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ARTICLE

Glucosamine-induced endoplasmic reticulum stress affects *GLUT4* expression via activating transcription factor 6 in rat and human skeletal muscle cells

G. A. Raciti · C. Iadicicco · L. Ulianich · B. F. Vind · M. Gaster · F. Andreozzi · M. Longo · R. Teperino · P. Ungaro · B. Di Jeso · P. Formisano · F. Beguinot · C. Miele

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Abstract
Aims/hypothesis Glucosamine, generated during hyperglycaemia, causes insulin resistance in different cells. Here we sought to evaluate the possible role of endoplasmic reticulum (ER) stress in the induction of insulin resistance by glucosamine in skeletal muscle cells.
Methods Real-time RT-PCR analysis, 2-deoxy-D-glucose (2-DG) uptake and western blot analysis were carried out in rat and human muscle cell lines.
Results In both rat and human myotubes, glucosamine treatment caused a significant increase in the expression of the ER stress markers immunoglobulin heavy chain-binding protein/glucose-regulated protein 78 kDa (*BIP/GRP78* [also known as *HSPA5*]), X-box binding protein-1 (*XBP1*) and activating transcription factor 6 (*ATF6*). In addition, glucosamine impaired insulin-stimulated 2-DG uptake in both rat and human myotubes. Interestingly, pretreatment of both rat and human myotubes with the chemical chaperones 4-phenylbutyric acid (PBA) or tauroursodeoxycholic acid (TUDCA), completely prevented the effect of glucosamine on both ER stress induction and insulin-induced glucose uptake. In both rat and human myotubes, glucosamine treatment reduced mRNA and protein levels of the gene encoding GLUT4 and mRNA levels of the main regulators of the gene encoding GLUT4 (myocyte enhancer factor 2 a [*MEF2A*] and peroxisome proliferator-activated receptor-γ coactivator 1α [*PGC1α*]). Again, PBA or TUDCA pretreatment prevented glucosamine-induced inhibition of *GLUT4* (also known as *SLC2A4*), *MEF2A* and *PGC1α* (also known as *PPARGC1A*). Finally, we showed that overproduction of ATF6 is sufficient to inhibit the expression of genes *GLUT4*, *MEF2A* and *PGC1α* and that ATF6 silencing with a specific small interfering RNA is sufficient to completely prevent glucosamine-induced inhi-

G. A. Raciti and C. Iadicicco contributed equally to this study.

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G. A. Raciti · C. Iadicicco · L. Ulianich · M. Longo · R. Teperino · P. Ungaro · P. Formisano · F. Beguinot · C. Miele
Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli Federico II,
Via Sergio Pansini, 5,
Naples 80131, Italy

G. A. Raciti · C. Iadicicco · L. Ulianich · M. Longo · R. Teperino · P. Ungaro · P. Formisano · F. Beguinot · C. Miele (✉)
Istituto di Endocrinologia ed Oncologia Sperimentale del CNR,
Via Sergio Pansini, 5,
Naples 80131, Italy
e-mail: c.miele@ieos.cnr.it

B. F. Vind · M. Gaster
KMEB, Department of Endocrinology,
Odense University Hospital,
Odense, Denmark

F. Andreozzi
Dipartimento di Medicina Sperimentale e Clinica,
Università di Catanzaro Magna Graecia,
Catanzaro, Italy

B. Di Jeso
Dipartimento di Scienze e Tecnologie Biologiche e Ambientali,
Università degli Studi di Lecce,
Lecce, Italy

bition of *GLUT4*, *MEF2A* and *PGC1α* in skeletal muscle cells.

Conclusions/interpretation In this work we show that glucosamine-induced ER stress causes insulin resistance in both human and rat myotubes and impairs GLUT4 production and insulin-induced glucose uptake via an ATF6-dependent decrease of the GLUT4 regulators *MEF2A* and *PGC1α*.

Keywords ER stress · Glucosamine · Insulin resistance · Skeletal muscle

Abbreviations

2-DG	2-Deoxy-D-glucose
ATF6	Activating transcription factor 6
BIP/GRP78	Immunoglobulin heavy chain-binding protein/glucose-regulated protein 78 kDa
ChIP	Chromatin immunoprecipitation assay
eIF2α	Eukaryotic translation initiation factor 2α
ER	Endoplasmic reticulum
GEF	GLUT4 enhancer factor
GlcN	Glucosamine
GFAT	Glutamine:fructose-6-phosphate amidotransferase
HBP	Hexosamine biosynthetic pathway
HG	High glucose
MEF2A	Myocyte enhancer factor 2 a
MnTBAP	Mn(III)tetrakis(4-benzoic acid) porphyrin chloride
NAC	*N*-Acetyl-cysteine
PBA	4-Phenylbutyric acid
PERK	Double-stranded RNA-activated protein kinase-like ER kinase
PGC1α	Peroxisome proliferator-activated receptor-γ coactivator 1 α
PUGNAc	*O*-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino)*N*-phenylcarbamate
SHP	Orphan nuclear receptor small heterodimer partner
siRNA	Small interfering RNA
Thap	Thapsigargin
TUDCA	Tauroursodeoxycholic acid
UPR	Unfolded protein response
XBP-1s	X-box binding protein-1, spliced active form
XBP-1t	X-box binding protein-1, total form

Introduction

The endoplasmic reticulum (ER) is the principal site of protein synthesis, and together with the Golgi apparatus it facilitates transport and release of correctly folded proteins. Under conditions of cellular stress leading to an impairment of ER function, proteins are unable to fold properly and accumulate in the ER lumen. It is because of these unfolded or misfolded proteins that the ER has evolved a coping system known as the unfolded protein response (UPR) [1, 2].

Cellular stresses that may elicit UPR activation include glucose and energy deprivation, increased protein synthesis, inhibition of protein glycosylation and imbalance of ER calcium levels [3, 4]. In mammalian cells, at least four functionally distinct responses have been identified and three ER-resident transmembrane proteins have been described as primary sensors and transducers of the UPR: the double-stranded RNA-activated protein kinase-like ER kinase (PERK), inositol requiring-1, and activating transcription factor 6 (ATF6) [5–7]. The first response, mediated by PERK, is translational attenuation, to reduce the load of new protein synthesis and prevent further accumulation of unfolded proteins [8]. The second response is upregulation of genes encoding ER chaperone proteins such as the immunoglobulin heavy chain-binding protein/glucose-regulated protein 78 kDa (BIP/GRP78) and the glucose-regulated protein 94 kDa, to increase the ER protein-folding capacity [7, 9]. The third response is transcriptional activation of genes involved in the degradation of misfolded protein in the ER by the ubiquitin–proteasome system, called ER-associated degradation [10]. The fourth response is apoptosis, which occurs when severe and prolonged ER stress impairs ER functions, to protect the organism by eliminating the damaged cells [4]. ER stress plays an important role in several human diseases, including type 2 diabetes; indeed, recent studies reported that ER stress is involved in both pancreatic beta cell dysfunction [11–13] and peripheral insulin resistance [14, 15].

While the consequences of ER stress have been widely studied in adipose tissue and liver, ER stress in skeletal muscle, the major site of glucose disposal, has not received equal attention. The hexosamine biosynthetic pathway (HBP) is a minor glucose metabolic pathway that metabolises ~3% of glucose entering the cell, and the final product of this pathway, UDP-*N*-acetylglucosamine, as other nucleotide hexosamines, is used in the ER as substrate for protein glycosylation [16, 17]. Although quantitatively using a small fraction of glucose, HBP is an important contributor to the insulin-resistant state. Several studies have shown, indeed, that chronic exposure to glucosamine (GlcN), a precursor of the HBP, impairs insulin responsiveness, thus contributing to the formation of an insulin-resistant state in cultured human skeletal muscle cells and rat adipocytes [17] as well as in vivo [18]. However, the precise mechanisms by which GlcN induces insulin resistance have not been conclusively established in these studies.

Methods

Materials DMEM, FBS, FCS, L-glutamine and BSA were from Invitrogen (Paisley, UK). Thapsigargin (Thap), GlcN, 4-phenylbutyric acid (PBA) and *N*-acetyl-cysteine (NAC), were from Sigma-Aldrich (St Louis, MO, USA). Mn(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) and tauroursodeoxycholic acid (TUDCA) were from Calbiochem (San Diego, CA, USA). Other reagents were as follows: Ultroser G (Pall Biosepra, Cergy, France), 2-deoxy-D-[^{14}C]glucose (2-DG) (Perkin Elmer, Waltham, MA, USA), insulin (Novo Nordisk, Bagsværd, Denmark), GLUT4 (Abcam, Cambridge, UK), myocyte enhancer factor 2 a (MEF2A), peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), eukaryotic translation initiation factor 2α (eIF2α) and phospho-eIF2α antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), small interfering RNA (siRNA) and siPORT NeoFX Transfection Agent (Applied Biosystems, Carlsbad, CA, USA)

Cell culture procedures and 2-DG uptake Human cell cultures from lean individuals were established as described previously [19–21] (Table 1). Cells were cultured in DMEM supplemented with 2% (vol./vol.) FCS, 2% (vol./vol.) Ultroser G and antibiotics. Human myotubes were allowed to differentiate under physiological conditions of insulin (25 pmol/l) and glucose (5.5 mmol/l) for 4 days. L6 rat skeletal muscle myoblasts were grown in DMEM supplemented with 10% (vol./vol.) FBS, 2 mmol/l L-glutamine and antibiotics. L6 myotubes were allowed to differentiate as described previously [22]. 2-DG uptake was measured as reported previously [23].

Real-time RT-PCR and western blot analysis Total RNA extraction, cDNA synthesis and real-time RT-PCR analysis were performed as described previously [24]. Primer sequences are in Electronic supplementary material (ESM) Table 1.

Table 1 Biochemical features of the euglycaemic individuals (n=5)

Variable	Value
Age (years)	55.3±2.2
BMI (kg/m^2)	23.0±1.1
Fasting plasma glucose (mmol/l)	5.4±0.2
Fasting plasma insulin (pmol/l)	35.2±7.8
HbA_{1c} (%)	5.6±0.1

Values represent means ± SD

Muscle biopsies were obtained from the vastus lateralis muscle of five lean individuals by needle biopsy under local anaesthesia [19]. Subjects had normal glucose tolerance and no family history of diabetes. Individuals gave written, informed consent, and the local ethics committee of Funen and Vejle county (Denmark) approved the study

Cell lysates and immunoblotting were carried out as described previously [22]. Antibodies against GLUT4, phospho-eIF2α and eIF2α were used for detection of proteins.

Chromatin immunoprecipitation (ChIP) assay ChIP assays were performed as reported [25]. Vehicle- or reagent-treated myotubes were fixed with 1% (vol./vol.) formaldehyde at 37°C. The fixed cells were lysed in a SDS lysis buffer (1% [wt/vol.] SDS, 10 mmol/l EDTA and 50 mmol/l TRIS–HCl, pH 8.1), incubated on ice, and sonicated to shear DNA. Sheared chromatin samples were taken as input control or used for immunoprecipitation with anti-MEF2A, anti-PGC1α or non-immune antibodies. DNA fragments were recovered and were subjected to real-time RT-PCR amplification by using specific primers for the analysed regions.

Atf6 siRNA-mediated knockdown Cells were transfected with 5 nmol/l of siRNA negative control and *Atf6* siRNA (GCUUGUCAGUCACGAAAGAtt) and antisense (UCUUUCGUGACUGACAAGCag) according to the manufacturer's recommendations and processed 48 h after transfection.

Statistical procedures Data were analysed with Statview software (Abacus Concepts, Piscataway, NJ, USA) by one-factor analysis of variance. $p<0.05$ was considered statistically significant.

Results

To investigate the role of GlcN in ER stress induction, differentiated L6 skeletal muscle cells were treated with different concentrations of GlcN. The classic ER stress inducer Thap, an inhibitor of sarcoplasmic/ER calcium-transporting ATPases, was used as a control of ER stress induction [2]. In L6 myotubes, Thap induced a 17-fold increase of the chaperone *Bip/grp78* (also known as *Hspa5*) mRNA (Fig. 1a), indicating that our cellular model was sensitive to ER stress. *Bip/grp78* mRNA levels were increased also by GlcN, with a maximal expression observed at 10 mmol/l GlcN for 24 h (Fig. 1b). Interestingly, pretreatment of cells with azaserine, a non-specific but commonly used inhibitor of the glutamine:fructose-6-phosphate amidotransferase (GFAT) [26, 27], the rate-limiting enzyme of the HBP, prevented high glucose (HG)-induced ER stress (ESM Fig. 1a). In addition, treatment of cells with the peptide *O*-acetylglucosamine-β-*N*-acetylglucosaminidase inhibitor *O*-(2-acetamido-2-deoxy-D-glucopyranosylidenamino)*N*-phenylcarbamate (PUGNAc), did not increase *Bip/grp78* mRNA levels, suggesting that enhanced O-linked glycosylation was not

responsible for the induction of ER stress (ESM Fig. 1a). To evaluate whether GlcN-induced ER stress could be mediated by oxidative stress, L6 myotubes were pretreated with two anti-oxidants, the glutathione precursor NAC and the superoxide dismutase mimetic MnTBAP. Pretreatment of cells with both NAC and MnTBAP did not affect GlcN-induced *Bip/grp78* mRNA increase, suggesting that GlcN-induced ER stress was not dependent on oxidative stress in skeletal muscle cells (Fig. 1b). Then we evaluated the effects of different concentrations of GlcN on ATP intracellular levels. GlcN depleted the ATP pool only at the highest concentrations, suggesting that GlcN-induced ER stress was not dependent on ATP depletion in skeletal muscle cells (ESM Fig. 2). As expected, xylose did not induce a *Bip/grp78* mRNA increase, even at high concentrations (Fig. 1b), thus excluding an osmotic stress effect caused by treatments. Time course analysis with 7.5 mmol/l GlcN showed that *Bip/grp78* expression was significantly increased as early as 2 h after the treatment and was elevated up to 16 h (Fig. 1c). To investigate whether chemical chaperones could prevent GlcN-induced ER stress, we analysed *Bip/grp78* mRNA in L6 myotubes treated with GlcN in the presence of either 10 mmol/l PBA, a low molecular weight non-specific chemical chaperone known to stabilise protein conformation and to improve ER folding capacity [28], or 5 mmol/l TUDCA, a bile acid derivative that also modulates ER function [28]. Both PBA and TUDCA almost completely prevented the effect of GlcN on *Bip/grp78* mRNA (Fig. 1d). These data suggest that GlcN is able to induce ER stress in skeletal muscle cells and that this effect is prevented by chemical chaperones.

To verify whether GlcN was able to induce UPR activation, L6 myotubes were treated with GlcN or Thap and different markers of UPR activation were analysed. The mRNA levels of the gene (*Xbp1*) encoding the spliced active form of the X-box binding protein-1 (XBP-1; *Xbp1 s*) increased significantly following both Thap (Fig. 2a) and GlcN treatments (Fig. 2b). Time course experiments showed that the increase of *Xbp1 s* peaked at 8 h of GlcN treatment and remained higher than basal level up to 24 h

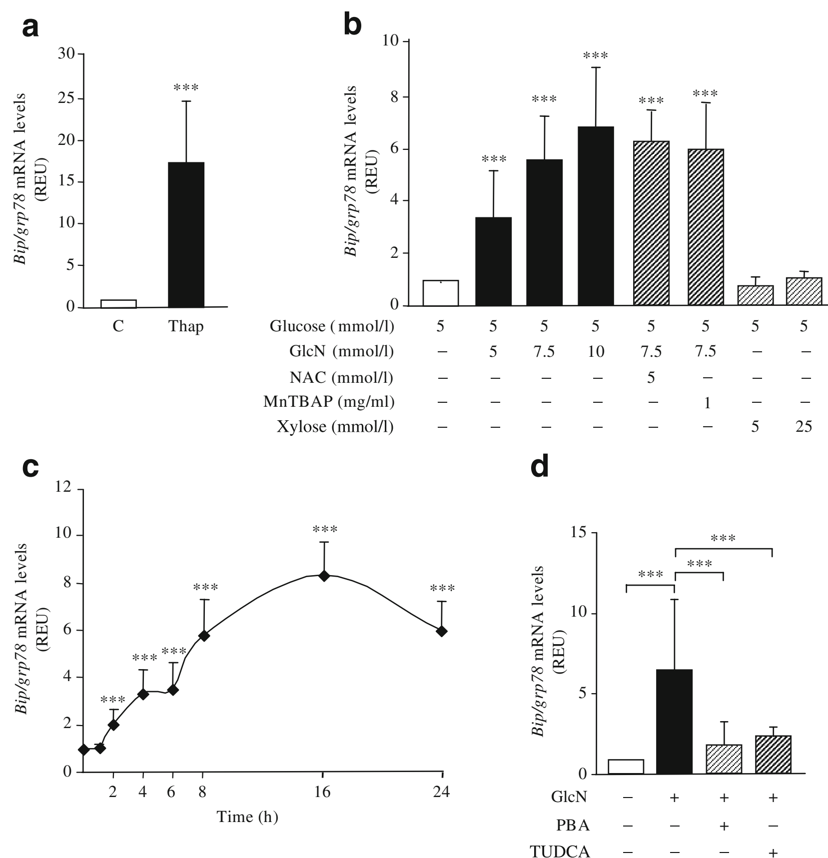

Fig. 1 GlcN induces BIP/GRP78 in L6 myotubes. **a–d** *Bip/grp78* mRNA was determined by real-time RT-PCR analysis of total RNA isolated from myotubes, using *Gapdh* as internal standard. mRNA levels in treated cells are relative expression units (REU) to those in control (C; mean ± SD; $n=8$). ***$p<0.001$. **a** L6 cells were treated with 0.5 µmol/l Thap for 30 min, followed by 24 h without Thap. **b** L6 cells were pretreated or not with 5 mmol/l NAC or with 1 mg/ml MnTBAP for 2 h and then cultured in the presence of GlcN or xylose, as indicated, for 24 h. **c** Time course of *Bip/grp78* mRNA in L6 cells cultured with 7.5 mmol/l GlcN for the indicated times. **d** L6 cells were pretreated or not for 1 h with 10 mmol/l PBA or 5 mmol/l TUDCA and then treated with 7.5 mmol/l GlcN for 24 h

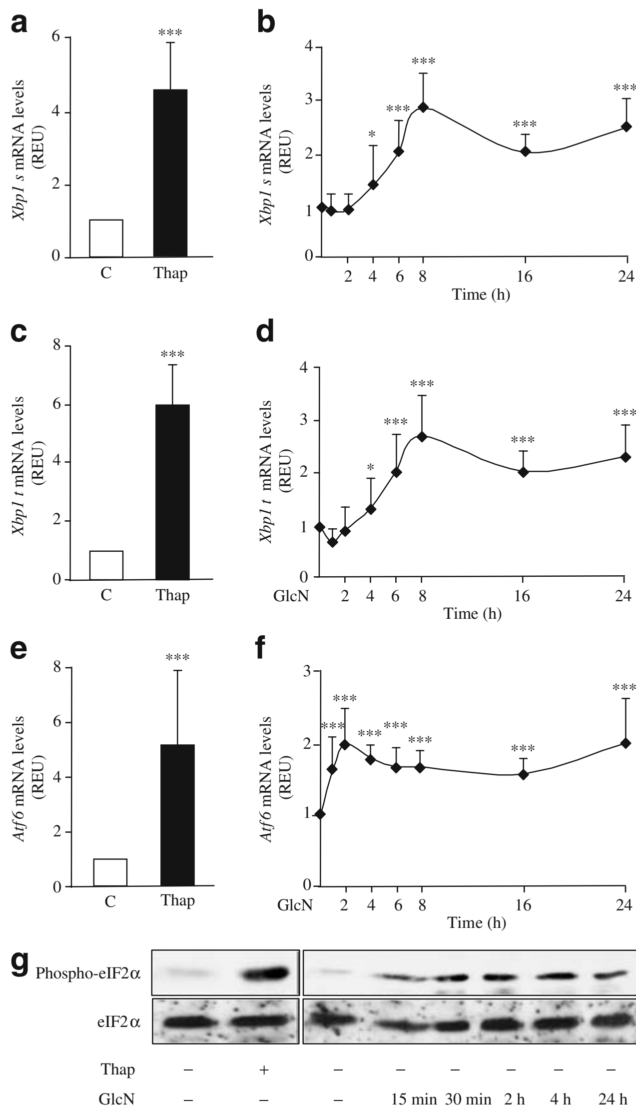

Fig. 2 GlcN induces UPR activation in L6 myotubes. **a–g** L6 cells were treated with 0.5 µmol/l Thap for 30 min, followed by 24 h without Thap, or with 7.5 mmol/l GlcN for the indicated times (**a**–**f**). *Xbp1 s* (**a**, **b**), *Xbp1 t* (**c**, **d**) and *Atf6* (**e**, **f**) mRNAs in treated cells are relative expression units (REU) to those in control (C; mean ± SD; $n=7$) and were determined by real-time RT-PCR analysis, using *Gapdh* as internal standard. $*p<0.05$; $***p<0.001$. **g** L6 cells treated with 0.5 µmol/l Thap for 30 min, followed by 24 h without Thap (left), or with 7.5 mmol/l GlcN (right) for the indicated times, were solubilised and equal amounts of proteins (80 µg per sample) were analysed by western blotting using phospho-eIF2α Ser51 and eIF2α specific antibodies ($n=5$)

(Fig. 2b). In addition, also the mRNA levels of the genes encoding the total form of XBP-1 (*Xbp1 t*) and ATF6 (*Atf6*) were significantly increased upon both Thap (Fig. 2c,e) and GlcN treatments (Fig. 2d,f). Furthermore, phosphorylation of eIF2α was evident as early as 30 min after both GlcN and Thap treatment, and it persisted up to 24 h following GlcN treatment (Fig. 2g). These data suggest that GlcN caused UPR activation in L6 myotubes.

To investigate the effect of GlcN-induced ER stress on the insulin sensitivity of skeletal muscle cells, insulin-induced glucose uptake was evaluated in L6 myotubes treated with GlcN for 24 h. GlcN treatment reduced the capability of L6 cells to take up the glucose analogue 2-DG upon insulin stimulation, compared with control cells (Fig. 3a). Similar results were obtained when cells were treated with Thap (data not shown) and HG (ESM Fig. 1b). To verify the hypothesis that GlcN and HG may impair glucose uptake in L6 cells through ER stress induction, we analysed insulin-induced glucose uptake in cells treated with either GlcN or HG in the presence of PBA or TUDCA. Interestingly, both PBA and TUDCA prevented GlcN (Fig. 3a) and HG (ESM Fig. 1b) effects on insulin-stimulated glucose uptake, suggesting that ER stress caused insulin resistance in skeletal muscle cells.

We then evaluated GlcN effects on the expression of GLUT4, since it is the main glucose transporter responsible for insulin-mediated glucose uptake in muscle [29]. Time course analysis showed that *Glut4* (also known as *Slc2a4*) mRNA levels were significantly decreased as early as 6 h after GlcN treatment, and were reduced by about 50% after 16 h of treatment (Fig. 3b). *Glut4* mRNA levels did not show any significant variation when L6 myotubes were treated with GlcN in the presence of PBA (Fig. 3c). In addition, the GlcN-dependent decrease of *Glut4* mRNA expression was paralleled by a similar reduction of GLUT4 protein levels and this was also prevented by pretreatment with PBA (Fig. 3d). As for GlcN, both Thap (data not shown) and HG treatment (ESM Fig. 1c), induced a significant decrease of both GLUT4 protein and mRNA levels compared with control cells. These data indicated that GlcN and HG reduced *Glut4* expression through the induction of ER stress.

Actinomycin D treatment did not further decrease *Glut4* mRNA upon GlcN treatment, suggesting that GlcN-induced ER stress determined a transcriptional inhibition of *Glut4* in skeletal muscle cells, without affecting its mRNA stability (data not shown). To gain further insight into the mechanisms leading to *Glut4* reduced transcription, we analysed the expression of genes relevant to *Glut4* transcriptional regulation by real-time RT-PCR analysis. Time course experiments showed that GlcN caused a significant reduction of *Mef2a* mRNA expression as early as 6 h after the treatment, with a 60% reduction at 16 h (Fig. 4a). Similarly, MEF2A coactivator *Pgc1α* (also known as *Ppargc1a*) was reduced by 50% after 16 h of treatment compared with control cells (Fig. 4c). As for GlcN, both Thap (data not shown) and HG treatment (ESM Fig. 1c) induced a significant decrease of *Mef2a* and *Pgc1α* expression levels. L6 cells treated with GlcN in the presence of PBA exhibited no differences in the mRNA levels of both *Mef2a* (Fig. 4b) and *Pgc1α* (Fig. 4d) compared with control cells, confirming that also *Mef2a* and *Pgc1α* reduced expression

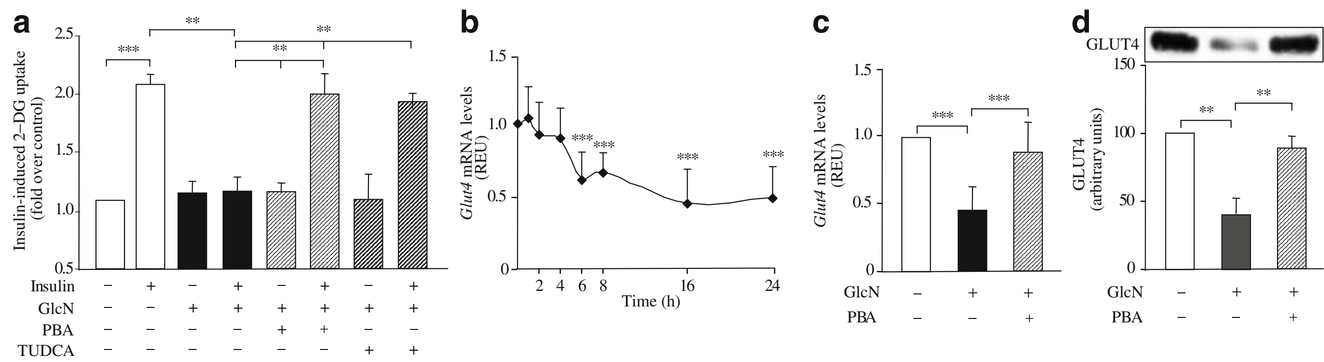

Fig. 3 Effect of ER stress on 2-DG uptake and *Glut4* expression in L6 myotubes. **a** L6 cells were pretreated or not for 1 h with 10 mmol/l PBA or 5 mmol/l TUDCA and then treated with 7.5 mmol/l GlcN for 24 h. 2-DG uptake was measured following 30 min of insulin stimulation (mean ± SD; n=5). **p<0.01; ***p<0.001. **b**, **c** *Glut4* mRNA was determined by real-time RT-PCR analysis of total RNA isolated from myotubes, using *Gapdh* as internal standard. mRNA levels in treated cells are relative expression units (REU) to those in control (mean ± SD; n=5). ***p<0.001. **b** Time course of *Glut4* mRNA in L6 cells treated with 7.5 mmol/l GlcN for the indicated times. **c** *Glut4* mRNA was detected in L6 cells pretreated or not with 10 mmol/l PBA for 1 h, and then treated with 7.5 mmol/l GlcN for 24 h. **d** L6 cells pretreated or not with 10 mmol/l PBA for 1 h, and then treated with 7.5 mmol/l GlcN for 24 h, were solubilised and equal amounts of proteins (80 µg/sample) were analysed by western blotting using GLUT4 specific antibody (n=5). A representative autoradiograph is shown. **p<0.01

was dependent on GlcN-induced ER stress. To establish whether the reduction of *Mef2a* and *Pgc1α* expression induced by ER stress was paralleled by a reduced binding of these two proteins to the GLUT4 promoter, we performed ChIP and re-ChIP experiments in L6 cells treated with GlcN. MEF2A binding to GLUT4 promoter showed a 60% decrease upon GlcN treatment compared with control cells (Fig. 4e). Similarly, PGC1α indirect binding to GLUT4 promoter measured by re-ChIP assay was reduced by 40% upon GlcN treatment compared with control cells (Fig. 4f). These data indicate that GlcN-induced ER stress causes the transcriptional inhibition of *Glut4*, at least in part by reducing both *Mef2a* and *Pgc1α* mRNA levels and their binding to the GLUT4 promoter.

To understand the mechanisms involved in the transcriptional inhibition of *Glut4*, *Mef2a* and *Pgc1α* by ER stress, we sought to evaluate the role of *Atf6*, a gene whose expression has been reported to be upregulated during ER stress and may cause inhibition of gene expression via upregulation of the orphan nuclear receptor small heterodimer partner (SHP) in pancreatic beta cells [30]. To test the hypothesis that the overexpression of ATF6 is sufficient to impair GLUT4 expression, we generated L6 cell lines stably overexpressing *Atf6* (Fig. 5a). In *Atf6*-overexpressing cells, *Glut4* mRNA levels were reduced by 60% in basal condition (Fig. 5b), and were further decreased by 75% upon GlcN treatment (Fig. 5b). *Atf6* overexpression also induced similar decreases of *Mef2a* and *Pgc1a* expression compared with control cells (data not shown). Treatment with a specific siRNA for *Atf6* significantly inhibited the mRNA level of *Atf6* in both untreated and GlcN-treated cells (Fig. 5c). As expected, the transfection of cells with an siRNA for a non-eukaryotic gene, used as negative control, did not affect the upregulation of *Atf6* induced by GlcN (Fig. 5c). In addition, the siRNA for *Atf6* completely prevented GlcN-induced downregulation of *Glut4*, *Mef2a* and *Pgc1α* (Fig. 5d). L6 cells were also transfected with an siRNA for *Shp* (also known as *Nr0b2*). As expected, the treatment with the siRNA for *Shp* significantly inhibited the mRNA level of *Shp* in both untreated and GlcN-treated cells (ESM Fig. 3a); by contrast, it was not able to prevent the GlcN-induced downregulation of *Glut4*, *Mef2a* and *Pgc1α* (ESM Fig. 3b). These data indicate that the activation of *Atf6* is responsible for the impairment of *Glut4* expression during GlcN-induced ER stress through a mechanism independent of *Shp* activation.

Finally, to evaluate GlcN effects on human skeletal muscle cells we used cultured human skeletal muscle cells that display several features of mature skeletal muscle and that have been previously used for studies of muscle metabolism [20]. In differentiated human muscle cells, GlcN induced a significant increase of both *BIP/GRP78* and *ATF6* mRNA levels (Fig. 6a, b), indicating that also the human skeletal muscle cells were sensitive to GlcN-induced ER stress. In addition, pretreatment with PBA completely prevented the increase of both *BIP/GRP78* and *ATF6* mRNAs observed upon GlcN treatment (Fig. 6a, b), confirming in human skeletal muscle cells the results obtained in the rat cells. In human myotubes, GlcN treatment reduced the expression of *GLUT4 MEF2A* and *PGC1α* mRNAs (Fig. 6c–e), and completely inhibited the uptake of 2-DG upon insulin stimulation compared with control cells (Fig. 6f). Furthermore, human myotubes treated with GlcN in the presence of PBA exhibited no differences in the mRNA expression of both *GLUT4* and its upstream regulators *MEF2A* and *PGC1α* (Fig. 6c–e) and a rescue of insulin-induced 2-DG uptake (Fig. 6f) compared

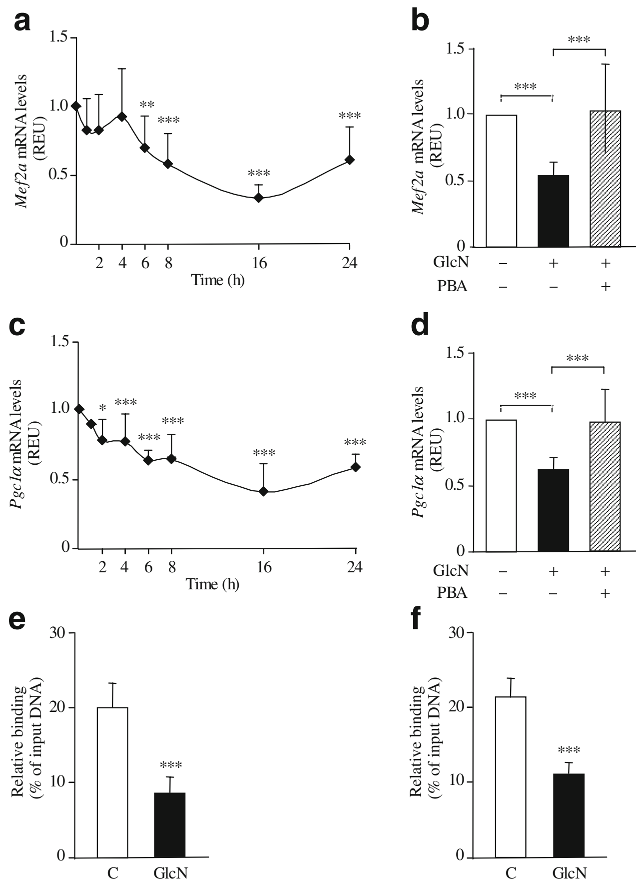

Fig. 4 Effect of ER stress on *Mef2a* and *Pgc1α* mRNA expression and GLUT4 promoter binding in L6 myotubes. **a–d** *Mef2a* and *Pgc1α* mRNAs were determined by real-time RT-PCR analysis, using *Gapdh* as internal standard. mRNA levels in treated cells are relative expression units (REU) to those in control (mean ± SD; n=4). *p< 0.05; **p<0.01; ***p<0.001. **a** Time course of *Mef2a* mRNA in L6 cells treated with 7.5 mmol/l GlcN for the indicated times. **b** *Mef2a* mRNA was detected in L6 cells pretreated or not with 10 mmol/l PBA for 1 h, and then treated with 7.5 mmol/l GlcN for 24 h. **c** Time course of *Pgc1α* mRNA in L6 cells treated with 7.5 mmol/l GlcN for the indicated times. **d** *Pgc1α* mRNA was detected in L6 cells pretreated or not with 10 mmol/l PBA for 1 h, and then treated with 7.5 mmol/l GlcN for 24 h. **e**, **f** Soluble chromatin was prepared from L6 cells vehicle treated or treated with 7.5 mmol/l GlcN for 24 h as described in Methods and immunoprecipitated with MEF2A antibody (**e**) or re-immunoprecipitated with PGC1α antibody (**f**). Immunoprecipitates were then amplified by real-time RT-PCR analysis using specific primers for the analysed regions (mean ± SD; n=3). ***p<0.001

with control cells. Thus, GlcN-induced ER stress impairs insulin sensitivity also in human skeletal muscle, at least in part by inhibiting *GLUT4*, *MEF2A* and *PGC1α* expression.

Discussion

Glucose toxicity may contribute to impaired insulin action in diabetes [22]. A widely accepted hypothesis regarding the mechanism responsible for glucose-induced insulin resistance is that glucose toxicity is mediated by increased flux of glucose into the HBP [17, 26, 27]. Other studies have shown that GlcN, but not HG, causes impairment of insulin-stimulated IRS-1 tyrosine phosphorylation and phosphoinositide-3-kinase activation, and that defective

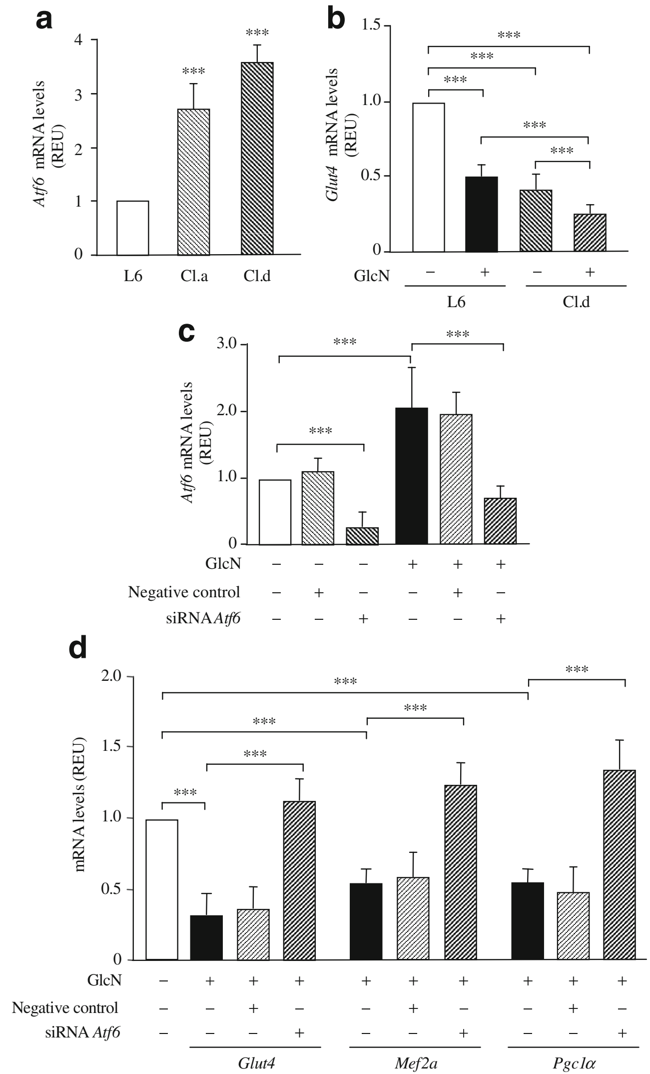

Fig. 5 Role of ATF6 on GlcN-induced ER stress in L6 myotubes. **a**, **b** L6 cells were stably transfected with *Atf6* cDNA. *Atf6* (**a**) and *Glut4* (**b**) mRNAs in clones are relative expression units (REU) to those in untransfected cells (L6) and were determined by real-time RT-PCR analysis using *Gapdh* as internal standard (mean ± SD; n=3). ***p< 0.001. **a** *Atf6* mRNA was detected in several clones. Two clones (termed clones Cl.a and Cl.d) overexpressing *Atf6* by 2.5- and 3.5-fold were further characterised. **b** *Glut4* mRNA was detected in L6 cells untransfected or stably transfected with *Atf6* cDNA and treated with 7.5 mmol/l GlcN for 24 h. **c**, **d** L6 cells were transiently transfected with *Atf6* siRNA or with a negative control siRNA, and then treated with 7.5 mmol/l GlcN for 24 h. Forty-eight hours after transfection, total RNA was extracted from transfected and non-transfected cells. mRNAs for *Atf6* (**c**), *Glut4, Mef2a* and *Pgc1α* (**d**) in treated cells are relative expression units (REU) to those in control cells and were determined by real-time RT-PCR analysis using *Gapdh* as internal standard (mean ± SD; n=3). ***p<0.001

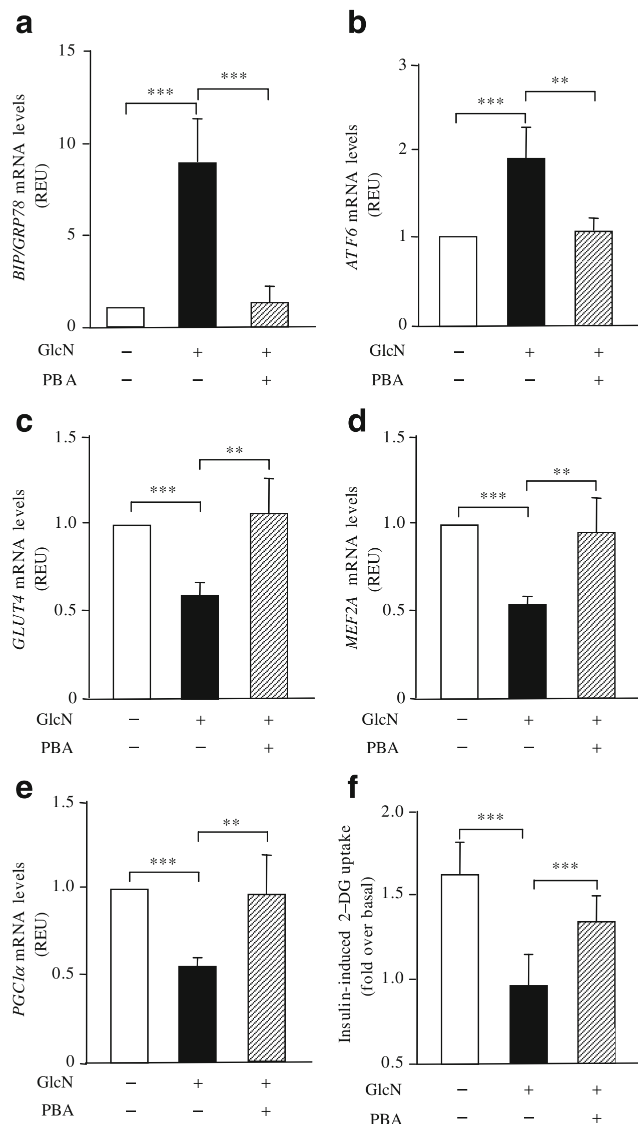

Fig. 6 GlcN effects in human myotubes. **a–f** Human myotubes were pretreated or not with 10 mmol/l PBA for 1 h, and then were treated with 7.5 mmol/l GlcN for 24 h. *BIP/GRP78* (**a**), *ATF6* (**b**), *GLUT4* (**c**), *MEF2A* (**d**) and *PGC1α* (**e**) mRNAs were determined by real-time RT-PCR analysis of total RNA isolated from human myotubes, using *GAPDH* as internal standard. mRNA levels in treated cells are relative expression units (REU) to those in control (mean ± SD; n=4). **p< 0.01; ***p<0.001. **f** 2-DG uptake was measured following 30 min of insulin stimulation (mean ± SD; n=5). ***p<0.001

protein kinase B activation by insulin is associated with glucose- but not GlcN-mediated insulin resistance in adipocytes [31]. Nevertheless, studies in several model systems, including overexpression of GFAT and infusion/treatment with GlcN have confirmed that increased flux through HBP can lead to impaired glucose metabolism [27, 32]. Thus it seems likely that a role for the HBP in the development of the metabolic syndrome and insulin resistance will prevail. According to this, it has been reported that GlcN infusion in rats induces insulin resistance to glucose uptake at the level of both the whole body and skeletal muscle [33]. Furthermore, GlcN induces insulin resistance in vitro in skeletal muscle [34] and adipose cells [35], by reducing the insulin-induced glucose uptake.

Many investigators have suggested the reversible *O*-acetylglucosamine modification of proteins as one possible mechanism by which increased HBP activity may cause insulin resistance and diabetes complications. However, the causal relationship between increased flux through HBP and insulin resistance has not been clearly established. Recently, it has been reported that elevated GlcN levels could interfere with correct protein folding in the ER, inducing ER stress and impairment of cellular function in hepatic cells, monocytes and smooth muscle cells [36]. On the other hand, it has been described that ER stress and UPR activation may play an important role in the pathogenesis of type 2 diabetes [37], affecting both beta cell function/survival [11–13] and obesity-induced peripheral insulin sensitivity in liver and adipose tissue [15, 28]. However, little is known about the role of ER stress in the development of insulin resistance in skeletal muscle tissue.

A very recent study hypothesised a molecular convergence of activated HBP and ER stress pathways leading to insulin-resistance in L6 cells [38]. However, the causal link between the O-glycosylation pathway and the ER stress pathway in determining insulin resistance remained elusive. Indeed, cells silenced for O-linked *N*-acetylglucosamine transferase, the enzyme responsible for the addition of UDP-*N*-acetylglucosamine to Ser/Thr residues of proteins, and treated with GlcN or HG, showed improved insulin-stimulated glucose uptake without any effect on ER chaperone regulation [38].

In the present work, we suggest that ER stress may represent the molecular link between GlcN and insulin resistance in skeletal muscle cells. We show that high GlcN concentrations, as well as the ER stress inducer Thap, cause ER stress and the activation of the UPR in L6 rat skeletal muscle cells, as demonstrated by increased expression of the chaperone *Bip/grp78* and of the transcription factor *Atf6*, the phosphorylation of eIF2α and the increase of both the expression and splicing of the transcription factor XBP-1. Furthermore, ER stress and UPR activation are induced by GlcN in a model of human skeletal muscle cells [20], suggesting that both rat and human skeletal muscle cells are sensitive to GlcN-induced ER stress. More interestingly, HG also induces ER stress in both L6 cells and human myotubes (data not shown). Pretreatment of cells with azaserine, a non-specific but commonly used inhibitor of GFAT [26, 27] prevented HG-induced ER stress, suggesting that at least in part HG levels cause ER stress through hexosamines production. In addition, others [34] and we have shown that L6 myotubes are insulin-resistant upon GlcN as well as HG treatment, as demonstrated by the significant decrease in their

capability to take up the glucose analogue 2-DG upon insulin stimulation. To gain further insight into the mechanisms leading to insulin resistance, Bailey and Turner [34] evaluated GlcN-induced insulin-resistance in L6 myotubes using three different insulin-sensitive acting agents. None of these agents was able to prevent GlcN-induced insulin resistance [34], suggesting that GlcN does not impair insulin sensitivity by altering the upstream steps of insulin signalling. It has been shown that inhibition of insulin-stimulated glucose uptake by GlcN is due to intracellular ATP depletion in rat skeletal muscle [31], adipocytes [39] and chondrocytes [40]. However, in other cell types, ATP depletion by exposure to sodium azide or dinitrophenol did not mimic the effects of GlcN to induce insulin resistance [41]. Thus, ATP depletion is not the sole mechanism underlying all of the effects of GlcN. Indeed, we show that GlcN does not induce ATP depletion at the concentrations used in this study, suggesting that GlcN effects on both ER stress and insulin-resistance were not dependent on ATP depletion in skeletal muscle cells. Interestingly, we have found that pretreatment of both rat and human myotubes with PBA or TUDCA, two chemical chaperones known to prevent ER stress and the UPR activation in different cellular systems [15, 28], completely prevents the effect of GlcN and HG on both ER stress induction and insulin-induced glucose uptake, suggesting that GlcN-induced insulin-resistance is, at least in part, dependent on ER stress.

Glucose uptake into skeletal muscle is primarily mediated by GLUT4 [29]. Since it is well documented that insulin resistance in type 2 diabetes can be associated with a marked reduction in GLUT4 expression [42] and/or translocation [43], we hypothesised that GlcN-induced ER stress might affect transcription of the gene for GLUT4 in both rat and human muscle cells. Here we show that this is the case, as both GLUT4 gene mRNA and protein levels are decreased by 50% upon GlcN as well as upon Thap and HG treatment. Both the human and the rat GLUT4 gene promoter are regulated through the cooperative function of two distinct regulatory elements, domain 1 and MEF2 domain, each required for the maximal transcription of GLUT4 promoter. Domain 1 binds the transcription factor GEF (GLUT4 enhancer factor); MEF2 domain binds transcription factor isoforms MEF2A and MEF2D [42]. MEF2A and GEF associate and function together to activate GLUT4 gene transcription [44]. Little is known about GEF, whilst the role of MEF2A as the main regulator of GLUT4 gene is well documented. Indeed, MEF2A reduced activity correlates with decreased *Glut4* transcription in skeletal muscle of diabetic mice and its activity is completely normalised after insulin treatment [45]. GLUT4 gene transcription can also be modulated in skeletal muscle by other proteins, such as PGC1α [46]. PGC1α is a coactivator of MEF2A. Indeed, MEF2D binds PGC1α, recruiting this transcriptional coactivator to MEF2A [42]. Moreover, *PGC1α* expression is reduced in skeletal muscle of prediabetic and diabetic individuals [47], and enhanced *GLUT4* mRNA expression coincides with increased *PGC1α* mRNA in human skeletal muscle cell culture after treatment with rosiglitazone [46]. Our data, obtained in both rat and human myotubes, show that both GlcN- and HG-induced GLUT4 inhibition is paralleled by a significant decrease of both *MEF2A* and *PGC1α* gene mRNA expression, indicating that GlcN-induced GLUT4 inhibition is exerted very likely at the transcriptional level. Furthermore, mRNA stability of those genes does not appear to be affected by GlcN-dependent ER stress (data not shown). These observations were confirmed by ChIP and re-ChIP experiments, showing a reduced binding of both MEF2A and PGC1α to *Glut4* promoter. Again, these effects appear to be mediated by ER stress signalling, since PBA or TUDCA pretreatment of myotubes is able to prevent *GLUT4*, *MEF2A* and *PGC1α* gene inhibition following both GlcN and Thap treatments. These observations prompted us to consider the GlcN-induced insulin resistance of skeletal muscle cells as a consequence of GLUT4 inhibition and, therefore, reduced membrane translocation (data not shown). However, the contribution of additional components known to be relevant to insulin resistance caused by ER stress in adipocytes and skeletal muscle cells [15, 38], such as JUN N-terminal kinase activation, cannot be excluded. Different proteins have been described to be activated by ER stress and to play a role in ER stress-mediated transcriptional repression. Very recently, indeed, ER stress-dependent activation of ATF6 has been reported to impair insulin gene expression in INS-1 pancreatic beta cells cultured in HG conditions or treated with different ER stressors, via upregulation of SHP [30]. We demonstrate that the overexpression of *Atf6* is sufficient to inhibit the expression of *Glut4*, *Mef2a* and *Pgc1α* both in basal conditions and upon GlcN treatment and that the silencing of *Atf6* expression with a specific siRNA is sufficient to completely prevent GlcN-induced downregulation of *Glut4*, *Mef2a* and *Pgc1α*. This effect seems to be independent of the upregulation of SHP, since its silencing does not modify *Glut4*, *Mef2a* and *Pgc1α* in skeletal muscle cells upon GlcN treatment.

It has been recently shown that ATF6 is responsible for cystic fibrosis transmembrane conductance regulator transcriptional repression by binding to its promoter [48]. Our analysis of the minimal promoter region of *Glut4*, *Mef2a* and *Pgc1α* (GenBank accession numbers: NC_005109.2, NW_001084766.1 and NC_005113.2), using MatInspector, identified several putative binding sites for ATF6 and for other UPR regulatory factors. Further studies will be necessary to understand if ATF6 could repress *Glut4*, *Mef2a* and *Pgc1α* expression in our cell models through a similar mechanism.

In conclusion, in this work we show that GlcN- as well as HG-induced ER stress causes insulin resistance in both human and rat myotubes and impairs GLUT4 gene expression and insulin-induced glucose uptake via an ATF6-dependent decrease of the GLUT4 regulator genes for *MEF2A* and *PGC1α*. Interestingly, treatment with the molecular chaperones PBA and TUDCA completely prevents HG- and GlcN-induced UPR activation and restores insulin sensitivity in myotubes. These findings are particularly relevant for understanding the molecular mechanisms of glucose toxicity in skeletal muscle and of the consequences of ER stress in the pathogenesis of type 2 diabetes.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

1. Kaufman RJ (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 13:1211–1233
2. Liang SH, Zhang W, McGrath BC, Zhang P, Cavener DR (2006) PERK (eIF2alpha kinase) is required to activate the stress-activated MAPKs and induce the expression of immediate-early genes upon disruption of ER calcium homoeostasis. Biochem J 393:201–209
3. Mori K (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 101:451–454
4. Oyadomari S, Araki E, Mori M (2002) Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells. Apoptosis 7:335–345
5. Shi Y, Vattem KM, Sood R et al (1998) Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. Mol Cell Biol 18:7499–7509
6. Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M, Ron D (1998) Cloning of mammalian Ire1 reveals diversity in the ER stress responses. EMBO J 17:5708–5717
7. Yoshida H, Haze K, Yanagi H, Yura T, Mori K (1998) Identification of the *cis*-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. J Biol Chem 273:33741–33749
8. Harding HP, Calfon M, Urano F, Novoa I, Ron D (2002) Transcriptional and translational control in the mammalian unfolded protein response. Annu Rev Cell Dev Biol 18:575–599
9. Kozutsumi MY, Segal M, Normington K, Gething MJ, Sambrook J (1988) The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature 332:462–464
10. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 101:249–258
11. Kaneto H, Matsuoka TA, Nakatani Y et al (2005) Oxidative stress, ER stress, and the JNK pathway in type 2 diabetes. J Mol Med 83:429–439
12. Scheuner D, Song B, McEwen E et al (2001) Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Mol Cell 7:1165–1176
13. Shi Y, Taylor SI, Tan SL, Sonenberg N (2003) When translation meets metabolism: multiple links to diabetes. Endocr Rev 24:91–101
14. Nakatani Y, Kaneto H, Kawamori D et al (2005) Involvement of endoplasmic reticulum stress in insulin resistance and diabetes. J Biol Chem 280:847–851
15. Ozcan U, Cao Q, Yilmaz E et al (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 306:457–461
16. Rossetti L (2000) Perspective: hexosamines and nutrient sensing. Endocrinology 141:1922–1925
17. Pirola L, Johnston AM, Van Obberghen E (2004) Modulation of insulin action. Diabetologia 47:170–184
18. Rossetti L, Hawkins M, Chen W, Gindi J, Barzilai N (1995) In vivo glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycaemic conscious rats. J Clin Invest 96:132–140
19. Bergstrom J (1975) Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. Scand J Clin Lab Invest 35:609–616
20. Gaster M, Kristensen SR, Beck-Nielsen H, Schroder HD (2001) A cellular model system of differentiated human myotubes. APMIS 109:735–744
21. Gaster M, Schroder HD, Handberg A, Beck-Nielsen H (2001) The basal kinetic parameters of glycogen synthase in human myotube cultures are not affected by chronic high insulin exposure. Biochim Biophys Acta 1537:211–221
22. Cassese A, Esposito I, Fiory F et al (2008) In skeletal muscle advanced glycation end products (AGEs) inhibit insulin action and induce the formation of multimolecular complexes including the receptor for AGEs. J Biol Chem 283:36088–36099
23. Miele C, Riboulet A, Maitan MA et al (2003) Human glycated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced insulin receptor substrate (IRS) signaling through a protein kinase C alpha-mediated mechanism. J Biol Chem 278:47376–47387
24. Ulianich L, Garbi C, Treglia AS et al (2008) ER stress is associated with dedifferentiation and an epithelial-to-mesenchymal transition-like phenotype in PC CI3 thyroid cells. J Cell Sci 121:477–486
25. Ungaro P, Teperino R, Mirra P et al (2008) Molecular cloning and characterization of the human PED/PEA-15 gene promoter reveal antagonistic regulation by hepatocyte nuclear factor 4alpha and chicken ovalbumin upstream promoter transcription factor II. J Biol Chem 283:30970–30979
26. Marshall S, Bacote V, Traxinger RR (1991) Discovery of a metabolic pathway mediating glucose induced desensitization of the glucose transport system: role of hexosamine biosynthesis in the induction of insulin resistance. J Biol Chem 266: 4706–4712
27. Buse MG (2006) Hexosamines, insulin resistance, and the complications of diabetes: current status. Am J Physiol Endocrinol Metab 290:E1–E8
28. Ozcan U, Yilmaz E, Ozcan L et al (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science 313:1137–1140

29. Klip A (2009) The many ways to regulate glucose transporter 4. Appl Physiol Nutr Metab 34:481–487
30. Seo HY, Kim YD, Lee KM et al (2008) Endoplasmic reticulum stress-induced activation of activating transcription factor 6 decreases insulin gene expression via up-regulation of orphan nuclear receptor small heterodimer partner. Endocrinology 149:3832–3841
31. Han DH, Chen MM, Holloszy JO (2003) Glucosamine and glucose induce insulin resistance by different mechanisms in rat skeletal muscle. Am J Physiol Endocrinol Metab 285:E1267–E1272
32. Ciaraldi TP, Carter L, Nikoulina S, Mudaliar S, McClain DA, Henry RR (1999) Glucosamine regulation of glucose metabolism in cultured human skeletal muscle cells: divergent effects on glucose transport/phosphorylation and glycogen synthase in non-diabetic and type 2 diabetic subjects. Endocrinology 140:3971–3980
33. Patti ME, Virkamäki A, Landaker EJ, Kahn CR, Yki-Järvinen H (1999) Activation of the hexosamine pathway by glucosamine in vivo induces insulin resistance of early postreceptor insulin signalling events in skeletal muscle. Diabetes 48:1562–1571
34. Bailey CJ, Turner SL (2004) Glucosamine-induced insulin resistance in L6 muscle cells. Diabetes Obes Metab 6:293–298
35. Heart E, Choi WS, Sung CK (2000) Glucosamine-induced insulin resistance in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 278:E103–E112
36. Werstuck GH, Khan MI, Femia G et al (2006) Glucosamine-induced endoplasmic reticulum dysfunction is associated with accelerated atherosclerosis in a hyperglycaemic mouse model. Diabetes 55:93–101
37. Sundar Rajan S, Srinivasan V, Balasubramanyam M, Tatu U (2007) Endoplasmic reticulum (ER) stress and diabetes. Indian J Med Res 125:411–424
38. Srinivasan V, Tatu U, Mohan V, Balasubramanyam M (2009) Molecular convergence of hexosamine biosynthetic pathway and ER stress leading to insulin resistance in L6 skeletal muscle cells. Mol Cell Biochem 328:217–224
39. Hresko RC, Heimberg H, Chi MM, Mueckler M (1998) Glucosamine-induced insulin resistance in 3T3-L1 adipocytes is caused by depletion of intracellular ATP. J Biol Chem 273:20658–20668
40. Shikhman AR, Brinson DC, Valbracht J, Lotz MK (2009) Differential metabolic effects of glucosamine and *N*-acetylglucosamine in human articular chondrocytes. Osteoarthritis Cartilage 17:1022–1028
41. Kang J, Heart E, Sung CK (2001) Effects of cellular ATP depletion on glucose transport and insulin signaling in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 280:E428–E435
42. Armoni M, Harel C, Karnieli E (2007) Transcriptional regulation of the GLUT4 gene: from PPAR-gamma and FOXO1 to FFA and inflammation. Trends Endocrinol Metab 18:100–107
43. Shepherd PR, Kahn BB (1999) Glucose transporters and insulin action: implications for insulin resistance and diabetes mellitus. N Engl J Med 341:248–257
44. Knight JB, Eyster CA, Griesel BA, Olson AL (2003) Regulation of the human GLUT4 gene promoter: interaction between a transcriptional activator and myocyte enhancer factor 2A. Proc Natl Acad Sci U S A 100:14725–14730
45. Handschin C, Rhee J, Lin J, Tarr PT, Spiegelman BM (2003) An autoregulatory loop controls peroxisome proliferator-activated receptor γ coactivator 1 α expression in muscle. Proc Natl Acad Sci USA 100:7111–7116
46. Al-Khalili L, Forsgren M, Kannisto K, Zierath JR, Lönnqvist F, Krook A (2005) Enhanced insulin-stimulated glycogen synthesis in response to insulin, metformin or rosiglitazone is associated with increased mRNA expression of GLUT4 and peroxisomal proliferator activator receptor gamma co-activator 1. Diabetologia 48:1173–1179
47. Patti ME, Butte AJ, Crunkhorn S (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A 100:8466–8471
48. Bartoszewski R, Rab A, Twitty G et al (2008) The mechanism of cystic fibrosis transmembrane conductance regulator transcriptional repression during the unfolded protein response. J Biol Chem 283:12154–12165