

Effect of insulin levels on the phosphorylation of specific amino acid residues in IRS-1: Implications for burn-induced insulin resistance

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Received April 22, 2009; Accepted June 16, 2009

DOI: 10.3892/ijmm_00000262

Abstract. Alterations in the phosphorylation and/or degradation of insulin receptor substrate-1 (IRS-1) produced by burn injury may be responsible, at least in part, for burn-induced insulin resistance. In particular, following burn injury, reductions in glucose uptake by skeletal muscle may be secondary to altered abundance and/or phosphorylation of IRS-1. In this study, we performed *in vitro* experiments with 293 cells transfected with IRS-1. These studies demonstrated that there is a dramatic change in the phosphorylation pattern of Tyr, Ser and Thr residues in IRS-1 as a function of insulin levels. Specifically, Ser and Thr residues in the C-terminal region were phosphorylated only at high insulin levels. SILAC (stable isotope labeling with amino acids in cell culture) followed by sequencing of C-terminal IRS-1 fragments by tandem mass spectrometry demonstrated that there is significant protein cleavage at these sites. These findings suggest that one of the biological roles of the C-terminal region of IRS-1 may be negative modulation of the finely coordinated insulin signaling system. Clearly, this could represent an important factor in insulin resistance, and identification of kinase inhibitors that are responsible for the phosphorylation may foster new lines of research for the development of drugs for treating insulin resistance.

Introduction

The maintenance of glucose levels represents one of the most tightly regulated systems in the body. All cells require glucose,

but glucose is only available from what is ingested or produced by the liver. Since glucose cannot be stored in significant amount except as glycogen in the liver and muscle, glucose transport into the cell by specific transporter proteins is critical for cell function. Insulin plays a major role in the maintenance of normal glucose levels. Thus, when blood glucose levels rise, insulin secretion is stimulated resulting in increased uptake by skeletal muscle via glucose transporter proteins and decreased glucose production by the liver. There appears to be several key proteins in the insulin/glucose regulatory cascade including insulin receptor substrate-1 (IRS-1). When levels of insulin and glucose are abnormally high in the fasting state, a condition called insulin resistance exists.

Hyperglycemia and glucose intolerance are frequently associated with the metabolic response to major trauma. Following injury (1,2), burn shock (3,4) or systemic infection (5,6), oral and intravenous glucose tolerance tests have demonstrated delayed disposal of glucose from plasma into tissues. This 'diabetes of injury' could be explained if there was an insulin deficiency, and several studies (1,3,7) have shown that early after trauma ('ebb phase') insulin concentrations are reduced even in the face of hyperglycemia. After resuscitation of trauma patients ('flow phase'), β -cell responsiveness to glucose administration normalizes, and plasma insulin levels are appropriate or even higher than expected (3,7). However, despite this appropriate acute insulin response to glucose administration, glucose intolerance and hyperglycemia continue. This finding suggests that certain tissues in trauma patients are relatively insensitive to the effects of insulin.

Although the number of investigations addressing the mechanism(s) of insulin resistance in trauma patients is limited, one significant study using the euglycemic insulin clamp technique demonstrated that i) the maximal rate of glucose disposal is reduced in trauma patients; ii) the metabolic clearance rate of insulin is almost twice as normal in these patients; and iii) post-trauma insulin resistance appears to occur in peripheral tissues, particularly skeletal muscle, and is consistent with a post-receptor effect (8). Unfortunately, the procedures used in this study were not capable of independently assessing the contributions of glucose transport, phosphorylation, and subsequent intracellular metabolism of glucose. Another study revealed that burn injury to rats

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Abbreviations: ESI, electrospray ionization; DDA, data-dependent acquisition; IRS-1, insulin receptor substrate-1; PTM, post-translational modification; Q-TOF, tandem quadrupole time-of-flight mass spectrometry; SILAC, stable isotope labeling with amino acids in cell culture

Key words: insulin receptor substrate-1, phosphorylation, degradation, kinase, MS/MS, SILAC

results in impaired insulin-stimulated transport of [^3H]2-deoxyglucose into soleus muscle strips *in vitro* (9). These investigators also demonstrated that insulin-stimulated phosphoinositide 3-kinase (PI 3-kinase) activity, pivotal for glucose transport in muscle by glucose transporter 4 (GLUT 4), was decreased by burn injury to rats as measured by its IRS-1-associated activity. These data are consistent with alterations in post-receptor signaling following burn injury, which results in burn-induced insulin resistance.

There are numerous reports that describe insulin resistance in burn patients and animal models (7,10-27). Insulin resistance is defined as elevated insulin levels in the presence of normal or elevated glucose levels. Direct measurements show that liver and skeletal muscle are resistant tissues (7). Lipolysis is not attenuated in trauma patients after glucose administration (15). Although the precise mechanism for insulin resistance produced by burns or other stressors is unknown, it is likely that insulin binding to membrane receptors is unchanged and post-receptor mechanism(s) are of greater importance (10).

Alterations in cytokine levels, such as TNF, IL-1 and IL-6, in burn patients and animal models of burn injury have been reported by our laboratory and by other investigators (28). Infusion of endotoxin, TNF and IL-1 can produce alterations in glucose metabolism and insulin resistance *in vivo* (29,30). In addition, it has also been shown that endotoxin (31) and IL-6 (32) can produce insulin resistance in isolated hepatocyte cultures. It was demonstrated that IL-6 inhibits the stimulation of glucokinase by insulin in isolated hepatocytes (32). Cortisol, glucagon and epinephrine can also produce insulin resistance (33). These molecules oppose the actions of insulin and are termed counter-regulatory hormones. Since these counter-regulatory hormones are elevated, at least initially after burn injury, it has been proposed that they may play a part in burn-induced insulin resistance. The levels of cytokines and counter-regulatory hormones are dramatically altered in burn patients and animal models (24,33-35). It also has been demonstrated that TNF suppresses insulin-induced tyrosine phosphorylation of insulin receptor and inhibits signaling from the insulin receptor (36,37). Furthermore, the insulin resistance produced in spontaneously obese rats can be overcome by pretreatment of the animals with antibodies to TNF (38).

Dynamic and stress-dependent multi-site phosphorylations of IRS-1 tyrosine, serine and threonine residues have been described to have both positive and negative insulin effects. Tyrosine phosphorylation, in the N- or C-terminal regions of IRS-1 are generally considered to be positive post-translational modifications (PTM) in insulin signaling pathways. In contrast, phosphorylation of serine and threonine residues at the C-terminal region is usually considered to be negative PTMs; however, some positive effects of serine phosphorylation have been reported. A number of IRS-1 phosphorylation sites have been identified using different approaches such as radiolabeling with [γ - ^{32}P]-ATP (39-43), immunoblotting with anti-phosphopeptide antibodies (44-53), studies with mutated IRS-1 (54-56) and on- or off-line HPLC interfaced with MALDI-TOF or ESI-TOF (57-61). The large variation and poor reproducibility of reported phosphorylation sites can be explained by method sensitivities, enzymatic and

chemical stabilities of the phosphorylated sites and the dosages/timing of stimuli. Proposed mechanisms for impairment of the insulin signaling system by phosphorylation of serine and threonine residues include feedback inhibition, cooperative interactions, uncoupling of the protein signaling network (62-66) and ubiquitin-proteasome degradation (67-73). It is fair to state that, to date, the ability of insulin to alter the phosphorylation patterns of IRS-1 is poorly understood. Since the negative biological effects of IRS-1 C-terminal phosphorylation may provide clues for exploring the mechanism of insulin resistance (74-78) after burn injury (79-82), evaluation of this issue has become a major focus of our laboratory.

In this study, we used nano-LC interfaced with tandem mass spectrometry to pin-point IRS-1 phosphorylation sites in a well-defined *in vitro* system. An N-terminal-specific mAb, generated in our laboratory, was used for studying the phosphorylation pattern of IRS-1 after treatment with varying concentrations of insulin. Stable isotope labeling with amino acids in cell culture (SILAC) measurements indicated that at least 50% of IRS-1 was degraded in our studies (insulin, 1 μM ; 10 min). Thus, the phosphorylation sites and SILAC results predicted that multiple fragmentations may occur in the middle of the C-terminus of IRS-1. In order to capture these informative IRS-1 fragments, a commercially available C-terminal-specific mAb was used in conjunction with SDS-PAGE. MS/MS sequence data unambiguously confirmed (for the first time) the two predicted IRS-1 fragments with apparent MW's of ~80 and ~65 kDa on SDS-PAGE. The multiple cleavages at the C-terminus of IRS-1 were attributed to phosphorylation at Thr⁴⁷⁵, Thr⁴⁷⁷ (80 kDa) and Ser⁶⁴¹ (65 kDa) by sequence analysis. These results may provide pharmaceutical targets for producing drugs that inhibit sequence-specific Ser/Thr kinases. These reagents may normalize metabolic alterations, such as abnormal cytokine release and altered protein catabolism, which are associated with insulin resistance in critically ill patients with burn injuries.

Materials and methods

Chemicals. Acetonitrile (ACN, LC-MS Chromasolv), formic acid (FA), glacial acetic acid, LC-MS grade water, [Glu¹]-fibrinopeptide B and 2-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO). SDS-PAGE Ready gels (7.5% Tris-HCl), Laemmli sample buffer (cat. no. 161-0737) and Coomassie brilliant blue R-250 (cat. no. 161-0436) were obtained from Bio-Rad. Trypsin profile IGD kits (cat. no. PP0100) were obtained from Sigma. Anti-IRS-1 monoclonal antibody (cat. no. 05-699) and recombinant IRS-1 were purchased from Upstate (Charlottesville, VA). Insulin, potassium bisperoxo(bipyridine)oxovanadate, calyculin A (*Discodermia calyx*) and SILAC phosphoprotein identification kits (cat. no. SP10001) were products of Invitrogen (Carlsbad, CA). Protein G agarose beads were purchased from Pierce (Rockford, IL).

Preparation of monoclonal antibodies to IRS-1. Monoclonal antibodies were generated using traditional methods. Briefly, BALB-C mice were immunized with 5-100 μg of protein in 25-50 μl of Freud's Complete Adjuvant (Sigma) in 4 to 6



SPANDIDOS PUBLICATIONS:ous sites. The material used for immunization was a recombinant protein containing 150 amino acids from the N-terminus of IRS-1 (MASPPESDGFSDVRKVGYLKPK SMHKRFFVLRAASEAGGPALREYYENEKKWRHK SSAPKRSIPLESCFNINKRADSKNHLVALYTRDEHFAI AADSEAEQDSWYQALLQLHNRAKGHHDGAAALGAG GGGGSCSGSSGLGEAGEDLS). The same immunogen (in 25-50 μ l of Freund's Incomplete Adjuvant) was used for 'booster' injections (4-6 subcutaneous sites) in the animals at two-week intervals. Once suitable blood titers were obtained, spleen cells were harvested and fused (PEG method) with SP 2/0 myeloma cells to generate hybridomas secreting monoclonal antibodies recognizing IRS-1 protein. These procedures, as well as all animal handling protocols, were reviewed and approved by the local animal study review board. The animals were housed and handled at the institution's AAALAC accredited facilities. Three mAb (3D10, 4C6 and 5G7) were protein G purified and stored (0.1 M bicarbonate buffer, pH 9.2) at -70°C . Screening of these antibodies was performed against recombinant IRS-1, and antibodies were selected using both the highest tryptic peptide coverage and MS/MS sequence scores.

Transfection of 293 mammalian cells with recombinant IRS-1. An appropriate plasmid was prepared by subcloning of IRS-1-tagged plasmid PCMVIRS transfected into DH5 α -competent *E. coli* (Invitrogen). The transformed *E. coli* were incubated on Luria Broth plates with ampicillin. Single colonies were picked and incubated in Luria Broth medium with ampicillin, and the plasmids were purified using the Purelink Hipure Plasmid DNA Purification Maxiprep System (Invitrogen). Mammalian 293 cells, supplied from the American Type Culture Collection, were transfected with the recombinant IRS-1-tagged plasmid using a liposome reagent (Invitrogen) and Opti-MEM medium (Gibco) to deliver the plasmid DNA into the cells. IRS-1 expression was checked by Western blot analysis. Anti-IRS-1 (mouse) monoclonal primary antibody (Sigma) and Cell Signaling anti-mouse IgG HRP-linked secondary antibody (GE Healthcare) as well as anti-IRS-1 primary (rabbit) (Upstate) and Cell Signaling anti-rabbit IgG HRP-linked secondary antibody (GE Healthcare) were used to probe the blots.

Insulin stimulation. Mammalian 293 cells transfected with recombinant IRS-1 were grown to confluence and placed in serum-free Dulbecco's modified Eagle's medium (DMEM) for 2 h. The cells were then treated with insulin (0, 50, 100, 150, 250, 500, 750 and 1000 nM) for 10 min. Twelve culture plates for each insulin concentration were used for phosphorylation studies. After insulin treatment, the medium was aspirated, and the cells were lysed in Reporter lysis buffer with phosphatase cocktail inhibitors containing 50 nM potassium bisperoxo(bipyridine)oxovanadate and 5 nM calyculin A (*Discodermia calyx*) at 4°C . SILAC was performed at an insulin concentration of 1 μM for 10 min according to the SILAC Phosphoprotein Identification Kit protocol (Invitrogen, SP10001) with [U- $^{13}\text{C}_6$]-L-lysine and [U- $^{13}\text{C}_6$]-L-arginine.

Immunoprecipitation of human IRS-1 and its C-terminal fragments. Intact IRS-1 was immunoprecipitated with

selected N-terminal-specific mAb (5G7, 5 μg) and prewashed with protein G agarose beads (50 μl packed volume). The reaction mixtures were gently stirred in PBS (200 μl) at 4°C for 1 h, and excess mAb was removed with PBS (1 ml x 3). Cell lysates (containing human IRS-1 with total proteins ~ 2.5 mg/ml, 3 ml for each IP) were centrifuged at $14,000 \times g$ for 2 min and filtered (0.22 μm) at 4°C . The processed cell lysates and washed immunobeads were gently stirred at 4°C for 2 h. The immunocomplex beads were centrifuged at $14,000 \times g$ for 15 sec and washed with PBS (1 ml x 3, for 2 min each). IRS-1 was released, and cysteine residues were alkylated with Laemmli sample buffer (with 5% 2-mercaptoethanol, 50 μl) for 5 min at 95°C and then 1 h at room temperature with stirring. The reaction mixtures were separated by SDS-PAGE (7.5% Tris-HCl, Ready gels). The IRS-1 bands stained with Coomassie brilliant blue R-250 were excised, and in-gel trypsin digestion was performed (0.2 μg trypsin in 70 μl reaction buffer, 37°C , overnight).

Fragments of IRS-1 C-terminal were immunoprecipitated with mAb raised to the first C-terminal 14 amino acid residues of IRS-1 (05-699, clone 1M92-7, Upstate) using the methods described above.

Nano-LC interfaced with Q-TOF tandem mass spectrometry. The phosphorylated peptides induced with various insulin dosages were analyzed by nano-LC coupled to a Q-TOF hybrid mass spectrometer from Micromass; equipped with an in-house fabricated electrospray (PicoTip emitter, FS360-20-10-D-20-C7, New Objective) and operated in the positive ion mode. Chromatographic separation was achieved using a C18 PepMap300 column (5- μm , 75- μm I.D. x 15-cm length, LC Packings). HPLC gradient elution (160 nl/min) was performed with mobile phase A (water 98%, acetonitrile 2%, formic acid 0.1%) and mobile phase B (acetonitrile/0.1% formic acid) starting with 2% B, 2 min isocratic, up to 80% B over 25 min (linear gradient), 10 min of isocratic elution with 80% B, and a return to 2% B over 5 min. The column was reconditioned with the starting mobile phase for 30 min before the next analysis. The digested peptide mixture (5 μl) was trapped and desalted with a μ -Precolumn Cartridge (PepMap C18, 5- μm , 300- μm I.D. x 5 mm, LC Packings) for 2 min at a flow rate of 15 $\mu\text{l}/\text{min}$. [Glu 1]-fibrinopeptide B (100 fmol/ μl constant infusion, Sigma) was used for mass calibration. Optimized Q-TOF conditions were: ESI capillary voltage, 3500 V; cone voltage, 35 V; source temperature 80°C . Quadrupole-1 was set to bandwidth bypass mode, and the TOF analyzer was scanned over m/z 400 to 1200 with a 1-sec integration time (mass resolution was 5500 FWHM at a mass of 785.84). Data were recorded in continuum DDA mode under CE 5V. Phosphorylated peptides were manually checked in order to reduce false-positive discoveries.

Results and Discussion

Screenings of the three anti-IRS-1 mAbs (3D10, 4C6 and 5G7) were performed based on each mAb's immunoprecipitated IRS-1 sequence coverage as well as sequence scores obtained with Q-TOF tandem mass spectrometry (Table I). A total of 11 tryptic peptides of human IRS-1 expressed in the cell line was found to have satisfactory

Table I. Screening results for 3 monoclonal antibodies.^a

Tryptic ID	Sequences	Charge	MS theory	3D10	4C6	5G7
T19	82-HLVALYTR-89	2	971.56			88
T23	162-EVWQVILKPK-171	2	1238.74			191
T28	197-LNSEAAAVVLQLMNIR-227	2	1740.96			56
T45-46	365-LHPPLNHSRIPMPASR-381	2	1909.01		31	
T51	444-SVTPDSLGHTPPAR-457	2	1434.73			166
T63	581-SYPEEGLEMHPLER-594	2	1685.77	53		
T66-67	627-KGSGDYMPMSPK-638	2	1296.58		25	
T68	639-SVSAPQQIINPIR-651	2	1421.80		173	154
T92	952-AAWQESTGVEMGR-964	2	1420.64		91	165
T96	999-QSYVDTSAPVSYADMR-1016	2	1956.89	37		58
T97	1017-TGIAAEEVSLPR-1028	2	1241.66	58	145	188

^aMS/MS scores were obtained from MassLynx 4.1.

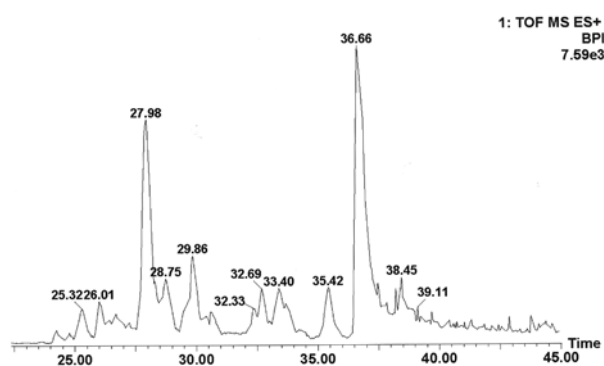


Figure 1. Nano-LC base peak ion chromatogram of human IRS-1 tryptic peptides. Column: C18 PepMap 300 column (5- μ m, 75- μ m I.D. x 15-cm length). Flow rate: 160 nL/min. Desalting column: μ -Precolumn Cartridge (PepMap C18, 5- μ m, 300- μ m I.D. x 5 mm, LC Packings) for 2 min at a flow rate of 15 μ L/min. Gradient conditions are described in the text.

MS/MS scores using PepSeq of MassLynx v4.1 software. Since the reproducibility of the sequencing results (Table I) indicated that mAb (5G7) was the optimal choice, it was used for the *in vitro* studies of insulin effects.

A total of 260 IRS-1 tryptic peptides, both doubly and triply charged under electrospray ionization, was located within the Q-TOF mass survey window (from m/z 400 to 1200). The chromatographic elution time window was \sim 30 min with the nano-LC gradient conditions that were employed; it was impossible to chromatographically separate the digested IRS-1 peptide mixture at the single peptide level since many of the tryptic peptides that were eluted overlapped. In other words, at any given retention time, several peptides were able to be recognized by the mass survey. To overcome these problems, 6 precursor ions which can be selected for MS/MS sequence analysis from a single MS survey scan (precursor ion charge state recognitions under ESI) were selected to provide secondary mass resolution as supplementation to the chromatographic separation. False-positive precursor ions attributed to contaminations were eliminated by MS/MS

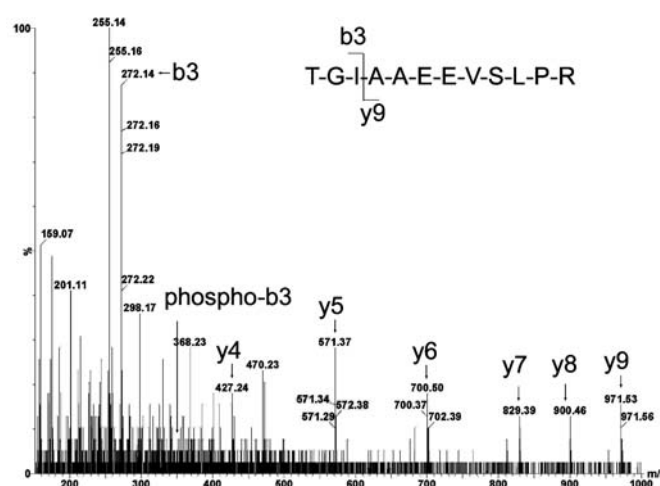


Figure 2. MS/MS sequence analysis of the pThr¹⁰¹⁷ peptide detected with DDA: doubly charged, $[M+2H]^{2+}$ (m/z = 621.84), 1017-TGIAAEEVSLPR-1028 ¹⁰¹⁷Thr-Gly-Ile, non-phosphorylated b3 ion (m/z = 272.16) and corresponding phosphorylated b3 ion (m/z = 352.13) were observed after insulin stimulation.

sequence analysis. The nano-LC Q-TOF settings described above do have false-negative discoveries; these missed precursor ions may have had weak hydrophobic properties (escaping from the trapping C18 column that was used for desalting), weak ionizations and/or chemical alterations in predictable peptide structures. However, the positively identified precursor ions under our experimental conditions represented desirable candidate peptides for the phosphorylation studies at the proteomic level. Thus, unambiguously discovered peptides were selected as relative MS fingerprints for IRS-1 protein expression and post-translational modification analysis in the insulin treatment studies. A representative nano-LC chromatogram of in-gel trypsin-digested IRS-1 is shown in Fig. 1.

The *in vitro* IRS-1 phosphorylation pattern as a function of increasing insulin concentration is shown in Table II. The



Insulin (nM)	0	50	100	150	250	500	750	1000
Sites	pSer ³²³ pThr ³⁰⁵	pSer ³⁰⁸ pThr ³⁰⁵ pTyr ¹⁰⁰¹ pTyr ¹⁰¹²	pSer ³²³ pSer ¹¹³² pTyr ¹⁰⁰¹ pTyr ¹⁰¹²	pSer ³⁰⁸ pSer ⁶⁴¹ pThr ¹¹¹⁶ pTyr ⁵⁵⁸ pTyr ¹⁰⁰¹ pTyr ¹⁰¹²	pSer ³²³ pSer ⁴⁸⁶ pSer ⁶⁴¹ pTyr ⁵⁵⁸ pTyr ¹⁰⁰¹ pTyr ¹⁰¹²	pSer ⁶⁴¹ pThr ⁴⁷⁵ pThr ¹⁰¹⁷ pTyr ⁵⁵⁸	pSer ⁶⁴¹ pThr ⁴⁷⁵ pThr ¹⁰¹⁷ pTyr ⁵⁵⁸	pSer ⁶⁴¹ pThr ⁴⁷⁵ pThr ⁴⁷⁷ pThr ¹⁰¹⁷

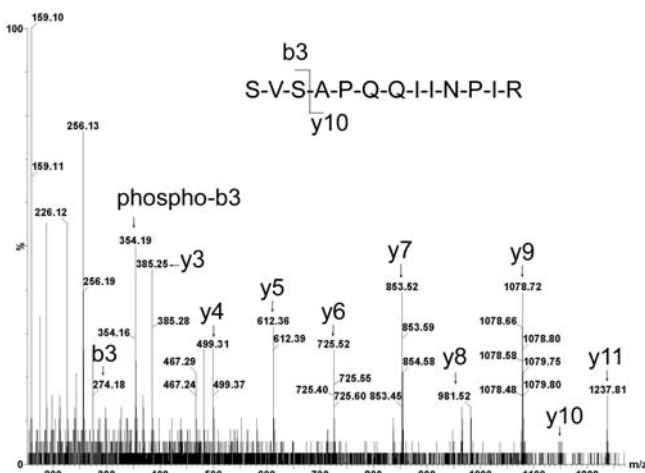


Figure 3. MS/MS sequence analysis of the pSer⁶⁴¹ peptide detected with DDA: doubly charged, [M+2H]²⁺ (m/z = 711.91), 639-SVSAPQIINPIR-651 ⁶³⁹Ser-Val, b2 ion (m/z = 187.12). No corresponding phosphorylated ⁶³⁹Ser b2 ion was observed (expected m/z = 267.09). ⁶³⁹Ser-Val-Ser⁶⁴¹, b3 ion (m/z = 274.14), phosphorylated b3 ion (m/z = 354.17) indicated phosphorylation at Ser⁶⁴¹ in control IRS-1.

phosphorylated sequence sites were assigned by 79.97-Da mass differences between phosphorylated b or y ions and corresponding non-phosphorylated ions at the 50-mDa mass accuracy of their centroid MS/MS spectra. Typical MS/MS sequences of insulin-dependent phosphorylation sites pSer⁶⁴¹ and pThr¹⁰¹⁷ of IRS-1 are shown in Figs. 2 and 3.

The striking insulin-dependent phosphorylation pattern indicated that pThr⁴⁷⁵, pThr⁴⁷⁷ and pSer⁶⁴¹ were induced by high levels of insulin. These phosphorylated sites provide the first clue toward understanding the negative effects of insulin (possibly via the ubiquitin-proteasome pathway) and suggest a possible mechanism for insulin resistance. SILAC was used to study the IRS-1 degradation at these phosphorylation sites at high insulin dosages (1 μ M, 10 min). Tryptic peptide T23 without phosphorylation (162-EVWQVILKPK-171) labeled with heavy isotopes (insulin stimulation) and light isotopes (without insulin) were used as a mass marker to evaluate IRS-1 phosphorylation under varying insulin conditions. Relative peak areas of precursor peptides indicated that at least 50% of IRS-1 was rapidly degraded. The critical question was whether IRS-1 degradation was triggered at the three phosphorylated sites. To address this question, two major C-terminal fragments at the cleavage sites pThr⁴⁷⁵, pThr⁴⁷⁷ and

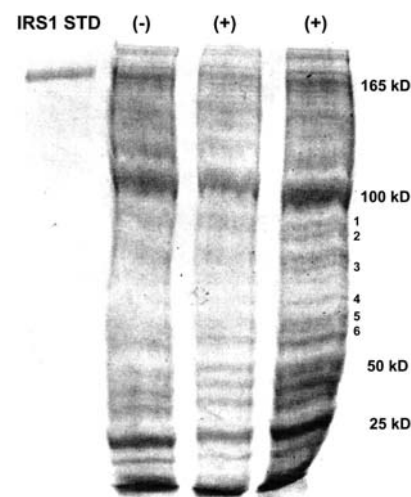


Figure 4. SDS-PAGE of fragmented human IRS-1 after insulin treatment (1000 nM, 10 min). C-terminal mAb, 05-699 (5 μ g); protein G beads, 50 μ l (packed). Immunobeads were prepared at 4°C for 1 h and washed with chilled PBS (1 ml x 3). Twelve cell culture plates were evaluated. Cell lysates were filtered through 0.22- μ m membranes. Immunoprecipitation was performed at 4°C for 2 h, and excess proteins were removed with three PBS washes. Ready gels (7.5%, Bio-Rad, 30 μ l per well) were used for separation, and protein bands were stained with Coomassie brilliant blue R-250. Left lane, rat IRS-1 as MW marker; next lane, 15 μ l of IP mixtures without insulin treatment (-), next lane 15 μ l of IP mixtures with insulin treatment (+); right lane, 30 μ l of IP mixtures with insulin treatment (+). Human IRS-1 bands from the *in vitro* experiments corresponded to a slightly lower MW compared with the standard rat IRS-1 band. Fragment band #1 (~80 kDa) was confirmed with two sequences: 639-SVSAPQIINPIR-651 and 1017-TGIAAEVSLPR-1028, and fragment band #4 (~65 kDa) was confirmed with 1017-TGIAAEVSLPR-1028.

pSer⁶⁴¹ must be obtained for 50% degradation. The predicated C-terminal fragment cleaved at sites pThr⁴⁷⁵ pThr⁴⁷⁷ was expected at 80,673 Da and the second fragment cleaved at site pSer⁶⁴¹ at 64,441 Da. When C-terminal-specific mAb (05-699, 5 μ g) was used in the insulin stimulation study, SDS-PAGE showed that the IRS-1 band was reduced by ~50% with 1000 nM insulin (for 10 min) as compared with the control; in agreement with the SILAC result. The multiple bands between 50 kDa (IgG heavy chain) and 100 kDa (dimer of IgG heavy chain; 2-mercaptoethanol is not sufficient for complete reduction of all disulfide bonds, Fig. 4) were excised, digested and subjected to nano-LC Q-TOF sequence analysis. Sequencing confirmed that the human IRS-1 bands in Fig. 4 were slightly lower in MW than rat IRS-1 standard (~165 kDa). The fragment band represented by band #1 (~80 kDa

estimated from SDS-PAGE) was confirmed with two human IRS-1 sequences: 639-SVSAPQIINPIR-651 and 1017-TGIAAEEVSLPR-1028. Also, the cleavage site precursor ion (472-GPS_pTL_pTAPNGHYILSR-487) was no longer detected. The fragment represented by band #4 (~65 kDa from SDS-PAGE) was confirmed only with 1017-TGIAAEEVSLPR-1028, whereas, the precursor ion (639-SV_pSAPQQIINPIR-651) corresponding to this cleavage was missing from our MS survey list. These three observations confirm an inhibitory mechanism at high insulin conditions; stressed cells treated with high concentrations of insulin activate unknown kinases to phosphorylate threonine and serine residues located in the middle of the C-terminus of IRS-1, and the multiple phosphorylated sites may trigger the ubiquitin-proteasome pathway. The C-terminus of IRS-1 cleaved around pThr⁴⁷⁵ and pThr⁴⁷⁷ afforded a fragment with MW ~80 kDa, while the fragment with MW ~65 kDa was related to pSer⁶⁴¹. The observations that serine phosphorylation decreases insulin-stimulated tyrosine phosphorylation of IRS-1 (83) and proteolytic turnover of the protein (84) suggest that the IRS-1 function is modulated by both positive (N-terminal) and negative (C-terminal) mechanisms. Negative modulation is associated with serine phosphorylation at the N-terminus of the protein; with the C-terminal boundary at residue 574 (85). Ser/Thr kinases, such as mTOR/S6k and Akt1, may be involved in C-terminal cleavages triggered by multi-site phosphorylation. In contrast, positive signaling effects at low insulin levels were associated with pSer³⁰⁸, pThr³⁰⁵ and pTyr¹⁰¹². Phosphorylated Tyr¹⁰¹² may be one of the downstream SH2 domain binding sites, since it possesses the characteristic YADM sequence. Thus, sequence-specific Ser/Thr kinases may be pharmaceutical targets for modulating insulin resistance.

In conclusion, the observed effects of insulin concentration on the phosphorylation pattern of IRS-1 represents an important factor in insulin resistance. C-terminal cleavages may be produced at specific phosphorylated Ser/Thr sites. Also, two major C-terminal fragments (sequenced with tandem MS) point to the left boundary of the C-terminus as an important region for negative modulatory effects. In the future, identification of kinase inhibitors which are responsible for phosphorylation may foster new lines of research for the development of drugs for treating insulin resistance (86).

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