REGULATION OF INSULIN/IGF-1 SIGNALING BY PROTEASOME-MEDIATED DEGRADATION OF IRS-2

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SUMMARY

Insulin and IGF-1 regulate metabolism and body growth through homologous receptor tyrosine kinases that phosphorylate the IRS-proteins. IRS-2 is an important IRS-protein as it mediates peripheral insulin action and β-cell survival. In this study, we show that insulin, IGF-1 or osmotic stress promoted ubiquitin/proteasome-mediated degradation of IRS-2 in 3T3-L1 cells, Fao hepatoma cells and mouse embryo fibroblasts; however, insulin/IGF-1 did not promote degradation of IRS-1 in 3T3-L1 preadipocytes or mouse embryo fibroblasts. MG132 or lactacystin, specific inhibitors of 26S proteasome, blocked insulin/IGF-1-induced degradation of IRS-2 and enhanced the detection of ubiquitinated IRS-2. Insulin/IGF1-induced ubiquitination and degradation of IRS-2 was blocked by inhibitors of PI 3-kinase (wortmannin or LY294002) or mTOR (rapamycin). Chronic insulin or IGF-1 treatment of IRS1-deficient mouse embryo fibroblasts inhibited IRS2-mediated activation of Akt and ERK1/2, which was reversed by lactacystin pretreatment. By contrast, IRS1-activation of Akt and ERK1/2 was not inhibited by chronic insulin/IGF-1 stimulation in IRS-2 deficient mouse embryo fibroblasts. Thus, we identified a novel negative feedback mechanism by which the ubiquitin/proteasome-mediated degradation of IRS-2 limits the magnitude and duration of the response to insulin or IGF-1.
INTRODUCTION

Insulin and insulin-like growth factor 1 (IGF-1) regulate a variety of biological functions through homologous tyrosine kinases that phosphorylate the insulin receptor substrate (IRS) proteins. IRS-proteins mediate signal specificity and diversity in various cellular backgrounds (1). Upon ligand binding, the activated insulin/IGF-1 receptors engage IRS-proteins and promote the phosphorylation of multiple tyrosine residues, which activate various downstream signaling pathways, including the phosphatidylinositol (PI) 3-kinase and MAP-kinase cascades (1,2). These signaling pathways coordinate a network of protein kinases that regulate the activity of cytoplasmic enzymes and nuclear transcription factors (1-4). Four IRS-proteins are known, including IRS-1 and IRS-2 that play a central role in many tissue and organ systems. Deletion of IRS-1 in mice impairs somatic growth and causes peripheral insulin resistance, but diabetes rarely develops owing to lifelong compensatory hyperinsulinemia (5,6). By contrast, IRS-2 is essential for normal nutrient homeostasis because it mediates both peripheral insulin action and the effect of IGF-1 on \(\beta\)-cells growth; mice lacking IRS-2 fail to maintain sufficient compensatory insulin secretion and develop diabetes as young adults (7). Moreover, female mice lacking IRS-2 are hyperplastic and infertile, owing to a failure of the hypothalamic-pituitary-ovarian axis (8).

Many mechanisms are proposed to explain the inhibition of IRS-protein signaling, including phosphotyrosine dephosphorylation, serine/threonine phosphorylation, and degradation (9-14). Recent studies suggest that proteasome-mediated degradation of IRS-1 might be involved in the down regulation of signaling by insulin and IGF-1 and contribute to insulin resistance (12,13,15-17). However, there is no convincing evidence showing that the decrease of endogenous IRS-1 via the ubiquitin/proteasome system contributes to an inhibition of insulin signaling.
Proteasome-mediated degradation regulates many biological processes including gene transcription and cell cycle progression (18-20). Proteins targeted for destruction by 26S proteasome are usually ubiquitinated by a complex containing an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) (20-22). In certain cases, phosphorylation promotes ubiquitination and degradation of signaling proteins, including IκBα and β-catenin (18,23-25). Overexpressed recombinant IRS-1 is ubiquitinated in response to IGF-1, and PI 3-kinase inhibitors block IRS-1 degradation (12,13,15-17). However, it is unclear whether insulin promotes ubiquitination of endogenous IRS-proteins or whether the ubiquitin/proteasome system inhibits IRS-protein signaling. In this work, we show that insulin and IGF-1 stimulate ubiquitination and degradation of IRS-2 in multiple cell types via a PI 3-kinase/Akt/mTOR-dependent pathway, which correlates closely with the inhibition of insulin signaling. This negative feedback mechanism might limit the magnitude and duration of IRS2-mediated signals, and contribute to insulin resistance associated with hyperglycemia and hyperinsulinemia.
EXPERIMENTAL PROCEDURES

Reagents—Human insulin and IGF-1 were a gift from Eli Li. Protein A-agarose was purchased from Repligen; 3-isobutylmethylxanthine, dexamethasone, aprotinin and leupeptin were purchased from Sigma. Enhanced ChemiLuminescence (ECL) detection system was purchased from Amersham Corp. Lactacystin, MG132, rapamycin, PD98059, LY294002, wortmannin and Nonidet P-40 were purchased from Calbiochem. Polyclonal anti-active ERK1/2 (αpERK) was purchased from Promega. Polyclonal anti-phospho-Akt (on Ser473) (αpAkt) was purchased from New England Biolabs Inc. (Beverly, MA); monoclonal anti-ubiquitin was purchased from Santa Cruz Inc; polyclonal anti-IRS-1 antibodies were raised against the full-length rat IRS-1 (JD#159, used at a dilution of 1:15,000 for immunoblotting). Polyclonal anti-IRS-2 antibodies were raised in the laboratory against the amino acids 976-1094 (JD#110) or 618-747 (JD#101) of rat IRS-2. Polyclonal anti-p85 antibodies were raised against the N-terminal SH2 domain of p85 regulatory subunit of PI 3-kinase.

Cell culture and differentiation—Fao cells were grown at 37°C in 5% CO2 in RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS). 3T3-L1 preadipocytes were grown at 37°C in 10% CO2 in Dulbecco's modified Eagle Medium (DMEM) containing 25 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% calf serum (HyClone Laboratories, Inc). For adipocyte differentiation, confluent preadipocytes were cultured for three days in differentiation medium (DMEM supplemented with 25-mM glucose, 1 μM insulin, 0.5 mM 3-isobutylmethylxanthine, 1 μM dexamethasone and 10% FBS), and three days in DMEM supplemented with 1 μM insulin and 10% FBS. The cells were then
grown for an additional 4-9 days in DMEM containing 25-mM glucose and 10% FBS without any other additives (>90% cells are adipocytes).

**Preparation of immortalized mouse embryo fibroblasts (MEF)** — The generation of IRS-1 and IRS-2 knockout mice has been previously described (5,6,6,7). IRS1−/− or IRS2−/− embryos were harvested on day 16 of gestation. After removal of the head and all internal organs, embryo carcasses were washed with PBS, minced with scissors and incubated with 0.25% trypsin on ice overnight. The embryos were incubated for an additional 20 min at 37°C to activate trypsin. Growth medium (DMEM supplemented with 25 mM glucose, 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin) was added to stop trypsin action, and the embryos were disrupted by vigorous pipetting. The resulting single cell suspension was centrifuged for 6 min at 1000 rpm. The cell pellet was resuspended in growth medium and cultured at 37°C in 5% CO2. The MEF cells reached confluence in two days, and were replated every 72 hours at a density of 5x10³ cells/cm² until the establishment of permanent cell lines.

**Immunoprecipitation and immunoblotting** — Confluent cells were deprived of serum overnight in DMEM (for 3T3-L1 and MEF cells) or RPMI-1640 (for Fao cells) containing 0.5% BSA, and treated with different ligands at 37°C. The cells were rinsed three times with ice-cold PBSV (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM Na3VO4), solubilized in lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na3VO4, 100 mM NaF, 10 mM Na4P2O7, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin), and centrifuged at 14,000xg for 10 min at 4°C. The supernatant (cell lysates) was boiled for 5 min in SDS-PAGE sample buffer (50 mM Tris.Cl, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and 0.004% bromophenol blue) and separated by SDS-PAGE. In some experiments, the supernatant was incubated with the indicated antibody on ice for 2 hours. The
immune complexes were collected on protein A-agarose during one-hour incubation at 4°C. The beads were washed 3 times with washing buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA) and boiled for 5 min in SDS-PAGE sample buffer. The solubilized proteins were separated by SDS-PAGE. Proteins on the gel were transferred to nitrocellulose membrane (Amersham) and detected by immunoblotting with the indicated antibody using ECL. Some membranes were subsequently incubated at 55°C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) to prepare them for a second round of immunoblotting. To make clear cell lysates, cells were scraped in PBS, centrifuged at 14,000xg for 1 min at 4°C, and boiled for 5 min in SDS-PAGE sample buffer.
RESULTS

Insulin, IGF-1 and osmotic stress reduce IRS-2 protein levels—To determine whether insulin and IGF-1 decrease IRS-2 protein levels, 3T3-L1 preadipocytes were treated for 6 h with either insulin (100 nM) or IGF-1 (100 ng/ml). Cell lysates prepared in 1% NP40 were resolved by SDS-PAGE and analyzed by immunoblotting with polyclonal antibodies against IRS-2 (αIRS2). IRS-2 was detected in control cell lysates, but not in insulin- or IGF-1-treated cells (Fig. 1A, top panel). To verify that this protein was IRS-2, 3T3-L1 preadipocytes were treated without or with insulin, and proteins in the cell lysates were immunoprecipitated and immunoblotted with αIRS2. Insulin significantly reduced the level of IRS-2 (Fig. 1B). By contrast, insulin and IGF-1 only slightly reduced the level of IRS-1, and did not change the level of the p85 regulatory subunit of PI 3-kinase (Fig. 1A).

To exclude the possibility that IRS-2 was redistributed into an inaccessible compartment, insulin-stimulated cells were lysed in buffer containing 2% SDS, and clear lysates were resolved by SDS-PAGE and immunoblotted with αIRS2. Consistent with the initial results, insulin reduced the level of IRS-2 but not p85 (Fig. 1C). Similar results were obtained with polyclonal antibodies raised against other regions of IRS-2, excluding the possibility that phosphorylation of IRS-2 inhibited its immunoblotting by αIRS2 (Fig 1D).

The degradation of IRS-2 was tested by other growth factors and in various cell backgrounds. 3T3-L1 preadipocytes were treated with epidermal growth factor (EGF) (100 ng/ml), platelet-derived growth factor-BB (PDGF) (50 ng/ml) or insulin. As expected, insulin stimulated reduction of IRS-2, but neither EGF nor PDGF altered the level of IRS-2; the level of p85 was not changed in response to insulin, EGF or PDGF (Fig. 1E). Both insulin and PDGF strongly
stimulated phosphorylation and activation of Akt, whereas EGF stimulated Akt to a less extent (Fig. 1E).

Insulin-induced degradation of IRS-1 or IRS-2 was compared in several cell types, including 3T3-L1 adipocytes, Fao hepatoma cells, or mouse embryonic fibroblasts (MEF) cells from wild-type, IRS1−/− or IRS2−/− knockout mice. The cells were treated with insulin (100 nM) for 6 h, and proteins in cell lysates were immunoblotted with αIRS1, αIRS2 or αp85. IRS-2 levels decreased in all three of the cell types whereas IRS-1 was not reduced in wild-type and IRS2−/− MEF cells; p85 did not change (Fig. 2).

Osmotic stress caused by hyperglycemia might exacerbate peripheral insulin resistance and β-cell dysfunction by impairing insulin/IGF-1 signaling (26). To examine whether osmotic stress induced reduction of IRS-2, Fao cells were treated for 60 min with various concentrations of D-sorbitol. Osmotic stress dramatically reduced the level of IRS-2 but not p85 in a dose-dependent fashion (Fig. 3). It also reduced IRS-1 protein levels, but to a less extent (Fig. 3). Together, these results suggest that hyperinsulinemia and/or hyperglycemia might promote degradation of IRS-2 that exacerbates insulin/IGF-1 resistance.

**Insulin/IGF-1 stimulates 26S proteasome-mediated degradation of IRS-2**—IRS-2 protein levels decreased after 1 h of insulin treatment, and reached the lowest level within 3 h (data not shown). This rapid reduction of IRS-2 was most likely caused by proteolytic degradation rather than by inhibition of transcription and/or translation. To confirm that protein degradation was involved, 3T3-L1 preadipocytes were pretreated without or with lactacystin or MG132, specific inhibitors of 26S proteasome (22). Without drug pretreatment, insulin reduced the levels of IRS-2, as previously described (Fig. 4A, lane 2 vs 1). However, both lactacystin and MG132 inhibited insulin-induced reduction of IRS-2 protein levels (Fig. 4A, upper panel); lactacystin and MG132
also inhibited IGF-1-induced reduction of IRS-2 (data not shown). Similarly, insulin-induced reduction of IRS-2 was inhibited by lactacystin in Fao cells (Fig. 4B) and MEF (data not shown). Lactacystin and MG132 did not alter the level of p85 (Fig. 4, A and B, lower panel). Insulin ordinarily stimulates a shift in mobility of IRS-2, owing largely to phosphorylation (Fig. 4A).

Lactacystin and MG132 did not significantly affect the insulin-induced mobility shift of IRS-2, suggesting that these inhibitors did not directly inhibit phosphorylation of IRS-2 (Fig. 4A and B). Consistent with this idea, lactacystin and MG132 did not alter tyrosyl phosphorylation of IRS-1 and IRS-2 induced during 5 min insulin stimulation (data not shown). Taken together, the data suggest that 26S proteasome mediates the degradation of IRS-2 during insulin/IGF-1 stimulation.

Ubiquitination targets proteins for degradation by 26S proteasome (27). To determine whether insulin promoted the ubiquitination of IRS-2, Fao cells were treated for 1 h with 100 nM insulin. IRS-2 was immunoprecipitated, resolved by SDS-PAGE and analyzed by immunoblotting with anti-ubiquitin antibody; however, ubiquitination of IRS-2 was not detected by this experimental protocol. However, when cells were pretreated with lactacystin or MG132, insulin-stimulated ubiquitination of IRS-2 was clearly detected by immunoblotting with anti-ubiquitin, and the migration of ubiquitinated IRS-2 was significantly retarded (Fig. 4C). Thus, under ordinary conditions, ubiquitinated IRS-2 might not accumulate to sufficient levels to be detected by immunoblotting, owing to rapid proteasome-mediated degradation.

The PI 3-kinase→Akt→mTOR pathway is required for insulin/IGF-1-induced degradation of IRS-2—IRS-proteins mediate the activation of the PI 3-kinase and the MAP-kinase cascades during insulin/IGF-1 stimulation (1,2). To determine whether these signaling pathways mediate degradation of IRS-2, 3T3-L1 preadipocytes or FAO cells were pretreated with wortmannin or LY294002 (inhibitors of type 1b PI 3-kinase), or PD98059 (an inhibitor of
MEK1/2). As expected, both wortmannin and LY294002 blocked insulin-stimulated activation of the PI 3-kinase/Akt pathways without altering the activation of the MEK/MAP kinase cascade, whereas PD98059 inhibited insulin-stimulated activation of ERK1/2 without effect on the PI 3-kinase/Akt pathways (data not shown). Moreover, wortmannin and LY294002, but not PD98059, inhibited insulin-induced degradation of IRS-2 (Fig. 5, A and B). Similarly, wortmannin and LY294002 but not PD98059 inhibited IGF-1-stimulated degradation of IRS-2 in 3T3-L1 preadipocytes (data not shown). These drugs did not change the level of p85 during these experiments (Fig. 5A). Consistent with these data, LY294002 inhibited insulin-induced ubiquitination of IRS-2 in the presence of lactacystin (Fig. 5C).

mTOR is a serine/threonine kinase downstream of the PI 3-kinase/Akt pathway that contributes to the activation of p70S6 kinase (28-32). To determine whether mTOR mediates the degradation of IRS-2, Fao cells were treated prior to insulin stimulation with rapamycin, an inhibitor of mTOR. Rapamycin inhibited insulin-stimulated degradation of IRS-2, as demonstrated by immunoblotting cell extracts with αIRS2 (Fig. 5B, lane 5). Similarly, rapamycin also blocked IGF-1-stimulated degradation of IRS-2 in 3T3-L1 preadipocytes (data not shown). As expected, rapamycin inhibited the activation of p70S6 kinase, as revealed by the inhibition of insulin-induced shift in mobility of p70S6 kinase that represents phosphorylation and activation of p70S6 kinase (Fig. 5B, lane 5). Rapamycin and insulin did not alter the level of p70S6 kinase (Fig. 5B, lower panel).

Proteasome-mediated degradation of IRS-2 down regulates signaling by insulin and IGF-1—IRS-1 and IRS-2 mediate common and unique signals during insulin/IGF-1 action. Differential degradation of these IRS-proteins might have important influence on metabolic regulation. However, this hypothesis is difficult to test in ordinary cell backgrounds that express
both IRS-1 and IRS-2. To overcome these technical difficulties, MEF cells derived from IRS-1 and IRS-2 knockout mice were used.

The expression of IRS-1 or IRS-2 was undetectable in IRS1−/− and IRS2−/− MEF cells, respectively (data not shown). Deletion of either IRS-1 or IRS-2 did not alter the level of insulin/IGF-1 receptors (data not shown). Both insulin and IGF-1 promoted activation of Akt as well as ERK1/2 in IRS1−/− or IRS2−/− MEF, as demonstrated by immunoblotting cell extracts with antibodies against phospho-Akt or phospho-ERK, respectively (Fig. 6, lanes 1-3). These results suggest that IRS-1 and IRS-2 are redundant in mediating insulin-stimulated activation of the PI 3-kinase/Akt and the MEK→ERK pathways in this cell background. However, insulin stimulated degradation of IRS-2 in IRS1−/− MEF, but insulin did not promote IRS-1 degradation in IRS2−/− MEF cells (Fig. 2C).

To determine whether degradation of IRS-2 inhibits signaling by insulin and IGF-1, IRS1−/− MEF cells were pretreated for 4 h with 100-nM insulin or 100 ng/ml IGF-1. Cells were then washed, incubated in serum-free medium for additional 2 h, and stimulated for 5 minutes with 100-nM insulin or 100 ng/ml IGF-1. Activation of the PI 3-kinase→Akt and the MEK→ERK1/2 pathways was measured by immunoblotting cell extracts with antibodies against phospho-Akt or phospho-ERK1/2, respectively. Insulin or IGF-1 pretreatment did not alter the levels of Akt or ERK1/2 in these cells (Fig. 6, A and B). However, insulin pretreatment significantly reduced the activation of both Akt and ERK1/2 in response to a subsequent insulin or IGF-1 stimulation (Fig. 6, A and B). Similarly, IGF-1 pretreatment inhibited the activation of Akt and ERK1/2 induced by a subsequent insulin/IGF-1 stimulation to a greater extent (Fig. 6A and B, lanes 7-9). IGF-1 pretreatment almost completely inhibited activation of Akt induced by subsequent insulin.
stimulation (Fig. 6A, lane 8). The difference in the magnitude of inhibition between insulin and IGF-1 might reflect a higher abundance of IGF-1 receptors in MEF cells (data not shown).

In the parallel experiments with IRS2<sup>−/−</sup> MEF cells, neither insulin nor IGF-1 pretreatment inhibited the activation of Akt and ERK1/2 induced by a subsequent acute stimulation with insulin or IGF-1 (Fig. 6, C and D). This resistance to the inhibition by insulin/IGF-1 pretreatment is consistent with the resistance of IRS-1 to degradation in IRS2<sup>−/−</sup> MEF cells. Moreover, these results revealed that insulin or IGF-1 pretreatment did not inhibit ligand-stimulated activation of the receptors for insulin or IGF-1, nor impaired the PI 3-kinase/Akt and MEK/ERK1/2 pathways. Therefore, the inhibition of Akt and ERK1/2 by insulin or IGF-1 pretreatment in IRS1<sup>−/−</sup> MEF cells is most likely caused by insulin/IGF1-induced degradation of IRS-2.

To confirm the hypothesis that proteasome-mediated degradation of IRS-2 contributes to down regulation of signaling by insulin and IGF-1, IRS1<sup>−/−</sup> MEF cells were incubated for 30 min with or without the proteasome inhibitor lactacystin prior to IGF-1 pretreatment. IGF-1 pretreatment inhibited activation of Akt and ERK1/2 in response to a subsequent IGF-1 stimulation as expected. However, lactacystin completely reversed the inhibition of Akt and ERK1/2 by IGF-1 pretreatment (Fig. 7).
DISCUSSION

The causes of prevalent forms of type 2 diabetes are poorly understood. Initially, patients develop mild insulin resistance and glucose intolerance. Hyperglycemia then prompts β-cells to secret compensatory insulin to overcome insulin resistance, resulting in hyperinsulinemia. While moderate hyperinsulinemia might be well tolerated in the short term, chronic hyperglycemia and hyperinsulinemia exacerbate insulin resistance; and if uncontrolled, this process continues until β-cells fail to compensate, resulting in diabetes (3,4,26). IRS-2 is critical in both insulin action and β-cell function, as demonstrated by the finding that deletion of IRS-2 in mice causes severe insulin resistance and β-cell failure (7). In ob/ob mice, IRS-2 protein is undetectable in liver, which correlates with hyperglycemia and hyperinsulinemia, hallmarks of insulin resistance (33,34). Exogenous leptin treatment not only reverses insulin resistance, but also increases concomitantly IRS-2 protein to a normal level (34). Thus, the decrease of hepatic IRS-2 might be a major determinant for overall insulin resistance (35).

In this study, we showed that insulin substantially reduces IRS-2 protein levels in multiple cell lines, which is blocked by specific inhibitors of the 26S proteasome. These results suggest that proteasome-mediated degradation of IRS-2, rather than inhibition of transcription and/or translation of IRS-2, determines IRS-2 protein levels and activation of IRS2-mediated signaling pathways. Consistent with this idea, insulin stimulates ubiquitination of IRS-2. During our experiments, the ubiquitinated IRS-2 is detected only in the presence of proteasome inhibitors, suggesting that ubiquitinated IRS-2 is degraded rapidly by the 26S proteasome. Consistent with these findings, deletion of insulin receptor in liver increases specifically hepatic IRS-2 by more than 5-fold (35),
suggesting that the ablation of the insulin signals might block IRS-2 degradation in a whole animal. Reduction of IRS-2 by ubiquitin/proteasome-mediated proteolysis in mouse embryo fibroblasts lacking IRS-1 dramatically inhibits the activation of Akt and ERK1/2 in response to insulin/IGF-1. Strikingly, proteasome inhibitors completely reverse this inhibition. Interestingly, inhibition of the PI 3-kinase→Akt→mTOR pathway, but not the MEK→ERK1/2 pathway, prevents insulin-induced degradation of IRS-2. These observations suggest that the initial activation of the PI 3-kinase→Akt→mTOR pathway by insulin induces ubiquitination and subsequent degradation of IRS-2 by 26S proteasome, resulting in inhibition of signaling pathways downstream of IRS-2 during subsequent insulin stimulation. This negative feedback mechanism is likely to modulate insulin action in the whole body by limiting the magnitude and duration of insulin action. Both insulin and osmotic stress stimulate proteasome-mediated degradation of IRS-2, and insulin and osmotic stress exhibit synergistic effect on destruction of IRS-2 (data not shown). Interestingly, the activity of the ubiquitin/proteasome system is elevated in diabetes, suggesting that IRS-2 degradation might be exacerbated (36,37). Thus, proteasome-mediated degradation of IRS-2 might be involved in the development of insulin resistance and diabetes.

Serine/threonine phosphorylation plays an important role in ubiquitin/proteasome-mediated destruction of signaling proteins. For instance, TNFα stimulates phosphorylation of IκBα at Ser32 and Ser36 by IKK complex, which enable β-TrCP, a F-box-containing subunit of the SCF complex, to recognize and bind to phosphorylated IκBα together with E2, resulting in the ubiquitination of IκBα. The ubiquitinated IκBα is subsequently degraded by the 26S proteasome (19,23,24). We propose a similar mechanism for IRS-2 degradation. Insulin or IGF-1 activates the PI 3-kinase→Akt→mTOR pathway. Kinase(s) within this pathway phosphorylate IRS-2, most likely on serines/threonine residues. This phosphorylated motif(s) in IRS-2 might serve as a signal tag for a
specific E2/E3 complex with F-box-containing subunits. Consistent with this hypothesis, we and others observed that insulin, IGF-1 and osmotic stress promote serine/threonine phosphorylation of both IRS-1 and IRS-2 (14,38-41). Serine/threonine phosphorylation of IRS-1 was reported recently to promote its degradation in some cell types (41), and we confirmed insulin-stimulated degradation of IRS-1 in 3T3-L1 adipocytes and Fao cells. However, insulin stimulates degradation of IRS-2 in 3T3-L1 preadipocytes and MEF, but fails to promote IRS-1 degradation. Thus, degradation of IRS-1 and 2 might be mediated by different mechanisms. In addition, these observations raise an intriguing possibility that in certain insulin/IGF-1 target cells, insulin/IGF-1-promoted degradation of IRS-2 might enhance IRS1-mediated signaling by reducing the competition by IRS-2 of common signaling molecules including receptors for insulin/IGF-1 and downstream effector molecules; therefore, proteasome-mediated degradation of IRS-2 might positively propagate insulin/IGF-1 signals in some physiological contexts.

In summary, insulin and IGF-1 stimulate substantial degradation of IRS-2 mediated by the ubiquitin/proteasome system in multiple cell types, leading to an inhibition of insulin/IGF-1 signaling. The PI 3-kinase→Akt→mTOR pathway is required for insulin/IGF-1-induced degradation of IRS-2. We propose that the ubiquitin/proteasome-mediated degradation of IRS-2 is a component of negative feedback inhibition modulating the action of insulin and IGF-1. Inhibitors of 26S proteasome significantly improve insulin sensitivity, suggesting that these types of agents might have clinical value in the treatment of patients with type 2 diabetes.
REFERENCES


5. Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y.,
   Ueki, K., Kaburagi, Y., Satoh, S., Sekihara, H., Yoshioka, S., Horikoshi, H., Furuta, Y.,


7. Withers, D. J., Gutierrez, J. S., Towery, H., Burks, D. J., Ren, J. M., Previs, S., Zhang, Y.,
   900-904

8. Burks, D. J., de Mora, J. F., Schubert, M., Withers, D. J., Myers, M. G., Towery, H. H.,

   Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C. C., Ramachandran, C., Gresser, M.


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FOOTNOTES

The abbreviations used are: IGF-1, insulin-like growth factor 1; IRS, insulin receptor substrate; PI, phosphatidylinositol; MEF, mouse embryo fibroblasts.
**FIGURE LEGENDS**

**Figure 1.** *Insulin and IGF-1 reduce the level of IRS-2 in 3T3-L1 preadipocytes.* A. Cells were treated with insulin (100 nM) or IGF-1 (100 ng/ml) for 6h, and lysed in a buffer containing 1% NP40. Cell extracts were resolved by 7% SDS-PAGE and immunoblotted with anti-IRS2 (#110, against full-length rat IRS-2), αIRS1 or αp85. B. Cells were treated with insulin and lysed as in A. Proteins in cell extracts were immunoprecipitated and immunoblotted with αIRS2. C. Cells were treated with insulin (100 nM) for 6h, and lysed in a buffer containing 2% SDS. Cell extracts were immunoblotted with αIRS2 or αp85. D. Cells were treated with insulin (100 nM) for 6h, and cell extracts were immunoblotted with αp85 or αIRS2 (#101, against the PH domain of rat IRS-2). E. Cells were treated for 6h (top two panels) or 10 min (bottom panel) with insulin (100 nM), EGF (100 ng/ml) or PDGF-BB (50 ng/ml). Cell extracts were immunoblotted with αIRS2, αp85 or antibodies against phosphorylated Akt (αpAkt). The migration of molecular standards, IRS-1, IRS-2 and p85 was indicated.

**Figure 2.** *Insulin decreases the level of IRS-2 in different cell types.* 3T3-L1 adipocytes (A), Fao cells (B) and MEF cells derived from wild-type (WT), IRS1−/− (C) or IRS2−/− mice (D) were treated with insulin (100 nM) for 6h. Cell extracts were resolved by 7% SDS-PAGE and immunoblotted with αIRS2, αp85 or αIRS1 as indicated.

**Figure 3.** *Osmotic stress decreases IRS-2 level.* Fao cells were treated for 60 min with the indicated concentration of D-sorbitol. Cell extracts were resolved by 7% SDS-PAGE and immunoblotted with αIRS2, αIRS1 or αp85 as indicated.
Figure 4. **Insulin stimulates ubiquitin/proteasome-mediated degradation of IRS-2.** 3T3-L1 preadipocytes (A) or Fao cells (B) were pre-incubated for 30 min with lactocystin (10 µM) or MG132 (50 µM) prior to insulin (100 nM) stimulation for 6h. Cell extracts were immunoblotted with αIRS2 or αp85. C. Fao cells were pretreated with lactocystin (10 µM) for 30 min prior to insulin (100 nM) stimulation for 1h. IRS-2 was immunoprecipitated with αIRS2 and immunoblotted with αubiquitin. The blot was reprobed with αIRS2.

Figure 5. **The PI 3-kinase/mTOR pathway but not the MEK/EKR1/2 cascade is required for insulin-induced degradation of IRS-2.** A and B. 3T3-L1 preadipocytes or Fao cells were pretreated for 30 min with 30 µM LY294002 (LY), 100 nM wortmannin (wort), 100 µM PD98059 (PD), or 10 µM rapamycin (Rap), prior to insulin (100 nM) stimulation for 6h. Cell extracts were immunoblotted with αIRS2, αp85 or αp70^{S6K}. C. Fao cells were pretreated with MG132 (50 µM) for 30 min in the absence or presence of LY294002 prior to insulin (100 nM) stimulation for 1h. Immunopurified IRS-2 was immunoblotted with αubiquitin.

Figure 6. **Degradation of IRS-2 downregulates signaling by insulin and IGF-1.** IRS1^{−/−} (A and B) or IRS2^{+/−} (C and D) MEF cells were pretreated with insulin (100 nM) or IGF-1 (100 ng/ml) for 4h. The cells were washed with PBS and incubated in serum-free medium for additional 2h. The treated cells were then stimulated for 5 min with insulin (100 nM) or IGF-1 (100 ng/ml). Cell extracts were resolved by 8% SDS-PAGE, and immunoblotted with antibodies against phosphorylated Akt (αpAkt) (A and C) or
phosphorylated ERK1/2 (αpERK) (B and D). The same blots were reprobed with αAkt or αERK2 as indicated. αERK2 reacts with both ERK1 and ERK2.

Figure 7. Inhibition of proteasome-mediated degradation of IRS-2 improves signaling by IGF-1. IRS1−/− MEF cells were pretreated for 30 min with or without lactocystin (30 µM) prior to IGF-1 (100 ng/ml) stimulation for 4h. Cells were then washed with PBS, and incubated in serum-free medium for additional 2h. The treated cells were then stimulated with IGF-1 (100 ng/ml) for 5 min. Cell extracts were resolved by 8% SDS-PAGE, and immunoblotted with antibodies against phosphorylated Akt (A) or ERK1/2 (B). The blots were reprobed with anti-Akt or anti-ERK2 as indicated.
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Regulation of insulin/IGF-1 signaling by proteasome-mediated degradation of IRS-2

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