Proteasome Dysfunction Associated to Oxidative Stress and Proteotoxicity in Adipocytes Compromises Insulin Sensitivity in Human Obesity

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Abstract

Aims: Obesity is characterized by a low-grade systemic inflammatory state and adipose tissue (AT) dysfunction, which predispose individuals to the development of insulin resistance (IR) and metabolic disease. However, a subset of obese individuals, referred to as metabolically healthy obese (MHO) individuals, are protected from obesity-associated metabolic abnormalities. Here, we aim at identifying molecular factors and pathways in adipocytes that are responsible for the progression from the insulin-sensitive to the insulin-resistant, metabolically unhealthy obese (MUHO) phenotype.

Results: Proteomic analysis of paired samples of adipocytes from subcutaneous (SC) and omental (OM) human AT revealed that both types of cells are altered in the MUHO state. Specifically, the glutathione redox cycle and other antioxidant defense systems as well as the protein-folding machinery were dysregulated and endoplasmic reticulum stress was increased in adipocytes from IR subjects. Moreover, proteasome activity was also compromised in adipocytes of MUHO individuals, which was associated with enhanced accumulation of oxidized and ubiquitinated proteins in these cells. Proteasome activity was also impaired in adipocytes of diet-induced obese mice and in 3T3-L1 adipocytes exposed to palmitate. In line with these data, proteasome inhibition significantly impaired insulin signaling in 3T3-L1 adipocytes.

Innovation: This study provides the first evidence of the occurrence of protein homeostasis deregulation in adipocytes in human obesity, which, together with oxidative damage, interferes with insulin signaling in these cells. Proteasome activity was also impaired in adipocytes of diet-induced obese mice and in 3T3-L1 adipocytes exposed to palmitate. In line with these data, proteasome inhibition significantly impaired insulin signaling in 3T3-L1 adipocytes. In line with these data, proteasome inhibition significantly impaired insulin signaling in 3T3-L1 adipocytes.

Conclusion: Our results suggest that proteasomal dysfunction and impaired proteostasis in adipocytes, resulting from protein oxidation and/or misfolding, constitute major pathogenic mechanisms in the development of IR in obesity. Antioxid. Redox Signal. 23, 597–612.

Introduction

Understanding fat cell biology has gained intensive scientific attention during the past decades as the prevalence of obesity is increasing worldwide and constitutes a major public health issue (2). It is well established that adipocyte hypertrophy and expansion of fat mass results in adipose tissue (AT) fibrosis and dysfunction, causing the activation of inflammatory cascades in adipocytes [i.e., c-Jun NH2-terminal kinase and nuclear factor κB (NF-κB)] and the
secretion of pro-inflammatory cytokines, which, in turn, trigger AT infiltration by macrophages and other immune cells (8, 36, 42, 64). Low-grade chronic inflammation and adipocyte dysfunction induce insulin resistance (IR), thus increasing the risk for the development of type 2 diabetes, cardiovascular disease, hepatic steatosis, hypertension, and cancer (21, 53). At the molecular level, obesity-related pathogenic factors causing adipocyte dysfunction include lipid and adipokine deregulation, mitochondrial malfunction, increased autophagy, and induction of cell stress processes [oxidative-, nitrosative-, and endoplasmic reticulum (ER)-stress] (8, 25).

A subset of obese individuals, known as “metabolically healthy obese” (MHO) subjects, seems to be resistant to obesity-associated metabolic complications (17). These individuals, which comprise between 20 and 30% of the adult obese population, are mainly characterized by (i) a lean-like adipokine pattern (i.e., normal adiponectin levels), (ii) reduced central body fat and AT fibrosis, (iii) diminished AT macrophage infiltration and inflammatory profile, and (iv) lower ectopic fat deposition (17, 39, 48), as compared with metabolically unhealthy obese (MUHO) individuals. Likewise, activation of pro-inflammatory signaling effectors challenging insulin sensitivity, such as NF-xB, in omental AT has been also proposed as a discriminative factor of MHO and MUHO individuals (6). However, the molecular components and processes underlying the favorable metabolic profile of MHO individuals are still poorly understood.

We, therefore, sought to (i) identify novel AT markers associated with the MUHO phenotype by comparing the proteomic profiles of mature adipocytes from human subcutaneous (SC) and omental (OM) fat of morbidly obese individuals with different degrees of insulin sensitivity, and (ii) to investigate the potential contribution of protein homeostasis deregulation to the pathogenesis of IR in obesity.

**Results**

**Baseline clinical and metabolic characteristics of subjects**

The clinical characteristics of normoglycemic (NG) (n = 18) and insulin-resistant (IR) (n = 13) morbidly obese subjects (body mass index [BMI] > 30 kg/m²) are shown in Table 1. Both groups were matched by age, BMI, and percent body fat. Average blood pressure values were similar in the two groups, yet some subjects in the IR group exhibited hypertension (4 out of 13 subjects). Fasting plasma glucose and insulin levels were significantly higher in insulin-resistant individuals. These subjects also exhibited higher homeostasis model assessment of insulin resistance (HOMA-IR) values and waist circumference than NG individuals. Plasma cholesterol, FFA, and triglycerides were higher in IR individuals than in NG subjects, although differences did not reach statistical significance.

**Proteomic profile of adipocytes from NG and IR individuals**

A multi-comparative proteomic analysis was employed to identify adipocyte proteins that are differentially expressed between SC and OM fat of NG versus IR individuals. From the 1687 protein spots identified in the master gel, 49 spots were differentially expressed between groups (p < 0.05) (Fig. 1A–C). Among them, 40 spots were identified by MALDI-TOF protein identification (Table 2). Of the identified proteins, 11 proteins were found to be differentially expressed between SC and OM adipocytes in NG subjects (3 upregulated and 8 down-regulated), and 3 proteins were down-regulated in SC adipocytes of IR individuals. Proteomic comparison of adipocytes from NG and IR subjects revealed significant changes in 20 proteins (12 upregulated and 8 downregulated) and 6 proteins (3 upregulated and 3 down-regulated) in SC and OM adipocytes, respectively. Of note, 16 out of the 20 differentially expressed proteins in SC adipocytes of NG and IR subjects were also regulated in OM adipocytes, although differences did not reach statistical significance (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars).
The PANTHER classification analysis revealed that the identified proteins belonged to four major functional groups: (i) catalytic activity: SUCB1, 3HIDH, GSHB, VAT1, ACADM, PRDX6, GSTM2, ACADV, NDUB9, PRDX4, PGM1, ALDOA, PRDX2, KAD1, EST1, PRDX1, ILK, DJ1, and PDIA3; (ii) antioxidant activity: PRDX1, PRDX2, PRDX4, and PRDX6; (iii) binding: FIBB, EST1, CALB2, and DJ1; and (iv) structural molecule activity: CO6A3, K2C6C, and VIME (Supplementary Fig. S2). Statistical differences in the expression of proteins with antioxidant (PRDX1, PRDX2, PRDX4, and PRDX6) or oxidoreductase (PRDX1, PRDX2, 3HIDH, VAT1, PRDX4, ACADV, ACADM, PRDX6, and NDUB9) activities were found between NG and IR individuals ($p < 0.05$).

Enhanced oxidative stress and failure of antioxidant defenses in adipocytes from IR subjects.

To confirm and extend the data on the oxidative status of adipocytes, we characterized the glutathione synthetase (GSHB)/glutathione system in SC and OM adipocytes from additional NG and IR subjects to those employed for the 2D-DIGE study. Consistent with the 2D-DIGE observations, GSHB expression was higher in SC adipocytes from IR individuals than from NG subjects (Fig. 2A). Accordingly, total glutathione levels were increased in both SC and OM adipocytes of IR subjects compared with NG subjects, although differences did not reach statistical significance due to interindividual variability within groups (Fig. 2D).

In view of these results, we analyzed oxidative-induced damage on adipocyte proteins. As shown in Figure 2E and F, the levels of carbonylated proteins and 4-Hydroxynonenal (4-HNE)-modified proteins were significantly increased in SC and OM adipocytes of IR individuals compared with NG subjects. A positive correlation in carbonylated protein content was found between SC and OM adipocytes (Supplementary Fig. S3A).

IR is associated with impaired protein folding and decreased proteasome activity in adipocytes

2D-DIGE analysis also revealed the differential expression of several molecular chaperones between groups (Table 2 and Supplementary Fig. S1). Quantitative immunoblotting showed that the protein levels of heat shock protein 70 (Hsp70/Hsc70) were decreased in OM adipocytes of IR subjects as compared with NG subjects (Fig. 3A). Contrary to that shown by 2D-DIGE, HSP70 levels in SC adipocytes of NG were not significantly different from those observed in IR subjects, likely due to interindividual variability (Fig. 3A).

We also investigated ER stress in SC and OM adipocytes of the two groups of subjects. As shown in Figure 3B, protein levels of the ER stress marker, C/EBP homologous protein (CHOP), were significantly higher in SC adipocytes of IR subjects than in IR subjects. In all, these data were suggestive of dysregulated protein folding in IR adipocytes. In line with this, we observed higher levels of ubiquitinated proteins in adipocytes from IR subjects (Fig. 3C). Ubiquitinated protein content in SC and OM adipocytes was positively correlated (Supplementary Fig. S3B). Therefore, we...
Table 2. Proteins Identified by MALDI-MS/MS Differentially Expressed in Mature Subcutaneous and Omental Adipocytes from Normoglycemic and Insulin-Resistant Morbidly Obese Individuals

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(continued)
# Table 2. (Continued)

| Spot<sup>a</sup> | T-test<sup>b</sup> | Av ratio<sup>c</sup> | Accession number<sup>d</sup> | Symbol | Name | Mw (kDa)/pI<sup>e</sup> | Pep<sup>f</sup> | % cover<sup>g</sup> | Expect<sup>h</sup> |
|-----------------|-----------------|-----------------|-----------------|--------|-----------------|-----------------|----------------|-----------------|
| 1572            | 0.025           | 1.9             | Q13418          | ILK    | Integrin-linked protein kinase | 51.8/8.3       | 9              | 16              | 4.0e-009         |
| 1044            | 0.019           | 1.99            | CAA35267        | CO6A3  | Collagen alpha-3 (VI) chain   | 34.5/8.9       | 1              | 4               | 0.00068           |
| 711             | 0.046           | 3.39            | NP_005504       | PDIA3  | Protein disulfide-isomerase A3 | 57.1/5.9       | 15             | 33              | 6.4e-013           |
| 1215            | 0.017           | -1.58           | NP_689953       | 3HIDH  | 3-hydroxyisobutyrate dehydrogenase, mitochondrial | 35.7/8.3       | 14             | 33              | 5.1e-009           |
| 1496            | 0.014           | -1.38           | CAG28580        | PRDX1  | Peroxiredoxin-1               | 22.3/8.2       | 19             | 68              | 4.0e-046           |
| 1496            | 0.014           | -1.38           | CAG46588        | PRDX2  | Peroxiredoxin-2               | 22.0/6.7       | 3              | 16              | 2.2e-006           |
| 1383            | 0.050           | -1.66           | NP_004896       | PRDX6  | Peroxiredoxin-6               | 25.1/6.0       | 26             | 83              | 1.6e-052           |

<sup>a</sup>Spot numbers correspond to those in Figure 1A.  
<sup>b</sup>p-value of the Student’s t-test. Only those proteins with a p ≤ 0.05 were considered.  
<sup>c</sup>Average volume ratio (a,b,c were calculated by the DeCyder analysis).  
<sup>d</sup>Accession numbers from the NCBInr database.  
<sup>e</sup>Theoretical molecular weight (kDa) and isoelectric point (pI).  
<sup>f</sup>Pep. corresponds to the number of peptides identified.  
<sup>g</sup>Coverage of all peptide sequences matched to the identified protein (%).  
<sup>h</sup>Mascot expected value sequence (a,b,c were determined by Mascot).  

**FIG. 2.** Analysis of antioxidant defenses and oxidative stress in SC and OM adipocytes from NG and IR subjects.  
(A) GSHB expression in isolated SC and OM adipocytes from NG and IR subjects. Standardized log. abundance of the spot was determined by DeCyder analysis of 2D-DIGE data (upper panels). Values indicate increased (+) or decreased (−) levels relative to the internal standards (n = 4 individuals/group). Immunoblot images (middle panel) and quantification of GSHB expression (lower panel) are shown (n = 5 individuals/group).  
(B) Total glutathione, reduced (GSH) and oxidized (GSSG) glutathione, and GSH/GSSG ratio in NG and IR adipocytes (n = 5 individuals/group).  
(C, D) Protein expression of DJ1 (C) and NQO1 (NAD(P)H dehydrogenase [quinone] 1) (D) in SC and OM adipocytes from NG and IR subjects (n = 4 individuals/group for 2D-DIGE and n = 5 individuals/group for immunoblotting studies).  
(E, F) Quantification of carbonylated proteins (DNP-protein adducts; E) and 4-HNE-modified proteins (F: dot blots) in NG and IR adipocytes (n = 5 individuals/group); protein levels in SC-NG adipocytes were set to 1 and used as reference for data normalization. *p < 0.05, **p < 0.01, and ***p < 0.001 versus NG.
next analyzed the capacity of IR adipocytes to preserve protein homeostasis and, in particular, we determined the activity of the proteasome. As shown in Figure 3D, IR adipocytes showed lower chymotrypsin-like (ChT-L) peptidase activities of the 26S and 20S proteasomes than NG adipocytes.

To validate the results obtained in human adipocytes, we examined the activity of the proteasome in adipocytes from mice fed a normal diet (ND) or a high-fat diet (HFD). As shown in Figure 4A, body weight was significantly higher in mice fed an HFD compared with ND-fed mice. Fasting plasma glucose levels were also increased in obese HFD-fed mice (Fig. 4B). Moreover, glucose (ipGTT; Fig. 4C) and insulin tolerance (ITT; Fig. 4D) tests demonstrated that mice fed an HFD exhibited lower glucose tolerance and insulin sensitivity than ND-fed animals. We next analyzed the activity of the proteasome in mature and in vitro differentiated adipocytes isolated from both SC and visceral AT of both groups. Our data showed that ChT-L peptidase activity of the 26S proteasome was significantly decreased in mature SC adipocytes of mice fed an HFD as compared with their ND counterparts (Fig. 4E). In contrast, the activity of the 26S proteasome was increased in mature visceral adipocytes of obese mice (Fig. 4E); 26S activity was markedly reduced in both SC and visceral in vitro differentiated adipocytes from HFD-fed mice (Fig. 4F).

**Activation of c-Jun N-terminal kinase in adipocytes of IR subjects**

We examined the expression of p54/p46 JNK1/2 and its phosphorylated forms in SC and OM adipocytes of NG and IR subjects. Figure 5 shows that either p54 JNKs or p46 JNKs protein levels were higher in SC adipocytes of IR subjects than in NG subjects. Notably, pJNK/JNK ratios were increased in OM adipocytes of IR subjects. SC adipocytes of IR individuals also exhibited increased pJNK/JNK ratio for p46. Together, these results indicated that IR was associated with an increase in JNK activity (Fig. 5).

**Proteasome inhibition impairs insulin signaling in 3T3-L1 cells**

To determine whether proteasomal dysfunction could mediate IR in adipocytes, we examined the effects of proteasome inhibition by treating 3T3-L1 cells with MG132 for 24 h. It is noteworthy that chronic exposure to insulin has been shown to induce degradation of the insulin receptor substrate 1 (IRS-1) by the proteasome (56), and that proteasome inhibition under chronic insulin stimulation evokes tyrosine phosphorylation of IRS-1 and glucose uptake in adipocytes (51). Nevertheless, whether proteasomal dysfunction impairs insulin signaling has not been yet examined. As shown in Figure 6A, 3T3-L1 cells exhibited enhanced HSP70 levels but reduced protein content of GSHB and NQO1 after 24 h of MG132 treatment. Notably, CHOP protein levels were significantly higher in MG132-treated cell cultures than in control cultures (Fig. 6A). However, MG132 did not modify the protein content of the pro-apoptotic factor Bax or the anti-apoptotic factor Bcl-2 (Fig. 6A). Measurement of DNP-protein adducts showed no differences between MG132-treated and untreated 3T3-L1 cells (Fig. 6B), while the proteasome inhibitor enhanced levels of ubiquitinated proteins (Fig. 6C).

MG132 treatment also reduced the expression of IRS-1 while increasing IRS-1 phosphorylation at Ser307 (Fig. 6A). Notably, the pJNK/JNK ratio was enhanced in cells exposed to MG132 (Fig. 6A). In all, these data supported the notion that inhibition of the proteasome could impair insulin signaling in 3T3-L1 cells.

**FIG. 3.** Analysis of the protein folding, ER stress and degradation machineries in SC and OM adipocytes from NG and IR subjects. (A) HSP70 protein content in SC and OM adipocytes of NG and IR subjects (n=5 individuals/group). Standardized log. abundance of the spots in the 2D-DIGE gels (upper panels; n=4 individuals/group). (B) Protein content of the ER marker, CHOP, in adipocytes of NG and IR subjects (n=5 individuals/group). (C) Quantification of ubiquitinated proteins in NG and IR adipocytes (n=5 individuals/group); protein levels in SC-NG adipocytes in the dot blots were set to 1 and used as reference for data normalization. (D) Measurement of the chymotrypsin-like (ChT-L) peptidase activity of the 26S proteasome (+ATP) (left panel) and 20S proteasome (+SDS) (right panel) in SC and OM adipocytes of NG and IR subjects (n=5 individuals/group). Data are expressed as a percentage of values in SC-NG adipocytes (100%). *p < 0.05 versus NG.
signaling. Indeed, insulin-induced phosphorylation of Akt was significantly reduced in MG132-treated cells when compared with control cells (Fig. 6D).

We then analyzed the activity of the proteasome in differentiated 3T3-L1 cells exposed to different treatments known to induce IR, including TNFα, a combination of high glucose and high insulin (HGHI), or palmitate (34). These experiments showed that exposure of 3T3-L1 adipocytes for 24 h to TNFα or HGHI had no effect on the ChT-L peptidase activity of the 26S proteasome (+ATP) (left panel) and 20S proteasome (+SDS) (right panel) in mature adipocytes isolated from subcutaneous and visceral fat of mice on ND or HFD (n = 3; 3–4 animals/pool). (F) Chymotrypsin-like (ChT-L) peptidase activity of the 26S proteasome (+ATP) (left panel) and 20S proteasome (+SDS) (right panel) in in vitro differentiated adipocytes from SVFCs of subcutaneous and visceral fat of mice on ND and HFD (n = 3; 3–4 animals/pool). *p<0.05, **p<0.01, and ***p<0.001 versus mice on ND.

Discussion

It has been proposed that metabolically healthy obesity, as compared with MUHO, is characterized by an improved adipogenic capacity and a healthy AT expansion, along with a low inflammatory profile (48). This study provides evidence demonstrating that defective protein folding and degradation, and the accumulation of oxidized and unfolded/misfolded proteins in adipocytes may contribute to the development of IR in obesity.

Here, we report the first comparative analysis of the proteome of paired SC and OM mature adipocytes of obese subjects with different degrees of insulin sensitivity. In agreement with the long established association between oxidative stress and IR in obesity (62), our 2D-DIGE data from isolated adipocytes indicated the existence of significant
differences in cellular oxidoreductase and antioxidant activities between MHO and MUHO individuals. In particular, when compared with adipocytes from NG subjects, IR adipocytes exhibited diminished levels of several antioxidant enzymes, including NQO1 and DJ1 (18, 57), as well as a lower GSH/GSSG ratio, which is a hallmark of oxidative stress (43). Together, these results support the notion that, contrarily to obese NG individuals, adipocytes from obese IR subjects are unable to cope with increased levels of oxidative stress in obesity.

Of note, both SC and OM adipocytes from IR subjects exhibited proteomic features of increased oxidative stress and impaired antioxidant defenses. Indeed, our proteomic and biochemical data suggest that SC and OM adipocytes undergo similar molecular rearrangements in the transition from the NG to the IR state, supporting the notion that adipocyte cell damage in both SC and OM fat may contribute to metabolic disease. We cannot comment on the relative association of whole OM or SC fat depots with the IR obese phenotype, inasmuch as our analysis did not include the other major component of AT, the stromal–vascular fraction (68), and we have no information on abdominal fat distribution in our subjects. Notwithstanding these limitations, our observations are in accordance with previous genomic data on human AT, demonstrating that molecular adaptations in abdominal SC AT prove as discriminating as those in OM AT with respect to obesity and metabolic complications (32).

When cellular antioxidant and repair pathways fail to restore protein oxidative damage, cells activate additional defense mechanisms to prevent the accumulation of oxidized proteins, which are toxic for cells and threaten cell viability (20). The major cellular proteolytic system responsible for the removal of oxidized proteins is the proteasomal system, predominantly the ATP/ubiquitin-independent 20S proteasome (47). Recently, a biphasic oxidative stress-dependent regulation of the proteasome proteolytic machinery has been proposed, with mild oxidative stress activating preexisting 20S proteasomes, and prolonged oxidative damage causing decreased proteolysis (3). Here, we found that the activity of the 20S proteasome was not upregulated in IR adipocytes but, instead, tended to diminish as compared with that observed in NG adipocytes. In line with this, our data revealed elevated protein carbonylation and 4-HNE-modified protein levels in human adipocytes from both fat depots under IR conditions, which is consistent with previous studies in both AT and isolated adipocytes, and further supports the link between enhanced protein carbonylation and obesity, IR, and diabetes (15). Specifically, carbonylation and 4-HNE modification of different proteasome subunits have been shown to suppress their activity (3). In this scenario, the increased levels of carbonylated and 4-HNE-modified proteins found in IR adipocytes might contribute, at least in part, to prevent the activation of the 20S proteasome in IR adipocytes, with this, in turn, causing accumulation of oxidatively modified proteins in these cells.

In accordance to that observed for the 20S proteasome, the activity of the 26S proteasome, which is composed by the core 20S proteasome and two 19S ATPase regulatory complexes (33), was also reduced in IR adipocytes. In line with these findings, we observed that the 26S proteasome was also decreased in freshly isolated mature adipocytes, and in vitro differentiated adipocytes of SC fat from IR mice induced by an HFD, as well as in palmitate-induced insulin-resistant 3T3-L1 adipocytes. The 26S proteasome is more vulnerable to oxidative damage than the 20S proteasome (50). In particular, it has been reported that sustained oxidative stress causes the dissociation of the 20S core from the regulatory subunits in the 26S proteasome, thus resulting in the loss of the 26S proteasome function (65). The 26S proteasome is responsible for removing misfolded proteins that have been previously tagged with ubiquitin, including both cytosolic proteins and proteins translocated from the ER during conditions of ER stress, as well as oxidized ubiquitinated proteins (55). The 26S proteasome also degrades structurally abnormal proteins in a ubiquitin-independent manner (69). Indeed, the ubiquitin/26S proteasome system
(UPS) represents the major intracellular pathway for selective extralysosomal protein degradation and thus, it is essential for protein quality control in the cells (55).

Together with the proteasome system, molecular chaperones play a major role in cellular protein homeostasis by assisting refolding of misfolded proteins or unfolded/partially folded newly synthesized proteins, which, if incorrectly folded, are finally targeted for degradation (12). Interestingly, it has been reported that insulin-resistant and hyperglycemic individuals have reduced expression of HSP72 in muscle, and that heat shock therapy, transgenic overexpression, or pharmacological activation of this chaperone protects against obesity-
adipocytes in response to TNF-like peptidase activity of the 26S proteasome (17). Data are expressed as a percentage of values in control cultures (100%). Mean ± SEM of five independent experiments. *p < 0.05, **p < 0.001 versus control.

FIG. 7. Effects of exposure of 3T3-L1 adipocytes on TNFα (5 nM; 24 h), to a combination of high glucose and high insulin (4.5 gr/L 100 nM, respectively; 24 h) (HGHI), or to palmitate (250 and 500 μM; 18 h). (A and C) Chymotrypsin-like peptidase activity of the 26S proteasome (+ ATP) (left panels) and 20S proteasome (+ SDS) (right panels) in 3T3-L1 adipocytes in response to TNFα or HGHI (A), and palmitate (C). (B and D) Insulin response (pAkt/Akt ratios) of 3T3-L1 cells exposed to TNFα or HGHI (B), and palmitate (D). After the treatments, control and TNFα-, HGHI-, and palmitate-treated 3T3-L1 cells were stimulated with insulin (100 nM) for 5 min. Cell lysates were analyzed for Akt and pAkt content. Data are expressed as a percentage of values in control cultures (100%). Mean ± SEM of five independent experiments. *p < 0.05, and **p < 0.001 versus control.

induced IR (13, 30). Along with the reduction in the activity of the 26S proteasome, we also show that OM adipocytes of IR subjects express diminished levels of HSP70, which binds and helps proteins fold to their functional state (29). Moreover, these changes were accompanied by the accumulation of ubiquitinated proteins in adipocytes of IR obese individuals. When viewed together, our data indicate that IR is associated with dysfunction of the proteolytic and protein-folding machinery in adipocytes, which leads to enhanced accumulation of abnormal proteins (oxidized and/or misfolded), thus linking proteotoxicity to impaired insulin response. Interestingly, it has been shown that proteasome inhibition induces the activation of JNK in several cell systems (19, 23, 37). In line with these observations, levels of phosphorylated JNK were upregulated in both OM and SC adipocytes of IR obese subjects as compared with those found in their counterparts from NG subjects, which is in agreement with the involvement of the kinase in IR (24).

In all, our data indicate that the transition from the insulin-sensitive to the insulin-resistant phenotype is linked to an imbalance in protein homeostasis in adipocytes. Noteworthy, it has been recently demonstrated that decreased proteasomal activity in mice aggravates HFD-induced obesity and hepatic steatosis (63), and that proteasome disruption further exacerbates IR in skeletal muscle cells from type 2 diabetic patients (4). Moreover, the expression of several components of the UPS is coordinately regulated in the liver of obese individuals, and proteasome dysregulation has been shown to mediate obesity-induced ER stress and IR in hepatocytes (41, 58).

Notably, human SC adipocytes exhibited both decreased 26S proteasome activity and increased levels of the ER stress marker, CHOP. In this scenario, our studies in 3T3-L1 cells demonstrate that proteasome dysfunction negatively regulates insulin response in adipocytes and this occurs through proteasome-mediated effects on key components of the insulin signaling pathway. Specifically, treatment of 3T3-L1 cells with the proteasome inhibitor, MG132, reduced IRS-1 levels, increased Ser307 IRS-1 phosphorylation, and inhibited insulin-induced phosphorylation of Akt, all of which are hallmark features of IR (60, 61, 67). Remarkably, JNK activation, which has been demonstrated to promote IRS-1 phosphorylation in Ser307 residues (1), was upregulated in MG132-treated 3T3-L1 cells, as it was also observed in human adipocytes from IR individuals. Likewise, proteasome inhibition induced a robust increase in CHOP protein levels in 3T3-L1 cells. It is thus likely that other cellular stress processes that are triggered in response to proteasome inhibition, including oxidative stress (16) and/or ER stress (41) (our results), directly or indirectly (i.e., via JNK activation) contribute toward negatively regulating insulin response in IR adipocytes.

It is now accepted that proteasome complexes are regulated by various mechanisms under different physiological and pathological conditions [reviewed in Kaake et al. and Schmidt and Finley (28, 54)]. In an attempt to dissect the factors and pathways responsible for the inhibition of the proteasome in IR adipocytes, we examined the activity of the proteasome in 3T3-L1 adipocytes after induction of IR by a variety of treatments. Specifically, the in vitro insulin-resistance models tested included exposure of 3T3-L1 cells to TNFα, a combination of HGHI, or palmitate (34), as a means to recapitulate the conditions observed in our human and/or animal models of MUHO, that is, inflammation, a combination of hyperglycemia and hyperinsulinemia, and systemic saturated fatty acid excess, respectively. Intriguingly, palmitate, but not TNFα or HGHI, inhibited the activity of
the 26S proteasome in 3T3-L1 cells. It has been reported that palmitate exposure alters multiple cellular processes, including from insulin signaling to fatty acid metabolism, autophagy, or apoptosis in several cell types (9, 14, 26). Importantly, this saturated fatty acid has been recently shown to modify genes regulating ubiquitin and protease functions in human pancreatic islets (14).

In adipocytes, palmitate has been shown to impair insulin signaling by inducing both oxidative and ER stress as well as inflammation (22, 31, 40). However, when we examined the effects of H2O2 and the ER stressor, thapsigargin, on 3T3-L1 cells, we observed that, at least at the conditions tested (1 and 50 μM, respectively, for 24 h), these compounds were unable to mimic the effects of palmitate on either 26S proteasome activity or pAkt/Akt ratio (data not shown). In all, these results, together with our data on TNFα and HGHI in 3T3-L1 cells, suggest that inhibition of proteasome activity in IR might be mediated by the combined activation of several cellular stress processes, which, in turn, are differentially activated by distinct environmental insults. In this vein, a growing body of evidence suggests that cellular stress pathways are closely linked and impact each other at various stages to functionally impair insulin response in metabolic disease (8, 10, 52, 71). As summarized in Figure 8, our data support the occurrence of a feed-forward cascade or a vicious cycle of cellular stress responses in adipocytes in obesity-associated IR, in which proteasome malfunction would impact other central pathways that contribute to IR.

Our study presents some limitations. First, it is limited by small sample size. However, the subjects were well matched for baseline anthropometric and clinical characteristics. Another limitation is that, as mentioned earlier, no information is available on the distribution of OM and SC intrabdominal fat and the regional distribution of body fat, both of which are markers of metabolic health (66). This information could provide a more integrated view of the contribution of adipocyte dysfunction to the development of IR in obesity.

In conclusion, our data suggest that proteasome dysfunction exacerbates the effects caused by the inability of adipocytes to activate efficient adaptive antioxidant and protein-folding responses to increased oxidative stress and ER stress occurring in obesity. Based on this and published data, we propose a model in which the proteasome would play a major role in maintaining adequate insulin responsiveness in adipocytes (Fig. 8). Thus, proteasome dysfunction and disruption of cell proteostasis in adipocytes appear as important contributors to the onset and/or progression of IR in obesity.

**Materials and Methods**

**Subjects**

Subjects were recruited at the Lipids and Atherosclerosis Unit of the Reina Sofia University Hospital (Cordoba, Spain). Blood samples were obtained by venipuncture after an overnight fast. Serum measurements of cholesterol, triglycerides, high-density lipoprotein cholesterol, glucose, and insulin were carried out as previously described (70). Subjects were not on insulin therapy or medication that was likely to influence endogenous insulin levels. Cardiovascular disease, diabetes, arthritis, acute inflammatory disease, and infectious or renal diseases were considered exclusion criteria. Obese subjects (BMI > 30 kg/m²) were subclassified into two groups [normoglycemia (NG): Glu < 100 mg/dl and HbA1c < 5.7%; and impaired fasting glucose (IFG): Glu 100–126 mg/dl and HbA1c: 5.7–6.4%] following the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes (5). IFG individuals exhibited significantly higher HOMA-IR values than NG individuals, as well as other clinic and plasma parameters that are within the cutoff points for identifying IR in hyperinsulinemic-euglycemic clamp studies (5, 59), and they will be referred to as insulin-resistant (IR) subjects. All participants gave written informed consent, and the study was reviewed and approved by the ethics and research committee of the Reina Sofia University Hospital.

**Isolation of mature adipocytes**

Paired OM and SC abdominal AT samples were obtained from individuals undergoing bariatric surgery. Fresh AT samples were dispersed by collagenase digestion to isolate mature adipocytes as previously described (44). Floating adipocytes were frozen in liquid nitrogen and stored at −80°C until further processing.

**FIG. 8.** Schematic representation of the intracellular pathways altered in adipocytes of IR individuals. Impairment of proteasome activity aggravates the effects caused by the inability of adipocytes to cope with oxidative stress and excess protein-folding load. The vicious cycle between proteasome dysfunction and oxidative stress in obesity worsens: (i) cellular protein homeostasis (i.e., ER stress and disruption of protein-folding responses), and (ii) the inflammatory state, which negatively impacts insulin responsiveness in adipocytes. The model is reproduced based on published molecular mechanisms (dashed arrows), and the main findings are shown in this article (solid arrows; see Discussion section).
**Protein extraction**

For proteomic studies, human adipocytes were homogenized in cold urea/thiourea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 45 mM Tris, pH 8.4, and Complete protease inhibitors (Roche)). The homogenate was incubated for 30 min on ice and centrifuged at 13,500 rpm for 30 min. The aqueous phase between the upper lipid phase and the lower cellular debris phase was collected. Proteins were precipitated using the 2D-CleanUp Kit (GE Healthcare) and quantified using the RC/DC Protein Assay (Bio-Rad Laboratories).

**2D-DIGE and data analysis**

Proteins were labeled according to the manufacturer’s instructions (GE Healthcare). Briefly, 50 μg of protein from SC and OM adipocytes of NG and IR subjects were randomly labeled with 400 pmol of the N-hydroxysuccinimide esters of Cy3 or Cy5 fluorescent cyanine dyes. An internal standard consisting of a mixture of equal amounts of all the samples was labeled with Cy2 dye and combined with the Cy3- or Cy5-labeled samples. The labeling reaction was performed on ice for 30 min and then quenched by adding 1 μl of lysine (10 mM) on ice for 10 min. Paired Cy2-, Cy3-, and Cy5-labeled samples (150 μg) were diluted in Rehydration buffer (GE Healthcare) to a final volume of 340 μl containing 0.8% (v/v) IPG buffer. Isoelectric focusing onto immobilized pH gradient strips (18 cm, pH 3–10 NL) and 2D-electrophoresis in 11% Tris-glycine gels were performed following the procedure previously described (45). Gels were scanned with a Typhoon 9400 scanner (GE Healthcare) at 100 μm resolution using appropriate excitation and emission wavelengths.

Relative quantification of differentially expressed proteins was performed using DeCyder software version 7.0 (GE Healthcare). After differential In-gel Analysis using the internal standard as a reference, spot abundance was measured in each image. Statistical analysis was carried out to determine protein expression changes. p-values lower than 0.05 as calculated from Student’s t-test were considered significant.

**MALDI-TOF-MS analysis**

Spots were excised automatically in a ProPic station (Genomic Solutions), digested automatically, and analyzed in a 4800 MALDI-ToF/ToF Analyzer (Applied Biosystems/MDS SCIEX) to obtain the MALDI-MS and MALDI-MS/MS spectra as previously reported (45).

MS and MS/MS spectra data were combined through the GPS Explorer Software v3.6 using Mascot software v2.2 (Matrix Science) to search against a nonredundant human database (SwissProt release 56.0), with 30 ppm precursor tolerance, and 0.2 Da MS/MS fragment tolerance. Analysis was limited to peptides of six or more amino acids and maximum one missed cleavage site. Oxidation of methionine was searched as variable modification, and carbamidomethylation of cysteine was set as fixed modification. All spectra and database results were manually inspected in detail. Protein scores greater than 56 were accepted as significant (p < 0.05), considering positive the identification when protein score CI% (Confidence Interval) was above 98. In the case of MS/MS spectra, total ion score CI% was above 95.

**3T3-L1 cell culture and treatment**

3T3-L1 adipocytes were differentiated as previously described (49). Differentiated 3T3-L1 adipocytes (day 9) were exposed for 24 h to Dulbecco’s modified Eagle’s medium (DMEM) alone or containing the proteasome inhibitor MG132 (50 μM). Thereafter, control and MG132-treated 3T3-L1 cells were stimulated with insulin (100 nM) for 5 min.

In another set of experiments, differentiated 3T3-L1 adipocytes were exposed for 24 h to the inflammatory cytokine TNFα (5 nM) or to a combination of high glucose (4.5 g/L) and high insulin (100 nM) (HGHI). Finally, the effects of 18-h exposure of 3T3-L1 cells to palmitate (sodium salt; 250 and 500 μM) were also explored. Palmitate solutions containing 2% fatty acid-free bovine serum albumin were prepared in DMEM containing no serum. After the treatments, control and TNFα-, HGHI-, and palmitate-treated 3T3-L1 cells were stimulated with insulin (100 nM) for 5 min.

At the end of the experiments, 3T3-L1 cells were processed for immunoblotting as previously described (49).

**Quantitative immunoblotting**

Mature adipocytes isolated from additional NG and IR subjects distinct from those employed for 2D-DIGE analysis were disrupted in buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA, and 1 μg/ml anti-protease cocktail. Thirty microgram of protein per sample were loaded into 4–20% precasted SDS-PAGE gels and transferred to nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). For dot-blot assays, 10 μL aqueous samples containing 10 μg of protein were laid in dots onto nitrocellulose membranes. Membranes were stained with Ponceau to ensure equal sample loading (Supplementary Fig. S4) and processed for immunoblotting with specific antibodies (Supplementary Table S1). Ponceau staining was employed as a loading control (27, 45).

**Analysis of oxidative markers**

Total glutathione and reduced (GSH) glutathione were quantified in human adipocytes, and the GSH/GSGG ratio was calculated as previously described (11).

Protein carbonyl levels were determined by 2,4-dinitrophenylhydrazine (DNPH) derivatization and immunoblotting using an anti-DNP antibody (Supplementary Table S1) (7). 4-HNE-modified proteins were analyzed by dot-blot using an anti-4-HNE antibody (Supplementary Table S1) (16).

**Proteasome activity assay**

Proteasome activity of adipocytes was measured by using synthetic fluorescent substrates (N-Suc-LLVY-AMC, ZLLE-AMC, and Boc-LRR-AMC) as previously described (35). Fluorescence of the released AMC products was kinetically followed for 1 h in a FlexStation 3 microplate reader (Molecular Devices) (excitation, 355 nm; emission, 460 nm). Parallel reactions containing 25 μM MG132 were run as controls for nonspecific peptide hydrolysis, and fluorescence background was subtracted from activity values. Proteasome peptidase activity was calculated as AMC values per hour and mg of protein.
Analysis of proteasome activity in adipocytes of lean and obese mice

Four-week-old male C57BL/6J mice were housed (five per cage) under a 12 h light/12 h dark cycle, in a temperature-controlled room (22°C), with food and water ad libitum. After weaning, mice were fed a standard chow diet, and after 4 weeks, they were divided into two groups (10 animals per group) with a similar average body weight and assigned either to a standard chow global diet (ND, #2918, 6.2% fat, 18% protein; 3.1 kcal/g; Harlan Laboratories) or to an HFD (D12492, 60% kcal% fat, 20% kcal% carbohydrates and 20 kcal% protein; 5.24 kcal/g; Research Diets) during 14 weeks. Blood glucose levels were measured with an Accucheck glucometer (Roche) after an intraperitoneal injection of either 2 mg/g D-glucose (Sigma) or 0.75 U/kg insulin (Sigma-Aldrich) as described (46). Tests were performed after 13 weeks of feeding mice with ND or HFD. All experiments were conducted under approval of the Animal Care and Use Committee of the University of Santiago de Compostela and the University of Córdoba (Spain). All the experiments were performed in agreement with the Rules of Laboratory Animal Care and International Law on Animal Experimentation.

Animals were sacrificed, and SC and visceral AT samples were dispersed by collagenase digestion to isolate mature adipocytes and stromal vascular fraction cells (SVFCs) as described (44). Cells (mature adipocytes or SVFCs) from 3 or 4 animals were pooled together for further analysis. Mature adipocytes were frozen in liquid nitrogen and stored to −80°C until analysis. SVFCs were seeded in 35-mm plastic plates (30,000 cells/plate) and grown in adipocyte medium (DMEM/F-12 [1:1] (Invitrogen), 16-80 mM ascorbate, and antibiotic-antimycotic) supplemented with 10% newborn calf serum (NCS) at 37°C in a humidified atmosphere with 95% air: 5% CO₂. After 3 days induction period, cells were cultured in differentiation medium lacking IBMX and BRL49653 for the remaining 7 days of adipocyte differentiation. Thereafter, proteasomal activities in the cell samples were measured as described for human adipocytes.

Statistical analysis

Correlations were analyzed using Pearson’s correlation test. Statistical differences were determined using Student’s t-test. Values considered significant at p < 0.05. Data are expressed as mean ± SEM. Proteins identified by the proteomic study were analyzed using the PANTHER database (www.pantherdb.org/) for classification according to their function (38). The Bonferroni correction for multiple testing was used for the calculation of PANTHER p-values. Statistical analyses were performed using SPSS statistical software (version 13.0).

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Author Disclosure Statement

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Abbreviations Used

4-HNE = 4-hydroxynonenal
AT = adipose tissue
BMI = body mass index
ChT-L = chymotrypsin-like
DMEM = Dulbecco’s modified Eagle’s medium
ER = endoplasmic reticulum
FFA = free fatty acid
GSH = reduced glutathione
GSHB = glutathione synthetase
GSPP = oxidized glutathione
GT = total glutathione
HbA1c = glycated hemoglobin
HDL = high-density lipoprotein
HFD = high-fat diet
HGHI = high glucose and high insulin

HOMA-IR = homeostasis model assessment of insulin resistance
IR = insulin resistance
IRS-1 = insulin receptor substrate 1
JNK = c-Jun NH2-terminal kinase
LDL = low-density lipoprotein
MHO = metabolically healthy obese
MUHO = metabolically unhealthy obese
ND = normal diet
NF-κB = nuclear factor κB
NG = normoglycemic
NQO1 = NADPH quinone oxidoreductase 1
OM = omental
proteostasis = protein homeostasis
SC = subcutaneous
SVFC = stromal vascular fraction cell
UPS = ubiquitin/26S proteasome system