Altered Autophagy in Human Adipose Tissues in Obesity

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Context: Autophagy is a housekeeping mechanism, involved in metabolic regulation and stress response, shown recently to regulate lipid droplets biogenesis/breakdown and adipose tissue phenotype.

Objective: We hypothesized that in human obesity autophagy may be altered in adipose tissue in a fat depot and distribution-dependent manner.

Setting and Patients: Paired omental (Om) and subcutaneous (Sc) adipose tissue samples were used from obese and nonobese (n = 65, cohort 1); lean, Sc-obese and intraabdominally obese (n = 196, cohort 2); severely obese persons without diabetes or obesity-associated morbidity, matched for being insulin sensitive or resistant (n = 60, cohort 3).

Results: Protein and mRNA levels of the autophagy genes *Atg5*, *LC3A*, and *LC3B* were increased in Om compared with Sc, more pronounced among obese persons, particularly with intraabdominal fat accumulation. Both adipocytes and stromal-vascular cells contribute to the expression of autophagy genes. An increased number of autophagosomes and elevated autophagic flux assessed in fat explants incubated with lysosomal inhibitors were observed in obesity, particularly in Om. The degree of visceral adiposity and adipocyte hypertrophy accounted for approximately 50% of the variance in omental *Atg5* mRNA levels by multivariate regression analysis, whereas age, sex, measures of insulin sensitivity, inflammation, and adipose tissue stress were excluded from the model. Moreover, in cohort 3, the autophagy marker genes were increased in those who were insulin resistant compared with insulin sensitive, particularly in Om.

Conclusions: Autophagy is up-regulated in adipose tissue of obese persons, especially in Om, correlating with the degree of obesity, visceral fat distribution, and adipocyte hypertrophy. This may co-occur with insulin resistance but precede the occurrence of obesity-associated morbidity. **(*J Clin Endocrinol Metab* 96: E268–E277, 2011)**

Autophagy is a highly evolutionary-conserved self-digestive process, through which intracellular components are contained in an intracellular double-membrane organelle (autophagosome) and delivered for degradation by fusing with lysosomes. In mammalian cells, autophagy likely represents a common name for sev-

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Abbreviations: BMI, Body mass index; CT, computed tomography; GIR, glucose infusion rate; IA, intraabdominally; IR, insulin resistant; IS, insulin sensitive; Om, omental; OMIM, online Mendelian inheritance in man; Sc, subcutaneous.

eral related but distinct pathways, each characterized by different levels of substrate selectivity and molecular machinery that executes it. The integrity of autophagic flux was shown to be required for basic and occasionally opposing biological processes, such as cell survival and death. In the regulation of energy metabolism, autophagy is thought to contribute to carbohydrate (glycogen) and protein degradation (1): the importance of lysosome-based degradation of glycogen is exemplified by the lethality of inherited metabolic disorders in which this pathway is defective [type 2 glycogen storage disease, online Mendelian inheritance in man (OMIM) 232300, in which acid maltase is defective, and Danon's disease, OMIM 309060, in which a lysosomal protein required for some forms of autophagy, lysosome-associated membrane protein-2, is mutated]. Moreover, autophagic degradation of dysfunctional mitochondria (a process termed mitophagy) in the liver has long been known (2, 3). More recently, interest in the possibility that autophagy may also contribute to the regulation of lipid metabolism has mounted (1): it has been proposed that intact autophagic machinery is required for either the formation of lipid droplets (4) or for breakdown (thus creating the term lipophagy) (5, 6). These processes, initially implicated in nonadipocytes, have now been complemented by studies on adipose tissue in autophagy gene 7 knockout mice (7, 8). As a consequence of inhibited autophagy, adipocytes of these mice adopt a phenotype resembling brown fat cells (increased mitochondria and small, multilocular lipid droplets). Consistently these mice have an enhanced metabolic rate and are resistant to the development of obesity, a finding that may suggest that, at least in adipose tissue, active (and potentially increased) autophagy contributes to the pathogenesis of obesity itself.

We therefore hypothesized that in human obesity autophagy may be altered in adipose tissue. To test this hypothesis, we used established biobanks of paired omental (Om) and subcutaneous (Sc) adipose tissue biopsies (9–11) to assess the expression of autophagy-related genes at the protein and mRNA levels, to estimate autophagy rate (flux), and to assess whether autophagy is altered in human adipose tissue in a depot-specific manner. Finally, we assessed which clinical and/or adipose tissue characteristics are most strongly associated with altered expression of autophagy markers and estimated the role of autophagy in the pathogenesis of obesity and its associated comorbidities.

Subjects and Methods

Study populations

Paired human Om and Sc fat tissue samples of three independent cohorts were from biobanks described previously in joint studies (9–11). Cohort 1 from Beer-Sheva (Israel) consisted of samples obtained from obese [body mass index (BMI) $\geq$30 kg/m^2, n = 50] and nonobese (BMI $<$30 kg/m^2, n = 15) men and women (n = 9 and 56, respectively), who underwent elective abdominal surgery (bariatric surgery or cholecystectomy) (Supplemental Table 1A, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Cohort 2 from Leipzig (Germany) consisted of samples obtained from 196 extensively characterized Caucasian lean (n = 66) and obese (n = 130) men and women (n = 74 and 122, respectively) who underwent open abdominal surgery for elective cholecystectomy, gastric sleeve resection, or explorative laparotomy (Supplemental Table 1B). Type 2 diabetes was defined based on oral glucose tolerance tests. Abdominal visceral and sc fat areas were calculated from computed tomography (CT) scans (L_4–L_5) as described elsewhere (12). Based on these measurements, obese subjects were further categorized as predominantly sc obese (n = 88) or intraabdominally (IA) obese (n = 42) (the latter with a ratio of IA to sc fat area $>$0.5). Patients with malignant diseases or any acute or chronic inflammatory disease were excluded. Cohort 3 consisted of 60 healthy individuals with a BMI of 45 $\pm$ 1.3 kg/m^2, who were strictly matched for age, gender, and body fat before elective surgery and divided into groups of insulin-sensitive (IS) or insulin-resistant (IR) obesity based on the glucose infusion rate (GIR) during euglycemic-hyperinsulinemic clamp studies (GIR $>$70 or $<$60 μmol/kg $\cdot$ min for IS or IR, respectively, as recently described (13) (Supplemental Table 1C).

All procedures were performed in accordance with the guidelines in the Declaration of Helsinki and approved by each institution's ethics committees. All patients gave a written informed consent in advance after the study's objectives and procedures were explained to them.

Handling of fat biopsies

Laboratory procedures in the two centers were matched and validated in previous joint studies (9–11). Briefly, immediately after excision, samples were transferred on ice to the laboratory. Samples were rinsed in saline and frozen in liquid nitrogen (for protein or mRNA analyses). For collagenase digestion or *ex vivo* incubation with lysosomal inhibitors, tissues were immediately used for added incubations, as detailed below, before freezing in liquid nitrogen.

Western blot analyses

For preparing adipose tissue lysates, frozen Om and Sc adipose tissue biopsies were powdered, and lysates were prepared using a glass homogenizer, with lysis buffer [150 mmol/liter NaCl, 50 mmol/liter Tris-HCl (pH 7.5), 1% (vol/vol) Nonidet P-40, 0.25% (wt/vol) sodium deoxycholate, 10 mmol/liter sodium β-glycerophosphate, 5 mmol/liter sodium pyrophosphate, 1 mmol/liter EGTA, 1 mmol/liter sodium vanadate, 1 mmol/liter NaF], supplemented with protease inhibitor cocktail (1:1000 dilution; Sigma, St. Louis, MO) (9). Next, protein content was determined by the bicinchoninic acid method (Pierce, Rockford, IL), and equal protein loading of paired Om and Sc samples was confirmed by repeated immunoblotting for β-actin (Sigma). Forty to 50 μg of lysate proteins were separated by SDS-PAGE on 10 or 12% polyacrylamide gels, transferred to nitrocellulose or PVDF membranes, and blotted overnight at 4 C with antibodies against Atg5, Atg12, LC3B (Cell Signaling Technology, Danvers, MA), anti-NBR1 (Abnova, Taiwan), and anti-p62

(Santa Cruz Biotechnology Inc., Santa Cruz, CA). In some experiments LC3 was also detected using self-made antibodies (14). Densitometry analysis of scanned films was performed using ImageGauge software (Fuji Photo Film Co., Ltd., Tokyo, Japan), as previously described (9). A standard sample was run in each blot to compare density values between blots by expressing each band density value as fold of this standard sample.

Autophagy flux analysis

Sc and Om adipose tissue explants were minced into small tissue fragments (2–3 mm^3) and preincubated for 1 h in a CO_2 incubator (37 C,5% CO_2) in complete medium-DMEM supplemented with 2 mM glutamine, 1% (vol/vol) antibiotic solution, and 10% (vol/vol) fetal bovine serum (all from Biological Industries, Beit-Haeemek, Israel). Thereafter tissue fragments were incubated for an additional 4 h in the same medium containing (or not) bafilomycin-A1 (0.1 μM; LC Laboratories, Woburn, MA) and leupeptin (10 μg/ml; Sigma). Lysates were immediately prepared as described above.

Immunohistofluorescence

Adipose tissue biopsies were carefully minced into small tissue fragments (0.5–1 mm^3) and fixed for 1 h in 4% paraformaldehyde. After extensive washing in PBS, explants were incubated for additional 30 min at room temperature in PBS supplemented with 2% BSA and 0.1% Triton X-100, followed by 1.5 h incubation with the rabbit polyclonal antibody directed against human LC3B (1:100). After extensive rinsing with PBS supplemented with 0.2% Tween 20 and 1 h incubation with antirabbit antibody coupled to AlexaFluor 555 (Invitrogen, Carlsbad, CA) (1:200), the tissues were placed on slides and examined using a Zeiss LSM-510 confocal microscope (Zeiss, Jena, Germany) (15). Acquisition parameters were kept constant and below background signal (as determined by samples incubated without primary antibody) to allow comparison between different samples.

Adipose tissue mRNA expression for autophagy marker genes

Human autophagy marker genes (*Atg5*, *LC3B*, *LC3A*) mRNA expression was measured by quantitative real-time RT-PCR in a fluorescent temperature cycler using the TaqMan assay, and fluorescence was detected on an ABI-PRISM 7000 sequence detector (Applied Biosystems, Darmstadt, Germany). Total RNA was isolated using TRIzol (Life Technologies, Grand Island, NY), and 1 μg RNA was reverse transcribed with standard reagents (Life Technologies). From each RT-PCR consisting of an initial denaturation at 95 C for 10 min, followed by 40 PCR cycles, each cycle consisting of 95 C for 15 sec, 60 C for 1 min, and 72 C for 1 min, 2 μl was amplified in a 26-μl PCR using the Brilliant SYBR Green QPCR core reagent kit from Stratagene (La Jolla, CA). The following primers were used: human *Atg5*, 5′-AACTGAAAGGGAAGCAGAACCA-3′ (sense) and 5′-CCATTTCAGTGGTGTGCCTTC-3′ (antisense); human *LC3B*, 5′-ACCATGCCGTCGGAGAAG-3′ (sense) and 5′-GGTTGGATGCTGCTCTCGAA-3′ (antisense); human LC3A, 5′-CCAGCAAAATCCCGGTGAT-3′ (sense) and 5′-TGGTC-

TABLE 1. Age-adjusted univariate correlations between expression of autophagy genes in Sc and Om human adipose tissue and various obesity-related and adipose tissue phenotypes (n = 196)

	Atg5		*LC3B*		*LC3A*	
	r	*P*	r	*P*	r	*P*
BMI						
Sc	0.323	<0.001	0.159	0.029	0.058	0.421
Om	0.442	<0.001	0.067	0.350	0.086	0.234
Adip diam						
Sc	0.392	<0.001	0.094	0.236	0.197	0.012
Om	0.564	<0.001	0.075	0.342	0.249	0.001
GIR						
Sc	−0.363	<0.001	−0.058	0.426	0.027	0.713
Om	−0.385	<0.001	−0.044	0.547	−0.203	0.005
TChol						
Sc	0.313	<0.001	0.108	0.134	−0.004	0.961
Om	0.361	<0.001	0.049	0.498	0.113	0.114
TG						
Sc	0.318	<0.001	0.023	0.749	0.023	0.745
Om	0.350	<0.001	0.007	0.917	0.107	0.138
FFA						
Sc	0.340	<0.001	−0.010	0.886	0.103	0.153
Om	0.345	<0.001	0.015	0.831	0.271	<0.001
Leptin						
Sc	0.408	<0.001	0.077	0.306	0.043	0.568
Om	0.424	<0.001	0.177	0.017	0.107	0.153
Adiponectin						
Sc	−0.409	<0.001	−0.090	0.224	−0.087	0.238
Om	−0.363	<0.001	−0.165	0.024	−0.228	0.002
IL-6						
Sc	0.390	<0.001	0.181	0.014	−0.008	0.919
Om	−0.310	<0.001	0.099	0.182	0.198	0.007

Adip diam, Adipocyte diameter; TChol, total cholesterol; FFA, free fatty acid.

CGGGACCAAAAACT-3′ (antisense). Human *Atg5*, *LC3B*, and *LC3A* mRNA expression was calculated relative to the mRNA expression of 18S rRNA, determined by a premixed assay on demand for human 18S rRNA (PE Applied Biosystems, Foster City, CA). Amplification of specific transcripts was confirmed by melting curve profiles at the end of each PCR. Specificity of PCR was verified by subjecting the amplification products to agarose gel electrophoresis.

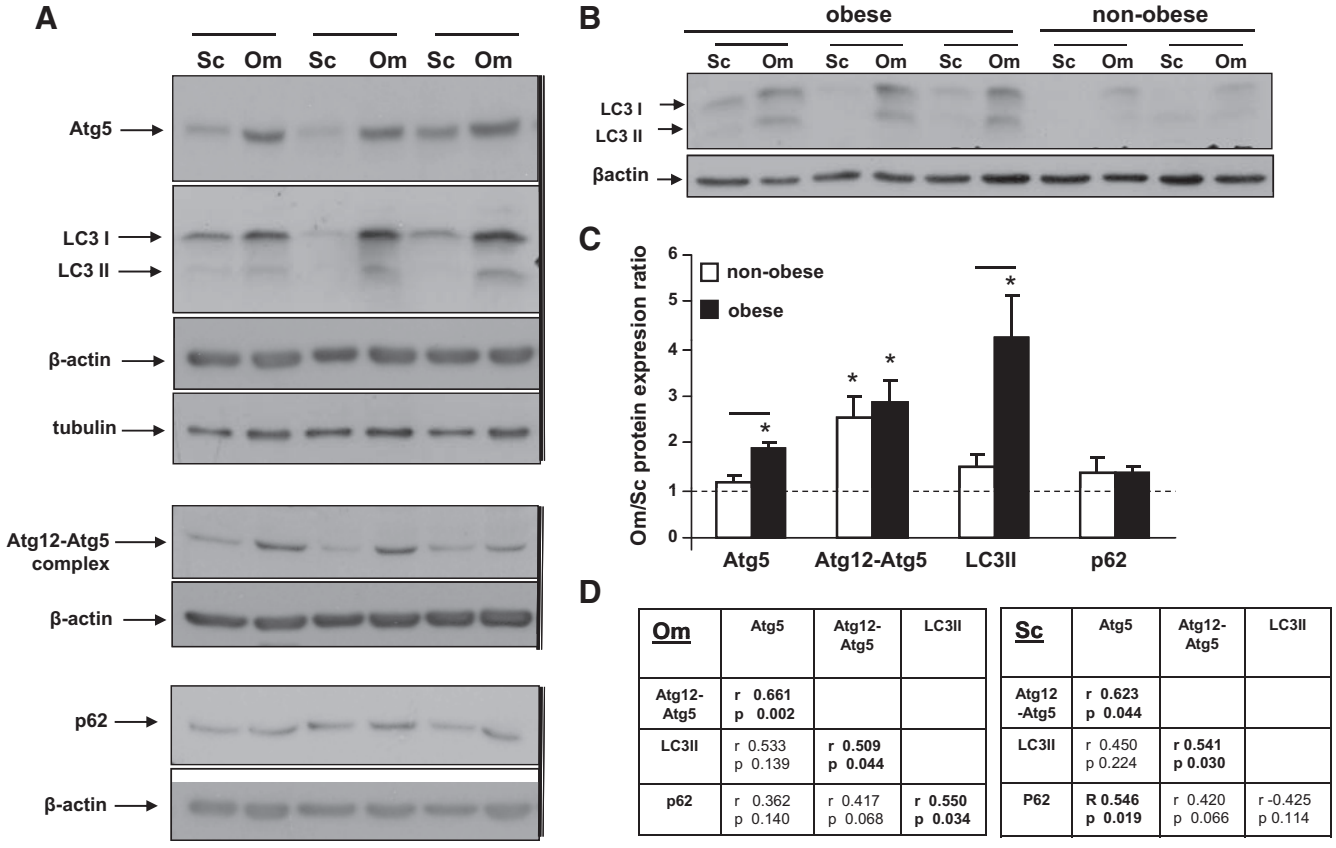
Statistical analyses

We used a group *t* test to compare variables within subgroups of cohorts 1 and 3 and an ANOVA in which all comparisons among the three groups of cohort 2 (lean, SC obese, IA obese) were performed using Tukey's Studentized range test. We used a paired *t* test to compare Sc and Om fat tissue biomarkers. We performed Spearman's test to analyze intercorrelations between the nonnormally distributed protein levels of the three proteins in each fat depot, between p62 ratio in Om fat and BMI, and used age-adjusted correlations for the correlation matrix with the mRNA levels of *Atg5*, *LC3A*, and *LC3B* (Table 1, Fig. 1D). To evaluate the factors most strongly associated with levels of *Atg5* mRNA in either Sc or Om fat, we performed stepwise multivariate regression analysis wherein the various optional confounders were introduced to the model. We estimated how well future outcomes are likely to be predicted by the model by the adjusted R^2 level. All analyses were performed with SPSS-17 statistical package program (SPSS Inc., Chicago, IL).

Results

Evidence for increased autophagic flux in human adipose tissue in obesity

Using three cohorts from previously described adipose tissue biobanks of paired Sc and Om human adipose tissue samples obtained during elective abdominal surgery (9–11, 16), we first assessed the level of expression of key autophagy genes. We assessed the protein levels of *Atg5*, *Atg12-Atg5* complex, *LC3* (*Atg8*), and p62, a protein predominantly degraded by autophagy. In obese persons (BMI ≥30 kg/m^2), Om fat exhibited higher protein levels of *Atg5*, *Atg12-Atg5* complex, and the lipidated/cleaved form of *LC3* (LC3II) compared with Sc fat (Fig. 1A). In nonobese persons, although Om exhibited higher LC3II than Sc, the levels were lower than in obese persons and among the proteins measured only *Atg12-Atg5* complex was significantly higher in Om compared with Sc (Fig. 1, B and C). Intercorrelations between the levels of expression of these authophagy-related proteins revealed significant correlations in both fat depots (Fig. 1D), suggesting that their expression is coregulated. We did not detect significant differences in the expression of these autoph-



D

Om	Atg5	Atg12-Atg5	LC3II
Atg12-Atg5	r 0.661 p 0.002		
LC3II	r 0.533 p 0.139	r 0.509 p 0.044	
p62	r 0.362 p 0.140	r 0.417 p 0.068	r 0.550 p 0.034

Sc	Atg5	Atg12-Atg5	LC3II
Atg12-Atg5	r 0.623 p 0.044		
LC3II	r 0.450 p 0.224	r 0.541 p 0.030	
P62	R 0.546 p 0.019	r 0.420 p 0.066	r -0.425 p 0.114

FIG. 1. Protein levels of autophagy genes and related proteins in human Sc and Om fat. Representative Western blots of Atg5, LC3, Atg12-Atg5 complex, and p62 in paired Sc and Om human adipose tissue biopsies of obese persons (A) or obese and nonobese (BMI <30 kg/m^2) (B). C, Results of densitometry analyses of the ratio of protein expression of each protein between Om and Sc fat. *, Om/Sc ratio significantly different than 1 ($P < 0.05$) (as indicated by the *broken line*). D, The result of intercorrelations between the protein levels of the four proteins in each fat depot (n = 21–51).

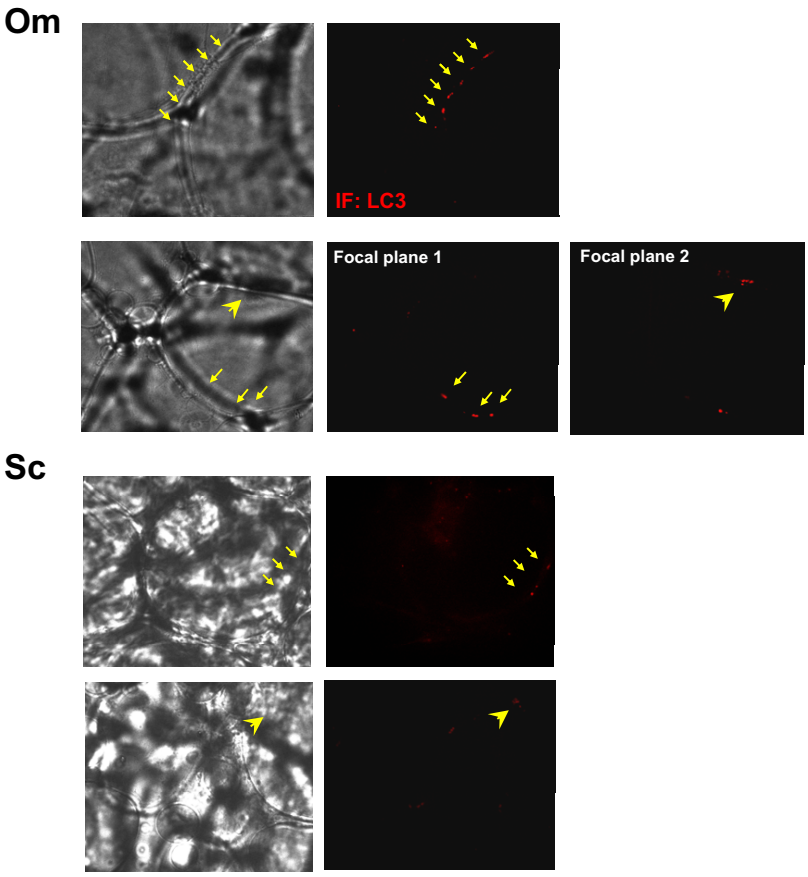


FIG. 2. LC3-positive dots in human adipose tissue. Human adipose tissue biopsies were fixed and subjected to indirect immunofluorescence (IF) analysis of LC3 using a laser confocal microscope, as detailed in the *Materials and Methods*. Shown are representative bright-field and fluorescence images of Om and Sc adipose tissue from an obese person. *Small arrows*, LC3 dots in adipocytes; *arrowheads*, LC3 dots in nonadipocytes. Additional images of Sc and Om adipose tissue from an overweight person, without and with incubation with bafilomycin A1, is provided in the supplemental material (Supplemental Fig. 1).

agy proteins between males and females nor between persons without or with type 2 diabetes (not shown). Immunofluorescence microscopy was used to assess whether *LC3* dots, a commonly used approach to image autophagosomes, are present in human adipose tissue (Fig. 2). *LC3*-positive dots could be observed in adipose tissue explants from obese persons, in both adipocytes and smaller, non-lipid-laden cells within adipose tissue. In both cell types, more dots were observed in Om compared with Sc fat.

Because cargo proteins and proteins participating in autophagosome generation (like LC3) are degraded during autophagy, increased levels of these proteins may either signify increased autophagosome production and a resulting elevated autophagic flux, or conversely, inhibition of this process that in turn stabilizes the autophagy-related proteins. To differentiate between these two possibilities, we used two approaches: 1) assessment of the effect of autophagy/lysosomal inhibitors; 2) determination of mRNA levels of autophagy genes in adipose tissue to ascertain whether increased protein levels is mirrored by increased gene expression.

In the first approach, assessing autophagic flux requires the determination of the effect of autophagy/lysosomal inhibitors on the expression of specific proteins that are degraded during the autophagy process: if autophagy is attenuated, inhibitors should have a small, if any, effect on such proteins. If, however, the steady-state levels of these proteins are increased due to enhanced production of autophagosomes, the level should increase even further after exposure to inhibitors. p62 is a protein almost exclusively degraded by autophagy and is thus commonly used to assess autophagy flux. Whereas its steady-state level in Om and Sc in both obese and nonobese persons were similar (Fig. 1), when adipose tissue explants were incubated with bafilomycin A1 and leupeptin that inhibit autophagosome acidification and lysosomal proteases, respectively, increased p62 levels were readily observable (Fig. 3, A and B). Similar effects were discernable with NBR1, another protein degraded by autophagy, and with LC3II, which participates in autophagosome generation but is also partially degraded during autophagy (LC3II that is located within the autophagosome lumen, Fig. 3A). These findings are all suggestive of an active autophagic process in human adipose tissue (17). The result with LC3 was further substantiated by demonstrating increased LC3-positive dots in adipose tissue explants incubated with bafilomycin A1 (Supplemental Fig. 1). Remarkably, in Sc fat, but significantly more so in Om, obese persons exhibited a larger increase in p62 on incubation of tissue explants with autophagy inhibitors compared with nonobese persons (Fig. 3B). Consistently, the fold increase in p62 upon exposure to autophagy inhibitors in Om correlated with the degree of visceral adiposity characterized by BMI (Fig. 3C) and WC ($r = 0.449$, $P = 0.035$). These observations are consistent with an elevated autophagic flux in human adipose tissue in obesity, which is higher in Om than Sc fat.

In the second approach, mRNA levels of autophagy genes were shown to correlate with increased autophagic flux in a variety of experimental systems (18–20). To gain further insight into autophagy regulation in relation to fat distribution, we used a large ($n = 196$) cohort in whom fat distribution was assessed by CT. Consistent with the dy-

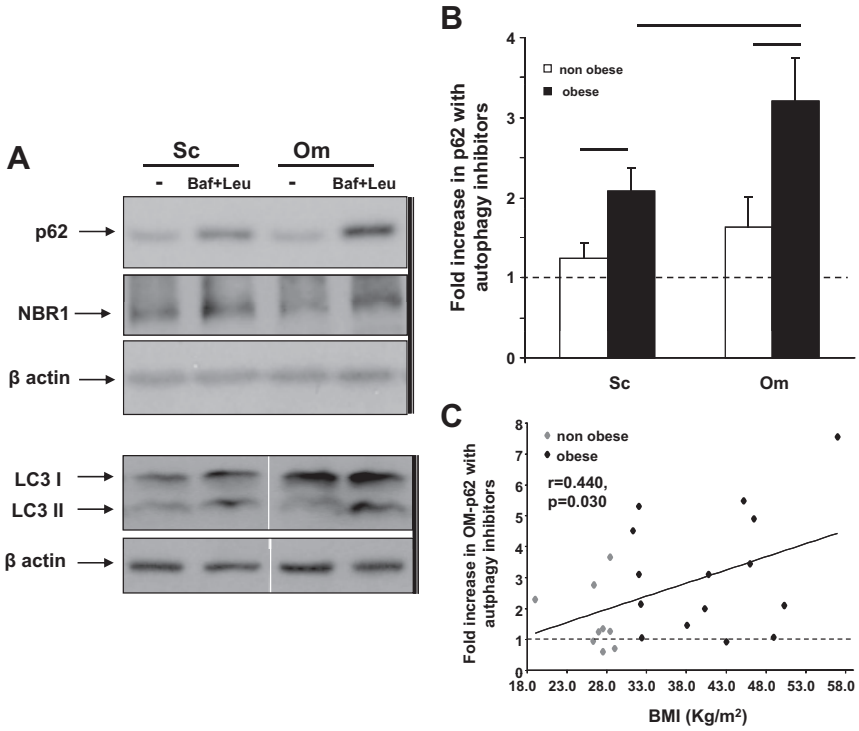


FIG. 3. Dynamic analyses of autophagic flux in human adipose tissue explants using autophagy/lysosomal inhibitors. A, Human adipose tissue biopsies were minced and incubated as explants without or with bafilomycin A1 and leupeptin (Baf+Leu), after which Western blot analyses were performed to assess the amount of p62 and NBR1, two proteins predominantly degraded by autophagy, and LC3. Blots are representative of 25 independent analyses. *Vertical white line* in the LC3 blot denotes image splicing to present bands in the same order as p62 and NBR1 for clarity (shown is a single blot). B, Densitometry analyses of p62 expressed as the ratio of p62 expression between samples incubated with *vs.* without inhibitors. A value of 1 was assigned to the intensity of the p62 band in the respective samples that were incubated without inhibitors. *Lines*, $P < 0.05$. C, Spearman's correlation analysis between p62 ratio in Om fat and BMI.

namic studies of autophagic flux, mRNA levels of *Atg5*, *LC3A*, and *LC3B* were significantly higher in Om than Sc fat. This was evident in lean persons, as well as in obese persons with a predominantly Sc or IA fat accumulation (Fig. 4). Intriguingly, the most robust change in the autophagy genes tested was observed in Om of predominantly IA obese persons. As with the protein levels, we did not detect differences in mRNA levels of these gene products between men and women, and only nonsignificant elevation was observed in persons with type 2 diabetes compared with nondiabetics (data not shown).

Obesity is associated with changes in the cellular composition of adipose tissue, as exemplified by the infiltration of macrophages, which is higher in Om particularly in IA obese persons (11). Thus, to verify that the expression of adipose tissue autophagy genes occurs in adipocytes and is not just contributed by the nonadipocyte components of adipose tissue (fibroblasts, endothelial cells, immune cells), adipose tissue was collagenase digested, and adipocytes were separated from the stromal-vascular fraction using established protocols (21). Adipocytes had higher relative mRNA expression of *Atg5*, *LC3A*, and *LC3B* than stroma vascular fraction (Supplemental Fig. 2A). Similarly, the protein levels of LC3B were higher in adipocytes than in stroma vascular fraction (Supplemental Fig. 2B). These results suggest that adipocyte autophagy gene expression at both the mRNA and protein level is a major, although not exclusive, contributor to overall adipose tissue expression of these genes.

Association of autophagy gene expression in human adipose tissue with obesity-related phenotypes

To gain further insight into the potential associations between elevated autophagy gene expression and obesity-related phenotypes, we undertook two complementary approaches. In the first approach, we used correlation and regression analyses to estimate the strongest associations between autophagy gene expression and obesity-related phenotypes or adipose tissue alterations. In the second approach, we measured expression of autophagy genes in adipose tissues of severely obese persons, matched for being either insulin sensitive or insulin resistant (cohort 3).

Age-adjusted correlation matrix with the mRNA levels of *Atg5*, *LC3A*, and *LC3B* revealed highly significant positive correlations between *Atg5* and BMI, adipocyte diameter, insulin resistance, circulating lipids and free fatty acids, and adipocytokines (Table 1). The correlations were occasionally stronger in Om but were also discernable and highly significant in Sc. Correlations with *LC3A* or *LC3B* were generally less robust but overall in the same direction as with *Atg5*. We therefore tried to assess which factors most strongly associate with levels of *Atg5* mRNA expression using stepwise multivariate regression analysis (Table 2). In addition to age and sex, we introduced parameters into the model that represent factors that could potentially induce autophagy in adipose tissue: 1) the visceral fat area determined from the CT scans (visceral adiposity); 2) adipocyte diameter (cell hypertrophy); 3) circulating IL-6 (systemic inflammation); 4) the GIR during hyperinsulinemic-euglycemic clamp (whole body insulin sensitivity); and 5) mRNA levels of the MAP3K *ASK1* [composite indicator of adipose tissue stress (10)]. In this multivariate

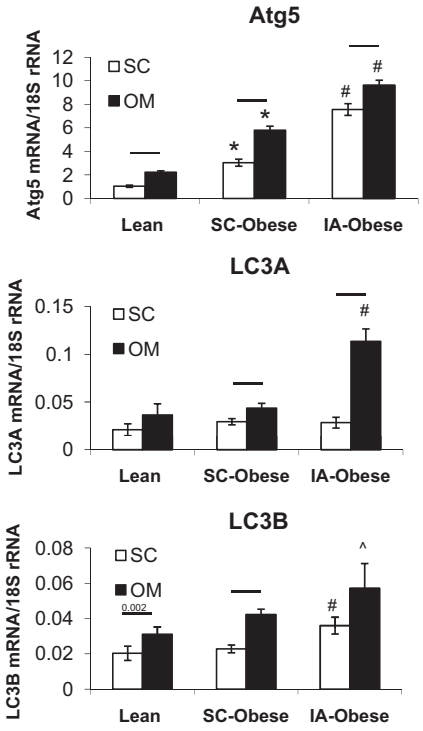


FIG. 4. mRNA levels of autophagy genes in human Sc and Om fat according to obese state and fat distribution. *Atg5*, *LC3A*, and *LC3B* mRNA levels were measured in paired Om and Sc fat tissue samples from lean persons, obese (BMI ≥30 kg/m^2) with predominant sc fat accumulation (Sc obese) or with predominantly intraabdominal fat accumulation (IA obese). Patients' characteristics are shown in Supplemental Table 1B. *, $P < 0.001$ *vs.* Sc or Om in lean; #, $P < 0.001$ *vs.* Sc or Om in lean and in Sc obese; ^, $P < 0.001$ *vs.* Om in lean only. *Line*, $P < 0.001$ between Om and Sc in the same group (unless different *P* is indicated).

model in Om, only the degree of visceral adiposity and adipocyte cell diameter remained as significant variables associated with *Atg5* mRNA, whereas other variables were excluded from the final predictive model. This suggests that visceral adiposity *per se* and visceral adipocyte hypertrophy are the strongest independent parameters associated with increased levels of *Atg5* mRNA, markedly attenuating the independent association between *Atg5* expression and age, sex, insulin resistance, inflammation, and fat cell stress, some of which correlated significantly in the univariate analysis (Table 1). Jointly, visceral adiposity and omental fat hypertrophy could explain approximately 50% of the variance (adjusted R^2 of the model) in *Atg5* mRNA expression in Om. In Sc fat adipocyte diameter was also an independent predictor of *Atg5* expression along with IL-6, whereas insulin resistance and *ASK1* expression nearly reached statistical significance, and this model collectively explained 18% of the variability (Table 2).

Finally, we thought to gain further insight into the possible role of increased autophagy in adipose tissue in the natural history of obesity-related morbidity by using a subcohort of severely obese persons who are nondiabetic, without a family history of diabetes nor with any severe obesity-related comorbidities and who were age and sex matched for insulin sensitivity or resistance (based on euglycemic-hyperinsulnemic clamp studies) (13). Remarkably, Om level of expression of *Atg5*, *LC3A*, and *LC3B* significantly differed from those in Sc (Fig. 5). Insulin-resistant obese persons exhibited a greater increase in the expression of *Atg5* and both *LC3*s in their Om fat. These results suggest that increased expression of autophagy genes, particularly in Om, is already apparent in obesity when whole-body insulin resistance has developed but is independent of (and potentially precedes) the development of obesity-related morbidity.

Discussion

In this study we demonstrate that human adipose tissue and adipocytes express autophagy genes and contain autophagosomes and that protein levels of autophagy markers are increased in obesity. The degree of (intraabdominal) fat accumulation and fat cell hypertrophy are the factors that most strongly associate with autophagy gene expression, and altered autophagy is accentuated when obesity-related insulin resistance had already developed but before cardiovascular and/or metabolic derangement ensued. Increased expression of autophagy markers in obesity appears to be sex independent and not significantly different in obese subjects with and without diabetes. However, it is clearly more evident in omental fat than in Sc adipose tissue, particularly when fat accumulation is predominantly intraabdominal.

Clearly, the most important questions raised by this study are what is the functional consequence of the observed altered autophagy gene expression in human obesity: 1) on autophagy itself; and 2) on whole-body (patho)physiology. The clinical observational design of this study poses inherent limitations to the ability to conclusively answer these important questions and determine cause-effect relationships between autophagy and obesity-related disease processes. Nevertheless, it is clear that steady-state expression of key autophagy genes is increased in human adipose tissue in obesity and that adipocytes and not solely nonadipocyte components of adipose tissue contribute to this altered expression. Very recently impaired autophagy with decreased expression of autophagy genes has been shown in mouse models of obesity in the liver and tied to insulin resistance (22). Our results in humans and a recent study in mice (23) suggest that obesity alters autophagy differently in adipose tissue than it does in the liver: first, gene expression of *Atg5* and the two human *LC3* genes are increased in adipose tissue

TABLE 2. Stepwise multivariate regression analysis of association between expression of *Atg5* and age, adiposity (fat area), fat cell hypertrophy (adipocyte diameter), IS (GIR), adipose tissue stress (*ASK1* mRNA), and systemic inflammation (IL-6)

	Atg5 mRNA		
	β-Coefficient	**Significance (*P*)**	**Adjusted R^2 for model**
Omental			
Visceral fat area	**0.582**	**<0.001**	**0.488**
Adipocyte diameter	**0.173**	**0.030**	
Excluded variables in final model			
Age	0.075	0.226	
Sex	0.067	0.274	
GIR	0.005	0.938	
Om-*Ask1* mRNA	0.040	0.680	
IL-6	−0.069	0.351	
Subcutaneous			
IL-6	**0.288**	**0.001**	**0.180**
Adipocyte diameter	**0.231**	**0.007**	
Excluded variables in final model			
Age	−0.003	0.965	
Sex	−0.091	0.240	
Subcutaneous fat area	−0.071	0.384	
GIR	−0.154	0.068	
SC-*Ask1* mRNA	0.156	0.066	

Bold values relate to variables that remained independent predictors of *Atg5* mRNA expression.

in obesity. In contrast to the observations in mouse liver (22), these changes in fat tissue correspond to elevated protein levels of Atg5, Atg12-Atg5 complex, and the active form of LC3 (LC3II), similar to the observations in *ob/ob* mice (23). Moreover, in high-fat-fed mice, we observed that although LC3II is indeed decreased in liver, it tends to be elevated in periepididymal fat pads (data not shown). In addition, our results suggest that autophagic flux is increased along with the higher protein levels of autophagy-related genes: increased mRNA levels of these proteins were shown in several different biological systems to associate with increased autophagy flux (18–20). Moreover, dynamic studies with autophagy/lysosomal inhibitors (Fig. 3) also support elevated autophagy flux, although it is important to acknowledge that adipose tissue explants may suffer from *ex vivo* stresses that could introduce various artifacts. Thus, although elevated protein levels of autophagy-related genes might signify inhibited autophagy, it seems plausible that in human obesity autophagy flux is enhanced in adipose tissue, particularly in intraabdominal fat and in relation to the degree of obesity (Fig. 3C) and to fat distribution. This is now also supported by a very recent study that will be discussed below (24).

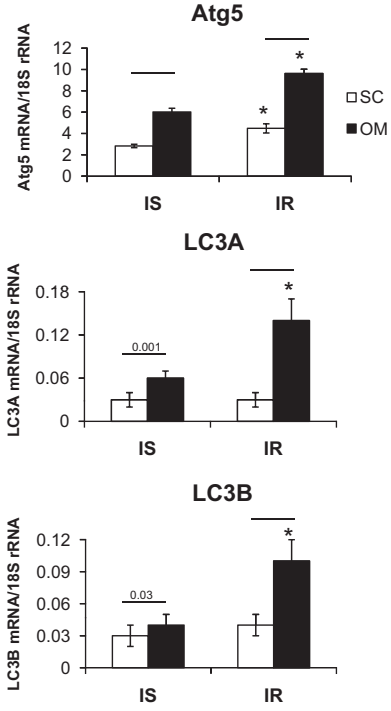


FIG. 5. mRNA levels of autophagy genes in severely obese insulin-sensitive *vs.* insulin-resistant humans (cohort 3). mRNA levels of *Atg5*, *LC3A*, or *LC3B* were measured as in Fig. 4 in Sc or Om adipose tissue biopsies of severely obese persons (BMI ~45 kg/m^2) who were either IS or IR based on GIR measurements during euglycemic-hyperinsulinemic clamp studies, matched for age, sex, and BMI [cohort 3, (13)]. Patients' characteristics are shown in Supplemental Table 1C. *Line*, $P < 0.001$ (unless otherwise specified) between Sc and Om. *, $P < 0.05$ compared with the respective depot in IS.

The functional significance of this finding at the whole-body level is the second key question. We could demonstrate using expression of key autophagy genes as the putative indicators of adipose tissue autophagy that the strongest associating factors with Om fat autophagy are the degree of visceral adiposity *per se* and fat cell hypertrophy (Table 2). Jointly, these two parameters could account for approximately 50% of the variance in expression of *Atg5*. Parameters that indicated the degree of systemic inflammation, adipose tissue stress, or insulin resistance were excluded from the multivariate regression model, although they correlated with autophagy gene expression in univariate analyses (Table 1). This is of interest

because inflammatory cytokines and various cellular stresses including hypoxia and endoplasmic reticulum stress that were shown to be elevated in obesity can directly elevate autophagy (20). Moreover, insulin is a major physiological inhibitor of autophagy, making insulin resistance a potential activator of this process. Indeed, insulin resistance in obese diabetic persons was shown to be associated with elevated adipocyte autophagy in Sc tissue, attributed to attenuated mammalian target of rapamycin (24). Nevertheless, the fact that these factors have been excluded from the multivariate model may suggest that they may not be independent activators, or consequences, of elevated autophagy in adipose tissue in human obesity. Interestingly, however, elevated autophagy gene expression, at least in Om, occurs along with obesity-related insulin resistance, even when other morbid manifestations of severe obesity have not occurred, as exemplified by the matched cohort 3 of insulin-sensitive *vs.* -resistant obese persons (Fig. 5). This cooccurrence of insulin resistance and increased autophagy gene expression may suggest that these two factors correlate but develop and/or contribute independently of each other and before the development of obesity-related diseases, such as type 2 diabetes or cardiovascular diseases.

Finally, it is important to acknowledge the double-edged sword view of autophagy as a homeostatic process necessary for cell survival on the one hand but as a mechanism for cell death on the other hand. In that sense, activated autophagy may represent a survival coping mechanism of (hypertrophied) adipocytes and/or stromal-vascular cells in the challenging environment that develops in the adipose tissue in obesity. Conversely, it is also possible that increased autophagy signifies a process underlying increased cell death of hypertrophied adipocytes (25). Future studies will have to address these possibilities and reflect on the potential therapeutic value of manipulating adipocyte autophagy in established obesity.

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