



Published in final edited form as:

J Endocrinol. 2010 August ; 206(2): 171–181. doi:10.1677/JOE-09-0456.

Glycogen synthase kinase 3 β 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 3 β (GSK3 β 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 GSK3 β was sufficient to induce IRS1 degradation. Our data reveal the molecular mechanism of HG-induced insulin resistance, and support the notion that activation of GSK3 β 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

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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.

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 *et al.* 1993, White 2006). These interactions lead to the activation of multiple signaling pathways required for insulin's pleiotropic actions (White 2006). In addition, IRS1 also contains multiple serine/threonine phosphorylation sites (Sun *et al.* 1991). It is subject to phosphorylation by a wide array of protein kinases that are involved in diverse signaling pathways including those stimulated by insulin, inflammation, nutrients, and cellular stress (Sun & Liu 2009). Many serine/threonine kinases that phosphorylate IRS1 have been identified, including glycogen synthase kinase 3 β (GSK3 β or GSK3B), p70^{S6K}, protein kinase C θ (PKC θ), 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 vivo* 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 (Taniguchi *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.* 1994, Ide *et al.* 1994, Pillay *et al.* 1996, Nakajima *et al.* 2000).

GSK3 β is a serine kinase negatively regulated by insulin that was originally identified as a key regulator of glycogen synthesis. Insulin activates AKT/protein kinase B through a well-defined mechanism mediated by the IRS1/PI3K pathway. This leads to the phosphorylation of GSK3 β at serine⁹, resulting in its inactivation (Cross *et al.* 1995, Shaw *et al.* 1997, Frame & Cohen 2001). In addition to glycogen synthesis, GSK3 β plays roles in many other biological functions including Wnt signaling (crucial in development) and NF κ B (or NFKB) signaling (a key mediator of the inflammatory response; Frame & Cohen 2001, Lee & Kim

2007). GSK3 β is also thought to be involved in insulin resistance. In type 2 diabetes, GSK3 β activity is elevated twofold in both basal and insulin-stimulated muscle (Nikoulina *et al.* 2000). Additionally, inhibition of GSK3 β improves insulin's action and glucose metabolism in human smooth muscle (Nikoulina *et al.* 2002). GSK3 β has been shown to directly phosphorylate IRS1 *in vitro* and *in vivo* at serine³³² and impair insulin signaling (Eldar-Finkelman & Krebs 1997, Liberman & Eldar-Finkelman 2005). Thus, GSK3 β may play a causative role in the development of insulin resistance.

In this study, we have investigated the molecular mechanism of HG-induced insulin resistance in both Chinese hamster ovary (CHO) cells and primary mouse hepatocytes. We report that HG promotes proteasome-mediated degradation of IRS1. Unlike insulin, HG-induced degradation is independent of IR signaling and PI3K activity. We have identified GSK3 β as a key kinase that mediates HG-induced degradation of IRS1.

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 (IRS1^{His6}), wild-type IRS1, IRS1 with mutation of serine³³² to alanine, or IRS1 with mutation of serine^{332/336} to alanine were obtained by transfection of CHO/IR cells with pCMV/His/IRS1^{His6}, pCMV/His/IRS1, pCMV/His/IRS1^{S332A}, or pCMV/His/IRS1^{S332/336A} 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 GSK3 β 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-GSK3 β -KD (kinase-deficient mutant), or AdV-GSK3 β ^{S9A} (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 μ M), LY294002 (50 μ M), 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 (IRS1^{His6}; Zhande *et al.* 2002) were washed once with ice-cold PBS containing 100 μ M Na₃VO₄ and 1 mM phenylmethyl-sulfonyl 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 μ M Na₃PO₄, 50 μ g/ml aprotinin, 50 μ g/ml leupeptin, and 50 μ g/ml microcystin) for 10 min. Lysates were centrifuged at 13 000 g for 15 min, and the supernatants were mixed with Ni-NTA beads (Qiagen; 200 μ l per 10 cm plate of cells). The beads with IRS1^{His6} 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 μ M Na₃PO₄, 50 μ g/ml aprotinin, and 50 μ g/ml leupeptin) three times, and were then 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): α PY (PY20), α IR β (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α PS⁹-GSK3 β α -P44/42 ERK1/2, α ERK1/2, α PT²⁴ FoxO1, α GSK3 β (Cell Signaling Technology, Inc.), α ubiquitin (FK2, Biomol International, LP, Plymouth Meeting, PA, USA), α IRS1, α IRS2, and α PS³³²IRS1. 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 GSK3 β 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%), threonine²⁴ phosphorylation of FoxO1 (20%), serine⁹ phosphorylation of GSK3 β threonine²⁰²/tyrosine²⁰⁴ phosphorylation of ERK (35%), and serine⁴⁷³ phosphorylation of AKT (65%; Fig. 2, lane c versus lane b).

GSK3 β 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 (GSK3 β), 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 GSK3 β in HG-induced degradation of IRS1. Since phosphorylation of GSK3 β at serine⁹ inactivates the kinase, we next checked the phosphorylation level of GSK3 β . Indeed, HG exposure led to a significant reduction in GSK3 β serine⁹ phosphorylation (activation; Fig. 3A, panel 2, lane b versus lane a), and LiCl enhanced the phosphorylation of GSK3 β (inactivation; Fig. 3A, panel 2, lanes c–d versus lanes a–b). The effect of LiCl on GSK3 β 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 GSK3 β 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 GSK3 β 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 GSK3 β is sufficient to induce IRS1 degradation, we overexpressed a GSK3 β kinase-deficient mutant as well as a constitutively active GSK3 β (serine⁹ mutated to alanine) by adenoviral infection of CHO/IR cells. Overexpression of the constitutively active GSK3 β mutant significantly reduced IRS1 protein levels (Fig. 3C, lane c versus lane a), whereas overexpression of the kinase-deficient mutant slightly increased the level of IRS1 protein (Fig. 3C, lane b versus lane a). Since high levels of endogenous GSK3 β are present in CHO/IR cells, the kinase-deficient mutant presumably served as a dominant negative mutant to block endogenous GSK3 β activity towards IRS1. This result demonstrates that activation of GSK3 β is sufficient to prompt the degradation of IRS1.

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 d and b versus lanes a and c). 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.

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.

The fact that HG and insulin imposed opposite effects on GSK3 β activity (HG activates GSK3 β while insulin inhibits GSK3 β ; 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 GSK3 β (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 GSK3 β 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 GSK3 β 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 of IRS1 by insulin were consistent with changes in GSK3 β activity (Fig. 4D, panel 2), further supporting the involvement of GSK3 β in HG-induced degradation of IRS1.

HG promotes the phosphorylation of serine³³² in IRS1

GSK3 β has been shown to negatively regulate insulin signaling by phosphorylating IRS1 at serine³³² after phosphorylation of the priming site serine³³⁶ by PKC β II (Eldar-Finkelmann & Krebs 1997, Liberman & Eldar-Finkelmann 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 GSK3 β 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 \rightarrow alanine mutants (Ser \rightarrow Ala³³² and Ser \rightarrow Ala^{332/336}) were overexpressed in CHO/IR cells (Liberman & Eldar-Finkelmann 2005), as well as in wild-type IRS1 control. Transfected cells were subjected to HG treatment and examined for IRS1 degradation. Both ser \rightarrow 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 over-expressed 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 GSK3 β is required for and appears to be the principle site with respect to HG-induced degradation of IRS1.

GSK3 β 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 (to preserve the ubiquitinated IRS1). Indeed, HG increased IRS1 ubiquitination (Fig. 6, lane b versus lane a). Consistent with our data, LiCl inhibited GSK3 β 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 GSK3 β .

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 GSK3 β is required for HG-induced degradation of IRS1. HG activates GSK3 β , 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 degrade IRS1, there exist at least two distinct upstream signaling chains that ultimately lead to ubiquitination of IRS1 (Fig. 7). Contrary to the insulin-induced degradation of IRS1, which requires both the IR tyrosine kinase and PI3K activities (Sun *et al.* 1999, Lee *et al.* 2000, Zhang *et al.* 2000, Rui *et al.* 2002), HG-induced proteasome degradation is an IR- and PI3K-independent process. This conclusion is supported by the fact that overexpression of the IR and/or inhibition of PI3K did not significantly affect HG-induced IRS1 degradation (Fig. 4). Although high concentrations of insulin and HG individually lead to the degradation of IRS1, insulin at low concentrations, for short periods of time, seems to inhibit HG-induced degradation of IRS1. This can be clearly explained by our finding that insulin and HG have opposing effects on GSK3 β phosphorylation. Insulin, through activation of PI3K/Akt, phosphorylates serine⁹ on GSK3 β , inhibiting kinase activity. In contrast, HG activates GSK3 β through unknown mechanism by reducing serine⁹ phosphorylation. Since insulin-induced degradation of IRS1 is a GSK3 β -independent process (LiCl cannot block insulin-induced IRS1 degradation, data not shown), long-term insulin exposure – even at lower concentrations that initially counter HG's effects on GSK3 β – would eventually trigger insulin-induced proteasome degradation (Fig. 4D, 6- and 12-h versus 24-h insulin co-treatment with HG).

The central finding in this study is the identification of GSK3 β as the key component in HG-induced degradation of IRS1. There are a few conflicting reports that describe the effect of HG on GSK3 β activity. In cancer cells, increasing glucose uptake and high rates of glucose utilization induced the phosphorylation of GSK3 α/β (inhibition) in a PKC-mediated process, leading to cell survival (Zhao *et al.* 2007). In glomerular mesangial cells, HG-stimulated GSK3 β activity augmented apoptosis by antagonizing Wnt signaling, an effect preventable by LiCl treatment (Lin *et al.* 2006). GSK3 β 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 GSK3 β (Singh & Crook 2000). Our current study clearly shows that HG reduces GSK3 β serine⁹ phosphorylation without changing GSK3 β protein levels. More importantly, we have demonstrated in several experiments that GSK3 β is necessary and sufficient for the mediation of HG-induced degradation of IRS1. First, an inhibitor of GSK3 β blocks HG-induced proteasome degradation of IRS1; secondly, HG reduces GSK3 β phosphorylation at serine⁹, which is indicative of activation; and finally, overexpression of constitutively active GSK3 β increases IRS1 degradation, whereas overexpression of kinase-deficient GSK3 β slightly blocks basal IRS1 degradation.

The mechanism by which GSK3 β mediates IRS1 degradation is unknown. Our results provide a possible mechanism explaining how GSK3 β 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). GSK3 β phosphorylates IRS1 at serine³³² and attenuates insulin's action (Lieberman & Eldar-Finkelman 2005). Our results clearly show that inhibition of GSK3 β 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 GSK3 β 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 GSK3 β remains to be revealed in a future study.

Much evidence in the literature suggests that GSK3 β 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 GSK3 β (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 GSK3 β knockout is embryonically lethal, suggesting that it plays an important role in early development (Hoeflich *et al.* 2000). However, GSK3 α knockout animals are viable, and demonstrate enhanced glucose and insulin sensitivity (MacAulay *et al.* 2007). Insulin-stimulated Akt and GSK3 β phosphorylation is higher in GSK3 α 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 GSK3 α , it is very likely that GSK3 α 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.

Acknowledgments

Funding

This work was supported by a grant from NIH Grant R01 DK060128 to XJS.

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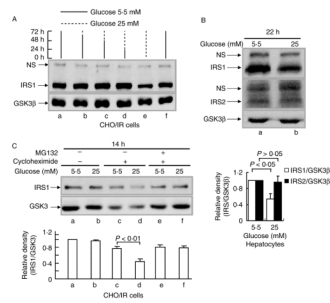


Figure 1.

Effect of high glucose on IRS1 protein levels. (A) CHO/IR cells were incubated in DMEM–0.25% BSA containing 5.5 mM (solid lines) or 25 mM glucose (dashed lines) for the indicated time periods. The medium was refreshed every 24 h. (B) Primary mouse hepatocytes were incubated in DMEM–10% FBS containing 5.5 or 25 mM glucose for 22 h. (C) CHO/IR cells were incubated in DMEM–0.25% BSA containing 5.5 or 25 mM glucose in the presence or absence of cycloheximide (0.5 mM) and MG132 (25 μ M) for 14 h. After treatment(s), all cells were lysed in Laemmli sample buffer containing 0.1 M DTT. IRS1 and IRS2 proteins in the cell lysates were detected by immunoblotting analysis using anti-IRS1 and anti-IRS2 antibodies respectively as described in Materials and methods. Results are representative of at least three separate experiments. The densities for western blot bands were quantified by Image J software. The ratio of density for IRS1 and GSK3 β proteins was calculated for each lane, and the value for normal (5.5 mM) was considered as 1 and compared with other treatments for each individual experiment. Data are expressed as means \pm S.E.M., $n=3$.

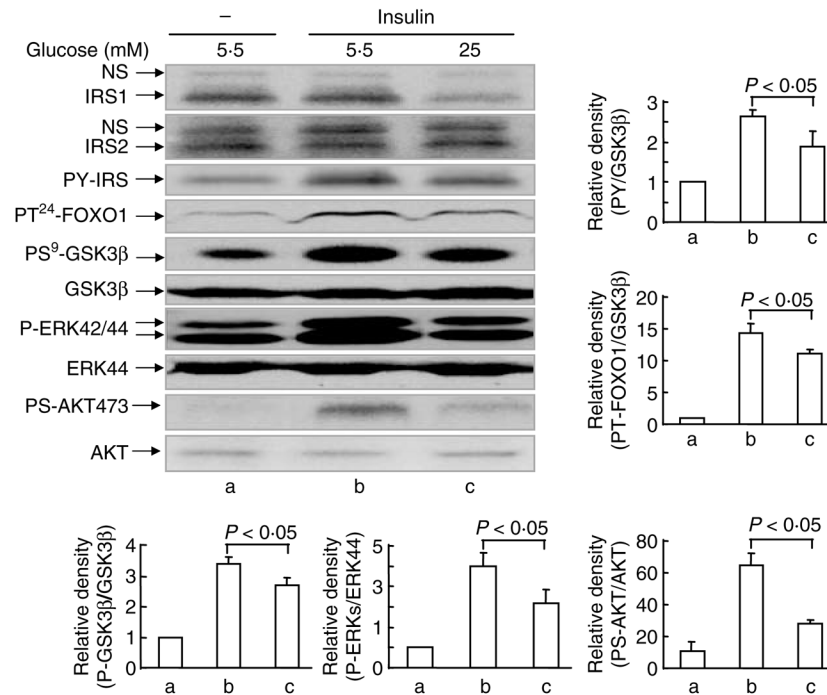
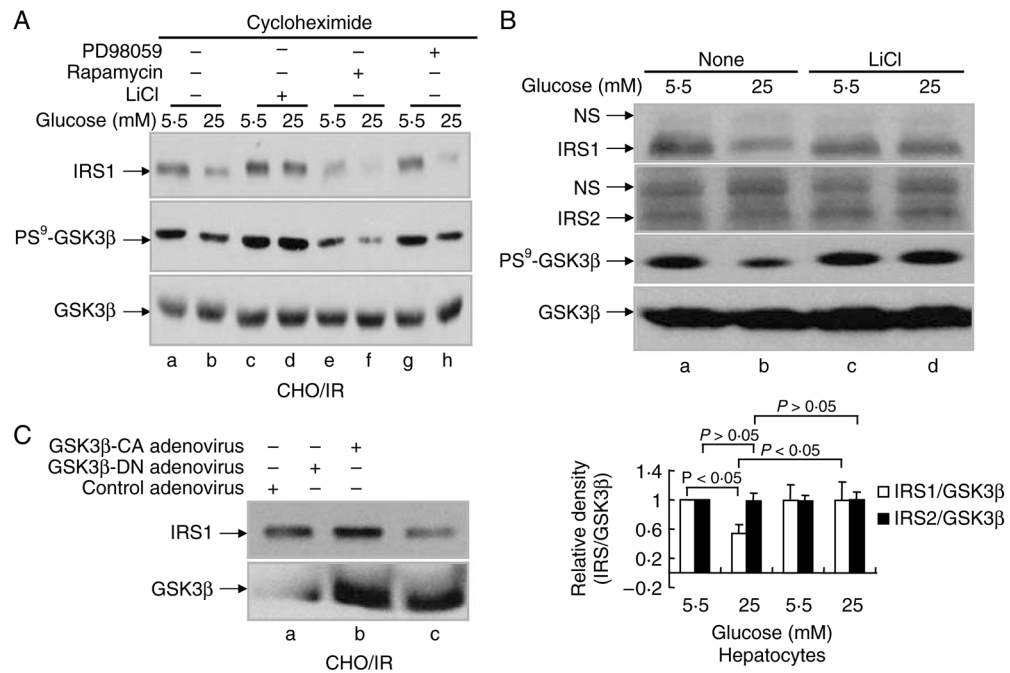
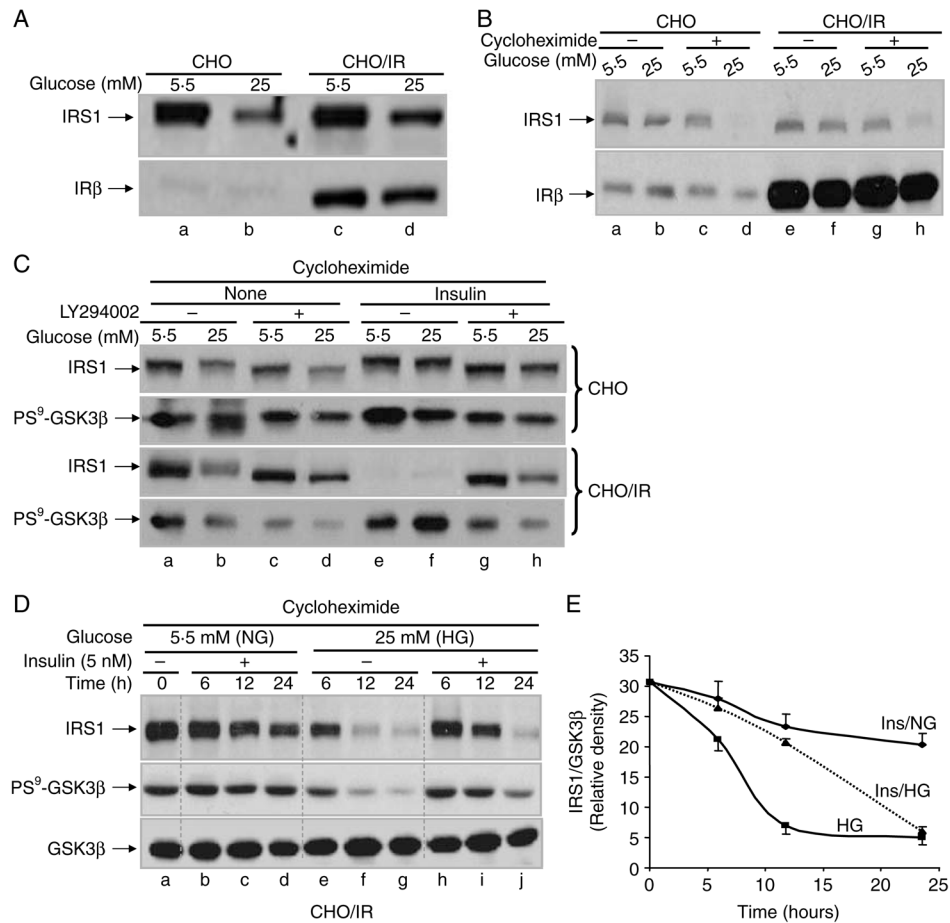


Figure 2. Effect of high glucose on insulin signaling. Primary mouse hepatocytes were incubated in DMEM–10% FBS containing 5.5 or 25 mM glucose for 22 h. Cells were challenged with insulin (10 nM) for 10 min before being lysed in Laemmli sample buffer containing 0.1 M DTT. Signal proteins in the cell lysates were detected by immunoblotting analysis using the corresponding antibodies. Quantification of western blot was done in the same way as described in Fig. 1. Data are expressed as means \pm S.E.M., $n = 3$.

**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., $n = 3$.

**Figure 4.**

Independence of insulin signaling and PI3K activity for HG-induced degradation of IRS1. (A) CHO and CHO/IR cells were incubated in DMEM–0.25% BSA containing 5.5 or 25 mM glucose for 72 h. (B) CHO and CHO/IR cells were incubated in DMEM–0.25% BSA containing 5.5 or 25 mM glucose in the presence or absence of cycloheximide (0.5 mM) for 16 h. (C) CHO and CHO/IR cells were incubated in DMEM–0.25% BSA containing cycloheximide (0.5 mM) and 5.5 or 25 mM glucose in the presence or absence of LY294002 (50 μM) and/or insulin (10 nM) for 16 h. (D) CHO/IR cells were incubated in DMEM–0.25% BSA containing cycloheximide (0.5 mM) and 5.5 or 25 mM glucose in the presence or absence of insulin (5 nM) for 6, 12, or 24 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. (E) Densities of IRS1 and GSK3β in (D) were quantified by Image J software. The ratio of the IRS1:GSK3β density was plotted against time. NG, normal glucose (5.5 mM) and HG, high glucose (25 mM).

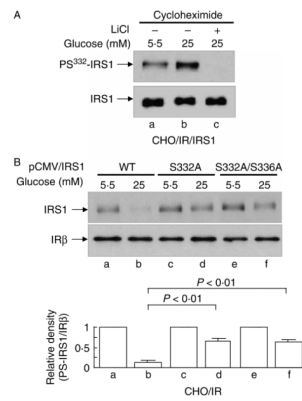


Figure 5. Serine³³² phosphorylation of IRS1 in HG-induced IRS1 degradation. (A) CHO/IR/IRS1 cells were incubated in DMEM–0.25% BSA containing cycloheximide (0.5 mM) and MG132 (25 μM) and 5.5 or 25 mM glucose in the presence or absence of LiCl (20 mM) for 24 h. (B) CHO/IR cells overexpressing wild-type IRS1, IRS1^{S332A}, or IRS1^{S332/336A} mutants were incubated in DMEM–0.25% BSA containing cycloheximide (0.5 mM) and 5.5 or 25 mM glucose for 16 h. After treatment(s), all cells were lysed in Laemmli sample buffer containing 0.1 M DTT. S332 phosphorylation of IRS1, IRS1 fprotein, 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.

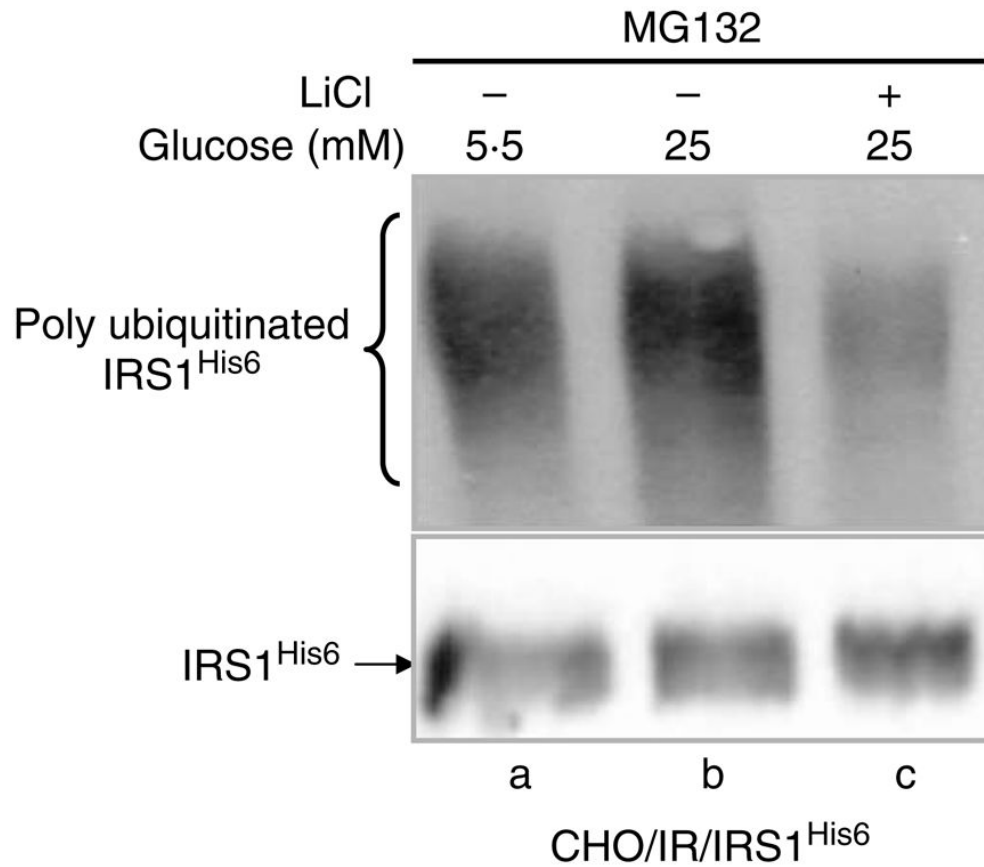


Figure 6. Ubiquitination of IRS1 induced by HG. CHO/IR/IRS1^{His6} cells were incubated in DMEM–0.25% BSA containing MG132 (25 μ M) and 5.5 or 25 mM glucose in the presence or absence of LiCl (20 mM) for 24 h. After treatments, His6-tagged IRS1 (IRS1^{His6}) was purified using Ni-NTA beads and detected by immunoblotting with ubiquitin antibody (FK2) and IRS1 antibody respectively. Results are representative of two separate experiments.

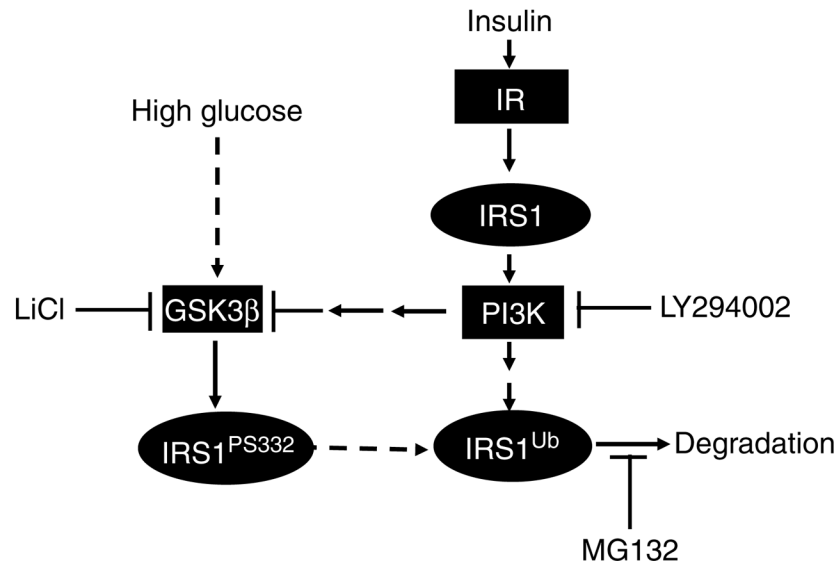


Figure 7. Model of HG- and insulin-induced proteasome degradation of IRS1. Insulin-induced degradation of IRS1 is mediated by a PI3K-dependent pathway; whereas high glucose-induced degradation of IRS1 depends on GSK3 β activity. Although both high glucose and insulin induce the degradation of IRS1 under certain conditions (low insulin concentration and short treatment times), insulin can block the high glucose-induced degradation of IRS1 through the inhibition of GSK3 β activity.