate. Duplicates of 50 μl homogenate were incubated with 250 μl assay buffer containing 12.5 mM DCFH-DA dissolved in methanol at 37°C for 15 min. DCF formation was recorded at the excitation wavelength of 480 nm and emission wavelength of 520 nm by using an Ascent fluorescence reader (Labsystems). Values were corrected against those obtained from auto-oxidation of DCFH-DA in buffer only.

Animal studies. These experimental protocols were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic. Male C57BL/6 mice, 20–25 grams of body weight (Jackson Laboratory, Bar Harbor, ME), were placed on either a high-fat (HFAT) diet (Teklad Mills) (control (CTL) diet consisting of 5% fat, for 2, 6, and 12 wk (TD 2918; provided by Dr. Ephraim Sehayek, Lerner Research Institute, Cleveland, OH), 20–25 grams of body weight, were fed either the HFAT diet or the CTL diet for 6 wk (= 5/time point in each group). Total body weight was measured weekly. In selected studies, C57BL/6 ctsb knockout (ctsbb−/−) mice and wild-type littermates (ctsbb+/+) (generously provided by Dr. Ephraim Sehayek, Lerner Research Institute, Cleveland, OH), 20–25 grams of body weight, were fed either the HFAT diet or the CTL diet for 6 wk (n = 4–8 in each group).

Histopathology and immunohistochemistry. Adipose (epididymal) tissue was fixed in 4% paraformaldehyde and embedded in Tissue Path (Fisher Scientific, Pittsburgh, PA). Tissue sections (4 μm) were prepared, and hematoxylin- and eosin-stained adipose specimens were evaluated by light microscopy. The presence of macrophage infiltration was assessed by immunofluorescence with primary antibodies, F4/80 (AbD Serotec, Raleigh, NC) and CD11b (Abcam, Cambridge, MA), followed by secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 594 (Invitrogen, Carlsbad, CA) and examined using a laser confocal microscopy (Leica SP5). Lysosomal permeabilization in AT was assessed by immunostaining with ctsb antibody and visualized using fluorescent microscopy.

Macrophage infiltration by flow cytometry. Epididymal AT were minced and centrifuged at 500 rpm for 5 min. Floating pieces of AT were incubated with 1 mg/ml of collagenase type II (Worthington, Lakewood, NJ) for 20 min at 37°C. After filtration through a 100-μm mesh and centrifugation at 1,000 rpm for 10 min, the pellet containing the stromal vascular fraction was recovered. Cells were incubated with erythrocyte-lysing buffer (eBioscience, San Diego, CA) and washed with PBS two times. Cells were finally suspended with 3% FBS-PBS and incubated with labeled antibodies, F4/80 (AbD Serotec) and CD11b (eBioscience). Macrophage infiltration was analyzed by Flow Cytometer (LSR II; Becton-Dickinson).

Assessment of cell death. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed following the manufacturer’s instructions (in-situ cell death detection kit; TREVIGEN, Gaithersburg, MD). Adipocyte apoptosis in AT sections was quantified by counting the number of TUNEL-positive cells in 10 random microscopic fields (×10). Caspase 3/7 activation in AT was assessed using ApoOne fluorometric assay (Promega) according to the manufacturer’s instructions.

Immunoblot analysis. For in vitro study, immunoblot analysis was performed using either whole cell lysates or cytosolic fractions. Cytosolic fractions were obtained using the Mitochondria Isolation Kit (Thermo Scientific, Rockford, IL) following the manufacturer’s instructions. For in vivo study, AT lysates were prepared using lysis buffer (50 mM Tris, 6.4 mM NaCl, 1 mM EDTA, and 1% Triton X-100). Protein samples were resolved by 12 or 15% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA), and blotted with appropriate primary antibodies. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; KPL, Gaithersburg, MD). The bands were visualized using a chemiluminescence system (Thermo Scientific, Waltham, MA). Primary antibodies were anti-cytochrome c antibody (1:1,000 dilution; Cell Signaling, Danvers, MA), anti-ctsb antibody (1:500 dilution; Millipore, Billerica, MA), anti-cathepsin D antibody (1:1,000 dilution; Genetex, Irvine, CA), anti-glyceroldehyde-3-phosphate dehydrogenase antibody (1:4,000 dilution; Ambion, Austin, TX), anti-α-tubulin (1:5,000 dilution; Sigma-Aldrich, St. Louis, MO), and anti-actin (1:1,000 dilution; Santa Cruz, Santa Cruz, CA).

Real-time PCR. Total RNA was isolated from AT using the RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA). Reverse transcript (the cDNA) was synthesized from 1 µg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR quantification was performed. Briefly, 25 μl reaction mix contained cDNA, Syber Green buffer, Gold Taq polymerase, dNTPs, and primers at final concentration of 200 nM. The sequences of the primers used for quantitative PCR were as follows: tumor necrosis factor (TNF)-α 5′-CTCCCACTACCATGATCTTCTCT and 5′-GCTACACGTTGGGCTAAG; interleukin (IL)-6 5′-TAGTGGCTTCTGACCCAATITCC and 5′-TGGTGGCTTCCAGAGCACCCTTC; F4/80 5′-CCCAGTGGCTCCTACAGAGT and 5′-GTGCGCCAGAGGATGCTCT; transforming growth factor-β 5′-CTCCGCGGT-GCTTCTAGTG and 5′-GCCATTTGGTTGCAAGATTCT; arginase 15′-CTCACACACCCAGAATAC and 5′-AGCAGCAGCCTCTCTCCACAG; inducible nitric oxide synthase (iNOS) 5′-TCTTGGACCGCCAACTATAC and 5′-GTGCGACCGTGATGTCA; IL-β 5′-GCAAGATGTCATCTGA-CACT and 5′-ATCTTCTGGTCCGCAACTC and CD11c 5′-GCAAACGTGGTCGCAACT and 5′-CTGGATAGC-TTCTCTTCTGCTG. RT-PCR was performed in the Mx3000P cycler (Stratagene): 95°C for 10 min, 40 cycles of 1 s at 95°C, 30 s at 60°C, 30 s at 72°C followed by 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. The fold change over control samples was calculated using cycle threshold (Ct), ΔCt, and ∆∆Ct values using MxPro software (Stratagene). 18S ribosomal RNA (Ambion) was used as an endogenous control.

Statistical analysis. All data were expressed as means ± SE. Differences between groups were compared by an ANOVA followed by a post hoc Bonferroni test to correct for multiple comparisons. Differences were considered to be statistically significant at P < 0.05.

RESULTS

Lipotoxicity in adipocytes is associated with lysosomal permeabilization, and release of ctsb into cytosol. Accumulating evidence suggests a central role for lysosomes in both necrotic and apoptotic cell death. We hypothesized that overaccumulation of lipotoxic lipids in adipocytes treated with FFA may result in lysosomal permeabilization and release of ctsb into the...
cytosol. ctsb is a key lysosomal cysteine protease that has been implicated in a variety of cell death signaling pathways. To test this hypothesis, differentiated mature adipocytes were initially incubated in the presence or absence of the FFA palmitic acid for up to 18 h. This process resulted in significant adipocyte hypertrophy (Fig. 1A). Lysosomal permeabilization was initially determined by staining with LysoTracker Red (577/590 nm), an acidophilic fluorescent dye that loads predominantly into lysosomes in unfixed live cells, and visualized under fluorescent microscopy. Lysosomal permeabilization was assessed subsequently using immunoblot analysis of cytosolic fractions for the presence of ctsb and ctsb activity assays (Fig. 1, B–D). We found a significant loss of LysoTracker Red punctuate staining pattern consistent with lysosomal permeabilization in cells treated with palmitic acid compared with controls treated with BSA alone (Fig. 1B). Moreover, treatment with palmitic acid resulted in activation and translocation of ctsb into the cytosol as detected by Western blot analysis of cytosolic fractions (Fig. 1C). Consistent with these results, a marked increase in ctsb activity was detected in adipocytes treated with palmitic acid (Fig. 1D). Similar findings were observed with treatment with another lipotoxic saturated FFA, stearic acid (data not shown). To gain further insight into the potential role of lysosome destabilization in adipocyte cell death in vivo, we used a common dietary murine model of obesity, adipocyte hypertrophy, and insulin resistance. C57BL/6 on a HFAT “Western type” diet over a period of 12 wk showed marked obesity and adipocyte hypertrophy compared with those animals kept on a standard low-fat CTL diet (Fig. 2, A–C). More importantly, the hypertrophied AT was associated with evidence of ctsb activation and lysosomal permeabilization, resulting in relocation of the lysosomal protease to the cytosol as detected by Western blot analysis and immunofluorescence (Fig. 2, D and E), respectively. Taken together, these data strongly suggest that lysosomal permeabilization and ctsb activation occur in hypertrophied adipocytes both in vitro and in vivo.

**Lysosomal permeabilization occurs early during weight gain and precedes macrophage infiltration to AT.** The findings of lysosomal breakdown and ctsb activation in the AT of obese mice led us to further investigate whether these events are a late consequence of the AT expansion and inflammation or an upstream event that initiates adipocyte cell death and induces recruitment of macrophages to AT. To address this question, we assessed the temporal relationship between lysosomal permeabilization, ctsb activation, adipocyte cell death, and ATM recruitment during weight gain. A significant increase in ctsb expression and activity was noted in AT in mice on the HFAT diet compared with those on the CTL diet as early as 2 wk after initiation of the diet (Fig. 3, A and B). These changes were associated with an increase in adipocyte cell death as detected by the presence of TUNEL-positive cells in the AT of these mice (Fig. 3, C and D). Furthermore, fluorescence-activated cell sorter analysis of AT...
from mice on the HFAT demonstrated no difference on the numbers of F4/80-CD11b positive macrophages compared with CTL mice at the 2-wk time point while a marked increase in the numbers of infiltrating macrophages was noted after 6 wk on the HFAT diet (Fig. 4, A and B). The increase in macrophage infiltration was associated with an increase in a variety of proinflammatory cytokines as well as an increase in the iNOS-to-arginase 1 ratio (Fig. 4, C and D). These data strongly suggest that lysosomal permeabilization and ctsb activation in adipocytes preceded the infiltration of AT of proinflammatory M1 type macrophages.

Lysosomal permeabilization is dependent on ctsb activation and results in mitochondrial dysfunction. Previous studies in hepatocytes and a variety of hepatic cell lines have demon-
stratified that lysosomal permeabilization induced by different stimuli such as the proinflammatory cytokine TNF-α, as well as lipotoxic lipids such as palmitic acid, is dependent on ctsb. Activated ctsb in the cytosol acts directly on mitochondria to induce mitochondrial dysfunction, increase ROS production, and release several mitochondrial intermembrane space proteins, including cytochrome c, into the cytosol. We next examined whether lysosomal permeabilization induced by palmitic acid in adipocytes was dependent on ctsb activation. Coincubation of adipocytes with palmitic acid and a selective ctsb inhibitor almost completely abrogated the loss of LysoTracker Red staining induced by palmitic acid (Fig. 5A).
Furthermore, this treatment resulted in a decreased in cytosolic cytochrome c as well as decreased ROS production (Fig. 5, C and D), suggesting a protective effect of ctsb inhibition on mitochondria.

Genetic inhibition of ctsb protects obese animals against lysosomal permeabilization and ATM recruitment. The findings of increased lysosomal permeabilization and ctsb activation during weight gain in conjunction with the in vitro evidence that ctsb inhibition prevents palmitic acid-induced lysosomal permeabilization and subsequent mitochondrial dysfunction in adipocytes led us to further examine the role of adipocyte ctsb by using ctsb null mice. ctsb−/− mice and their wild-type littermates were placed on either a HFAT or CTL diet for 6 wk. ctsb knockouts gained more weight than their wild-type littermates when placed on the HFAT diet (Fig. 6A). However, genetic inactivation of ctsb resulted in a marked decrease in recruitment of F4/80-CD11b-positive macrophages to AT and inflammatory cytokines induced by this diet (Fig. 6, B–E) and protected against AT ROS production (Fig. 6F). Collectively, these data suggest that adipocyte ctsb activation is an early...
event that results in lysosomal permeabilization and recruitment of macrophages to AT.

**DISCUSSION**

The principal findings of this study relate to the mechanisms linking obesity-associated AT expansion to adipocyte cell death and subsequent recruitment of macrophages to AT. The results demonstrate that lysosomal permeabilization and ctsb activation occurs in adipocytes exposed to palmitic acid in vitro as well as in adipocytes from mice with diet-induced obesity. These are early events that are upstream of mitochondrial dysfunction in adipocytes and precede macrophage infiltration to AT. Genetic inhibition of ctsb in vivo protects against macrophage infiltration into AT.

Obesity has reached epidemic proportions in most of the Western world (2, 8). Many lines of evidence have shown that a state of chronic low-grade inflammation is a key link between obesity and its associated metabolic dysregulation (5, 15, 24, 26). An important initiator of this inflammatory response is the AT, which actively secretes a variety of products such as cytokines, adipokines, and fatty acids into the circulation (20). Macrophages that infiltrate the AT of obese mice and humans are a major source of proinflammatory cytokines such as TNF-α and IL-6 (17, 20, 28) and appeared to be a key link...
between obesity and insulin resistance. However, the mechanisms that initiate macrophage recruitment to AT remain incompletely understood.

Recently, increased adipocyte cell death has been proposed as an attractive mechanism that initiates the recruitment of macrophages into AT (1). Consistent with this concept, recent studies have reported the presence of crown-like structures in AT of obese mice and obese humans that are formed by clusters of macrophages surrounding death adipocytes (4, 16, 22). However, it has remained unclear whether adipocyte cell death is a late event and a consequence of inflammation or a process that can actually initiate ATM recruitment. Moreover,
the molecular signaling events resulting in adipocyte cell death remain incompletely understood.

Lysosomes are a key organelle involved in both necrotic and apoptotic cell death (11). In both circumstances, lysosomal permeabilization appears to be an early event that precedes other features characteristic of the cell death process (11). Lysosomal permeabilization results in release of lysosomal proteases into the cytosol. The lysosomal cysteine proteases, also called cathepsins, represent the largest group of proteolytic enzymes in the lysosomes. ctsb is one of the most stable proteases at physiologic pH and has been shown to be an essential mediator of cell death triggered by a variety of stimuli, including TNF-α, toxic bile acids, and fatty acids (6, 9, 10). The release of lysosomal enzymes may set off a cascade of events culminating in cell death. Indeed, a growing body of evidence suggests that lysosomal proteases induce cell death by acting directly or indirectly on mitochondria to induce mitochondrial dysfunction (14). ROS generated following mitochondrial damage and possible other factors of mitochondria origin could also feed back to the lysosome, resulting in further lysosomal breakdown and cell damage.

We have now shown that incubation of mature differentiated adipocytes with the saturated fatty acid palmitic acid results in lysosomal destabilization, ctsb activation, and release into the cytosol. Inhibition of ctsb with a selective inhibitor prevented palmitic acid-induced lysosomal permeabilization, protected against mitochondrial dysfunction, and decreased ROS production. The in vitro observations were corroborated using an in vivo model. Feeding mice a HFAT diet results in extensive adipocyte hypertrophy and infiltration of AT with macrophages and is also associated with lysosomal permeabilization and ctsb activation in adipocytes. Moreover, our data suggest that these are early events during weight gain that are associated with an increase in adipocyte cell death and precede the increased macrophage infiltration and expression of various proinflammatory cytokines. The importance of these changes is supported by the findings that inhibition of ctsb activation in ctsb knockout mice was sufficient to prevent macrophage infiltration of AT associated with diet-induced obesity.

In summary, the current studies elucidate a key pathogenic role for lysosomal permeabilization and ctsb activation in adipocyte cell death and subsequent recruitment of macrophages to AT. The results support a model in which, during the development of obesity, overflow of lipotoxic lipids to adipocytes triggers lysosomal permeabilization in a ctsb-dependent manner. ctsb then acts on mitochondria to induce mitochondrial dysfunction and ROS production, resulting in cell death and subsequent recruitment of macrophages into AT. Therapy targeted at inhibiting the lysosomal pathway, such as ctsb inhibitors, may be a new therapeutic strategy for treatment of obesity-associated metabolic complications.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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