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

Diabetes 2014;63:2977–2983 | DOI: 10.2337/db14-0055

We recently showed 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 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-h hyperglycemic-hyperinsulinemic clamping in 13 normal subjects and in 6 chronically insulin-resistant patients with type 2 diabetes mellitus (T2DM). In normal subjects, hyperglycemia-hyperinsulinemia increased after/before 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 did not 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 suggests, but does not prove, that these two phenomena were causally related.

Endoplasmic reticulum (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 type 2 diabetes mellitus (T2DM), hypertension, atherogenic dyslipidemia, and nonalcoholic 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 recently showed 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 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 phosphoinositide 3-kinase (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 mitogen-activated protein kinase 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 examined effects of hyperinsulinemia on ER stress markers in

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Received 13 January 2014 and accepted 10 April 2014.

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subcutaneous adipose tissue of normal subjects in whom the metabolic/PI3K pathway was inhibited with lipid infusion and in subcutaneous adipose tissue of insulin-resistant patients with T2DM, in whom the metabolic/PI3K pathway is known to be inhibited.

RESEARCH DESIGN AND METHODS

Subjects and Studies

We studied 13 healthy subjects (9 male/4 female) and 6 patients (3 male/3 female) 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 h before admission. Body weight of all study volunteers was 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 ~8:00 A.M. after an overnight fast. The following three studies were performed.

Study 1: 8-Hour Hyperglycemic-Hyperinsulinemic (No Insulin Infusion) Clamps in Healthy Subjects (n = 6)

In this study, endogenous hyperinsulinemia was the result of infusions with 20% glucose i.v. (to produce glucose levels of ~200–250 mg/dL, ~11.1–13.9 mmol/L). Plasma electrolytes, body weight, and fluid balances were monitored

every 4 h. Potassium (20 mg) and magnesium (400 g) were given orally if needed.

Study 2: 8-Hour Hyperglycemic-Hyperinsulinemic (No Insulin Infusion) Clamps With Coinfusion of Lipid/Heparin in Healthy Subjects (n = 7)

Lipid/heparin infusions were started together with the glucose infusions at 0 h and consisted of intravenous infusion of Intralipid III, a 20% fat emulsion (1.5 mL/min; Abbott Laboratories, Chicago, IL) and heparin (a 200-unit bolus followed by infusion of 0.4 units/kg/min).

Study 3: 8-Hour 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 h, open subcutaneous fat biopsies were obtained from the lateral aspect of the upper thigh under local anesthesia by a surgeon as previously described (13).

RT-PCR

Total RNA was isolated from frozen adipose tissues, and real-time RT-PCR was performed with an SYBR Green One-Step qRT-PCR kit (cat. no. 75770; Affymetrix, Santa Clara, CA) and an Eppendorf Mastercycler ep realplex cyclor as previously described (4). Primers used were as follows: human GRP78 sense gttggtgctcgtactcgaat, antisense cgctacagcttcatctggg; human/mouse XBP1s sense ttgagaaccaggagtaa, antisense cctgcacgtctgctggact; human activating transcription factor 4 (ATF4) sense ccacgttgatgacac, antisense ggcttctatctctctcag; human CHOP sense ggagaaccaggaaacggaaac, antisense tcttcagctagctgtgccac; human phosphodiesterase 3b (PDE3B) sense gccacagatgttgctacatgc, antisense gacaggcagccataactctc; human protein disulfide-isomerase A3 (PDI-A3) sense ctgtggcatcatcttggt, antisense ggtgtggtcactgtaagaacct; human calreticulin sense accctgagtacaagggtgag, antisense agatggtgccagacttgacc; human calnexin sense cagaccagtggtatggagat, antisense gactgacagtgccacatct; and human/mouse 18S (cat. no. 5103G; Ambion). Triplicate samples were normalized with 18s or β -actin.

Immunoprecipitation

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 μ g).

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 previously described (4). The primary antibodies used were as follows: a rabbit antiserum (Upstate) that recognizes the N-SH₂ region of PI3K and the regulatory p85 subunit of PI3K.

Table 1—Studies and study subjects

	1 HG/HI	2 HG/HI+L	3 HG/HI
DM	No	No	Yes
Sex	4M/2F	5M/2F	3M/3F
Age, years	27 \pm 3	31 \pm 5	53 \pm 1
WT, kg	81.0 \pm 6.4	87.7 \pm 8.6	90.5 \pm 9.2
HT, cm	170 \pm 4	174 \pm 3	169 \pm 5
BMI, kg/m ²	27.9 \pm 2.0	29.2 \pm 3.3	31.8 \pm 2.8
FBG, mg/dL	85.7 \pm 2.6	89.6 \pm 3.3	162 \pm 3
FBG, mmol/L	4.8 \pm 0.1	5.0 \pm 0.2	9.0 \pm 0.2
A1C, %	5.4 \pm 0.2	5.6 \pm 0.2	8.5 \pm 0.7
A1C, mmol/mol	36 \pm 1	38 \pm 1	69 \pm 5
DM duration, years	—	—	14 \pm 4

DM, diabetes mellitus; F, female; FBG, fasting blood glucose; HG, high glucose (hyperglycemia); HG/HI+L, hyperglycemia/hyperinsulinemia plus lipid; HI, high insulin (1 mU/kg/min); HT, height; M, male; WT, weight.

Analytical Procedures

Plasma glucose was measured with a glucose analyzer (YSI, Yellow Springs, OH). Insulin was determined in serum by radioimmunoassay with a specific antibody that cross-reacts minimally (0.2%) with proinsulin (Linco, St. Charles, MO). Free fatty acids (FFAs) were measured in plasma, containing Paraoxon (Sigma-Aldrich, St. Louis, MO), a lipoprotein lipase inhibitor, with a kit from Wako Pure Chemical (Richmond, VA).

Statistical Analysis

All data are expressed as means ± SE. Pre- and postbiopsy values in Fig. 1E–G were compared using the two-tailed *t* test. Normality was tested with the Kolmogorov-Smirnov test. The Wilcoxon signed rank test was used to determine significance of the data that were not normally distributed. Two-way ANOVA was used in Fig. 1D 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 glucose infusion rate (GIR) across time, 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 (studies 1 and 2), (Table 1) resulted in similar degrees of hyperglycemia and hyperinsulinemia but different levels of plasma FFA (Fig. 1A–C). Lipid infusion resulted in systemic (Fig. 1D) and adipose

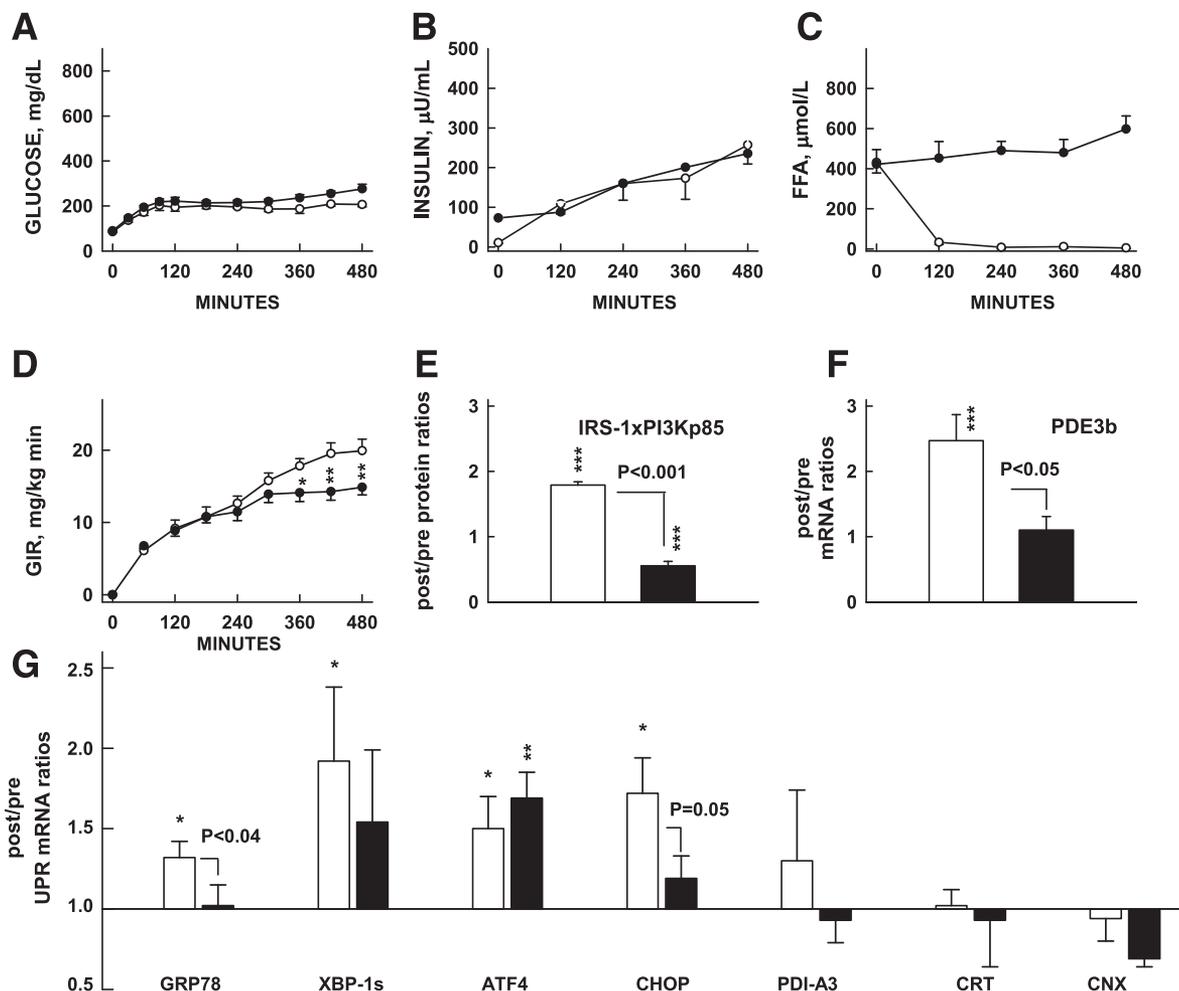


Figure 1—Effects of lipid-induced insulin resistance on UPR mRNA. Effects of 8-h hyperglycemic-hyperinsulinemic clamps with (closed symbols) or without (open symbols) lipid/heparin infusions on plasma glucose, insulin, and FFA concentrations and GIRs needed to maintain hyperglycemia (GIR) (A–D) on after/before ratios of IRS-1-associated PI3K (p85) protein and after/before mRNA of PDE3b, two insulin targets in adipose tissue (E and F) and seven UPR after/before mRNA ratios (G). Shown are means ± SE; **P* < 0.05, ***P* < 0.02, ****P* < 0.01 compared with before/after ratios of 1.0 (paired Student *t* test). CNX, calnexin; CRT, calreticulin; IRS1xPI3Kp85, IRS1-associated PI3K 85 KD protein; PDI-A3, protein disulfide-isomerase A3.

tissue insulin resistance (inhibition of insulin-stimulated PI3K activation [Fig. 1E] and PDE3B mRNA [Fig. 1F]).

Hyperglycemic-hyperinsulinemia increased after/before mRNA ratios of GRP78, XBP1s, ATF4, and CHOP.

Hyperglycemic-hyperinsulinemia with lipid infusion-induced insulin resistance was associated with significantly reduced insulin-mediated increases in after/before mRNA ratios of GRP78 and Chop (Fig. 1G).

Table 2—Effects of insulin and lipid-induced insulin resistance on differentially expressed UPR mRNA

Gene bank accession no.	Symbol	Description	HG+HI (study 1) increase over basal >1.5 fold	HG+HI+lipid (study 2)
UP binding, protein folding				
NM014278	HSPA4L	Heat shock 70kDa protein 4-like	2.95	1.15
NM030752	T-CP1	T-complex 1	1.77	1.11
NM198334	GANAB	Glucosidase, α ; neutral AB	1.71	0.77
NM021130	PPIA	Peptidylprolyl isomerase A	1.58	1.15
NM013247	HTRA2	Htr A serine peptidase 2	1.52	1.04
NM019891	EROILB	Erol-like β	1.53	1.18
Translation regulation				
NM032025	eIF2 α	Eukaryotic translation initiation factor 2A	1.78	1.47
NM004836	eIF2 α K3	Eukaryotic translation initiation factor-2 α 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	4.12	1.31
NM014685	HERPUD1	Homocysteine-inducible ER stress-inducible ubiquitine-like domain member-1	1.67	1.23
NM013247	HTRA2	HtrA serine peptidase 2	1.52	1.04
Transcription factors				
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 β	1.68	0.76
HSP				
NM005527	HSP AIL	Heat shock 70 kDa protein 1-like	2.50	0.52
NM006644	HSP 1 ^H	Heat shock 105 kDa protein 1	1.78	1.02
NM006260	DNAJC3	Hsp40 homolog, subfamily C, member 3	1.76	1.42
			(mean 1.98 \pm 0.14, decrease to <0.25 of basal)	(mean 1.08 \pm 0.07; $P < 0.001$)
NM005194	CEBPB	CCAAT/enhancer-binding protein β , C/EBP β	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 \pm 0.08)	(mean 1.07 \pm 0.13; $P < 0.02$)

Subcutaneous upper-thigh fat biopsies were obtained before and after 8-h hyperglycemic-hyperinsulinemic clamps without (HG+HI) and with (HG+HI+lipid) coinfusion of lipid. Shown are after/before mRNA ratios from pooled fat biopsies ($n = 4$) determined with a human UPR pathway array (cat. no. PAH-098Z; SABiosciences Co.).

UPR Pathway Array Confirms UPR mRNA Stimulation by Insulin and the Prevention of This Effect by Lipid Infusion

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, after/before mRNA ratios of 20 of the 84 UPR markers in the array rose >1.5-fold above basal, whereas 3 decreased to <75% of basal. Coinfusion 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 (Table 2).

Insulin Stimulation of the UPR Is Reduced in Chronically Insulin-Resistant Patients With T2DM

To examine effects of chronic insulin resistance on ER stress, we compared UPR responses with comparable hyperglycemic-hyperinsulinemia in six insulin-resistant patients

with T2DM with those in six healthy control subjects (study 1 vs. 3) (Table 1). Insulin-stimulated glucose uptakes (GIR) were significantly lower in T2DM patients than in control subjects, 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 control subjects 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 subcutaneous adipose tissue of patients with T2DM (Fig. 2).

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.

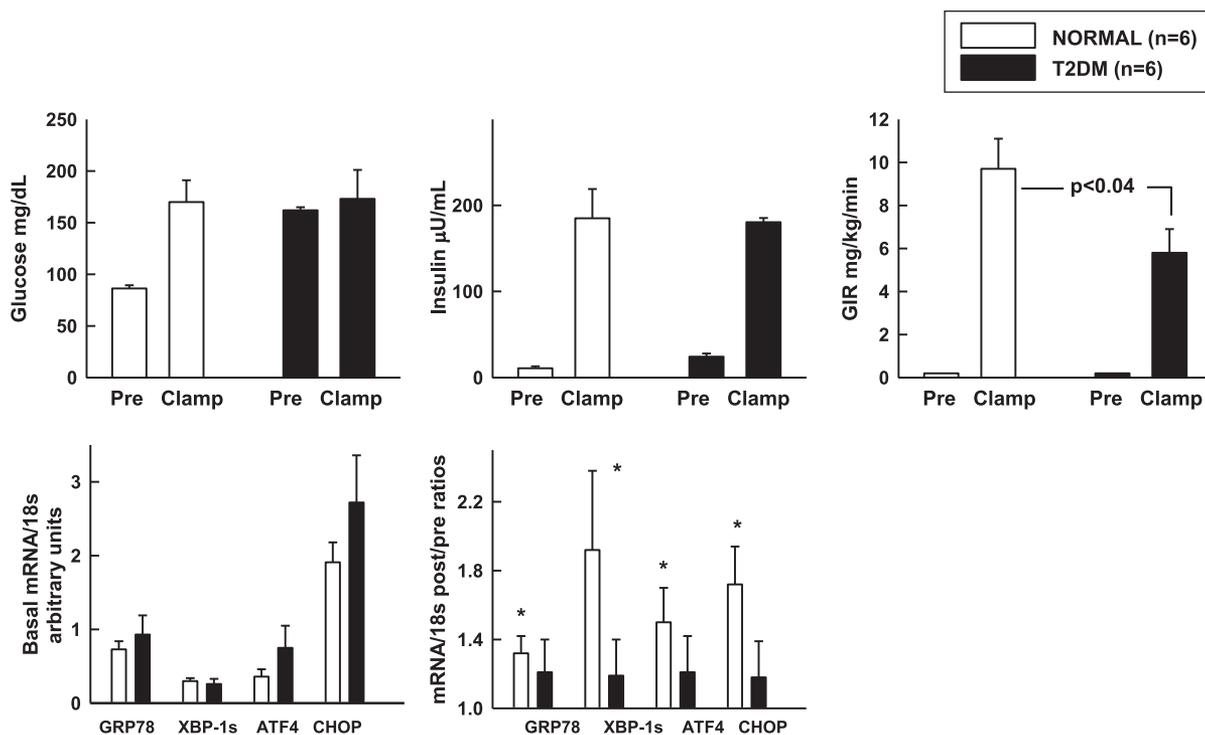


Figure 2—Effects of comparable hyperglycemia hyperinsulinemia for 8 h on GIR and UPR mRNA in patients with T2DM and nondiabetic control subjects. Insulin was infused at 2 mU/kg/min in T2DM patients and in nondiabetic control subjects. In T2DM patients, glucose was clamped at isoglycemic levels (i.e., at their postabsorptive hyperglycemic levels). Shown are means ± SE preclamp and clamp (60–240 min) glucose, insulin, and GIR values. Basal UPR mRNA/18s and after/before UPR mRNA/18s ratios, **P* < 0.05 compared with an after/before ratio of 1.0.

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 preadipocytes (22–24). 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 posttranslational 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 (26–29). Nevertheless, even though insulin resistance may be an appropriate and adaptive cellular response, it will become ineffective and overall maladaptive if its cause, for instance, excess caloric intake, continues and the nutrients that cannot enter the cells accumulate extracellularly.

In summary, we showed 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 suggest, but do 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 nondiabetic and diabetic subjects and could be an adaptive defense against further increase in ER stress.

Acknowledgments. The authors thank Maria Mozzoli and Yan Chen for excellent technical assistance and Constance Harris Crews for typing the manuscript.

Funding. This work was supported by grants from the National Institutes of Health (R01-DK58895 and DK090588) and a grant from the American Diabetes Association (1-10-CT-06) (all to G.B.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. G.B. designed the study and wrote the manuscript. P.C. and C.H. contributed and researched data. K.K., B.P., and L.F.

contributed data and contributed to the discussion. G.B. 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.

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