Caspase Induction and BCL2 Inhibition in Human Adipose Tissue

A potential relationship with insulin signaling alteration

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OBJECTIVE—Cell death determines the onset of obesity and associated insulin resistance. Here, we analyze the relationship among obesity, adipose tissue apoptosis, and insulin signaling.

RESEARCH DESIGN AND METHODS—The expression levels of initiator (*CASP8/9*) and effector (*CASP3/7*) caspases as well as antiapoptotic B-cell lymphoma *(BCL)2* and inflammatory markers were assessed in visceral (VAT) and subcutaneous (SAT) adipose tissue from patients with different degrees of obesity and without insulin resistance or diabetes. Adipose tissue explants from lean subjects were cultured with TNF-α or IL-6, and the expression of apoptotic and insulin signaling components was analyzed and compared with basal expression levels in morbidly obese subjects.

RESULTS—SAT and VAT exhibited increased *CASP3/7* and *CASP8/9* expression levels and decreased BCL2 expression with BMI increase. These changes were accompanied by increased inflammatory cytokine mRNA levels and macrophage infiltration markers. In obese subjects, *CASP3/7* activation and *BCL2* downregulation correlated with the IRS-1/2–expression levels. Expression levels of caspases, *BCL2*, *p21*, *p53*, *IRS-1/2*, *GLUT4*, protein tyrosine phosphatase 1B, and leukocyte antigen-related phosphatase in TNF-α– or IL-6–treated explants from lean subjects were comparable with those found in adipose tissue samples from morbidly obese subjects. These insulin component expression levels were reverted with *CASP3/7* inhibition in these TNF-α– or IL-6–treated explants.

CONCLUSIONS—Body fat mass increase is associated with *CASP3/7* and *BCL2* expression in adipose tissue. Moreover, this proapoptotic state correlated with insulin signaling, suggesting its potential contribution to the development of insulin resistance.

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The prevalence of obesity has increased dramatically in the last decades—so much so that it is now considered a major health problem. Obesity is very often accompanied by other diseases, with the most common being type 2 diabetes and cardiovascular complications (1–3).

Both type 2 diabetes and obesity include genetic, environmental, and lifestyle factors. However, progression to overt diabetes in obese subjects is not always predictable. Thus, while some obese individuals progress to type 2 diabetes, others may only have mild metabolic abnormalities, suggesting that the absolute amount of fat stored may not be the most important factor determining the relationship between obesity and type 2 diabetes (4–7). Indeed, other factors such as adipose tissue inflammation are viewed as key promoters of progression to type 2 diabetes (7–9).

Adipose tissue expandability in response to a positive energy balance has been considered classically as an adaptive passive process. However, recent evidence suggests that the expandability of adipose tissue is not an unlimited process. Furthermore, it may be an important factor determining the appearance of obesity-associated comorbidities.

Apoptosis is a fundamental mechanism for the homeostasis of mammalian tissues and has been linked to a variety of disorders. Apoptosis is a form of programmed cell death that occurs under certain physiological and pathological conditions as a common mechanism of cell replacement, tissue remodeling, and elimination of damaged cells. The caspase family is the largest enzyme involved in this process, synthesized as proenzymes, and appears distributed in multiple locations, including the cytoplasm, mitochondrial intermembrane space, or nuclear matrix (10). Another large protein family involved in this process is B-cell lymphoma 2 (BCL2) proteins, which regulate mitochondrial permeability processes and therefore constitute a key point for the mitochondrial pathway of apoptosis. BCL2 is well-known to be a potent prosurvival advocate with antiapoptotic effects (11).

The extrinsic apoptotic pathway involves "death receptors" (i.e., Fas, tumor necrosis factor [TNF]-αR, death receptor [DR]3, DR4, and DR5) and is exclusively controlled by caspases (12). This process is initiated by extracellular ligands that, upon binding to their corresponding DRs, cause the recruitment of initiator caspase (CASP)8. CASP8 activates via autocatalysis and then cleaves and activates its effector CASP3, which leads to

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cleavage of certain cellular substrates (13,14), allowing apoptosis. CASP9 is known to be the effector of CASP3/7 (10). The intrinsic apoptotic pathway leads to cell death without the involvement of membrane receptors, and after exposure to certain stimuli the balance between pro- and antiapoptotic BCL2 family proteins determines the choice between survival and cell death by release of cytochrome c from the mitochondria to the cytosol (15). The activation of some caspases is not always only conducive to an apoptotic mechanism but also to other forms of cell death, such as one morphologically and mechanistically distinct from apoptosis called pyroptosis or CASP1-dependent programmed cell death, in which CASP1 activation can result not only in the production of activated inflammatory cytokines but also in rapid cell death characterized by plasma-membrane rupture and release of proinflammatory intracellular contents (16).

Recently, a relationship between adipose tissue inflammation and apoptosis was proposed (17,18). A better understanding of the mechanisms that affect adipose tissue mass increase, including apoptotic cell death, is crucial for dealing with obesity and related diseases. The goal of this study was to analyze the potential changes in the gene expression profile of proteins that mediate apoptosis, including caspase and BCL2 family members in adipose tissue from lean and obese subjects with different degrees of obesity and displaying comparable insulin resistance. In addition, we aimed to decipher whether inflammatory cytokines known to be produced by adipose tissue (i.e., TNF-α and interleukin [IL]-6) could be associated with caspase and BCL2 activation and to determine whether these cellular effectors interfere with insulin signaling.

RESEARCH DESIGN AND METHODS

—Patients and adipose tissue collection are described in detail in Supplementary Data. Real-time PCR and Western blot are also described in detail in Supplementary Data.

Adipose tissue culture

Visceral (VAT) and subcutaneous (SAT) adipose tissue explants were prepared by cutting samples into 5-mg portions, which were subsequently incubated for 30 min in PBS supplemented with 5% BSA (3 mL/g). After 30 s of centrifugation (400*g*; samples incubated in M199 medium [Gibco, Invitrogen] supplemented with 10% FBS, 100 unit/mL penicillin, and 100 μg/mL streptomycin, with Ac-DEVD-CHO previously added or not to adipose tissue explants at the concentration of 10 μmol/L/mL), a preincubation with this CASP3/7-specific inhibitor of 1 h was done, and then TNF-α or IL-6 were added to milieu at a concentration of 50 ng/mL for 24 h at 37°C. After these treatments, tissue explants were collected and homogenized in PBS containing TritonX-100 (0.5%) and protease inhibitors. Protein concentrations were determined by spectrophotometry using Bradford reagent.

Separation of stromal vascular fraction from adipocytes

Adipose tissue samples were washed twice with PBS and minced removing the blood vessels. Samples were then digested in a water bath with collagenase I (1 μg/mL) at 37°C for 30 min followed by several washes in PBS, filtrations, and centrifugations (800 rpm) with 250- and 100-μm nylon mashes. Erythrocytes were removed with erythrocyte lysis buffer (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 2 mmol/L NaEDTA), and cells were centrifuged for 5 min at 800 rpm. Stromal vascular fraction (SVF) and adipocyte fraction were used for gene expression analysis

CASP3/7 activity assay

Apoptosis was measured using the Caspase-Glo3/7 assay (Promega). Proteins were transferred to a white opaque 96-well plate, and 10 μg protein in 100 μL total volume was mixed with 100 μL equilibrated Caspase-Glo3/7 reagent. After 24 h at 37°C, luminescence was measured using the GloMax-Multi Detection system (Promega). Each sample was measured in triplicate.

Statistical analyses

The statistical analysis was done with SPSS (version 15.0). Anthropometric, biochemical, and hormonal variables were compared between the different groups using the Kruskal-Wallis test. Statistical differences in mRNA expression levels between obese and lean subjects were determined using the Mann-Whitney *U* test. In the explant culture experiments, comparisons between the groups of explants incubated with IL-6 and TNF-α with lean control explants (from lean subjects without any addition) and obese control explants (adipose tissue from obese subjects) were assessed using the Mann-Whitney *U* test. In experiments treated with CASP3/7 inhibitor and subsequently with TNF-α/IL-6, comparisons were done between lean control and TNF-α/IL-6 treatment and between treatments in the absence and presence of CASP3/7 inhibition. The existence of correlation between different measured variables was determined through Spearman correlation coefficient (r_s).

RESULTS

—The subject clinical and biochemical characteristics are shown in Supplementary Table 1. Overweight and obese subjects displayed higher waist circumference and glucose levels compared with lean subjects. Morbidly obese patients presented low levels of HDL cholesterol and high levels of leptin compared with control subjects. Serum adiponectin was lower in obese patients than in lean subjects. All of the studied groups displayed the same degree of insulin resistance (homeostasis model assessment of insulin resistance <4).

Caspase and BCL2 expression profile in VAT and SAT from the studied group

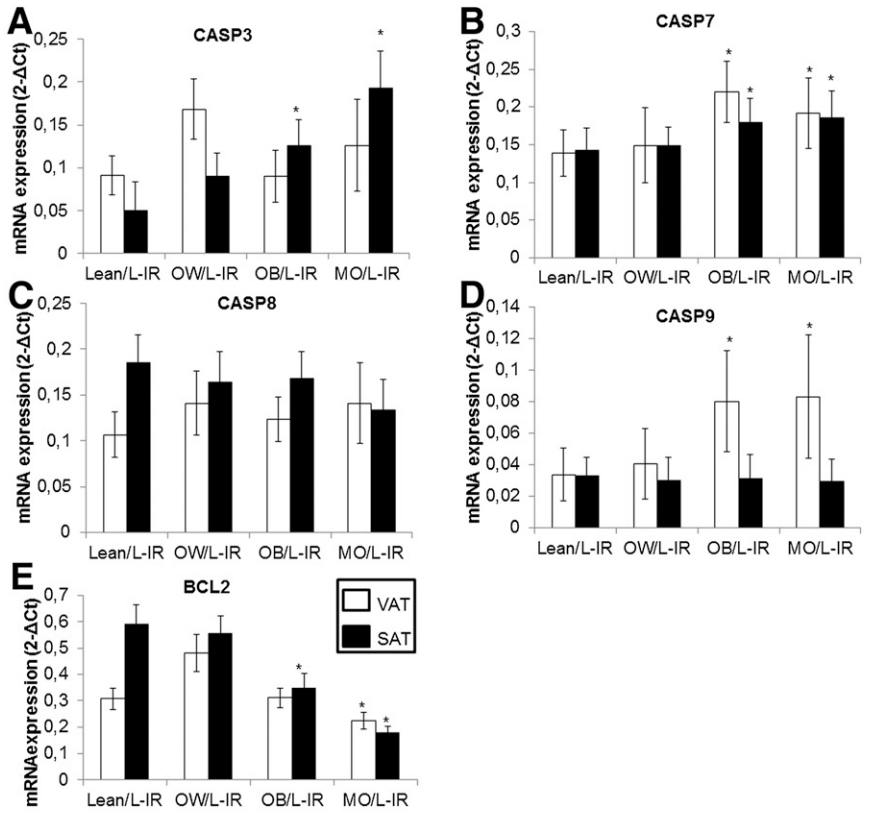
Gene expression and cleaved-forms protein expression of CASP3 and CASP7, which are known to play a central role in the execution phase of cell apoptosis and to interact with CASP8 and -9 (10), were significantly higher in both SAT and VAT from obese and morbidly obese subjects than in control subjects (Fig. 1*A*, *B*, and *F*). CASP9 was significantly enhanced in VAT of obese and morbidly obese subjects (Fig. 1*D* and *F*). Gene expression and phosphorylated form protein expression of BCL2 were significantly decreased in obese and morbidly obese subjects compared with lean subjects in both VAT and SAT (Fig. 1*E* and *F*).

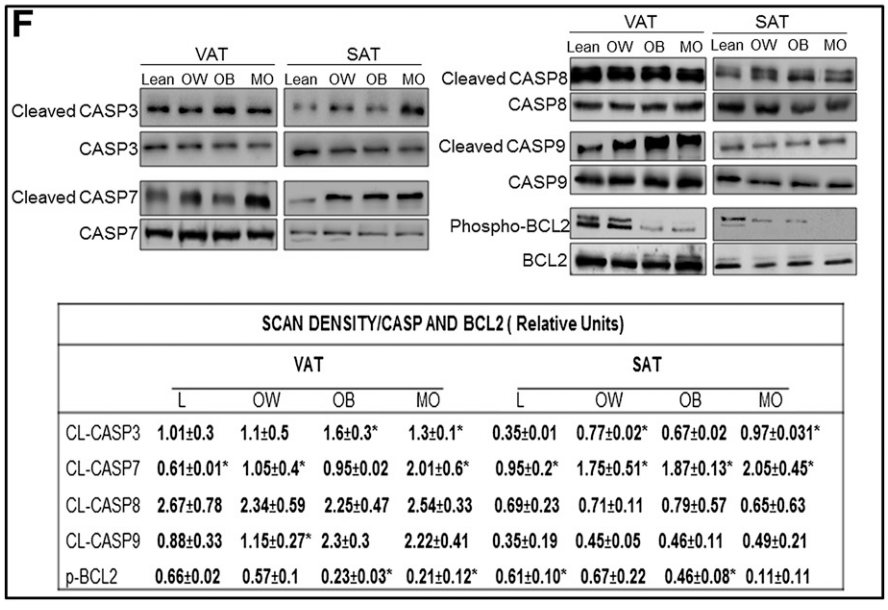
Correlative analysis of caspase and *BCL2* gene expression with BMI

Visceral *CASP9* and subcutaneous *CASP3* correlated positively with BMI ($r_s = 0.327$, $P < 0.05$, and $r_s = 0.696$, $P < 0.005$, respectively), while subcutaneous *BCL2* correlated negatively ($r_s = -0.524$, $P < 0.005$).

Differences in the gene expression profiles of proinflammatory cytokines (*TNF-α* and *IL-6*) and macrophage markers (*CD11b* and colony-stimulating factor-3) in VAT and SAT between the studied groups

TNF-α and *IL-6* gene expression levels were significantly increased in obese and morbidly obese subjects compared with





SCAN DENSITY/CASP AND BCL2 (Relative Units)

	VAT				SAT			
	L	OW	OB	MO	L	OW	OB	MO
CL-CASP3	1.01±0.3	1.1±0.5	1.6±0.3*	1.3±0.1*	0.35±0.01	0.77±0.02*	0.67±0.02	0.97±0.031*
CL-CASP7	0.61±0.01*	1.05±0.4*	0.95±0.02	2.01±0.6*	0.95±0.2*	1.75±0.51*	1.87±0.13*	2.05±0.45*
CL-CASP8	2.67±0.78	2.34±0.59	2.25±0.47	2.54±0.33	0.69±0.23	0.71±0.11	0.79±0.57	0.65±0.63
CL-CASP9	0.88±0.33	1.15±0.27*	2.3±0.3	2.22±0.41	0.35±0.19	0.45±0.05	0.46±0.11	0.49±0.21
p-BCL2	0.66±0.02	0.57±0.1	0.23±0.03*	0.21±0.12*	0.61±0.10*	0.67±0.22	0.46±0.08*	0.11±0.11

Figure 1—*Caspase and* BCL2 *gene and protein expression profile in VAT and SAT from lean, overweight, obese, and morbidly obese subjects* (A–E). *CASP3, CASP7, CASP8, CASP9, and BCL2 mRNA expression analysis was performed on VAT and SAT adipose tissues from four groups of subjects: lean, overweight (OW/L-IR), obese (OB/L-IR), and morbidly obese (MO/L-IR)—all with low degrees of insulin resistance (L-IR). mRNAs were normalized to cyclophilin levels. Western blotting analysis of human adipose tissue was carried out using antibodies against cleaved/active caspase and phosphorylated (Ser70) BCL2. Cleaved caspase expression levels were compared in relation to total caspase and phosphorylated BCL2 in reference to total BCL2* (F). *The blots are representative of three independent experiments with different samples. Density analysis was carried out by normalizing the samples to each corresponding caspase and BCL2 using NIH ImageJ software. Results were obtained in triplicate and are expressed as means ± SEM* (n = *13 for lean,* n = *13 for OW/L-IR,* n = *12 for OB/L-IR, and* n = *12 for MO/L-IR subjects*). **P < 0.05 comparing OW, OB, and OM with lean.*

lean individuals in both adipose tissue depots. Moreover, *TNF-α* gene expression was significantly increased in overweight subjects compared with lean subjects in VAT. In both adipose tissue depots, colony-stimulating factor *(CSF)-3* was higher in morbidly obese compared with control subjects, while *CD11b* was increased in overweight, obese, and morbidly obese subjects (Table 1).

Correlations between gene expression levels of caspases, *BCL2*, and inflammatory markers

CASP8 correlated positively with *TNF-α* in VAT ($r_s = 0.464$, $P < 0.05$) and with *CSF-3* in SAT ($r_s = 0.621$, $P < 0.05$). *BCL2* negatively correlated with IL-6 and *CD11b* ($r_s = -0.331$ and $r_s = -0.480$, respectively; $P < 0.05$) in VAT and with *TNF-α* and *CD11b* ($r_s = -0.385$ and $r_s = -0.480$, respectively; $P < 0.05$) in SAT.

Effects of TNF-α and IL-6 on CASP3/7 activity in VAT and SAT explants from healthy lean subjects

VAT and SAT adipose tissue explants from healthy lean subjects were incubated with TNF-α (50 ng/mL) or IL-6 (50 ng/mL) for 24 h, and then CASP3/7 activity was measured. The resulting values were compared with those observed in untreated VAT and SAT samples from morbidly obese subjects.

As shown in Fig. 2*A*, IL-6 and TNF-α induced a significant increase in CASP3/7 activity in both VAT and SAT explants compared with control subjects, approaching the levels observed in the morbidly obese.

Effects of TNF-α and IL-6 on expression levels of *CASP3*, *CASP7*, *CASP8*, *CASP9*, and *BCL2* in VAT and SAT explants from healthy lean subjects

IL-6 and TNF-α induced significant increases in *CASP3*, *CASP7*, *CASP8*, and *CASP9* mRNA levels in VAT and SAT explants (similar to those observed in adipose tissue from morbidly obese) (Fig. 2*B–E*). In contrast, *BCL2* gene expression was significantly decreased, approaching basal *BCL2* mRNA values in adipose tissue samples from morbidly obese subjects (Fig. 2*F*). Protein analysis confirmed mRNA expression data. In these explants, BCL-2 phosphorylation in Ser70 decreased in comparison with control subjects (Fig. 2*O*).

Table 1—*mRNA expression ($2^{-\Delta Ct}$) of inflammatory cytokines and macrophage markers in VAT and SAT*

	Lean/L-IR	OW/L-IR	OB/L-IR	MO/L-IR
VAT				
TNF-α	0.032 ± 0.002	0.043 ± 0.002*	0.049 ± 0.002*	0.145 ± 0.009*
CSF-3	0.004 ± 0.000	0.001 ± 0.000	0.003 ± 0.000	0.053 ± 0.002*
IL-6	0.013 ± 0.002	0.011 ± 0.002	0.016 ± 0.001*	0.051 ± 0.003*
CD11b	0.036 ± 0.002	0.053 ± 0.002*	0.049 ± 0.002*	0.209 ± 0.036*
SAT				
TNF-α	0.021 ± 0.001	0.022 ± 0.002	0.029 ± 0.001*	0.200 ± 0.022*
CSF-3	0.017 ± 0.002	0.013 ± 0.000	0.020 ± 0.002*	0.032 ± 0.004*
IL-6	0.024 ± 0.001	0.026 ± 0.002	0.028 ± 0.002*	0.102 ± 0.030*
CD11b	0.075 ± 0.002	0.136 ± 0.020*	0.447 ± 0.026*	0.906 ± 0.031**

Data are means ± SEM. Differences in mRNA expression levels between groups were analyzed using the Mann-Whitney *U* test. L-IR, low insulin resistance; MO, morbidly obese; OB, obese; OW, overweight. *$P < 0.05$, **$P < 0.01$: significant differences between lean patients and the other groups of patients.

Effects of TNF-α and IL-6 on p21 and p53 gene expression in adipose tissue explants from healthy lean subjects

Proteins p21 and p53 are responsible for causing apoptosis through the activation and/or suppression of the transcription of target genes that in turn are well-known to be associated with the development of insulin resistance. Figure 2*G* and *H* shows that both inflammatory cytokines induced significant increases in p21 and p53 gene expression in VAT and SAT explants from lean subjects to levels similar to those in morbidly obese subjects (Fig. 2*G* and *H*). Protein expression analysis confirmed mRNA expression data (Fig. 2*O*).

Effects of TNF-α and IL-6 on gene expression of markers of insulin signaling cascade in VAT and SAT explants from healthy lean subjects

We assessed mRNA levels of GLUT4, insulin receptor substrate (IRS)-1 and -2, and the phosphatases protein tyrosine (PTP)1B and leukocyte antigen related (LAR) in VAT and SAT explants from lean subjects exposed to TNF-α and IL-6 (Fig. 2*I*–*M*). *GLUT4* and *IRS-1/2* gene expression decreased, reaching the basal levels observed in adipose tissue from morbidly obese subjects. Moreover, TNF-α and IL-6 significantly diminished tyrosine phosphorylation of IRS-1/2 in both SAT and VAT explants from lean subjects (Fig. 2*N*), which were similar to those observed in adipose tissue from morbidly obese subjects.

Gene and protein expression levels of phosphatases *PTP1B* and *LAR* were elevated in both VAT and SAT explants from lean subjects incubated in the presence of TNF-α and IL-6, which, in turn, were comparable with the basal levels obtained in adipose tissue from morbidly obese subjects (Fig. 2*I*, *J*, and *O*).

Effects of TNF-α and IL-6 on apoptotic and insulin signaling components in SVFs and adipocyte fractions separately from healthy lean VAT and SAT explants

After incubation of adipose tissue explants, SVF was separated from adipocyte, and mRNA expression levels of apoptotic and insulin signaling components were evaluated in both fractions separately (Fig. 1*A* and B and Supplementary Data). With the exception of *CASP3*, no significant changes were observed in SVF, however, while in adipocytes the effects of TNF-α and IL-6 were clearly comparable with those observed in whole adipose tissues.

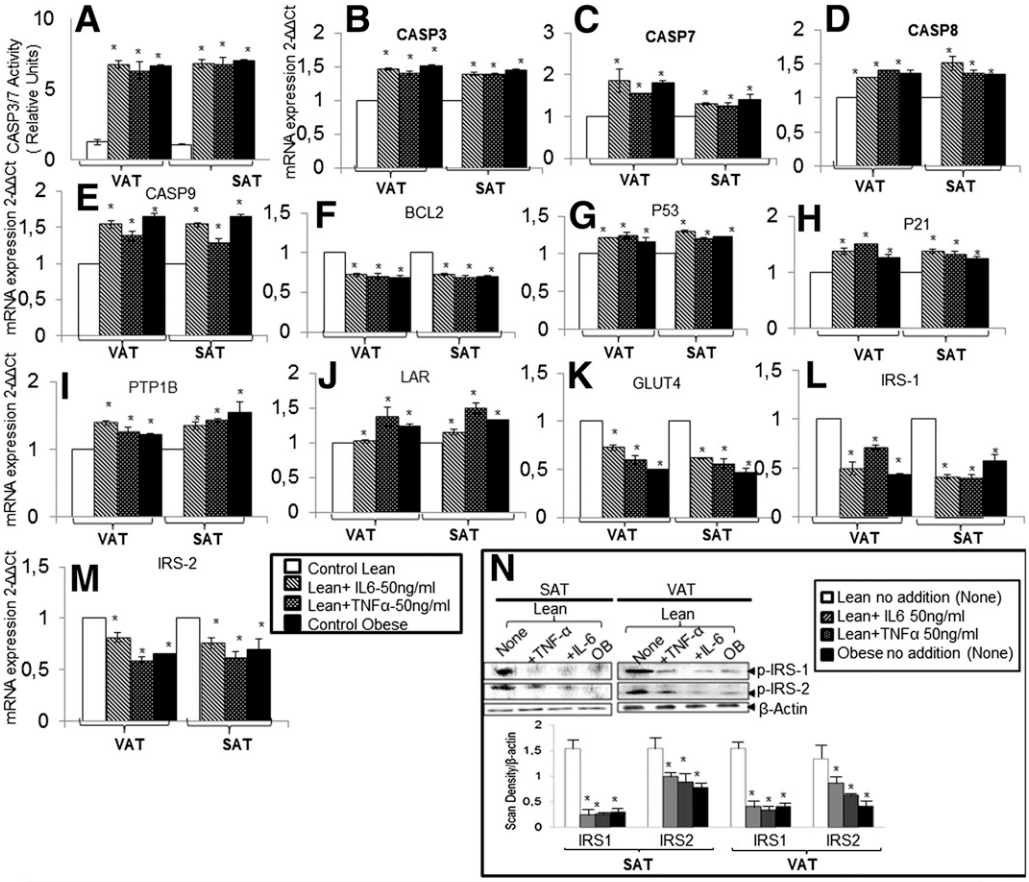
Alterations of insulin signaling marker expression induced by TNF-α and IL-6 in adipose tissue explants were reverted by inhibiting CASP3/7

To ascertain whether caspases are involved in the alterations observed in presence of TNF-α and IL-6, specific inhibitor of CASP3/7 was used. Figure 3*A* and *B* clearly shows that in presence of Ac-DEVD-CHO, downregulations of IRS-1/2 and GLUT4 observed in presence of inflammatory cytokines were markedly reverted. This effect was especially detected in adipocyte fraction of both adipose tissues, where *caspase/BCL2* and insulin signaling markers were altered by TNF-α and IL-6. However, it is not clear whether CASP3/7 is partially or totally involved in IRS-2 alteration by inflammation because in the presence of Ac-DEVD-CHO, IRS-2 mRNA levels overcome those detected in control subjects, which could be due to other parallel effects that may have CASP3/7 inhibition on IRS-2.

Correlation analysis of caspase and *BCL2* gene expression with inflammatory and insulin signaling marker (*IRS-1/2*) gene expression in VAT and SAT from obese subjects

CASP3 negatively correlated with *IRS-1* and *IRS-2* in SAT ($r_s = -0.202$ and $r_s = -0.425$, respectively; $P < 0.05$). *CASP7* negatively correlated with IRS-2 in VAT ($r_s = -0.595$, $P < 0.05$) and with *IRS-1* in SAT ($r_s = -0.699$ $P < 0.05$). *CASP8* positively correlated with *TNF-α* in VAT ($r_s = 0.464$, $P < 0.05$) and with *CSF-3* in SAT ($r_s = 0.621$, $P < 0.05$). *BCL2* correlated with *IRS-1* in both fat depots ($r_s = 0.446$, $P < 0.05$, and $r_s = 0.480$, respectively; $P < 0.005$). Moreover, *BCL2* negatively correlated with *IL-6* and *CD11*b in VAT ($r_s = -0.331$ and $r_s = -0.480$, respectively; $P < 0.05$) and with *TNF-α* ($r_s = -0.385$, $P < 0.05$) and *CD11b* ($r_s = -0.480$, $P < 0.01$) in SAT.

CONCLUSIONS—Apoptosis of adipose tissue is a relatively poorly studied phenomenon compared with other tissues; yet, dysregulation of this process has recently been proposed to contribute to obesity, differences in regional fat distribution, or lipodystrophy (19,20). The main finding of this study is that adipocyte fraction from both SAT and VAT displays an increase in gene expression of proapoptotic caspases including *CASP3*, *CASP7*, *CASP8*, and *CASP9* and decreased antiapoptotic (*BCL2*) gene expression with BMI increase. In addition, these changes were paralleled by an increase in gene expression of inflammatory cytokines and macrophage infiltration markers in both fat depots. In TNF-α/IL-6–incubated lean fat depots, the interference with apoptosis initiation via CASP3/7 inhibition involved insulin signaling pathway alteration. These data point to the fact that in morbidly obese subjects, altered expression of mediators of the insulin signaling pathways is likely to be related to the activation of CASP3/7 and the downregulation of BCL2, given that these two processes can be triggered by the inflammatory state (i.e., increased TNF-α and IL-6). Together, these data support a relationship between the apoptotic pathway (proapoptotic caspases and antiapoptotic BCL2) and



SCAN DENSITY/CASP, BCL2 AND β-ACTIN(Relative Units)

	VAT				SAT			
	Lean AT explant				Lean AT explant			
	None	+TNF-α	+IL-6	OB	None	+TNF-α	+IL-6	OB
CL-CASP3	0.61±0.06	1.4±0.2*	2.16±0.4*	1.86±0.1	0.15±0.02	0.94±0.02	0.67±0.02	0.98±0.031
CL-CASP7	0.32±0.01*	0.95±0.2	0.87±0.04	0.81±0.1*	0.35±0.03	1.05±0.32*	0.89±0.11*	0.75±0.34
CL-CASP8	0.27±0.04	1.12±0.22*	2.15±0.38*	1.94±0.51	0.64±0.16	1.21±0.23	1.19±0.24	1.76±0.46*
CL-CASP9	0.26±0.04	1.67±0.37	2.09±0.56*	1.29±0.27*	0.97±0.21	1.54±0.37*	1.86±0.31	1.79±0.29
p-BCL2	1.27±0.19	0.85±0.23*	0.63±0.16*	0.42±0.07	1.31±0.15	0.97±0.26	0.69±0.16*	0.51±0.08
P21	0.35±0.021	1.21±0.18	1.11±0.03	0.89±0.01	0.21±0.01	0.87±0.14	0.94±0.21	0.87±0.16
P53	0.52±0.07	1.65±0.25*	1.59±0.31*	1.11±0.14	0.43±0.08	1.67±0.21*	1.04±0.36	1.38±0.25*
LAR	0.41± 0.13	0.51±0.21	0.86±0.13	1.06±0.21*	0.22±0.02	0.31±0.01	0.54±0.07	0.61±0.11
PTP1B	0.31±0.04	2.07±0.54*	1.89±0.27	1.43±0.21	0.39±0.21	0.78±0.01	1.13±0.12*	1.12±0.24

Figure 2—*Effects of TNF-α and IL-6 on CASP3/7 activity; caspase,* BCL2, p53, p21, *and insulin signaling markers; and GLUT4, IRS-1, IRS-2, PTP1B, and LAR gene expression and protein activation in VAT and SAT explants from healthy lean subjects. Adipose tissue explants (VAT and SAT) (5 mg) were incubated in the presence of TNF-α (50 ng/mL) or IL-6 (50 ng/mL) for 24 h at 37°C. A: CASP3/7 activity was measured in homogenate of incubated explants and of adipose tissue from morbidly obese (OB) subjects as indicated in each panel.* B–H: CASP3, CASP7, CASP8, CASP9, BCL2, p53, and p21 *mRNA expression analysis was analyzed in lysed explants and lysed adipose tissue from morbidly obese subjects. mRNAs were normalized to cyclophilin levels.* I–M: GLUT4, IRS-1, IRS-2, PTP1B, *and* LAR *mRNA expression analysis was examined in lysed explants and lysed*

insulin signaling dysfunction associated with the proinflammatory state of adipose tissue, which may underlie the increased susceptibility of morbidly obese subjects to developing insulin resistance (21). In line with our hypothesis, it has recently been described that hyperglycemia induced by a sustained proinflammatory state activates CASP1, which may be conducive to insulin resistance in human adipose tissue (22)

Our studies show that in healthy subjects with different degrees of obesity, there was a clear increase in apoptotic markers associated with body fat mass increase. These changes were paralleled by a decrease in the expression of BCL2. This antiapoptotic protein, localized to mitochondria, endoplasmic reticulum, and the nuclear envelope, interferes with the activation of caspases by preventing the release of cytochrome c (23). In accordance with our data, it has recently been reported that during the development of obesity, the expansion of adipose tissue results in the activation of apoptotic signaling, including DR and mitochondrial pathways. These cytotoxic signaling pathways lead to the activation of effector caspases and adipocyte apoptosis (24). In our study, executive components of apoptosis, especially CASP3, highly correlated with BMI, while BCL2 correlated negatively. These data highlight the relationship of these caspases and BCL2 with the functionality of adipose tissue. In line with this notion, a recent report demonstrated that CASP3 activation was significantly enhanced in adipocytes from obese mice on either high-fat or high-sucrose diets compared with lean mice on a control diet (24). Moreover, other studies showed that peroxisome proliferator–activated receptor γ, a key regulatory adipogenesis factor that also plays a relevant role in obesity (25), is a substrate of CASP3 and CASP8 during TNF-α receptor signaling in adipocytes, and the consequent peroxisome proliferator–activated receptor γ cleavage disrupts its nuclear localization (26). Other research suggests that adiposity regulation by guggulsterone, the active substance in guggulipid, is done by reducing the number of mature adipocytes via the inhibition of differentiation of 3T3-L1 preadipocytes and the induction of apoptosis through the activation of CASP3 and CASP7 (27). In a recent study, Spalding et al. (28) showed that neither adipocyte death nor generation rate is altered in early-onset obesity, but at the same time they recognize that their study did not rule out the fact that delayed obesity in subjects may increase the rate of recruitment of adipocytes and, consequently, of cell death. Moreover, selected subjects in their study did not clarify whether this affirmation is also related to morbidly obese subjects. In line with our hypothesis, other studies are showing that the frequency of adipocyte death is increased 30-fold in a mouse (*db/db*) model of obesity-associated white adipose tissue inflammation as well as in obese humans. These observations suggest that adipocyte death promotes macrophage recruitment, accumulation, and persistence in white adipose tissue of obese individuals (17). On the other hand, the downregulation of *BCL2* gene expression with BMI increase supports the notion of the occurrence of a shift in the balance between pro- and antiapoptotic proteins in response to increased fat mass in morbidly obese subjects. No studies on adipocyte or adipose tissue have clearly stated the relation of BCL2 adipose tissue levels with obesity. With regard to this issue, the only thing that has been proven is that in obesity, free fatty acids released from adipose tissue induce the apoptosis of pancreatic β cells via an endoplasmic stress response and by inhibiting the expression of antiapoptotic factor BCL2 (29). Here, we found a significant negative association between subcutaneous *BCL2* mRNA levels and BMI, which suggests a role for this antiapoptotic protein in the regulation of adipose tissue homeostasis.

As the abnormal function of adipocytes may play an important role in the development of the chronic low-grade proinflammatory state associated with obesity, we wondered whether these inflammatory mediators could also affect the expression of caspases and BCL2 in VAT and SAT and whether this could be related to impaired insulin signaling in morbidly obese subjects who are known to be susceptible to developing insulin resistance.

It has become clear that a state of low-grade chronic inflammation typically associated with obesity and characterized by macrophage infiltration of adipose tissue and increased production of proinflammatory cytokines plays a crucial role in the development of insulin resistance (30). In obesity, adipose tissue harbors predominantly M1 macrophages, which secrete TNF-α and IL-6, thereby increasing inflammation (31). TNF-α is a major proapoptotic stimulant in adipocytes (32). Moreover, in obesity the expression of TNF-α in adipocytes is increased, and this is believed to represent a major factor in the development of insulin resistance and type 2 diabetes (33). TNF-α directly interferes with the insulin signaling cascade, impairs insulin-stimulated glucose transport, and may act as an important autocrine/paracrine regulator of fat cell function to limit adipose tissue expansion. This could be mediated, at least in part, through triggering adipocyte apoptosis (32,34). Elevated IL-6 expression in adipose tissue is associated with obesity and insulin resistance (35,36). Moreover, there is a hierarchy of these two cytokines within adipose tissue, and in fact, TNF-α is a key regulator of the synthesis of IL-6 in adipose tissue (37). In agreement with these data, here we observed that both TNF-α and IL-6 gene expression levels were significantly enhanced with the increase in the obesity grade. Furthermore, incubation of adipose tissue explant from lean and healthy subjects with TNF-α or IL-6 significantly diminished *GLUT4*, *IRS-1*, and *IRS-2* gene expression to levels comparable with those observed in adipose tissue from morbidly obese subjects. Moreover, expression of phosphatases PTP1B and LAR, which are known to have negative regulation of the insulin receptor and are implicated in negatively regulating insulin signal transduction, increased upon treatment of adipose tissue

VAT and SAT from morbidly obese subjects. mRNAs were normalized to cyclophilin levels. Results were obtained in triplicate for each patient and are expressed as means ± SEM (n = *13 lean and* n = *12 obese patients). *P < 0.05. Immunoblotting analysis of tyrosine phosphorylation (p) of IRS-1 and IRS-2* (M) *and of cleaved/activated caspase and phosphorylated (Ser70) BCL-2 in explants from lean subjects incubated in the presence of TNF-α (50 ng/mL) or IL-6 (50 ng/mL) for 24 h at 37°C and in adipose tissue from morbidly obese subjects* (O). *The resulting blot of each isoform was compared and normalized to β-actin constitutive protein for IRS-1/2, total caspase for cleaved (CL) caspase, and total BCL-2 for phosphorylated BCL-2. Density analysis was performed using NIH Image J software. The blot is representative of three independent experiments with different samples* (n = *13 lean and* n = *12 obese subjects). *P < 0.05 comparing obese TNF-α/IL-6 treatment subjects with lean subjects without treatment.*

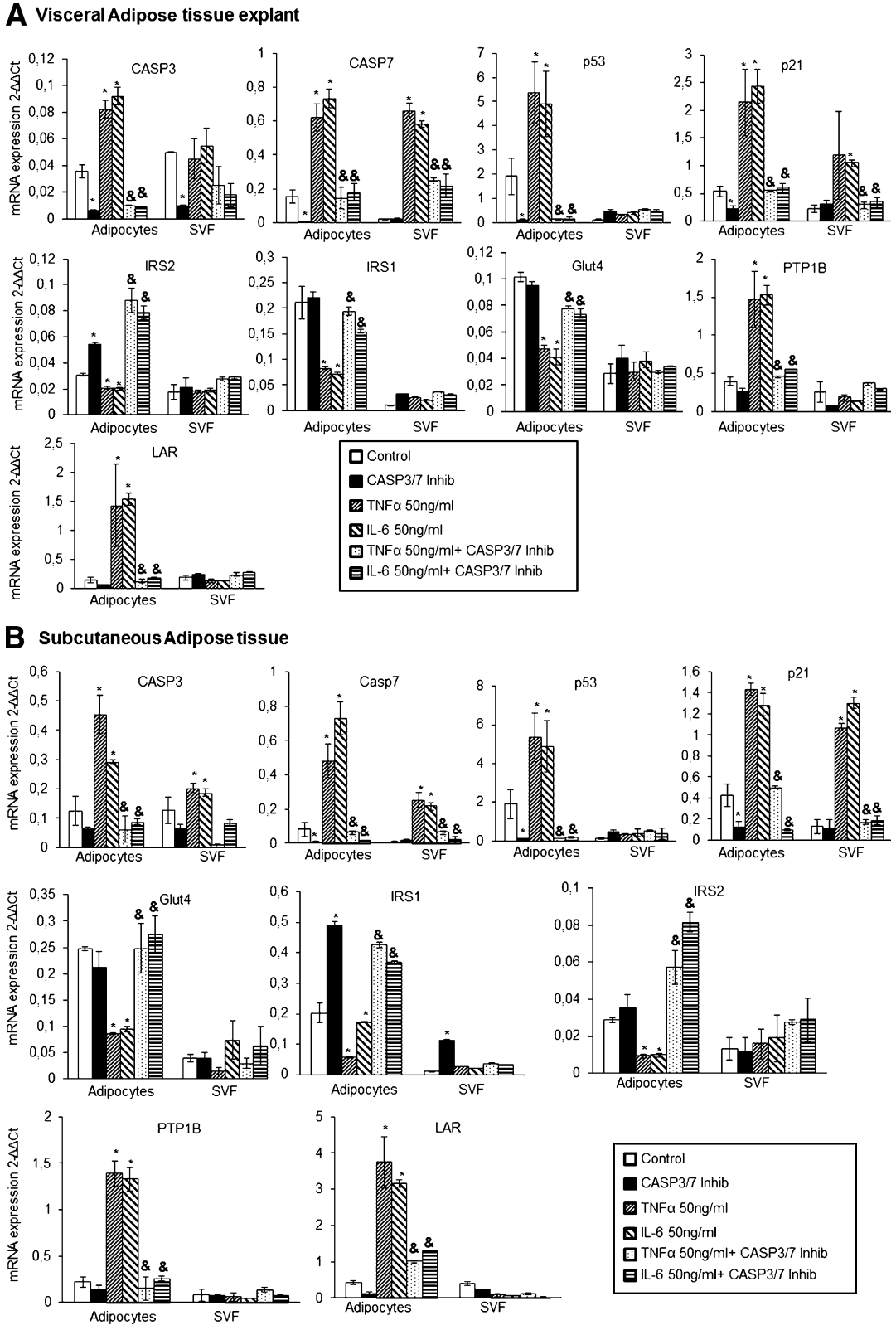


Figure 3—*Effects of CASP3/7 inhibition (Inhib) on TNF-α– and IL-6–induced p21, p53, PTP1B, and LAR gene expression upregulation and IRS-1/2 and GLUT4 gene expression downregulation in human adipose tissue (VAT and SAT) explants from healthy lean subjects. Adipose tissue explants (VAT and SAT) (5 mg) were previously incubated in the absence or presence of 10 μmol/L/mL Ac-DEVD-CHO for 1 h, and then TNF-α (50 ng/mL) or IL-6 (50 ng/mL) was added and explant was incubated for 24 h at 37°C.* A: CASP3, CASP7, p53, p21, IRS-1/2, GLUT4, PTP1B, *and* LAR mRNA *expression analysis was carried out in SVF and adipocyte fraction separately from VAT* (A) *and SAT* (B). *mRNAs were normalized to cyclophilin levels. Results were obtained in triplicate for each patient and are expressed as means ± SEM* (n = *8). *P < 0.05 comparing CASP3/7 inhibition and TNF-α/IL-6 treatment with untreated control subjects;* **&**P *< 0.05 comparing TNF-α/IL-6 plus CASP3/7 inhibition with TNF-α/IL-6 treatment.*

explants from lean subjects with IL-6/TNF-α, reaching levels similar to those found in adipose tissue from morbidly obese subjects. These findings suggest that TNF-α/IL-6 could increase the susceptibility to develop insulin resistance by altering the insulin signaling mechanism in adipose tissue.

TNF-α or IL-6 increased the expression of proapoptotic caspase expression levels, as well as CASP3/7 activity, while decreasing the expression of BCL2. Interestingly, we observed significant correlations between these apoptotic genes and insulin transduction mediators. In fact, CASP3/7 showed a significant negative association with IRS-1/2 in both SAT and VAT. On the other hand, BCL2 correlated positively with both IRS-1/2. These data suggest a potential relationship between the alteration in insulin signaling and apoptotic proteins in adipose tissue of morbidly obese subjects. Tumor suppressor genes *p21* and *p53* have been identified as responsible for preventing division of stressed cells and as causing apoptosis through activation and/or suppression of the transcription of target genes (38,39). Specifically, p53 activates the transcription of genes such as *p21* through binding to its response element at the *p21* promoter site (38–40). Both *p21* and *p53* have been shown to cause an inflammatory response that leads to insulin resistance in adipose tissue (41). Moreover, it has been described that p53 expression in adipose tissue is critical for insulin resistance development (41). Also, p21 is involved in both adipocyte differentiation and protecting hypertrophied adipocytes against apoptosis. Through these mechanisms, p21 promotes adipose tissue expansion with high-fat diets, leading to increased downstream pathophysiological consequences such as insulin resistance (42). In line with these data, VAT and SAT explants from healthy lean subjects exposed to TNF-α or IL-6 showed an increase in both *p21* and *p53* gene expression levels in a manner similar to that found in adipose tissue from morbidly obese subjects.

In summary, current studies uncover a key pathogenic role for adipose tissue CASP3/7 activation and BCL2 inhibition in the impairment of insulin signaling and subsequent development of insulin resistance. Moreover, a high inflammatory status seems to be involved in insulin signaling alteration in adipose tissue. Although further research is required, the results of this study suggest that a therapy controlling these apoptotic proteins may represent a useful strategy for the treatment or prevention of morbid obesity and associated insulin resistance.

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