The ghrelin *O*-acyltransferase-ghrelin system reduces TNF-α-induced apoptosis and autophagy in human visceral adipocytes

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ABSTRACT

Proinflammatory and proapoptotic cytokines, such as TNF-*α*, are up-regulated in human obesity. We evaluated the association of ghrelin isoforms (acylated and desacyl ghrelin) and TNF-α in obesity and obesity-associated type 2 diabetes (T2D) in 194 subjects as well as the potential role of ghrelin in the control of apoptosis and autophagy in human adipocytes. Circulating concentrations of acylated ghrelin and TNF-α were increased, whereas desacyl ghrelin levels were decreased in obesity-associated T2D. Ghrelin and GOAT were expressed in both omental and subcutaneous adipose tissue. Obese T2D patients showed higher expression of GOAT in visceral adipose tissue, without changes in ghrelin expression. Visceral adipose tissue from obese patients with T2D showed increased adipocyte apoptosis as well as increased expression of autophagy-related genes *ATG5, BECN1* and *ATG7*. In differentiating human omental adipocytes, incubation with both acylated and desacyl ghrelin reduced the TNF-α-induced activation of caspase-8, caspase-3 and cell death. Additionally, acylated ghrelin reduced the basal expression of the autophagy-related molecules *ATG5* and *ATG7*, while desacyl ghrelin inhibited the TNF-α-induced increased expression in *ATG5, BECN1* and *ATG7*. Apoptosis and autophagy are up-regulated in visceral adipose tissue of patients with T2D. Acylated and desacyl ghrelin reduce TNF-α-induced apoptosis and autophagy in human visceral adipocytes.

Keywords: Ghrelin • Obesity • Type 2 diabetes • Visceral adipocytes • Tumor necrosis factor α • Apoptosis • Autophagy

Ghrelin, a 28 amino-acid peptide with an *n*-octanoyl group at the serine 3 residue, is the endogenous ligand of the growth hormone secretagogue receptor (GHS-R) (1). Ghrelin is synthesized predominantly in X/A cells of the oxyntic glands in the mucosa layer of the gastric fundus, and secreted to the bloodstream as two major forms, acylated (~5% of total ghrelin) and desacyl ghrelin (the form lacking *n*-octanoylation and representing ~95% of total ghrelin) (1-3). The porcupine-like enzyme ghrelin *O*-acyltransferase (GOAT) catalyzes the octanoylation of ghrelin in the endoplasmic reticulum (ER) (3, 4) and belongs to the family of membrane-bound *O*-acyltransferases (MBOAT) (4). The expression sites of GOAT in rodents include stomach, intestine, colon and testis, while in humans it is mainly expressed in stomach, intestine and pancreas (3-5). Acylated and desacyl ghrelin increase adiposity and body weight through central and peripheral mechanisms (5). On the one hand, ghrelin increases food intake through the activation of hypothalamic neuropeptide Y/agouti related peptide neurones expressing GHS-R type 1a via the modulation of the fatty acid metabolism (6). On the other hand, adipose tissue also constitutes an important target for the adipogenic actions of ghrelin in rodents and humans (7, 8). The ghrelin (*GHRL*) gene expression increases during adipogenesis with preproghrelin knockdown reducing insulin-mediated adipogenesis in 3T3-L1 adipocytes (8). Moreover, acylated and desacyl ghrelin directly stimulate the expression of several fat storage-related proteins, including acetyl-CoA carboxylase, fatty acid synthase, lipoprotein lipase and perilipin in human visceral adipocytes, thereby stimulating intracytoplasmic lipid accumulation (7, 9).

The excessive expansion of the adipose tissue during the onset of obesity results in activation of death receptors and mitochondrial pathways, leading to the activation of effector caspases and adipocyte apoptosis (10, 11). Other types of cell death, namely autophagy, have been recently described to be altered in adipose tissue in obesity (12). Autophagy represents a dynamic self-degradative process characterized by sequestering cytosolic organelles and proteins in double-membrane vesicles, termed autophagosomes, that translocate to lysosomes for fusion and content degradation (13). The proteins encoded by autophagy-related genes (ATG) are required for the formation of autophagic vesicles. Given the anabolic properties of ghrelin in other tissues (5), it seems plausible that ghrelin operates as a survival factor to prevent obesity-associated apoptosis and autophagy in human adipocytes. Therefore, the aim of the present study

was to characterize the expression and regulation of the ghrelin-GOAT system in human adipose tissue as well as to analyze the effect of acylated and desacyl ghrelin on basal and tumor necrosis factor α (TNF-α)-induced apoptosis and authophagy in human visceral adipocytes.

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RESEARCH DESIGN AND METHODS

For detailed Research Design and Methods see **Online Supplemental Data**.

Patient selection and study design. The study of plasma acylated and desacyl ghrelin was performed in a population of 194 participants (111 men and 83 women) recruited from healthy volunteers and patients attending the Departments of Internal Medicine and Endocrinology & Nutrition of the Clínica Universidad de Navarra (Pamplona, Spain) for a medical check-up. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Obesity was defined as a BMI≥30 kg/m^2, and normal weight as a BMI<25 kg/m^2. Obese patients were sub-classified into three groups [normoglycemia (NG), impaired glucose tolerance (IGT) or type 2 diabetes (T2D)] following the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes (14). T2D subjects were not on insulin therapy or medication likely to influence endogenous insulin levels. The clinical characteristics of the subjects are shown in **Table 1.**

In addition, the presence of ghrelin and GOAT as well as the effect of the ghrelin forms was assessed in paired omental and subcutaneous adipose tissue samples from 53 patients undergoing either laparoscopic Roux-en-Y gastric bypass (n=43) or Nissen funduplication (n=10) at the Clínica Universidad de Navarra. Clinical characteristics of this cohort were essentially similar to the cohort for the study of circulating ghrelin isoforms (**Supplemental Table 1**). All reported investigations were carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008. The experimental design was approved, from an ethical and scientific standpoint, by the Hospital's Ethical Committee responsible for research (028/2009) and the informed consent from all volunteers was obtained.

Analytical procedures. Biochemical and hormonal assays performed in the study subjects were measured as previously described (7, 15) (for details see supplementary information).

RNA extraction and real-time PCR. RNA isolation and purification were performed as described earlier (7, 15). Transcript levels for ghrelin (*GHRL*), GOAT (*MBOAT4*) and autophagy-related genes ATG5 (*ATG5*), Beclin-1/ATG6 (*BECN1*) and ATG7

(*ATG7*) were quantified by real-time PCR (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). Primers and probes (**Supplemental Table 2**) were designed using the software Primer Express 2.0 (Applied Biosystems).

Western-blot studies. Samples (30 μg) were run out in 10% SDS-PAGE, transferred to a nitrocellulose membrane and blocked in Tris-buffer saline (TBS) with 0.05% Tween 20 (TBS-T) containing 5% non-fat dry milk (ghrelin, GOAT, β-actin) or 5% BSA (caspases) for 1 h at room temperature (RT). Blots were then incubated overnight at 4 ºC with primary antibodies diluted in blocking solution. The antigen-antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibodies for 1 h at RT diluted in blocking solution and the enhanced chemiluminescence ECL Plus detection system (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry of ghrelin and GOAT. The immunodetection of ghrelin and GOAT in histological sections of omental and subcutaneous adipose tissue was performed by the indirect immunoperoxidase method (7). Negative control slides without primary antibody were included to assess non-specific staining.

Confocal immunofluorescence microscopy of ghrelin and GOAT. Differentiated 3T3-L1 adipocytes grown on glass coverslips were incubated with rabbit polyclonal anti-ghrelin (Alpha Diagnostic International, San Antonio, TX, USA) or anti-GOAT (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies diluted 1:50 (ghrelin) or 1:250 (GOAT) in blocking solution overnight at 4 ºC. Thereafter, cells were washed with PBS and incubated with Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (Invitrogen, Paisley, UK) diluted 1:500 in blocking solution for 2 h at RT. After washing, coverslips were mounted on microscope slides and examined under a TCS-SP2-AOBS confocal laser scanning microscope (Leica Corp., Heidelberg, Germany).

Cell cultures. Human stromovascular fraction cells (SVFC) were isolated from omental adipose tissue from obese normoglycemic subjects and differentiated as previously described (7). Differentiated human omental adipocytes were serum-starved for 24 h and then treated with increasing concentrations of insulin (Sigma, Steinhein, Germany), leptin (PeproTech EC, Inc., Rocky Hill, NJ, USA), TNF-α (PeproTech), acylated ghrelin (Tocris, Ellisville, MO, USA) or desacyl ghrelin (Tocris) for 24 h. In a subset of

experiments quiescent cells were incubated with TNF-α (100 ng/mL) in the presence or absence of acylated ghrelin (100 pmol/L) or desacyl ghrelin (100 pmol/L) for 24 h.

TUNEL assay. The TUNEL assay was performed using the *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics Corp., Indianapolis, IL, USA) following the manufacturer's instructions. TUNEL-positive cells were analyzed under the light microscope.

Statistical analysis. Data are expressed as mean ± SEM. Statistical differences between mean values were determined using Student's t test, χ^2 test, and one-way ANOVA followed by Scheffé's or Dunnet's tests, where appropriate. A P value <0.05 was considered statistically significant. In the analysis of Pearson's correlation coefficients (r), Bonferroni's correction was applied when multiple comparisons were performed simultaneously, establishing the significance level at P=0.003.

RESULTS

Plasma acylated and desacyl ghrelin as well as TNF-α concentrations in obesity and obesity-associated type 2 diabetes. Obese subjects showed increased circulating concentrations of acylated ghrelin (obese 5.1±0.3, lean 3.9±0.3 pmol/L, *P*=0.013) and decreased circulating concentrations of desacyl ghrelin (obese 90.9±4.7, lean 168.1±15.6 pmol/L, *P*<0.001) compared with lean individuals. Furthermore, acylated ghrelin levels were higher (*P*<0.01) in obese individuals with T2D (6.7±0.7 pmol/L) compared with obese NG (4.6±0.3 pmol/L) and IGT (4.3±0.5 pmol/L) patients (**Fig. 1A**). No effect of glucose intolerance or diabetes was observed on circulating desacyl ghrelin concentrations (*P*=0.999). Plasma desacyl ghrelin concentrations were increased in women compared with men in the lean group (women 192.5±13.6, men 133.3±13.8 pmol/L, *P*<0.001) as well as in the obese group (women 104.3±12.1, men 88.6±9.4 pmol/L, *P*<0.001). This sexual dimorphism was neither found in plasma acylated ghrelin of the lean subjects (women 3.8±0.4, men 4.2±0.5 pmol/L, *P*=0.171) nor in the obese patients (women 4.7±0.4, men 5.3±0.3 pmol/L, *P*=0.261).

T2D was associated with increased circulating levels of well-known inflammatory markers, including TNF-α (**Table 1**). Interestingly, a highly significant positive correlation was observed between acylated ghrelin and TNF-α, whereas desacyl ghrelin showed a strong negative correlation with this proinflammatory cytokine (**Fig. 1B**).

Localization and expression of ghrelin and GOAT in human adipose tissue. To analyze whether adipose tissue constitutes an expression site of ghrelin and its acylating enzyme GOAT, the presence of both molecules in biopsies of omental and subcutaneous adipose tissue of lean and obese patients was analyzed by real-time PCR and western-blot. Visceral adipose tissue of obese subjects showed similar gene expression of ghrelin (P=0.800) but increased transcript levels of GOAT (*P*<0.01) compared with lean individuals (**Fig. 2A**). Furthermore, transcript levels of GOAT were higher (*P*<0.05) in obese individuals with T2D (12.86±3.13 A.U.) compared with those of obese NG patients (1.96±0.78 A.U.). No differences were found in the subcutaneous fat *GHRL* (P=0.444) and *MBOAT4* (P=0.994) mRNA levels between the lean and obese groups (**Fig. 2B**). The protein expression of ghrelin and GOAT in both omental and

subcutaneous fat depots exhibited a similar pattern to that observed in the gene expression analyses (**Fig. 2C and 2D**). A positive correlation between *MBOAT4* mRNA levels in omental adipose tissue and serum LDL-cholesterol (r=0.71, P=0.002) was found.

To sort out the cell type responsible for ghrelin and GOAT expression, the presence of both molecules in omental adipose tissue was evaluated by immunohistochemistry and real-time PCR. A positive black staining for ghrelin and GOAT was observed in fully mature adipocytes as well as in the SVFC of both omental and subcutaneous fat depots of obese patients (**Fig. 2E and 2F**). Adipocytes and SVFC showed similar transcript levels of ghrelin and GOAT in omental (*GHRL*: 1.00±0.67 *vs.* 0.38±0.26 A.U., P=0.415; *MBOAT4*: 1.00±0.40 *vs.* 0.70±0.43 A.U., P=0.333) and subcutaneous (*GHRL*: 1.00±0.45 *vs.* 1.52±0.36 A.U., P=0.130; *MBOAT4*: 1.00±0.53 *vs.* 0.82±0.33 A.U., P=0.531) adipose tissue. In order to gain more insight into the localization of ghrelin and GOAT, the subcellular localization of both proteins was studied in differentiated 3T3-L1 adipocytes by confocal immunofluorescence microscopy. Under basal conditions, ghrelin showed a yuxtanuclear immunolocalization as well as a positive staining in small secretion vesicles next to the plasma membrane, whereas GOAT was present in perinuclear vesicle structures in the cytoplasm of adipocytes (**Fig. 2G**).

Regulation of ghrelin and GOAT in human omental adipocytes. Based on the fact that the expression of GOAT was increased in omental adipose tissue and given the relevance of this fat depot in obesity-associated metabolic disturbances (16), the subsequent experiments were focused on this fat depot. To gain insight into the regulation of ghrelin and GOAT, the role of insulin and leptin, two hormones tightly associated to obesity and obesity-associated T2D and with opposite effects on fat deposition, and TNF-α, a proinflammatory cytokine, was analyzed as plausible regulators of the gene expression of *GHRL* and *MBOAT4* in human omental adipocytes (**Fig. 3A-C**). *GHRL* gene expression was reduced (P<0.05) by increasing concentrations of insulin and leptin, while *GHRL* expression levels augmented (P<0.05) in response to TNF-α in human visceral fat cells. *MBOAT4* transcript levels increased (P<0.05) in response to insulin, while neither leptin nor TNF-α (P=0.399 and P=0.511, respectively) modified them. The self-regulation of ghrelin in human visceral fat cells

was also tested (**Fig. 3D-E**). *GHRL* gene expression was down-regulated (P<0.01) by desacyl ghrelin and up-regulated (P=0.019) by acylated ghrelin. *MBOAT4* transcript levels in human visceral adipocytes were significantly increased (P<0.01) by acylated ghrelin treatment at all the concentrations tested.

Acylated and desacyl ghrelin prevent TNF-α-induced apoptosis in human visceral adipocytes. TUNEL assays were initially performed to analyze adipocyte apoptosis in human omental adipose tissue of a well-characterized group of lean and obese subjects. Adipocyte apoptosis was markedly increased (P<0.0001) in the visceral adipose tissue of obese subjects (**Fig. 4A**), which is in agreement with previous reports (11). Quantification of TUNEL-positive cells showed that adipocyte apoptosis was further increased (P<0.05) in adipose tissue of obese T2D patients compared to obese NG subjects.

We evaluated the effect of acylated and desacyl ghrelin on TNF-α-induced caspase activation, by the appearance of cleaved caspase-8 and caspase-3, while apoptosis was explored by detecting DNA fragmentation with the TUNEL assay. As expected, the incubation of human visceral fat cells with different concentrations of TNF-α for 24 h significantly (P<0.001) increased the activation of caspase-8, caspase-3 and apoptotic cell death (**Fig. 4B**). The co-incubation with either desacyl ghrelin (100 pmol/L) or acylated ghrelin (100 pmol/L) significantly reduced the cleavage of caspase 3 as well as the apoptotic indices induced by TNF-α (100 ng/mL) (**Fig. 4C**).

Acylated and desacyl ghrelin prevent basal autophagy in human visceral adipocytes. To study the regulation of autophagy in obesity, we first examined the expression patterns of several molecular indicators of autophagy (ATG5, ATG7 and Beclin-1/ATG6) in human visceral adipose tissue from lean and obese subjects. Obese patients with T2D showed significantly (P<0.05) increased transcript levels of *ATG7* and *BECN1* in omental adipose tissue compared to lean controls as well as obese NG individuals (**Fig. 5B-C**). The gene expression of *ATG5* showed a similar trend in obesity-associated T2D, but differences fell out of statistical significance (P=0.062) (**Fig. 5A**). Adipocytes and SVFC isolated from omental adipose tissue of morbidly obese patients were used to identify which cell type expressed the autophagy-related genes in this fat depot. No statistically significant changes were observed in the transcript levels of *ATG5, ATG7* and *BECN1* in adipocytes and SVFC (**Fig. 5D-F**).

Taken together, our data suggest that the altered expression profile of autophagy-related genes in human adipose tissue is more related to insulin resistance than obesity.

To determine whether acylated and desacyl ghrelin and TNF-α directly affect autophagy in adipose tissue, differentiated human adipocytes were exposed for 24 h to increasing concentrations of acylated and desacyl ghrelin in the presence or absence of TNF-α and the gene expression of *ATG5, ATG7* and *BECN1* was analyzed. TNF-α up-regulated ($P<0.05$) these autophagy-related genes at the highest concentration tested (100 ng/mL) (**Fig. 6A**). The stimulation of human visceral adipocytes with desacyl ghrelin did not modify the basal expression of autophagy-related genes, but acylated ghrelin decreased ($P<0.05$) the transcript levels of *ATG5* and *ATG7* in human visceral adipocytes (**Fig. 6B and 6C**). The coincubation of TNF-α with acylated ghrelin returned the expression of *BECN1* to basal levels ($P=0.711$ *vs* control adipocytes), while coincubation with desacyl ghrelin inhibited the TNF-α-induced increase in *ATG5, ATG7* and *BECN1* ($P=0.913$, $P=0.969$, $P=0.911$, respectively, *vs* control adipocytes) (**Fig. 6D**).

DISCUSSION

Circulating total ghrelin levels are decreased in obesity, insulin resistance or the metabolic syndrome, all well-known low-grade chronic inflammatory diseases (17-19). Nevertheless, our findings show that obesity-associated T2D is related with low desacyl ghrelin concentrations (the most abundant form of the hormone) and high acylated ghrelin levels, which is in accordance with previously published data (7, 9). Moreover, our present study provides evidence that acylated ghrelin correlates positively with TNF-α, while desacyl ghrelin shows a negative correlation with the proinflammatory factor. A similar relationship of ghrelin forms with TNF-α has been found in other pathological inflammatory conditions, such as juvenile idiopathic arthritis, rheumatoid arthritis or congenital heart disease (19-21). Since TNF-α and ghrelin exert opposite effects on the immune system and metabolism (22), the present study focused on the interaction of both molecules in human adipose tissue. In spite of the negative correlation of TNF-α with plasma desacyl ghrelin, we observed that TNF-α increased the mRNA expression of the *GHRL* gene in human visceral adipocytes, suggesting an autocrine/paracrine role of both molecules on fat cells. In this sense, as described below in detail, our data show that acylated and desacyl ghrelin directly act on visceral adipose tissue to regulate TNF-α-induced apoptosis and autophagy in humans.

The human adipose tissue expresses all the components of the ghrelin system, namely ghrelin, obestatin, GOAT and the receptors of ghrelin-related peptides, GHS-R type 1a and GPR39 (7, 15, 23, 24). Our findings show for the first time the simultaneous presence of ghrelin and GOAT in both human visceral adipocytes and SVFC. Ghrelin immunoreactivity exhibited a yuxtanuclear distribution as well as in small vesicles next to the plasma membrane, while GOAT was present in perinuclear vesicle structures in the cytoplasm. In this sense, it has been suggested that GOAT is located in the membrane of the ER and mediates the translocation of the octanoyl-CoA from the cytosolic side to the ER lumen prior to the transport of acylated ghrelin to the Golgi (25-27). The subcellular localization of ghrelin and GOAT found in the present study is similar to that observed in human myometrial smooth muscle cells and in the human embryonic kidney (HEK) 293 cell line (28-30). Further investigation is warranted to ascertain the mechanisms of ghrelin trafficking in human adipocytes.

Since human obesity and obesity-associated T2D are associated with altered circulating acylated and desacyl ghrelin, the potential contribution of human adipose tissue to this dysregulation was analyzed. Ghrelin mRNA levels in omental and subcutaneous adipose tissue were similar and comparable within groups, which is in accordance with data published by Knerr and colleagues (23). To our knowledge, the present study shows, for the first time, that insulin resistance rather than obesity increases the expression of GOAT in human visceral adipose tissue. Moreover, transcript levels of *MBOAT* mRNA levels in omental adipose tissue were positively associated with serum LDL-cholesterol. In this regard, acylated ghrelin has been shown to be mainly transported by triglyceride-rich lipoproteins (VLDL and, to some extent, LDL) in the bloodstream (31). Thus, the increased expression of visceral adipose GOAT in patients with T2D may accelerate the acylation of ghrelin in visceral adipocytes and its transport into the bloodstream, thereby contributing to the high circulating acylated ghrelin in insulin resistance.

We also investigated the regulation of ghrelin and GOAT in human visceral adipocytes. Several nutritional and hormonal factors as well as the autonomic central system are involved in the regulation of the ghrelin/GOAT system in other tissues (29, 30). Our data show that insulin and leptin suppress ghrelin mRNA levels in human visceral fat cells. Growing experimental and clinical observations support this possibility. Perfusion of the rat stomach with either insulin or leptin induces a dose-dependent inhibition of ghrelin release (32). Infusion of insulin at physiological doses throughout prolonged (more than 240 min) euglycemic-hyperinsulinemic clamps significantly decreases desacyl ghrelin, as expected, but not acylated ghrelin, which remains unchanged or even tended to increase in healthy subjects (33) and in pregnant women with type 2 diabetes (12, 34). Our data also show that insulin increases GOAT mRNA transcript levels in human visceral adipocytes. Current existing literature about the effect of insulin on GOAT is scarce and controversial, since an inhibition of GOAT by insulin in INS-1 pancreatic cells, but also a lack of effect of insulin on murine primary pituitary cell cultures has been reported (35, 36). Although acylated as well as desacyl ghrelin act on fat cells to stimulate adipogenesis (7), both ghrelin forms exert opposite effects on *GHRL* and *MBOAT4* gene expression in human visceral adipocytes. We herein show that acylated ghrelin clearly stimulates GOAT transcript levels, with similar results having been reported by Gahete and colleagues (36) in murine primary

pituitary cell cultures. On the other hand, desacyl ghrelin represses *GHRL* expression, but does not alter GOAT mRNA levels. In this sense, *Mboat4* knockout mice, which lack acylated ghrelin but otherwise exhibit physiological levels of desacyl ghrelin, showed a decreased body weight without changes in fat mass when fed a high-fat diet (37). This lack of increase in fat mass in *Mboat4* knockout mice may be explained by the negative regulation of desacyl ghrelin on the *GHRL* gene evidenced in the present study. Thus, our data suggest that, acylated ghrelin promotes ghrelin expression and acylation in human adipocytes, while desacyl ghrelin constitutes a reppressor of ghrelin transcription. Taken together, insulin, leptin, TNF-α and ghrelin forms themselves embody transcriptional regulators of *GHRL* and *MBOAT4* in human visceral adipocytes.

Acylated and desacyl ghrelin were further observed to significantly reduce adipocyte apoptosis induced by TNF-α, a well-known regulator of apoptosis in the adipose tissue. Upon binding to its receptor, TNF-α proapoptotic signaling results in caspase-8 cleavage and activation, which further activates caspase-3, leading to adipocyte cell death (38). Our findings provide evidence that ghrelin forms reduced the activation of caspase-8, caspase-3 and apoptosis induced by TNF-α. In this regard, ghrelin reportedly prevents apoptosis in murine 3T3-L1 and rat adipocytes (39, 40) as well as in other cell types, such as murine adult cardiomyocyte cell line HL-1, porcine aortic endothelial cells, rat hypothalamic neurons and cortical oligodendrocytes, rat INS-1E pancreatic β-cells and human adrenal gland carcinoma cell line (41-45). In the present study, we also confirmed the increased adipocyte apoptosis in human obesity and obesity-associated T2D. Since adipocyte apoptosis constitutes a key initial event that contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis associated with human obesity (11), the inhibition of adipocyte apoptosis by ghrelin forms may represent a novel potential therapeutic strategy for the treatment of obesity-associated metabolic complications (**Fig. 7**).

In addition to the turnover increase of cell constituents, autophagy is also required for either the formation or breakdown of lipid droplets (13, 46, 47). *Atg5* and *Atg7* knockdown in 3T3-L1 adipocytes inhibits lipid accumulation and decreases the expression of adipogenic transcription factors PPARγ and C/EBPα, revealing the important role of autophagy in the regulation of adipose mass and differentiation (47). Human adipose tissue expresses the autophagy-related molecules LC3-I, LC3-II and

ATG5 at the same time as containing autophagosomes (12). Our data provide evidence for the presence of ATG5, beclin-1/ATG6 and ATG7 in human adipocytes and SVFC. A similar expression of these autophagy-related molecules was found in adipocytes and SVFC in the present study, suggesting that both fractions contribute to the overall adipose tissue expression of these autophagic genes. Human obesity has been associated with altered expression of LC3-I, LC3-II and ATG5 (12). Our data show that insulin resistance rather than obesity is associated with an altered gene expression of *BECN1* and *ATG7* in adipose tissue of obese patients with T2D. In this sense, insulin constitutes a major inhibitor of autophagy with insulin resistance being a potential activator of this process, since patients with T2D show elevated formation of autophagosomes in subcutaneous adipose tissue (48). It has been suggested that an increased autophagy in insulin resistance may reflect a process underlying cell death of hypertrophied adipocytes (12). In this regard, Beclin-1/ATG6 has been proposed as a potential link between apoptosis and autophagy (49). Moreover, TNF-α reportedly induces the expression of Beclin-1/ATG6 in vascular smooth muscle cells in atherosclerotic plaques and Beclin-1/ATG6 is associated with cell death in the plaque (50). In this context, we analyzed the role of the pro-apoptotic TNF-α and the anti-apoptotic ghrelin in the regulation of autophagy-related genes. We found that TNF-α increases the transcript levels of *ATG5*, *BECN1* and *ATG7*, while acylated ghrelin reduced *ATG5* and *ATG7* in human visceral adipocytes. Additionally, desacyl ghrelin reduced TNF-α-induced expression of *ATG5, ATG7* and *BECN1*. Taken together, we herein show for the first time that acylated ghrelin and TNF-α exert opposite effects on the regulation of autophagy in human visceral adipocytes (**Fig. 7**). Furthermore, the imbalance of both molecules in insulin resistance may contribute to the altered autophagy observed in patients with T2D.

In summary, the main findings of the present study are 1) that GOAT expression, but not ghrelin, is increased in human visceral adipose tissue in patients with T2D, 2) that insulin, leptin, TNF-α as well as ghrelin itself constitute transcriptional regulators of *GHRL* and *MBOAT* in human visceral adipocytes, and 3) that acylated and desacyl ghrelin reduce TNF-α-induced apoptosis and basal autophagy in human visceral adipocytes. These results broaden the current understanding of defective mechanisms underlying insulin resistance.

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Table 1. Clinical characteristics of the subjects included in the study.

	Lean NG	Obese NG	Obese IGT	Obese T2D	*P*
n	55	66	37	36	-
Sex (male/female)	25/30	41/25	21/16	24/12	0.166
Age (years)	56 ± 2	55 ± 1	59 ± 1	59 ± 2	0.154
Weight (kg)	66 ± 2	92 ± 3[a]	90 ± 3[a]	93 ± 3[a]	**<0.00001**
BMI (kg/m^2)	23.1 ± 0.3	32.5 ± 0.5[a]	33.5 ± 0.8[a]	33.4 ± 0.6[a]	**<0.00001**
Waist circumference (cm)	82.1 ± 1.5	106.9 ± 1.7[a]	106.9 ± 1.8[a]	109.2 ± 1.9[a]	**<0.00001**
Glucose (mg/dL)	92 ± 1	93 ± 1	109 ± 2[a,b]	132 ± 3[a,b]	**<0.00001**
Insulin (μU/mL)	3.7 ± 0.4	7.5 ± 0.5[a]	10.4 ± 0.9[a]	14.4 ± 1.7[a,b]	**<0.00001**
HOMA	0.9 ± 0.1	1.7 ± 0.1[a]	2.7 ± 0.2[a]	4.9 ± 0.4[a]	**0.017**
QUICKI	0.42 ± 0.02	0.37 ± 0.02	0.34 ± 0.01[a,b]	0.31 ± 0.01[a,b]	**0.012**
FFA (mg/dL)	15 ± 1	21 ± 3	21 ± 2	26 ± 6	0.121
Triglycerides (mg/dL)	71 ± 9	110 ± 9[a]	120 ± 15[a]	123 ± 13[a]	**<0.00001**
Total cholesterol (mg/dL)	202 ± 7	219 ± 8	210 ± 10	180 ± 4	0.106
LDL-cholesterol (mg/dL)	122 ± 5	141 ± 7	132 ± 9	106 ± 7	0.071
HDL-cholesterol (mg/dL)	68 ± 4	55 ± 3[a]	54 ± 3[a]	49 ± 2[a]	**0.0001**
AST (IU/L)	13 ± 1	17 ± 1[a]	14 ± 1[a]	17 ± 2[a]	**0.007**
ALT (IU/L)	13 ± 1	19 ± 3[a]	19 ± 2[a]	33 ± 6[a,b]	**0.0001**
Alkaline phosphatase (IU/L)	81 ± 4	101 ± 3	84 ± 5	90 ± 11	0.067
γ-GT (IU/L)	13 ± 1	27 ± 6	25 ± 4[a]	53 ± 14[a]	**0.0001**
CRP (mg/L)	1.2 ± 0.1	5.7 ± 1.0	4.6 ± 1.2[a]	5.0 ± 1.1[a]	**0.008**
Uric acid (mg/dL)	5.2 ± 0.4	6.4 ± 0.4[a]	5.9 ± 0.2[a]	6.2 ± 0.3[a]	**0.0001**
Leptin (ng/mL)	7.3 ± 0.5	25.9 ± 2.8[a]	21.3 ± 1.7[a]	20.0 ± 1.9[a]	**0.0001**
TNF-α (pg/mL)	2.3 ± 0.5	2.2 ± 0.3	2.6 ± 0.4	4.0 ± 0.8[a,b]	**0.041**

Values presented as the mean ± SEM. Bold values are statistically significant *P* values. NG, normoglycemia; IGT, impaired glucose tolerance; T2D, type 2 diabetes; BMI, body mass index; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; FFA, free fatty acids; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, γ-glutamyltransferase; CRP, high-sensitivity C-reactive protein; TNF-α, tumor necrosis factor α. Differences between groups were analyzed by one-way ANOVA followed by Scheffé's test or χ^2 test, where appropriate. [a] $P<0.05$ *vs* lean NG; [b] $P<0.05$ *vs* obese NG.

FIGURE LEGENDS

FIG. 1. Impact of obesity and obesity-associated type 2 diabetes on plasma concentrations of acylated and desacyl ghrelin. (A) Fasting plasma concentrations of acylated and desacyl ghrelin in lean volunteers (n=55), obese normoglycemic (NG) subjects (n=66), obese individuals with impaired glucose tolerance (IGT) (n=37) and obese patients with type 2 diabetes (T2D) (n=36). Values are the mean ± SEM. Differences between groups were analyzed by one-way ANOVA followed by Scheffé's test. ***P<0.001 *vs* lean NG subjects; ††P<0.01, *vs*. obese NG subjects. (B) Correlations between plasma TNF-α with plasma acylated ghrelin (left) and desacyl ghrelin (right). The Pearson's coefficient of correlation (r) and P value are indicated.

FIG. 2. Impact of obesity and obesity-associated type 2 diabetes on the expression of ghrelin and GOAT in omental and subcutaneous adipose tissue. Bar graphs show the expression of ghrelin and ghrelin-*O*-acyltransferase (GOAT) mRNA and protein in omental (A, C) and subcutaneous (B, D) adipose tissue obtained from lean volunteers, obese normoglycemic (NG) subjects, obese individuals with impaired glucose tolerance (IGT) and obese patients with type 2 diabetes (T2D). Representative blots are shown at the bottom of the histograms. The gene and protein expression in lean subjects was assumed to be 1. Values are the mean ± SEM (n=10 per group). Differences between groups were analyzed by one-way ANOVA followed by Scheffé's test. *P<0.05, **P<0.01 *vs* lean NG subjects; †P<0.05 *vs* obese NG subjects. (E, F) Immunohistochemical detection of ghrelin and GOAT in omental adipose tissue obtained from obese patients (magnification, X100 in the *central panels*, X400 in the *right panels*). No immunoreactivity was found without primary antibody (*left panels*). (G) Immunocytochemical detection of ghrelin and GOAT proteins in differentiated 3T3-L1 adipocytes (day 10) under basal conditions. Images were taken from the basal planes of the cells. Representative images of at least 3 separate experiments are shown.

FIG. 3. Regulation of ghrelin and GOAT in human omental adipocytes. Effect of incubation of differentiated omental fat cells for 24 h with different concentrations of insulin (A), leptin (B), TNF-α (C), desacyl ghrelin (D) and

acylated ghrelin (E) on *GHRL* and *MBOAT4* gene expression. Differences among groups were analyzed by one-way ANOVA followed by Dunnet's test. **P*<0.05, ***P*<0.01 *vs* unstimulated adipocytes.

FIG. 4. Effect of acylated and desacyl ghrelin on TNF-α-induced apoptosis. (A) Bar graphs show the adipocyte apoptosis observed in omental adipose tissue obtained from lean volunteers, obese normoglycemic (NG) subjects, obese individuals with impaired glucose tolerance (IGT) and obese patients with type 2 diabetes (T2D) (n=5 per group). Differences between groups were analyzed by one-way ANOVA followed by Scheffé's test. **P*<0.05, ***P*<0.01, ****P*<0.001 *vs* lean subjects; †*P*<0.05 *vs* obese NG. (B) Histograms illustrate the effect of TNF-α (0, 1, 10 and 100 ng/mL) treatment for 24 h on the activation of caspase-8, caspase-3 and apoptosis. (C) Effect of coincubation of acylated ghrelin (100 pmol/L) or desacyl ghrelin (100 pmol/L) on the TNF-α (100 ng/mL)-induced activation of caspase-8, caspase-3 and apoptosis. Values are the mean ± SEM (n=8 per group). Differences between groups were analyzed by one-way ANOVA followed by Dunnet's test. **P*<0.05, ****P*<0.001 *vs* unstimulated adipocytes.

FIG. 5. Impact of obesity and obesity-associated type 2 diabetes on indicators of autophagy in human omental adipose tissue. Bar graphs show the gene expression of *ATG5* (A), *ATG7* (B) and *BECN1* (C) mRNA in omental adipose tissue obtained from lean volunteers, obese normoglycemic (NG) subjects, obese individuals with impaired glucose tolerance (IGT) and obese patients with type 2 diabetes (T2D). Values are the mean ± SEM (n=9-13 per group). The gene expression in lean subjects was assumed to be 1. Differences between groups were analyzed by one-way ANOVA followed by Scheffé's test. **P*<0.05 *vs* lean NG subjects; †*P*<0.05 *vs* obese NG. Comparison of *ATG5* (D), *ATG7* (E) and *BECN1* (F) gene expression in freshly isolated adipocytes and stromal vascular fraction cells (SVFC) from omental adipose tissue of obese patients (n=15). Differences between groups were analyzed by two-tailed unpaired Student's *t* test.

FIG. 6. Regulation of autophagy induced by TNF-α and ghrelin isoforms in human omental adipocytes. Histograms illustrate the effect of TNF-α (0, 1, 10 and 100 ng/mL) treatment for 24 h on the induction of *ATG5* (A), *ATG7* (B) and *BECN1* (C) mRNA in human omental adipocytes. (D) Effect of acylated ghrelin (100 pmol/L) or desacyl ghrelin (100 pmol/L) on TNF-α (100 ng/mL)-induced activation of *ATG5, ATG7* and *BECN1* gene expression. Values are the mean ± SEM (n=8-10 per group). Differences between groups were analyzed by one-way ANOVA followed by Dunnet's test. **P*<0.05 *vs* unstimulated adipocytes.

FIG. 7. Schematic diagram of the effect of ghrelin isoforms on TNF-α-induced autophagy and apoptosis in human visceral adipocytes. Acylated and desacyl ghrelin inhibit the activation of caspase-3, caspase-8 and apoptosis induced by TNF-α upon binding to its receptor TNFR1. Desacyl ghrelin reduces TNF-α-induced transcription of *BECN1*, an autophagy-related gene required for the initiation of the formation of the autophagosome in autophagy, as well as *ATG7* and *ATG5*, autophagy-related genes that participate in the conjugation cascades for the autophagosome elongation. Acylated ghrelin reduces the basal expression of the autophagic-related genes *ATG5* and *ATG7*. The imbalance of ghrelin isoforms and TNF-α in insulin resistance may contribute to the altered apoptosis and autophagy observed in patients with T2D.

SUPPLEMENTAL RESEARCH DESIGN AND METHODS

Patient selection and study design. The study of plasma acylated and desacyl ghrelin was performed in a population of 194 participants (111 men and 83 women) recruited from healthy volunteers and patients attending the Departments of Internal Medicine and Endocrinology & Nutrition of the Clínica Universidad de Navarra (Pamplona, Spain) for a medical check-up. Patients underwent a clinical assessment including medical history, physical examination, body composition analysis, comorbidity evaluation and nutritional interviews performed by a multidisciplinary consultation team. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Obesity was defined as a BMI ≥ 30 kg/m^2, and normal weight as a BMI < 25 kg/m^2. Obese patients were sub-classified into three groups [normoglycemia (NG), impaired glucose tolerance (IGT) or type 2 diabetes (T2D)] following the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes (1). T2D subjects were not on insulin therapy or medication likely to influence endogenous insulin levels. It has to be stressed that the patients included in our obese T2D group did not have a long diabetes history (less than 2-3 years or even *de novo* diagnosis as evidenced from their anamnesis and biochemical determinations). The clinical characteristics of the subjects are shown in **Table 1.**

In addition, the presence of ghrelin and GOAT as well as the effect of the ghrelin forms was assessed in paired omental and subcutaneous adipose tissue samples from 53 patients undergoing either laparoscopic Roux-en-Y gastric bypass (n=43) or Nissen funduplication (n=10) at the Clínica Universidad de Navarra. Clinical characteristics of this cohort were essentially similar to the cohort for the study of circulating ghrelin isoforms (**Supplemental Table 1**). A portion of each biopsy of fat was fixed in 10% formalin solution, with the rest being immediately frozen in liquid nitrogen and stored at -80 ºC for subsequent gene and protein expression analyses. All reported investigations were carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008. The experimental design was approved, from an ethical and scientific standpoint, by the Hospital's Ethical Committee responsible for research (028/2009) and the informed consent from all volunteers was obtained.

Analytical procedures. Blood samples were collected after an overnight fast. Glucose, uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase and γ-glutamyltransferase (γ-GT) were measured by enzymatic tests (Hitachi Modular P800, Roche, Basel, Switzerland). Free fatty acids (FFA), total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol and TG concentrations (Roche) were calculated as previously described (2, 3). High sensitivity C-reactive protein (CRP) concentrations were determined as previously reported (2, 3). Insulin was measured by means of an enzyme-amplified chemiluminiscence assay (IMMULITE; Diagnostics Products Corp., Los Angeles, CA, USA); intra- and inter-assay coefficients of variation were 4.2 and 5.7%, respectively. Insulin resistance and sensitivity were calculated using the homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI), respectively (4, 5). Leptin was quantified by a double-antibody RIA method (Linco Research, Inc., St. Charles, MO, USA); intra- and inter-assay coefficients of variation were 6.7 and 7.8%, respectively. TNF-α was measured with a high-sensitivity ELISA (R&D Systems Inc., Minneapolis, MN, USA); intra- and inter-assay coefficients of variation were 5.0 and 5.7%, respectively. Acylated and desacyl ghrelin were determined by commercially available ELISA kits (Linco Research Inc.) following the manufacturer's guidelines, with intra- and inter-assay coefficients of variation being 5.5 and 2.6%, respectively, for the former, and 4.7 and 4.2%, respectively, for the latter.

RNA extraction and real-time PCR. RNA isolation and purification were performed as described earlier (2, 6). Transcript levels for ghrelin (*GHRL*), GOAT (*MBOAT4*) and autophagy-related genes ATG5 (*ATG5*), Beclin-1/ATG6 (*BECN1*) and ATG7 (*ATG7*) were quantified by real-time PCR (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). Primers and probes (Supplemental Table 2) were designed using the software Primer Express 2.0 (Applied Biosystems) and purchased from Genosys (Sigma, Steinhein, Germany). Primers or TaqMan® probes encompassing fragments of the areas from the extremes of two exons were designed to ensure the detection of the corresponding transcript avoiding genomic DNA amplification. The cDNA was amplified at the following conditions: 95 ºC for 10 min, followed by 45 cycles of 15 s at 95 ºC and 1 min at 59 ºC, using the TaqMan® Universal PCR Master Mix (Applied Biosystems). The primer and probe concentrations were 300 and 200 nmol/L, respectively. All results were normalized for the expression of *18S* rRNA

(Applied Biosystems), and relative quantification was calculated using the $\Delta\Delta C_T$ formula (7). Relative mRNA expression was expressed as fold expression over the calibrator sample. All samples were run in triplicate and the average values were calculated.

Western-blot studies. Cells and tissues were homogenized and protein content was measured as previously described (2). Thirty micrograms of total protein were separated by SDS-PAGE under denaturing conditions, transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked in Tris-buffer saline (TBS) with 0.05% Tween 20 (TBS-T) containing 5% non-fat dry milk (ghrelin, GOAT, β-actin) or 5% BSA (caspases) for 1 h at room temperature (RT). Blots were then incubated overnight at 4 ºC with a rabbit polyclonal anti-ghrelin (Alpha Diagnostic International, San Antonio, TX, USA), rabbit polyclonal anti-GOAT (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-caspase-3 (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit monoclonal anti-cleaved caspase-3 (Cell Signaling), mouse monoclonal anti-caspase-8 (Cell Signaling), rabbit anti-cleaved caspase-8 (Cell Signaling) antibodies (diluted 1:1,000 in blocking solution) or mouse monoclonal anti-β-actin (Sigma) antibody (diluted 1:5,000 in blocking solution). The antigen-antibody complexes were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (1:5,000) and the enhanced chemiluminescence ECL Plus detection system (Amersham Biosciences, Buckinghamshire, UK). The intensity of the bands was determined by densitometric analysis with the Gel Doc™ gel documentation system and Quantity One 4.5.0 software (Bio-Rad) and normalized with β-actin density values. All assays were performed in duplicate.

Immunohistochemistry of ghrelin and GOAT. The immunodetection of ghrelin and GOAT in histological sections of omental and subcutaneous adipose tissue was performed by the indirect immunoperoxidase method (2). Sections of formalin-fixed paraffin-embedded adipose tissue (6 μm) were dewaxed in xylene, rehydrated in decreasing concentrations of ethanol and treated with 3% H_2O_2 (Sigma) in absolute methanol for 10 min at RT to quench endogenous peroxidase activity. Slides were blocked during 30 min with 1% murine serum (Sigma) diluted in TBS (50 mmol/L Tris, 0.5 mol/L NaCl; pH 7.36) to prevent non-specific adsorption. Sections were incubated

overnight at 4 ºC with rabbit polyclonal anti-ghrelin (Alpha Diagnostic International) or rabbit polyclonal anti-GOAT (Santa Cruz Biotechnology, Inc.) antibodies diluted 1:50 in TBS. After washing with TBS, slides were incubated with Dako Real™ EnVision™ horseradish peroxidase-conjugated anti-rabbit/mouse (Dako, Glostrup, Denmark) for 1 h at RT. After washing in TBS, peroxidase reaction was visualized with a 3,3'-diaminobenzidine (DAB, Amersham Biosciences)/H_2O_2 solution (0.5 mg/mL DAB, 0.03% H_2O_2 diluted in 50 mmol/LTris-HCl, pH 7.36), as chromogen and Harris hematoxylin solution (Sigma) as counterstaining. Sections were dehydrated, coverslipped and observed under a Zeiss Axiovert 40 CFL optic microscope (Zeiss, Göttingen, Germany). Negative control slides without primary antibody were included to assess non-specific staining.

Confocal immunofluorescence microscopy of ghrelin and GOAT. Differentiated 3T3-L1 adipocytes grown on glass coverslips were serum-starved for 2 h and fixed in 4% paraformaldehyde for 15 min at RT, washed with PBS (pH 7.40) (3 x 5 min), and permeabilized with blocking buffer [PBS containing 0.1% Triton X-100 and 0.5% bovine seroalbumine (BSA) for ghrelin detection; PBS containing 0.1% Triton X-100 and 0.1 mol/L glycine for GOAT detection] for 30 min at RT. Cells were then incubated with rabbit polyclonal anti-ghrelin (Alpha Diagnostic International) or anti-GOAT (Santa Cruz Biotechnology, Inc.) antibodies diluted 1:50 (ghrelin) or 1:250 (GOAT) in blocking solution overnight at 4 ºC. Thereafter, cells were washed with PBS (3 x 5 min) and incubated with Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (Invitrogen, Paisley, UK) diluted 1:500 in blocking solution for 2 h at RT. After washing, coverslips were mounted on microscope slides and examined under a TCS-SP2-AOBS confocal laser scanning microscope (Leica Corp., Heidelberg, Germany). For each cell, 2-3 stacks per channel were collected and projected in a single image. Image stacks underwent deconvolution off-line with the software package Huygens Essential 2.4.4 (SVI, Hilversum, The Netherlands). To verify the specificity of the immunoreaction, the primary or secondary antibodies were substituted with PBS.

Cell cultures. Human stromovascular fraction cells (SVFC) were isolated from omental adipose tissue from obese normoglycemic subjects as previously described (2). SVFC were seeded at 2 x 10^5 cell/cm^2 and grown in adipocyte medium [DMEM/F-12 [1:1] (Invitrogen), 17.5 mmol/L glucose, 16 μmol/L biotin, 18 μmol/L panthotenate, 100

μmol/L ascorbate and antibiotic-antimycotic] supplemented with 10% newborn calf serum (NCS). After 4 days, the medium was changed to adipocyte medium supplemented with 3% NCS, 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 0.1 μmol/L dexamethasone, 1 μmol/L BRL49653 and 10 μg/mL insulin. After a 3-day induction period, cells were fed every 2 days with the same medium but without IBMX and BRL49653 supplementation for the remaining 7 days of adipocyte differentiation. Differentiated human omental adipocytes were serum-starved for 24 h and then treated with increasing concentrations of insulin (1, 10 and 100 nmol/L) (Sigma), leptin (1, 10 and 100 nmol/L) (PeproTech EC, Inc., Rocky Hill, NJ, USA), TNF-α (1, 10 and 100 ng/mL) (PeproTech), acylated ghrelin (10, 100 and 1,000 pmol/L) (Tocris, Ellisville, MO, USA) or desacyl ghrelin (10, 100 and 1,000 pmol/L) (Tocris) for 24 h. In a subset of experiments quiescent cells were incubated with TNF-α (100 ng/mL) in the presence or absence of acylated ghrelin (100 pmol/L) or desacyl ghrelin (100 pmol/L) for 24 h. One sample per experiment was used to obtain control responses in the presence of the solvent.

TUNEL assay. The TUNEL assay was performed using the *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics Corp., Indianapolis, IL, USA). Differentiated adipocytes were fixed with 4% formaldehyde for 1 h at RT. After washing three times with PBS, cells were treated with 3% H_2O_2 (Sigma) in absolute methanol for 10 min at RT to quench endogenous peroxidase activity and, then, incubated with the permeabilisation solution (freshly prepared PBS containing 0.1% Triton X-100 and 0.1% sodium citrate) for 2 min at 4 ºC. After washing three times with PBS, cells were incubated with 50 μL in a reaction mixture containing fluorescein-labelled dNTPs and terminal deoxynucleotidyl transferase (TdT) for 1 h at 37 ºC in a humidified atmosphere in the dark. Negative controls were incubated in the absence of TdT, and positive controls were pretreated for 10 min at RT with 100 μL of 100 U/mL DNase I from bovine pancreas (Roche) in 50 mmol/L Tris-HCl (pH 7.36) and 1 mg/mL BSA to induce DNA breaks, prior to labelling procedures. Cells were rinsed three times with PBS and, then, incubated with 50 μL of anti-fluorescein antibody conjugated with horseradish peroxidase for 20 min at 37 ºC in a humidified atmosphere in the dark. After washing three times with PBS, peroxidase reaction was visualized with 50 μL of 3,3'-DAB/H_2O_2 solution for 10 min at RT. Cells were rinsed three times with PBS and analyzed under the light microscope.

Statistical analysis. Data are expressed as mean ± SEM. Statistical differences between mean values were determined using Student's t test, χ^2 test, and one-way ANOVA followed by Scheffé's or Dunnet's tests, where appropriate. A P value < 0.05 was considered statistically significant. In the analysis of Pearson's correlation coefficients (r), Bonferroni's correction was applied when multiple comparisons were performed simultaneously, establishing the significance level at P=0.003. The statistical analyses were carried out by the SPSS/Windows version 15.0 software (SPSS Inc., Chicago, IL, USA).

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Supplemental Table 1. Clinical characteristics of the subjects included in the gene and protein expression study.

	Lean NG	Obese NG	Obese IGT	Obese T2D	*P*
n	10	17	13	13	-
Sex (male/female)	3/7	10/7	7/6	7/6	0.519
Age (years)	49 ± 5	40 ± 3	43 ± 2	45 ± 2	0.125
Weight (kg)	63 ± 4	130 ± 6[a]	132 ± 5[a]	136 ± 7[a]	**<0.00001**
BMI (kg/m^2)	22.8 ± 0.7	46.2 ± 2.0[a]	47.5 ± 2.1[a]	48.9 ± 3.1[a]	**<0.00001**
Body fat (%)	26.5 ± 2.8	48.0 ± 2.3[a]	51.7 ± 2.2[a]	50.7 ± 1.9[a]	**<0.00001**
Glucose (mg/dL)	87 ± 3	91 ± 3	106 ± 3	133 ± 16[a,b]	**0.004**
Insulin (μU/mL)	8.2 ± 1.6	20.4 ± 3.7	15.0 ± 3.1	21.4 ± 3.3	0.125
HOMA	1.7 ± 0.3	3.1 ± 0.8	3.6 ± 1.0	6.5 ± 2.0	0.083
QUICKI	0.36 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.200
Triglycerides (mg/dL)	68 ± 11	137 ± 39	135 ± 35	234 ± 98	0.319
Total cholesterol (mg/dL)	192 ± 10	181 ± 9	190 ± 8	188 ± 5	0.772
LDL-cholesterol (mg/dL)	117 ± 16	115 ± 10	122 ± 9	126 ± 13	0.882
HDL-cholesterol (mg/dL)	58 ± 2	38 ± 2[a]	44 ± 3[a]	37 ± 2[a]	**<0.00001**
AST (IU/L)	13 ± 3	15 ± 1	15 ± 1	19 ± 3	0.381
ALT (IU/L)	13 ± 3	28 ± 4	29 ± 4	35 ± 6	0.079
Alkaline phosphatase (IU/L)	93 ± 12	126 ± 8	127 ± 9	105 ± 9	0.167
γ-GT (IU/L)	9 ± 2	23 ± 3	27 ± 3	36 ± 17	0.438
CRP (mg/L)	1.8 ± 0.3	5.3 ± 0.9[a]	7.7 ± 1.1[a]	9.6 ± 1.3[a]	**0.002**
Uric acid (mg/dL)	4.1 ± 0.5	6.5 ± 0.5[a]	6.9 ± 0.4[a]	6.7 ± 0.5[a]	**0.008**
Leptin (ng/mL)	7.8 ± 1.5	34.9 ± 6.5	43.4 ± 9.7	56.0 ± 12.7[a]	**0.049**

Values presented as the mean ± SEM. NG, normoglycemia; IGT, impaired glucose tolerance; T2D, type 2 diabetes; BMI, body mass index; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, γ-glutamyltransferase; CRP, high-sensitivity C-reactive protein. Differences between groups were analyzed by one-way ANOVA followed by Scheffé's test or χ^2 test, where appropriate. [a] $P<0.05$ vs. lean NG; [b] $P<0.05$ vs. obese NG.

Supplemental Table 2. Sequences of primers and TaqMan® probes

Gene (GenBank accesion no.)	Oligonucleotide sequence (5'-3')	Nucleotides
GHRL		
(NM_016362)		
Forward	AAGAAGCCACCAGCCAAGCT	173-192
Reverse	CGTTGAACCGGACTTCCAGTT	261-281
Probe	FAM-CCGGAAGATGGAGGTCAAGCAGAAGG-TAMRA	224-249
MBOAT4		
(NM_001100916)		
Forward	TTCCCTTTGCACTTCTCTTCAATT	142-167
Reverse	CTCCAGTCAGGAGAAAGAGGTACCT	201-225
Probe	FAM-TGCATCATGGATTCATTCTCCACTCGTG-TAMRA	171-198
ATG5		
(NM_004849)		
Forward	GCAGAAAAAAGATCACAAGCAACTC	788-812
Reverse	CCATTTTCTTCTGCAGGATATTCC	872-895
Probe	FAM-AATGACAGATTTGACCAGTTTTGGG-TAMRA	828-852
ATG7		
(NM_ 006395)		
Forward	CTGCAGATAAGAAGCTCCTTTTGG	410-433
Reverse	GTTTTCAAGAGCAGTGCCTGATTT	466-489
Probe	FAM-CAAGCAGCAAATGAGATATGGGAATCC-TAMRA	436-462
BECN1		
(NM_003766)		
Forward	TCCATGCTCTGGCCAATAAGA	1103-1123
Reverse	TGTCAGAGACTCCAGATATGAATGGT	1166-1191
Probe	FAM-ATTTCAGAGATACCGACTTGT-TAMRA	1134-1143

GHRL, ghrelin; *MBOAT4*, membrane bound O-acyltransferase domain containing 4; *ATG5*, ATG5 autophagy related 5 homolog (*S. cerevisiae*); *ATG7*, autophagy related 5 homolog (*S. cerevisiae*); *BECN1*, beclin-1, autophagy related.

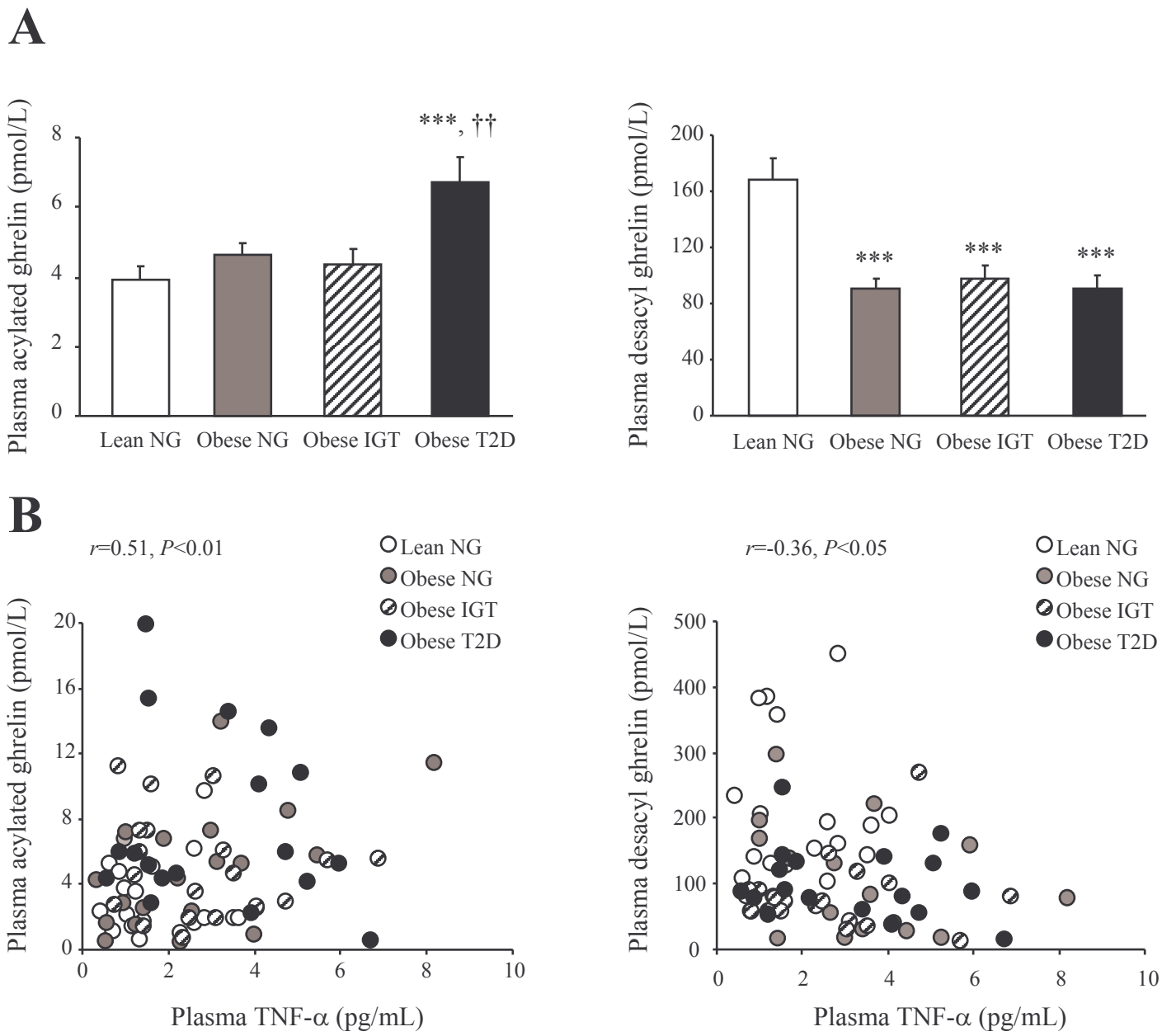

A
Plasma acylated ghrelin (pmol/L)
8
6
4
2
0
***, ††
Lean NG
Obese NG
Obese IGT
Obese T2D
Plasma desacyl ghrelin (pmol/L)
200
160
120
80
40
0
***
***
***
Lean NG
Obese NG
Obese IGT
Obese T2D
B
r=0.51, P<0.01
Lean NG
Obese NG
Obese IGT
Obese T2D
Plasma acylated ghrelin (pmol/L)
20
16
12
8
4
0
0
2
4
6
8
10
Plasma TNF-α (pg/mL)
r=-0.36, P<0.05
Lean NG
Obese NG
Obese IGT
Obese T2D
Plasma desacyl ghrelin (pmol/L)
500
400
300
200
100
0
0
2
4
6
8
10
Plasma TNF-α (pg/mL)

Figure 1

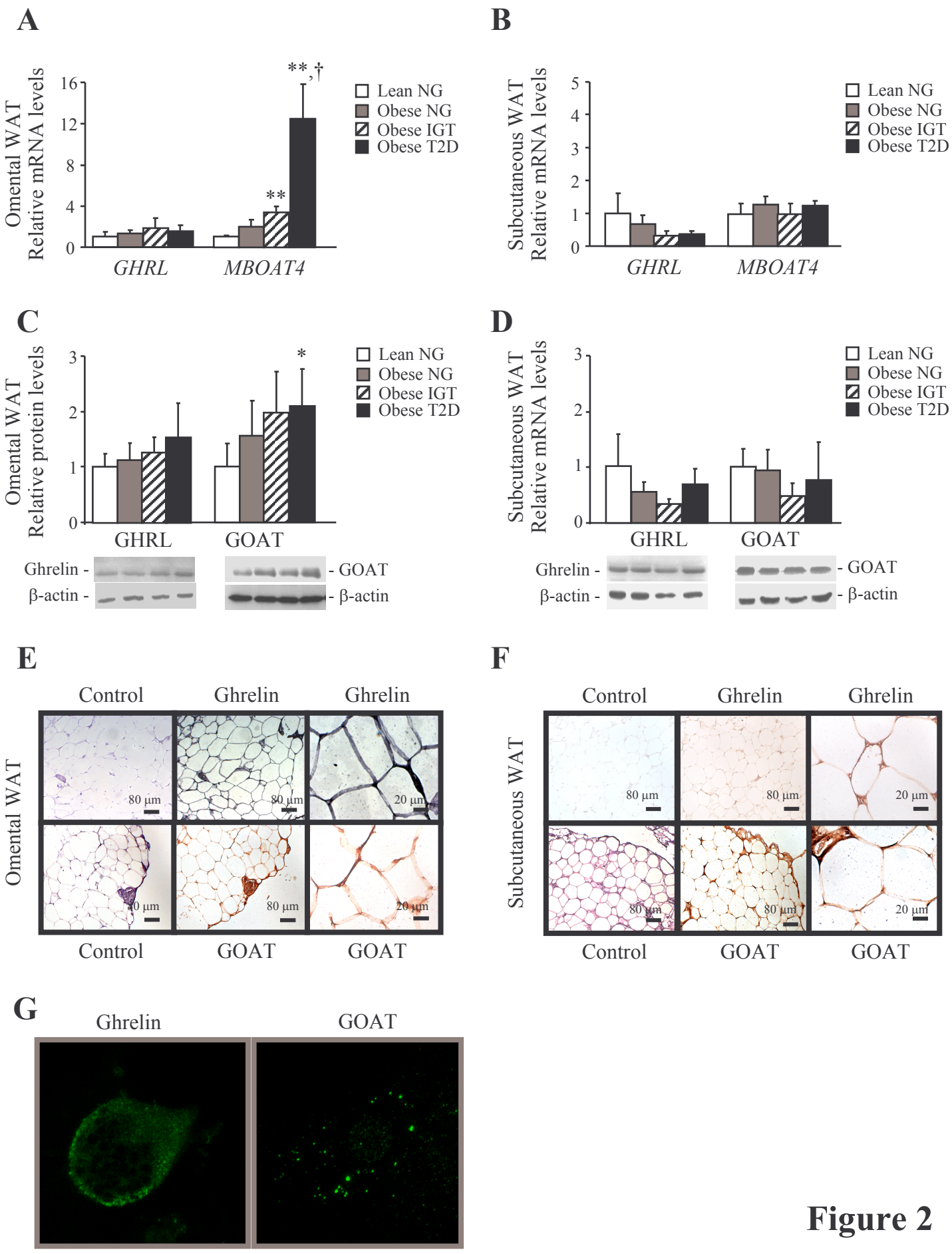
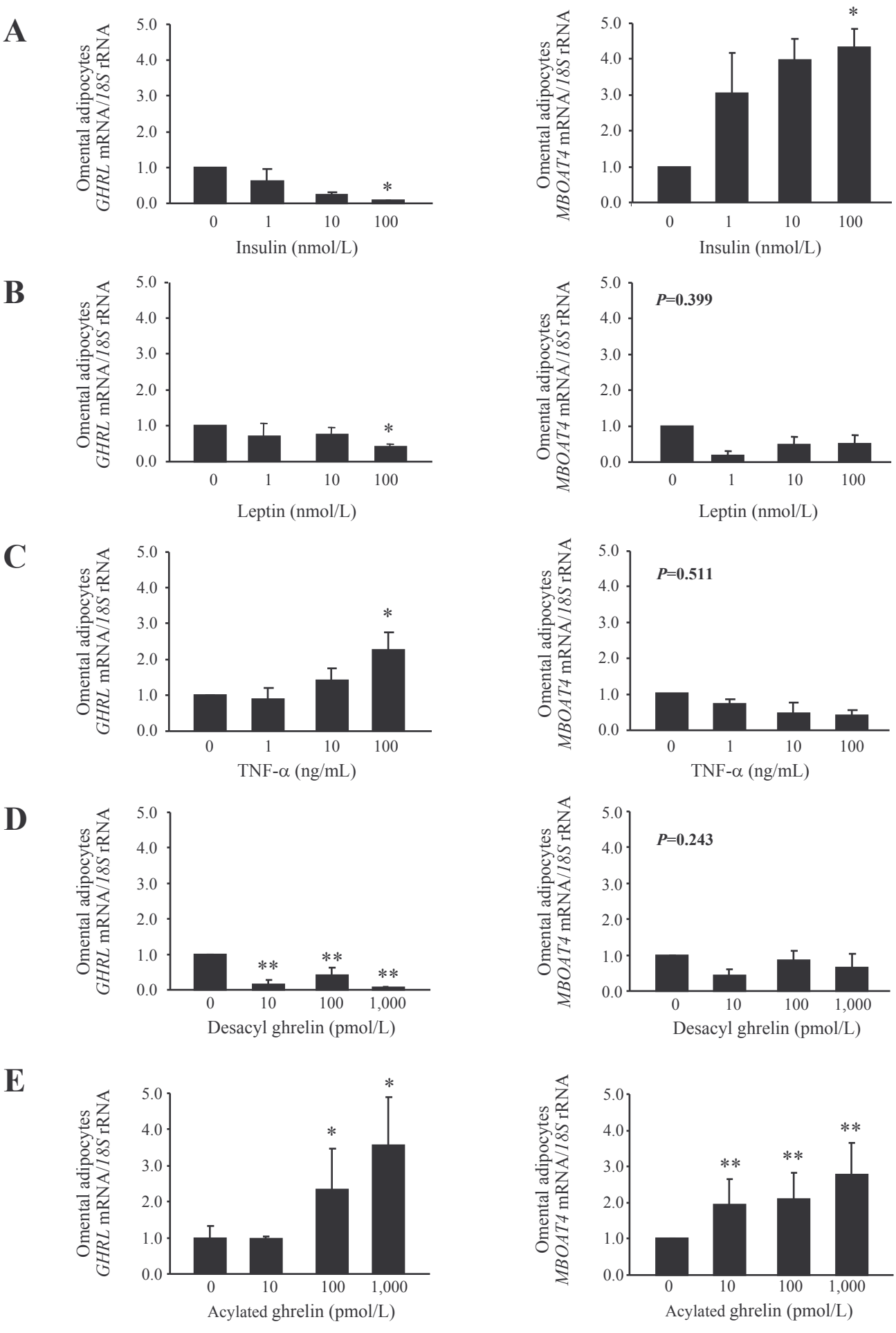

A
Omental WAT
Relative mRNA levels
16
12
8
4
0
**
**,†
GHRL
MBOAT4
Lean NG
Obese NG
Obese IGT
Obese T2D
B
Subcutaneous WAT
Relative mRNA levels
5
4
3
2
1
0
GHRL
MBOAT4
Lean NG
Obese NG
Obese IGT
Obese T2D
C
Omental WAT
Relative protein levels
3
2
1
0
*
GHRL
GOAT
Lean NG
Obese NG
Obese IGT
Obese T2D
Ghrelin -
β-actin -
- GOAT
- β-actin
D
Subcutaneous WAT
Relative mRNA levels
3
2
1
0
GHRL
GOAT
Lean NG
Obese NG
Obese IGT
Obese T2D
Ghrelin -
β-actin -
- GOAT
- β-actin
E
Control
Ghrelin
Ghrelin
Omental WAT
80 µm
80 µm
20 µm
80 µm
80 µm
20 µm
Control
GOAT
GOAT
F
Control
Ghrelin
Ghrelin
Subcutaneous WAT
80 µm
80 µm
20 µm
80 µm
80 µm
20 µm
Control
GOAT
GOAT
G
Ghrelin
GOAT

Figure 2


A
Omental adipocytes GHRL mRNA/18S rRNA
5.0
4.0
3.0
2.0
1.0
0.0
*
0
1
10
100
Insulin (nmol/L)
Omental adipocytes MBOAT4 mRNA/18S rRNA
*
Insulin (nmol/L)
B
Leptin (nmol/L)
P=0.399
C
TNF-α (ng/mL)
P=0.511
D
**
0
10
100
1,000
Desacyl ghrelin (pmol/L)
P=0.243
E
Acylated ghrelin (pmol/L)

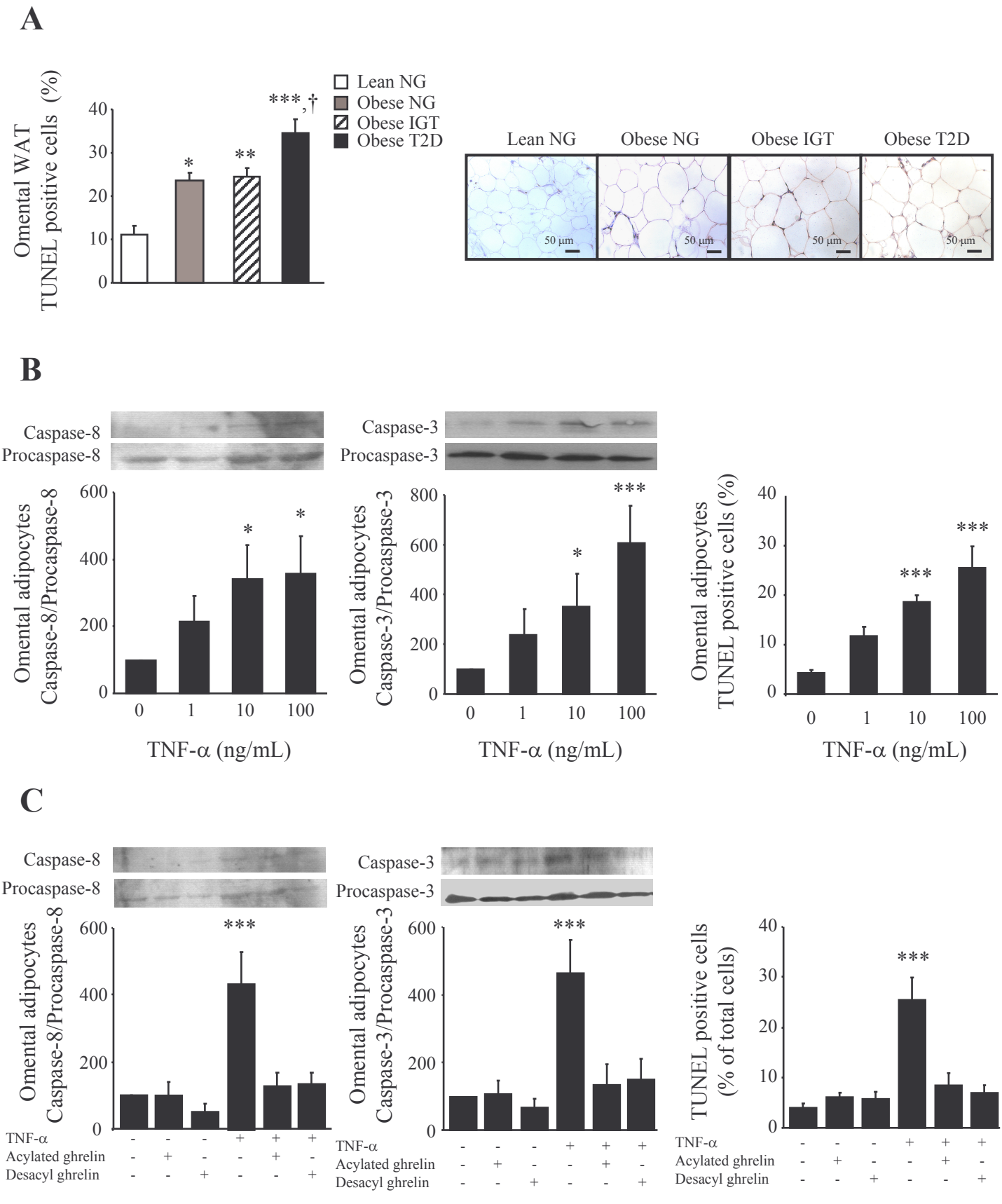

A
Omental WAT TUNEL positive cells (%)
Lean NG
Obese NG
Obese IGT
Obese T2D
Lean NG
Obese NG
Obese IGT
Obese T2D
50 µm
B
Caspase-8
Procaspase-8
Caspase-3
Procaspase-3
Omental adipocytes Caspase-8/Procaspase-8
Omental adipocytes Caspase-3/Procaspase-3
Omental adipocytes TUNEL positive cells (%)
TNF-α (ng/mL)
C
TUNEL positive cells (% of total cells)
TNF-α
Acylated ghrelin
Desacyl ghrelin

Figure 4

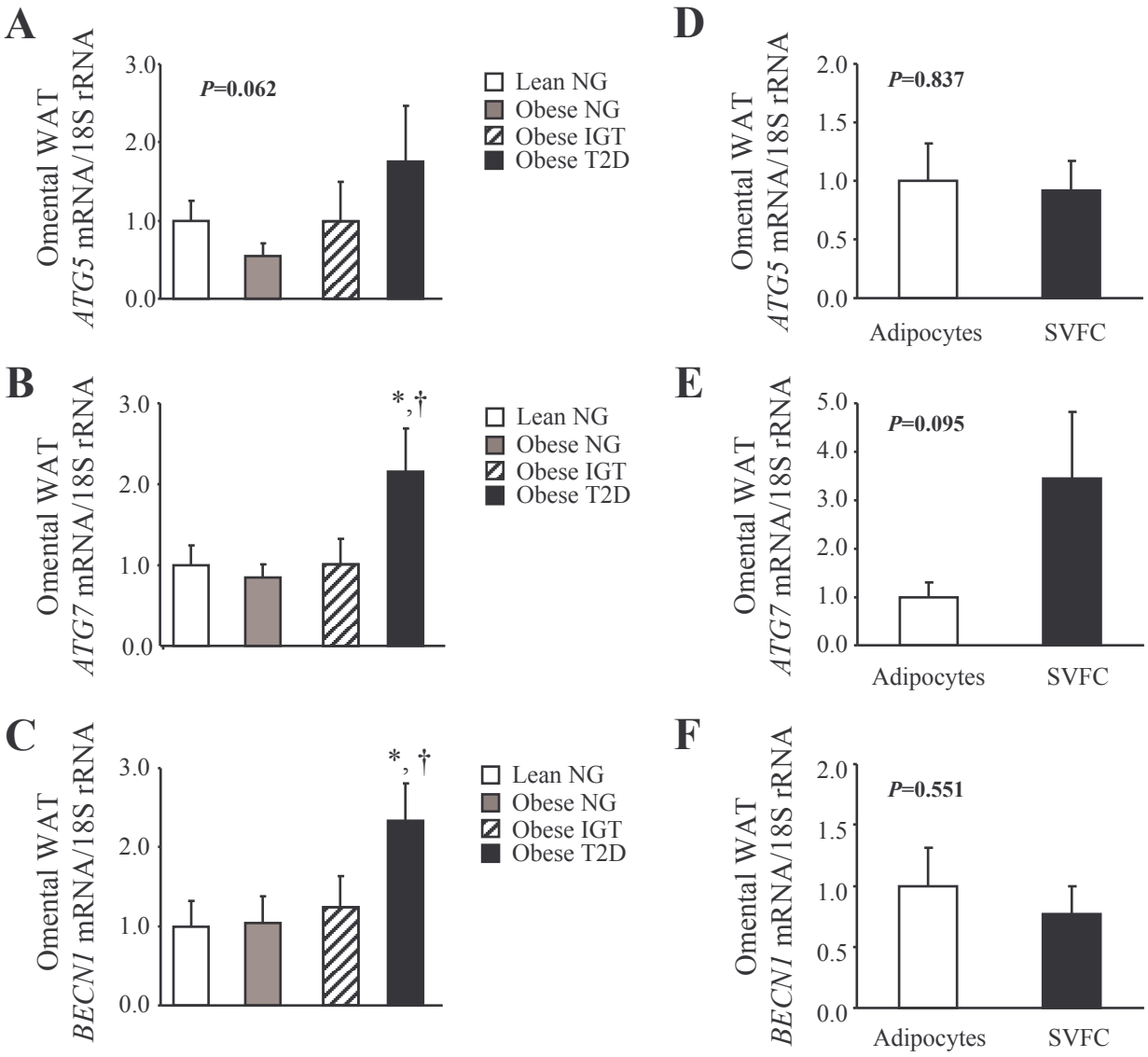

A
Omental WAT
ATG5 mRNA/18S rRNA
3.0
2.0
1.0
0.0
P=0.062
Lean NG
Obese NG
Obese IGT
Obese T2D
B
Omental WAT
ATG7 mRNA/18S rRNA
3.0
2.0
1.0
0.0
*,†
Lean NG
Obese NG
Obese IGT
Obese T2D
C
Omental WAT
BECN1 mRNA/18S rRNA
3.0
2.0
1.0
0.0
*, †
Lean NG
Obese NG
Obese IGT
Obese T2D
D
Omental WAT
ATG5 mRNA/18S rRNA
2.0
1.5
1.0
0.5
0.0
P=0.837
Adipocytes
SVFC
E
Omental WAT
ATG7 mRNA/18S rRNA
5.0
4.0
3.0
2.0
1.0
0.0
P=0.095
Adipocytes
SVFC
F
Omental WAT
BECN1 mRNA/18S rRNA
2.0
1.5
1.0
0.5
0.0
P=0.551
Adipocytes
SVFC

Figure 5

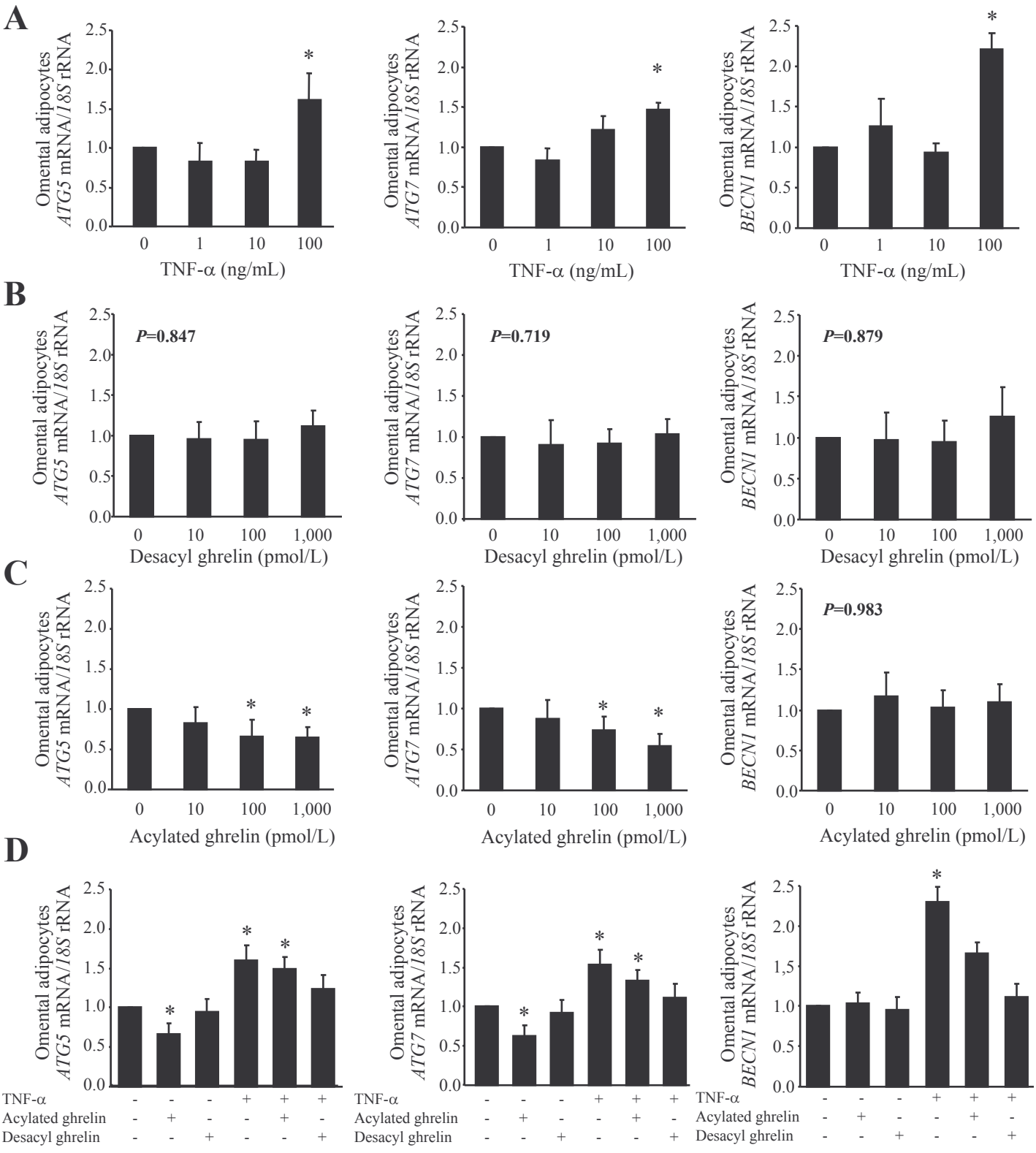

A
Omental adipocytes ATG5 mRNA/18S rRNA
Omental adipocytes ATG7 mRNA/18S rRNA
Omental adipocytes BECN1 mRNA/18S rRNA
TNF-α (ng/mL)
B
P=0.847
P=0.719
P=0.879
Desacyl ghrelin (pmol/L)
C
P=0.983
Acylated ghrelin (pmol/L)
D
TNF-α
Acylated ghrelin
Desacyl ghrelin

Figure 6

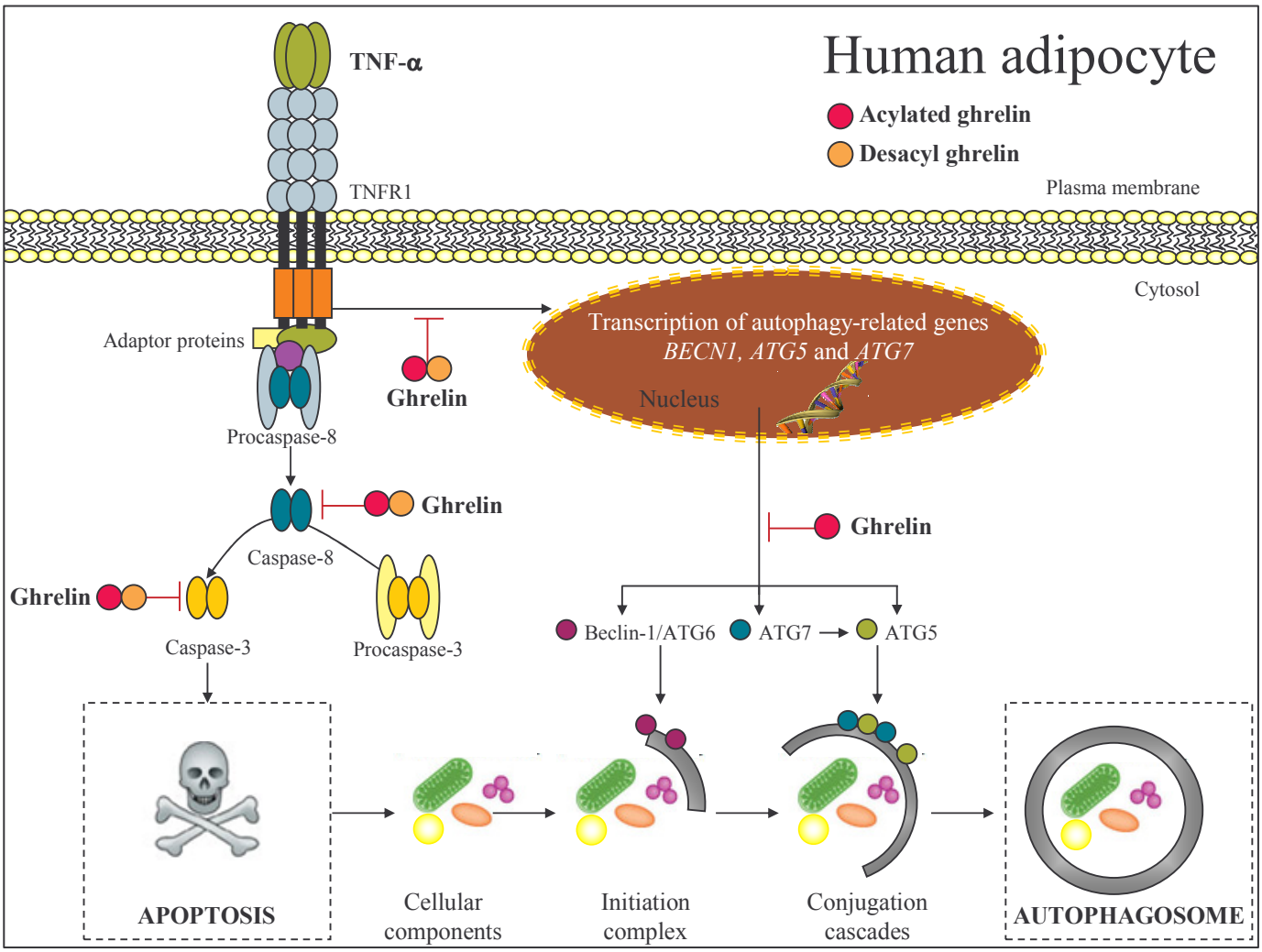

Human adipocyte
Acylated ghrelin
Desacyl ghrelin
TNF-α
TNFR1
Plasma membrane
Cytosol
Adaptor proteins
Procaspase-8
Ghrelin
Transcription of autophagy-related genes
BECN1, ATG5 and ATG7
Nucleus
Ghrelin
Ghrelin
Caspase-8
Ghrelin
Caspase-3
Procaspase-3
Beclin-1/ATG6
ATG7
ATG5
APOPTOSIS
Cellular components
Initiation complex
Conjugation cascades
AUTOPHAGOSOME

Figure 7