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**“Mechanisms linking glucotoxicity to the
development of insulin resistance: a role for the
endoplasmic reticulum stress”**



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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 ER stress in the induction of insulin-resistance by glucosamine in skeletal muscle cells. **Methods:** Real-time RT-PCR analysis, 2-deoxy-D-glucose uptake and western blot analysis were carried out in rat and human muscle cell lines. **Results:** Glucosamine treatment causes both in rat and human myotubes a significant increase in the expression of the ER stress markers *BIP*, *XBP1* and *ATF6*. In addition, glucosamine impairs insulin-stimulated 2-deoxyglucose uptake both in rat and human myotubes. Interestingly, pre-treatment of both rat and human myotubes with the chemical chaperones PBA or TUDCA, completely prevents the effect of glucosamine on both ER stress induction and insulin-induced glucose uptake. Glucosamine treatment reduces *GLUT4* mRNA and protein levels and the mRNA levels of the main regulators of *GLUT4* gene, *MEF2A* and *PGC1 α* , both in rat and human myotubes. Again, PBA or TUDCA pre-treatment prevents glucosamine-induced inhibition of *GLUT4*, *MEF2A* and *PGC1 α* . Finally, we show that overexpression of ATF6 is sufficient to inhibit the expression of *Glut4*, *Mef2a* and *Pgc1 α* and that ATF6 silencing with a specific siRNA is sufficient to completely prevent glucosamine-induced inhibition of *Glut4*, *Mef2a* and *Pgc1 α* in skeletal muscle cells. **Conclusions/interpretation:** In this work we show that glucosamine-induced ER stress causes insulin-resistance both in human and rat myotubes and impairs *GLUT4* expression and insulin-induced glucose uptake *via* an ATF6-dependent decrease of the *Glut4* regulators *Mef2a* and *Pgc1 α* .

Background

1. Glucose homeostasis

Despite periods of feeding and fasting, plasma glucose remains in a narrow range between 4 and 7mM in normal individuals. This tight control is governed by the balance between glucose absorption from the intestine, the production by the liver and the uptake and metabolism by peripheral tissues (Saltiel and Kahn 2001). Carbohydrate metabolism is regulated by several hormones, and also by sympathetic and parasympathetic nervous system activity (Table 1). The increasing glucose concentration after feeding (80/150 mg/dl) determines an increase of insulin release from pancreatic beta cells and a decrease of glucagon release from pancreatic alpha cells (Kahn 1994). Insulin lowers blood glucose levels both by suppressing glycogenolysis and gluconeogenesis in the liver (thereby decreasing hepatic glucose output), and by stimulating glucose uptake into skeletal muscle and adipose tissue. These actions are opposed by the “counter-regulatory” hormones, which are secreted continuously but whose release is enhanced during physiological “stress” (Pickup 2005). Thus, the correct efficiency of pancreatic beta cells in insulin synthesis and secretion represents a fundamental role in glucose homeostasis maintenance.

	Liver Gluconeogenesis	Glycogenolysis	HGO	Peripheral Glucose uptake
Insulin	↓	↓↓	↓↓	↑↑
Glucagon	↑↑§	↑↑	↑↑	-
Catecholamines	↑↑*§	↑	↑↑	↓
Growth hormone	↑*	-	↑	↓
Cortisol	↑*§	-	↑	↓

Table 1. Main hormones affecting carbohydrate metabolism

HGO, hepatic glucose output.

*Indirect enhancement of gluconeogenesis due to increased supply of glycerol and fatty acids by enhanced lipolysis

§Increased gluconeogenesis by effects on hepatic enzymes and increased supply of glucogenic amino acids

2. Insulin

Insulin is the most potent anabolic hormone known. Secreted by pancreatic beta cells in response to the increase in plasmatic glucose and amino acids levels after feeding, insulin promotes the synthesis and storage of carbohydrates, lipids and proteins, while inhibiting their degradation and release into the circulation. Insulin stimulates the uptake of glucose, amino acids and fatty acids into cells, and increases the expression and the activity of enzymes that catalyse glycogen, lipid and protein synthesis, while inhibiting the activity or the expression of those that catalyse degradation. Insulin increases glucose uptake in muscle and fat, and it inhibits hepatic glucose production (glycogenolysis and gluconeogenesis) in liver, thus serving as the primary regulator of blood glucose concentration. Insulin also stimulates cell growth and differentiation, and promotes the storage of substrates in fat, liver and muscle by stimulating lipogenesis, glycogen and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein breakdown (Figure 1) (Saltiel and Kahn 2001).

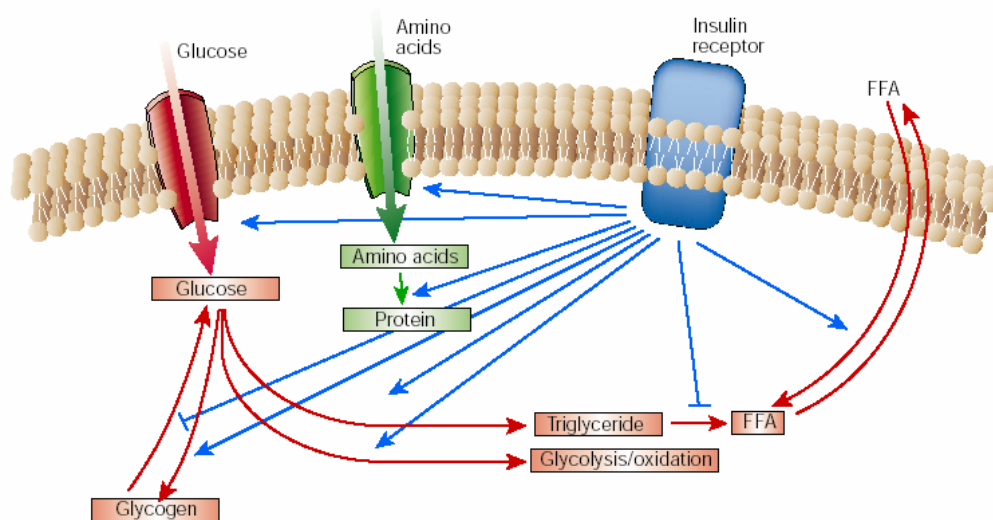


Figure 1 The regulation of metabolism by insulin.

Insulin promotes the synthesis and the storage of carbohydrates, lipids and proteins. Indeed, insulin stimulates the uptake of glucose, amino acids and fatty acids into cells, and increases the expression or the activity of enzymes that catalyse glycogen, lipid and protein synthesis, while inhibiting the activity or the expression of those that catalyse degradation.

Insulin is an hormone of 51 amino acids and is synthesized as a precursor protein (preproinsulin) in the beta cells. Preproinsulin presents a signal sequence at the N-terminus that allows its entrance in

secretion vesicles. The cut of the signal sequence by proteolytic enzymes produces proinsulin. When the increase in plasmatic glucose concentrations stimulates insulin secretion, proinsulin is converted in the mature hormone by specific proteases that hydrolyse two peptide bonds and generate mature insulin (Taylor, et al. 1999). Insulin is a little protein formed by two polypeptide chains, termed respectively "A" of 21 amino acids, and "B" of 30, linked by two interchain sulphide bond. Once beta cells are stimulated by glucose, insulin is released in blood circulation and is distributed to target tissues, where it can bind a specific receptor at high affinity, essential for insulin signalling transduction.

3. Insulin signaling system

Insulin action is mediated through the insulin receptor (IR), a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity. The insulin receptor belongs to a subfamily of receptor tyrosine kinases that includes the insulin-like growth factor (IGF)-I receptor and the insulin receptor-related receptor (IRR). These receptors are tetrameric proteins consisting of two α - and two β -subunits that function as allosteric enzymes where the α -subunit inhibits the tyrosine kinase activity of the β -subunit. Insulin binding to the α -subunit leads to derepression of the kinase activity of the β -subunit followed by transphosphorylation of the β -subunits and conformational change of the α subunits that further increases kinase activity (Patti and Kahn 1998). Several intracellular substrates of the insulin receptor kinases have been identified (Figure 2). Four of these belong to the family of insulin-receptor substrate (IRS) proteins (White, et al. 1998). Other substrates include Gab-1 and isoforms of Shc10 (Pessin and Saltiel 2000). The phosphorylated tyrosines in these substrates act as 'docking sites' for proteins that contain SH2 (Src homology-2) domains. Many of these SH2 proteins are adaptor molecules, such as the p85 regulatory subunit of PI(3)K and Grb2, or CrkII, which activate small G proteins by binding to nucleotide exchange factors. Others are themselves enzymes, including the phosphotyrosine phosphatase SHP2 and the cytoplasmic tyrosine kinase Fyn. PI(3)K has a pivotal role in the metabolic and mitogenic actions of insulin (Shepherd, et al. 1995). It consists of a p110 catalytic subunit and a p85 regulatory subunit that possesses two SH2 domains that interact with tyrosinephosphorylated motifs in IRS proteins (Myers MG Jr, et al. 1992). PI(3)K catalyses the phosphorylation of phosphoinositides on the 3-position to produce phosphatidylinositol-3-phosphates, especially PtdIns(3,4,5)P3, which bind to the pleckstrin homology (PH) domains of a variety of signalling molecules thereby altering their activity, and subcellular localization (Lietzke, et al. 2000). Phosphotidylinositol-3-phosphates regulate three main classes of signalling molecules: the AGC family of

serine/threonine protein kinases, the Rho family of GTPases, and the TEC family of tyrosine kinases. PI(3)K also might be involved in regulation of phospholipase D, leading to hydrolysis of phosphatidylcholine and increases in phosphatidic acid and diacylglycerol. The best characterized of the AGC kinases is phosphoinositide-dependent kinase 1 (PDK1), one of the serine kinases that phosphorylates and activates the serine/threonine kinase Akt/PKB (Alessi, et al. 1997). Akt/PKB has a PH domain that also interacts directly with PtdIns(3,4,5)P₃, promoting membrane targeting of the protein and catalytic activation. Akt/PKB has a pivotal role in the transmission of the insulin signal, by phosphorylating the enzyme GSK-3, the forkhead transcription factors and cAMP response element-binding protein. Other AGC kinases that are downstream of PI(3)K signaling include the atypical PKCs, such as PKC- ζ . Akt/PKB and/or the atypical PKCs are required for insulin stimulated glucose transport (Standaert, et al. 1997).

As is the case for other growth factors, insulin stimulates the mitogenactivated protein (MAP) kinase extracellular signal regulated kinase (ERK) (Figure 2). This pathway involves the tyrosine phosphorylation of IRS proteins and/or Shc, which in turn interact with the adapter protein Grb2, recruiting the Son-of-sevenless (SOS) exchange protein to the plasma membrane for activation of Ras. The activation of Ras also requires stimulation of the tyrosine phosphatase SHP2, through its interaction with receptor substrates such as Gab-1 or IRS1/2. Once activated, Ras operates as a molecular switch, stimulating a serine kinase cascade through the stepwise activation of Raf, MEK and ERK. Activated ERK can translocate into the nucleus, where it catalyses the phosphorylation of transcription factors such as p62TCF, initiating a transcriptional programme that leads to cellular proliferation or differentiation (Boulton, et al. 1991).

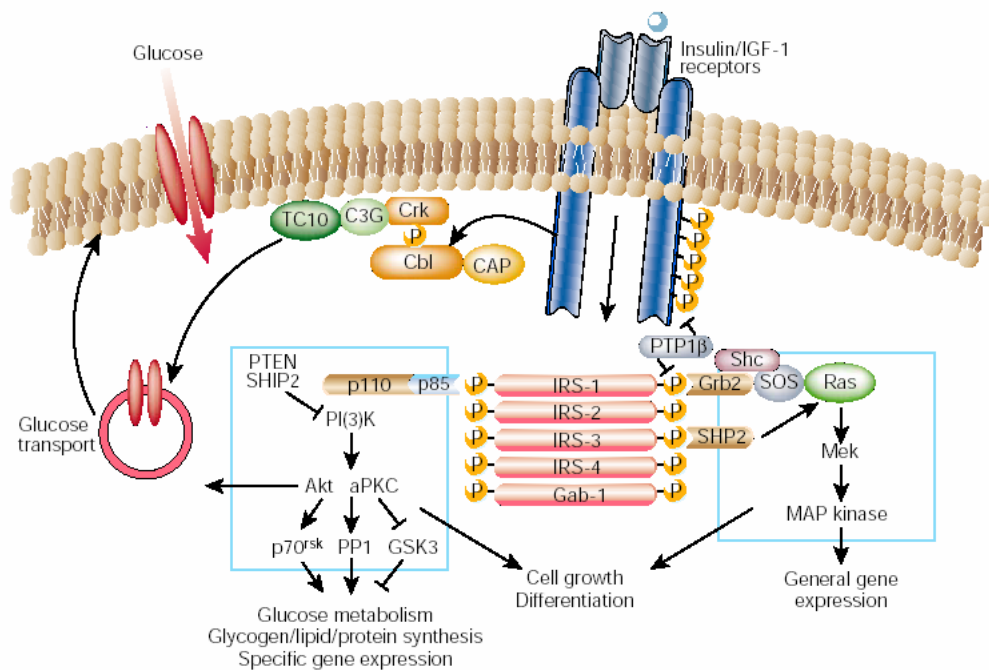


Figure 2 Signal transduction in insulin action.

The insulin receptor is a tyrosine kinase that undergoes autophosphorylation, and catalyses the phosphorylation of cellular proteins such as members of the IRS family, Shc and Cbl. Upon tyrosine phosphorylation, these proteins interact with signaling molecules through their SH2 domains, resulting in a diverse series of signaling pathways, including activation of PI(3)K and downstream PtdIns(3,4,5)P3 dependent protein kinases, Ras and the MAP kinase cascade. These pathways act in a concerted fashion to coordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation and inactivation, and gene expression, which results in the regulation of glucose, lipid and protein metabolism.

4. Skeletal muscle glucose uptake regulation: a role for GLUT4

Insulin-stimulated glucose uptake by skeletal muscle plays an important role in the maintenance of whole-body glucose homeostasis. Glucose transporter 4 (GLUT4) is the main glucose transporter isoform expressed in skeletal muscle that mediates glucose uptake in response to hormones such as insulin, to stimuli such as exercise/contraction and hypoxia, and to pharmacological interventions that alter mitochondrial energy output. Under basal conditions, GLUT4 is predominantly sequestered in several intracellular compartments. Both insulin and muscle contraction cause a net gain of GLUT4 at the plasma membrane to increase glucose uptake, but they utilize distinct signalling molecules and mobilize GLUT4 from different intracellular pools. It is well accepted that activation of the insulin receptor substrates (IRS)/phosphatidylinositol 3-kinase (PI3K) axis is indispensable to insulin-stimulated GLUT4 translocation and glucose uptake (Figure 3). Similarly, it is well documented that 2 serine/threonine kinases (Akt and

the atypical protein kinase C [αPKC] zeta/lambda downstream of PI3K) participate in mediating insulin's metabolic actions in skeletal muscle and fat (Klip 2009).

The GLUT4 gene is subject to complex tissue-specific and metabolic regulation, with a profound impact on insulin mediated glucose disposal. The human GLUT4 promoter is regulated through the cooperative function of two distinct regulatory elements, domain 1 and the myocyte enhancer factor 2 (MEF2) domain. The MEF2 domain binds transcription factors MEF2A and MEF2D *in vivo*; domain 1 binds the transcription factor GEF (GLUT4 enhancer factor). MEF2A and GEF coimmunoprecipitated and function together to activate *GLUT4* transcription. No much is known about GEF (Knight et al. 2003). By contrast, it is well established that MEF2 plays a role in the transcriptional regulation of different cellular functions in skeletal and cardiac muscle, neurons, and T cells. To respond to diverse developmental and physiological cues, MEF2 is structurally organized to receive and respond to multiple signals from several intracellular signaling pathways. Consistent with its role as a signal sensor, putative phosphoacceptor motifs in the carboxyl terminal MEF2 transactivation domain may prove to further modulate MEF2 stability and function in response to extracellular cues. By contrast, the ubiquitous phosphorylation of Ser-255 by endogenous kinases is detected in several cell types (C2C12, COS, and HeLa) and is coupled with its role in the degradation of MEF2. This phosphorylation points tantalizingly toward a role for glycogen synthase kinase 3. This ubiquitous kinase is now known to be involved in numerous cellular signaling functions and to phosphorylate several proteins, which are subsequently targeted for degradation (Cox et al. 2003). Finally, GLUT4 transcription can be modulated by other proteins, such as peroxisome proliferators-activated receptor-γ coactivator 1 (PGC-1) (Al-Khalili et al. 2005). Indeed, MEF2D binds PGC1 recruiting this transcriptional coactivators to MEF2A.

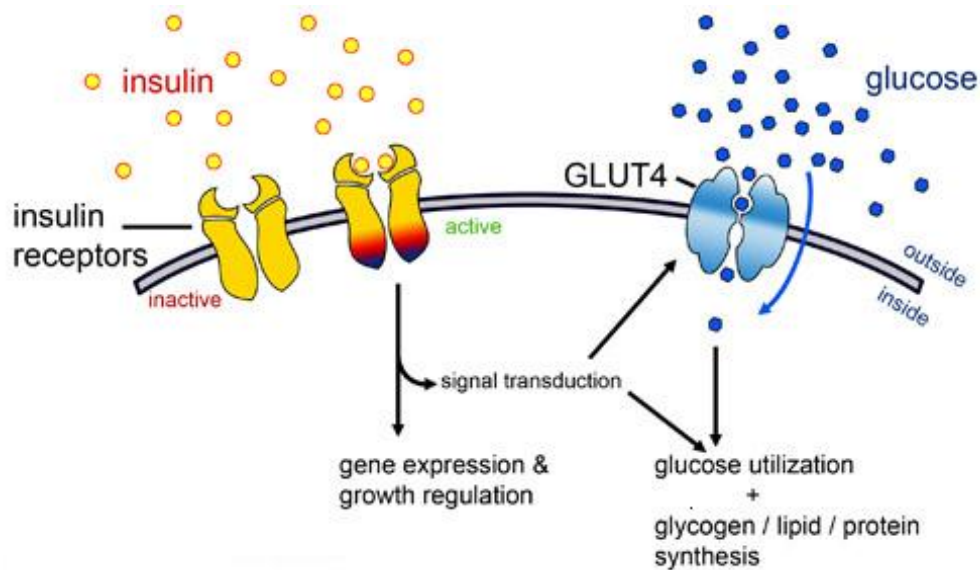


Figure 3 Insulin-induced glucose uptake in muscle. Insulin regulates glucose uptake into muscle cells by recruiting membrane vesicles containing the GLUT4 glucose transporters from the interior of cells to the cell surface, where it allows glucose to enter cells by facultative diffusion. Once in the cytoplasm, the glucose is phosphorylated and thereby trapped inside cells. The effect of insulin on GLUT4 distribution is reversible. Within an hour of insulin removal, GLUT4 is removed from the membrane and restored intracellular in vesicles ready to be re-recruited to the surface by insulin. Thus, glucose uptake by muscle cells is regulated by modulating the number of GLUT4 glucose transporters on the surface of cells.

5. Diseases with altered glucose homeostasis

Maturity-onset diabetes of the young (MODY), Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI), mitochondrial diabetes, Type 1 (T1D) and Type 2 Diabetes (T2D) are a heterogeneous group of disorders characterized by high blood glucose levels and by insulin secretory abnormalities. In subjects with MODY, PHHI, or mitochondrial diabetes, the elevation in blood glucose levels is due to genetically determined defects in processes that are critical for the normal signaling pathways in the beta cell. The effects of these mutations on insulin secretion are so severe that diabetes develops in people with normal or relatively normal insulin sensitivity and without the contribution of environmental factors. In T1D, instead, insulin secretory abnormalities results from an absolute deficiency of insulin due to autoimmune destruction of the insulin-producing pancreatic beta cell. Finally, in T2D, more moderate abnormalities of secretion are present that cause glucose intolerance only if insulin resistance is also present. The genetic basis of beta cell dysfunction in this form of diabetes is more complex, involving both multiple interacting genes and environmental factors, which determine whether diabetes will develop and at what age. Here no genetic abnormalities profoundly

affect beta-cell function, but rather the inability of the pancreatic beta cell to adapt to the reductions in insulin sensitivity that occur over a lifetime precipitates the onset of hyperglycaemia (Bell and Polonsky 2001).

5.1 MODY, PHHI, and mitochondrial diabetes

MODY is a clinically heterogeneous group of disorders characterized by nonketotic diabetes mellitus, an autosomal dominant mode of inheritance, onset usually before 25 years of age and frequently in childhood or adolescence, and a primary defect in pancreatic beta cell function. MODY can result from mutations in any one of at least six different genes that encode the glycolytic enzyme glucokinase and five transcription factors: hepatocyte nuclear factor (HNF)-4 α , HNF-1 α , insulin promoter factor-1 (IPF-1), HNF-1 β and neurogenic differentiation 1/beta cell E-box transactivator 2 (NeuroD1/BETA2) (Table 2). All the mentioned genes are expressed in the pancreatic beta-cell and mutations lead to beta cell dysfunction and diabetes mellitus in the heterozygous state (Bell and Polonsky 2001).

MODY	Gene involved
MODY 1	HNF-4 α
MODY 2	GK
MODY 3	HNF-1 α
MODY 4	IPF-1
MODY 5	NEURO D1/BETA 2

Table 2 MODY classification and genes

GK is expressed at highest levels in the pancreatic beta cell and in the liver. It catalyses the transfer of phosphate groups from ATP to glucose to generate glucose-6-phosphate: the first rate-limiting step in glucose metabolism. GK functions as the glucose sensor in the beta cell and influences the ability to store glucose as glycogen in the liver. Heterozygous mutations leading to partial deficiency of GK are associated with MODY and homozygous mutations usually result in complete deficiency of this enzyme, leading to PHHI (Bell and Polonsky 2001). The transcription factors HNF-1 α , HNF-1 β and HNF-4 α are involved in the tissue-specific regulation of gene expression in the liver, pancreatic beta cells and other tissues. HNF-1 α and HNF-1 β are members of the homeodomain containing family of transcription factors and HNF-4 α is an orphan nuclear receptor. They are part of a network of transcription factors that controls gene expression during embryonic development and also in adult tissues where they are co-expressed. In the pancreatic beta cell, they regulate the expression of genes important in the regulation of insulin secretion. Mutations in these genes produce defects in insulin secretory responses to a variety of factors,

particularly glucose, which are present before the onset of hyperglycaemia. In mice lacking the gene for HNF-1 α , beta cell mass fails to increase despite the presence of hyperglycaemia, suggesting that this transcription factor may regulate beta cell mass (Bell and Polonsky 2001).

IPF-1 is a homeodomain-containing transcription factor involved in pancreatic development, transcriptional regulation of a number of beta cell genes including insulin, glucokinase, islet amyloid polypeptide and GLUT2, and mediation of glucose-stimulated insulin gene transcription. Mutations in the heterozygous state are associated with MODY, whereas mouse and human homozygotes fail to develop a pancreas and suffer congenital diabetes mellitus. IPF-1 mutations have also been discovered in a small fraction of patients with typical adult-onset type 2 diabetes. Subjects with heterozygous mutations in IPF-1 demonstrated reduced insulin secretory responses to glucose and glucagon-like peptide-1, consistent with a defect in the signalling pathways that regulate secretion in the beta cell and/or a defect in beta cell mass. The basic helix-loop-helix transcription factor NeuroD1/BETA2 is involved in the regulation of transcription of the insulin gene and is required for normal pancreatic islet development. Mutations in NeuroD1 are a rare cause of MODY and result in reduced serum insulin concentrations, either due to a signalling defect in the beta cell, or a reduction in beta cell mass, or both (Bell and Polonsky 2001). PHHI, also known as familial hyperinsulinism, pancreatic nesidioblastosis or hyperinsulinemic hypoglycemia, is a rare genetic disease, that can be inherited in a dominant or recessive way. It is mainly characterized by the presence of inappropriately high levels of insulin in parallel with low or very low glucose levels. This disease affects newborns and infants and it is as frequent as 1:50000 for unselected populations. Mutations in four different genes have been identified: the KATP channel genes *SUR1* and *Kir6.2*, glucokinase (*GK*), and glutamate dehydrogenase (*GLUD1*). Nevertheless, in as many as 60% of patients, the genetic basis of the condition has not been elucidated. The most common and most severe forms of PHHI arise from *SUR1* (40 different mutations) and/or *Kir6.2* (three mutations) gene defects and alterations of beta cellular KATP channels are responsible for PHHI physiopathology. Using both recombinant expression systems and beta cells isolated from patients after surgery, investigators have shown these mutations to lead to impaired trafficking or assembly of KATP channels or to cause defects in the ADP dependent regulation of channel activity, and it results in depolarized beta cells with unregulated Ca²⁺ channel activity, that determines hypoglycaemia and impaired insulin secretion (Kane et al. 1996; Straub et al. 2001). In addition to mutations in the nuclear genome, abnormal mitochondrial function resulting from mutations in the mitochondrial genome can lead to diabetes. The most common

diabetes-associated mutation is an A-to-G transition in the mitochondrial tRNA^{Leu} (UUR) gene at base pair 3,243. This mutation results in defects in insulin secretion including failure of glucose to prime the insulin secretory response and abnormal insulin secretory oscillation (Bell and Polonsky 2001).

5.2 Type 1 diabetes

T1D accounts for 10% of all diabetes cases. The disease can affect people of any age, but usually occurs in children or young adults. T1D is caused by an auto-immune reaction against the insulin-producing cells. The reason why this occurs is not fully understood. T1D pathogenesis involves environmental triggers that may activate autoimmune mechanisms in genetically susceptible individuals. It is characterized by insulinitis, an infiltration of the pancreatic islets by lymphocytes, macrophages and neutrophil granulocytes, resulting in auto-immune destruction of the beta cells since chronic exposure to proinflammatory cytokines such as IL-1, TNF α , and interferon γ is cytotoxic to beta cells. The clinical manifestation of T1D is typically late-stage, when majority of beta cells (90%) have been destroyed and its onset is often sudden and dramatic. The lives of people with this form of diabetes are entirely dependent on injections of insulin every day for survival in order to prevent the development of ketoacidosis. (Diabetes Atlas 2006).

5.3 Type 2 diabetes

T2D is the most common type of diabetes and accounts for 90% of all forms of diabetes. T2D is most common in people older than 45 who are overweight. However, as a consequence of increased obesity among young people, it is becoming more common in children and young adults. T2D is a heterogeneous syndrome with many possible causes. This is due to the interaction of environmental factors with a genetic susceptibility to the disease (Table 3), and it is becoming more and more evident that the relative contribution of genes and environment can differ considerably, even among individual whose clinical phenotype is similar (Diabetes Atlas 2006).

The maintenance of normal glucose homeostasis depends on a precisely balanced and dynamic interaction between tissue sensitivity to insulin and insulin secretion. Type 2 diabetes develops because of defects in both insulin secretion and action, both of these with a genetic as well as an acquired component. Thus, T2D is made up of different forms each of which is characterized by variable degrees of insulin resistance and beta cell dysfunction, and which together lead to hyperglycaemia. Insulin resistance, typically, is an early feature of T2D. It results from a genetically determined reduction in insulin sensitivity, compounded by exposure to the environmental factors, which further

impair insulin action. Major sites of insulin resistance include liver and the peripheral tissues, skeletal muscle and fat. In muscle and fat, insulin resistance is manifested by decreased glucose uptake; in muscle, it impairs glucose utilization by nonoxidative pathways as well as by a decrease in glucose oxidation; in the liver, insulin resistance leads to failure of insulin to suppress hepatic glucose production, which is followed by glycogen breakdown and particularly by gluconeogenesis (Pickup 2005) (Figure 4).

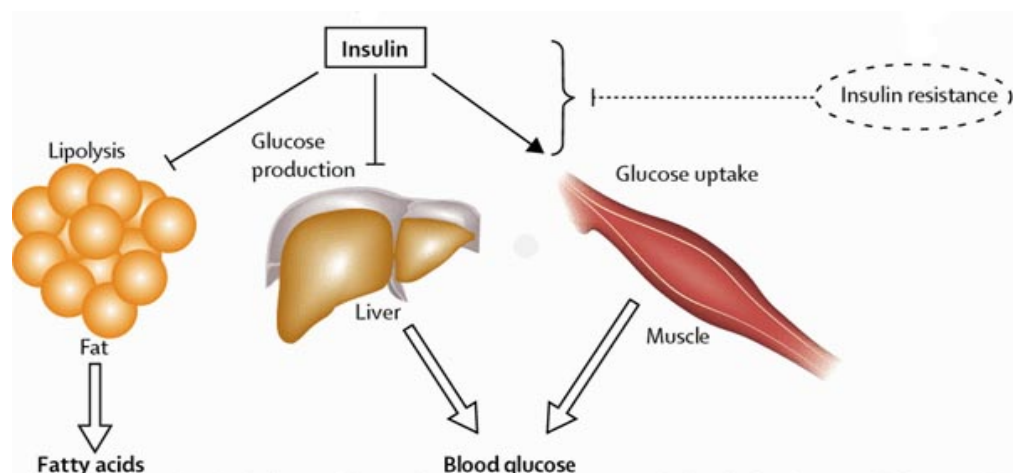


Figure 4 Insulin resistance on tissue targets.

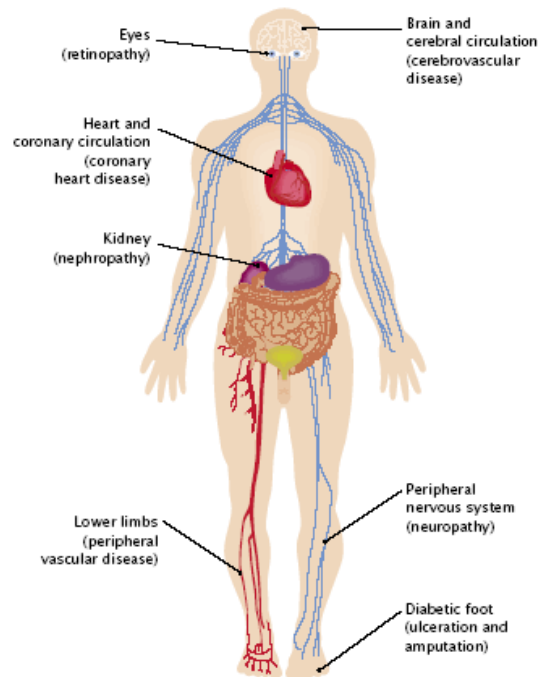
The main sites of insulin resistance are liver and the peripheral tissue, skeletal muscle and fat. Insulin resistance is manifested by decreased glucose uptake in muscle and fat, and by failure of insulin to suppress hepatic glucose production in the liver.

The beta cell dysfunction, the other key component of T2D pathophysiology, involves a relatively selective defect in the ability of glucose to stimulate insulin secretion by beta cells, a temporal irregularity in the pulse and oscillations of insulin secretion, and a loss of the tight coupling between pulses of insulin secretion and pulse in glucose. This defect accounts for the failure of beta cells to compensate for increasing insulin-resistance and for the ultimate development of overt hyperglycaemia.

The disease often remains asymptomatic and undetected for years. People with T2D do not usually require injections of insulin, and they control their blood glucose levels by watching their diet, taking regular exercise, oral medication, and possibly insulin. But if T2 diabetic people are not diagnosed and treated, they can develop serious complications, which can result in early death. Indeed, as glycaemic control deteriorates over time, patients are at increased risk of microvascular complications (disease of the small blood vessels) including retinopathy, neuropathy and nephropathy, and macrovascular

complications (disease of the large blood vessels) including coronary heart disease, myocardial infarction and stroke. As a result, diabetes affects almost every element of the human body, (Figure 5).

The major diabetic complications



Source: *Diabetes Atlas* second edition, ©International Diabetes Federation, 2003

Figure 5 The major diabetic complications.

Diabetic patients are at increased risk of microvascular complications (disease of the small blood vessels) including retinopathy, neuropathy and nephropathy, and macrovascular complications (disease of the large blood vessels) including coronary heart disease, myocardial infarction and stroke.

Major risk factor
Demographic factors - Race and ethnic background (native American, Polynesian islanders, African-American) - Age \geq 45 years Family history: first-degree relatives with type 2 diabetes Obesity: BMI \geq 27 Kg/m ² with central fat distribution History of impaired fasting glucose or impaired glucose tolerance Hypertension (blood pressure \geq 140/90 mmHg) HDL cholesterol level \leq 0.90 mmol/l and/or a triglyceride level \geq 2.8 mmol/l
Other risk factors
Malnutrition in the first year of life, and particularly in utero Lifestyle factors - Physical inactivity - High-fat, low-carbohydrate diet - Alcohol (heavy consumption) - Smoking

Table 3. Main risk factors for T2D

6. “Glucotoxicity ” and its role in the induction of insulin resistance.

As said before, chronic hyperglycemia is a characteristic of the diabetic condition, while glucose toxicity is the main cause of diabetic complications, which are often observed only several years after the beginning of the illness (Reusch 2003)

Glucose toxicity, in its narrow sense, can indicate a clinical condition where control of diabetes in particular is poor, since hyperglycemia itself reduces the insulin secretion capacity of pancreatic β -cells, and the resultant increase in insulin resistance leads to further hyperglycemia. This vicious circle finally leads to the total incapacity of β -cells to secrete insulin (LeRoith 2002; Dubois, Vacher 2007). Despite the recognized implications of glucotoxicity in generation of tissue damage in diabetes, still little is known about the possible impact of hyperglycemia on insulin resistance.

It is well established that chronic hyperglycaemia *per se* promotes insulin resistance (Hager 1991; Davidson 1994) and a number of mechanisms have been proposed to explain hyperglycemia-induced insulin resistance; these include activation of oxidative stress (Evans 2007), increased formation of advanced glycosylation end products (AGE) or their precursor methylglyoxal (Miele 2003; Van Obberghen 2006) and increased flux through the hexosamine pathway (Buse 2006) by chronically elevated glucose concentrations. However, the molecular mechanism(s) through which hyperglycaemia exacerbates insulin resistance in diabetes have only partially been elucidated.

6.1 Glucotoxicity, oxidative stress and insulin-resistance

Glucotoxicity participates in insulin resistance of insulin-sensitive tissues, which include liver, skeletal muscle, and adipose tissue and oxidative stress is strongly suspected to be involved in this process. (Eriksson 2007). Indeed, it is known that incubation of primary adipocyte cells with chronic high glucose concentration can induce oxidative stress (Lu 2001). Moreover, it was demonstrated that oxidative stress induces insulin resistance in the 3T3-L1 adipocyte cell line by inhibiting the translocation of Glut 4 to the plasma membrane (Rudich 1998). Finally, it was found that oxidative stress can induce insulin resistance in intact rat muscle (Dokken 2008). Through *in vitro* studies and in animal models of diabetes, it has been found that antioxidants, especially α -lipoic acid (LA), improve insulin sensitivity (Maddux 2001). Several clinical trials, even if small and of short duration, have also demonstrated that treatment with vitamin E, vitamin C, or glutathione improves insulin sensitivity in insulin-resistant individuals and/or patients with type 2 diabetes (Evans 2003).

In vitro, ROS and oxidative stress lead to the activation of multiple serine kinase cascades (Kyriakis 1996). The insulin signaling pathway

offers a number of potential targets (substrates) of these activated kinases, including the insulin receptor (IR) and the family of IR substrate (IRS) proteins. For IRS-1 and -2, an increase in serine phosphorylation decreases the extent of tyrosine phosphorylation and is consistent with the attenuation of insulin action. In Chinese hamster ovary cells, stress activation of JNK/SAPK increased serine phosphorylation (at Ser307) and inhibited insulin-stimulated tyrosine phosphorylation of IRS-1. In L6 muscle cells, H₂O₂-mediated inhibition of insulin-stimulated glucose transport was accompanied by activation of p38 MAPK by H₂O₂ (Blair 1999). Insulin-stimulated glucose transport could be restored by LA or by a specific inhibitor of p38 MAPK (Blair 1999).

Activation of IKK- β , a serine kinase that regulates the NF- κ B pathway, also inhibits insulin action (Yuan 2001). Salicylates lower blood glucose (Yuan 2001), augment glucose-induced insulin secretion in normal subjects, and restore insulin secretion in patients with type 2 diabetes. In addition, salicylates inhibit IKK- β activity and restore insulin sensitivity, both in vitro and in vivo. Treatment with aspirin or salicylates alters the phosphorylation patterns of IRS proteins, resulting in decreased serine phosphorylation, increased tyrosine phosphorylation, and improved insulin action (Yuan 2001). Further support for the importance of IKK- β in insulin resistance is provided by results of recent gene knockout experiments in mice. IKK- β (+/-) heterozygotes were more insulin sensitive compared with their normal (+/+) littermates (Yuan 2001). Treatment of nine type 2 diabetic patients for 2 weeks with high dosages of aspirin (7g/day) resulted in reduced hepatic glucose production and fasting hyperglycemia and increased insulin sensitivity. Although these latter data are preliminary and require confirmation in an expanded study, they are consistent with a role for activation of IKK- β in the pathogenesis of insulin resistance. Furthermore, they suggest that inhibition of IKK- β might be an attractive pharmacological approach to increasing insulin sensitivity.

6.2 Glucotoxicity , increased intracellular formation of advanced glycation end-products, methylglyoxal and insulin resistance.

An additional deleterious effect of chronic hyperglycemia is the increased production of advanced glycation end products (AGEs). AGEs can arise from intracellular auto-oxidation of glucose to glyoxal (Wells-Knecht 1995), decomposition of the Amadori product (glucose-derived 1-amino-1 deoxyfructose lysine adducts) to 3-deoxyglucosone (perhaps accelerated by an amadoriase), and fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal (Thornalley 1990). These reactive intracellular dicarbonyls, glyoxal, methylglyoxal and 3-deoxyglucosone, react with amino groups of intracellular and extracellular proteins to form AGEs. Methylglyoxal and glyoxal are detoxified by the glyoxalase system

(Thornalley 1990). All three AGE precursors are also substrates for other reductases (Suzuki 1998). The modifications of proteins that lead to their glycation induce alterations in their biological properties as compared with their non-glycated counterparts. Production of intracellular AGE precursors damages target cells by three general mechanisms. First, intracellular proteins modified by AGEs have altered function. Second, extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with the receptors for matrix proteins (integrins) on cells. Third, plasma proteins modified by AGE precursors bind to AGE receptors on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production of reactive oxygen species. This AGE receptor ligation activates the pleiotropic transcription factor NF- κ B, causing pathological changes in gene expression (Hattori 2001).

Although glycated albumin has initially thought to be involved only in the generation of diabetes complications (Bronwlee 2005), there are accumulating evidences that strongly support the idea that glucose-derived products also affect insulin signaling. As said before, glycated albumin and AGEs exert their effects through specific cellular receptors found in different cell types and through the activation of several signaling pathways (Schmidt 2000; Wu 2001). Recently, Naitoh *et al.* (Naitoh 2001) demonstrated that, in human monocytic cells, glycated albumin induces the release of TNF- α , a factor involved in insulin resistance (Peraldi 1998).

Moreover in L6 skeletal muscle cells, exposure to human glycated albumin selectively inhibits the PI3K/PKB branch of the insulin signaling cascade, while leaving the Ras-ERK pathway and mitogenic action of the hormone unaltered. Mechanistically, HGA-mediated PI3K/PKB inhibition is dependent on a PKC α -mediated serine/threonine phosphorylation of IRS-1/2 proteins that leads to a strong decrease in insulin-regulated metabolic responses, such as glucose uptake and glycogen synthesis. Interestingly, activation of PKC α by chronic HGA treatment appears to be independent of ROS production. Thus, by deregulating intracellular insulin signaling, human glycated albumin exacerbates the insulin-resistant state (Miele 2003).

In addition it has been demonstrated that, in L6 skeletal muscle cells, methylglyoxal induces alterations of IRS-1 proteins and this represents a new concept of negative regulation of insulin signaling as they appear to be essentially based on chemical modifications of IRS proteins induced by a metabolite (Van Obberghen 2006).

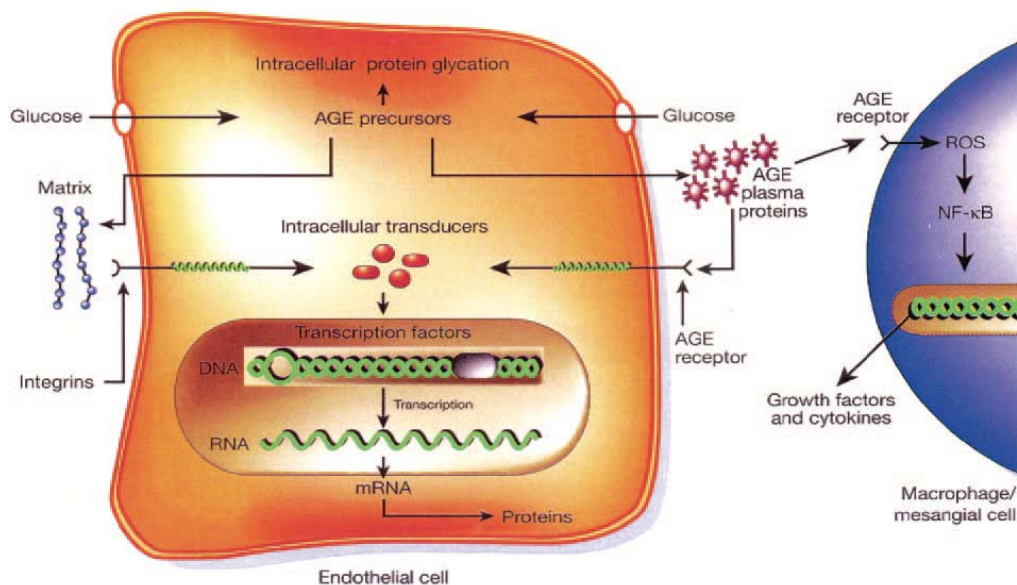


Figure 6 Mechanisms by which intracellular production of a advanced glycation end-product (AGE) precursors damages cells. Covalent modification of intracellular proteins by dicarbonyl AGE precursors alters several cellular functions. Modification of extracellular matrix proteins causes abnormal interactions with other matrix proteins and with integrins. Modification of plasma proteins by AGE precursors creates ligands that bind to AGE receptors, inducing changes in gene expression in different cells type.

6.3 Glucotoxicity, increased flux through the hexosamine pathway and insulin resistance.

The HSP is a relatively minor branch of the glycolytic pathway, encompassing about 3% of total glucose utilized (Marsahl 1991). Entry into the HSP is catalyzed by the first and rate limiting enzyme glutamine:fructose-6-phosphate (F-6-P) amidotransferase (GFAT), which converts F-6-P and glutamine to glucosamine-6-phosphate (GlcN-6-P) and glutamate. Subsequent steps metabolize GlcN-6-P to UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-N-acetylgalactosamine (UDP-GalNAc) and CMP-sialic acid, essential building blocks of the glycosyl side chains of glycoproteins, glycolipids, proteoglycans and gangliosides (Figure 7). UDP-GlcNAc is of particular interest because a) quantitatively it is the major end product of the HSP, b) it is an allosteric feedback inhibitor of GFAT, which regulates glucose entry into the pathway and c) it is the obligatory substrate of O-GlcNAc transferase (OGT). The latter is a cytosolic and nuclear enzyme, which catalyzes a reversible post-translational protein modification, whereby N-acetylglucosamine (GlcNAc) is transferred in O-linkage to specific

serine/threonine residues of numerous proteins (Buse 2006). The sites of O-GlcNAc modification (O-GlcNAcylation) are often identical or adjacent to known phosphorylation sites, suggesting a regulatory function (Comer 2001). Functional significance of O-GlcNAcylation has been reported for several proteins (Wells 2001), including the transcription factors Sp1 (Buse 2006), c-myc (Kamemura 2002), CREB (Lamarre-Vincent N 2003) and PDX-1 (Gao Y 2003), as well as cytosolic and nuclear enzymes, e.g. glycogen synthase (Parker 2004; Parker 2003) and RNA polymerase II (Comer 2001). Of particular interest in the context of insulin resistance is that insulin receptor substrates (IRS)-1 and 2 (Andreozzi 2004; Buse 2006) and probably also GLUT4 (Buse 2002) are subject to O-GlcNAcylation. The reversible, O-GlcNAc modification of proteins has been suggested by many investigators as a mechanism by which increased HSP activity could cause insulin resistance.

A role for excess glucose flux via HSP in insulin resistance was first proposed by Marshall et al in 1991, based on experiments using isolated rat adipocytes (Buse 2006). In this system, pre-exposure of the cells to insulin and high glucose act synergistically to induce resistance of glucose transport to subsequent acute stimulation by insulin.

There is considerable evidence indicating that increased activity of the HSP can cause insulin resistance in cell culture models and in rodents *in vivo*. In the model mentioned above, where sustained exposure to high glucose in the presence of insulin caused insulin resistance in adipocytes, treatment of the cells with inhibitors of GFAT activity prevented this effect. Furthermore, glucosamine, (GlcN) which enters the HSP bypassing GFAT, also caused insulin resistance. The effect of GlcN infusions on the development of insulin resistance in rodents, undergoing insulin clamp studies has been extensively studied (Buse 2006; Rossetti 1995). Rossetti reported in 1995, that infusion of GlcN increased the concentrations of UDP-GlcNAc in muscle, and markedly decreased insulin stimulated total body glucose utilization in healthy control, but not in diabetic rats, which were already insulin resistant (Rossetti 1995). Previous studies demonstrated that *in vitro* treatment of isolated muscles with GlcN inhibited the insulin response of glucose transport, without affecting insulin receptor and GLUT4 expression (Robinson 1993). Sustained hyperglycemia, which causes insulin resistance, also increased UDP-HexNAc concentrations in muscles (Robinson 1995). Transgenic mice overexpressing GFAT in skeletal muscle and adipocytes develop peripheral insulin resistance as they age, as determined by euglycemic insulin clamp studies. GLUT4 expression in muscle is unchanged, and the insulin resistance likely represents defective translocation of GLUT4 to the muscle cell membrane, or a defect in docking and/or incorporation of GLUT4 into the plasmalemma of muscle *in vivo* (Hebert 1996). The mechanism by

which GlcN or the increased glucose flux via the HSP determines insulin resistance is still debated. Several studies, performed both “in vitro” and “in vivo” , showed a defect in GLUT4 translocation in response to insulin (Buse 2006) or a block in signal transduction downstream of PI-3-kinase, at the level of Akt activation (Buse 2006). Alternatively it has been well documented that increased glucose flux into cells and via HSP promotes the O-GlcNAc modification on Ser/Thr residues of selected proteins, and this process is believed to mediate the effects of HSP. The process is catalyzed by a cytosolic/nuclear enzyme, O-GlcNAc transferase (OGT), which is responsive to UDP-GlcNAc concentrations in the physiological range (Buse 2006). Numerous investigators have reported increased O-GlcNAc modification of proteins, (demonstrated immunologically), in tissue culture or in experimental animals, under conditions of insulin resistance where glucose flux into cells was chronically increased, or after exposure to GlcN or in cells overexpressing GFAT (Buse 2002; McClain 2002; Patti 1999; Rossetti 2000; Yki-Jarvinen 1998). However, the proteins were only rarely identified, and a causal connection was not established. More recently, more direct evidence has emerged. The enzyme, which removes the O-GlcNAc modification from Ser/Thr, is O-GlcNAcase, has been cloned (Buse 2006) and a pharmacological agent, which is a competitive inhibitor of O-GlcNAcase has been developed, (PUGNAC) (Haltiwanger 1998). When 3T3-L1 adipocytes are incubated with PUGNAC, the removal of O-GlcNAc from proteins is inhibited, and the cells develop insulin resistant glucose transport, as well as inhibition of insulin stimulated Akt activation.

There are a very few clinical studies examining the role of HSP in insulin resistance. Yki Jarvinen reported in 1996 that GFAT activity was increased in muscle biopsies obtained from insulin resistant patients with type 2 diabetes (Yki-Jarvinen 1996). Two papers studied the effect of GlcN infusions on insulin responsiveness in humans. In one paper, minimal effects were observed, *i.e.* blunted insulin secretory response in response to a glucose load and mildly increased fasting glucose levels (which could represent the known inhibitory effect of GlcN on islet glucokinase), but in neither paper were any effects noted on glucose utilization during an euglycemic insulin clamp or on hepatic glucose production. Thus, humans may be less sensitive to the insulin resistance promoting effect of GlcN than rodents (Pouwels 2001). The role of the HSP as a determinant in the development of insulin resistance is more problematic. While it is clear that overexpression of GFAT or of O-GlcNAc transferase can produce insulin resistance in mice, the requirement for increased HSP flux for the establishment of glucose induced insulin resistance has been questioned. Nevertheless, with the rapidly increasing knowledge of the O-GlcNAc modification of selected proteins and their regulation, and the fact that uncontrolled

diabetes affects this process, it seems likely that a role for the HSP in the development of the metabolic syndrome and insulin resistance will prevail.

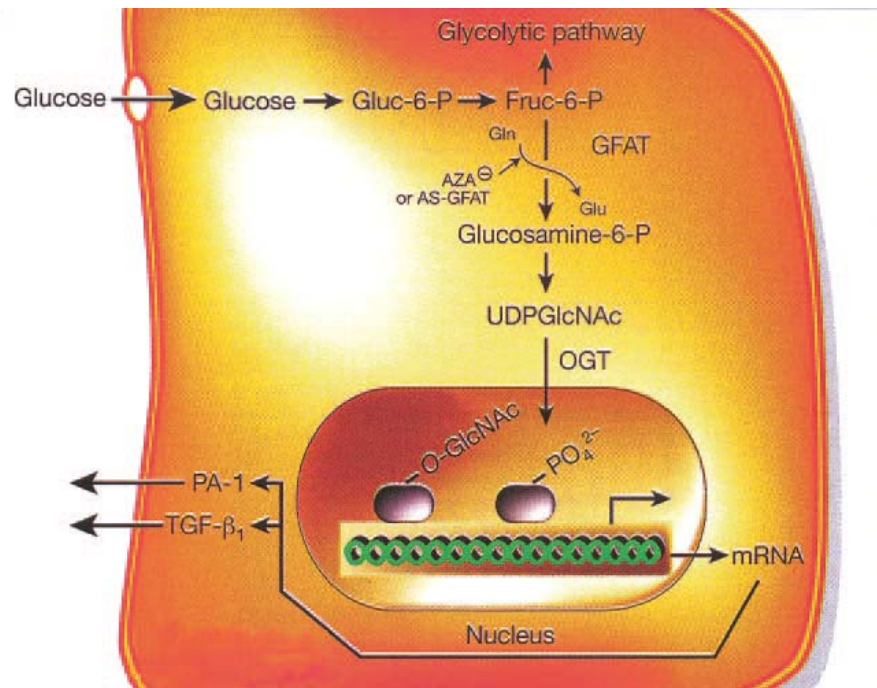


Figure 7 The hexosamine pathway. The glycolytic intermediate fructose-6-phosphate (Fru-6-P) is converted to glucosamine-6-phosphate by the enzyme glutamine:fructose-6 phosphate amidotransferase (GFAT). Intracellular glycosylation by the addition of N acetylglucosamine (GlcNAc) to serine and threonine is catalysed by the enzyme O GlcNAc transferase (OGT). Increased donation of GlcNAc moieties to serine and threonine residues of transcription factors such as Sp1, often at phosphorylation sites, increases the production of factors as PAI-1 and TGF-β₁. AZA, azaserine; AS-GFAT, antisense to GFAT.

6.4 Glucotoxicity , Endoplasmic Reticulum stress and insulin resistance.

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 due to these unfolded or misfolded proteins that the ER has evolved a coping system known as the Unfolded Protein Response (UPR) (Kaufman 1999; Liang 2006). 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 (Mori 2000; Oyadomari 2002). 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), the inositol requiring-1 (IRE-1) and the activating transcription factor 6 (ATF6) (Shi 1998; Wang 1998; Yoshida 1998). The first response, mediated by PERK, is translational attenuation, to reduce the load of new protein synthesis and prevent further accumulation of unfolded proteins (Harding 2002). The second response is upregulation of genes encoding ER chaperone proteins such as the immunoglobulin heavy chain-binding protein/glucose-regulated protein 78kDa (BiP/GRP78) and the glucose-regulated protein 94kDa (GRP94), to increase the ER protein-folding capacity (Yoshida 1998; Kozutsumi 1988). The third response is transcriptional activation of genes involved in the degradation of misfolded protein in the ER by the ubiquitin-proteasome system, called the ER-associated degradation (Travers 2000). 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 (Oyadomari 2002) (Figure 8).

ER stress plays an important role in several human diseases, including Type 2 diabetes; indeed, recent studies reported that ER stress is involved both in pancreatic beta-cell dysfunction (Kaneto 2005; Scheuner 2001; Shi 2003) and peripheral insulin resistance (Nakatani 2005; Ozcan 2004). 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 metabolizes ~3% of glucose entering the cell, and the final product of this pathway, UDP-N-acetylglucosamine, as other nucleotide hexosamines, are used in the ER as substrates for protein glycosylation (Rossetti 2000; Pirola 2004). Although quantitatively utilising a small fraction of glucose, HBP is an important contributor to the insulin-resistant state. Several studies showed, 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 (Pirola 2004) as well as *in vivo* (Rossetti 1995). However, the precise mechanisms by which GlcN induces insulin-resistance have not been conclusively established in these studies.

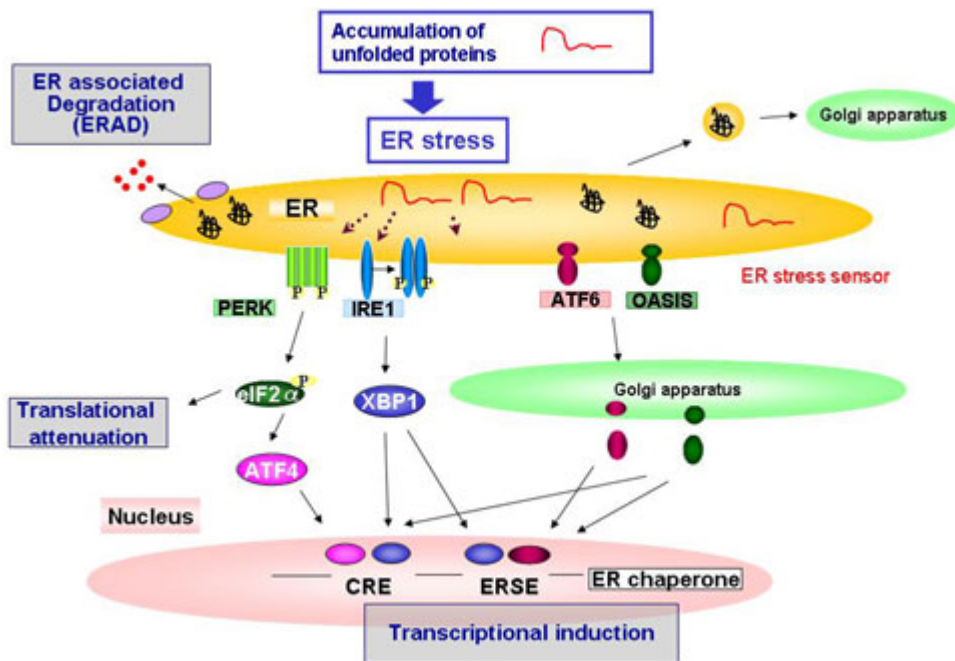


Figure 8 Mechanism of UPR activation. In mammalian cells, the UPR is characterized by transcriptional activation of chaperone and protein-folding genes, regulation of growth arrest and apoptosis genes, and repression of global protein synthesis. The ER chaperone protein BiP/GRP78 acts as the principal regulator of three ER-resident transmembrane proteins, PERK (double-stranded RNA-activated protein kinase-like ER kinase), IRE-1 (inositol requiring-1) and ATF6 (activating transcription factor 6) which are primary sensors and transducers of the UPR.

Aim of the study

Recently it has been demonstrated that chronic hyperglycaemia induces ER stress in cultured pancreatic β cells, causing the increase in Bip/GRP78 and CHOP/GADD153 and activating SREBP-1 (Sterol Regulatory Element Binding Protein-1). *In vivo* and *in vitro* experiments established that chronic hyperglycaemia in diabetic humans and rats could induce or worsen the insulin-resistance state and, in some cases, the process seem to be linked to the UPR activation. Glucosamine (GlcN) is one of the molecule that mediate glucotoxicity effects. Indeed recent data suggest that in hyperglycaemia conditions, glucose, through GlcN production, induces insulin-resistance; although the precise mechanism by which it happens is not yet clear. Recent evidences show that high GlcN levels induce ER stress in cultured hepatocytes, monocytes and smooth muscle cells (Werstuck 2006). Even if different studies support the role of ER stress in the alteration of insulin sensitivity in the liver and adipose tissue there are few evidences regarding the effect of ER stress in skeletal muscle. Thus, in this work we sought to evaluate the possible role of ER stress in the induction of insulin resistance by glucosamine in skeletal muscle cells.

Methods

Materials. DMEM, FBS, FCS, L-glutamine and BSA were from Invitrogen (Paisley, UK). Thapsigargin (Thap), GlcN, 4-phenyl butyric acid (PBA), 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: Ultrosor G (Pall Biosepra, Cergy, France), [¹⁴C]2-deoxy-glucose (2-DG) (Perkin Elmer, Waltham, MA, USA), insulin (Novo Nordisk, Bagsværd, Denmark), GLUT4 (Abcam, Cambridge, UK), myocyte enhancer factor 2 a (MEF2A), peroxisome proliferators-activated receptor- γ coactivator 1 α (PGC1 α), eukaryotic translation initiation factor 2 α (eIF2 α) and phospho-eIF2 α antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), siRNA and siPORT™ NeoFX™ Transfection Agent (Applied Biosystems, Carlsbad, CA, USA). Azaserine (Aza), O-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino) and N-phenylcarbamate (PUGNAc) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ApoSENSOR ADP/ATP Ratio Assay Kit was from BioVision, Inc (Mountain View, CA, USA).

Cell culture procedures. Human cell cultures from lean subjects were established as described in (Bergstrom 1975; Gaster 2001) (Table 3). Cells were cultured in DMEM supplemented with 2% (vol/vol) FCS, 2% (vol/vol) Ultrosor 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 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 in (Cassese 2008).

2-DG uptake. 2-DG uptake was measured as reported previously (Miele 2003). Briefly, myotubes were incubated in DMEM supplemented with 0.25% (wt/vol) BSA for 24 h in the presence or absence of different compounds. Cells were incubated in glucose-free HEPES buffer [5 mmol/L KCl, 120 mmol/L NaCl, 1.2 mmol/L MgSO₄, 10 mmol/L NaHCO₃, 1.2 mmol/L KHPO₄, and 20 mmol/L HEPES, pH 7.8, 2% (wt/vol) albumin], then stimulated with 100 nmol/L insulin for 30 min, supplemented during the final 10 min with 0.2 mmol/L [¹⁴C]2-DG. Cells were then solubilized and the 2-DG uptake was quantified by liquid scintillation counting.

RNA extraction and Real-time RT-PCR

Total cellular RNA was isolated from human and rat muscle cells using the RNeasy kit (QIAGEN Sciences, Germany). For Real-time RT-PCR analysis, 1 µg of cell RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen CA). PCR reactions were analyzed using Platinum SYBR Green qPCR Super-UDG using an iCycler IQ multicolour Real Time PCR Detection System (Biorad, CA). All reactions were performed in triplicate and GAPDH was used as an internal standard.

Western Blot analysis . For Western blot analysis, cells were solubilized in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $\text{Na}_2\text{P}_2\text{O}_7$, 2 mM Na_3VO_4 , 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 10 µg/ml aprotinin) for 2 h at 4°C. Cell lysates were clarified by centrifugation at 5,000 x *g* for 20 min, separated by SDS-PAGE (or firstly immunoprecipitated), and transferred into 0.45-µm Immobilon-P membranes (Millipore, Bedford, MA). Upon incubation with primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions.

ATP Measurement. ATP concentrations were determined according to manufacturer's recommendations. Briefly, differentiated myotubes were treated or not with different concentrations of GlcN (5, 7.5, 10, 20 and 40 mmol/L) for 24 h. Cells were then incubated with the Nucleotide Releasing Buffer for 5 minutes at room temperature and solubilised. After adding the ATP Monitoring Enzyme in the cell lysates ATP concentrations were quantified in a luminometer.

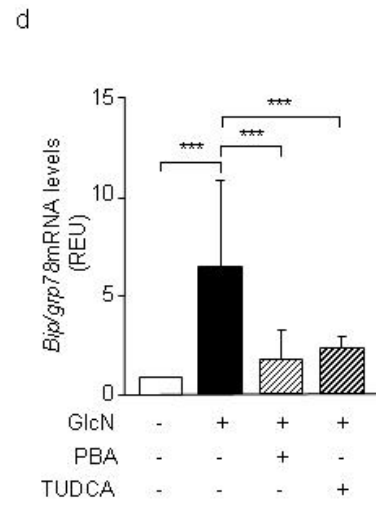
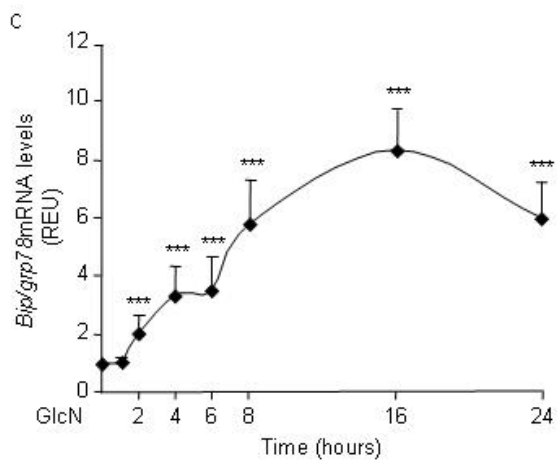
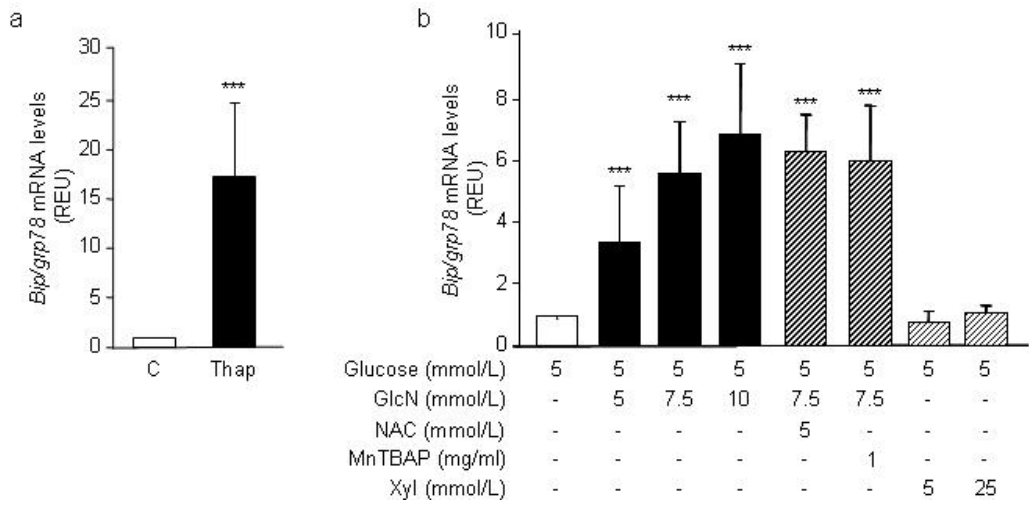
Chromatin Immunoprecipitation Assay . ChIP assay was performed as reported (Ungaro 2008). Vehicle- or treated myotubes were fixed with 1% (vol/vol) formaldehyde at 37 °C. The fixed cells were lysed in a SDS lysis buffer [1% (wt/vol) SDS, 10 mmol/L EDTA, and 50 mmol/L Tris-HCl, pH 8.1], incubated on ice, 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 analyzed regions.

ATF6 siRNA-mediated Knockdown. Cells were transfected with 5 nmol/L of siRNA negative control and siRNA-Atf6 Sense (GCUUGUCAGUCACGAAAGAtt) and Antisense (UCUUUCGUGACUGACAAGCag) according to manufacturer's recommendations and processed 48 h after transfection.

Statistical procedures. Data were analysed with Statview software (Abacus-concepts) by one-factor analysis of variance. *p* values of less than 0.05 were considered statistically significant.

Results

To investigate the role of GlcN in the ER stress induction, differentiated L6 skeletal muscle cells were treated with different concentrations of GlcN. The classical ER stress inducer Thap, an inhibitor of SERCA ATP-ases, was used as control of ER stress induction (Liang 2006). In L6 myotubes, Thap induced a 17-fold increase of the chaperon *Bip/grp78* mRNA (Figure 9a), 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 (Figure 9b). Interestingly, pre-treatment of cells with azaserine, a non-specific but commonly used inhibitor of the Glutamine:fructose 6 phosphate amidotransferase (GFAT) (Marshall 1991; Buse 2006), the rate-limiting enzyme of the HBP, prevented high glucose (HG)-induced-ER stress (Figure 9e). In addition, treatment of cells with the peptide O-GlcNAc- β -N acetylglucosaminidase inhibitor PUGNAc, did not increase *Bip/grp78* mRNA levels, suggesting that enhanced O-linked glycosylation was not responsible for the induction of ER stress (Figure 9e). To evaluate whether GlcN-induced ER stress could be mediated by oxidative stress, L6 myotubes were pre-treated with two anti-oxidants, the glutathione precursor NAC and the superoxide dismutase mimetic MnTBAP. Pre-treatment 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 from oxidative stress in skeletal muscle cells (Figure 9b). Then we evaluated the effects of different concentrations of GlcN on ATP intracellular levels. GlcN depleted ATP pool only at the highest concentrations, suggesting that GlcN-induced ER stress was not dependent from ATP depletion in skeletal muscle cells (Figure 9f). As expected, xylose did not induce *Bip/grp78* mRNA increase even at high concentrations (Figure 9b), 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 raised up to 16 h (Figure 9c). To investigate whether chemical chaperones could prevent GlcN induced ER stress, we analyzed *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 stabilize protein conformation and to improve ER folding capacity (Ozcan 2006), or 5 mmol/L TUDCA, a bile acid derivative that also modulates ER function (Ozcan 2006). Both PBA and TUDCA almost completely prevented the effect of GlcN on *Bip/grp78* mRNA (Figure 9d). These data suggest that GlcN is able to induce ER stress in skeletal muscle cells and that this effect is prevented by chemical chaperones.



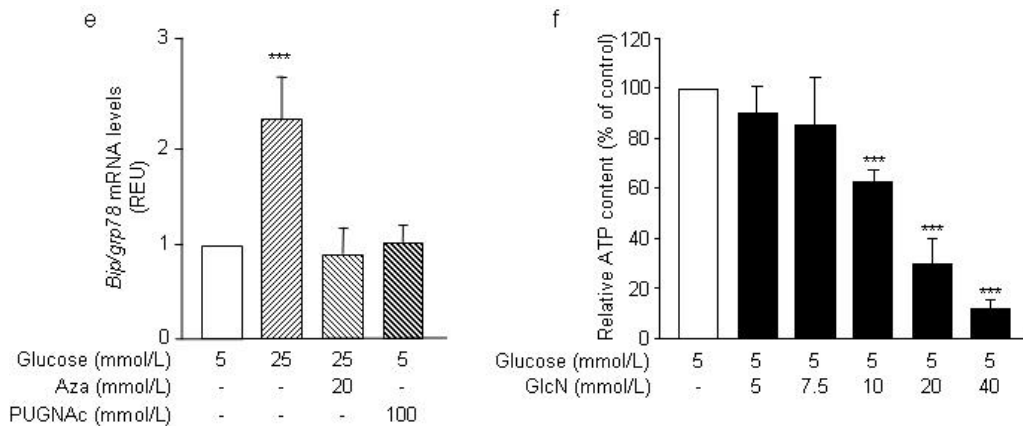
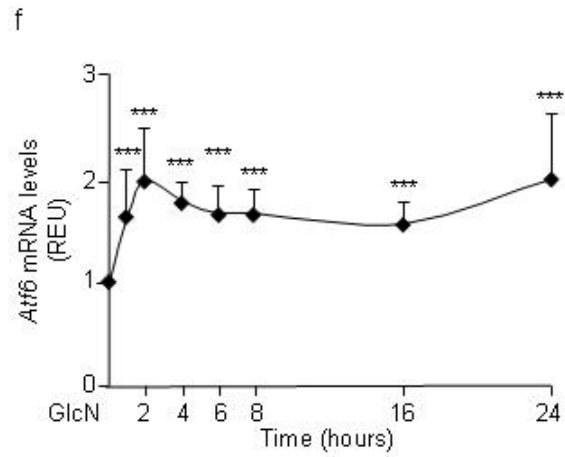
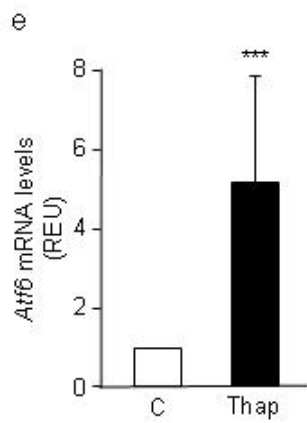
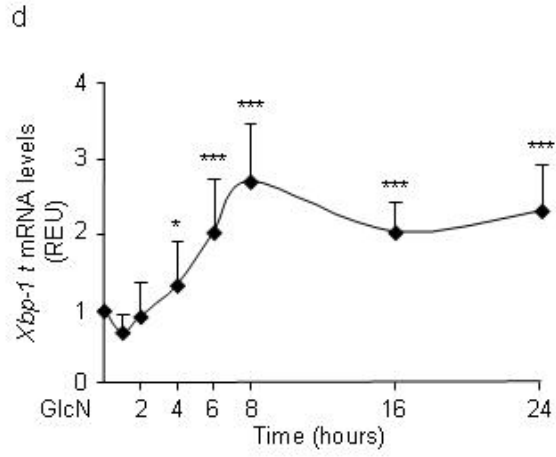
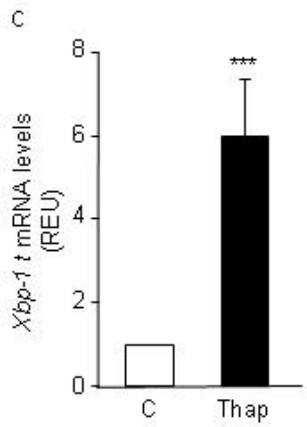
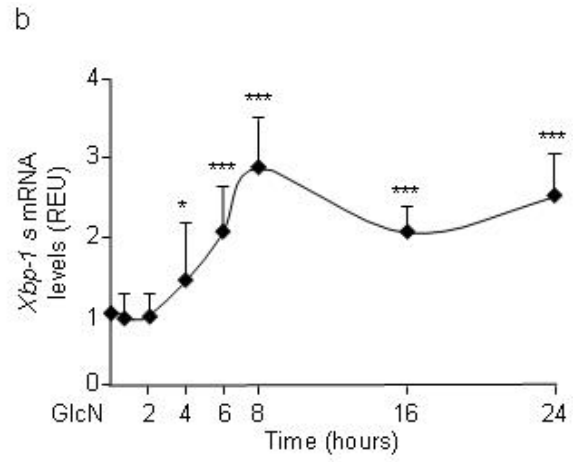
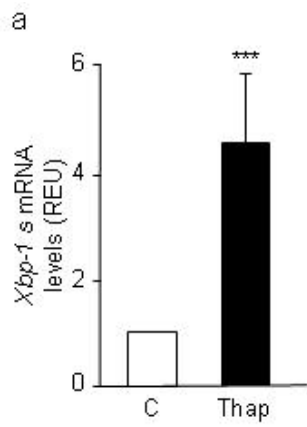


Figure 9 *GlcN induces BIP/GRP78 in L6 myotubes*. **a–e** *Bip/grp78* mRNA was determined by real-time RT-PCR analysis of total RNA isolated from myotubes, using *Gapdh* as internal standard. mRNA levels in treated cells are relative expression units (REU) to those in control (C; mean \pm SD; $n = 8$). *** $p < 0.001$. **a** L6 cells were treated with 0.5 μ mol/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. **e** L6 cells were pre-treated or not with 20 μ mol/L Aza for 20 min and then cultured in presence of 25 mmol/L glucose or 100 μ mol/L PUGNAc, as indicated above, for 24 h. **f** L6 cells were treated or not with different concentrations of GlcN (5, 7.5, 10, 20 and 40 mmol/L) for 24 h. ATP content in treated cells is relative to those in control cells in which is expressed as 100% (mean \pm SD; $n=3$). ***, $P < 0.001$.

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 analyzed. The mRNA levels of the spliced active form of the X-box binding protein-1 (*Xbp-1 s*) increased significantly following both Thap (Figure 10a) and GlcN treatments (Figure 10b). Time-course experiments showed that the increase of *Xbp-1 s* peaked at 8 h of GlcN treatment and remained higher than basal level up to 24 h (Figure 10b). In addition, also the mRNA levels of the total form of *Xbp-1* (*Xbp-1 t*) and *Atf6* were significantly increased upon both Thap (Figure 10c, e) and GlcN treatments (Figure 10d, f). Furthermore, phosphorylation of eIF2 α was evident as early as 30 min after both GlcN and Thap treatment, and it persisted up to 24 h following GlcN treatment (Figure 10g). These data suggest that GlcN caused UPR activation in L6 myotubes.



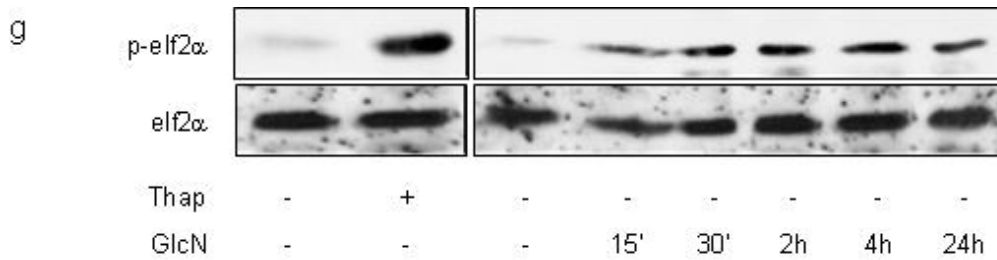


Figure 10 GlcN induces UPR activation in L6 myotubes. a–g L6 cells were treated with 0.5 $\mu\text{mol/l}$ Thap for 30 min, followed by 24 h without Thap, or with 7.5 mmol/l GlcN for the indicated times (a–f). *Xbp1 s* (a, b), *Xbp1 t* (c, d) and *Atf6* (e, f) mRNAs in treated cells are relative expression units (REU) to those in control (C; mean \pm 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 $\mu\text{mol/l}$ 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-eIF2 α Ser51 and eIF2 α specific antibodies ($n = 5$)

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 uptake the glucose analogue 2-DG upon insulin stimulation, compared to control cells (Figure 11a). Similar results were obtained when cells were treated with Thap (data not shown) and HG (Figure 11b). To verify the hypothesis that GlcN may impair glucose uptake in L6 cells through ER stress induction, we analyzed insulin-induced glucose uptake in cells treated with GlcN in the presence of PBA or TUDCA. Interestingly, both PBA and TUDCA prevented GlcN (Figure 11a) and HG (Figure 11b) effects on insulin-stimulated glucose uptake suggesting that ER stress caused insulin resistance in skeletal muscle cells.

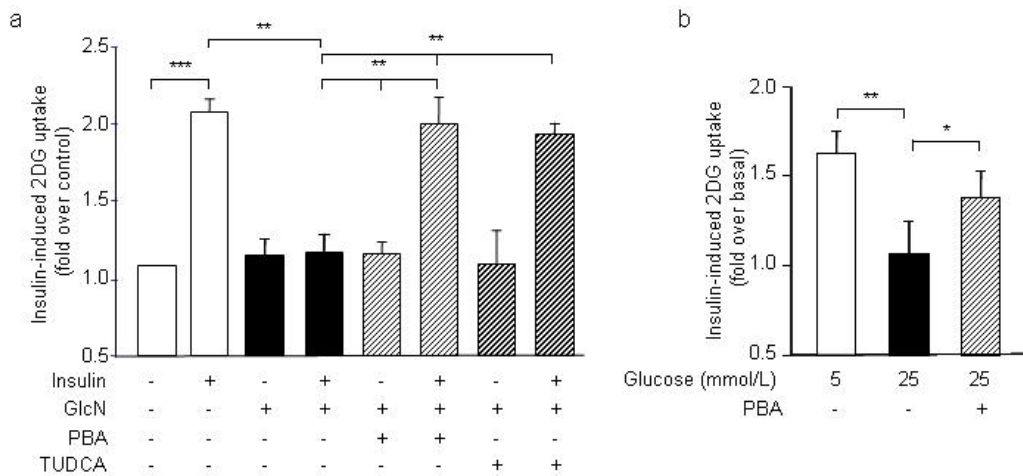


Figure 11 Effect of ER stress on 2-DG glucose uptake and *Glut4* expression in L6 myotubes. a-b L6 cells were pre-treated or not for 1 h with 10 mmol/L PBA or 5 mmol/L TUDCA and then treated with 7.5 mmol/L GlcN or 25mmol/L Glu for 24 h. 2-DG uptake was measured following 30 min of insulin stimulation (mean±SD; n=5). **, $P < 0.01$; ***, $P < 0.001$.

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 (Klip 2009). Time-course analysis showed that *Glut4* mRNA levels were significantly decreased as early as 6 h after GlcN treatment, and were reduced by about 50% after 16 h of treatment (Figure 12a). *Glut4* mRNA levels did not show any significant variation when L6 myotubes were treated with GlcN in the presence of PBA (Figure 12b). 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 pre-treatment with PBA (Figure 12c). As for GlcN, both Thap and HG treatment (data not shown), induced a significant decrease of both *Glut4* protein and mRNA levels compared to control cells. These data indicated that GlcN and HG reduced *Glut4* expression through the induction of ER stress.

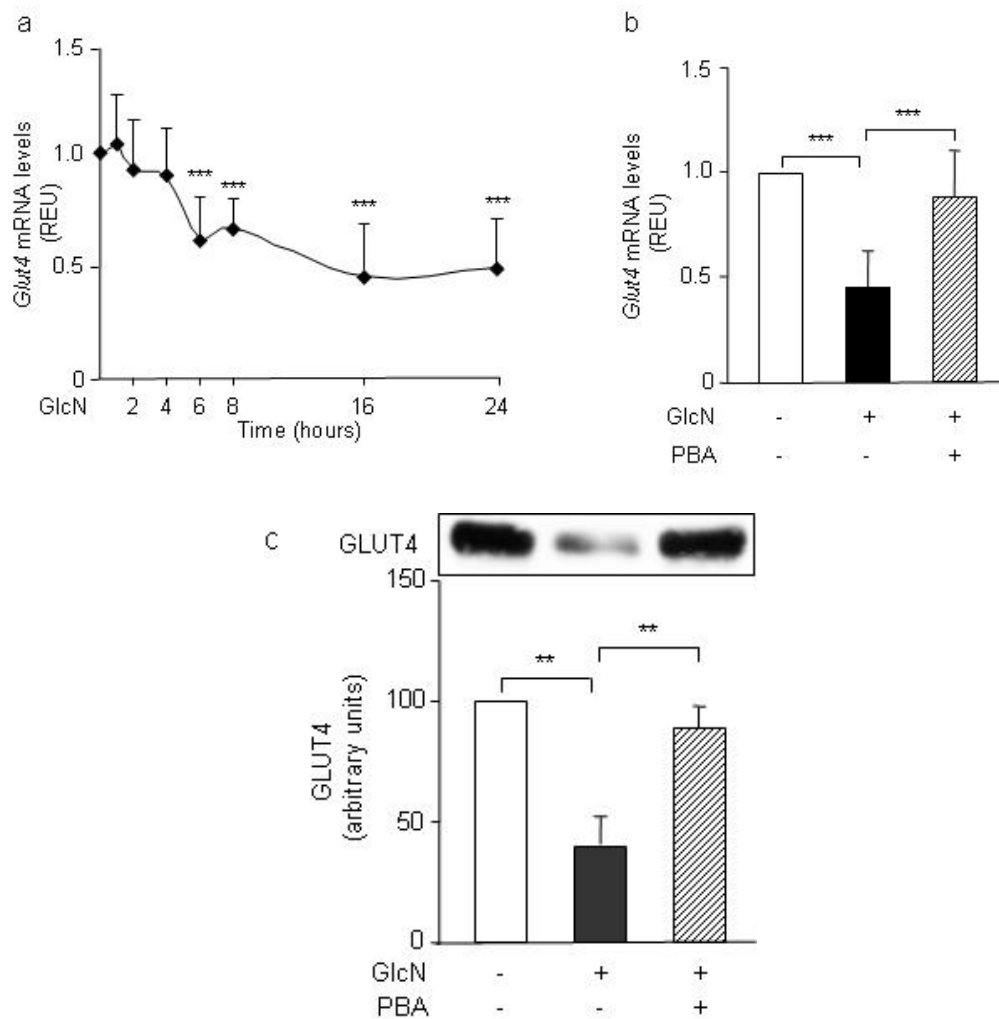
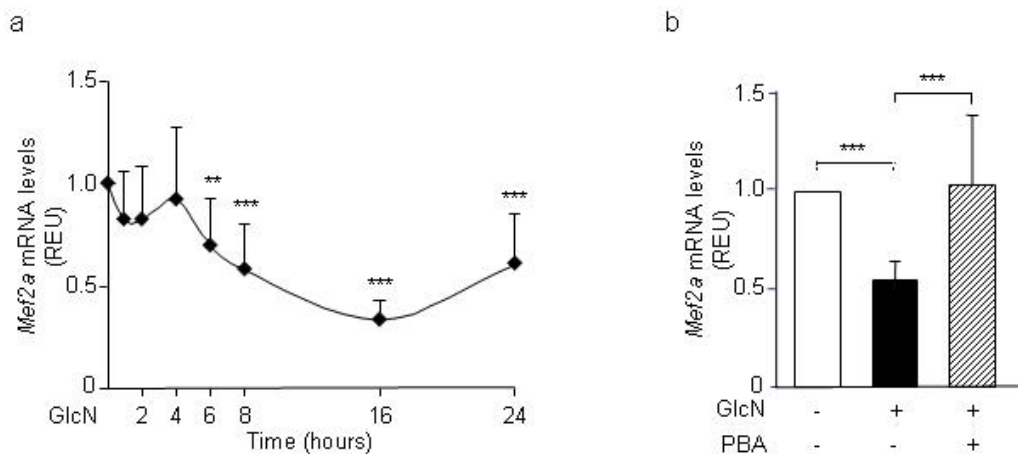


Figure 12 *GlcN* effect on *Glut4* expression (a-c) *Glut4* mRNA was determined by real-time RT-PCR analysis of total RNA isolated from myotubes, using *Gapdh* as internal standard. mRNA levels in treated cells are relative to those in control (mean±SD; $n=5$). ***, $P < 0.001$. (a) Time-course of *Glut4* mRNA in L6 cells treated with 7.5 mmol/L *GlcN* for the indicated times. (b) *Glut4* mRNA was detected in L6 cells pre-treated or not with 10 mmol/L PBA for 1 hour, and then treated with 7.5 mmol/L *GlcN* for 24 h. (c) L6 cells pre-treated or not with 10 mmol/L PBA for 1 hour, and then treated with 7.5 mmol/L *GlcN* for 24 h, were solubilised and equal amounts of proteins (80 μ g/sample) were analyzed by WB using GLUT4 specific antibody ($n=5$). A representative autoradiograph is shown. **, $P < 0.01$.

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). Then, to gain further insight into the mechanisms leading to *Glut4* reduced transcription, we analyzed 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 the *Mef2a* mRNA expression as early as 6 h after the treatment with a 60% reduction upon 16 h (Figure 13a). Similarly, MEF2A coactivator peroxisome *Pgc1 α* was reduced by 50 % upon 16 h of treatment when compared to control cells (Figure 13c). As for GlcN, both Thap (data not shown) and HG treatment (data not shown) induced a significant decrease of *Mef2a* and *Pgc1 α* expression levels. L6 cells treated with GlcN in the presence of PBA exhibited no differences in the mRNA levels of both *Mef2a* (Fig. 13b) and *Pgc1 α* (Figure 13d) when compared to control cells, confirming that also *Mef2a* and *Pgc1 α* reduced expression was dependent on GlcN-induced ER stress. To establish whether the reduction of *Mef2a* and *Pgc1 α* expression induced by ER stress was paralleled by a reduced binding of these two proteins to the *Glut4* promoter, we performed ChIP and ReChip experiments in L6 cells treated with GlcN. *Mef2a* binding to *Glut4* promoter showed a 60% decrease upon GlcN treatment compared to control cells (Figure 13e). Similarly, *Pgc1 α* indirect binding to *Glut4* promoter measured by ReChip assay was reduced by 40% upon GlcN treatment compared to control cells (Figure 13f). These data indicate that GlcN-induced ER stress causes the transcriptional inhibition of *Glut4* at least in part by reducing both *Mef2a* and *Pgc1 α* mRNA levels and their binding to the *Glut4* promoter.



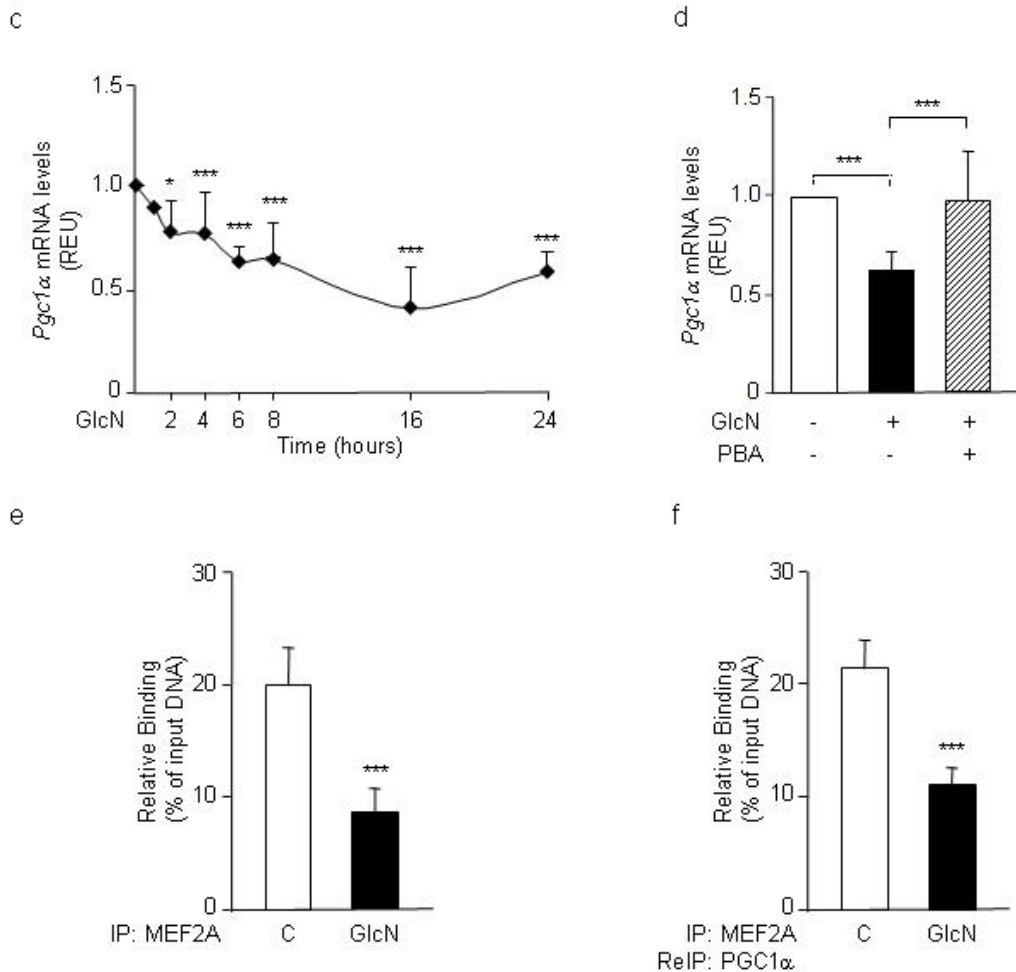
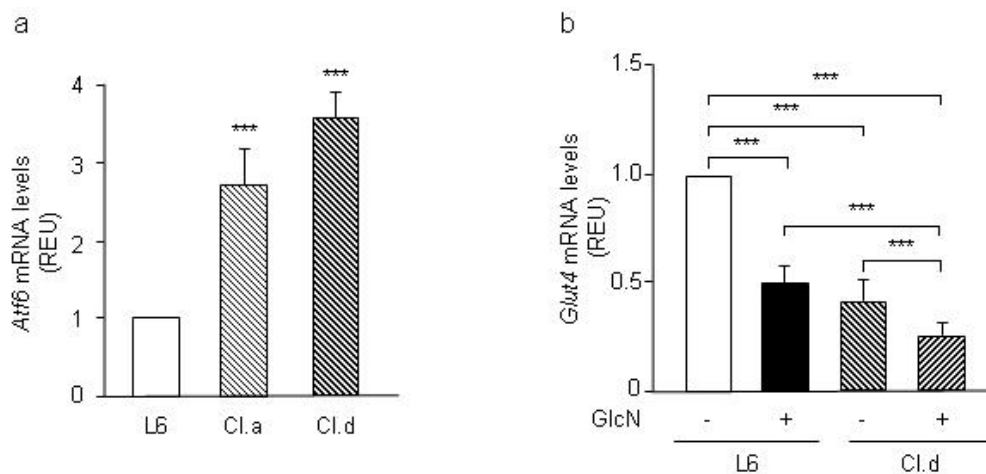


Figure 13 Effect of ER stress on *Mef2a* and *Pgc1α* mRNA expression and *Glut4* promoter binding in L6 myotubes. (a-d) *Mef2a* and *Pgc1α* mRNAs were determined by real-time RT-PCR analysis, using *Gapdh* as internal standard. mRNA levels in treated cells are relative to those in control (mean±SD; n=4). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (a) Time-course of *Mef2a* mRNA in L6 cells treated with 7.5 mmol/L GlcN for the indicated times. (b) Time-course of *Pgc1α* mRNA in L6 cells treated with 7.5 mmol/L GlcN for the indicated times. (c) *Mef2a* mRNA was detected in L6 cells pre-treated or not with 10 mmol/L PBA for 1 hour, and then treated with 7.5 mmol/L GlcN for 24 h. (d) *Pgc1α* mRNA was detected in L6 cells pre-treated or not with 10 mmol/L PBA for 1 hour, 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 hours as described in Methods and immunoprecipitated (IP) with MEF2A antibody (e) or re-immunoprecipitated (RelIP) with PGC1α antibody (f). IP were then amplified by Real Time RT-PCR analysis using specific primers for the analyzed regions (mean±SD; n=3). ***, $P < 0.001$.

To understand the mechanisms involved in the transcriptional inhibition of *Glut4*, *Mef2a* and *Pgc1 α* by ER stress, we sought to evaluate the role of the activating transcription factor 6 (*Atf6*), a gene whose expression has been reported to be up-regulated during ER stress and may cause inhibition of gene expression via up-regulation of the orphan nuclear receptor small heterodimer partner (SHP) in pancreatic beta-cells (Seo 2008). To test the hypothesis that the overexpression of ATF6 is sufficient to impair *Glut4* expression, we generated L6-cell lines stably overexpressing *Atf6* (Figure 14a). In *Atf6* overexpressing cells, *Glut4* mRNA levels were reduced by 60% in basal condition (Figure 14b), and were further decreased by 75% upon GlcN treatment (Figure 14b). *Atf6* overexpression also induced similar decreases of *Mef2a* and *Pgc1 α* expression compared to control cells (data not shown). Treatment with a specific small interference RNA (siRNA) for *Atf6* significantly inhibited the mRNA level of *Atf6* both in untreated and GlcN treated cells (Figure 14c). As expected, the transfection of cells with a siRNA for a non eukaryotic gene, used as negative control, did not affect the up-regulation of *Atf6* induced by GlcN (Figure 14c). In addition, the siRNA for *Atf6* completely prevented GlcN-induced down-regulation of *Glut4*, *Mef2a* and *Pgc1 α* (Figure 14d). L6 cells were also transfected with a siRNA for *Shp*. As expected, the treatment with the siRNA for *Shp* significantly inhibited the mRNA level of *Shp* both in untreated and GlcN treated cells (data not shown); by contrast, it was not able to prevent the GlcN-induced down-regulation of *Glut4*, *Mef2a* and *Pgc1 α* (data not shown). 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 from *Shp* activation.



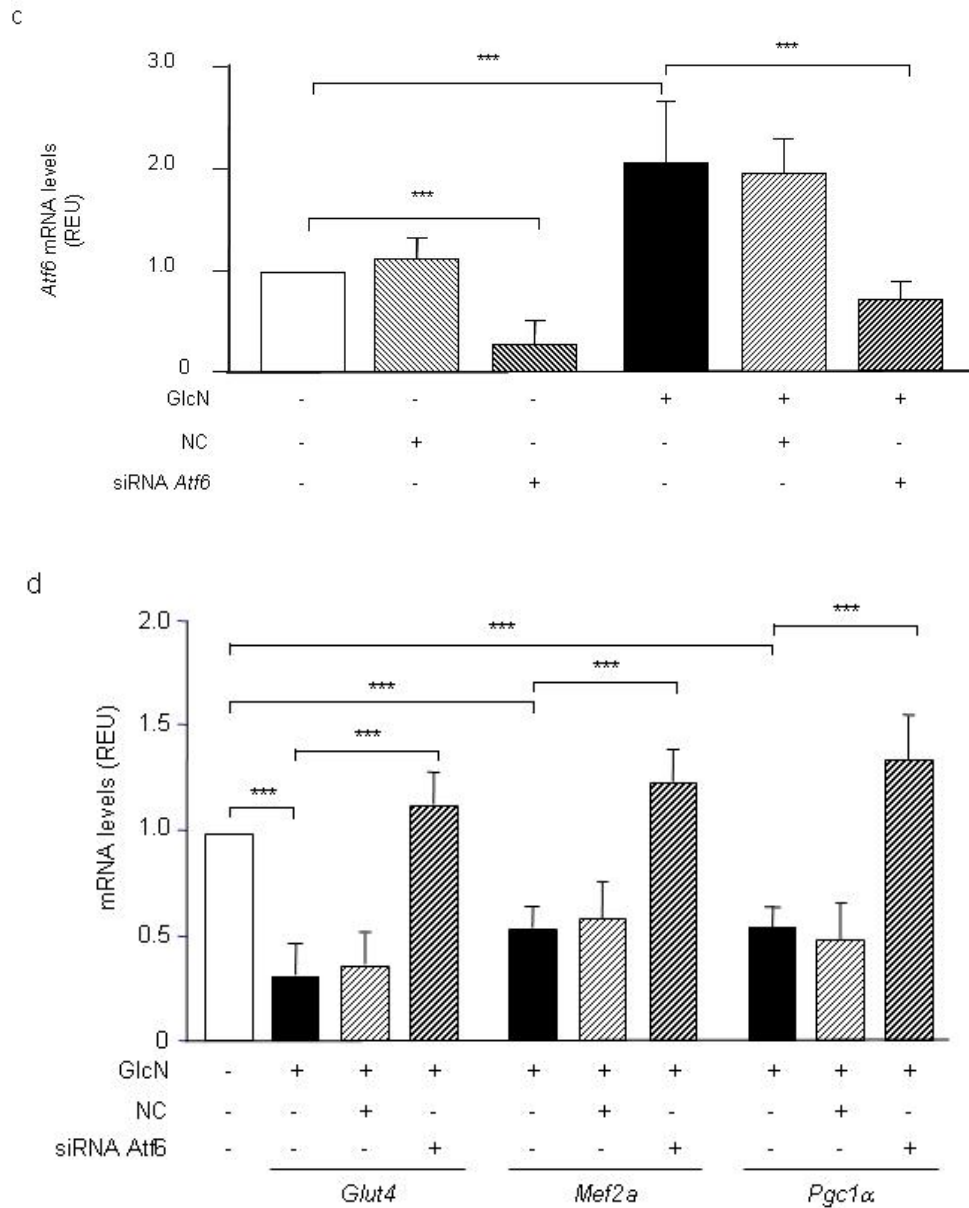
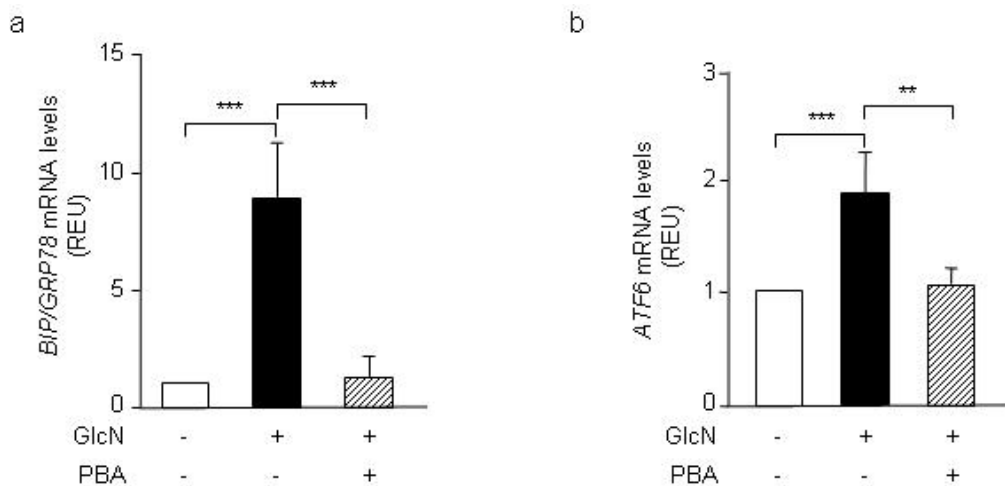


Figure 14 Role of Atf6 on GlcN-induced ER stress in L6 myotubes. (a-b) L6 cells were stably transfected with *Atf6* cDNA. *Atf6* (a) and *Glut4* (b) mRNAs in clones are relative to those in untransfected cells (L6) and were determined by real-time RT-PCR analysis using *Gapdh* as internal standard (mean±SD; $n=3$). ***, $P < 0.001$. (a) *Atf6* mRNA was detected in several clones. Two clones (termed clones a and d) overexpressing *Atf6* by 2.5- and 3.5-fold were further characterized. (b) *Glut4* mRNA was detected in L6 cells untransfected or stably transfected with *Atf6* cDNA treated with 7.5 mmol/L GlcN for 24 h. (c-d) L6 cells were transiently transfected with siRNA-*Atf6* or with a negative control siRNA, and then treated with 7.5 mmol/L GlcN for 24 h. 48 h upon 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 to those in control cells and were determined by real-time RT-PCR analysis using *Gapdh* as internal standard (mean±SD; $n=3$). ***, $P < 0.001$.

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 (Gaster 2001). In differentiated human muscle cells, GlcN induced a significant increase of both *BiP/GRP78* and *ATF6* mRNA levels (Figure 15a, b), indicating that also the human skeletal muscle cells were sensitive to GlcN-induced ER stress. In addition, pre-treatment with PBA completely prevented the increase of both *BiP/GRP78* and *ATF6* mRNAs observed upon GlcN treatment (Figure 15a, b), confirming in human skeletal muscle cells the results obtained in the rat cells. In human myotubes GlcN treatment reduced the expression both of *GLUT4*, *MEF2A* and *PGC1 α* mRNAs (Figure 15c-e), and completely inhibited the uptake of 2DG upon insulin stimulation compared to control cells (Figure 15f). 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 *PGC1 α* (Figure 15c-e) and a rescue of insulin-induced 2DG uptake (Figure 15f) when compared to control cells. Thus, GlcN-induced ER stress impairs insulin-sensitivity in human skeletal muscle, at least in part by inhibiting *GLUT4*, *MEF2A* and *PGC1 α* expression.



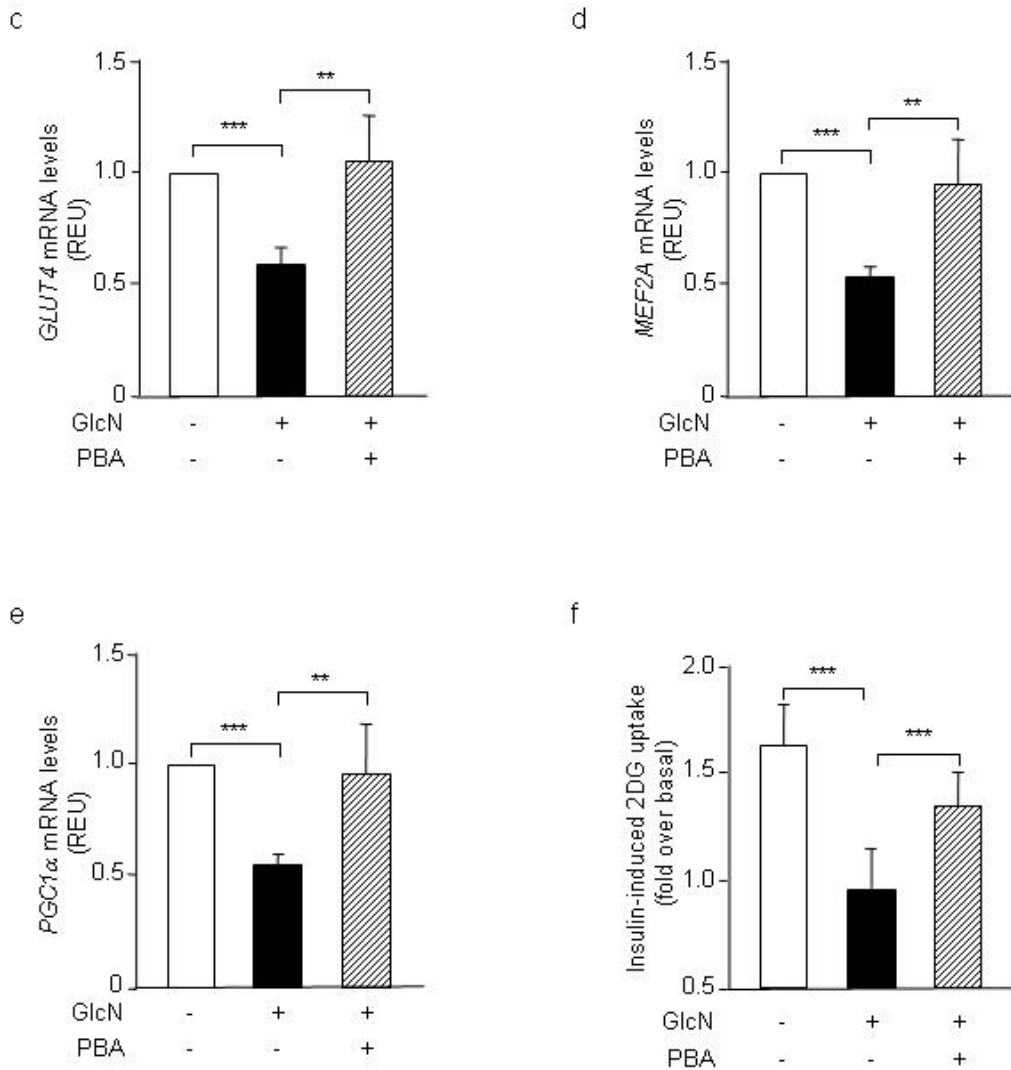


Figure 15 *GlcN* effects in human myotubes. (a-f) Human myotubes were pre-treated or not with 10 mmol/L PBA for 1 h, and then were treated with 7.5 mmol/L GlcN for 24 h. *BIP/GRP78* (a), *ATF6* (b), *GLUT4* (c), *MEF2A* (d) and *PGC1 α* (e) mRNAs were determined by real-time RT-PCR analysis of total RNA isolated from human myotubes, using GAPDH as internal standard. mRNA levels in treated cells are relative to those in control (mean \pm SD; $n=4$). **, $P < 0.01$; ***, $P < 0.001$. (f) 2-DG uptake was measured following 30 min of insulin stimulation (mean \pm SD; $n=5$). ***, $P < 0.001$.

Discussion

Glucose toxicity may contribute to impaired insulin action in diabetes (Cassese 2008). 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 (Pirola 2004; Marshall 1991; Buse 2006). 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 protein kinase B activation by insulin is associated with glucose, but not GlcN-mediated insulin resistance, in adipocytes (Han 2003). 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 (Buse 2006; Ciaraldi 1999). 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 (Patti 1999). Furthermore, GlcN induces insulin resistance *in vitro* in skeletal muscle (Bailey 2004) and adipose cells (Heart 2000), by reducing the insulin-induced glucose uptake. Many investigators have suggested the reversible O-GlcN 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 the correct protein folding in the ER, inducing ER stress and impairment of cellular function in hepatic cells, monocytes and smooth muscle cells (Werstuck 2006). 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 (Sundar Rajan 2007), affecting both beta-cell function/survival (Kaneto 2005; Scheuner 2001; Shi 2003) and obesity-induced peripheral insulin-sensitivity in liver and adipose tissue (Ozcan 2004; Ozcan 2006). However, little is known about the role of ER stress in the development of insulin-resistance in skeletal muscle tissue. A very recent study hypothesized a molecular convergence of activated HBP and ER stress pathways leading to insulin-resistance in L6 cells (Srinivasan 2009). However, the causal link between O-glycosylation pathway and the ER stress pathway in determining insulin-resistance remained elusive. Indeed, cells silenced for O-linked *N*-acetylglucosamine transferase (OGT), 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 chaperones regulation

(Srinivasan 2009). 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 eIF2 α and the increase of both the expression and splicing of the transcription factor *Xbp1*. Furthermore, ER stress and UPR activation are induced by GlcN in a model of human skeletal muscle cells (Gaster 2001), suggesting that both rat and human skeletal muscle cells are sensitive to GlcN-induced ER stress. More interestingly, HG also induces ER stress both in L6 cells and in human myotubes (data not shown). Pre-treatment of cells with azaserine, a non-specific but commonly used inhibitor of the GFAT (Marshall 1991; Buse 2006) prevented HG-induced-ER stress, suggesting that at least in part HG levels cause ER stress through hexosamines production. In addition, others (Bailey 2004) 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 uptake the glucose analogue 2-DG upon insulin stimulation. To gain further insight into the mechanisms leading to insulin resistance, Bailey and Turner (Bailey 2004) tested GlcN-induced insulin-resistance in L6 myotubes against three different insulin-sensitive acting agents. Neither metformin, that promotes insulin receptor tyrosine activity, nor peroxovanadium, a protein tyrosine phosphate inhibitor, nor D-pinitol, that partially mimics the PI3K pathway, were able to prevent GlcN-induced insulin resistance (Bailey 2004), suggesting that GlcN impairs insulin sensitivity with a mechanism different from that represented by an alteration of 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 (Han 2003), adipocytes (Hresko 1998) and chondrocytes (Shikhman 2009). 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 (Kang 2001). 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 for the study, suggesting that GlcN effects both on ER stress and insulin-resistance were not dependent from ATP depletion in skeletal muscle cells. Interestingly, we have found that pre-treatment 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 (Ozcan 2004; Ozcan 2006), 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 by ER stress.

Glucose uptake into skeletal muscle is primarily mediated by GLUT4. Since it is well documented that insulin resistance in Type 2 diabetes can be associated with a marked reduction in *GLUT4* expression (Armoni 2007) and/or translocation (Shepherd 1999), we hypothesized that GlcN-induced ER stress might affect *GLUT4* gene transcription in both rat and human muscle cells. Here we show that this is the case, as both *GLUT4* 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* 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 (Armoni 2007). MEF2A and GEF associate and function together to activate GLUT4 transcription (Knight 2003). 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 (Handschin 2003). *GLUT4* transcription can also be modulated in skeletal muscle by other proteins, such as PGC1 α (Al-Khalili 2005). PGC1 α is a coactivator of MEF2A. Indeed, MEF2D binds PGC1 α recruiting this transcriptional coactivator to MEF2A (Armoni 2007). Moreover, PGC1 α expression is reduced in skeletal muscle of prediabetic and diabetic subjects (Patti 2003), and enhanced *GLUT4* mRNA expression coincides with increased PGC1 α mRNA in human skeletal muscle cell culture after treatment with rosiglitazone (Al-Khalili 2005). Our data, obtained both in rat and human myotubes, show that both GlcN- and HG-induced *GLUT4* inhibition is paralleled by a significant decrease of both MEF2A and PGC1 α 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 PGC1 α to *Glut4* promoter. Again, these effects appear to be mediated by ER stress signalling, since PBA or TUDCA pre-treatment of myotubes are able to prevent *GLUT4*, MEF2A and PGC1 α 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 the adipocytes and skeletal muscle cells (Ozcan 2004; Srinivasan 2009), such as JNK 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 high glucose conditions or treated with different ER stressors, via up-regulation of SHP (Seo 2008). We demonstrate that the overexpression of *Atf6* is sufficient to inhibit the expression of *Glut4*, *Mef2a* and *Pgc1 α* both in basal condition and upon GlcN treatment and that the silencing of *Atf6* expression with a specific siRNA is sufficient to completely prevent GlcN-induced down-regulation of *Glut4*, *Mef2a* and *Pgc1 α* . This effect seems to be independent from the up-regulation of SHP since its silencing does not modify *Glut4*, *Mef2a* and *Pgc1 α* 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 (Bartoszewski 2008). Our analysis of the minimal promoter region of *Glut4*, *Mef2a* and *Pgc1 α* (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 *Pgc1 α* 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 cause insulin-resistance both in human and rat myotubes and impair *GLUT4* expression and insulin-induced glucose uptake via an ATF6-dependent decrease of the *GLUT4* regulators *MEF2A* and *PGC1 α* . Interestingly, treatment with the molecular chaperones, PBA and TUDCA, completely prevent HG- and GlcN-induced UPR activation and restore insulin sensitivity in myotubes. These findings are particularly relevant for the understanding of the molecular mechanisms of glucose-toxicity in skeletal muscle and of the consequences of ER stress to the pathogenesis of Type 2 Diabetes.

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List of Publications

1. Cassese A, Esposito I, Fiory F, Barbagallo APM, Paturzo F, Mirra P, Ulianich L, Giacco F, **Iadicicco C**, Lombardi A, Oriente F, Van Obberghen E, Beguinot F, Formisano P, Miele C. *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. 2008 Dec 26;283(52):36088-99.
2. Raciti GA, **Iadicicco C**, Ulianich L, Vind BF, Gaster M, Andreozzi F, Longo M, Teperino R, Ungaro P, Di Jeso B, Formisano P, Beguinot F, Miele C. *Glucosamine-induced ER stress affects GLUT4 expression via Activating Transcription Factor 6 in skeletal muscle cells*. Diabetologia. 2010 May;53(5):955-65.

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In Skeletal Muscle Advanced Glycation End Products (AGEs) Inhibit Insulin Action and Induce the Formation of Multimolecular Complexes Including the Receptor for AGEs*

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Chronic hyperglycemia promotes insulin resistance at least in part by increasing the formation of advanced glycation end products (AGEs). We have previously shown that in L6 myotubes human glycated albumin (HGA) induces insulin resistance by activating protein kinase C α (PKC α). Here we show that HGA-induced PKC α activation is mediated by Src. Coprecipitation experiments showed that Src interacts with both the receptor for AGE (RAGE) and PKC α in HGA-treated L6 cells. A direct interaction of PKC α with Src and insulin receptor substrate-1 (IRS-1) has also been detected. In addition, silencing of IRS-1 expression abolished HGA-induced RAGE-PKC α co-precipitation. AGEs were able to induce insulin resistance also *in vivo*, as insulin tolerance tests revealed a significant impairment of insulin sensitivity in C57/BL6 mice fed a high AGEs diet (HAD). In tibialis muscle of HAD-fed mice, insulin-induced glucose uptake and protein kinase B phosphorylation were reduced. This was paralleled by a 2.5-fold increase in PKC α activity. Similarly to *in vitro* observations, Src phosphorylation was increased in tibialis muscle of HAD-fed mice, and co-precipitation experiments showed that Src interacts with both RAGE and PKC α . These results indicate that AGEs impairment of insulin action in the muscle might be mediated by the formation of a multimolecular complex including RAGE/IRS-1/Src and PKC α .

Insulin resistance is genetically determined, but it may also be affected by environmental conditions and by factors secondary to diseases (1). These acquired and secondary factors further impair insulin action in diabetic individuals. For instance, chronic hyperglycemia *per se* promotes insulin resistance (2, 3). A number of mechanisms have been proposed to explain hyperglycemia-induced insulin resistance. These include abnormalities in the protein kinase C (PKC)³ signaling system (4) and activation of the NF- κ B transcription factors by chronically elevated glucose concentrations (5, 6). Chronic hyperglycemia also leads to the production of Amadori products through the nonenzymatic glycation reactions between glucose and reactive amino groups of serum proteins. Depending on the protein turnover rate and glucose concentration, Amadori products undergo further irreversible reactions to form advanced glycation end products (AGEs). The modifications of proteins that lead to their glycation induce alterations in biological properties as compared with their non-glycated counterparts. Several studies have shown that elevated concentrations of Amadori products such as glycated albumin (GA) are associated with diabetic atherogenesis by activating vascular smooth muscle cells (7). GA has also been implicated in the development of diabetic retinopathy (8) by induction of vascular endothelial growth factor expression (9, 10) and the stimulation of choroidal endothelial cell proliferation (11). Finally, GA has been shown to participate in the development of diabetic nephropathy by the induction of cytokines and growth factors (12), which may themselves contribute to diabetic renal disease (13). In addition to those endogenously formed, AGEs are abundant in exogenous sources such as foods, especially when prepared under elevated temperatures (14, 15). After ingestion, 10% of preformed AGEs are absorbed into the human or rodent circulation (16, 17), $\frac{2}{3}$ of which are retained in tissues. Also, reduced intake of dietary AGEs has been shown to

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³ The abbreviations used are: PKC, protein kinase C; PKB, protein kinase B; AGE, advanced glycation end product; RAGE, receptor for AGE; sRAGE, soluble RAGE; GA, glycated albumin; HGA, human GA; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; CML, carboxymethyllysine; LAD, low AGE diet; HAD, high AGE diet; PLD, phospholipase D; HA, human serum albumin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; IRS-1, insulin receptor substrate-1; PBS, phosphate-buffered saline; DAG, diacylglycerol; DN, dominant negative.

decrease the incidence of type 1 diabetes in non-obese diabetic mice (18) as well as the formation of atherosclerotic lesions in diabetic apolipoprotein E-deficient mice (19). Furthermore, Vlassara and co-workers (20) have shown that reduced AGE intake leads to lower levels of circulating AGEs and to improved insulin sensitivity in *db/db* mice. Several AGE-binding proteins have been identified, including lactoferrin, galectin-3 (AGE-R3), lysozyme, and the receptor for AGE (RAGE) (21). RAGE is a multiligand member of the immunoglobulin superfamily and is expressed on the surface of a variety of cell types. By their binding to RAGE, AGEs trigger a range of cellular responses. RAGE has been reported to activate intracellular signals including the MAPK cascade and the *cdc42/Rac* pathway (22), leading to amplification or progression of various diseases including diabetic vascular complications (23), inflammation (24), and tumor growth/metastasis (25). The cytoplasmic region of RAGE is considered to be responsible for the binding of the signaling molecule(s) (26). It has been demonstrated that ERK1/2 interacts with the cytoplasmic region of RAGE after stimulation with amphotericin (27). Furthermore, recent studies have shown that STAT5 becomes activated and physically interacts with RAGE upon glycated low density lipoprotein stimulation (28). However, RAGE is devoid of intrinsic catalytic activity. Src tyrosine kinase is required for signaling events downstream of several receptors lacking intrinsic tyrosine kinase activity, such as cytokine receptors. Cho *et al.* (29) have shown that glycated low density lipoprotein (LDL) activates ERK1/2 via Src-, phospholipase C (PLC)-, and PKC-dependent pathways, whereas native and non-glycated LDL activate ERK1/2 by an Src-independent mechanism. It has been recently reported that in vascular smooth muscle cells derived from insulin-resistant and diabetic *db/db* mice, RAGE expression and Src activity are increased compared with those from control mice. Further studies showed that the RAGE ligand S100B induced Src activation in a RAGE-dependent manner. Moreover, in vascular smooth muscle cells Src activation is necessary for the S100B-induced RAGE downstream signaling involving several targets such as caveolin-1, MAPKs, NF- κ B, and STAT3. Interestingly, a PKC inhibitor could block S100B-induced activation of MAPKs, suggesting a role for PKC in this event (30). Recently, we have demonstrated that human glycated albumin (HGA) pretreatment induces a selective activation of PKC α . Insulin receptor substrate (IRS) serine/threonine phosphorylation by HGA-activated PKC α inhibits insulin-stimulated glucose metabolism without changes in growth-related pathways regulated by insulin (31). However, the intracellular signaling mechanism by which HGA induces PKC α activation remains to be determined. In this work we report that HGA and dietary AGEs induce the formation of a complex including RAGE, PKC α , and Src in L6 cells and in skeletal muscle from insulin-resistant HAD-fed mice, respectively. In L6 cells HGA-mediated complex formation requires the presence of IRS-1. We suggest that the formation of this complex may mediate the AGE-dependent inhibition of insulin action in skeletal muscle cells *in vitro* and, possibly, *in vivo*.

EXPERIMENTAL PROCEDURES

General—Media, sera, antibiotics for cell culture, and the Lipofectamine reagent were from Invitrogen (Invitrogen). Phospho-PKB and phospho-Src antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). PKB, IRS-1, phospho-Ser⁶⁵⁷ PKC α , phospho-Tyr antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). PKC α , PKC β , PKC δ , PKC ζ , and RAGE antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Src antibody was from Calbiochem (EMD Chemicals, Inc. San Diego, CA). The PKC assay system was from Promega (Madison, WI). Protein electrophoresis reagents were purchased from Bio-Rad, and ECL reagents were from GE Healthcare. The IRS-1 ribozyme and control ribozyme were generous gifts of M. Quon (National Institutes of Health, Bethesda, MD). PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) was from Alexis Biochemicals (San Diego CA). Soluble RAGE was kindly provided by A. Bierhaus (University of Heidelberg, Heidelberg, Germany). All other chemicals were from Sigma.

Cell Culture and Transfection—The L6 skeletal muscle cells were plated (6×10^3 cells/cm²) and grown in Dulbecco's modified Eagle's medium containing 1 g/liter glucose supplemented with 2% (v/v) fetal bovine serum and 2 mM glutamine. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO₂. Under these culture conditions, L6 myoblasts spontaneously differentiate into myotubes upon confluence. Transient transfections of IRS-1 and control ribozymes and of the kinase-inactive Src were performed by the Lipofectamine method according to the manufacturer's instruction. The cells were incubated for the appropriated times with 0.1 mg/ml of HGA or non-glycated human serum albumin (HA) as a control. Where indicated, cells were pretreated for 1 h with 50 μ M PD98059, 30 min with 50 nM wortmannin, 2 h with 5 μ M PP1, or 30 min with 10 μ M U73122 or 5 μ M U73343 before the incubation with HGA. Where indicated, 50 μ g/ml of soluble RAGE (sRAGE) were added together with HGA for the appropriated times (32).

Characterization of Glycated Human Serum Albumin—Glycated and nonglycated human serum albumin were purchased from Sigma. The glycated serum albumin contained 2–5 mol of fructosamine/mol of albumin. The human glycated and nonglycated albumin preparations were tested for carboxymethyllysine (CML) concentrations and the extent of lysine and arginine modifications as already described (31). Each batch was tested for possible insulin-like growth factor-I contamination by IGF-I-D-RIA-CT (BioSource Europe, Nivelles, Belgium) and the absence of endotoxin (lipopolysaccharide) by the use of Limulus amoebocyte lysates assay (Sigma). Each human glycated albumin batch was reconstituted at 10 mg/ml with sterile PBS and, to avoid glycooxidation, thereafter immediately frozen at –30 °C until use. The results obtained are summarized in Table 1 and show the absence of significant modifications in the extent of free lysine and arginine residues between the two preparations. Furthermore, the batches were found not to contain IGF-I and to be bacterial endotoxin-free. The physicochemical properties determined for our HGA preparation demonstrate that the effects observed in L6 myotubes are the

AGEs and Insulin Resistance

TABLE 1

Characteristics of glycated human albumin

Modification ratio of HGA was expressed as a percent of non-glycated human albumin modifications used as control.

	Non-glycated human albumin	Glycated human albumin
CML/mg protein	55 ng	195 ng
Lys modification (%)	100	94.9 ± 3.2
Arg modification (%)	100	91.6 ± 1.5
IGF-1	Undetectable	Undetectable
LPS	Undetectable	Undetectable

TABLE 2

Characteristics of HAD and LAD

Nutrients	HAD	LAD
Protein (%)	20.5	18.5
Fat (%)	4.7	4.0
Carbohydrate (%)	54.8	58.6
Fiber (%)	5.5	6.0
Niacin (mg/kg)	93	55
Folic acid (mg/kg)	2.9	1.92
Pantothenic acid (mg/kg)	17	14.4
Biotin (mg/kg)	0.1	0.279
Cholin (mg/kg)	1000	1000
Vitamin A (IU/kg)	2500	4400
Vitamin D3 (IU/kg)	2200	1260
Vitamin E (IU/kg)	99	49.5
Vitamin B1 (mg/kg)	18	13.5
Vitamin B2 (mg/kg)	8	6.74
Vitamin B6 (mg/kg)	7.8	5.98
Vitamin B12 (mg/kg)	0.018	0.027
Caloric profile (kcal/g)	3.4	3.7

consequence of glycated albumin present essentially as a glycated Amadori product.

Immunoblotting, Overlay Blot, PKC Assay, and Determination of Diacylglycerol (DAG) Cellular Content—Cell lysates were solubilized as described in Miele *et al.* (31). Mice were sacrificed by cervical dislocation, and tibialis and soleus muscle samples were collected rapidly and homogenized as previously reported (33). Tissue homogenates and cell lysates were then separated by SDS-PAGE and analyzed by Western blot as described (34). Cell lysates immunoprecipitations were accomplished as described in Oriente *et al.* (35). Overlay blotting with biotinylated PKC α was performed as reported previously (34). Filters were revealed by ECL and autoradiography. Upon immunoprecipitation with anti-PKC α or anti-RAGE antibodies, PKC activity was assayed using the SignaTECT PKC assay system (Promega) according to the manufacturer's instructions as described in Oriente *et al.* (35). DAG content was quantified radioenzymatically by incubating aliquots of the lipid extract with DAG kinase and [32 P]ATP as described previously (36, 37).

Animals and Treatment—4-Week-old C57/BL6 female mice (n 20) were purchased from the Charles River Laboratories (Milan, Italy). Animals were kept under a 12-h light/12-h dark cycle, and all experimental procedures and euthanasia described below were approved by Institutional Animal Care and Utilization Committee. After 1 week of adjustment, C57/BL6 mice were randomly divided into two groups and placed for 20 weeks on two commercially available standard rodent diets similar in nutritional and caloric content (Table 2) but with different AGEs content. Research Diets formula D12328 (Research Diets, Inc., New Brunswick, NJ) was used for the low AGE diet (LAD), and PicoLab Rodent Diet 20 exposed to an additional step of autoclaving at 120 °C for 30 min (Labdiet;

Purina Mills, St. Louis, MO) was used for the high AGE diet (HAD) (20). Fasting blood glucose and body weights were monitored biweekly. After 1 week of adjustment, food intake (in grams of food) of individual mice was recorded daily for 1 week at week 1, 11, and 22.

Skeletal muscles (quadriceps) of a group of 12 age-matched C57/BL6 mice were injected with 50 mg/kg glycated albumin (4 mice), non-glycated albumin (4 mice), or PBS (4 mice). Another group of 12 mice were treated with the same concentrations of glycated and non-glycated albumin, but the compounds were delivered by tail vein injection. Mice were sacrificed after 24 h by cervical dislocation, and muscle samples were collected rapidly and homogenized as previously reported (33).

AGEs Determination, Metabolite Assays, and 2-Deoxy-D-[1- 3 H]Glucose Uptake in Vivo—CML concentrations were determined by using a CML-enzyme-linked immunosorbent assay kit as described in Miele *et al.* (31). Fasting and fed blood glucose levels were measured with glucometer (A. Menarini Diagnostics, Florence, Italy). Insulin tolerance tests, glucose tolerance tests, and serum insulin measurements were performed as previously described (33). Fasting plasma free fatty acid levels were measured with Wako NEFA C kit (Wako Chemicals, Richmond, VA), and triglycerides were measured with the Infinity triglyceride reagent (Sigma-Aldrich). For analyzing glucose utilization by skeletal muscle, an intravenous injection of 1 μ Ci of the non-metabolizable glucose analog 2-deoxy-D-[1- 3 H]glucose (GE Healthcare) and an intraperitoneal injection of insulin (0.75 milliunits/kg of body weight) were administered to random-fed mice. The specific blood 2-deoxy-D-[1- 3 H]glucose clearance was determined with 25- μ l blood samples collected from the tail vein obtained 1, 15, and 30 min after injection as previously reported (33).

Statistical Analysis—Data were analyzed with Statview software (Abacus Concepts) by one-factor analysis of variance and are expressed as the means \pm S.D. Statistical significance was evaluated using Student's t test for unpaired comparison. A value of $p < 0.05$ was considered statistically significant. The total area under the curve for glucose response during the insulin tolerance tests and the glucose tolerance tests was calculated by the trapezoidal method.

RESULTS

Role of HGA-activated Src in PKC α Activation in L6 Skeletal Muscle Cells—To investigate the molecular mechanisms of PKC α activation by AGEs, HGA-induced PKC α activity was measured in L6 cells treated with different pharmacological inhibitors to selectively block ERK1/2, phosphatidylinositol 3-kinase, and Src. PKC α activity in response to HGA was unchanged upon treatment of L6 cells with PD98059 and wortmannin, which inhibit ERK1/2 and phosphatidylinositol 3-kinase, respectively (Fig. 1a). At variance, preincubation with the Src inhibitor PP1 or the expression of a dominant negative kinase-inactive Src (SrcDN) (38) reduced by almost 70% HGA-induced PKC α activity (Fig. 1a). Therefore, the levels of Src phosphorylation on Tyr 416 have been evaluated in L6 myotubes upon incubation for 24 h with HGA in the presence or absence of either PP1 or SrcDN. Src phosphorylation was increased by

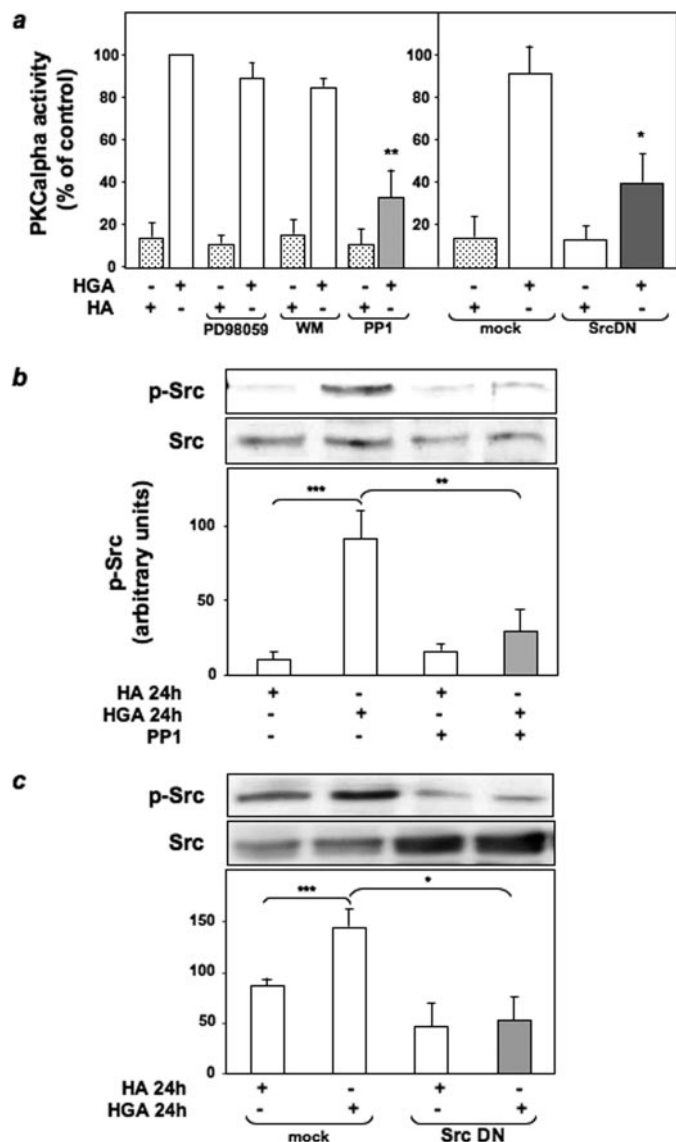


FIGURE 1. HGA-activated Src mediates PKC α activation in L6 skeletal muscle cells. *a*, L6 skeletal muscle cells were incubated with 0.1 mg/ml HA or HGA for 24 h in the absence or in the presence of 50 μ M PD98059, 50 nM wortmannin (WM), or 5 μ M PP1. PKC α activity was assayed in the immunoprecipitates as described under "Experimental Procedures." PKC α activity was measured also in L6 cells transfected with the empty vector pSG5 (*mock*) or with the vector pSG5 expressing a kinase-inactive Src protein (*SrcDN*) and then incubated with 0.1 mg/ml HA or HGA for 24 h. Bars represent the mean \pm S.D. of three independent experiments. *b*, L6 cells were incubated with 0.1 mg/ml HA or HGA for 24 h in the absence or in the presence of 5 μ M PP1, lysed as described under "Experimental Procedures"; lysates were then separated by SDS-PAGE followed by immunoblotting with antibodies to phosphotyrosine 416 Src or total Src. The autoradiographs shown are representative of four independent experiments. *c*, cell lysates of L6 skeletal muscle cells transfected with the empty vector pSG5 (*mock*) or with the vector pSG5 expressing a kinase-inactive Src protein (*SrcDN*) and treated as in *b* were then separated by SDS-PAGE followed by immunoblotting with antibodies to phosphotyrosine 416 Src or total Src. The autoradiographs shown are representative of three independent experiments. *, statistically significant differences (*, $p < 0.1$; **, $p < 0.01$; ***, $p < 0.001$).

HGA, but this effect was prevented by PP1 treatment (Fig. 1*b*) and by overexpression of SrcDN (Fig. 1*c*). Furthermore, both HGA-induced PKC α (Fig. 2*a*) and Src (Fig. 2*b*) activation were inhibited by the addition of an excess of sRAGE. To investigate the possibility that HGA-activated Src could directly regulate PKC α activity, we first measured in L6 myotubes PKC α tyro-

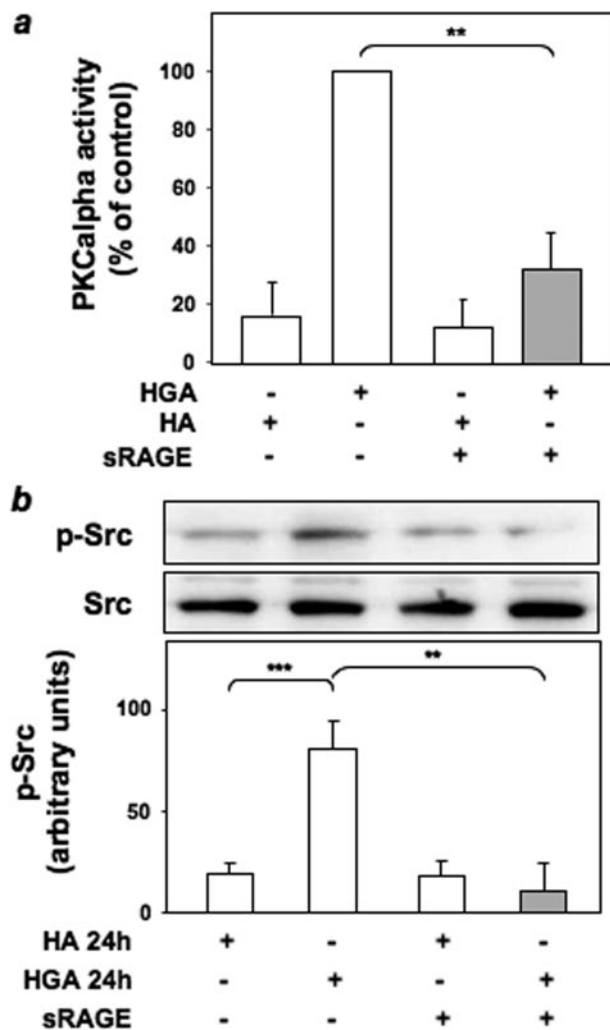


FIGURE 2. sRAGE abrogates HGA-activated Src and PKC α activation in L6 skeletal muscle cells. *a*, L6 skeletal muscle cells were incubated with 0.1 mg/ml HA or HGA for 24 h in the absence or in the presence of 50 μ g/ml sRAGE. PKC α activity was assayed in the immunoprecipitates as described under "Experimental Procedures." Bars represent the mean \pm S.D. of three independent experiments. *b*, L6 skeletal muscle cells were incubated with 0.1 mg/ml HA or HGA for 24 h in the presence or in the absence of 50 μ g/ml sRAGE, lysed as described under "Experimental Procedures"; lysates were then separated by SDS-PAGE followed by immunoblotting with antibodies to phosphotyrosine 416 Src or total Src. The autoradiographs shown are representative of three independent experiments. *, statistically significant differences (**, $p < 0.01$; ***, $p < 0.001$).

sine phosphorylation after HGA incubation for 24 h. As shown in Fig. 3*a*, HGA induced a 3-fold increase of the tyrosine phosphorylation of PKC α . This effect was abolished in the presence of PP1.

Role of PLC and Phospholipase D (PLD) in HGA-induced PKC α Activation in L6 Skeletal Muscle Cells—DAG is the main endogenous activator of classical PKCs (37). We, therefore, performed thin-layer chromatography to evaluate DAG levels in response to HGA treatment for 24 h in the presence or absence of PP1. HGA caused a >5-fold increase ($p < 0.001$) in the amount of DAG in L6 skeletal muscle cells. Interestingly, the HGA effect on DAG levels was significantly reduced ($p < 0.001$) when cells were treated with PP1 (Fig. 3*b*). In response to extracellular stimuli, DAG is mainly generated through the action of PLC and PLD (37). To clarify the role of PLC and PLD in HGA-

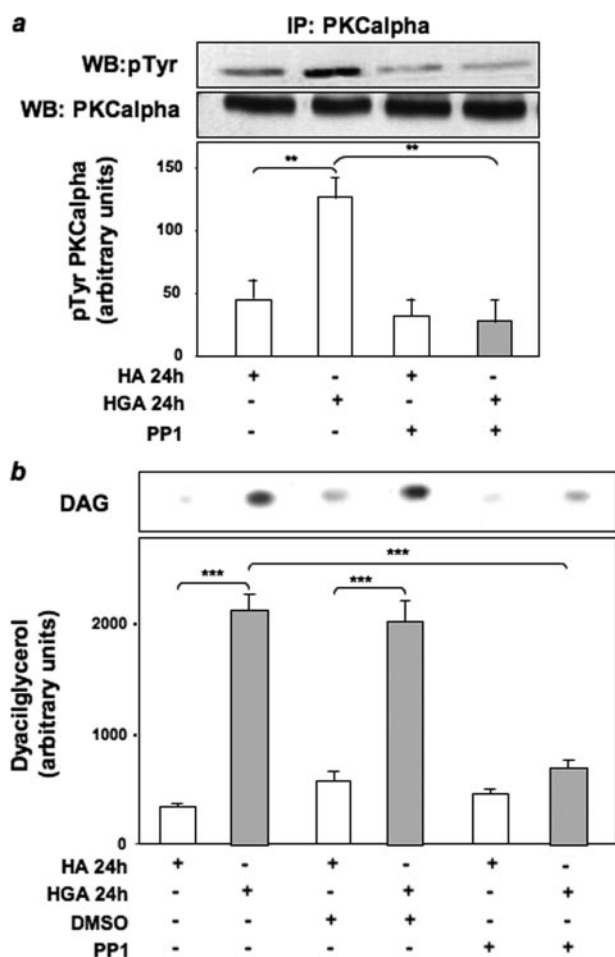


FIGURE 3. Role of Src in HGA-induced PKC α activation in L6 skeletal muscle cells. *a*, lysates from L6 skeletal muscle cells treated or not with 0.1 mg/ml HA or HGA for 24 h in the absence or in the presence of 5 μ M PP1 were precipitated (IP) with selective anti-PKC α antibodies and immunoblotted (WB) with anti-phosphotyrosine or anti-PKC α antibodies. The autoradiographs shown are representative of three independent experiments. *b*, L6 skeletal muscle cells were incubated with 0.1 mg/ml HA or HGA for 24 h in the absence or in the presence of 5 μ M PP1. Lipids were extracted, and determination of DAG levels was performed as described under "Experimental Procedures." [32 P]Phosphatidic acid was separated by TLC and quantitated by densitometric analysis. Bars represent the mean \pm S.D. of values obtained from three independent experiments in duplicate. The autoradiographs shown are representative of three independent experiments. *, statistically significant differences (**, $p < 0.01$; ***, $p < 0.001$).

induced PKC α activation, we evaluated PKC α phosphorylation levels in L6 cells treated or not with the PLD1 inhibitor 1-butanol, the PLC inhibitor U73122, and its inactive structural analogue U73343. PKC α activation, measured as Ser⁶⁵⁷ phosphorylation, was unaffected by preincubation with 1-butanol but was abolished by treatment with U73122 (Fig. 4*a*). By contrast, treatment with U73343 did not modify HGA effect on PKC α phosphorylation (Fig. 4*b*).

HGA-induced Association of PKC α , Src, and RAGE in L6 Skeletal Muscle Cells—To investigate the ability of HGA to induce the formation of a molecular complex between Src, RAGE, and/or PKC α , lysates from HGA-stimulated or untreated L6 myotubes were precipitated with anti-RAGE antibody and blotted with anti-Src or anti-PKC α antibodies. As shown in Fig. 5*a*, HGA treatment increased RAGE coprecipitation with both Src and PKC α .

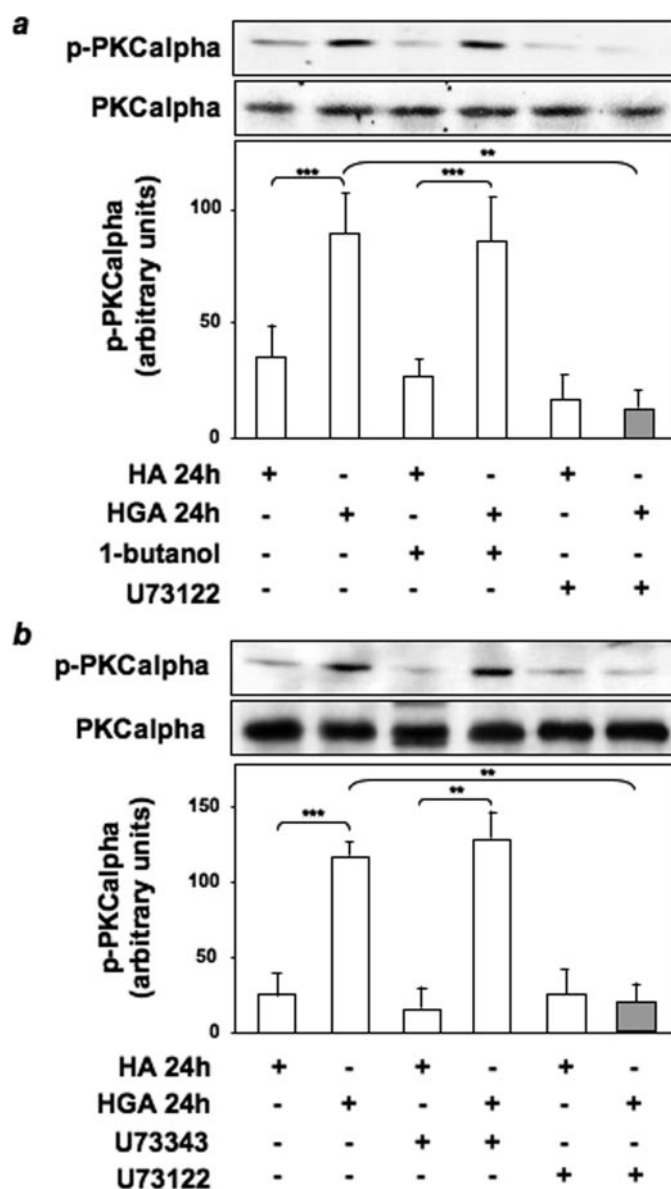


FIGURE 4. Role of PLC and PLD in HGA-induced PKC α activation in L6 skeletal muscle cells. Cell lysates of L6 skeletal muscle cells incubated with 0.1 mg/ml HA or HGA for 24 h in the absence or in the presence of 0.3% 1-butanol or 10 μ M U73122 (*a*) or 5 μ M U73343 (*b*) were then immunoprecipitated with anti-PKC α antibody and immunoblotted with antibodies to phospho-PKC α or to PKC α . The autoradiographs shown are representative of three independent experiments. *, statistically significant differences (**, $p < 0.01$; ***, $p < 0.001$).

Western blotting of RAGE immunoprecipitates with antibodies against PKC β , PKC ζ , and PKC δ revealed that these PKC isoforms did not co-precipitate with RAGE after HGA treatment for 24 h. By contrast, HGA induced the association of RAGE with PKC α (Fig. 5*b*). Furthermore, RAGE-associated PKC α activity increased in a time-dependent manner after HGA incubation, reaching a 2-fold increase after 24 h (Fig. 5*c*). Thus, upon AGEs treatment RAGE may form a multimolecular complex including Src and PKC α . Next, cell lysates were precipitated with RAGE antibodies and tested in overlay assays for their ability to bind recombinant biotinylated PKC α . Two major bands of 180 and 65 kDa were revealed upon HGA stimulation of the cells, whereas no bands corresponding to the

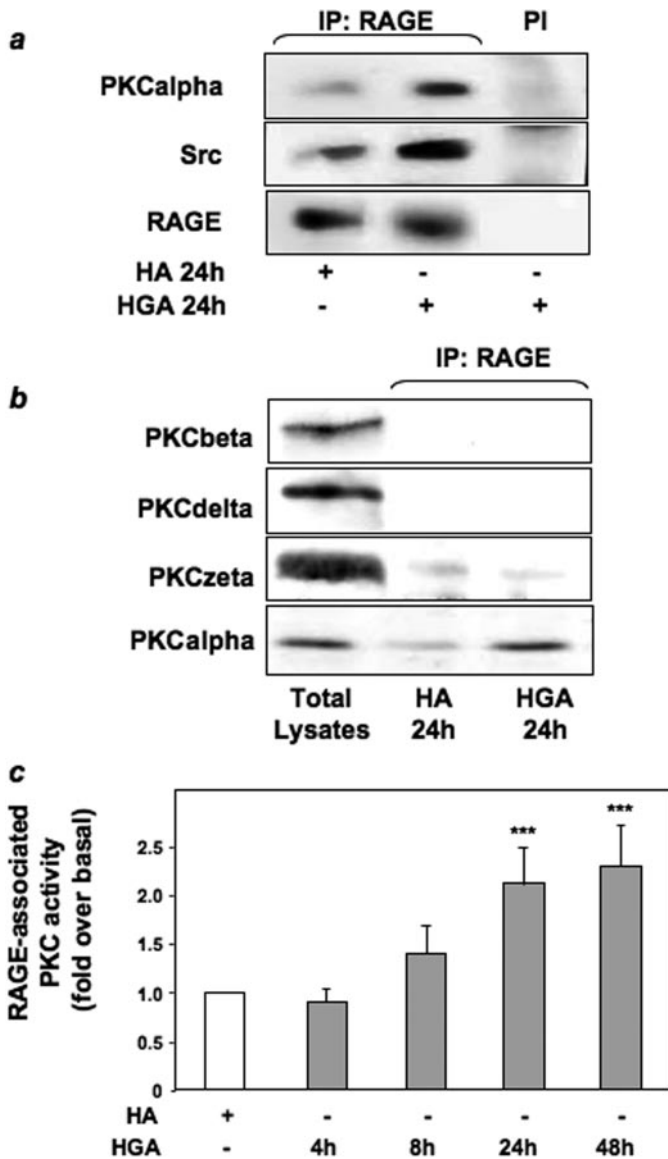


FIGURE 5. HGA-induced association of PKC α , Src, and RAGE in L6 skeletal muscle cells. *a*, lysates from L6 skeletal muscle cells treated or not with 0.1 mg/ml HA or HGA for 24 h were precipitated (IP) with preimmune serum (PI) or with selective anti-RAGE antibodies and immunoblotted with anti-PKC α , anti-Src, or anti-RAGE antibodies. The autoradiographs shown are representative of three independent experiments. *b*, lysates from L6 skeletal muscle cells treated as above were precipitated with selective anti-RAGE antibodies and immunoblotted with PKC β , PKC δ , PKC ζ , or PKC α antibodies. The autoradiographs shown are representative of three independent experiments. *c*, L6 skeletal muscle cells were treated with 0.1 mg/ml HA or HGA for the indicated times. Cells were lysed, cell lysates were then precipitated with selective anti-RAGE antibodies, and PKC activity was measured as described under "Experimental Procedures." Bars represent the mean \pm S.D. of values obtained from three independent experiments in duplicate. *, statistically significant differences (***, $p < 0.001$).

molecular mass of RAGE were detected (Fig. 6*a*). This indicates that RAGE/PKC α is not a direct interaction. Both the apparent molecular mass and mass spectrometric analysis indicated that the 180-kDa species corresponded to IRS-1, whereas the 65-kDa was identified as c-Src. Western blot with anti-IRS-1 and anti-PKC α antibodies performed on immunoprecipitates anti-RAGE revealed that HGA treatment increased RAGE co-precipitation with both IRS-1 and PKC α (Fig. 6*b*).

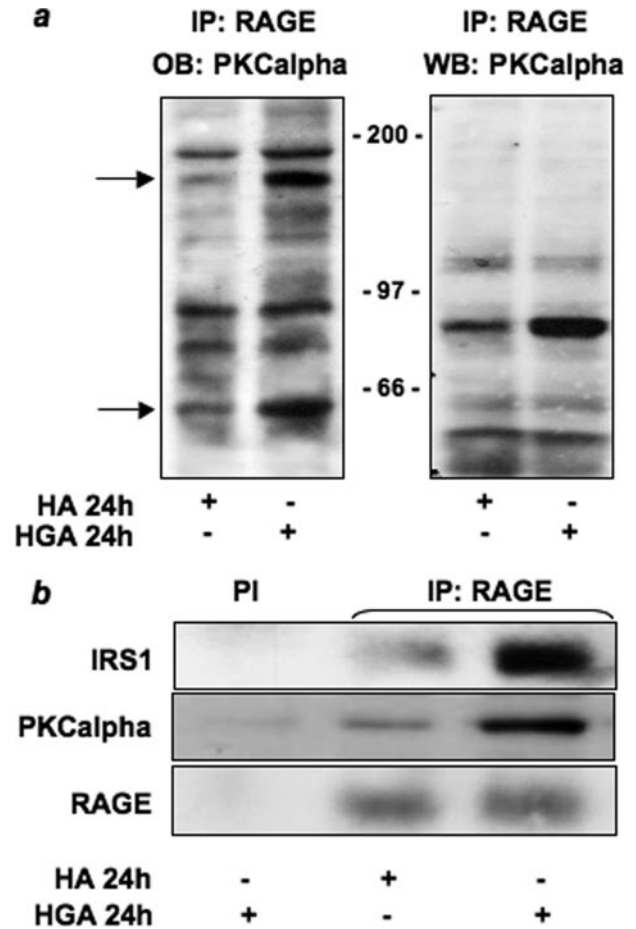


FIGURE 6. HGA induced the association of IRS-1 with PKC α and RAGE in L6 cells. *a*, L6 skeletal muscle cells were treated with 0.1 mg/ml HA or HGA for 24 h. Cells were lysed as described under "Experimental Procedures," and lysates were then precipitated (IP) with selective anti-RAGE antibodies and loaded on SDS-PAGE. Gels were then transferred onto nitrocellulose filters and incubated with recombinant biotinylated PKC α (OB PKC α) as described under "Experimental Procedures" (left panel). Cell lysates precipitated with selective anti-RAGE antibodies were also immunoblotted (WB) with PKC α antibody (right panel). The autoradiographs shown are representative of three independent experiments. *b*, lysates from L6 skeletal muscle cells treated as above were precipitated with pre-immune serum (PI) or selective anti-RAGE antibodies and loaded on SDS-PAGE. Gels were then transferred on nitrocellulose filters and immunoblotted with anti-IRS1, anti-PKC α , or anti-RAGE antibodies. The autoradiographs shown are representative of three independent experiments.

Role of IRS-1 and Src in PKC α and RAGE Association in L6 Skeletal Muscle Cells—To address the functional role of IRS-1 and Src in the activation of PKC α induced by HGA, we evaluated the effect of IRS-1 or Src inhibition on RAGE/PKC α association. L6 cells were transfected with a specific IRS-1-ribozyme (39), which specifically inhibited IRS-1 expression by about 80% without affecting PKC α levels (Fig. 7*a*). In untransfected L6 cells, 24 h of HGA treatment induced the association of RAGE with PKC α (Fig. 7*b*). Interestingly, the transfection of IRS-1-ribozyme abolished HGA-induced RAGE/PKC α co-precipitation (Fig. 7*b*). Furthermore, ribozyme-mediated inhibition of IRS-1 expression blocked the RAGE-associated PKC activity induced by HGA (Fig. 7*c*). The general PKC inhibitor bisindolyl maleimide was used as a control of PKC activity (Fig. 7*c*).

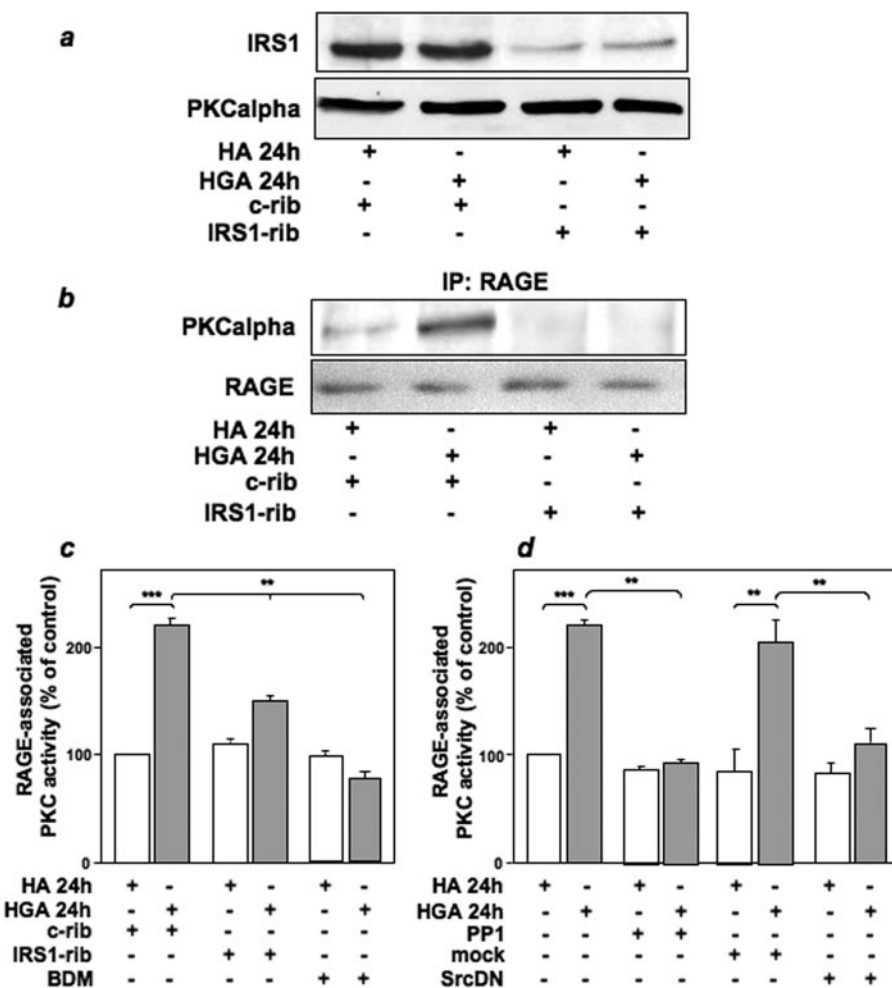


FIGURE 7. IRS-1 and Src are necessary for HGA-induced association of PKC α and RAGE in L6 cells. *a*, L6 skeletal muscle cells were transfected with a specific IRS-1-ribozyme (*IRS1-rib*) or a control ribozyme (*c-rib*) as described under "Experimental Procedures." Cells were then treated or not with 0.1 mg/ml HA or HGA for 24 h. Cells were lysed as described under "Experimental Procedures," and lysates were then immunoblotted with IRS1 or PKC α antibodies. The autoradiographs shown are representative of three independent experiments. *b*, lysates from L6 skeletal muscle cells treated as above were precipitated (IP) with selective RAGE antibodies and loaded on SDS-PAGE. Gels were then transferred on nitrocellulose filters and immunoblotted with PKC α or RAGE antibodies. The autoradiographs shown are representative of three independent experiments. *c*, L6 skeletal muscle cells were transfected with a specific IRS-1-ribozyme (*IRS1-rib*) or a control ribozyme (*c-rib*) as described under "Experimental Procedures" then treated or not with 0.1 mg/ml HA or HGA for 24 h in the absence or presence of 100 nM bisindolyl maleimide. Cells were lysed, cell lysates were then precipitated with selective anti-RAGE antibodies, and PKC activity was measured as described under "Experimental Procedures." *d*, L6 skeletal muscle cells were incubated with 0.1 mg/ml HA or HGA for 24 h in the absence or in the presence of 5 μ M PP1. Alternatively, L6 skeletal muscle cells were also mock-transfected or transfected with a kinase-inactive dominant negative Src (*SrcDN*), as described under "Experimental Procedures" and then incubated with 0.1 mg/ml HA or HGA for 24 h. Cells were lysed, cell lysates were then precipitated with selective anti-RAGE antibodies, and PKC activity was measured as described under "Experimental Procedures." Bars represent the mean \pm S.D. of three independent experiments. *, statistically significant differences (**, $p < 0.01$, ***, $p < 0.001$).

These results suggest a key role of IRS-1 in mediating RAGE/PKC α interaction. Moreover, inhibition of Src activity by PP1 or the SrcDN prevented the stimulatory effect of HGA on RAGE-associated PKC α activity (Fig. 7*d*).

Effect of Dietary AGEs on Body Weight, Food Intake, and Fasting Blood Metabolite Levels—C57/BL6 mice were fed either LAD or HAD. As assessed by detection of CML-like immunopitopes, AGE content was about 3-fold higher in HAD compared with LAD (data not shown). Because the daily food intake was equal between HAD- and LAD-fed mice (Table 3), HAD-fed mice ingested \sim 3-fold more CML than the LAD-fed mice.

This was reflected in part in the fasting serum CML-adducts levels, which were 2-fold higher in HAD-fed animals than in LAD-fed animals (LAD-fed mice 68 ± 8 units/ml versus HAD-fed mice 131 ± 6 units/ml; $n = 8$ /group, $p < 0.02$). Thus, steady-state serum levels of AGEs were consistent with the estimated content in the dietary formulas. Mice from either diet group showed no significant differences in body weight measured during the entire period of diet (Table 3). Within 11 weeks of treatment, fasting glucose levels in HAD-fed mice were significantly increased by 1.3-fold as compared with LAD-fed mice and became 1.5-fold higher up to 22 weeks of diet. At week 22, HAD-fed mice also featured a 1.4-fold increase in fasting insulin levels compared with LAD-fed animals (Table 3). Similarly, fasting non-esterified free fatty acid and triglyceride blood concentrations in HAD-fed mice were increased by 1.25- and 1.60-fold, respectively, suggesting the presence of insulin resistance in these animals (Table 3). Interestingly, fasting non-esterified free fatty acid and triglycerides in mice were also increased by 1.3-fold after 9 weeks of HAD despite no significant differences in fasting glucose levels (Table 3).

Glucose Tolerance and Insulin Sensitivity in HAD- and LAD-fed Mice—We then investigated the effect of HAD on glucose tolerance by intraperitoneal glucose loading (2 mg/kg) at week 22. In HAD-fed mice, blood glucose levels remained significantly higher than in LAD-fed mice (area under the curve, 7680 ± 3200 in HAD-fed mice versus 4791 ± 1770 in LAD-fed mice, $p <$

0.05). To determine possible alterations in insulin sensitivity, we performed insulin tolerance tests. In LAD-fed mice, intraperitoneal injection of insulin (0.75 milliunits/g of body weight) caused a severe decrease in blood glucose levels. This decrease achieved a maximum (70%; $p = 0.005$) after 45 min and was maintained for further 75 min. At variance, insulin reduced glucose levels by only 50% after 45 min in HAD-fed mice followed by a progressive rescue of the initial blood glucose concentration over the following 75 min (area under the curve, 9848 ± 3000 in HAD-fed mice versus 5267 ± 1700 in LAD-fed mice, $p < 0.0001$) (Fig. 8*a*). Insulin sensitivity was already

TABLE 3

Mice were analyzed as described under "Experimental Procedures." Data are the the means \pm S.D. of determinations in 12 LAD- and 12 HAD-fed mice

	LAD				HAD			
	Base line	Week 9	Week 11	Week 22	Base line	Week 9	Week 11	Week 22
Food intake (g/day)	3.30 \pm 0.10	3.46 \pm 0.20	3.44 \pm 0.14	4.30 \pm 0.24	3.50 \pm 0.22	3.88 \pm 0.15	3.58 \pm 0.15	4.04 \pm 0.20
Weight (g)	14.3 \pm 2.2	20.4 \pm 1.0	20.8 \pm 1.6	23.1 \pm 1.7	14.4 \pm 1.8	20.4 \pm 0.9	20.5 \pm 1.6	22.2 \pm 0.8
Fasting blood glucose (mg/dl)	69 \pm 13	69 \pm 22	79 \pm 16	80 \pm 12	68 \pm 10	78 \pm 12	103 \pm 15 ^a	117 \pm 12 ^b
Fasting serum insulin (mmol/liter)	ND	ND	ND	4.99 \pm 1.18	ND	ND	ND	7.48 \pm 2.70 ^a
Fasting NEFA (mmol/liter)	ND	0.35 \pm 0.07	ND	0.43 \pm 0.08	ND	0.45 \pm 0.02 ^c	ND	0.53 \pm 0.09 ^c
Fasting triglycerides (mg/ml)	ND	0.49 \pm 0.02	ND	0.59 \pm 0.12	ND	0.64 \pm 0.07 ^c	ND	0.95 \pm 0.27 ^c

^a*p* < 0.01.

^b*p* < 0.001.

^c*p* < 0.05.

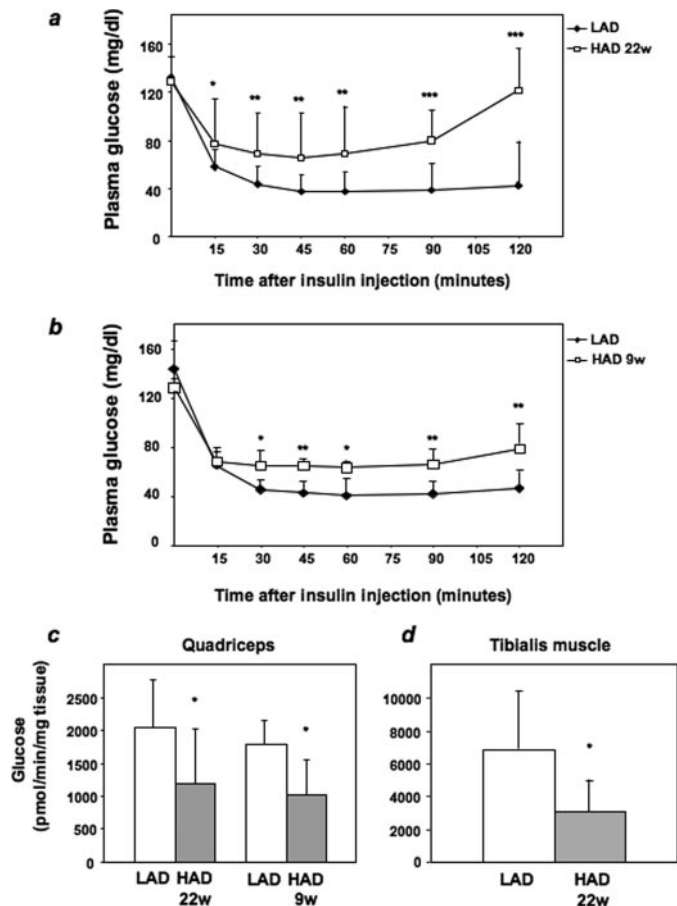


FIGURE 8. Insulin sensitivity and glucose transport in LAD- and HAD-fed mice. *a*, 4-week-old female mice of the C57/BL6 strain were fed a LAD or a HAD diet as described under "Experimental Procedures." At week (w) 22 (*a*) and week 9 (*b*) mice were injected intraperitoneally with insulin (0.75 milliunits/g of body weight) followed by determinations of blood glucose levels at the indicated times. Values are expressed as the means \pm S.D. *c* and *d*, mice fed LAD or HAD for 9 or 22 weeks were subjected to intravenous injection of 1 μ Ci of 2-deoxy-D-[1-³H]glucose and intraperitoneal injection of insulin. Quadriceps (*c*) or tibialis (*d*) muscles were removed 30 min after and snap-frozen in liquid nitrogen. 2-Deoxy-D-[1-³H]glucose accumulated in muscle tissues was quantitated as described under "Experimental Procedures." Bars represent mean values \pm S.D. of determinations in at least seven mice/group. *, statistically significant differences (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001).

reduced in mice after 9 weeks of HAD, as shown in Fig. 8*b* (area under the curve, 8522 \pm 2419 in HAD-fed mice versus 5920 \pm 1215 in LAD-fed mice, *p* < 0.01).

Effect of HAD on Glucose Uptake, PKB/Akt, and PKC α Activation in Vivo—In cultured L6 skeletal muscle cells, incubation with HGA causes resistance to insulin action on glucose uptake

(31). We, therefore, tested whether HAD also impairs insulin stimulation of the glucose transport machinery *in vivo*. Insulin-stimulated glucose uptake in the quadriceps and tibialis muscles from 22-week HAD-fed animals was decreased by 42 and 56%, respectively, compared with the LAD-fed mice (Fig. 8, *c* and *d*). Insulin-stimulated glucose uptake was reduced also in the quadriceps from 9-week HAD-fed mice (Fig. 8*c*). In parallel with the decreased glucose uptake, phosphorylation of PKB/Akt in response to insulin was also significantly reduced in skeletal muscles from HAD-fed mice (Fig. 9, *a* and *b*). This abnormality was accompanied by no significant change in the total PKB/Akt levels. We have previously demonstrated that the negative effects of HGA on insulin metabolic signaling in L6 myotubes are specifically mediated by PKC α (31). We, therefore, measured PKC α -specific activity in muscles from both HAD- and LAD-fed mice. As shown in Fig. 9*c*, PKC α specific activity measured in fasting conditions was increased by 2.3- and 1.8-fold in muscles from 22- and 9-week HAD-fed mice, respectively, compared with LAD-fed mice. Thus, AGEs modulate insulin signaling and PKC α activity *in vivo* as well as in cultured cells.

HAD-induced Src Activation and Association of PKC α , Src, and RAGE in Mice Skeletal Muscles—To investigate the ability of HAD to activate Src and to induce the formation of the complex between Src, RAGE, and/or PKC α *in vivo*, muscle tissue lysates from HAD- or LAD-fed mice were blotted with anti-phospho-Src Tyr⁴¹⁶ antibody. An \sim 2-fold increase of Src phosphorylation was observed in tibialis muscle from both 9- and 22-week HAD-fed mice compared with LAD-fed mice (Fig. 10). Furthermore, as shown in Fig. 11, RAGE coprecipitation with both Src and PKC α was increased in lysates from muscles from C57/BL6 HAD-fed mice when compared with LAD-fed mice. An increase of RAGE-associated PKC α activity was also observed in HAD fed mice (data not shown).

Glycated Albumin-induced PKC α and Src Activation in Mice Skeletal Muscles—To further support the role of AGEs in the activation of both PKC α and Src *in vivo*, C57/BL6 mice were injected with HGA either directly in the skeletal muscle (GA-Im) or in the tail vein (GA-Iv) as described under "Experimental Procedures." Muscle tissue lysates from GA-Im and GA-Iv mice were blotted with either anti-phospho PKC α (Fig. 12, *a*, *c*, *e*, and *g*) or Src Tyr⁴¹⁶ antibody (Fig. 12, *b*, *d*, *f*, and *h*). An \sim 2-fold increase of both PKC α (Fig. 12, *a* and *c*) and Src phosphorylation (Fig. 12, *b* and *d*) was observed in quadriceps from GA-Im mice compared with mice treated with vehicle alone

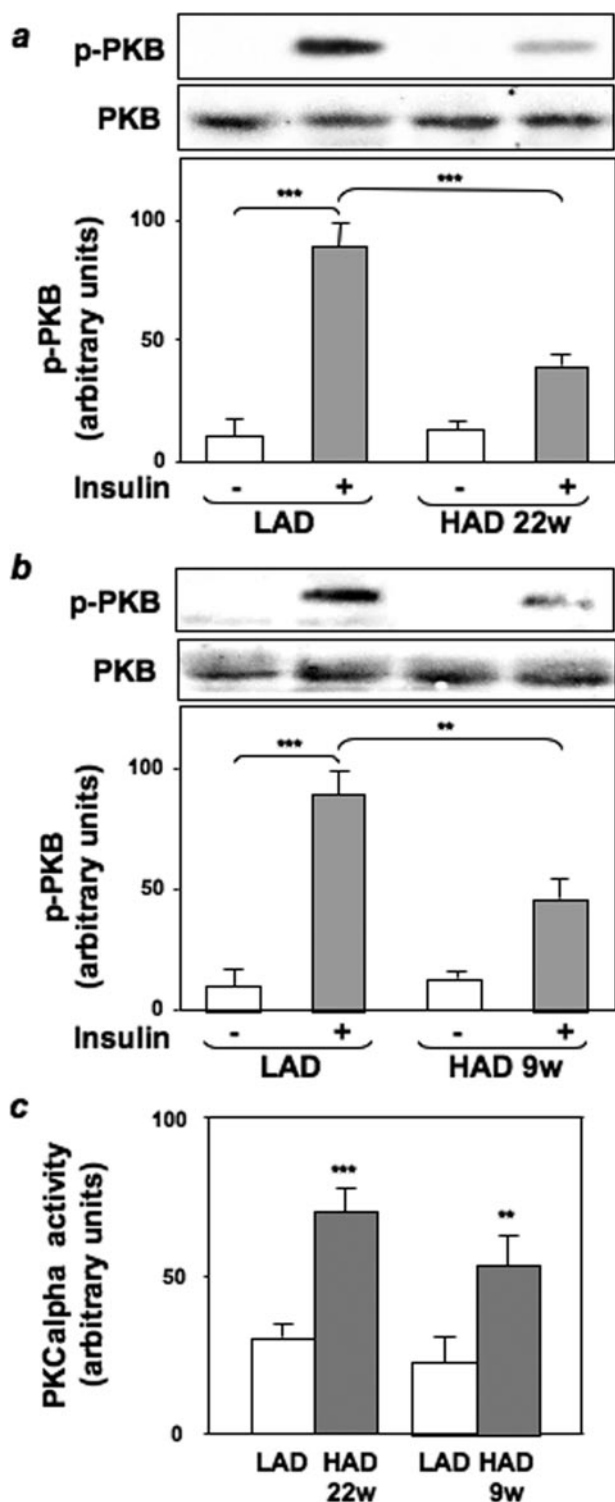


FIGURE 9. Effect of LAD and HAD on PKB/Akt and PKC α activation *in vivo*. *a*, 4-week-old female mice of the C57/BL6 strain were fed a LAD or a HAD diet as described under "Experimental Procedures." At week (w) 22 (*a*) and week 9 (*b*), mice were injected intraperitoneally with insulin (0.125 milliunits/g of body weight), and muscle tissues were collected and homogenized. Tissue homogenates were subjected to SDS-PAGE followed by immunoblotting with antibodies to phosphoserine 473 PKB/Akt and to PKB/Akt as a control. PKB/Akt phosphorylation was analyzed by densitometry. Bars represent the mean \pm S.D. of three different experiments. *c*, at week 9 or at week 22 muscle tissues homogenates were subjected to immunoprecipitation with PKC α antibodies, and specific PKC α activity was measured as described under "Experimental Procedures." Bars represent the mean \pm S.D. of three independent experiments. *, statistically significant differences (**, $p < 0.01$; ***, $p < 0.001$).

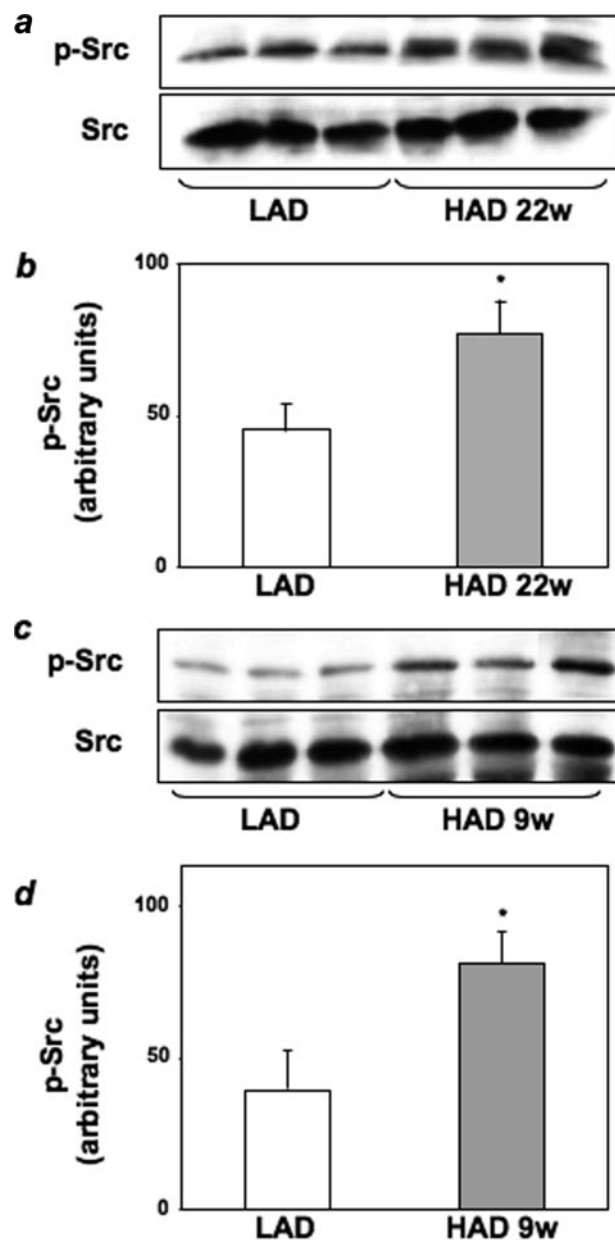


FIGURE 10. Effect of LAD and HAD on Src activation *in vivo*. *a*, tibialis muscle homogenates from 22 weeks (w) LAD- or HAD-fed mice were separated by SDS-PAGE followed by immunoblotting with antibodies to phosphotyrosine 416 Src or total Src. The autoradiographs shown are representative of three independent experiments. *b*, densitometric analysis of phosphotyrosine 416 Src normalized to total Src. *c*, tibialis muscle homogenates from 9-week LAD- or HAD-fed mice were separated by SDS-PAGE followed by immunoblotting with antibodies to phosphotyrosine 416 Src or total Src. The autoradiographs shown are representative of three independent experiments. *d*, densitometric analysis of phosphotyrosine 416 Src normalized to total Src. *, statistically significant differences (*, $p < 0.05$).

(PBS) or with non-glycated albumin (HA). Furthermore, both PKC α (Fig. 12, *e* and *g*) and Src phosphorylation (Fig. 12, *f* and *h*) were increased by 2.5- and 1.8-fold, respectively, in muscle lysates from GA-IV mice when compared with PBS or HA-treated mice.

DISCUSSION

We have previously shown that in L6 skeletal muscle cells chronic exposure to HGA selectively inhibits the phosphatidyl-

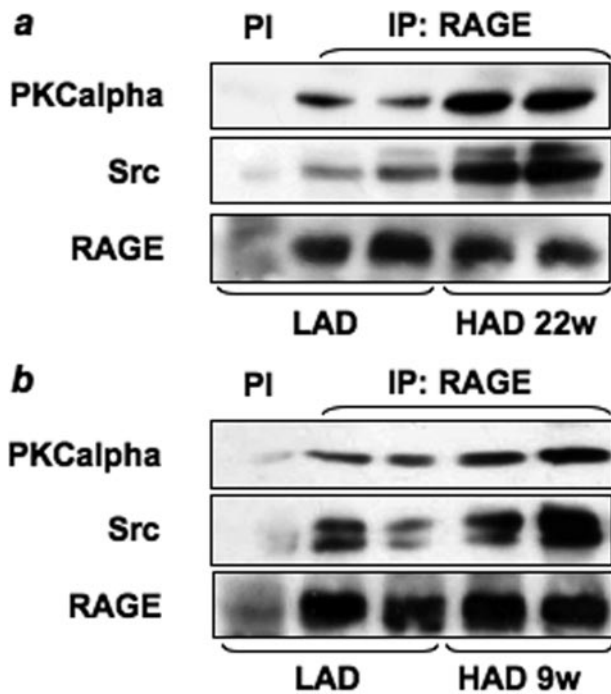


FIGURE 11. Effect of LAD and HAD on PKC α , Src, and RAGE association *in vivo*. Tibialis muscle homogenates from mice fed LAD or HAD for 22 weeks (w) (a) or for 9 weeks (b) were precipitated (IP) with preimmune serum (PI) or with selective anti-RAGE antibodies and immunoblotted with anti-PKC α , anti-Src or anti-RAGE antibodies. The autoradiographs shown are representative of four independent experiments.

inositol 3-kinase/PKB pathway in the insulin signaling cascade while leaving the Ras-ERK activation and mitogenic action of the hormone unaltered (31). In L6 myotubes the HGA-induced alteration of insulin metabolic signals is paralleled by an increase of serine/threonine phosphorylation of the IRSs specifically mediated by the activation of PKC α independently of reactive oxygen species (31). Previous studies have shown that the PKC family of serine/threonine kinases is implicated in development of insulin resistance (40, 41). The PKC family is composed of several isoforms, divided into three groups according to their structure and activation mechanisms (42). The atypical isoforms play a positive role in glucose transport (43), whereas classical isoforms are involved in generation of insulin resistance. It is well established that PKC α and β are able to phosphorylate insulin receptor and IRSs on serine and threonine residues, inhibiting their tyrosine phosphorylation (44–46).

Importantly, our studies revealed that in L6 cells inhibition of Src kinase, either by a specific pharmacological inhibitor such as PP1 or by transfecting a dominant negative form of the kinase, prevents the HGA-induced increase in PKC α activity, suggesting a key role for Src in this event. Interestingly, PKC α is tyrosine-phosphorylated upon HGA treatment, and its tyrosine phosphorylation is abolished by preincubation of L6 cells with PP1. Thus, Src may directly regulate PKC α activity in response to HGA. Furthermore, these effects appear to be RAGE-mediated as a soluble form of RAGE was able to prevent both HGA-induced Src and PKC α activation. PKC activity is implicated in v-Src-induced intracellular signals. In the rat large intestine 1,25-dihydroxyvitamin stimulates the physical association of

activated c-Src with PLC γ and activates two Ca²⁺-dependent PKC isoforms (47). Similarly, activation of v-Src in BALB/c 3T3 cells rapidly increases the intracellular second messenger, DAG via a type D phospholipase/PA phosphatase-mediated signaling pathway (48). These results are consistent with our findings that HGA caused a Src-dependent increase in the amount of DAG in L6 skeletal muscle cells. Moreover, we show that treatment of L6 cells with the pharmacological inhibitor of PLC, U73122, but not its inactive structural analogue U73343, almost completely abolishes HGA-induced PKC α phosphorylation, indicating that, in response to HGA, Src may increase the amount of DAG via PLC. Thus, Src may control PKC α activity by direct phosphorylation and by regulation of DAG intracellular levels via PLC.

HGA-induced increase in DAG levels is not sufficient to activate other PKC isoforms, as we have already described (31). Previous work by Zang *et al.* (49) demonstrated that in cells expressing the oncogenic v-Src, increased production of DAG is accompanied by a selective activation of the α and δ isoforms of PKC, whereas the ϵ isoform is not activated. Furthermore, Pula *et al.* (50) have shown that in platelets PKC α but not PKC β was activated in an Syk- and phospholipase C-dependent manner. It has been proposed that specific DAG species are generated to activate specific PKC isoforms. Indeed, several reports have demonstrated that different stimuli generate different DAG species, and PKC isotypes are differentially sensitive to the fatty acid composition of DAG (51). Thus, it is possible that in L6 cells the lack of activation of other PKC isoforms is due to specific DAG produced by HGA stimulation.

We then hypothesized that HGA could activate Src via RAGE. Reddy *et al.* (30) showed that S100B activates Src kinase in a RAGE-dependent manner and that Src kinase is required for the S100B-induced RAGE signaling, leading to migration and inflammatory gene expression in vascular smooth muscle cells. Co-localization of RAGE and Src has been evidenced in caveolae (30), where Cav-1 plays an important role in the assembly and integration of signaling complexes (52, 53). It is well known that RAGE can activate several downstream signaling pathways, including ERK1/2, phosphatidylinositol 3-kinase, PKC, the Janus tyrosine kinases (JAKs), and transcription factors, including STAT3, AP1, and NF- κ B (22, 26). Moreover, the cytoplasmic region of RAGE is responsible for the binding of the signaling molecule(s). In intact cells, ERK1/2 have been identified as RAGE-binding proteins. Binding to the intracellular region of RAGE localizes ERK under the proximal region of the plasma membrane, enabling the interaction between ERK and its substrates (27). Our results are consistent with the hypothesis that the formation of a multimolecular complex, involving RAGE, PKC α , and Src kinase, occurs in response to chronic HGA treatment. Indeed, coprecipitation experiments showed that in L6 cells Src kinase interacts with both RAGE and PKC α . RAGE cytoplasmic domain might also be involved in the interaction of RAGE with both Src and PKC α . Fine mapping of the interaction sites between Src, PKC α , and RAGE is currently under investigation in our laboratory.

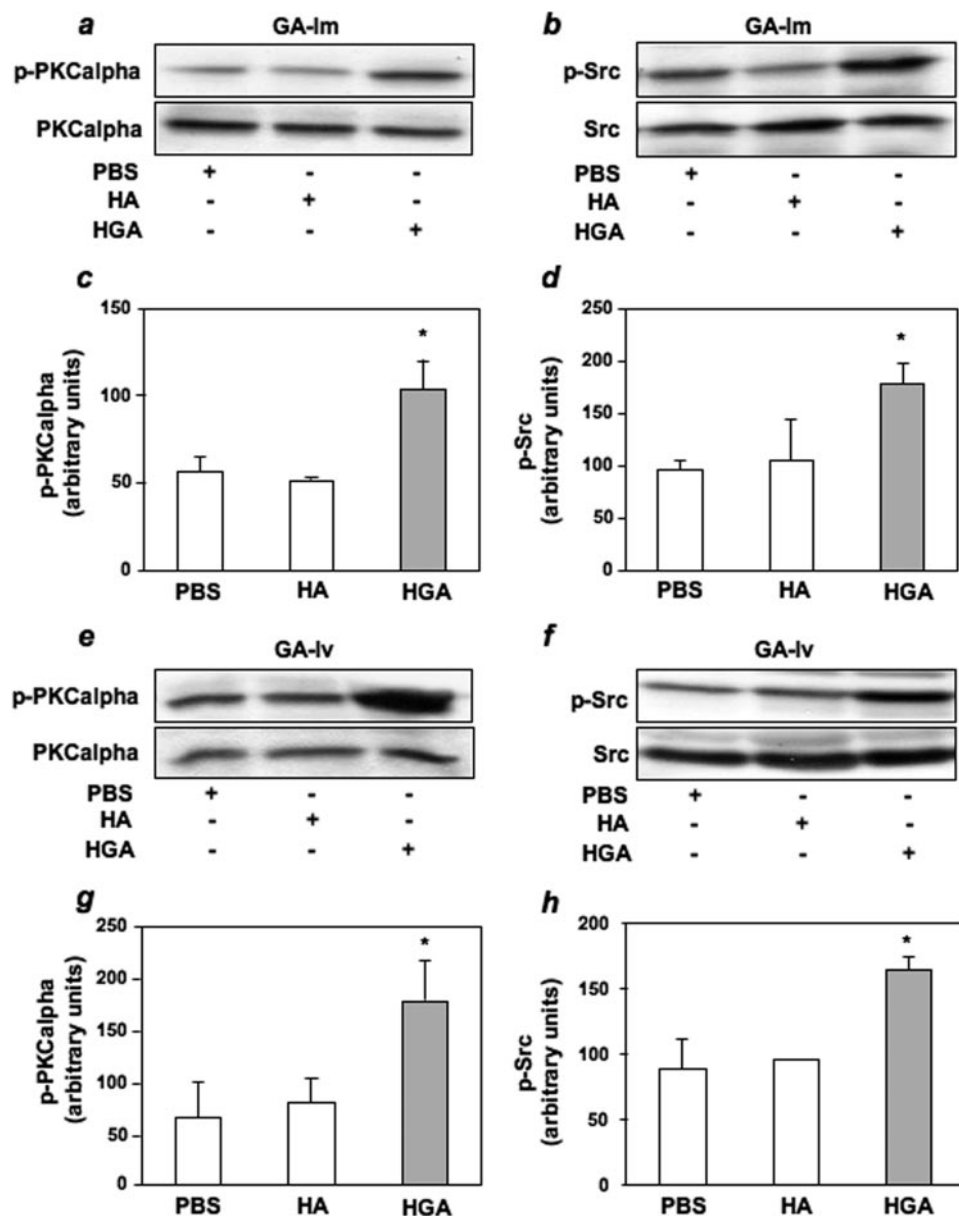


FIGURE 12. Effect of glycated albumin on PKC α and Src activation *in vivo*. 4-Week-old female mice of the C57/BL6 strain were injected with 50 mg/kg of HGA or HA or PBS either directly in the quadriceps (GA-Im: a, b, c, and d) or in the tail vein (GA-Iv: e, f, g, and h) as described under "Experimental Procedures. a and c, muscle tissue lysates from muscle-injected mice were blotted with anti-phospho PKC α , and PKC α phosphorylation was analyzed by densitometry. b and d, muscle tissue lysates from muscle-injected mice were blotted with anti-phospho Src Tyr⁴¹⁶ antibody and Src phosphorylation was analyzed by densitometry. e and g, muscle tissue lysates from tail vein-injected mice were blotted with anti-phospho PKC α , and PKC α phosphorylation was analyzed by densitometry. f and h, muscle tissue lysates from tail vein-injected mice were blotted with anti-phospho-Src Tyr⁴¹⁶ antibody, and Src phosphorylation was analyzed by densitometry. A representative autoradiograph is shown. Bars represent the mean \pm S.D. of three different experiments. *, statistically significant differences (*, $p < 0.05$).

Overlay blot assays revealed that, in response to HGA, a 180-kDa protein, identified as IRS-1, and Src may directly bind PKC α . Thus, HGA may induce direct interaction of PKC α with IRS1 and Src. IRS1, or less likely Src, in turn may bridge RAGE interaction with PKC α , the latter two having no direct interaction. Indeed, our experiments in L6 cells transfected with IRS-1 ribozyme indicate that IRS-1 is required for RAGE-PKC α coprecipitation and activation in response to HGA. This is consistent with our previous findings showing that IRS-1 but not IRS-2 expression is necessary to allow classical PKC activation

in response to insulin (39). Thus, one might speculate that, in response to different stimuli, PKC α is involved in the formation of different specific multimolecular complexes, which modulate insulin sensitivity. In this paper we also provide evidence that dietary AGEs are able to affect insulin sensitivity and glucose tolerance *in vivo*. These effects were AGEs-specific and not caused by hyperglycemia, as C57/BL6 mice fed a HAD for 9 weeks show a reduction of insulin sensitivity despite no significant differences in fasting glucose levels (Table 3). Prolonged exposure to HAD subsequently led to higher fasting glucose levels after 11 weeks of diet up to the end of the treatment compared with mice fed a LAD despite equal food intake and weight gain. Throughout this period HAD-fed mice appear consistently hyperinsulinemic in fasting conditions and exhibit increased non-esterified free fatty acid and triglycerides levels compared with LAD-fed mice. Because glucose tolerance is also slightly impaired at 22 weeks of HAD, it is conceivable that dietary AGEs affect insulin secretion in addition to insulin action. This is consistent with the evidence that accumulation of islet AGEs could be important for glucotoxicity toward β -cells (54). Furthermore, insulin-induced PKB phosphorylation and glucose uptake are dramatically reduced in tibialis and quadriceps muscles of HAD-fed mice. Impairment of insulin metabolic signaling is accompanied by an increase in PKC α specific activity in tibialis muscles from the HAD-fed mice, showing again that the mechanism by which HGA exacerbates the insulin-resistant state in intact cells also occurs *in vivo*. Moreover, an increase in PKC α specific activity in tibialis muscles from the HAD-fed mice appeared to be associated to the increase of the RAGE-PKC α -Src complex formation. Increases of both PKC α and Src phosphorylation were also observed when HGA was delivered either by direct intramuscular or by intravenous injection in mice. These observations further support the *in vivo* relevance of HGA and the conclusion that the differences in LAD- and HAD-fed mice are largely due to AGEs. In summary, our data suggest that AGEs-induced PKC α activity in the muscle might be mediated by the forma-

tion of a RAGE-PKC α -Src-IRS1 complex both *in vitro* and *in vivo*.

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

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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 (*XBPI*) 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 1 α [*PGC1 α*]). Again, PBA or TUDCA pretreatment prevented glucosamine-induced inhibition of *GLUT4* (also known as *SLC2A4*), *MEF2A* and *PGC1 α* (also known as *PPARGC1A*). Finally, we showed that overproduction of ATF6 is sufficient to inhibit the expression of genes *GLUT4*, *MEF2A* and *PGC1 α* and that ATF6 silencing with a specific small interfering RNA is sufficient to completely prevent glucosamine-induced inhi-

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bition of *GLUT4*, *MEF2A* and *PGC1 α* 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 *PGC1 α* .

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
eIF2 α	Eukaryotic translation initiation factor 2 α
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	<i>N</i> -Acetyl-cysteine
PBA	4-Phenylbutyric acid
PERK	Double-stranded RNA-activated protein kinase-like ER kinase
PGC1 α	Peroxisome proliferator-activated receptor- γ coactivator 1 α
PUGNAc	<i>O</i> -(2-Acetamido-2-deoxy-D-glucopyranosylideneamino) <i>N</i> -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: Ultrosor 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 1 α (PGC1 α), eukaryotic translation initiation factor 2 α (eIF2 α) and phospho-eIF2 α 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.) Ultrosor 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 \pm 2.2
BMI (kg/m ²)	23.0 \pm 1.1
Fasting plasma glucose (mmol/l)	5.4 \pm 0.2
Fasting plasma insulin (pmol/l)	35.2 \pm 7.8
HbA _{1c} (%)	5.6 \pm 0.1

Values represent means \pm 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-eIF2 α and eIF2 α 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-PGC1 α 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-glucopyranosylideneamino)-*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; *Xbp1s*) increased significantly following both Thap (Fig. 2a) and GlcN treatments (Fig. 2b). Time course experiments showed that the increase of *Xbp1s* peaked at 8 h of GlcN treatment and remained higher than basal level up to 24 h

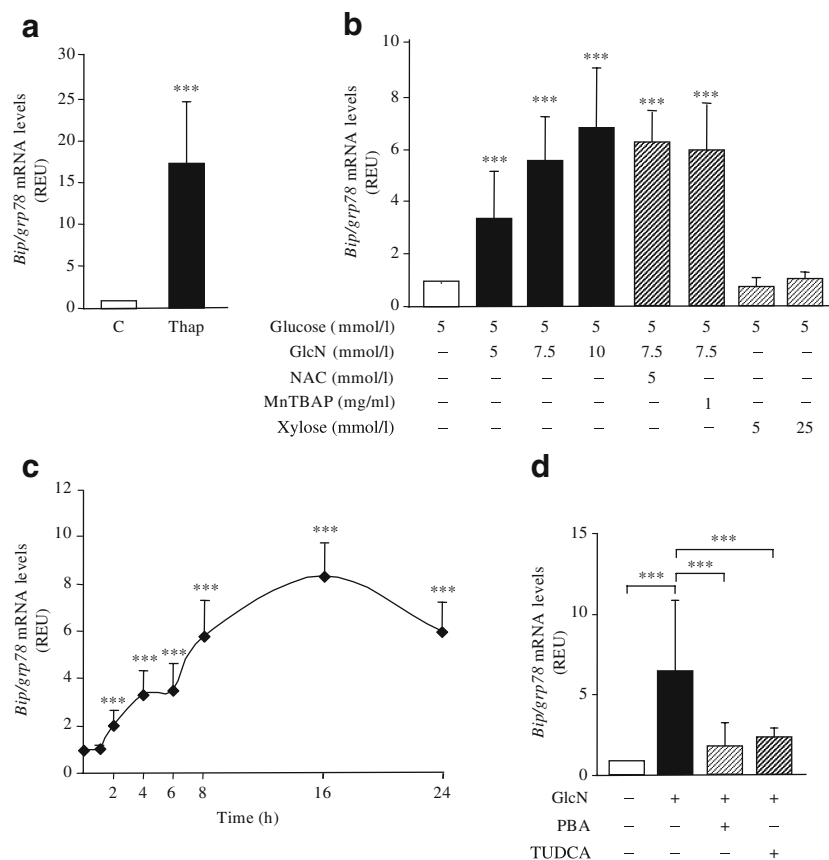


Fig. 1 GlcN induces BIP/GRP78 in L6 myotubes. **a–d** *Bip/grp78* mRNA was determined by real-time RT-PCR analysis of total RNA isolated from myotubes, using *Gapdh* as internal standard. mRNA levels in treated cells are relative expression units (REU) to those in control (C; mean \pm SD; $n=8$). *** $p<0.001$. **a** L6 cells were treated with 0.5 μ mol/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

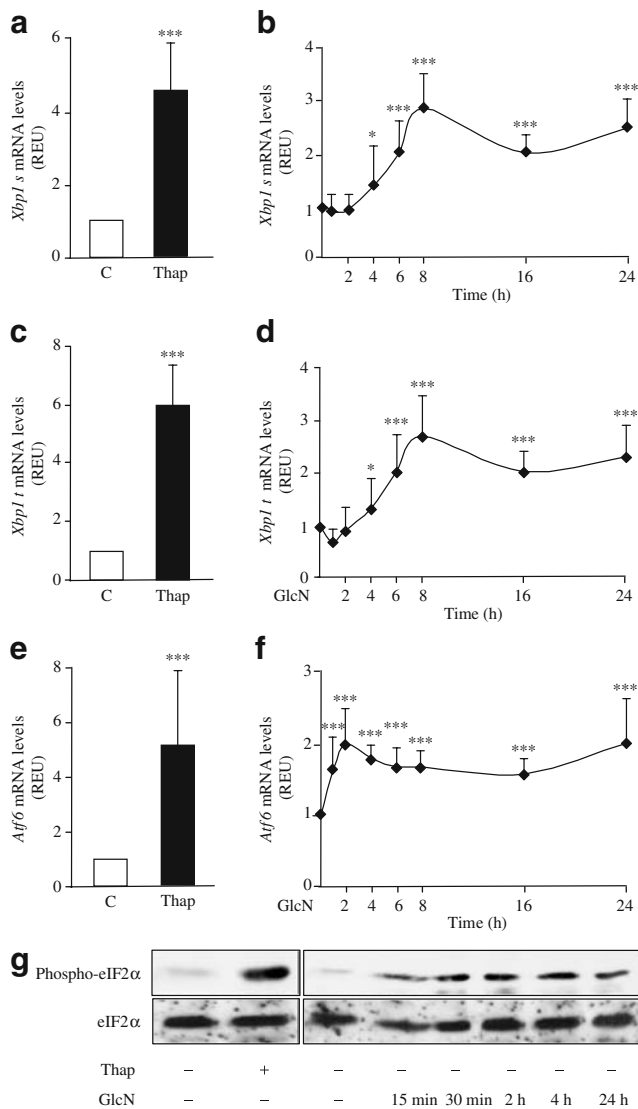


Fig. 2 GlcN induces UPR activation in L6 myotubes. **a–g** L6 cells were treated with 0.5 $\mu\text{mol/l}$ Thap for 30 min, followed by 24 h without Thap, or with 7.5 mmol/l GlcN for the indicated times (**a–f**). *Xbp1 s* (**a, b**), *Xbp1 t* (**c, d**) and *Atf6* (**e, f**) mRNAs in treated cells are relative expression units (REU) to those in control (C; mean \pm 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 $\mu\text{mol/l}$ 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-eIF2 α Ser51 and eIF2 α 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 eIF2 α 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 *Pgc1 α* (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 *Pgc1 α* 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 *Pgc1 α* (Fig. 4d) compared with control cells, confirming that also *Mef2a* and *Pgc1 α* reduced expression

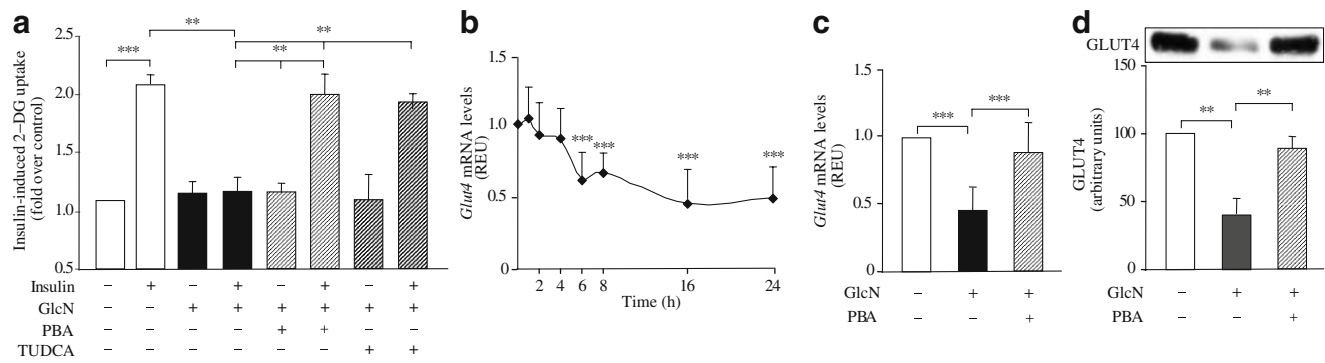


Fig. 3 Effect of ER stress on 2-DG uptake and *Glut4* expression in L6 myotubes. **a** L6 cells were pretreated or not for 1 h with 10 mmol/l PBA or 5 mmol/l TUDCA and then treated with 7.5 mmol/l GlcN for 24 h. 2-DG uptake was measured following 30 min of insulin stimulation (mean \pm SD; $n=5$). $**p<0.01$; $***p<0.001$. **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 \pm 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 μ g/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 *Pgc1 α* 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, PGC1 α 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 *Pgc1 α* mRNA levels and their binding to the GLUT4 promoter.

To understand the mechanisms involved in the transcriptional inhibition of *Glut4*, *Mef2a* and *Pgc1 α* 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 *Pgc1 α* 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 *Pgc1 α* (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 *Pgc1 α* (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 *PGC1 α* 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 *PGC1 α* (Fig. 6c–e) and a rescue of insulin-induced 2-DG uptake (Fig. 6f) compared

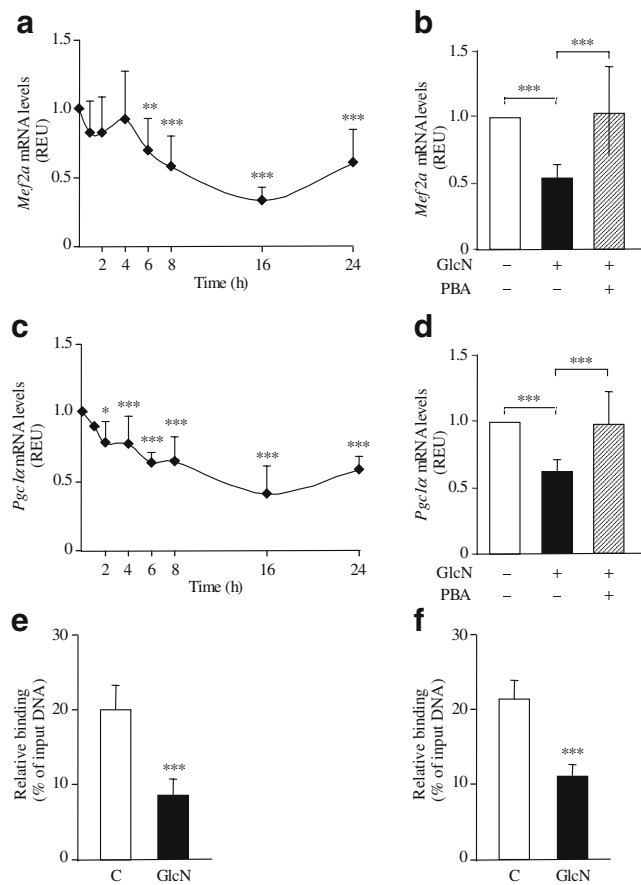


Fig. 4 Effect of ER stress on *Mef2a* and *Pgc1α* mRNA expression and GLUT4 promoter binding in L6 myotubes. **a–d** *Mef2a* and *Pgc1α* mRNAs were determined by real-time RT-PCR analysis, using *Gapdh* as internal standard. mRNA levels in treated cells are relative expression units (REU) to those in 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

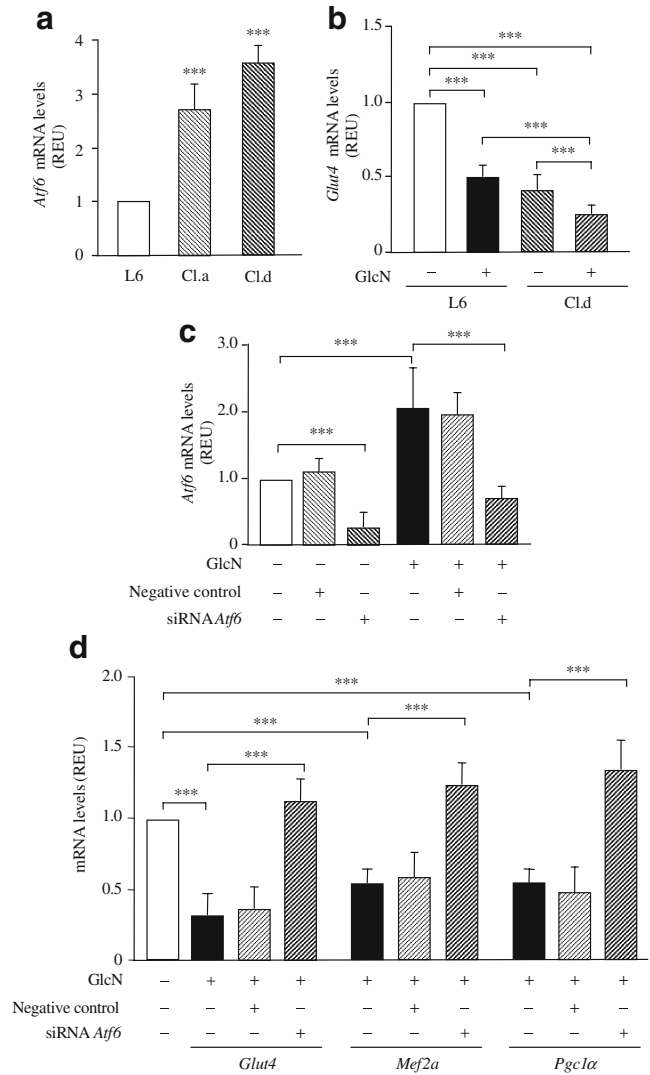


Fig. 5 Role of ATF6 on GlcN-induced ER stress in L6 myotubes. **a, b** L6 cells were stably transfected with *Atf6* cDNA. *Atf6* (**a**) and *Glut4* (**b**) mRNAs in clones are relative expression units (REU) to those in untransfected cells (L6) and were determined by real-time RT-PCR analysis using *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; $n=3$). *** $p < 0.001$

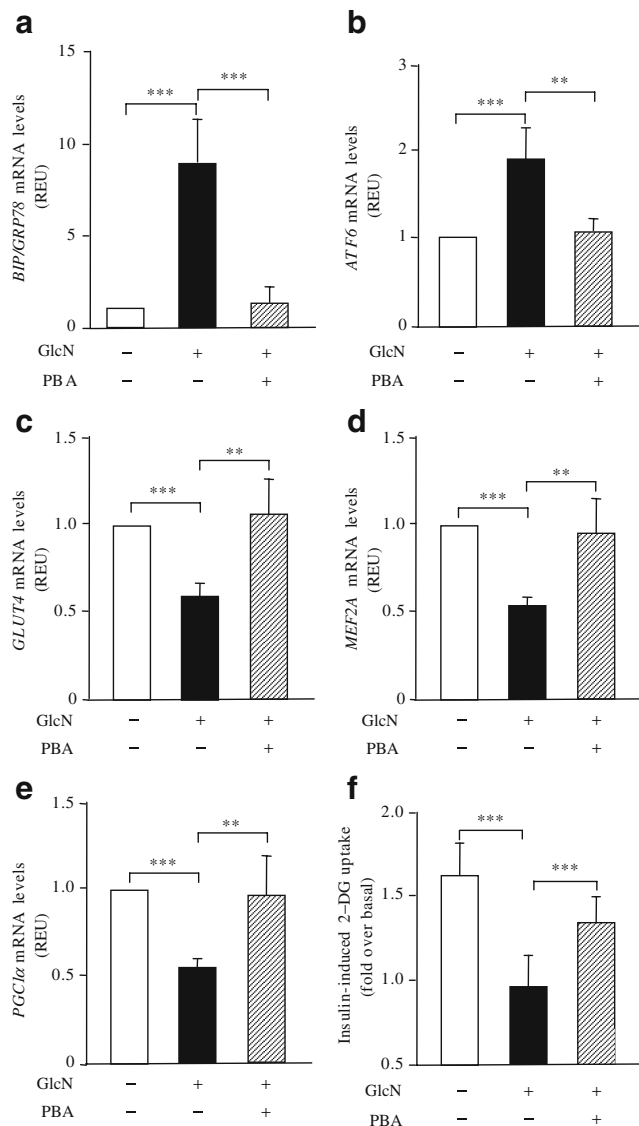


Fig. 6 GlcN effects in human myotubes. **a–f** Human myotubes were pretreated or not with 10 mmol/l PBA for 1 h, and then were treated with 7.5 mmol/l GlcN for 24 h. *BIP/GRP78* (**a**), *ATF6* (**b**), *GLUT4* (**c**), *MEF2A* (**d**) and *PGC1α* (**e**) mRNAs were determined by real-time RT-PCR analysis 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 \pm SD; $n=4$). ** $p < 0.01$; *** $p < 0.001$. **f** 2-DG uptake was measured following 30 min of insulin stimulation (mean \pm 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 eIF2 α 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 PGC1 α [46]. PGC1 α is a coactivator of MEF2A. Indeed, MEF2D binds PGC1 α , recruiting this transcriptional coactivator to MEF2A [42].

Moreover, *PGC1 α* expression is reduced in skeletal muscle of prediabetic and diabetic individuals [47], and enhanced *GLUT4* mRNA expression coincides with increased *PGC1 α* 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 *PGC1 α* 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 PGC1 α 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 *PGC1 α* 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 *Pgc1 α* 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 *Pgc1 α* . This effect seems to be independent of the upregulation of SHP, since its silencing does not modify *Glut4*, *Mef2a* and *Pgc1 α* 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 *Pgc1 α* (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 *Pgc1 α* 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 *PGC1 α* . 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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