Repression of GLUT4 expression by the endoplasmic reticulum stress response in 3T3-L1 adipocytes

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Abstract

Expression of GLUT4 is decreased in adipocytes in obesity and type 2 diabetes, contributing to the insulin resistance of these states. Recent investigations suggest a role for activation of the ER stress response in the pathophysiology of type 2 diabetes. We investigated activation of the ER stress response in 3T3-L1 adipocytes. We show that activation of the ER stress response decreased GLUT4 expression at the level of gene transcription. Activation of the ER stress response also increased the expression of CHOP10, an inhibitor of the activity and expression of C/EBPα. As expected, activation of the ER stress response decreased expression of C/EBPα, an activator of GLUT4 expression, providing a mechanism to account for the repression of GLUT4 by ER stress activation. Our studies identify repression of GLUT4 expression as another potential mechanism for obesity-induced activation of the ER stress response to contribute to the insulin resistance of obesity.
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GLUT4 is the major insulin-responsive glucose transporter, and is primarily expressed in adipose tissue, skeletal muscle, and cardiac muscle [1,2]. Insulin binding to its receptor initiates a cascade of events resulting in translocation of GLUT4 from an intracellular compartment and insertion into the plasma membrane. The insulin resistance of type 2 diabetes mellitus includes a blunting of the insulin-stimulated increase in glucose uptake into fat and muscle. In muscle, this is due to a defective stimulation of GLUT4 translocation [2,3]. In adipocytes, the decreased insulin sensitivity is due to both this defect in insulin-stimulated translocation, as well as to decreased expression of GLUT4 [2–4].

GLUT4 protein and mRNA levels are decreased in adipocytes in human obesity and type 2 diabetes, as well as in numerous rodent models of insulin resistance [3]. Although adipose tissue accounts for only a small fraction of glucose disposal after a meal, decreased GLUT4 expression in adipocytes can affect glucose homeostasis [4]. Mice lacking GLUT4 in adipocytes display insulin resistance in muscle and liver, resulting in abnormal glucose control and overall impaired insulin sensitivity [5]. In addition, when GLUT4 expression is selectively increased in adipose tissue of mice, the animals show increased insulin sensitivity and enhanced glucose tolerance [6].

A number of transcription factors that regulate GLUT4 expression have been identified, each of which could play a role in the repression of GLUT4 expression in adipocytes in insulin resistant states [1,7–10]. MEF2, C/EBP, KLF15, LXR, and GEF have all been characterized as activators of GLUT4 expression. O/E and NF1 proteins have not been directly demonstrated to either activate or repress GLUT4 expression, but we have demonstrated that they participate in the down-regulation of GLUT4 expression in 3T3-L1 adipocytes when these cells are treated with insulin or cyclic-AMP analogs.

In our prior investigations, we found that proteasome inhibition represses GLUT4 expression in 3T3-L1

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adipocytes [11]. The ability of proteasome inhibition to repress GLUT4 expression at the level of transcription suggested that such treatments alter the expression of transcription factors that regulate GLUT4 expression. Therefore, we examined changes in expression of these transcription factors. Through these investigations, we have found that activation of the endoplasmic reticulum (ER) stress response decreases GLUT4 expression in 3T3-L1 adipocytes, and that this repression is likely mediated through the well-described increase in expression of CHOP10 that occurs with activation of the ER stress response.

Materials and methods

Cell culture. 3T3-L1 cells were maintained in culture as previously described [11]. 3T3-L1 adipocytes were treated with proteasome inhibitors or vehicle beginning 8–10 days after differentiation was induced. Actinomycin D, cycloheximide, lactacystin, thapsigargin, tunicamycin, and MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) were obtained from Sigma (St. Louis, MO). They were dissolved in dimethylsulfoxide (DMSO, Sigma) with the final concentration of DMSO that cells were exposed to being no greater than 0.2%.

Western immunoblot analysis. Nuclei from three identically-treated 10-cm dishes were harvested together as previously described [12], with nuclear proteins extracted in NUN buffer (0.3 M NaCl, 1 M urea, 1% Igepal CA-630, 25 mM Hepes (pH 7.9), and 1 mM dithiothreitol) and stored at −80 °C in NUN buffer with 10% glycerol. Protein was quantitated by the Bradford assay (Biorad, Hercules, CA). Western immunoblot was performed as previously described [13]. Antibodies to the NF1 isoforms were prepared by inoculating rabbits with KLH-conjugated isoform-specific polypeptides (A: ADIKDQPENGHLC; B: RTWHERDQDM; C: GSDQEDSKPITLC, and X: CNQQGDA-DIKPLP; Covance, Denver, PA and Sigma Genosys, The Woodlands, TX). PPARγ antibody was provided by Dr. Mitchell Lazar (University of Pennsylvania) and C/EBPα antibody was provided by Dr. M. Daniel Lane (Johns Hopkins University). The remaining antibodies were obtained from commercial sources: GLUT4: Chemicon Int., Temecula, CA; MeF2A, LXR, and CHOP10: Santa Cruz Biotechnology, Inc., Santa Cruz, CA; MeF2D: BD Bioscience, San Jose, CA. The Western immunoblots were developed with peroxidase-conjugated antibody (Sigma) and an enhanced chemiluminescent reagent (Amersham, Piscataway, NJ).

Nuclear run-on assay. Nuclear run-on assays were performed using the quantitative RT-PCR-based assay described by Patrone et al. [14]. Three 100-mm dishes of control or treated 3T3-L1 adipocytes were pooled and nuclei were isolated by lysis with Igepal CA-630 [15]. The DNA content of the nuclei was quantitated by lysing an aliquot in 1% SDS, 40 mM Tris, pH 8.0 and measuring the UV absorbance at 260 nm. Nascent RNA transcripts from aliquots of nuclei with equivalent DNA content were extended in the presence of biotin-16-UTP. RNA was purified using Trizol (Invitrogen, Carlsbad, CA), and the extended RNA was isolated using streptavidin-coupled Dynabeads M-280 and a magnetic particle concentrator (Invitrogen, Calsbad, CA.). RNA levels were quantitated by real-time RT-PCR from equal aliquots of purified RNA. Run-on expression levels were calculated after normalization with run-on 18 S expression.

Quantitative RT-PCR. Total RNA was isolated from cells using the Nucleospin II Kit (Clonetech, Mountain View, CA.). cDNA was synthesized and then amplified using the Brilliant SYBR Green QRT-PCR Master Mix Kit, 1-Step (Stratagene, La Jolla, CA) and template-specific primers. Forty nanograms of total RNA per reaction was used to quantitate all templates except 18S RNA, where 160 pg per reaction was used. Primer concentration was 200 nM for GLUT4, 100 nM for all other reactions. The RT reaction was at 50 °C for 30 min., inactivation at 95 °C for 15 min; the PCR protocol was denaturation at 94 °C for 15 s, annealing at the appropriate temperature (Supplemental Table 1) for 30 s,

extension at 72 °C for 30 s During assay development, RT-PCR reaction products were separated on an agarose gel to verify product size. All reactions included a dissociation curve analysis at the end of the amplification to confirm a single product at the expected melting temperature. Quantitative real-time PCR reactions were run on the Stratagene MX4000 quantitative PCR system. Relative quantitation of gene expression was performed using the threshold cycle (C_t) and a standard curve for each reaction, as described by the manufacturer. Changes in mRNA expression level were calculated after normalization with 18S expression. Primers used for the QRT-PCR reactions are given in Supplemental Table 1.

Results and discussion

Proteasome inhibition increases CHOP10 protein levels

We had previously demonstrated that proteasome inhibition in 3T3-L1 adipocytes decreased expression of GLUT4 at the level of transcription. To explore the mechanism of this regulation, we used Western immunoblot analysis to investigate the effect of proteasome inhibition on the level of known regulators of GLUT4 expression. There was no significant change in the protein level of LXR, PPARγ, MEF2D or the A, B, and X isoforms of NF1 in 3T3-L1 adipocytes treated with the proteasome inhibitor MG132 for 24 h (Supplementary Fig. 1). Immunoblots for PGC-1, GEF, O/E-1 (EBF-1), and KLF-15 did not give strong predominating signals at the expected size for the protein, suggesting low level of expression of these proteins in 3T3-L1 adipocytes; however, there was no apparent change in the level of expression of these proteins with proteasome inhibition. NF1-C protein expression was decreased by approximately 50% after 24 h of proteasome inhibition ($P < 0.05$, Supplementary Fig. 1.). Western immunoblot using MEF2A antibody detected multiple proteins in 3T3-L1 adipocytes migrating between 56 and 65 kDa. Treatment with MG132 for 6 or 24 h resulted in a change in the pattern of proteins detected: there was a predominance of a faster migrating isoform in control cells, while there was a predominance of a slower migrating isoform after proteasome inhibition (Supplementary Fig. 2). These data suggest that a change in MEF2 isoform expression or in the protein level of NF1-C may play a role in regulating GLUT4 expression in response to proteasome inhibition. These possibilities were not further investigated in these studies, but warrant future investigation.

C/EBP proteins regulate numerous adipocyte genes, including GLUT4; C/EBPα is the predominant family member in mature adipocytes. Expression of C/EBPα protein is markedly decreased by proteasome inhibition by either MG132 or lactacystin in 3T3-L1 adipocytes (Fig. 1). As C/EBP proteins are known to activate expression of GLUT4, this marked repression of C/EBPα could be a significant contributor to the decreased expression of GLUT4 that occurs with proteasome inhibition. However, in our prior studies, and confirmed here (Fig. 2), GLUT4 mRNA level was decreased after 6 h of proteasome inhibition, and GLUT4 transcription was decreased after 3 h
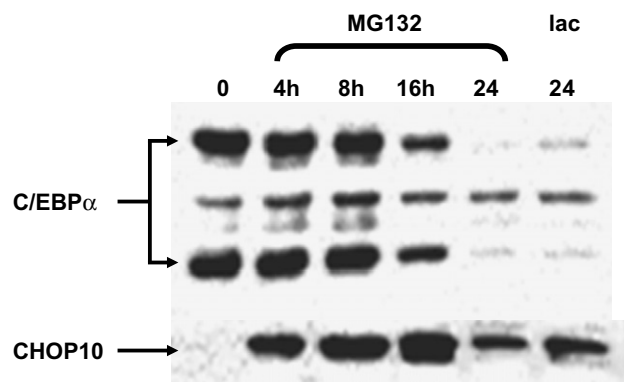
Fig. 1. Proteasome inhibition represses C/EBPα expression and increases CHOP10 expression in 3T3-L1 adipocytes. Nuclear proteins were extracted from 3T3-L1 adipocytes treated with 10 μM MG132 or 10 μM lactacystin (lac), for the indicated times. Western immunoblot was performed using antibodies to C/EBPα and CHOP10. Three independent experiments were performed; a representative result is shown.
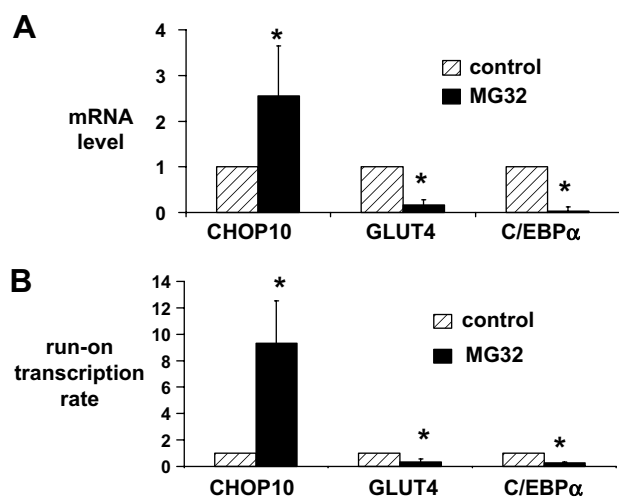

Fig. 2. Proteasome inhibition induces expression of the CHOP10 gene, and represses expression of the GLUT4 and C/EBPα genes in 3T3-L1 adipocytes. (A) Total RNA was isolated from 3T3-L1 adipocytes treated with 10 μM MG132 or vehicle (control) for 6 h. Quantitative real-time RT-PCR was used to quantitate CHOP10, GLUT4, and C/EBPα mRNA levels. mRNA levels are expressed relative to levels in vehicle-treated cells, after controlling for 18S RNA expression level. (B) Nuclei were isolated from 3T3-L1 adipocytes treated with 10 μM MG132 or vehicle (control) for 3 h. Run-on transcription was performed in the presence of biotin-16-UTP, and newly formed transcripts were purified using streptavidin-coupled Dynabeads M-280. Quantitative real-time RT-PCR was used to quantitate CHOP10, GLUT4, and C/EBPα transcript levels, which are expressed relative to levels in vehicle-treated cells, after controlling for 18S RNA expression level. Results represent the mean of 5 (A) or 3 (B) independent experiments; error bars indicate one standard deviation. *$P < 0.05$ vs. control.

hours of proteasome inhibition, while C/EBPα protein levels showed no decrease at 8 h (Fig. 1). Thus, another mechanism must account for the early decrease in GLUT4 expression.

CHOP10, (C/EBP-homologous protein 10; also called gadd153—growth arrest and DNA damage inducible 153)

is another member of the C/EBP family, having a high degree of sequence similarity to C/EBP proteins in the basic leucine zipper region, allowing it to heterodimerize with other C/EBP members. However, CHOP10 lacks an amino-terminal transactivation domain, and contains two prolines in the place of basic residues in the DNA binding domain—because of this, heterodimers of CHOP10 with C/EBP proteins are unable to bind to DNA, so that CHOP10 acts as an endogenous inhibitor of C/EBP gene activation [16]. Therefore, we considered whether the repression of GLUT4 expression occurring with proteasome inhibition might be due to an increase in CHOP10 expression. As seen in Fig. 1, by 4 h (the earliest time point examined) there is a dramatic increase in CHOP10 protein level in 3T3-L1 adipocytes treated with proteasome inhibitor. Because C/EBPα activates its own expression, the increased expression of CHOP10 can decrease GLUT4 expression by two mechanisms: initially CHOP10 can decrease GLUT4 expression by forming heterodimers with C/EBPα, blocking activation of the GLUT4 gene by C/EBPα; subsequently, the effect of CHOP10 to decrease C/EBPα expression by inhibiting C/EBPα activation of its own gene results in decreased quantities of C/EBPα protein available to activate the GLUT4 gene.

Proteasome inhibition increases CHOP10 gene transcription

The increase in CHOP10 protein level could be a direct effect of proteasome inhibition decreasing the rate of its degradation. However, there was no increase in CHOP10 protein expression with proteasome inhibition if new protein synthesis was simultaneously inhibited with cycloheximide (Fig. 3). In addition, proteasome inhibition increased CHOP10 mRNA level 2.5-fold at 6 h and increased CHOP10 transcription 9.3-fold at 3 h (Fig. 2). When 3T3-L1 adipocytes were treated with the RNA synthesis inhibitor actinomycin D to block the increase in CHOP10 mRNA, there was a marked attenuation in the increase in CHOP10 protein level with proteasome inhibition (Fig. 3). Thus, proteasome inhibition increases CHOP10 protein levels through an increased expression of the CHOP10 gene, rather than simply through decreasing its rate of degradation.
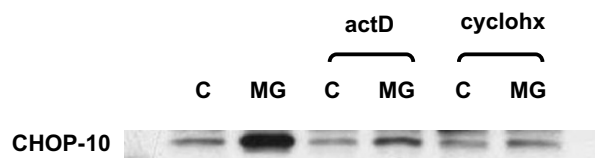

Fig. 3. Induction of CHOP10 in 3T3-L1 adipocytes by proteasome inhibition is attenuated by inhibition of either new RNA or new protein synthesis. Nuclear proteins were extracted from 3T3-L1 adipocytes treated for 24 h with 10 μM MG132 (MG) or vehicle (control, C) in the presence or absence of 5 μg/ml actinomycin D (actD) or 10 μg/ml cycloheximide (cyclohx). Western immunoblot was performed using CHOP10 antibody. Three independent experiments were performed; a representative result is shown.

*Activation of the endoplasmic reticulum stress response
represses GLUT4 expression*

After finding evidence that proteasome inhibition
represses GLUT4 expression in 3T3-L1 adipocytes through
activation of CHOP10 expression, we wished to investigate
possible mechanisms for this regulation to act *in vivo*. The
endoplasmic reticulum (ER) stress response, also called the
unfolded protein response (UPR), is a coordinated
response to an accumulation of misfolded proteins in the
endoplasmic reticulum [17,18]. This process helps to dimin-
ish the accumulation of misfolded proteins by: (1) decreas-
ing the overall rate of protein translation leading to a
decrease in the amount of protein delivered to the ER;
(2) increasing the capacity for correct folding of proteins
in the ER by increasing the protein levels of chaperone pro-
teins and other proteins involved in the folding process
(through an increase in protein translation of specific pro-
teins as well as increased gene expression of specific tar-
gets); and (3) increasing the elimination of misfolded
proteins by increasing their degradation in the proteasome.
Proteasome inhibition, by decreasing the degradation of
misfolded proteins, activates the ER stress response [18].
In addition, CHOP10 is one of the genes whose expression
is increased by the ER stress response [19,20]. Thus, we
were interested in determining whether activation of the
ER stress response might be responsible for the repression
of GLUT4 that we observed in 3T3-L1 adipocytes treated
with proteasome inhibitors.

We first investigated whether treatment with MG132
activated the ER stress response in 3T3-L1 adipocytes.
BiP is the major chaperone protein of the ER lumen. Its
expression is increased with activation of the ER stress
response [21,22]. BiP mRNA expression, determined by
quantitative real-time RT-PCR, was increased 20.9-fold
in 3T3-L1 adipocytes treated with 10 μM MG132. Thus,
treatment of 3T3-L1 adipocytes with MG132 at a concen-
tration that represses GLUT4 expression activates the ER
stress response.

We next investigated whether activation of the ER
stress response itself would repress GLUT4 expression.
To do this, we treated cells with compounds that activate
the ER stress response through mechanisms other than
proteasome inhibition. 3T3-L1 cells were treated with
the glycosylation inhibitor tunicamycin, or with thapsi-
gargin, which induces ER calcium release; both are
potent inducers of the ER stress response [21]. BiP
mRNA was increased 17.9-fold when 3T3-L1 adipocytes
were treated for 6 h with 2.5 μg/ml tunicamycin and was
increased 21.6-fold when the cells were treated for 6 h
with 2.5 μM thapsigargin, confirming activation of the
ER stress response by these compounds in 3T3-L1 adipo-
cytes. Similar to the response with the proteasome inhib-
itor MG132, activation of the ER stress response with
either tunicamycin or thapsigargin decreased GLUT4
expression ($P < 0.05$; Fig. 4). And just as was seen with
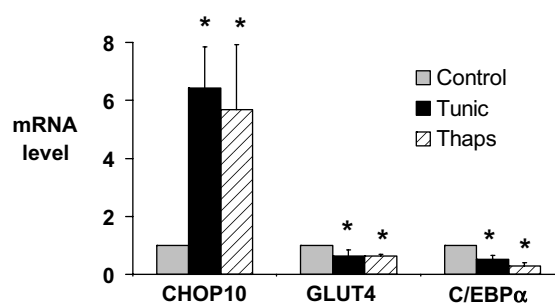proteasome inhibition, these activators of ER stress also

Fig. 4. Activation of the ER stress response in 3T3-L1 adipocytes
increases expression of CHOP10, and represses expression of GLUT4 and
C/EBPα. Total RNA was purified from 3T3-L1 adipocytes treated with
2.5 μg/ml tunicamycin (Tunic), 2.5 μM thapsigargin (Thaps), or vehicle
(Control) for 6 h. Quantitative real-time RT-PCR was used to quantitate
CHOP10, GLUT4, and C/EBPα mRNA levels. mRNA levels are
expressed relative to levels in vehicle-treated cells, after controlling for
18S RNA expression level. Results represent the mean of 4 independent
experiments; error bars indicate one standard deviation. $^*P < 0.05$ vs.
control.

increased CHOP10 expression, and decreased C/EBPα
expression ($P < 0.05$ for both; Fig. 4).

Recent investigations have demonstrated a potential
role of the ER stress response in type 2 diabetes. These
studies have identified mechanisms through which the ER
stress response could contribute to β-cell dysfunction
[23,24], as well as demonstrating involvement of the ER
stress response in insulin resistance [25]. Most significant
to the studies reported here, Özcan et al. demonstrated that
the ER stress response is activated in liver and adipose tis-
sue of obese, insulin resistant mice [25]. They found that
that the ER stress response activates JNK1—which partic-
ipates in insulin resistance by phosphorylating IRS-1 on
serine residues, leading to impaired insulin signaling [25].
They also found that an intact ER stress response was nec-
essary to limit obesity-induced insulin resistance, as mice
with a depletion of the ER stress response signaling protein
XBP-1 had an increased susceptibility to insulin resistance
induced by a high-fat diet. More support for the role of the
ER stress response in insulin resistance comes from studies
of ORP 150, a molecular chaperone that protects cells from
ER stress. Nakatani et al. found that over-expression of
ORP150 in mouse liver improves insulin sensitivity, while
depletion of ORP150 worsens insulin resistance [26].
Finally, Özcan et al. demonstrated that insulin resistance
and hyperglycemia can be alleviated in mice treated with
agents that decrease ER stress [27].

While the ER stress response helps to eliminate the accu-
mulation of misfolded proteins, prolonged or excessive ER
stress is linked to the development of programmed cell
death. This is a potential mechanism connecting ER stress
with β-cell dysfunction in type 2 diabetes, as there is evi-
dence that ER stress contributes to the free fatty acid-
induced apoptosis of β-cells [23]. Although the mechanisms
linking ER stress and apoptosis are incompletely under-
stood, the induction of CHOP10 plays an important role,
including ER stress-induced apoptosis of β-cells [28].

However, since diet-induced obesity activates the ER stress response in adipocytes [25] without increasing adipocyte apoptosis, activation of the ER stress response in this case does not appear to activate the apoptosis pathway.

We have found that activation of the ER stress response represses GLUT4 expression in 3T3-L1 adipocytes. Combined with the evidence that the ER stress response is activated in adipocytes in diet-induced obesity [25], these findings indicate that the activation of the ER stress response may be a significant contributor to the repression of GLUT4 expression in adipocytes in insulin resistant states. In addition these studies suggest that repression of GLUT4 expression in adipocytes is an additional mechanism through which activation of the ER stress response induces insulin resistance in obesity.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007. 07.176.

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