Insulin regulates the unfolded protein response (UPR) in human adipose tissue

Guenther Boden\textsuperscript{1,2}, Peter Cheung\textsuperscript{1,2}, Sajad Salehi\textsuperscript{1,2}, Carol Homko\textsuperscript{1,2}, Catherine Loveland-Jones\textsuperscript{3}, Senthil Jayarajan\textsuperscript{3}, T. Peter Stein\textsuperscript{4}, Kevin Jon Williams\textsuperscript{2}, Ming-Lin Liu\textsuperscript{2}, Carlos A. Barrero\textsuperscript{5}, Salim Merali\textsuperscript{5}

\textsuperscript{1}Division of Endocrinology/Diabetes/Metabolism, Temple University School of Medicine
\textsuperscript{2}Clinical Research Center, Temple University School of Medicine, Philadelphia, PA
\textsuperscript{3} Department of Surgery, Temple University School of Medicine, Philadelphia, PA
\textsuperscript{4} Department of Surgery, University of Medicine and Dentistry New Jersey, Stratford, NJ
\textsuperscript{5} Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA

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Corresponding author:
Guenther Boden, M.D.
Temple University Hospital
3401 North Broad Street
Philadelphia, PA 19140
Tel: 215-707-8984
Fax: 215-707-1560
Email: bodengh@tuhs.temple.edu
ABSTRACT

Endoplasmic reticulum (ER) stress is increased in obesity and is postulated to be a major contributor to many obesity related pathologies. Little is known about what causes ER stress in obese people. Here, we show that insulin upregulated the unfolded protein response (UPR), an adaptive reaction to ER stress, in vitro in 3T3-L1 adipocytes and in vivo, in subcutaneous (sc) adipose tissue of non-diabetic subjects, where it increased the UPR dose dependently over the entire physiologic insulin range (from ~35 to ~1450 pmol/l). The insulin induced UPR was not due to increased glucose uptake/metabolism and oxidative stress. It was associated, however, with increased protein synthesis, with accumulation of ubiquitination associated proteins, and with multiple post-translational protein modifications (acetylations, methylations, nitrosylations, succinylation, ubiquitinations), some of which are potential causes for ER stress. These results reveal a new physiologic role of insulin and provide a putative mechanism for the development of ER stress in obesity. They may also have clinical and therapeutical implications, for instance in diabetic patients treated with high doses of insulin.
INRODUCTION

Obesity greatly increases the risk for several medical problems including type 2 diabetes (T2DM), hypertension, atherogenic dyslipidemia and non-alcoholic fatty liver disease (NAFLD), conditions collectively called the metabolic syndrome (1). Exactly how obesity leads to these problems remains incompletely understood. On one hand, it has been established that increased release of fatty acids and proinflammatory cytokines from the expanded and dysfunctional adipose tissue plays an important role (2). On the other hand, mounting evidence supports a major role for endoplasmic reticulum (ER) stress. In the ER, proteins are folded into their native confirmation and undergo post-translational modifications which are important for their structure and activity. When protein folding is disturbed, ER stress develops and several signal transduction pathways, collectively called the unfolded protein response (UPR), are activated to restored ER homeostasis (3). The evidence linking ER stress with obesity and obesity related pathologies is strong and consists of a) the fact that ER stress is increased in adipose tissue of obese rodents (4-6) and humans (7,8) and decreases with weight loss (9) and b) that ER stress has been associated with several obesity related pathologies including insulin resistance, T2DM, hypertension, NAFLD and abnormalities in lipid metabolism (4-6, 10-14). However, little is known about what causes ER stress in obesity. Hypoxia and inflammation have been proposed as possible causes because they are present in adipose tissue of obese rodents and people and both have been shown to develop together with ER stress in adipose tissue (15,16). Other suspects are excess nutrient intake, the main cause for obesity, and the associated hyperinsulinemia.

Surprisingly, there is currently no information on effects of insulin on ER stress in human subjects despite the fact that hyperinsulinemia and ER stress are both common in obesity and
that insulin has been shown to produce ER stress *in vitro* in cultured murine macrophages (17) and in human neuroblastoma cells (18).

The objective of the current study, therefore, was to examine the effects of insulin on ER stress responses in vivo, in human subcutaneous adipose tissue from healthy subjects and in vitro, in 3T3-L1 adipocytes.
RESEARCH DESIGN AND METHODS

Subjects and Studies (Table 1)

44 healthy subjects (30 males/14 females) were studied. Their characteristics are shown in Table 1. Informed written consent was obtained from all subjects after explanation of the nature, purpose and potential risks of these studies. The study protocol was approved by the Institutional Review Board of Temple University Hospital. None of the subjects had a family history of diabetes or other endocrine disorders or were taking medications. Their body weights were stable for at least 2 months before the studies. Subjects were admitted to Temple University Hospital’s Clinical Research Center on the evening before the studies. The studies began at ~ 8 AM after an overnight fast with the subjects reclining in bed. A short polyethylene catheter was inserted into an antecubital vein for infusion of test substances. Another catheter was placed into the contralateral forearm vein for blood sampling. This arm was wrapped with a heating blanket (~70°C) to arterialize venous blood.

The following 8 studies were performed:

4 hour studies:

Study #1. Basal (Postabsorptive) insulin (no insulin infusion) - euglycemic clamps (n=4).
Study #2. Hyperinsulinemic (insulin infusion, 1 mU/kg min)-euglycemic clamps (n=6).
Study #3. Hyperinsulinemic (insulin infusion, 2 mU/kg min)-euglycemic clamps (n=6).

8 hour studies

Study #4. Basal (postabsorptive) insulin (no insulin infusion)-euglycemic clamps (n=3).
Study #5. Hyperinsulinemic (insulin infusion, 1 mU/kg min)-euglycemic clamps (n=7).
Study #6. Hyperinsulinemic (insulin infusion, 1.5 mU/kg min)-euglycemic clamps (n = 6).
In studies #1-6, euglycemia was maintained with variable rate glucose infusions. In studies #1 and 4, basal insulin was maintained by euglycemia.

Study #7. Hypoinsulinemic-euglycemic clamps (n=6).

Hypoinsulinemia in Study 7 was produced by infusion of saline only, which resulted in a small decrease in blood glucose (from 5.1 to 4.6 mmol/l over 8 h) but a large decrease in plasma insulin (from 73 to 35 pmol/l).

Study #8. Hyperinsulinemic (no insulin infusion)-hyperglycemic clamps (n=6).

In study #8, endogenous hyperinsulinemia was the result of glucose infusions (to produce glucose levels of ~ 14-16 mmol/l).

**Fat Biopsies**

Immediately before the infusions and again after 4 or 8 hours, open subcutaneous fat biopsies were obtained from the lateral aspect of the upper thigh (~ 15 cm. above the patella) under local anesthesia by a surgeon as described (7). The frozen fat was stored at -80°C until analyzed.

**RT-PCR**

Total RNA was isolated from frozen adipose tissues, and real-time RT-PCR was performed with a SYBR Green One-Step qRT-PCR kit (75770 Affymetrix, Santa Clara, CA) and an Eppendorf Mastercycler ep realpex cycler as described (7). Primers used were human GRP78 sense gttgggtgcagctgact, anti-sense cgtgaagcgcattccttggg;

human/mouse XBP1s sense tggagaacqgggctttaa, anti-sense ccctgcacctgctggccggttcgtgcagactccttcttggtggtggctcacttgcttggtggtgcagctgact; anti-sense cgtgaagcgcattccttggg;

human ATF4 sense ccaaggtggtgactagct, anti-sense cgtgaagcgcattccttggg;

human CHOP sense ggagaacqgggctttgtcactcattgcttcttggtggtggcagctcattggtggtgtggtggctcacttgcttggtggtggctcacttcttcttggtggtgcagctgact; anti-sense cgtgaagcgcattccttggg;

human PDIA3 sense cctgtgggtgtgtcactcattgcttcttggtggtgcagctcattggtggtgtggtggctcacttcttcttggtggtgcagctgact; anti-sense cgtgaagcgcattccttggg;
human Calreticulin sense accctgagtacaaggtgag, anti-sense agatggtgccagacttgacc;
human Calnexin sense cagaccagtgggtatgagat, anti-sense gactgacagtgcaccactct;
human Nrf2 sense acacgtcaggtctcatac, anti-sense tgcctccaaagaatgtcaatca;
human HO-1 sense ctaacttcacaagaagctgc, anti-sense ctgggcaatctttttgagcac;
human VEGFa sense ctccaggagtacctgatg, anti-sense ctatgtgctggccttgggcttg;
human CX3CL1 sense ccaccattctgccatctgac, anti-sense atgtgcattttgctcagacc;
human MCP-1 sense tggcagcttataagaatac, anti-sense tgcctgcaggtgctcattcag;
human Beta-actin sense cagccatgtacgatcattggtggtcc, anti-sense aggtccagacgcaggatgeat;
human JNK1 SuperArray Catalog#PPH00720B; human IL-1beta SuperArray Catalog#PPH00171B; TNF-1alpha SuperArray Catalog#PPH00341E;
human/mouse 18S Ambion catalog#5103G;
mouse ATF4 sense gagcttcctgaacagcgaag, anti-sense tgcctccatgcctctgatc;
mouse GRP78 sense gcaaggattgaaattgagtc, anti-sense gaggcagagctgcatgctc;
mouse CHOP sense agtgccagctcagctg, antisense ctcagatgctgctgct;
mouse INSIG sense tgcaagatcagctgagaata, antisense ccaggcaggagagaatg.

Triplicate samples were normalized with 18s or β-actin.

**Western Blot Analysis**

Proteins (30-80 µg) from adipose tissue lysates were separated by 10-14% gradient SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane in a semidry blotting chamber according to the manufacturer’s protocol (Bio-Rad, Hercules, CA).
Blots were blocked with 5% milk in Tris-buffered saline solution (pH 7.6) containing 0.05% Tween-20 and probed with a mouse antibody against human GRP78 from Bioscience (BD 610979) and the following rabbit anti-human antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) at a concentration of 0.4 µg/ml: protein disulfide isomerase A3 (PDI) (SC-20132), calreticulin (CRT) (SC-11398), calnexin (CNX) (SC-11397) and ATF4 (SC-200); the mouse anti-human antibodies CHOP (Sc-7351), ATF6 (SC-166659) and eIF2α (SC-133132) the goat anti-human antibodies p-eIF2α (Sc-12412) and rabbit anti-human antibodies against JNK (Cell Signaling #9252) and against phospho-JNK (Cell Signaling #9251). Blots were incubated with primary antibody overnight at 4°C with gentle shaking and then incubated with a goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10,000) for 1 h at room temperature. Blots were exposed using a chemiluminescent detection method (Immuno Cruz, SC).

**Proteomics Analysis**

Cytoplasmic proteins from adipose tissue from subjects infused with and without insulin for 4 hrs were extracted under hypotonic conditions. These proteins were processed for gel electrophoresis-liquid chromatography-mass spectroscopy (GeLC-MS/MS) proteomics analysis as previously described (19).

Mass spectra data processing was performed using Mascot Distiller (Version 2.4.3.3) with search and quantitation toolbox options. The generated deisotoped peak list was submitted to an in-house Mascot server 2.4 for searching against the Swiss-Prot database (version 2012_10 of 31-Oct-2012, 538,259 sequences,189,901,164 residues). Mascot search parameters were set as follows: species *Homosapiens* (20,255 sequences); enzyme, trypsin with maximal 2 missed cleavage; fixed modification, cysteine carbamidomethylation; variable modification, methionine
oxidation; 0.60 Da mass tolerance for precursor peptide ions; and 0.4 Da for MS/MS fragment ions. All peptide matches were filtered using an ion score cutoff of 20. The results were analyzed with MS Data Miner. Label-free quantified proteins with higher than 1.8 fold increases were selected and clustered by biological functions using ingenuity pathway analysis as described recently (20).

Post-translational modifications occurrence were specifically analyzed using the following search parameters: maximal 4 missed cleavage, with variable modification: Acetyl (K), methyl (K), dimethyl (K), trimethyl (K), methyl (DE), methyl (C-term), carbamidomethyl (C), ubiquitinylation (GlyGly) (K), nitrosyl (C), methyl (R), dimethyl (R), oxidation (M), succinyl (K), phospho (ST), phospho (Y) ADP-Ribosyl (R) deamidated (R); 0.90 Da mass tolerance for precursor peptide ions; and 0.6 Da for MS/MS fragment ions. Post-translational modified peptides identified with score ≥35 were selected.

3T3-L1 adipocytes studies

Murine 3T3-L1 adipocytes were cultured and differentiated into adipocytes as previously described (21). Briefly, murine 3T3-L1 cells were grown to 100% confluence, and then stimulated with FBS/DMEM medium containing 100 nM insulin, 0.5 mmol/l IBMX (3-Isobutyl-1-methylxanthine), 0.25 µM dexamethasone, and 1 µM rosiglitazone for a 2-day differentiation. Cells were then maintained in FBS/DMEM medium with 100 nM insulin for another 2 days, after which > 90% of cells were mature adipocytes with accumulated fat droplets. Afterward, adipocytes were transferred to FBS/DMEM medium without insulin for 40-hour insulin wash-out period.

After that ~ 10⁶ mature 3T3-L1 adipocytes in each well of a 6-well plate were treated without or with 10 nM of human insulin (in DMEM with 0.2% BSA) for 6 h. Then, cells were
homogenized with either Trizol or Roth lysis buffer respectively for mRNA or protein determination.

**Analytical Procedures**

Plasma glucose was measured with a glucose analyzer (YSI, Yellow Springs, OH). Insulin was determined in serum by RIA with a specific antibody that cross-reacts minimally (0.2%) with proinsulin (Linco, St. Charles, MO). 8-iso-PGF2α was determined with a modification of the liquid chromatography tandem – mass spectrometry method described by Saenger et al. (22).

**Statistical Analysis**

All data are expressed as means ± SE. Post/pre biopsy ratios were compared using the paired 2 tailed t test. Normality was tested with the Kolmogorov Smirnov test. The Wilcoxon’s signed rank test was used to determine significance of the data that were not normally distributed.
RESULTS

*Raising serum insulin increases UPR mRNA and proteins.*

**4 h studies:** To examine effects of acute hyperinsulinemia on UPR markers, 4 h euglycemic (5.5 mmol/l) clamps were performed with 3 different insulin concentrations (basal ~ 70 pmol/l, medium postprandial ~ 480 pmol/l and high postprandial ~ 1450 pmol/l) (Figure 1a + 1b) in 16 healthy subjects (Table 1, studies 1-3). UPR mRNA levels in sc fat biopsies, obtained before and after the clamps (post/pre mRNA ratios), did not change when basal insulin levels were maintained nor in response to medium postprandial hyperinsulinemia (~ 480 pmol/l). However, GRP78, XBP-1s and ATF4 mRNA rose in response to high postprandial insulin levels (~ 1450 pmol/l) (Figure 1c). Hyperinsulinemia had no significant effect on PDI-A3, CRT and CNX mRNA levels.

Protein abundance (post/pre ratios) of GRP78, ATF-4, ATF-6 (p45, the activated form) and phospho-eIF2α (the activated form of eIF2α) also rose in response to the high postprandial insulin levels (Figure 1 d).

**8 h studies.** To examine effects of longer insulin infusions, we performed 8 h clamps in 16 healthy subjects (Table 1, Studies 4-6). Plasma glucose and insulin levels and mRNA responses were comparable to those in the 4 h studies.

Thus, acutely raising insulin for 4 h to high postprandial levels increased several UPR marker mRNAs and proteins. Prolonging exposure to insulin to 8 h had similar effects except for an increase in XBP-1s mRNA at the medium postprandial insulin levels, which was not seen after 4 h.
Post/pre mRNA ratios of the UPR markers measured were similar in 6 normal weight (BMI < 25), 13 overweight (BMI 25-30) and 12 obese (BMI > 30) subject receiving insulin infusions (data not shown).

*Lowering serum insulin decreases UPR mRNA and proteins (Figure 2).*

To examine chronic effects of insulin on the UPR, we acutely lowered insulin in 6 healthy subjects (Table 1, Study 7). This was done by infusing saline for 8 h without supporting glucose levels. This resulted in a small decrease in plasma glucose (from 5.1 to 4.6 mmol/l, p < 0.01) which, although remaining within the normal range, nevertheless caused a more than 50% decline in basal serum insulin (from 75 to 35 pmol/l, p < 0.01) (Figure 2 a + b). In control studies (Table 1, Study 4), a decrease of plasma glucose and insulin was prevented, and basal levels of both were maintained by infusion of small amounts of glucose. The acute hypoinsulinemia resulted in a significant decrease in GRP78, XBP-1s, ATF-4, PDI-A3 and CRT post/pre mRNA ratios (Figure 2 c), as well as similar decreases in post/pre protein ratios (Figure 2 d).

Thus, acutely lowering insulin to below basal levels decreased UPR mRNA and proteins. These results showed that there had been post-absorptive UPR activity, which had been supported by basal insulin levels and that lowering of this basal insulin support resulted in lowering of the UPR, thus providing evidence for longterm effects of insulin on the UPR. Combining results of the 4 h and 8 h studies revealed a close and dose dependent relationship between insulin and UPR marker mRNA and proteins over the entire physiologic range of serum insulin concentrations (Figure 2 e).

To confirm these in vivo insulin effects in an in vitro system, we incubated 3T3-L1 adipocytes without and with insulin (10 nmol/l) for 6 hours. As seen in Figure 3, insulin
increased GRP78, ATF4 and XBP-1s mRNAs, 1.7, 2.7 and 1.6 fold, respectively (p < 0.001) and had similar effects on ATF4 and CHOP proteins, whereas GRP78 increased only with 100 nmol/l insulin (data not shown).

**Insulin increased protein synthesis, protein ubiquitination and posttranslational protein modifications (Tables 2 and 3).**

Insulin, by increasing protein synthesis, could have stimulated the UPR by generating an amount of nascent polypeptides large enough to exceed the ER folding capacity. This would produce ER stress via accumulation of unfolded/misfolded and ubiquitinated proteins. To test the effect of insulin on protein synthesis, we determined protein abundance by mass spectrometry, in pooled fat biopsy samples taken pre and post exposure to 4 h of euglycemic-hyperinsulinemia from 4 of the 6 subjects in Study 3 (Table 1). The results showed that 25 proteins increased from 1.8 – 21.2-fold in response to euglycemic-hyperinsulinemia. Among those proteins were 5 ubiquitination pathway proteins, which increased from 1.8 – 3.0 fold, suggesting accumulation of unfolded proteins (Table 2).

To test for effects of insulin on ubiquitination of proteins, we performed immunoblots of fat biopsy samples obtained from 4 individual subjects before and after 4 h of euglycemic-hyperinsulinemia (~1,500 pmol/l). The results showed that protein ubiquitination rose 3.4 ± 1.3 fold (p = 0.05) in response to insulin (Supplementary document, Figure 1).

Using our mRNA and protein data, we performed a canonical pathway analysis with the Ingenuity Pathway Analysis program as described recently (20). This analysis showed a 13.5 fold increase in ER stress pathway activity (including increases in ERN1, XBP1, DNAJC3, ATF4, ATF6, Hsp A5 and eIF2α K3) and a 4.1 fold increase in the protein ubiquitination
pathway (including increases in DNAJC3, Hsp A5, P34931, Hsp H1 and Hsp A4L). Together, these results further support the concept of insulin stimulation of ER stress.

In addition, we identified several insulin induced posttranslational protein modifications including acetylations, methylations, nitrosylations, succinylation and ubiquitinations in 8 proteins (Table 3).

*Insulin induced UPR changes were not associated with changes in glucose uptake and oxidation (Figure 4)*

Raising insulin increased glucose uptake as well as intracellular glucose metabolism. For instance, euglycemic-hyperinsulinemia (Figure 4 a + b, open circles) increased rates of glucose uptake ~ 9 fold (from ~ 12 to ~ 110 µmol/kg min) and carbohydrate oxidation rates ~ 5 fold (from ~3 to ~ 15 µmol/kg min). As an increase in glucose uptake/metabolism can produce ER stress via generation of ROS and changes in the intracellular redox state (23,24), we examined effects of glucose uptake on the UPR by comparing 2 studies with identical degrees of hyperinsulinemia but different rates of glucose uptake (Table 1, Studies 6 and 8, Figure 4 b-d). Despite the large difference in glucose uptakes, however, there were no significant differences in UPR mRNA post/pre ratios (Figure 4 e).

We also examined the effects of different rates of glucose uptakes on markers of ROS production and oxidative stress. Urinary excretion of 8-iso PGF2α, a sensitive and specific indicator of whole body lipid peroxidation and oxidative stress (25), as well as adipose tissue Nrf2, HO1 and VEGF post/pre mRNA ratios, all indicators of oxidative stress (26), also did not rise above basal in response to increased glucose uptakes in adipose tissue (Figure 4 f-i).

Since oxidative stress is known to activate the NFκB and c-jun N terminal kinase (JNK) stress pathways (26), we examined effects of insulin stimulated glucose uptake on adipose tissue
mRNA expression of JNK1, phosphorylation (activation) of JNK1 to phospho-JNK1 and the NFκB targets, TNFα, IL-1β, MCP1 and CX3CL1. Hyperinsulinemia induced a 10 fold increase in glucose uptake (from ~ 100 to ~ 1000 Kcal/8h) but did not increase expression of any of these factors (Table 4).

Thus, the observed insulin induced increases in UPR mRNA and proteins in adipose tissue were unlikely to be associated with changes in glucose uptake and/or oxidative stress.
DISCUSSION

Insulin controls the unfolded protein response dose dependently

A main finding in this study was that insulin regulated several UPR marker mRNAs and proteins dose-dependently over the entire range of physiologic serum insulin levels (from ~ 35 to ~ 1450 pmol/l) in sc adipose tissue of non-diabetic subjects (Figure 2e).

These results were supported by 4 studies showing that increasing serum insulin increased the UPR and by another study showing that lowering insulin action decreased the UPR, and by a canonical pathway analysis (with the Ingenuity Pathway Program, (ref. 20) using mRNA and protein data, which showed a 13.4 fold increase in ER stress pathway activation (and a 4.1 fold increase in protein ubiquitination pathway activation (suggesting accumulation of un and/or misfolded proteins)).

These UPR changes were caused by insulin, rather than by other insulin induced effects (for instance changes in plasma FFA levels), as evidenced by in vitro experiments, which showed that insulin increased UPR mRNA and proteins in 3T3-L1 adipocytes (Figure 3).

Several other results of our in vivo studies are noteworthy.

1. The observation that UPR activity decreased when insulin was lowered to below basal levels indicated that basal (postabsorptive) UPR activity had been supported by basal insulin levels and therefore, that insulin regulated the UPR long term as well as acutely; 2. that the effect of insulin on UPR mRNA was fully established after 4 h and changed little after 8 h (Figure 1); 3. that insulin affected all 3 proximal ER stress sensor pathways (p-eIF2α and ATF4 for the PERK pathway, XBP-1s for IRE1 and ATF6 p45 for the ATF6 pathway); 4. that the responses were not uniform as CRT and CNX changed little or not at all, supporting the notion that the UPR is differentially regulated in response to variable metabolic demands (3).
We believe that these are the first data on *in vivo* effects of insulin on UPR markers in human subjects. They were obtained in adipose tissue, a tissue where ER stress was found in obese rodents to be very prominent, leading to its dysfunction and to many obesity related pathologies (27). They are also in accord with a recent report from our laboratory which showed that insulin increased UPR markers in rat liver (28).

*How does insulin stimulate ER stress responses?*

The dose dependent UPR activation by insulin was accompanied by an equally dose dependent stimulation of glucose uptake (and oxidation) which is known to increase intracellular ROS production, oxidative and ER stress (23,24,29). Thus, insulin could have increased ER stress and activated the UPR by increasing glucose uptake/metabolism and oxidative stress. However, when we compared changes in UPR markers during studies with similar hyperinsulinemia but markedly different rates of glucose uptake, higher glucose uptakes did not result in higher UPR mRNA (Figure 4).

Other findings also did not support the notion that the observed insulin induced UPR changes were caused by oxidative stress. Thus, higher insulin levels and glucose uptakes did not produce greater urinary excretion of 8-Iso-PGF-2α, a sensitive and specific indicator of lipid peroxidation (25). They also did not increase Nrf-2, HO1 and VEGF mRNA in adipose tissue, all indicators of oxidative stress (26), nor did they increase expression of the NFκB and c-jun N terminal kinase (JNK) stress pathways, (see Table 4) which are known to be activated by oxidative stress (29,30). These findings are further supported by the lack of change of these proteins in our proteomic analysis.

Taken together, these results did not support a role for an insulin stimulated increase in glucose uptake/metabolism as a cause for the increase in UPR in our studies. A likely reason for
the absence of oxidative stress may be that the available antioxidant defenses were sufficient to prevent development of oxidative stress in response to the rather modest physiologic increases in glucose uptake (1000-2000 Kcal/4-8 h).

On the other hand, our results supported the notion that insulin caused ER stress by increasing protein synthesis which could have resulted in an increase in un or misfolded proteins and in accumulation of ubiquitinated proteins. Stimulation of protein synthesis and suppression of protein breakdown by insulin is well established (31,32). Here, we showed that insulin increased the abundance of a large number of proteins, including 5 involved in protein ubiquitination, between ~ 2 and 21 fold (Table 2) and increased protein ubiquitination ~ 3 fold (Supplementary document Figure 1). Further, Ingenuity Pathway analysis showed a 4.1 fold increase in ubiquitination pathway and a 13.5 fold increase in ER stress activity. Another new finding was that insulin produced multiple protein post-translational modifications (Table 3). These results support the hypothesis that the appearance of a large number of nascent polypeptides resulted in accumulation of un or misfolded and ubiquitinated proteins and the presence of post-translational protein modifications, some of which may have interfered with protein folding (3, 33), may have exceeded the ER folding capacity, initiating the UPR.

Our data do not rule out that insulin directly activated at least some part of the UPR. For instance, it has recently been reported that insulin can activate XBP-1, i.e., the IRE1α sensor pathway of the ER (34,35). In that case, insulin would activate one part of the UPR proactively, i.e., even before the development of ER stress. This could occur in addition to the reactive, i.e., ER stress in initiated activation, shown in our studies. In fact, a combined proactive and reactive UPR activation would be of physiological advantage under conditions where a rise of insulin and insulin induced ER stress are predictable, for instance during a meal (36).
Another mechanism by which insulin could have increased ER stress may be inhibition of autophagy. Acute hyperinsulinemia, via mTorc1 activation not only stimulates protein synthesis (see Table 2) but also inhibits autophagy (37). Thus, by inhibiting an inhibitor of ER stress, insulin may have increased ER stress.

In summary, we have shown here that insulin in vivo and in vitro, upregulated the UPR in human adipose tissue. The insulin mediated UPR increase could not be explained by an increase in insulin stimulated glucose uptake, oxidative stress, inflammation or insulin resistance, but was associated with an increase in protein synthesis, protein ubiquitination and various post-translational protein modifications. We conclude that acute physiological increases in circulating insulin produced a modest degree of ER stress and resulted in an increase in UPR, which, presumably, was sufficient to relieve the ER stress and prevented development of insulin resistance and inflammation. The effect on ER stress and the UPR of severe and/or long lasting hyperinsulinemia, for instance, due to chronic excessive caloric intake or in diabetic patients who are treated with very high doses of insulin, remains to be explored. Under these conditions, the UPR may not be able to relieve the more severe ER stress which then may result in chronically unrelieved ER stress and insulin resistance.
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AUTHOR CONTRIBUTIONS

G.B. conceived all studies and wrote the manuscript. P.C., S.S. and T.P.S. performed and analyzed serum, urine and biopsy samples and contributed to the Discussion. C.H., C.L.J. and S.J. performed human studies, analyzed results and contributed to the Discussion. K.J.W. and M.L. performed and analyzed the in vitro studies and contributed to the Discussion. S.M. and C.B. performed and analyzed proteomic studies and contributed to the Discussion.

CONFLICT OF INTEREST

None of the authors declares a conflict of interest.

GUARANTOR’S STATEMENT

Dr. Guenther Boden is the guarantor of this work and as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
REFERENCES


FIGURE LEGENDS

Figure 1 (left panels): Effects of 3 levels of euglycemic-hyperinsulinemia for 4 h on UPR mRNA and proteins.
Panels a and b – steady state (120-240 min) plasma glucose and insulin concentrations in non-diabetic volunteers during three 4 h euglycemic clamp studies with 3 different steady state insulin levels.
Panel c - subcutaneous (sc) fat biopsies were obtained surgically immediately pre and post the clamp studies. Shown are post/pre mRNA ratios of 6 UPR markers in response to euglycemia associated with basal (n=4, ~ 70 pmol/l), medium postprandial (n=6, ~ 480 pmol/l) and high postprandial (n=6, ~ 1450 pmol/l) insulin concentrations. Shown are the means ± SE of 18s normalized mRNA values. * p < 0.05, ** p < 0.02, *** p < 0.01 compared to post/pre ratios of 1.0.
Panel d – mean of 6 post/pre UPR protein ratios (left) and 2 representative Western blots each of the UPR proteins (right) in response to high postprandial insulin concentrations. Shown are means ± SE. * p < 0.05, ** p < 0.02; compared to post/pre ratios of 1.0 (paired Student’s t-test).

Figure 1 (right panels): Effects of 3 levels of euglycemic-hyperinsulinemia for 8 h on UPR mRNA.
Panels e and f – steady state plasma glucose and insulin concentrations during three 8 h euglycemic clamp studies with 3 different steady state insulin levels.
Panel g – mean post/pre mRNA ratios of 6 UPR markers in response to basal (n=3), medium postprandial (n=6) and high (n=7) postprandial insulin concentrations. Shown are mean ± SE. * p < 0.05 compared to post/pre ratios of 1.0 (paired Student’s t-test).
Abbreviations: ATF 4, activating transcription factor 4; ATF6 (p45), activated (p45) activating transcription factor 6; CRT, calreticulin; CNX, calnexin; p-eIF2α, phospho-eukaryotic translational initiation factor 2α; GRP78, glucose regulated protein 78; PDI-A3, protein disulfide isomerase A3; XBP1s, X box binding protein 1 s.

**Figure 2: (left panels) - Effects of acute euglycemic-hypoinsulinemia on UPR mRNA and proteins.**

Panels a and b – plasma glucose and insulin concentrations during 8 h of euglycemic-hypoinsulinemia and euglycemic-basal insulinemia. During the euglycemic-hypoinsulinemia studies, saline was infused without glucose. During the euglycemic-basal insulinemia studies, saline was infused with glucose * p < 0.05; *** p < 0.01 compared to 0 min (paired Student’s t-test).

Panel c – post/pre mRNA ratios of 6 UPR markers in response to euglycemic-hypoinsulinemia and basal insulinemia.

Panel d – post/pre protein ratios of UPR markers in response to hypoinsulinemia (Study #7) and euinsulinemia (Study #8) and 2 representative Western blots (from Study #7). Because of scarcity of tissue, obtained from the euglycemic-basal insulin studies only 4/7 UPR protein measurements could be performed. Shown are means ± SE.* p <0.05; *** p < 0.01 compared to post/pre ratios of 1.0 (paired Student’s t-test).

**Figure 2 e (right panels) – Correlation between UPR mRNA or protein and serum insulin**

Relationship between GRP78, ATF4 and XBP-1s post/pre mRNA ratios and serum insulin levels from 7 euglycemic clamp studies (three 4 h and four 8 h studies) (left) and GRP 78, phospho-eIF2α and ATF6 (p45) post/pre protein ratios and serum insulin levels from 3
euglycemic clamp studies (one 4 h and two 8 h studies) (right). Shown are means ± SE of UPR makers (vertical bars) and insulin concentrations (horizontal bars).

**Figure 3: Insulin increases UPR mRNA and proteins in 3T3-L1 adipocytes.**

Effect of 6 h incubations with insulin (10 nmol/l) or without insulin (-) on UPR mRNA (upper panel) and proteins (lower panel). Shown are means ± SE (n=6), *p < 0.001 compared to no insulin.

**Figure 4: Effects of similar hyperinsulinemia but different glucose uptakes on UPR mRNA and oxidative stress markers.**

Panels a-d – plasma glucose and insulin concentrations, glucose infusion rates (GIR) and insulin stimulated glucose uptakes (in Kcal/8 h) during 8 h euglycemic-hyperinsulinemic and hyperglycemic-hyperinsulinemic clamps (Table 1, Studies 6 and 8) and euglycemic-basal insulinemic controls (Table 1, Study 4). Despite GIR and glucose uptakes which were 2 times higher with hyperglycemic-hyperinsulinemia than with euglycemic-hyperinsulinemia, UPR mRNA levels were not different.

Panel e – post/pre mRNA ratios of 7 UPR markers. Shown are mean ± SE; * p < 0.05; ** p < 0.001 compared to post/pre ratios of 1.0.


Panels g-i - adipose tissue post/pre mRNA ratios of Nrf-2, HO-1 and VEGF. Shown are means ± SE.

Abbreviations: 8-iso-PGFα, 8 iso-prostaglandin 2α; Nrf2, nuclear factor erythroid 2-related factor 2; HOI, hemeoxygenase 1; VEGF, vascular endothelial growth factor.
Table 1: Studies and study subjects

Subcutaneous upper thigh fat biopsies were obtained pre and post 8 h hyperinsulinemic (~ 1000 pmol/l) –hyperglycemic (~ 11 mmol/l) clamps without (HI + HG) and with co-infusion of lipid (HI + HG + Lipid). Shown are post/pre mRNA ratios from pooled fat biopsies (n = 3) determined with a human UPR pathway array (SA Biosciences Co. Catalog Number (PAH-098Z)).

<table>
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<tr>
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<tr>
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<td></td>
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<tr>
<td>HG/HI</td>
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<td>41 ± 6</td>
<td>31 ± 5</td>
<td>48 ± 2</td>
<td>36 ± 6</td>
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<td>78.9 ± 8.4</td>
<td>86.4 ± 6.4</td>
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<td>80.1 ± 3.4</td>
<td>81.0 ± 6.4</td>
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<td>HT, cm</td>
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<td>171 ± 5</td>
<td>175 ± 5</td>
<td>173 ± 7</td>
<td>171 ± 4</td>
<td>174 ± 4</td>
<td>173 ± 4</td>
<td>170 ± 4</td>
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<td>BMI, kg/m²</td>
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<td>28.8 ± 0.9</td>
<td>26.1 ± 1.0</td>
<td>29.4 ± 1.4</td>
<td>32.1 ± 2.1</td>
<td>27.1 ± 1.3</td>
<td>27.9 ± 2.0</td>
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<td>FBG, mg/dL</td>
<td>99.8 ± 4.9</td>
<td>95.9 ± 4.4</td>
<td>97.8 ± 5.3</td>
<td>87.3 ± 6.9</td>
<td>91.3 ± 1.1</td>
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<td>96.9 ± 3.3</td>
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<td>A1c, %</td>
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<td>5.7 ± 0.2</td>
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<td>5.6 ± 0.2</td>
<td>5.5 ± 0.2</td>
<td>5.4 ± 0.2</td>
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BG = basal glucose (euglycemia), HG = high glucose (hyperglycemia), BI = basal insulin, HI = high insulin (1mU/kg/min), HIx1.5 = high insulin (1.5mU/kg/min), HIx2 = high insulin (2mU/kg/min)
Table 2. Increase in protein expression levels four hours after insulin.

SC upper thigh fat biopsies were obtained pre and post 4 h euglycemic-hyperinsulinemia (~ 1450 pmol/l) clamps. Shown are post/pre protein expression ratios from pooled biopsy samples (n = 4). Differential expression analysis using label free proteomics was performed to quantitate a change in protein expression.

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<td>78 kDa glucose-regulated protein</td>
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<td>ER-associated protein</td>
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<td>protein catabolic process</td>
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<td>Signaling by Rho</td>
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<td>Moesin</td>
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<td>Family GTPases</td>
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<td>Phosphoglycerate kinase 1</td>
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<td>Fructose-bisphosphate aldolase A</td>
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<td>ALDOC_HUMAN</td>
<td>Fructose-bisphosphate aldolase C</td>
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<td>Cellular Movement</td>
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<td>Alpha-1-acid glycoprotein 1</td>
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<td>Hsp H1 human</td>
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<td>Heat shock 105 kDa/110kDa protein</td>
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Table 3. Insulin induced protein post-translational modifications.
SC upper thigh fat biopsies were obtained pre and post 4 h euglycemic-hyperinsulinemia (~ 1450 pmol/l) clamps. Shown are unique post-translational modifications in pooled biopsy samples (n=4) as identified by mass spectroscopy. Residues modified are underlined within each peptide sequence. Modifications identified included Ace: Acetylation; Met: Methylation; Nit: Nitrosylation; Suc:Succinylation and Ubi:Ubiquitination.

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<tr>
<th>Protein ID</th>
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<th>Description</th>
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<td>MURC_HUMAN</td>
<td>48</td>
<td>Muscle-related coiled-coil protein</td>
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<td>LRKSGKEHIDNIK</td>
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<td>TGFA1_HUMAN</td>
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<td>Transforming growth factor-beta receptor-associated protein 1</td>
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<td>QIQDLLASR</td>
<td>Met_D319</td>
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<td>PERI_HUMAN</td>
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<td>TERA_HUMAN</td>
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<td>2A5D_HUMAN</td>
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<td>Serine/threonine-protein phosphatase 2A (56 kDa regulatory subunit delta isoform)</td>
<td>37</td>
<td>VLLPLHK</td>
<td>Ace_K332</td>
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Table 4: Effect of Hyperinsulinemia on Inflammatory Stress Markers

Subcutaneous upper thigh fat biopsies were obtained pre and post euglycemic-basal insulin (Studies 1 + 4) (~70pmol/l) and euglycemic-hyperinsulinemic (~1200 pmol/l) clamps (Study 5). Shown are means ± SE of post/pre mRNA and protein ratios

ns = not significant

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<th>Elevated insulin (n=7)</th>
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</thead>
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<tr>
<td>IL-1β</td>
<td>1.02 ± 0.33</td>
<td>0.74 ± 0.14</td>
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<tr>
<td>TNF-α</td>
<td>1.10 ± 0.22</td>
<td>1.42 ± 0.35</td>
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<tr>
<td>MCP1</td>
<td>1.04 ± 0.06</td>
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<td>CX3CL1</td>
<td>1.04 ± 0.13</td>
<td>1.08 ± 0.19</td>
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<tr>
<td>JNK</td>
<td>1.25 ± 0.16</td>
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post/pre mRNA/18 ratios (arbitrary units)

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<td>JNK</td>
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<td>phospho-JNK</td>
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post/pre protein/β-actin ratios (arbitrary units)
**Figure 2**

(a) Glucose levels over time with different treatments.
(b) Insulin levels over time with different treatments.
(c) UPR mRNA ratios with different treatments.
(d) UPR protein ratios with different treatments.
(e) Correlation analysis of UPR mRNA and protein levels with insulin levels.
Figure 3

Diabetes

mRNA fold increase

Protein fold increase

Insulin (nmol/L)    -        10       10        10         10

GRP78    ATF4   Chop  XBP-1s

INSULIN
nmol/L

-            10

GRP78
ATF4
Chop

β-actin

Figure 3