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Excessive caloric intake acutely causes oxidative stress, GLUT4 carbonylation, and insulin resistance in healthy men

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Abstract

Obesity-linked insulin resistance greatly increases the risk for type 2 diabetes, hypertension, dyslipidemia, and non-alcoholic fatty liver disease, together known as the metabolic or insulin resistance syndrome. How obesity promotes insulin resistance remains incompletely understood. Plasma concentrations of free fatty acids and proinflammatory cytokines, endoplasmic reticulum (ER) stress, and oxidative stress are all elevated in obesity and have been shown to induce insulin resistance. However, they may be late events that only develop after chronic excessive nutrient intake. The nature of the initial event that produces insulin resistance at the beginning of excess caloric intake and weight gain remains unknown. We show that feeding healthy men with ~6000 kcal/day of the common U.S. diet [~50% carbohydrate (CHO), ~35% fat, and ~15% protein] for 1 week produced a rapid weight gain of 3.5 kg and the rapid onset (after 2 to 3 days) of systemic and

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SUPPLEMENTARY MATERIALS

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Table S1. Individual daily measurements for Fig. 1A and individual clamp measurements for Fig. 1B.

Table S2. Individual measurements for Fig. 2 (B and C).

Table S3. Differentially expressed proteins in adipose tissue in response to overnutrition identified by GeLC-MS/MS (provided as an Excel file).

Table S4. Overnutrition-induced increases in proteins related to generation or detoxification of ROS.

Table S5. Identification of ROS-related posttranslational modifications in adipose tissue in response to overnutrition (provided as an Excel file).

Table S6. Overnutrition-induced modifications in GLUT4 immunoprecipitated from adipose tissue protein extracts.

Table S7. Primer sets used for RT-PCR studies.

Competing interests: The authors declare that they have no competing interests.

Author contributions: G.B. designed the study, analyzed the data, and wrote the manuscript. C.H. performed the clinical studies. C.A.B., C.F., and S.M. performed and analyzed the proteomic studies. X.C. determined and analyzed plasma cytokines, T.P.S. determined and analyzed urinary 8-iso-PGF2 α , S.K. performed the surgical fat biopsies, and P.C. performed RT-PCR analysis.

adipose tissue insulin resistance and oxidative stress but no inflammatory or ER stress. In adipose tissue, the oxidative stress resulted in extensive oxidation and carbonylation of numerous proteins, including carbonylation of GLUT4 near the glucose transport channel, which likely resulted in loss of GLUT4 activity. These results suggest that the initial event caused by overnutrition may be oxidative stress, which produces insulin resistance, at least in part, via carbonylation and oxidation-induced inactivation of GLUT4.

INTRODUCTION

Obesity-linked insulin resistance greatly increases the risk for several medical problems including type 2 diabetes, hypertension, atherogenic dyslipidemia, and non-alcoholic fatty liver disease, conditions collectively called the metabolic syndrome (1). Exactly how obesity promotes insulin resistance and other associated problems remains incompletely understood, although several potential mechanisms have been proposed. For instance, insulin resistance in obesity is associated with increased plasma concentrations of free fatty acids (FFAs) (2), elevated plasma concentrations of proinflammatory cytokines (3–5), increased endoplasmic reticulum (ER) stress in liver and adipose tissue (6–8), and increased oxidative stress (9–11). All of these have been postulated to cause insulin resistance, but they may be late events that develop after months of excessive nutrient intake. What would be important to know, but remains unknown, is the nature of the initial event that produces insulin resistance at the very beginning of excess caloric intake and weight gain. To obtain this information, we fed healthy, insulin-sensitive men with ~6000 kcal/day of the common U.S. diet for 1 week to reproduce the early phases of insulin resistance and identify its cause.

RESULTS

Overnutrition caused rapid development of systemic and adipose tissue insulin resistance

To produce insulin resistance, we fed 6206 ± 256 kcal/day of a regular diet [50% carbohydrate (CHO), 35% fat, and 15% protein] for 7 days to six normal to overweight, otherwise healthy men (Table 1) who were hospitalized in the Temple University Hospital Clinical Research Unit, where they remained at bed rest during the entire study. This increased their body weight from 79 ± 2.7 kg to 82.5 ± 2.8 kg, all of it as fat (Table 2). Resting energy expenditure (REE) (determined daily by indirect calorimetry) increased by 16% (from 1482 ± 99 kcal/day before to 1719 ± 127 kcal/day after the study, $P = 0.045$) and was $11,248 \pm 510$ kcal for the 7-day study. The thermic response to food (TRF), estimated as 8% of ingested CHO, 2% of ingested fat, and 25% of ingested protein calories, was 2977 ± 61 kcal/7 days. The excess caloric intake, calculated as the 7-day caloric intake minus REE minus the thermic effect of food, was $28,729 \pm 1140$ kcal. The expected gain in fat, on the basis of an excessive caloric intake of $28,729 \pm 1140$ kcal, was 3.19 ± 0.13 kg, which was not significantly different from the observed gain in fat of 3.5 ± 0.3 kg. Blood collected each day at ~8:00 a.m., 9 to 10 hours after the last food intake, showed no significant changes in fasting plasma glucose and FFA concentrations (Fig. 1 and table S1). Plasma triglycerides increased from 67 ± 10.5 mg/dl to 121 ± 20.7 mg/dl ($P = 0.0436$). However, we observed a rapid rise (on day 2), followed by a continuous rise, in serum insulin and a similar rise in insulin resistance, as determined by the homeostasis model of assessment of

insulin resistance (HOMA-IR) (Fig. 1 and table S1). The development of systemic insulin resistance, apparent from the rise in HOMA-IR, was confirmed with euglycemic-hyperinsulinemic clamps, performed immediately before and after the study, which showed an ~50% decrease in insulin-stimulated glucose uptake [glucose infusion rate (GIR)] after 7 days of overnutrition (Fig. 1B). Development of a similarly rapid onset of insulin resistance in adipose tissue was apparent from the failure of the increased serum insulin to appropriately suppress FFA levels (Fig. 1 and table S1).

Overnutrition-induced insulin resistance was associated with increased oxidative stress

We next examined whether an increase in plasma FFA, in proinflammatory cytokines, or in ER stress could have contributed to the observed insulin resistance. Plasma FFA did not increase (Fig. 1 and table S1). Tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and IL-10 serum concentrations remained at basal levels throughout the study in all six subjects. For unknown reasons, IL-8 concentration rose transiently on day 2 in subject 1, IL-10 on day 2 in subject 3, and IL-6 on day 6 in subject 5 (Fig. 2A). There were no significant changes in any of the measured adipose tissue ER stress marker mRNA and proteins (Fig. 2B and table S2). Moreover, a proteomic screen in adipose tissue did not detect increases in proinflammatory cytokines and ER stress markers (table S3). These results did not support major roles for FFAs, pro-inflammatory cytokines, or ER stress in the initial development of overnutrition-induced insulin resistance. They did not, however, rule out that these factors may play important roles during later phases of development of obesity. In obese people, plasma FFA concentrations are elevated and contribute to insulin resistance (2) and ER stress (8), whereas oxidative stress can result in inflammation (12), which can produce ER stress and vice versa (13). In our study population, urinary 8-iso-PGF2 α , a well-established marker of oxidative stress-induced lipid peroxidation (14), rose rapidly, indicating development of systemic oxidative stress (Fig. 2C and table S2).

Overnutrition produced oxidative stress in adipose tissue

To identify a tissue source for the increase in urinary 8-iso-PGF2 α , we focused on adipose tissue, which is particularly stressed during periods of overnutrition because it needs to take up and store the excess calories (15). Analyzing fat biopsy extracts with label-free proteomics, we identified over 1000 proteins, 38 of which were linked to the generation or detoxification of reactive oxygen species (ROS) and which were up-regulated during overnutrition (tables S3 and S4). For instance, there were significant ($P = 0.022$) increases in SOD2 (superoxide dismutase 2), the mitochondrial enzyme that generates hydrogen peroxide from superoxide; in catalase, which reduces H₂O₂ accumulation; and in glutathione peroxidase and peroxiredoxin, which reduce hydroperoxides (11). Moreover, there were increases in aldehyde dehydrogenase, aldo-keto reductase, and glutathione *S*-transferase, all of which degrade oxidized lipid products (16). ROS could not be measured directly because of their extreme instability. Nevertheless, the rise of these pro- and antioxidative stress proteins is a response to and therefore reflects an increase in ROS generation. The idea that the generation of ROS in adipose tissue exceeded their detoxification, that is, produced oxidative stress, was supported by the observation of extensive ROS-mediated protein oxidation and carbonylation (Fig. 3). Moreover, proteomic analyses identified 153 adipose tissue proteins that were posttranslationally modified. Of those, 130 were oxidized, 19 were

carbonylated (as glutamic semialdehyde), 17 were lipid-peroxidated [with 4-hydroxynonenal (4-HNE) or oxononenal adductions], and 2 were nitrosylated (table S5).

Oxidative stress produced oxidation and carbonylation of GLUT4 in adipose tissue

Oxidative stress has previously been implicated in the etiology of insulin resistance (9–11); however, exactly how oxidative stress produces insulin resistance has remained uncertain. In this respect, it was of considerable interest that we found that oxidative stress was associated with several GLUT4 posttranslational modifications (Fig. 4 and table S6). Specifically, we found extensive GLUT4 carbonylation as well as adduction of HNE and glutamic semialdehyde in close proximity to the glucose transport channel (Fig. 4, B to D). Carbonylation typically causes protein cross-linking and loss or alteration of protein function (17) and can target the affected proteins for selective degradation by the 26S proteasome (18).

DISCUSSION

Here, we have shown that feeding a common U.S. diet to healthy non-obese men at 2 to 2.5 times their regular caloric intake for as little as 1 to 2 days produced severe systemic and adipose tissue insulin resistance in every one of six study subjects. Our subjects were confined to their rooms and had no physical activity. This might have had a small effect (~5%) on their weight gain and onset of insulin resistance. Much less rapid and drastic results have been previously reported in studies using smaller excess caloric intakes for longer periods of time. Thus, Johannsen *et al.* increased the caloric intake of 29 healthy men and women by 40% for 8 weeks, which increased insulin resistance by 18% (19), whereas Samocha-Bonet *et al.* found an 8% decrease in insulin-stimulated glucose uptake after overfeeding 1040 kcal/day for 28 days to 40 healthy men and women (16).

We tried to determine the nature of the initial event that produced the insulin resistance in adipose tissue, which is highly stressed during periods of overnutrition (15). Our results showed that FFA, inflammatory cytokines, and ER stress were not involved in the initial development of overnutrition-induced insulin resistance, but it was tightly linked to an increase in urinary 8-iso-PGF2 α , a reliable marker for oxidative stress (14). Our once-daily blood collections did not allow us to determine whether oxidative stress or insulin resistance was the initial event. However, several studies have shown unequivocally that oxidative stress preceded insulin resistance in different models of insulin resistance (20, 21). We therefore believe that oxidative stress likely preceded development of insulin resistance and that oxidative stress, not inflammatory or ER stress, was the initial event that occurred after overnutrition.

Oxidative stress has previously been implicated in the etiology of insulin resistance (9–11, 22). Several human studies have attempted to support this concept using antioxidant treatments, but the results have been controversial (23–27). More direct evidence came from in vitro studies showing that hydrogen peroxide caused insulin resistance in 3T3-L1 cells (28–30) and in soleus muscle of lean Zucker rats (31). Additional evidence came from studies in humans showing that a high-fat diet increased mitochondrial H₂O₂ production and insulin resistance (22) and from studies in rodents showing that treatment with a

mitochondrial-targeted antioxidant or overexpression of catalase prevented high-fat diet-induced insulin resistance (15, 21). We found that subcutaneous adipose tissue was one likely source for the increase in urinary 8-iso-PGF2 α . Whereas superoxide and other ROS could not be measured directly because of their extreme instability, we identified 38 proteins that were linked to the generation or detoxification of ROS and which were up-regulated in the adipose tissue during overnutrition. The increased concentration of these proteins is normally a response to and reflects an increase in ROS generation. Moreover, the finding of extensive ROS-mediated protein oxidation and carbonylation showed that the generation of ROS exceeded their detoxification and had produced oxidative stress.

To explore a possible mechanism by which oxidative stress could have produced insulin resistance, we focused on changes in GLUT4, the major insulin-facilitated glucose transporter in adipose tissue. We found extensive GLUT4 oxidation and carbonylation in close proximity to the glucose channel. In as much as similar changes have been reported to result in protein cross-linking and loss of function, we consider it likely that the carbonylation near the glucose transport channel of the GLUT4 transporters made them dysfunctional, causing a defect in insulin stimulation of glucose uptake. This would provide a direct mechanistic link between oxidative stress and insulin resistance, which, however, needs to be confirmed by in vitro studies. In addition, other mechanisms are likely involved in this process. For instance, oxidative stress has been shown to stimulate several stress-activated kinases, which can then inhibit insulin signaling by inactivating insulin receptor substrate 1/2 (IRS 1/2) (32). In addition, treatment of 3T3-L1 adipocytes with 4-HNE caused carbonylation-mediated degradation of IRS 1/2 protein and reduced metabolic actions of insulin (33, 34). Last, in as much as insulin promotes intracellular glucose uptake and metabolism, insulin resistance reduces glucose uptake and metabolism and therefore ROS production and oxidative damage.

In summary, our results show that in healthy men, as little as 1 to 2 days of excess nutrient intake produced systemic oxidative stress and insulin resistance but no inflammatory or ER stress. In adipose tissue, the oxidative stress caused extensive oxidation and carbonylation of many proteins, including the glucose transporter GLUT4. The observation of carbonylation in proximity of the GLUT4 glucose transport channel strongly suggested that GLUT4 had become dysfunctional, resulting in insulin resistance and providing a likely causal link between overnutrition and insulin resistance. Furthermore, our results show a need to develop agents that can effectively reduce oxidative stress.

MATERIALS AND METHODS

Study design

The objective of the study was to explore the effects of overnutrition with a common U.S. diet (50% CHO, 35% fat, and 15% protein) on the initial stages of the development of insulin resistance. All study subjects were admitted to the Temple University Hospital Clinical Research Unit, where they remained at bed rest for the duration of the study. Subjects were given three meals plus three snacks totaling ~6000 kcal/day for 7 days. This was ~2.5 times their usual caloric intake and was well tolerated. A member of the research staff monitored each meal and snack to ensure that caloric goals were met. Subcutaneous fat

biopsies and 4-hour euglycemic-hyperinsulinemic clamps were performed the day before and after the study. The subjects' body weight and body composition, basic metabolic rates, and blood samples for determination of glucose, insulin, C-peptide, and FFA concentrations were obtained daily at ~8:00 a.m., 9 to 10 hours after the last food intake, which was at ~11:00 p.m. the night before.

Study subjects

We studied six normal to overweight, otherwise healthy male volunteers [three normal weight (BMI 23.0, 23.9, and 24.9) and three overweight (BMI 26.8, 28.7, and 28.1)]. The subjects were middle aged (mean, 50 ± 1.4 years; range, 46 to 55 years). All subjects had normal fasting glucose (mean, 90.7 ± 5.1 mg/dl) and A1c levels (mean, 5.04 ± 0.12 ; range, 5.0 to 5.8). None had a family history of diabetes or other endocrine disorders, and none were taking any medications. Informed written consent was obtained from all subjects after explanation of the nature, purpose, and potential risks of the study. The study protocol was approved by the Institutional Review Board at Temple University Hospital.

Euglycemic-hyperinsulinemic clamping

Euglycemic-hyperinsulinemic clamps were performed immediately before and after the study. Regular human insulin (Eli Lilly and Company) was infused intravenously at a rate of $7 \text{ pmol kg}^{-1} \text{ min}^{-1}$ ($1 \text{ mU kg}^{-1} \text{ min}^{-1}$) for 4 hours. Plasma glucose concentrations were clamped at ~5.5 mM by a feedback-controlled glucose infusion. Blood samples were obtained before (−30 and 0 min) and at hourly intervals after insulin infusion for the determination of glucose and insulin concentrations.

Indirect calorimetry

Respiratory gas exchange was determined at 30-min intervals during the clamps with a metabolic measurement cart (DeltaTrac II, SensorMedics) as previously described (35). Rates of protein oxidation were estimated from urinary nitrogen excretion with correction for changes in urine nitrogen pool size (36). Rates of protein oxidation were used to determine the nonprotein respiratory quotient. Rates of carbohydrate oxidation were determined with the tables of Lusk (37), which are based on a nonprotein respiratory quotient of 0.707 for 100% fat oxidation and 1.00 for 100% carbohydrate oxidation. REE was calculated using the abbreviated Weir equation: $\text{REE} = [(3.9 \times \text{VO}_2 + 1.1 \times \text{VCO}_2)] \times 1.44$.

Fat biopsies

Immediately before and after overfeeding, open subcutaneous fat biopsies were obtained by a surgeon from the lateral aspect of one thigh (~15 cm above the patella). An incision (~1 inch) was made through the skin, and 200 to 300 mg of subcutaneous fat was excised. The excised fat was immediately dropped into isopentane and frozen at -160°C by liquid nitrogen. The frozen fat was then transferred to a freezer and stored at -80°C until analyzed.

Body weight and composition

Body weight was measured to the nearest 0.1 kg every day at ~8:00 a.m. with the subject standing without shoes. Body composition was determined every day at ~8:00 a.m. using bioelectrical impedance analysis (RJL Systems) (38). The measurements were performed with subjects in the fasted state and in the supine position after voiding and resting for 10 min. FM and FFM were determined according to the following formulas:

$$\text{FFM} = (1.06 \times \text{extracellular fluid}) + (1.521 \times \text{intracellular fluid})$$

$$\text{FM} = \text{BW} - \text{FFM}$$

Serum cytokine concentrations

Cytokines were analyzed using the Luminex xMAP technology (Luminex Corporation) on the MAGPIX system. Serum concentrations of IL-1 β , IL-6, IL-8, and IL-10 and TNF- α were determined simultaneously by the MILLIPLEX Human High Sensitivity T cell 5-plex Magnetic Bead Panel (catalog #HTSCMAG-28SK) from EMD Millipore Corporation. According to the notes in the manufacturer's protocol, there was no or negligible cross-reactivity between the antibodies for each analyte and any of the other analytes in this panel. The minimal detectable concentrations were 0.14, 0.11, 0.13, 0.56, and 0.16 pg/ml for IL-1 β , IL-6, IL-8, IL-10, and TNF- α , respectively. The intra-assay coefficients of variation were between 3.9 and 6.3% for the cytokines assayed.

Isoprostane analyses

We used a modification of the liquid chromatography–tandem mass spectrometry (LC-MS/MS) method described by Saenger *et al.* (39). Briefly, 20 μ l of the internal standard, $^2\text{H}_4$ -8-iso-PGF2 α (100 ng/ml; Cambridge Isotopes), was added to 2 ml of urine in a screw cap tube and vortexed. Then, 50 μ l of β -glucuronidase (1250 U/ml; Sigma-Aldrich) was added, and the mixture was briefly vortexed and then incubated at 37°C for 15 min. After cooling on ice, the urine was extracted with a mixture of cold ethyl acetate (3 ml) and cold saturated KH_2PO_4 (2 ml). The ethyl acetate layer was transferred to a tube containing ~600 mg of Na_2SO_4 powder, vortexed, and centrifuged. After centrifuging at 1200 rpm for 2 min at 4°C, the ethyl acetate layer was transferred to a clean tube and dried under argon. The residue was reconstituted in 200 μ l of mobile phase A and 10 μ l was injected into an Agilent 6410 Triple Quad LC-MS/MS with Agilent 1200 Series LC (Agilent Instruments) fitted with an Agilent ZORBAX SB-C18 Rapid Resolution Cartridge 2.1 $\times$ 30 mm 3.5 μ m. Chromatography conditions were gradient over 15 min, starting with 95% mobile phase A (water/ammonium hydroxide, 1000:1) and ending with 95% mobile phase B (acetonitrile/ammonium hydroxide, 1000:1) at 40°C. The 357.3-to-197.2 and 353.3-to-193.3 transitions were monitored for $^2\text{H}_4$ -8-iso-PGF2 α and 8-iso-PGF2 α , respectively.

Analytical procedures

Plasma glucose was measured with a glucose analyzer (YSI). Insulin was determined in serum after protein precipitation with polyethylene glycol by radioimmunoassay with a

specific antibody that minimally cross-reacts (0.2%) with proinsulin (Linco). Plasma FFAs were determined enzymatically with a kit from Wako.

Fat tissue sample preparation

Cytosolic proteins were extracted from the tissue using a hypotonic lysis buffer consisting of 0.1× phosphate-buffered saline (PBS), 0.1% Triton X-100, and 1× protease inhibitor (Pierce Halt Protease Inhibitor Cocktail 100×). After incubation on ice for 10 min to allow cells to swell, the samples were homogenized for 2 min and were centrifuged at 10,000g for 15 min to separate the cytosolic fraction (supernatant) from the nuclei-enriched fraction (pellet). Membrane proteins from the pellet fraction were extracted with Mem-PER Plus Membrane Protein Extraction Kit (Thermo Scientific). Samples from before (day 1) and after the period of excess nutrients (day 7) were either pooled together for GLUT4 immunoprecipitation (IP) or processed individually for gel electrophoresis–LC-MS/MS (GeLC-MS/MS) proteomics analysis.

GLUT4 IP

GLUT4 IP was performed with a mouse monoclonal antibody, anti-GLUT4 (sc-53566, Santa Cruz Biotechnology). In brief, anti-GLUT4 antibody was incubated with beads for 24 hours at 4°C (Dynabeads Pan Mouse IgG from Invitrogen). Beads were washed using PBS with Triton X-100 (0.2%). The washed beads were then incubated with 1% of bovine serum albumin for 1 hour at room temperature. The antibody was fixed to beads using 0.05% formaldehyde for 5 min. Finally, beads were washed and incubated with the pool of membrane proteins extracted for 24 hours at 4°C. After incubation, beads were washed again, and GLUT4 was eluted using urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM tris-HCl, 100 mM dithiothreitol) or Laemmli buffer for evaluation on 2D Western blots and GeLC-MS/MS, respectively.

Carbonylation detection with DNPH derivatization

The presence of carbonyls on protein samples was tested using OxyBlot Protein Oxidation Detection Kit (Chemicon International) according to the manufacturer's instructions. Briefly, samples from before or after excess nutrition were pooled together and denatured with 12% SDS. After the addition of equal volume of dinitrophenylhydrazine (DNPH) solution, the samples were incubated at room temperature for 15 min. Finally, neutralization solution was used to stop the reaction. Samples were diluted with DeStreak reagent buffer (GE Biosciences) for 2D electrophoresis.

2D electrophoresis

The 2D electrophoresis methods are as previously described (40, 41), except that samples were diluted to 120 µl with rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS, 5% glycerol, 15% 2-propanol, 1.2% DeStreak reagent (GE Healthcare)], 0.5% immobilized pH gradient (IPG) buffer (pH 3 to 10), and 0.2% methylcellulose, and pipetted into an Ettan IPGphor strip holder (Amersham Biosciences).

Real-time RT-PCR

Total RNAs were extracted from each sample by Qiagen RNeasy Lipid Tissue Mini Kit (catalog 74804, Qiagen) and stored at -70°C in ribonuclease-free water. Concentrations were determined by Nano-Drop (Thermo Scientific). One-step real-time reverse transcription polymerase chain reaction (RT-PCR) was performed with HotStart-IT SYBR Green Master Mix Kit (catalog 75770, USB Affymetrix) and StepOnePlus Real-Time PCR System (Life Technologies). Relative concentrations were determined by dilution standard curves for each target and normalized to that of 18S. Ratios of post- to pretreatment were calculated from the average of triplicates. The primer sets are presented in table S7.

Western blot

Protein extract (50 μg) of each sample was separated by SDS–polyacrylamide gel electrophoresis (4 to 15%) and electroblotted onto 0.2- μm PVDF (polyvinylidene difluoride) membrane. The following primary antibodies were used for incubations: ATF4 (sc-200; rabbit; 1:500), calreticulin (sc-11398; rabbit; 1:500), XBP-1s (sc-32155; goat; 1:500), PDI (protein disulfide isomerase) (sc-20132; rabbit; 1:500), GAPDH (glyceraldehyde phosphate dehydrogenase) (sc-25778; rabbit; 1:2,000), GLUT4 (sc-53566; mouse; 1:1,000), all from Santa Cruz; GRP78 (BD610979, BD Biosciences; mouse; 1:500); calnexin (#2433, Cell Signaling; rabbit; 1:500); and anti-dinitrophenyl (90451, OxyBlot, Chemicon; 1:150).

The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies. Signals were developed by Luminol Reagents (sc-2048, Santa Cruz Biotechnology), and x-ray images were analyzed by ImageJ software. GAPDH signals were used as loading control.

Proteome analysis

The methods for label-free proteomics (GeLC-MS/MS) analysis were as previously described (42), except that the nanoelectrospray ionization (ESI) tandem MS was performed with a Q Exactive Orbitrap mass spectrometer (Thermo Scientific). ESI was delivered with a stainless steel emitter (30- μm inside diameter, 40-mm length, Thermo Scientific) at a spray voltage of -1800 V . MS/MS fragmentation was performed on the five most abundant ions on each spectrum using collision-induced dissociation with dynamic exclusion (excluded for 10.0 s after one spectrum), with automatic switching between MS and MS/MS modes. The complete system was fully controlled by Xcalibur software.

Mass spectra processing was performed using Proteome Discoverer 1.4.0.288 (DB version 79). The generated de-isotoped peak list was submitted to an in-house Mascot Server 2.2.07 for searching against the Swiss-Prot database (release 2013_01, version 56.6, 538,849 sequences). Mascot search parameters were set as follows: species, *Homo sapiens* (20,233 sequences); enzyme, trypsin with maximal two missed cleavage; fixed modification, cysteine carboxymethylation; variable modification, methionine oxidation; 10 ppm mass tolerance for precursor peptide ions; and 0.2 dalton tolerance for MS/MS fragment ions. All peptide matches were filtered using an ion score cutoff of 20. Quantified proteins were selected and clustered by biological functions, using Ingenuity Pathway Analysis software (www.ingenuity.com) for bioinformatics analysis.

Posttranslational modification identification

Protein modification occurrences were analyzed using the same program and database as described above for the data analysis, except for the following search parameters: maximal four missed cleavage, with variable modification: carbamidomethyl (C); nitrosyl (C, Y); oxidation (M, H, W, Y, C, D, K, F, R, P, N); phospho (S, T); phospho (Y); 4-ONE (K, C, H); HNE (C, A, K, L, H,); glutathione (C); Arg→GluSA (R); 20 ppm mass tolerance for precursor peptide ions; 0.4 dalton tolerance for MS/MS fragment ions.

Modeling of GLUT4 structure

GLUT4 protein sequence (P14672) was used to generate a protein structure homology model using the SWISS-MODEL workspace in automatic modeling mode (<http://swissmodel.expasy.org/workspace/>) (43, 44). The carbonylations are indicated within the amino acid residue identified in this study for GLUT4 in fat tissue after the excess caloric intake (Fig. 4).

Statistical analysis

All data were expressed as means $\pm$ SE. Values in pre- and post-studies were compared using a two-tailed paired Student's *t* test. Normality was tested with the Kolmogorov-Smirnov test. The Wilcoxon signed-rank test was used to determine significance if the data were not normally distributed. The pre- and posttreatment data were compared using a one-way repeated-measures analysis of variance (ANOVA) with the Student-Newman-Keuls test used for multiple comparisons. The Friedman repeated-measures ANOVA on ranks was used when the data were not normally distributed. Non-normal data were tested using the Mann-Whitney rank sum test. For GIR and HOMA-IR, a sample size of six detected a difference of 2.0 ± 1.0 with 90% power and an α of 0.01.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Development of insulin resistance in response to overnutrition in healthy men
 (A) Daily basal concentrations of plasma glucose, insulin, and FFA, as well as HOMA-IR during 7 days of overnutrition ($n=6$). (B) Glucose, insulin, and FFA concentrations, as well as GIRs, during euglycemic-hyperinsulinemic clamps performed immediately before and after overnutrition. Inserted box in (B) shows GIR before and after overnutrition in six individual subjects. Shown are means $\pm$ SE. Table S1 shows individual data for all study subjects. * $P < 0.05$, ** $P < 0.01$ compared to day 1. Day 1, before overnutrition.

Fig. 2. Connection between overnutrition-induced insulin resistance and oxidative stress
(A) Individual daily basal plasma cytokine concentrations of the six study participants during the 7 days of overnutrition. **(B)** Unfolded protein response (UPR) marker mRNA and proteins in subcutaneous adipose tissue biopsies obtained from the six study subjects immediately before and after overnutrition. Shown are means $\pm$ SE. **(C)** Daily urinary 8-iso-PGF_{2 α} concentration and HOMA-IR during 7 days of overnutrition. $n = 6$; shown are means $\pm$ SE. Table S2 shows individual data for all study subjects shown in (B) and (C). * P < 0.05 compared to day 1. ATF4, activating transcription factor 4; BIP, immunoglobulin binding protein; CHOP, C/EBP homology protein; CNX, calnexin; CRT, calreticulin; XBP-1s, X-box binding protein 1s; GRP94, 94 kDa glucose-regulated protein.

Fig. 3. Overnutrition-induced ROS production and posttranslational modifications of adipose tissue proteins

Two-dimensional (2D) gel electrophoresis oxyblot analysis of carbonylated proteins before (left panel) and after (right panel) overnutrition. The extent of posttranslational modifications, as indicated by carbonylation, is shown in table S4. Results were obtained from pooled lysates of the six individual fat biopsies. pI, isoelectric point.

Fig. 4. Identification of GLUT4 posttranslational modifications in response to overnutrition (A) 2D gel electrophoresis Western blots showing multiple isoforms of GLUT4 before (left panel) and after (right panel) excess nutrient uptake. The posttranslational modifications associated with these isoforms were identified by mass spectroscopy analysis (table S6). Results were obtained from pooled lysates of six individual fat biopsies. IgG, immunoglobulin G. (B) Homology-based modeling of GLUT4 structure showing the localization of posttranslationally modified amino acids (R246, R265, and K264) in the cytosolic helices. The amino acids in red were carbonylated, and the amino acids in blue contained 4-HNE adduction. (C) Space-filling model of GLUT4 showing the close proximity of the modified amino acids to the glucose transport channel (marked with red arrowheads). (D) GLUT4 putative topology showing the 12 transmembrane regions (TM) and the intracellular helix (IC). The posttranslational modifications are indicated by red stars (carbonylation) and blue triangle (HNE).

Table 1

Study subjects.

	Day 1	Day 7	Change	<i>P</i>
Age (years)	50.0 ± 1.4	50.2 ± 3.5	–	–
Gender (male/female)	6/0	6/0	–	–
Height (cm)	174 ± 2	174 ± 2	–	–
Weight (kg)	79.0 ± 2.7	82.5 ± 2.8	+3.5 ± 0.3	<0.0001
BMI (kg/m ²)	25.9 ± 0.9	27.1 ± 1.0	+1.13 ± 0.11	<0.0001
FM (kg)	15.1 ± 1.6	18.6 ± 1.9	+3.5 ± 1.0	<0.0001
FFM (kg)	63.9 ± 1.3	63.9 ± 1.6	+0.0 ± 0.8	NS
REE (kcal/24 hours)	1482 ± 99	1719 ± 127	237 ± 80	0.045

BMI, body mass index; FM, fat mass; FFM, fat-free mass; NS, not significant.

Table 2

Energy balance.

Daily caloric intake (kcal/24 hours)	6,206 ± 256
TCI (kcal/7 days)	42,955 ± 805
REE (kcal/7 days)	11,248 ± 510
TRF (kcal/7 days)	2,977 ± 61
Total energy expenditure (TEE = REE + TRF) (kcal/7 days)	14,226 ± 487
Total caloric excess (TCI – TEE)	28,729 ± 1,140
Expected fat gain (kg)	3.19 ± 0.13
Observed fat gain (kg)	3.50 ± 0.3

TCI, total caloric intake.