Glycogen synthase kinase 3β mediates high glucose-induced ubiquitination and proteasome degradation of insulin receptor substrate 1

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Abstract

High glucose (HG) has been shown to induce insulin resistance in both type 1 and type 2 diabetes. However, the molecular mechanism behind this phenomenon is unknown. Insulin receptor substrate (IRS) proteins are the key signaling molecules that mediate insulin’s intracellular actions. Genetic and biological studies have shown that reductions in IRS1 and/or IRS2 protein levels are associated with insulin resistance. In this study we have shown that proteasome degradation of IRS1, but not of IRS2, is involved in HG-induced insulin resistance in Chinese hamster ovary (CHO) cells as well as in primary hepatocytes. To further investigate the molecular mechanism by which HG induces insulin resistance, we examined various molecular candidates with respect to their involvement in the reduction in IRS1 protein levels. In contrast to the insulin-induced degradation of IRS1, HG-induced degradation of IRS1 did not require IR signaling or phosphatidylinositol 3-kinase/Akt activity. We have identified glycogen synthase kinase 3β (GSK3β or GSK3B as listed in the MGI Database) as a kinase required for HG-induced serine332 phosphorylation, ubiquitination, and degradation of IRS1. Overexpression of IRS1 with mutation of serine332 to alanine partially prevents HG-induced IRS1 degradation. Furthermore, overexpression of constitutively active GSK3β was sufficient to induce IRS1 degradation. Our data reveal the molecular mechanism of HG-induced insulin resistance, and support the notion that activation of GSK3β contributes to the induction of insulin resistance via phosphorylation of IRS1, triggering the ubiquitination and degradation of IRS1.


Introduction

Insulin resistance is a major pathophysiological problem in both type 1 and type 2 diabetes mellitus, and is a key component of the metabolic syndrome (DeFronzo et al. 1982, Fourlanos et al. 2004). Insulin resistance is characterized by a progressive reduction in the response of insulin-sensitive tissues to a normal level of insulin (Kahn 1994). The molecular events that mediate insulin’s actions in its target tissues have been well detailed in recent years due to extensive genetic and molecular studies (Virkamaki et al. 1999, Lee & White 2004, Taniguchi et al. 2006). In comparison, the molecular basis of insulin resistance is considerably less clear.

Insulin receptor substrate (IRS) proteins are major docking proteins that mediate insulin/insulin-like growth factor 1 (IGF1) signaling in various insulin-sensitive tissues (White 2006). IRS1, like other IRS proteins, contains many tyrosyl phosphorylation sites for IR tyrosine kinase (Sun et al. 1993). Tyrosine phosphorylation of IRS1 creates binding sites for proteins containing the Src homology–2 domain including phosphatidylinositol 3-kinase (PI3K), the GRB2/SOS complex, SHP-2 (or PTPN11 as listed in the MGI Database), c-fyn, and NCK (Sun & Liu 2009). Many serine/threonine kinases that phosphorylate IRS1 have been identified, including glycogen synthase kinase 3β (GSK3β or GSK3B), p70S6K, protein kinase Cα (PKCα), ERK, JNK, MTOR, and AMPK among others (Sun & Liu 2009). Most IRS1 serine/threonine phosphorylation events attenuate insulin signaling, and are thought to induce insulin resistance.

Studies in humans, animals (Saad et al. 1992, Rondinone et al. 1997), and cultured cells (Lee et al. 2000, Zhande et al. 2002)
have reported an inverse correlation between IRS1 protein levels and insulin resistance. Mice deficient in IRS1 are insulin resistant and growth retarded (Araki et al. 1994, Lee et al. 2000); diabetes develops when the loss of IRS1 is combined with a heterozygous null IR mutation (Bruning et al. 1997). Chronic insulin treatment, a well-established method for inducing insulin resistance (Del Prato et al. 1994, Koopmans et al. 1997), induces ubiquitin-mediated proteasome degradation of IRS1, leading to cellular insulin resistance (Sun et al. 1999, Lee et al. 2000, Rui et al. 2002, Zhande et al. 2002). Recently, an in vitro siRNA-based study demonstrated the essential role of IRS1 in the regulation of gluconeogenic gene expression in the liver, such that knockdown of IRS1 resulted in the upregulation of gluconeogenic genes (Taniyuchi et al. 2005). These studies emphasize the importance of cellular levels of IRS1 in insulin sensitivity.

It has been reported that chronic hyperglycemia can induce insulin resistance in both humans and animals (Vuorinen-Markkola et al. 1992, Buren et al. 2003). In contrast to that induced by hyperinsulinemia, hyperglycemia-catalyzed resistance is less straightforward. It is unclear whether high glucose (HG) inhibits insulin signaling directly through metabolite effects, secondarily by stimulation of excessive insulin secretion, via a combination of the two aforementioned mechanisms, or through some yet undiscovered mechanism. From cell-based studies and research in type 1 diabetes, it can be concluded that hyperglycemia certainly does not require secondary hyperinsulinemia to antagonize insulin function. However, even in insulin-free systems, the mechanism of hyperglycemia-induced insulin resistance is unclear. Activation of PKC, degradation of IRS2, and generation of reactive oxygen species have all been proposed, with no conclusive model to date (Berti et al. react). Chronic insulin treatment, a well-established method for inducing insulin resistance (Del Prato et al. 1994, Koopmans et al. 1997), induces ubiquitin-mediated proteasome degradation of IRS1, leading to cellular insulin resistance (Sun et al. 1999, Lee et al. 2000, Rui et al. 2002, Zhande et al. 2002). Recently, an in vitro siRNA-based study demonstrated the essential role of IRS1 in the regulation of gluconeogenic gene expression in the liver, such that knockdown of IRS1 resulted in the upregulation of gluconeogenic genes (Taniyuchi et al. 2005). These studies emphasize the importance of cellular levels of IRS1 in insulin sensitivity.

Materials and Methods

Cells and transfection

CHO cells and CHO/IR cells (CHO cells overexpressing the IR) were grown in F12 medium supplemented with 10% fetal bovine serum (FBS). They were cultured at 37 °C in a humidified incubator maintained at 5% CO₂.

CHO/IR cells overexpressing His-tagged IRS1 (IRS1His6), wild-type IRS1, IRS1 with mutation of serine332 to alanine, or IRS1 with mutation of serine332/336 to alanine were obtained by transfection of CHO/IR cells with pCMV/His/IRS1His6, pCMV/His/IRS1, pCMV/His/IRS1S332A, or pCMV/His/IRS1S332/336A constructs respectively using lipofectamine (Invitrogen; Zhande et al. 2002, Liberman & Eldar-Finkelman 2005). Transfected cells were selected for resistance to 10 mM histidinol for 3 weeks before being used in the experiments.

Adenoviral infection

For the expression of GSK3β variants, recombinant adenoviruses were generated and used as previously described (Finlay et al. 2004). In total, 40% confluent CHO/IR cells were incubated with 200 MOI of the following adenoviruses: AdV-Fluc (virus control), AdV-GSK3β-KD (kinase-deficient mutant), or AdV-GSK3βSA (constitutively active mutant). Infections were performed in F12 medium containing 2% FBS for 2 h. The medium was then removed, and the cells were washed twice with PBS. F12 medium containing 10% FBS was added again, and the cells were allowed to recover for at least 20 h. The adenovirus-infected CHO/IR cells were then used in the experiments as described.

Primary hepatocyte isolation and culture

Primary mouse hepatocytes (C57/Bj6) were isolated by a modified noncirculating collagenase perfusion method as described (Berry & Friend 1969, Zhande et al. 2006).

Prolonged HG exposure

For glucose exposure in CHO cells, the cells were cultured to 80–85% confluency, and were then incubated with low glucose (5.5 mM or 1 g/l) or HG (25 mM or 4.5 g/l) DMEM containing 0.25% BSA for various time points. The medium was refreshed every 24 h if incubation was done for longer...
than 24 h. For glucose exposure in primary mouse hepatocytes, the cells were cultured in low glucose (5-5 mM) or HG (25 mM) DMEM with 10% FBS. In some cases, insulin (5 or 10 nM) or inhibitors, including cycloheximide (0-5 mM), MG132 (25 pM), LY294002 (50 nM), LiCl (20 mM), rapamycin (200 nM), and PD98059 (0-1 mM), were also included in the medium. At the end of the incubation, cells were left unstimulated or stimulated with 10 nM insulin for 10 min before being lysed in an appropriate buffer.

His-tagged protein purification

CHO/IR cells overexpressing His6-tagged IRS1 (IRS1His6; Zhande et al. 2002) were washed once with ice-cold PBS containing 100 μM Na3VO4 and 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysed in 1 ml lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1% Tween 20, 1 mM PMSF, 100 μM Na3PO4, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 50 μg/ml microcin) for 10 min. Lysates were centrifuged at 13,000 g for 15 min, and the supernatants were mixed with Ni-NTA beads (Qiagen; 200 μl per 10 cm plate of cells). The beads with IRS1His6 were washed with a wash buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1% Tween 20, 1 mM PMSF, 100 μM Na3PO4, 50 μg/ml aprotinin, and 50 μg/ml leupeptin) three times, and then were denatured in Laemmli sample buffer containing 0.1 M dithiothreitol (DTT) and boiled for 5 min.

Whole cell lysate preparation

CHO cells, CHO/IR cells, or primary mouse hepatocytes were lysed directly in Laemmli sample buffer containing 0.1 M DTT, sonicated, and boiled for 5 min.

Immunoblotting analysis

Samples from Ni-NTA purification or whole cell lysates were separated by SDS-7-5% or 10% PAGE, and transferred onto nitrocellulose membranes. Membranes were briefly washed with tris buffered saline (TBS; 20 mM Tris–HCl, pH 8.0, and 0.15 M NaCl) and blocked (5% skim milk in TBST (TBS containing 0.05% Tween 20)) for 2 h at room temperature or overnight at 4 °C, followed by incubation with the corresponding antibodies in the appropriate blocking solution (5% skim milk–TBST or 5% BSA–TBST): αPY (PY20), αIRβ (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), αPS5-GSK3β-P-P44/42 ERK1/2, αERK1/2, αPT24 FoxO1, αGSK3β (Cell Signaling Technology, Inc.), αubiquitin (FK2, Biomas International, LP, Plymouth Meeting, PA, USA), αIRS1, αIRS2, and αPS3232 IRS1. Membranes were then washed three times with TBST and probed with HRP-conjugated protein A (Santa Cruz) for 30 min, or HRP-conjugated rat anti-mouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) or HRP-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc.) for 1 h. After incubation with secondary antibodies, membranes were washed three times with TBST and once with TBS. Specific proteins were visualized using ECL (GE Healthcare, Pittsburg, PA, USA; Zhande et al. 2002).

Results

HG induces degradation of IRS1, but not of IRS2

We have shown previously that prolonged insulin treatment can induce degradation of IRS1, but not of IRS2, in CHO cells overexpressing the human IR (CHO/IR; Sun et al. 1999, Zhande et al. 2002). To investigate if HG could lead to the same effect, CHO/IR cells were exposed to normal glucose (5-5 mM) or HG (25 mM) for various lengths of time. Reductions in IRS1 protein levels were observed after 72 h of exposure (Fig. 1A, lanes a–d and f versus lane e). This was not due to changes in cell number since nonspecific bands (NS) and GSK3β bands were constant at all time points. To ensure that this effect was not limited to CHO/IR cells, we performed similar experiments using primary mouse hepatocytes. We obtained comparable data, although the reduction (50%) in IRS1 protein levels was observed after 22 h of exposure to HG (Fig. 1B, panel 1). Interestingly, this effect was specific to the IRS1 molecule, since HG exposure had no effect on IRS2 protein levels under the same conditions (Fig. 1B, panel 2).

Intracellular protein levels are determined by the balance between protein synthesis and degradation. To evaluate the contribution of impaired synthesis versus enhanced degradation to HG-induced reduction of IRS1 protein levels, CHO/IR cells were coincubated with HG and cycloheximide, a protein translation inhibitor, for 14 h. Without cycloheximide, the reduction in IRS1 protein levels by HG was not detectable after 14 h of exposure (Fig. 1C, lane b versus lane a). When cycloheximide was present, a significant reduction in IRS1 protein levels by HG was observed (Fig. 1C, lane d versus lane c). This suggests that the effect of HG on IRS1 is not mediated by suppression of IRS1 gene transcription or mRNA translation, but rather that HG acts by augmenting IRS1 protein degradation. Indeed, MG132, a relatively specific proteasome inhibitor, significantly blocked HG-induced degradation of IRS1 (Fig. 1C, lanes e–f versus lanes c–d), implying the involvement of the ubiquitin-mediated proteasome degradation pathway.

To test if the reduction in IRS1 protein levels could impact insulin signaling, we examined insulin–stimulated tyrosyl phosphorylation of IRS proteins and downstream signaling events in primary hepatocytes. HG impaired insulin signaling, including insulin-induced tyrosine phosphorylation of IRS proteins (30%), threonine32 phosphorylation of FoxO1 (20%), serine phosphorylation of GSK3β threonine202/tyrosine204 phosphorylation of ERK (35%), and serine phosphorylation of AKT (65%; Fig. 2, lane c versus lane b).
GSK3β is required for HG-induced IRS1 degradation

To investigate the signaling pathway that mediates HG-induced degradation of IRS1, we used several specific inhibitors for various serine/threonine kinases that are involved in the modulation of insulin sensitivity. These include LiCl (GSK3β), rapamycin (MTOR), PD98059 (MEK), and LY294002 (PI3K). Among these tested inhibitors, LiCl was the only inhibitor that was capable of completely blocking HG-induced degradation of IRS1 (Fig. 3A, panel 1, lane d versus lanes b, f, and h; Fig. 4C, panels 1 and 3, lane b versus lane d), suggesting the involvement of GSK3β in HG-induced degradation of IRS1. Since phosphorylation of GSK3β at serine9 inactivates the kinase, we next checked the phosphorylation level of GSK3β. Indeed, HG exposure led to a significant reduction in GSK3β serine9 phosphorylation (activation; Fig. 3A, panel 2, lane b versus lane a), and LiCl enhanced the phosphorylation of GSK3β (inactivation; Fig. 3A, panel 2, lanes c–d versus lanes a–b). The effect of LiCl on GSK3β phosphorylation and HG-induced degradation of IRS1, but not of IRS2, was also observed in primary hepatocytes (Fig. 3B, panels 1–3, lanes c–d versus lanes a–b). PD98059, rapamycin, and LY294002 were unable to block the reduction of GSK3β phosphorylation or degradation of IRS1 induced by HG (Fig. 3A, panels 1–2, lanes e–h versus lanes a–b; Fig. 4C, panels 1–4, lanes a–b versus lanes c–d). These data strongly suggest a specific role for GSK3β in the mediation HG-induced degradation of IRS1.

It is well known that many kinase inhibitors are not as specific as commercially advertised (Bain et al. 2007). To verify if activation of GSK3β is sufficient to induce IRS1 degradation, we overexpressed a GSK3β kinase-deficient...
HG and insulin induce degradation of IRS1 through distinct signaling pathways

Insulin-induced IRS1 proteasome degradation depends on the activity of the IR tyrosine kinase and PI3K (Sun et al. 1999, Zhang et al. 2000). We first tested whether the IR was also required for HG-induced degradation by comparing CHO/IR cells to parental CHO cells. Although CHO/IR cells expressed significant quantities of IR when compared with CHO cells, a comparable degree of degradation of IRS1 was observed in both cell lines after 72 h of HG exposure (Fig. 4A, lanes b versus lane a). This pattern was maintained even when protein synthesis was blocked with cycloheximide (Fig. 4B, lanes d and h versus lanes c and g). Thus, IR is not required for HG-induced degradation of IRS1.

Figure 2

The fact that HG and insulin imposed opposite effects on GSK3β activity (HG activates GSK3β while insulin inhibits GSK3β; Fig. 4C, panels 2 and 4) prompted us to examine whether insulin signaling had any effect on HG-induced degradation of IRS1. In CHO/IR cells, although 10 nM insulin induced the inhibition of GSK3β (increased phosphorylation; Fig. 4C, panel 4, lanes e–f), it induced the degradation of IRS1 under both normal and HG conditions (Fig. 4C, panel 3, lanes e–f), suggesting a mechanism independent of GSK3β activity. However, blocking PI3K activity by LY294002 only prevented insulin-induced degradation under normal glucose conditions, but not under HG conditions (Fig. 4C, panel 3, lanes g–h versus lanes e–f), which is consistent with the fact that HG-induced degradation of IRS1 is independent of PI3K activity. Thus, HG and insulin induce degradation of IRS1 via distinct pathways.

Although 10 nM insulin failed to prevent the HG-induced degradation of IRS1 in CHO/IR cells (sensitive to insulin), it did prevent HG-induced IRS1 degradation to a modest extent in CHO cells (less sensitive to insulin; Fig. 4C, panels 1 and 3, lanes e–f versus lanes a–d). There is a possibility for the effect of insulin on HG-induced degradation of IRS1 being minimized by the insulin-induced degradation of IRS1 during overnight exposure of CHO/IR cells to high-dose insulin (10 nM). To investigate if short-term exposure to a low dose of insulin could reveal any effect of insulin on HG-induced activation of GSK3β and degradation of IRS1, CHO/IR cells were treated with normal and HG in conjunction with cycloheximide in the presence or absence of a low dose of insulin (5 nM) for various time periods. Five-nanomolar insulin in normal glucose induced degradation of IRS1 at a slow rate. In comparison, HG induced IRS1 degradation at a much faster rate (Fig. 4D, panel 1, lanes b–d versus lanes e–g, and Fig. 4E). Interestingly, HG-induced degradation of IRS1 was greatly reduced in the presence of 5 nM insulin (Fig. 4D, lanes h–j versus lanes b–d and e–g; Fig. 4E). The dynamic changes in HG-induced degradation

Next, we tested whether PI3K activity was required for HG-induced degradation of IRS1 by stimulating cells with insulin, or blocking PI3K activity with LY294002, a potent PI3K inhibitor. CHO and CHO/IR cells were incubated with or without insulin (10 nM) in the presence of normal glucose (5·5 mM) or HG (25 mM) for 16 h in the presence of cycloheximide (Fig. 4C). As expected, IR was required for insulin-induced degradation under normal glucose conditions, which was evidenced by the observation that insulin induced IRS1 degradation only in CHO/IR cells, but not in CHO cells (Fig. 4C, panels 1 and 3, lane e versus lane a). LY294002 completely blocked insulin-induced degradation of IRS1 (Fig. 4C, panel 3, lane g versus lanes e and a), suggesting that PI3K mediates the insulin-induced degradation of IRS1. In contrast, HG alone reduced IRS1 in both CHO and CHO/IR cell lines even when PI3K activity was blocked (Fig. 4C, panels 1 and 3, lanes b and d versus lanes a and c), indicating that IR and PI3K are not required for HG-induced degradation of IRS1.

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of IRS1 by insulin were consistent with changes in GSK3β activity (Fig. 4D, panel 2), further supporting the involvement of GSK3β in HG-induced degradation of IRS1.

HG promotes the phosphorylation of serine332 in IRS1

GSK3β has been shown to negatively regulate insulin signaling by phosphorylating IRS1 at serine332 after phosphorylation of the priming site serine336 by PKCβII (Eldar-Finkelman & Krebs 1997, Liberman & Eldar-Finkelman 2005, Liberman et al. 2008). To investigate if IRS1 is phosphorylated at this specific site during HG exposure, we used a phospho-specific antibody against the site to monitor the specific phosphorylation of IRS1. Indeed, serine332 phosphorylation was increased in HG-treated cells, and was completely blocked by LiCl (Fig. 5A, lanes b and c versus lane a), suggesting that GSK3β is the potential kinase that mediates the phosphorylation of IRS1 by HG. To further investigate the role of serine332 in the HG-induced degradation of IRS1, two nonphosphorylatable serine→alanine mutants (Ser→Ala332 and Ser→Ala332/336) were overexpressed in CHO/IR cells (Liberman & Eldar-Finkelman 2005), as well as in wild-type IRS1 control. Transfected cells were subjected to HG treatment and examined for IRS1 degradation. Both ser→ala mutants prevented HG-induced degradation of IRS1 to a significant extent (Fig. 5B, lanes d and f versus lane b). Considering the high ratio of endogenous versus overexpressed IRS1 proteins, the degree to which IRS1 was protected from degradation by the mutants is potentially greater than that suggested by the immunoblots. Thus, serine332 phosphorylation in IRS1 by GSK3β is required for and appears to be the principle site with respect to HG-induced degradation of IRS1.

GSK3β activity is required for HG-induced ubiquitination of IRS1

As shown in Fig. 1C, HG-induced IRS1 degradation was blocked by MG132, a 26S protease inhibitor, indicating the involvement of ubiquitin-mediated proteasome degradation. To confirm the ubiquitination of IRS1 during HG exposure, ubiquitination of IRS1 was measured in the presence of MG132. Indeed, HG increased IRS1 ubiquitination (Fig. 6, lane b versus lane a). Consistent with our data, LiCl inhibited GSK3β activity (Fig. 3A), reduced phosphorylation of IRS1 at serine332 (Fig. 5A), and reduced IRS1 ubiquitination (Fig. 6, lane b versus lanes a and b). These data support the conclusion that HG-induced ubiquitination and degradation of IRS1 are mediated by GSK3β.

**Figure 3** Involvement of GSK3β in HG-induced IRS1 degradation. (A) CHO/IR cells were incubated in DMEM–0.25% BSA with cycloheximide (0–5 mM) and 5.5 or 25 mM glucose containing various inhibitors: LiCl (20 mM), rapamycin (200 nM), or PD98059 (0–1 mM) for 16 h. (B) Primary mouse hepatocytes were incubated in DMEM–10% FBS with 5–5 or 25 mM glucose for 22 h. In lanes c–d, LiCl (20 mM) was added to the medium and incubated for the last 8 h. (C) CHO/IR cells were infected with blank adenovirus or adenovirus containing the kinase-deficient GSK3β mutant or the constitutively active GSK3β mutant (200 MOI) for 20 h. After treatment(s), all cells were lysed in Laemmli sample buffer containing 0–1 M DTT. Lysates were processed for immunoblotting analysis as described in Materials and Methods. Results are representative of at least two separate experiments. Quantification of western blots (B) was done in the same way as described in Fig. 1. Data are expressed as means ± S.E.M., n = 3.
Discussion

IRS1 is one of the major IRSs in the insulin signaling pathway, and is required for normal insulin signaling (Lee & White 2004, Taniguchi et al. 2006). IRS1 protein levels are controlled by many factors including insulin/IGF1, glucocorticoids, oxidative stress, and certain nutrients (free fatty acids, amino acids, and glucose). The ubiquitin-mediated proteasome degradation of IRS1 is an important cellular control mechanism for insulin sensitivity (Sun et al. 1999, Rui et al. 2002, Zhande et al. 2002). Here, we have shown that long-term HG exposure (mimicking chronic hyperglycemia) also induces ubiquitin-mediated proteasome degradation of IRS1. Interestingly, HG-induced degradation of IRS1 is mediated by a mechanism distinct from that of insulin, in that it is IR independent and does not require PI3K activity. More importantly, we have shown that the activation of GSK3β is required for HG-induced degradation of IRS1. HG activates GSK3β, which phosphorylates IRS1 at serine332, leading to the ubiquitination and proteasome-mediated degradation of IRS1. Thus, our study, for the first time, provides direct evidence that phosphorylation of IRS1 is linked to its ubiquitin-mediated proteasome degradation, and reveals a molecular mechanism for HG-induced degradation of IRS1.

We and others have reported previously that insulin/IGF1 can induce IRS1 degradation through a ubiquitin-mediated proteasome degradation pathway (Sun et al. 1999, Lee et al. 2000, Rui et al. 2002, Zhande et al. 2002). In this study, we have clearly shown that although both insulin and HG activate the ubiquitin-mediated proteasome pathway to...
Laemmli sample buffer containing 0.1 M DTT. S332 phosphorylation occurs in CHO/IR/IRS1 cells overexpressing wild-type IRS1, IRS1S332A, or IRS1S332/336A mutants were incubated in DMEM–0.25% BSA containing cycloheximide (0.5 mM) and MG132 (25 mM) and 5.5 or 25 mM glucose in the presence or absence of LiCl (20 mM) for 24 h. After treatments, all cells were lysed in Laemmli sample buffer containing 0.1 M DTT. S332 phosphorylation of IRS1, IRS1 protein, and the β-subunit of insulin receptor in the lysates were detected by immunoblotting analysis using the corresponding antibodies as described in Materials and Methods. Results are representative of at least three separate experiments. Quantification of western blot (B) was done in the same way as described in Fig. 1. Data are expressed as means ± S.E.M., n = 3.

Figure 5 Serine332 phosphorylation of IRS1 in HG-induced IRS1 degradation. (A) CHO/IR/IRS1 cells were incubated in DMEM–0.25% BSA containing cycloheximide (0.5 mM) and MG132 (25 μM) and 5.5 or 25 mM glucose for 16 h. After treatment(s), all cells were lysed in desalted sample buffer containing 0.1 M DTT. S332 phosphorylation of IRS1, IRS1 protein, and the β-subunit of insulin receptor in the lysates were detected by immunoblotting analysis using the corresponding antibodies as described in Materials and Methods. Results are representative of at least three separate experiments. Quantification of western blot (B) was done in the same way as described in Fig. 1. Data are expressed as means ± S.E.M., n = 3.

Contrary to the insulin-induced degradation of IRS1, there exist at least two distinct upstream signaling chains that ultimately lead to ubiquitination of IRS1 (Fig. 7). The central finding in this study is the identification of GSK3β as the key component in HG-induced degradation of IRS1. There are a few conflicting reports that describe the effect of HG on GSK3β activity. In cancer cells, increasing glucose uptake and high rates of glucose utilization induced the phosphorylation of GSK3α/β (inhibition) in a PKC-mediated process, leading to cell survival (Zhao et al. 2007). In glomerular mesangial cells, HG–stimulated GSK3β activity augmented apoptosis by antagonizing Wnt signaling, an effect preventable by LiCl treatment (Lin et al. 2006). GSK3β activity was found to be higher in STZ-treated hyperglycemic rats due to decreased AKT activity (Gurusamy et al. 2006). HG has also been shown to downregulate both basal and insulin-stimulated glycogen synthase in rat-1 fibroblasts secondary to the stimulation of GSK3β (Singh & Crook 2000). Our current study clearly shows that HG reduces GSK3β serine9 phosphorylation without changing GSK3β protein levels. More importantly, we have demonstrated in several experiments that GSK3β is necessary and sufficient for the mediation of HG-induced degradation of IRS1. First, an inhibitor of GSK3β blocks HG-induced proteasome degradation of IRS1; secondly, HG reduces GSK3β phosphorylation at serine9, which is indicative of activation;
and finally, overexpression of constitutively active GSK3β increases IRS1 degradation, whereas overexpression of kinase-deficient GSK3β slightly blocks basal IRS1 degradation.

The mechanism by which GSK3β mediates IRS1 degradation is unknown. Our results provide a possible mechanism explaining how GSK3β mediates the ubiquitination of IRS1. Serine/threonine phosphorylation is known to be an important signal for the ubiquitination and proteasome degradation of cellular proteins (Nash et al. 2001). GSK3β phosphorylates IRS1 at serine332 and attenuates insulin’s action (Liberman & Eldar-Finkelman 2005). Our results clearly show that inhibition of GSK3β not only blocks the proteasome degradation of IRS1, but also prevents IRS1 serine332 phosphorylation and its subsequent ubiquitination. Furthermore, mutation of serine332 to alanine prevents IRS1 degradation induced by HG. Taken together, the data support a model where HG activates GSK3β promoting serine332 phosphorylation of IRS1. This phosphorylation tags IRS1 for ubiquitination, leading to proteasome-mediated destruction.

Glucosamine, a glucose metabolite, has also been shown to induce insulin resistance in different cells and animals (DeFronzo et al. 1982, Rossetti et al. 1995, Patti 1999, Heart et al. 2000). We were unable to detect the effect of glucosamine on IRS1 ubiquitination and proteasome degradation in our cell systems (data not shown), indicating that glucose and glucosamine might induce insulin resistance via different mechanisms as suggested in the literature (DeFronzo et al. 1982, Nelson et al. 2000, 2002, Han et al. 2003, Duan et al. 2005). Exactly how glucose activates GSK3β remains to be revealed in a future study.

Much evidence in the literature suggests that GSK3β is involved in insulin resistance. GSK3 is constitutively active in unstimulated cells, and is inactivated by a variety of extracellular stimuli, notably insulin (Woodgett 1990). Elevated GSK3 activity and expression have been observed in obese and diabetic humans and rodents (Eldar-Finkelman et al. 1999, Nikoulina et al. 2000, Ciaraldi et al. 2002) and in the adipose tissue of high-fat-fed mice (Eldar-Finkelman et al. 1999). Overexpression of human GSK3β (fivefold) in the skeletal muscle of mice results in hyperglycemia, hyperinsulinemia, hyperlipidemia, a reduction in glycogen levels, and a significant decrease in IRS1 protein levels (Pearce et al. 2004). Homozygous GSK3β knockout is embryonically lethal, suggesting that it plays an important role in early development (Hoeflich et al. 2000). However, GSK3α knockout animals are viable, and demonstrate enhanced glucose and insulin sensitivity (MacAulay et al. 2007). Insulin-stimulated Akt and GSK3β phosphorylation is higher in GSK3α KO livers than in the wild-type littermates. Interestingly, hepatic IRS1 expression is markedly increased in these animals (MacAulay et al. 2007). Although our experiments did not test GSK3α, it is very likely that GSK3α is also involved in HG-induced IRS1 degradation. Thus, our studies are consistent with these reports, and further provide the molecular mechanism through which GSK3 is involved in insulin resistance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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