Insulin Resistance is Associated with Diminished Endoplasmic Reticulum Stress Responses in Adipose Tissue of Healthy and Diabetic Subjects

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ABSTRACT

We have recently shown that insulin increased ER stress in human adipose tissue. The effect of insulin resistance on ER stress is not known. It could be decreased, unchanged or increased, depending on whether insulin regulates ER stress via the metabolic/phosphoinositide 3-kinase (PI3K) or alternate signaling pathways.

To address this question, we have examined effects of lipid induced insulin resistance on insulin stimulation of ER stress.

mRNAs of several ER stress markers were determined in fat biopsies obtained before and after 8 hour hyperglycemic-hyperinsulinemic clamping in 13 normal subjects and in 6 chronically insulin resistant patients with type 2 diabetes (T2DM).

In normal subjects, hyperglycemia-hyperinsulinemia increased post/pre mRNA ratios of several ER stress markers (determined by ER stress pathway array and by individual RT-PCR). Lipid infusion was associated with inhibition of the PI3K insulin signaling pathway and with a decrease of hyperinsulinemia induced ER stress responses. In chronically insulin resistant patients with T2DM, hyperglycemic-hyperinsulinemia was unable to increase ER stress response marker mRNAs.

In summary, insulin resistance, either produced by lipid infusions in normal subjects or chronically present in T2DM patients, was associated with decreased hyperinsulinemia induced ER stress responses. This suggested, but did not prove, that these 2 phenomena were causally related.
INTRODUCTION

ER stress is increased in adipose tissue of obese rodents (1-3) and humans (4-6) and has been associated with several obesity related pathologies including T2DM, hypertension, atherogenic dyslipidemia and non-alcoholic fatty liver disease (1-3, 7-11). The reason why ER stress is increased in obesity is complex and includes hypoxia, inflammation (12,13) and hyperinsulinemia. We have recently shown that short term physiologic increases in circulating insulin upregulated the unfolded protein response (UPR), an adaptive ER stress response that reflects ER stress, in subcutaneous (s.c.) adipose tissue of normal subjects, dose-dependently over the entire physiological insulin range (14). Whether the chronic hyperinsulinemia in insulin resistant subjects has similar effects on ER stress responses is not known and depends on the mechanism through which insulin stimulates ER stress. Hence, if insulin signaling occurred through the so called metabolic, i.e., the PI3K pathway, one would expect little or no insulin effect on ER stress in obese subjects or in patients with T2DM, in whom this pathway is inhibited. If, on the other hand, insulin signaling occurred via alternate pathways, collectively called MAPK pathways, insulin could increase ER stress even in “insulin resistant” subjects. Instances of such “selective insulin resistance” i.e., resistance in the metabolic/PI3K pathway and normal or increased activity in an alternate insulin signaling pathway, are increasingly being recognized (15-17).

To differentiate between these possibilities, we have examined effects of hyperinsulinemia on ER stress markers in s.c. adipose tissue of normal subjects in whom the metabolic/PI3K pathway was inhibited with lipid infusion and in s.c. adipose tissue of insulin resistant patients with T2DM, in whom the metabolic/PI3K pathway is known to be inhibited.
RESEARCH DESIGN METHODS

Subjects and Studies (Table 1)

We have studied 13 healthy subjects (9 M/4F) and 6 patients (3M/3F) with T2DM. 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 healthy subjects had a family history of diabetes or other endocrine disorders or were taking medications. The patients with T2DM were treated with long acting insulin (3/6), short acting insulin (2/6), sulfonylureas (2/6), metformin (5/6), blood pressure lowering drugs (5/6) and lipid lowering drugs (4/6). All drugs except insulin were discontinued 2 days before admission. The last insulin dose was taken 2 hours before admission. Body weight of all study volunteers 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, which began at ~ 8 AM after an overnight fast.

The following 3 studies were performed:

Study #1. 8 h hyperglycemic - hyperinsulinemic (no insulin infusion) clamps in healthy subjects (n=6).

In this study, endogenous-hyperinsulinemia was the result of i.v. infusions with 20% glucose (to produce glucose levels of ~ 200-250 mg/dl, ~ 11.1-13.9 mmol/L). Plasma electrolytes were monitored every 6 h; body weight every 12 h; and fluid balances every 6 h. Potassium (20 mg) and magnesium (400 g) were given orally every 12 h.

Study #2. 8 h hyperglycemic-hyperinsulinemic (no insulin infusion) clamps with co-infusion of lipid/heparin in healthy subjects (n=7). Lipid/heparin infusions which were started
together with the glucose infusions at 0 h, consisted of IV infusion of Intralipid III, a 20% fat emulsion (1.5 ml/min Abbott Labs, Chicago, IL) and heparin (a 200 unit bolus followed by infusion of 0.4 U/kg min).

Study #3. Isoglycemic-hyperinsulinemic (insulin infusion at a rate of 2 mU/kg min) clamps in 6 patients with T2DM.

Glucose was clamped at the patient’s postabsorptive glucose level (isoglycemic clamp) by a variable rate infusion of 20% glucose.

**Fat Biopsies**

Immediately before the infusions and again after 8 hours, open s.c. fat biopsies were obtained from the lateral aspect of the upper thigh under local anesthesia by a surgeon as described (13).

**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 (4). Primers used were:

- human GRP78 sense gttggtgctgactgaat, anti-sense cgctacagcttcatctggg;
- human/mouse XBP1s sense ttgagaaccaggagttaa, anti-sense cctgcacctgctgcggact;
- human ATF4 sense ccacgttggatgacac, anti-sense ggcttcctatctccttcag;
- human CHOP sense ggagaaccaggaaacggaaac, anti-sense tcttcagctagctgtgccac;
- human PDE3B sense gccacagatgttgctacatgs, anti-sense gacaggcagccataactctc;
- human PDIA3 sense cttggcatccatcttgct, anti-sense gtttggtgctactgaagaacct;
- human Calreticulin sense accctgagtacaagggtgag, anti-sense agatggtgcagacttgacc;
- human Calnexin sense cagaccagtgtatgagat, anti-sense gactgacagtgccaccatct;
human/mouse 18S Ambion catalog#5103G;

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

**Immmuoprecipitation**

Rabbit anti-IRS-1 sera (from Upstate, Lake Placid, NY) and protein A-agarose beads were used to immunoprecipitate IRS-1 associated PI3K from fat extracts (100 µg).

**Western blots**

**Adipose** tissues were extracted and protein content was measured using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Sample preparation and performance of Western blots were as described (4). The primary antibodies used were: a rabbit antiserum (Upstate) that recognizes the N-SH₂ region of PI3K and the regulatory p85 subunit of PI3K.

**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). FFAs were measured in plasma, containing Paroxon (Sigma, St. Louis, MO), a lipoprotein lipase inhibitor, with a kit from Wako Pure Chemicals (Richmond, VA).

**Statistical Analysis**

All data are expressed as means ± SE. Pre- and postbiopsy values in Figure 1 e, f and g were compared using the 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. A two-way ANOVA was used in Figure 1 d to test for significant differences between studies with Student-Newman-Keuls post hoc analysis. If data were not normally distributed, the Kruskal-Wallis one-way ANOVA with Dunn post hoc analysis was
used. To test the differences in GIR across time, a one-way repeated measures ANOVA with Student-Newman-Keuls post hoc analysis was used. If data were not normally distributed, the Friedman repeated-measures ANOVA on ranks was used.
RESULTS

Lipid induced acute insulin resistance inhibits insulin stimulation of UPR mRNA in adipose tissue of healthy subjects

Infusion of glucose, either without or with infusion of lipid in healthy subjects (Table 1, Studies 1 and 2) resulted in similar degrees of hyperglycemia and hyperinsulinemia, but different levels of plasma FFA (Figure 1 a – c). Lipid infusion resulted in systemic (Figure 1 d), and adipose tissue insulin resistance (inhibition of insulin stimulated PI3K activation, Figure 1 e and PDE 3b mRNA Figure 1 f).

Hyperglycemic-hyperinsulinemia increased post/pre mRNA ratios of GRP-78, XBP-1s, ATF4 and Chop. Hyperglycemic-hyperinsulinemia with lipid infusion induced insulin resistance was associated with significantly reduced insulin mediated increases in post/pre mRNA ratios of GRP78 and Chop (Figure 1 g).

UPR pathway array confirms UPR mRNA stimulation by insulin and the prevention of this effect by lipid infusion (Table 2).

To confirm the effects of insulin and lipid induced insulin resistance on a larger number of UPR markers, we performed a UPR pathway associated gene expression PCR array with pooled adipose tissue (aliquots of 4 biopsy samples each) from studies #1 and #2. In response to hyperinsulinemia, post/pre mRNA ratios of 20 of the 84 UPR markers in the array rose more than 1.5-fold above basal, whereas 3 decreased to less than 75% of basal. Co-infusion of lipid decreased the insulin induced rise in all 20 UPR markers (from 1.98 ± 0.14 to 1.08 ± 0.07 fold, p < 0.001) and increased the insulin induced decrease in all 3 UPR markers (from 0.25 ± 0.08 to 1.07 ± 0.13, p < 0.02).
These results supported in a large number of UPR markers the finding that insulin stimulated, whereas lipid mediated metabolic/PI3K insulin resistance was associated with inhibition of insulin stimulated UPR transcription.

*Insulin stimulation of the UPR is reduced in chronically insulin resistant patients with T2DM (Figure 2)*

To examine effects of chronic insulin resistance on ER stress, we compared UPR responses to comparable hyperglycemic-hyperinsulinemia in 6 insulin resistant patients with T2DM with those in 6 healthy controls (Table 1, Studies 1 vs. 3). Insulin stimulated glucose uptakes (GIR) were significantly lower in T2DM patients than in controls, indicating systemic insulin resistance. Whereas basal UPR mRNA levels were not significantly different, in response to hyperinsulinemia, GRP78, XBP1s, ATF4 and Chop mRNAs rose in controls but not in patients with T2DM. This showed that chronic metabolic/PI3K insulin resistance was associated with inhibition of insulin stimulation of UPR mRNA in s.c. adipose tissue of patients with T2DM.
DISCUSSION

This study showed that in normal subjects, lipid infusions, known to inhibit the metabolic/PI3K insulin signaling pathway (18-20), not only inhibited systemic insulin actions on glucose uptake and plasma FFA levels, but also decreased ER stress responses in their adipose tissue. Moreover, in obese T2DM patients, in whom the metabolic/PI3K pathway is chronically inhibited (21), ER stress responses to insulin were similarly inhibited. Together these results suggested, but did not prove, that inhibition of the PI3K insulin signaling and inhibition of insulin induced ER stress responses may be causally related.

Inasmuch as insulin induced lowering of plasma FFA levels was associated with a rise in ER stress marker mRNA, our results seem to disagree with studies showing stimulation of ER stress by an increase in FFAs in 3T3-L1 pre-adipocytes (23,24). However, these in vitro data cannot easily be compared with results obtained under the more complex in vivo situation. In fact, the in vivo results may reflect two counteracting effects, namely, UPR stimulation by FFAs on one hand, and a larger reduction of ER stress caused by FFA induced inhibition of insulin action, on the other hand.

The finding that inhibiting the metabolic insulin actions was associated with decreased insulin mediated ER stress responses, suggested that the ER stress responses were the consequence of metabolic insulin actions, for instance on glucose uptake/metabolism and protein synthesis. Supporting this conclusion were our previous findings (14), that insulin induced ER stress was associated with increased protein synthesis, known to be stimulated by insulin (25) and increased production of post-translational protein modifications including ubiquitinations, suggesting an increase in unfolded proteins, a major cause for ER stress.
Inasmuch as ER stress can be produced by insulin, the development of insulin resistance could be considered an adaptive defense to prevent further increase in insulin induced ER stress. Similar views, i.e., that insulin resistance may be an appropriate cellular defense against stressful overnutrition and hyperinsulinemia, have recently been proposed by others (26-29). Nevertheless, even though insulin resistance may be an appropriate and adaptive cellular response, it will become ineffective and overall maladaptive, if it’s cause, for instance excess caloric intake, continues and the nutrients that cannot enter the cells accumulate extracellularly.

In summary, we have shown 1) that lipid infusions produced acute insulin resistance (inhibition of insulin mediated activation of PI3K) and diminished insulin mediated ER stress responses in adipose tissue of normal subjects and 2) that insulin was unable to increase ER stress responses in chronically insulin resistant patients with T2DM. These results suggested, but did not prove, that inhibition of the metabolic PI3K insulin pathway was responsible for the decrease in insulin mediated ER stress responses in adipose tissue of non-diabetic and diabetic subjects could be an adaptive defense against further increase in ER stress.
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Author Contributions

GB designed the study and wrote the paper; PC contributed and researched data, KK contributed data and contributed to the discussion, CH contributed and researched data, PB and LF contributed data and contributed to discussion.

Guarantor’s statement

GB is the guarantor of this paper and takes responsibility for it’s content.

Disclosure Summary

None of the authors declares any conflict of interest.
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Table 1: Studies and study subjects

| Study # | Study type | 1 HG/HI | 2 HG/HI+L | 3 HG/HI |
|---------|------------|---------|-----------|---------|
| Diabetes | No | No | Yes |
| Gender | 4M/2F | 5M/2F | 3M/3F |
| Age | 27 ± 3 | 31 ± 5 | 53 ± 1 |
| WT, kg | 81.0 ± 6.4 | 87.7 ± 8.6 | 90.5 ± 9.2 |
| HT, cm | 170 ± 4 | 174 ± 3 | 169 ± 5 |
| BMI, kg/m² | 27.9 ± 2.0 | 29.2 ± 3.3 | 31.8 ± 2.8 |
| FBG, mg/dL | 85.7 ± 2.6 | 89.6 ± 3.3 | 162 ± 3 |
| mmol/L | 4.8 ± 0.1 | 5.0 ± 0.2 | 9.0 ± 0.2 |
| A1c, % | 5.4 ± 0.2 | 5.6 ± 0.2 | 8.5 ± 0.7 |
| (mmol/mol) | (36 ± 1) | (38 ± 1) | (69 ± 5) |
| Duration of DM, yrs | ---- | ---- | 14 ± 4 |

HG = high glucose (hyperglycemia), HI = high insulin (1mU/kg/min), HG/HI+L = hyperglycemia/hyperinsulinemia + lipid
Table 2 – Effects of insulin and lipid induced insulin resistance on differentially expressed UPR mRNA

| Gene Bank | Symbol   | Description                                      | HG+ HI (Study #8) increase over basal | HG + HI + Lipid (Study #9) |
|-----------|----------|--------------------------------------------------|--------------------------------------|---------------------------|
|           |          | **UP binding protein folding**                   |                                      |                           |
| NM014278  | HSPA4L   | Heat shock 70kDa protein 4-like T-complex 1      | > 1.5 fold                           | 1.15                      |
| NM030752  | T-CP1    | Glucosidase, alpha; neutral AB Peptidyl/prolyl Isomerase A | 1.77                                 | 1.11                      |
| NM198334  | GANAB    |                                                  | 1.71                                 | 0.77                      |
| NM021130  | PPIA     |                                                  | 1.58                                 | 1.15                      |
| NM013247  | HTRA2    | Htr A serine Peptidase 2 Erol-like Beta          | 1.52                                 | 1.04                      |
| NM019891  | EROILB   |                                                  | 1.53                                 | 1.18                      |
|           |          | **Translation Regulation**                       |                                      |                           |
| NM032025  | eIF2α    | Eukaryotic translation initiation factor 2A      | 1.78                                 | 1.47                      |
| NM004836  | eIF2αK3  | Eukaryotic translation initiation factor-2α kinase 3 PERK | 1.58                                 | 1.22                      |
| NM14330   | PPP1R15A | Protein phosphatase 1, regulatory subunit 15A GADD 34 | 1.55                                 | 0.61                      |
|           |          | **ER associated destruction (ERAD)**             |                                      |                           |
| NM153692  | HTRA4    | HtrA serine peptidase 4 Homocysteine-inducible ER stress inducible ubiquitin-like domain member-1 | 4.12                                 | 1.31                      |
| NM014685  | HERPUD1  |                                                   | 1.67                                 | 1.23                      |
| NM013247  | HTRA2    | HtrA serine peptidase 2                          | 1.52                                 | 1.04                      |
| NM001433  | ERN1     | ER to nucleus signaling 1, IRE1                  | 2.50                                 | 0.38                      |
| NM005080  | XBP1     | X-box binding protein 1                          | 2.22                                 | 1.29                      |
| NM004083  | DDIT3    | DNA-damage-inducible transcript 3, CHOP          | 2.12                                 | 1.72                      |
| NM001675  | ATF4     | Activating transcription factor 4                | 1.76                                 | 1.23                      |
| NM004381  | ATF6B    | Activating transcription 6 beta                  | 1.68                                 | 0.76                      |
|           |          | **Heat shock proteins**                          |                                      |                           |
| NM005527  | HSP A1L  | Heat shock 70kDa protein 1-like                  | 2.50                                 | 0.52                      |
| NM006644  | HSP H1   | Heat shock 105 kDa protein 1                     | 1.78                                 | 1.02                      |
| NM006260  | DNAJC3   | Hsp40 homolog, sub family C, member 3           | 1.76                                 | 1.42                      |
|           |          |                                                 | mean 1.98 ± 0.14                     | 1.08 ± 0.07, p < 0.001    |
|           |          |                                                 | **decrease to < 0.25 of basal**      |                            |
| NM005194  | CEBPB    | CCAAT/enhancer binding protein beta, C/EBP beta | 0.20                                 | 1.34                      |
| NM025191  | EDEM3    | ER degradation enhancer mannosidase α-like 3    | 0.14                                 | 0.94                      |
| NM005346  | HSPA1B   | Heat shock 70 kDa protein-1 B                    | 0.41                                 | 0.94                      |
|           |          |                                                 | mean 0.25 ± 0.08                     | 1.07 ± 0.13, p < 0.02     |
Legend to Table 2

Subcutaneous upper thigh fat biopsies were obtained pre and post 8 h hyperglycemic-hyperinsulinemic clamps without (HG + HI) and with co-infusion of lipid (HG + HI + Lipid). Shown are post/pre mRNA ratios from pooled fat biopsies (n = 4) determined with a human UPR pathway array (SA Biosciences Co. Catalog Number (PAH-098Z)).
Fig 1

**A** Glucose, mg/dL

**B** Insulin, uU/mL

**C** FFA, umol/L

**D** GIR, mg/kg min

**E** IRS-1xPI3Kp85 mRNA ratios

**F** PDE3b mRNA ratios

**G** UPR mRNA ratios

- GRP78
- XBP-1s
- ATF4
- CHOP
- PDI-A3
- CRT
- CNX

8 hr high glu+high ins (n=6)
- high glu+high ins+lipid (n=7)

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Fig 2
Figure 1: Effects of lipid induced insulin resistance on UPR mRNA

Effects of 8 h hyperglycemic-hyperinsulinemic clamps with (closed symbols) or without lipid/heparin infusions (open symbols) on plasma glucose, insulin and FFA concentrations and glucose infusion rates needed to maintain hyperglycemia (GIR) (Figure 3 a-d), on post/pre ratios of IRS-1 associated PI3 kinase (p85) protein and post/pre mRNA of PDE3b, two insulin targets in adipose tissue (Figure 3 e + f) and 7 UPR post/pre mRNA ratios. Shown mean ± SE; * p < 0.05, ** p < 0.02, *** p < 0.01 compared to post/pre ratios of 1.0 (paired Student’s t-test).
Abbreviations: ATF-4 = activating transcription factor 4; Chop = C/EBP homologous protein; CNX = calnexin; CRT = calreticulin; GRP78 = glucose responsive protein 78; IRS1xPI3Kp85 = insulin receptor substrate 1 associated PI3 kinase 85 KD protein; PDE 3b = phosphodiesterase 3 b; PDI-A3 = protein disulfide-isomerase A3; XBP-1s = X box binding protein 1-s.

Figure 2: Effects of comparable hyperglycemia-hyperinsulinemia for 8 h on glucose infusion rates (GIR) and UPR mRNA in patients with T2DM and non-diabetic controls.

Insulin was infused at 2 mU/kg min in T2DM patients and in non-diabetic controls. In T2DM patients, glucose was clamped at isoglycemic levels (i.e., at their post-absorptive hyperglycemic levels). Shown are means ± SE pre-clamp and clamp (60-240 min) glucose, insulin and GIR values, basal UPR mRNA/18s and post/pre UPR mRNA/18s ratios, * p < 0.05 compared to a post/pre ratio of 1.0.