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Article

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Insulin/IGF-1 and TNF-α stimulate phosphorylation of IRS-1 at inhibitory Ser^{307} via distinct pathways

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Introduction

Insulin resistance occurs throughout life; but when combined with impaired insulin secretion, it contributes to type 2 diabetes (1, 2). Initially, β cells of the pancreas increase insulin secretion to compensate for mild hyperglycemia owing to insulin resistance; however, persistent hyperinsulinemia and hyperglycemia might impair insulin action and exacerbate the demand for insulin and promote the development of diabetes (2–4). Recent evidence reveals a close relationship between pancreatic β-cell function and insulin signaling. Disruption of the insulin receptor in pancreatic β cells impairs glucose-stimulated insulin secretion (5). Moreover, mice lacking IRS-2 develop uncompensated peripheral insulin resistance owing at least partially to reduced β-cell mass, suggesting that IRS-2 is a common element for insulin action and β-cell proliferation or survival (6).

Insulin binds to the α subunit of the insulin receptor to promote tyrosine autophosphorylation of the β subunit, which activates the catalytic domain and stimulates phosphorylation of cellular substrates, including the IRS proteins and Shc (7, 8). Phosphorylation of IRS-1 or IRS-2 on multiple tyrosine residues creates an active signaling complex by recruiting various proteins, including the phosphatidylinositol 3 (PI3) kinase, Grb2, Nck, Crk, Fyn, SHP2, and possibly others (9, 10). In humans, mutations of the insulin receptor contribute rarely to type 2 diabetes, and IRS-protein mutations that are directly responsible for diabetes in people are difficult to find (11); however, dysregulation of the insulin receptor and IRS proteins are common occurrences in type 2 diabetes (11–13). Several mechanisms might be involved in the inhibition of insulin-stimulated tyrosine phosphorylation of the insulin receptor and the IRS proteins, including proteasome-mediated degradation (14, 15); phosphatase-mediated dephosphorylation (16, 17), or kinase-mediated serine/threonine phosphorylation (18).

Acute and chronic stress causes insulin resistance, which might be mediated through cytokine-stimulated protein kinase cascades, including those activated by TNF-α or IL1-β (19). TNF-α, a potential mediator of insulin resistance, promotes serine/threonine phosphorylation of IRS-1 and IRS-2 (20–22). Serine/threonine phosphorylation of IRS-1 impairs the ability of IRS-1 to associate with the insulin receptor, which inhibits subsequent insulin-stimulated tyrosine phosphorylation (23). Moreover, during serine phosphorylation, IRS-1 might inhibit insulin-stimulated tyrosine phosphorylation of the insulin receptor itself (20–22, 24, 25). However, the phosphorylation sites in IRS-1 that mediate the inhibitory effects of TNF-α are difficult to determine. The c-Jun NH_2-terminal kinase

(JNK) is activated by diverse inflammatory stimuli, including TNF-α, IL1-β, double-stranded RNA and lipopolysaccharide. Recently, we reported that JNK-1 forms a stable complex with IRS-1 and phosphorylates Ser^{307}, which inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 (26).

The phosphorylation of Ser^{307} is difficult to study in intact cells or tissues because the methods used to radioactively label cultured cells induce JNK activity (26). To investigate the mechanism of Ser^{307} phosphorylation in cells, we developed a phosphospecific polyclonal antibody that specifically immunoblots the phosphorylated Ser^{307} residue in IRS-1. Using this antibody, we demonstrate that insulin/IGF-1 and TNF-α utilize distinct mechanisms in 3T3-L1 cells to mediate phosphorylation of IRS-1 on Ser^{307}. Moreover, insulin-stimulated phosphorylation of Ser^{307} is likely to be physiologically important, as it occurs in skeletal muscle of mice, rats, and humans in response to insulin. Given that phosphorylation of Ser^{307} inhibits insulin-stimulated tyrosyl phosphorylation of IRS-1, we propose that Ser^{307} integrates feedback and heterologous signals to attenuate IRS-1–mediated signals and contribute to insulin resistance.

Methods

Reagents. Murine TNF-α was purchased from R&S Systems Inc. (Minneapolis, Minnesota, USA). Human insulin and IGF-1 were a gift from Eli Lilly and Co. (Indianapolis, Indiana, USA). Protein A-agarose was purchased from RepliGen Corp. (Needham, Massachusetts, USA). The 3-isobutylmethylxanthine, dexamethasone, aprotinin, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). An enhanced chemiluminescence (ECL) detection system was purchased from Amersham Pharmacia Biotech Inc. (Piscataway, New Jersey, USA). Fugene 6 was purchased from Roche Molecular Biochemicals (Indianapolis, Indiana, USA). Okadaic acid, PD98059, LY294002, wortmannin, and Nonidet P-40 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, California, USA). Monoclonal anti-phosphotyrosine antibody (PY20) was purchased from Upstate Biotechnology Inc. (Lake Placid, New York, USA). Polyclonal anti-active MAPK (αpMAPK) was purchased from Promega Corp. (Madison, Wisconsin, USA). Polyclonal anti-phospho-Akt (Ser^{473}) was purchased from New England Biolabs Inc.(Beverly, Massachusetts, USA). Monoclonal anti-hemagglutinin (HA) antibody and anti–JNK-1 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Polyclonal anti-IRS-1 antibodies were raised against the PTB domain (JD no. 228, used at a dilution of 1:100 for immunoprecipitation) or the full length (JD no.159, used at a dilution of 1:15,000 for immunoblotting) of rat IRS-1. Polyclonal anti-phospho-Ser^{307} (αpS^{307}) was raised against a synthetic peptide (ESITATpS^{307}PASMVGGK) flanking Ser^{307}in IRS-1 that is conserved among mouse, rat, and human.

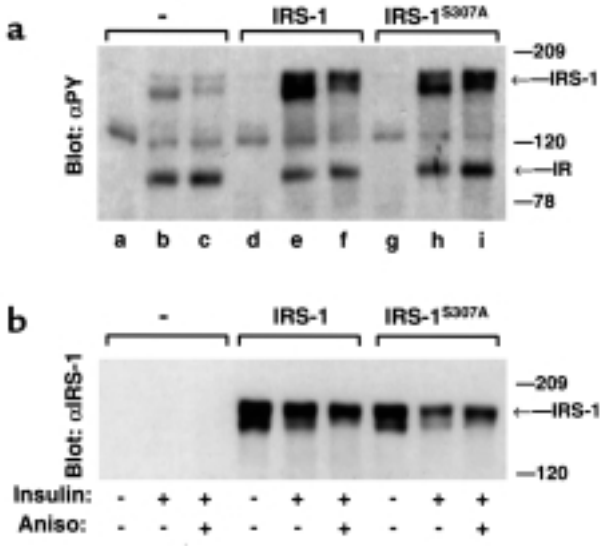
Plasmid construction. Ser^{307} in rat IRS-1 was mutated to Ala (IRS-1^{S307A}) with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, California, USA) using the primer 5′-GCATCACTGCCACCGCCCCTGCCAGTA-3′. IRS-1 and IRS-1^{S307A} were tagged with HA at the COOH-terminus via the PCR-based mutagenesis; the mutations were verified by DNA sequencing. The cytomegalovirus promoter controlled the expression of HA-tagged IRS-1 and IRS-1^{S307A}.

Cell culture, transfection, and differentiation. Human embryonic kidney 293 cells were grown at 37°C in 5% CO2 in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Chinese hamster ovary (CHO) cells were grown at 37°C in 5% CO_2 in F12 medium supplemented with 100 U/ml penicillin, 100-μg/ml streptomycin, and 10% FBS. Confluent CHO cells were split at the ratio of 1:10 (100-mm cell culture dish) 24 hours before transfection. The cells were transfected with 6 μg plasmid per dish, using Fugene 6 according to the protocol recommended by the manufacturer, and used for experiments 24 hours after transfection.

3T3-L1 preadipocytes were grown at 37°C in 10% CO_2 in DMEM containing 25 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% calf serum. 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 3 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).

Immunoprecipitation and immunoblotting. Confluent cells were deprived of serum overnight in DMEM (for 3T3-L1 and 293 cells) or F12 (for CHO cells) containing 1% 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, and 1 mM Na_3VO_4), solubilized in lysis buffer (50 mM Tris [pH 7.5], 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, 50 nM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and centrifuged at 14,000 *g* for 10 minutes at 4°C. The supernatant (cell lysates) was incubated with the indicated antibody on ice for 2 hours. The immune complexes were collected on protein A-agarose during 1-hour incubation at 4°C. The beads were washed three times with washing buffer (50 mM Tris [pH 7.5], 1% Nonidet P-40, 150 mM NaCl, and 2 mM EGTA) and boiled for 5 minutes in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 10% β-mercaptoethanol, 40% glycerol, and 0.01% bromophenol blue). The solubilized proteins were separated by SDS-PAGE (7% gels). Proteins on the gel were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech Inc.) and detected by immunoblot-

Figure 1
Ser^{307} in IRS-1 mediates the inhibitory effect of anisomycin on insulin-induced tyrosyl phosphorylation of IRS-1. CHO cells were transiently transfected with plasmids (10 μg) encoding IRS-1 or IRS-1^{S307A}. Twenty-four hours later, cells were deprived of serum overnight and preincubated for 30 minutes with 5 μg/ml anisomycin before 50 nM insulin stimulation for an additional 5 minutes. (**a**) Proteins (30 μg) in cell lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine. (**b**) The same blot was reprobed with αIRS-1. The migration of molecular weight standards, IRS-1, and IR is indicated.



ting with the indicated antibody using ECL. Some membranes were subsequently incubated at 55°C for 30 minutes in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl [pH 6.7]) to prepare them for a second round of immunoblotting.

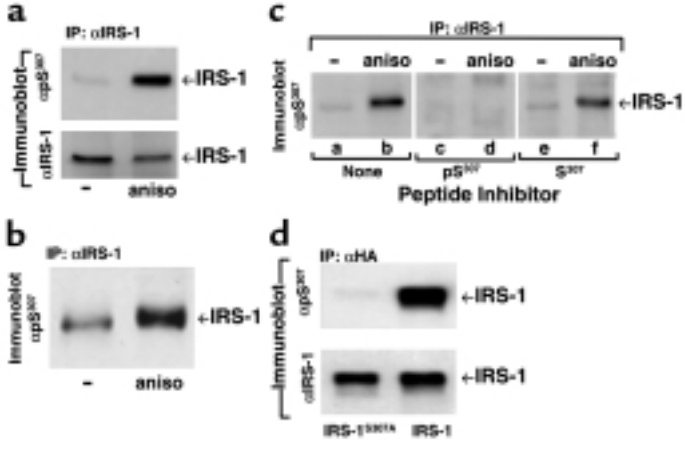
JNK in vitro kinase assay. Cells were treated and lysed as already described here. JNK was immunopurified using anti–JNK-1, and incubated with 2 μg GST-cJun (amino acids 1-79) at 30°C for 30 minutes in the kinase reaction buffer (25 mM HEPES [pH 7.4], 50 nM okadaic acid, 25 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.5 mM DTT, 10 μM ATP, and 2 μCi [γ-32P]-ATP). The reaction was stopped by boiling for 5 minutes in SDS-PAGE sample buffer. GST-cJun was then resolved by 12% SDS-PAGE, and subjected to autoradiography.

Preparation of muscle samples of mice, rats, and humans. Human insulin was injected as a bolus into the inferior vena cava of anesthetized mice or into rats fasted overnight. Soleus muscles were isolated and homogenized in lysis buffer (50 mM Tris [pH 7.5], 1% Nonidet P-40, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, 50 nM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Human vastus lateralis muscle biopsies were obtained at baseline and then at 15 minutes during a hyperinsulinemic euglycemic clamp (insulin dose, 400 mU/m^2/min). Approximately 50 mg muscle was pulverized and homogenized in ice-cold buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM EGTA, 1% IGEPAL CA-630, 10 mM $Na_4P_2O_7$, 100 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM Na_3VO_4, 1 mM PMSF, and 50 μM okadaic acid). One milligram of total protein in cell lysates was used for immunoprecipitation.

Results

Phosphorylation of Ser^{307} mediates the inhibitory effect of anisomycin on IRS-1 tyrosyl phosphorylation. We reported previously that anisomycin activates JNK and inhibits insulin action (26). To confirm that anisomycin inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 by promoting phosphorylation of Ser^{307}, we prepared CHO cells expressing wild-type IRS-1 or IRS-1 containing the Ser^{307}→Ala substitution (IRS-1^{S307A}). IRS-1 or IRS-1^{S307A} were expressed transiently in CHO^{IR} cells that stably overexpress the insulin receptor. Anisomycin did not alter tyrosyl phosphorylation of the insulin receptor during insulin stimulation; however, it inhibited insulin-stimulated tyrosyl phosphorylation of both endoge-

Figure 2
Anisomycin stimulates phosphorylation of IRS-1 on Ser^{307}. (**a** and **b**) CHO (**a**) or 293 (**b**) cells were treated with 5 μg/ml anisomycin (aniso) for 30 minutes. IRS-1 was immunoprecipitated (IP) with αIRS-1 and immunoblotted with αpS^{307}. The same blot was stripped and reprobed with αIRS-1. (**c**) αpS^{307} was preincubated on ice for 15 minutes with 5-μg phospho-Ser^{307} peptide (pS^{307}) or non-phospho-Ser^{307} (S^{307}) peptide. αIRS-1 immunoprecipitates from control or anisomycin-stimulated CHO cells were immunoblotted with αpS^{307} in the presence of pS^{307} or S^{307}, respectively. (**d**) CHO cells were transfected transiently with 5-μg plasmids encoding HA-tagged IRS-1 or IRS-1^{S307A}. Cells were treated with 5 μg/ml anisomycin for 30 minutes. HA-tagged IRS-1 and IRS-1^{S307A} in cell lysates were immunoprecipitated with αHA. The αHA immunoprecipitates were divided evenly into two and immunoblotted with αpS^{307} and αIRS-1, respectively.



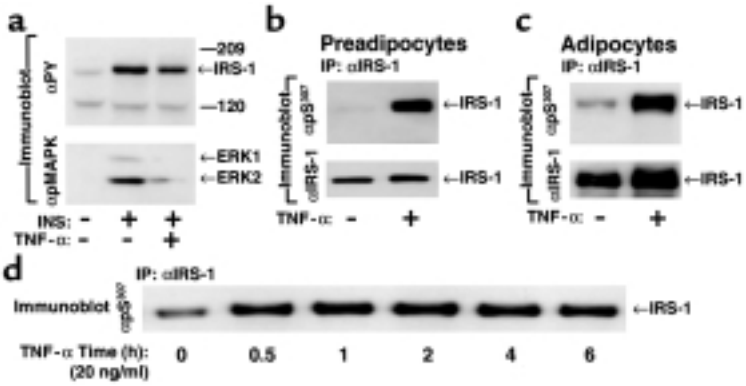


Figure 3
TNF-α stimulates phosphorylation of IRS-1 on Ser^{307} and inhibits insulin-promoted tyrosyl phosphorylation of IRS-1 and activation of ERK1/2. (**a**) 3T3-L1 adipocytes were pretreated with 20 ng/ml TNF-α for 5 hours before 50 nM insulin stimulation for 10 minutes. Proteins (40 μg) in cell lysates were immunoblotted with anti-phosphotyrosine and anti–phospho-MAPK, respectively. (**b** and **c**) 3T3-L1 preadipocytes (**b**) and adipocytes (**c**) were stimulated with 20 ng/ml TNF-α for 30 minutes. IRS-1 in cell lysates was immunoprecipitated with αIRS-1 and immunoblotted with αpS^{307}. The same blots were stripped and reprobed with αIRS-1. (**d**) The 3T3-L1 adipocytes were stimulated with 20 ng/ml TNF-α for the indicated time. IRS-1 immunoprecipitates were immunoblotted with αpS^{307}.

nous and recombinant IRS-1 (Figure 1). In contrast, anisomycin did not inhibit insulin-stimulated tyrosyl phosphorylation of IRS-1^{S307A}, confirming that Ser^{307} mediates the inhibitory action of anisomycin, as shown previously (26).

Phosphospecific antibodies reveal anisomycin-stimulated Ser^{307} phosphorylation. Polyclonal antibodies against a synthetic phospho-Ser^{307} peptide based on the amino acid sequence around Ser^{307} in IRS-1 were prepared in rabbits. The ability of this antibody (αpS^{307}) specifically to recognize IRS-1 phosphorylated on Ser^{307} was evaluated in CHO and HEK293 cells stimulated with anisomycin for 30 minutes. αIRS-1 immunoprecipitates from untreated CHO cells did not react with αpS^{307} during immunoblotting. However, after incubation with 5-μg/ml anisomycin for 30 minutes, IRS-1 from both CHO and 293 cells was strongly immunoblotted with αpS^{307} (Figure 2, a and b); dephosphorylation with alkaline phosphatase of IRS-1 from anisomycin-stimulated cells prevented αpS^{307} immunoblotting (data not shown). Moreover, incubation of αpS^{307} with the phosphorylated antigen used to generate αpS^{307} blocked immunoblotting, whereas incubation with the unphosphorylated peptide did not inhibit immunoblotting (Figure 2c). To verify that αpS^{307} only immunoblots phospho-Ser^{307}, HA-tagged IRS-1 or IRS-1^{S307A} was transiently expressed to the same level in CHO cells (data not shown). After 30 minutes of stimulation with 5 μg/ml anisomycin, recombinant IRS-1 or IRS-1^{S307A} was immunoprecipitated with HA-specific antibodies and immunoblotted with αpS^{307}. Under these experimental conditions, IRS-1 was strongly immunoblotted with αpS^{307}, whereas IRS-1^{S307A} was not recognized (Figure 2d). These results suggest that αpS^{307} binds specifically to phospho-Ser^{307} and does not cross-react with other phosphoserine residues in IRS-1.

TNF-α stimulates phosphorylation of IRS-1 on Ser^{307} and inhibits insulin-stimulated tyrosyl phosphorylation. TNF-α promotes insulin resistance during trauma and chronic obesity, and several reports suggest that this occurs through serine phosphorylation of IRS proteins (18, 21). In agreement with these reports, TNF-α inhibited insulin-stimulated tyrosyl phosphorylation of IRS-1 and insulin-stimulated activation of ERK1/2 in 3T3-L1 adipocytes (Figure 3a). However, TNF-α–stimulated serine phosphorylation of IRS-1 has never been observed directly, leaving open the possibility that other mechanisms are involved. Because TNF-α activates JNK, Ser^{307} might be an important site in the inhibition of IRS-1 signaling. Therefore, we used αpS^{307} to determine whether Ser^{307} is phosphorylated in TNF-α–stimulated cells.

3T3-L1 preadipocytes were treated for 30 minutes with TNF-α (20 ng/ml), and IRS-1 in cell lysates was immunoprecipitated with αIRS-1 and immunoblotted

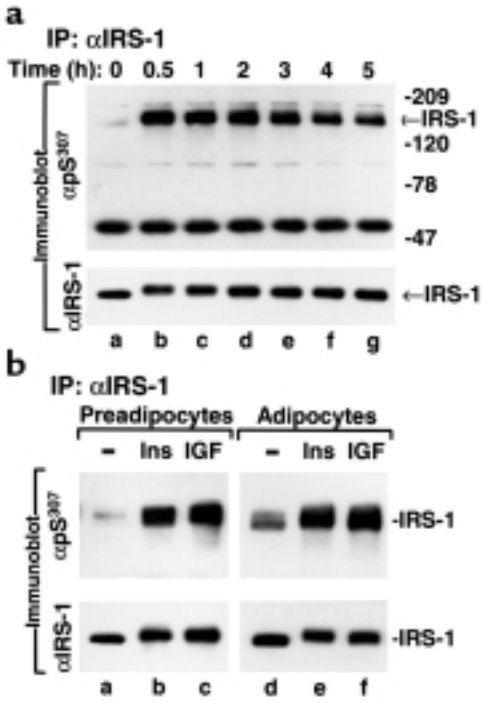


Figure 4
Insulin and IGF-1 stimulate phosphorylation of IRS-1 on Ser^{307} in both 3T3-L1 preadipocytes and adipocytes. (**a**) 3T3-L1 preadipocytes were stimulated with 100 nM insulin for the indicated time. IRS-1 in cell lysates was immunoprecipitated with αIRS-1 and immunoblotted with αpS^{307}. The same blot was stripped and reprobed with αIRS-1. (**b**) 3T3-L1 preadipocytes and adipocytes were stimulated for 30 minutes with 100 nM insulin (Ins) and 100 ng/ml IGF-1 (IGF), respectively. IRS-1 immunoprecipitates were immunoblotted with αpS^{307}. The same blot was stripped and reprobed with αIRS-1.

with αpS^{307}. Before TNF-α stimulation, IRS-1 was not immunoblotted with αpS^{307}, whereas after TNF-α stimulation, IRS-1 reacted strongly with αpS^{307} (Figure 3b), indicating that TNF-α stimulates Ser^{307} phosphorylation. Similarly, TNF-α stimulated phosphorylation of Ser^{307} in 3T3-L1 adipocytes (Figure 3c). The effect of TNF-α (20 ng/ml) on the phosphorylation of Ser^{307} was detected after 5 minutes, reached a maximum within the first 15 minutes, and remained elevated during 6 hours of TNF-α treatment (Figure 3d). Interestingly, compared with fibroblasts, the basal phosphorylation of Ser^{307} was slightly elevated in 3T3-L1 adipocytes before TNF-α stimulation (Figure 3, c and d). Because adipocytes produce TNF-α, the increase in the basal phosphorylation of Ser^{307} might represent autocrine action, as suggested previously (27–29).

Insulin and IGF-1 stimulated phosphorylation of IRS-1 on Ser^{307}. Chronic insulin stimulation inhibits insulin signal transduction in many experimental systems, which might exacerbate insulin resistance during compensatory hyperinsulinemia (2, 4, 23, 30). To determine whether insulin stimulates phosphorylation of IRS-1 on Ser^{307}, 3T3-L1 preadipocytes or adipocytes were treated with insulin (100 nM) for various time intervals, and immunopurified IRS-1 was immunoblotted with αpS^{307}. Insulin strongly stimulated phosphorylation of IRS-1 on Ser^{307}, which reached maximal levels within 30 minutes (Figure 4a). Ser^{307} phosphorylation remained elevated during 3 hours of insulin stimulation and then decreased slightly, even though the level of IRS-1 was unchanged during the 5-hour experiment (Figure 4a). Similarly, IGF-1 strongly stimulated phosphorylation of IRS-1 on Ser^{307} in both 3T3-L1 preadipocytes and adipocytes (Figure 4b). By contrast, EGF did not stimulate phosphorylation of IRS-1 on Ser^{307} (data not shown).

Phosphorylation of Ser^{307} during prolonged insulin stimulation might contribute to desensitization of the insulin response or cause insulin resistance especially during compensatory hyperinsulinemia. To determine whether insulin-stimulated phosphorylation of IRS-1 on Ser^{307} occurs in the whole organism, soleus muscle from male and female mice was isolated and homogenized and IRS-1 was immunopurified with αIRS-1. Immunoblotting with αpS^{307} revealed insulin-stimulated phosphorylation of Ser^{307} in skeletal muscle from both male and female mice (Figure 5a). Similarly, insulin increased phosphorylation of IRS-1 on Ser^{307} in muscle of rat more than twofold (Figure 5b).

To determine whether insulin also promotes phosphorylation of IRS-1 on Ser^{307} in human skeletal muscle, normal volunteers were subjected to hyperinsulinemic euglycemic clamps for 0 and 15 minutes. IRS-1 was immunopurified from homogenized vastus lateralis muscle biopsy samples and immunoblotted with αpS^{307}. Under these conditions, insulin strongly stimulated phosphorylation of IRS-1 on Ser^{307} (Figure 5c). Interestingly, phosphorylation of IRS-1 on Ser^{307} increased after prolonged insulin treatment (data not shown).

Insulin and TNF-α stimulate distinct pathways that converge at Ser^{307}. Distinct signaling pathways mediate insulin and TNF-α signal transduction, but both ligands promote Ser^{307} phosphorylation. LY294002 or wortmannin were used to determine whether PI 3-kinase–dependent pathways mediate Ser^{307} phosphorylation during insulin/IGF-1 or TNF-α stimulation. As expected, the PI-3 kinase inhibitors LY294002 or wortmannin inhibited activation of PKB/Akt in insulin or IGF-1 stimulated 3T3-L1 preadipocytes, but were without effect on ERK1/2. LY294002 or wortmannin abolished the phosphorylation of Ser^{307} induced by insulin/IGF-1 but not by TNF-α (Figure 6a). Both TNF-α and insulin/IGF-1 stimulated phosphorylation and activation of ERK1/2, which was blocked by PD98059 (Figure 6a, and data not shown). TNF-α–stimulated activation of ERK1/2 was reduced to the basal level within 2 hours (data not shown). However, PD98059 inhibited Ser^{307} phosphorylation induced by TNF-α, but not by insulin/IGF-1 (Figure 6b). The inhibition of TNF-α–induced Ser^{307} phosphorylation by PD98059 was dose-dependent and was correlated with the inhibition of ERK1/2 (Figure 6c). These results suggest that elements of the PI 3-kinase cascade mediate insulin/IGF-1–stimulated phosphorylation of Ser^{307}, whereas MEK1 or another PD98059-sensitive kinase might mediate the effects of

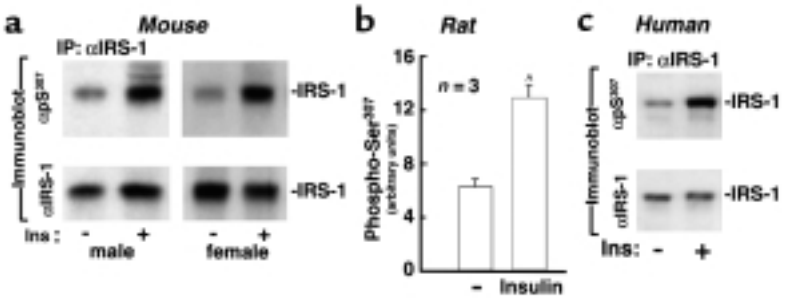


Figure 5
Insulin promotes phosphorylation of IRS-1 on Ser^{307}in mouse, rat, and human skeletal muscle. (**a**) Saline or 5 U human insulin was injected as a bolus into the inferior vena cava of anesthetized mice. Soleus muscle was isolated 5 minutes later and homogenized. IRS-1 was immunoprecipitated with αIRS-1 and immunoblotted with αpS^{307}. The same blots were stripped and reprobed with αIRS-1. (**b**) Insulin (5 U) was injected intraperitoneally into anesthetized rat. Saline was used as control. Soleus muscle was isolated 5 minutes later and homogenized. IRS-1 immunoprecipitates were immunoblotted with αpS^{307} or αIRS-1. Phospho-Ser^{307} was quantitated by phosphoimaging and normalized to IRS-1 level. The data were presented as mean ± SEM (three rats for each group; $^{A}P < 0.02$). (**c**) Normal volunteer subjects were subjected to hyperinsulinemic euglycemic clamp. Muscle biopsies were obtained after insulin-clamp for 0 or 15 minutes and homogenized. IRS-1 immunoprecipitates were immunoblotted with αpS^{307}. The data represent one of three independent experiments.

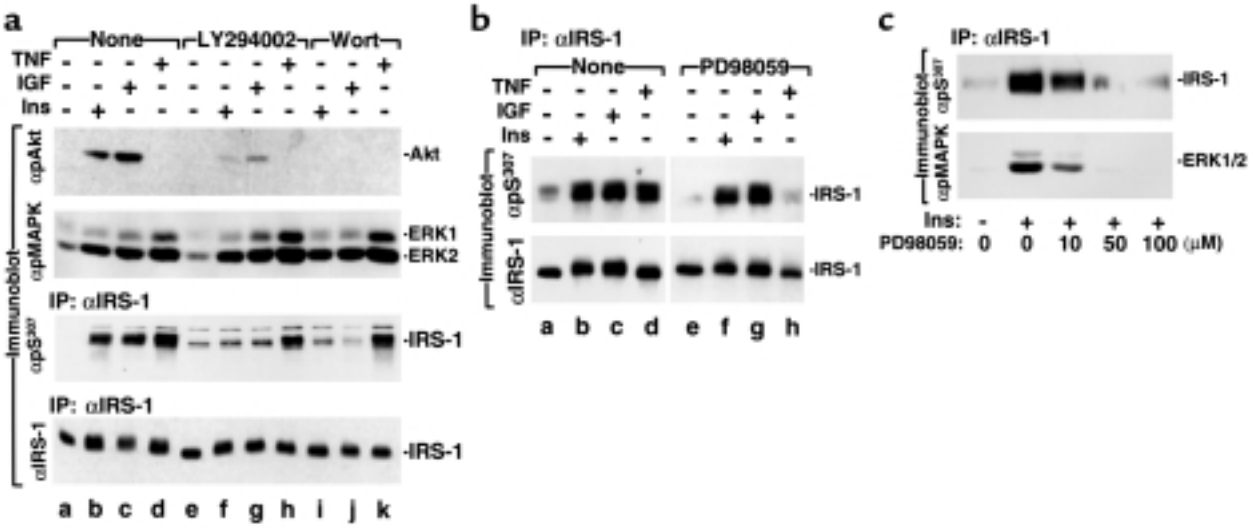


Figure 6

Two distinct pathways mediate Ser^{307} phosphorylation on IRS-1 induced by insulin/IGF-1 and TNF-α. (**a**) The 3T3-L1 preadipocytes were preincubated for 30 minutes with 20 μM LY294002 or 100 nM wortmannin (Wort) before stimulation for an additional 30 minutes with 100 nM insulin, 100 ng/ml IGF-1, or 20 ng/ml TNF-α. Proteins (50 μg) in the cell lysates were immunoblotted directly with antibodies against phospho-Akt or phospho-MAPK; or IRS-1 was immunoprecipitated from the lysates with αIRS-1 and immunoblotted with αpS^{307} or with αIRS-1. (**b**) The 3T3-L1 preadipocytes were preincubated for 30 minutes with 100 μM PD98059 before stimulation for an additional 30 minutes with 100 nM insulin, 100 ng/ml IGF-1, or 20 ng/ml TNF-α. IRS-1 in cell lysates was immunoprecipitated with αIRS-1. Half of the αIRS-1 immunoprecipitates were immunoblotted with αpS^{307}, and half were immunoblotted with αIRS-1. (**c**) The 3T3-L1 preadipocytes were preincubated for 30 minutes with the indicated concentration of PD98059 before stimulation for an additional 30 minutes with 20 ng/ml TNF-α. Immunopurified IRS-1 was immunoblotted with αpS^{307}. Cell extracts were immunoblotted with anti–phospho-MAPK.

TNF-α. Consistent with the idea that two distinct signaling pathways mediate the phosphorylation of Ser^{307} in IRS-1 by insulin and TNF-α, treatment of cells simultaneously with both TNF-α and insulin stimulates much stronger phosphorylation of Ser^{307} than does treatment with either insulin or TNF-α alone (Figure 7).

The close relation between MEK1 activity and Ser^{307} phosphorylation was unexpected, because JNK-1 was thought to mediate Ser^{307} phosphorylation during TNF-α stimulation (26). To determine whether JNK is involved in phosphorylation of Ser^{307} in response to TNF-α, 3T3-L1 preadipocytes were treated with 20 ng/ml TNF-α in the presence or absence of PD98059. JNK-1 was immunoprecipitated with anti–JNK-1, and subjected to an in vitro kinase assay using GST-cJun as a substrate. TNF-α stimulated robust activation of both JNK-1 and ERK1/2. PD98059 inhibited the activation of ERK1/2, but not JNK (Figure 8a). Because PD98059 inhibits Ser^{307} phosphorylation without any effect on JNK-1 activation, JNK-1 is unlikely to mediate TNF-α–induced Ser^{307} phosphorylation. Insulin only slightly stimulated JNK-1 activation, and the PI 3-kinase inhibitor LY294002 did not inhibit JNK-1, suggesting that JNK-1 is not the kinase to phosphorylate Ser^{307} in response to insulin (Figure 8b).

Discussion

In addition to type 2 diabetes, insulin resistance is associated with a variety of physiological states, including trauma and infection, hypertension, glucose intolerance, and obesity; however, the molecular mechanisms that modulate insulin signaling under these conditions are difficult to resolve. Many studies suggest that serine phosphorylation of the IRS proteins might inhibit insulin signaling, but the identification of physiologically relevant sites and the regulation of their phosphorylation and dephosphorylation has progressed slowly (26, 32–34). Recently, we discovered that JNK-1 associates with IRS-1 and promotes phosphorylation of Ser^{307}. Although our current results show that JNK is not the only kinase that phosphorylates Ser^{307}, several results including a Ser^{307}→Ala substitution reveals that the phosphorylation of Ser^{307} inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 in cultured cells (26).

The phosphorylation of specific residues in IRS-1 is an attractive regulatory mechanism because it provides specificity at multiple levels, including the interaction between specific serine kinases and IRS-1, regulation of the associated kinase activity, and selection of specific phosphorylation sites. However, it is difficult to demonstrate that serine phosphorylation of IRS-1 changes under specific physiological conditions, because IRS-1 contains nearly 100 potential serine phosphorylation sites that complicate ordinary biochemical analysis. Immunodetection of specific serine or threonine phosphorylation sites is successful in many cases. Applying this strategy to IRS-1, we prepared αpS^{307}, a polyclonal antiserum generated in rabbits against the peptide sequence around phosphorylated Ser^{307} in IRS-1. Several experiments demonstrate that αpS^{307} recognizes specifically the phosphorylated

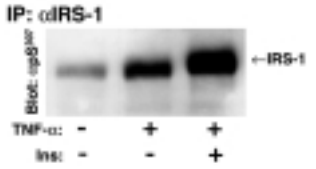


Figure 7
TNF-α and insulin act synergistically to promote phosphorylation of Ser^{307} in IRS-1. The 3T3-L1 preadipocytes were treated for 30 minutes with 20 ng/ml TNF-α and 100 nM insulin either separately or simultaneously. IRS-1 in cell lysates was immunoprecipitated with αIRS-1 and immunoblotted with αpS^{307}.

Ser^{307} residue: αpS^{307} fails to immunoblot IRS-1 immunoprecipitated from quiescent cells; αpS^{307} strongly immunoblots IRS-1 immunoprecipitated from cells stimulated with anisomycin, TNF-α, IGF-1, or insulin; under stimulated conditions, dephosphorylation of IRS-1 immunoprecipitates with alkaline phosphatase inhibits immunoreactivity of αpS^{307}; and phosphorylated peptides used to generate αpS^{307} inhibit immunoblotting, whereas unphosphorylated peptides have no inhibitory effect. Finally, αpS^{307} fails to recognize the mutant IRS-1 molecule containing the Ser^{307}→Ala substitution. Together these results demonstrate that αpS^{307} reacts specifically with phosphorylated Ser^{307} in IRS-1 from mouse, rat, and human tissues; it is unreactive with other IRS proteins because the peptide sequence around Ser^{307} is unique to IRS-1.

The use of αpS^{307} provides clear evidence that Ser^{307} phosphorylation is regulated by several mechanisms. Although previous work suggested indirectly that anisomycin and TNF-α stimulate Ser^{307} phosphorylation, immunoblots with αpS^{307} confirm this conclusion for the first time. Moreover, these results support the hypothesis that the inhibitory effect of anisomycin and TNF-α on insulin-stimulated tyrosine phosphorylation is mediated by the phosphorylation of Ser^{307} (26). Moreover, immunoblotting with αpS^{307} can help establish biologic relevance, by assessing Ser^{307} phosphorylation in IRS-1 isolated from tissues. For example, Ser^{307} is phosphorylated in liver, fat, and muscle isolated from rats subjected to thermal injury, suggesting that TNF-α or other inflammatory cytokines might also have effects in animals (data not shown).

Surprisingly, insulin and IGF-1 stimulate phosphorylation of Ser^{307} in 3T3-L1 fibroblasts and adipocytes. This unexpected result appears to be physiologically important, as injection of insulin into mice or rats promotes Ser^{307} phosphorylation in muscle. In humans, pharmacological insulin levels achieved during a hyperinsulinemic euglycemic clamp stimulate Ser^{307} phosphorylation on IRS-1 immunopurified from muscle biopsies. Thus, the regulated phosphorylation of Ser^{307} might be a common pathway to counterregulate the insulin signal during traumatic stress or chronic hyperinsulinemia.

The phosphorylation of Ser^{307} in IRS-1 was first observed during in vitro phosphorylation experiments with recombinant IRS-1 and JNK-1. JNK-1 associates specifically with IRS-1 and, during this association, mediates phosphorylation of Ser^{307} (26). Because JNK-1 is activated by TNF-α or anisomycin, these results reveal a pathway to regulate IRS-1 serine phosphorylation. However, the finding that insulin stimulates Ser^{307} phosphorylation of IRS-1 suggests that other kinases might also be involved. Interestingly, inhibition of PI 3-kinases by LY294002 and wortmannin abrogates phosphorylation of Ser^{307} induced by insulin and IGF-1, but not by TNF-α. In contrast, inhibition of MEK1 by PD98059 abolishes phosphorylation of Ser^{307} induced by TNF-α, but not by insulin or IGF-1; this finding is unexpected because PD98059 is not reported to inhibit JNK or kinases upstream of JNK activation and suggests that another kinase might mediate the effect of TNF-α on IRS-1. Thus, at least three kinases mediate phosphorylation of Ser^{307}, including JNK, serine kinases in the PI 3-kinase cascade that are activated by insulin or IGF-1, and MEK1-sensitive kinase cascades during TNF-α stimulation. Thus, Ser^{307} phosphorylation might integrate signals mediated through multiple serine kinases, including those promoted by heterologous ligands as well as insulin itself.

Interestingly, although PD98059 blocks the

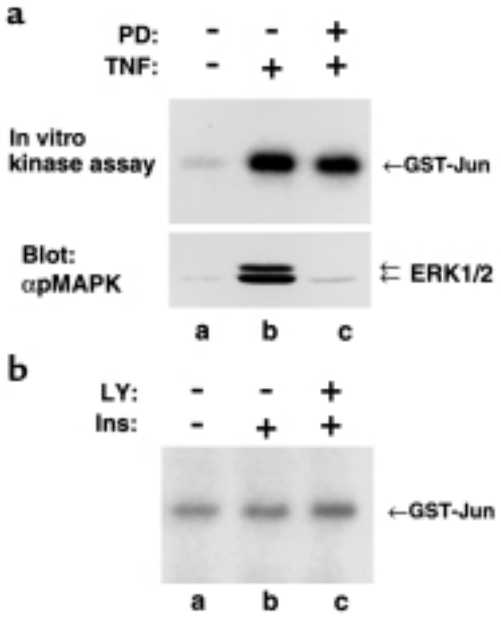


Figure 8
Kinase(s) other than JNK phosphorylate Ser^{307} in IRS-1. (**a**) The 3T3-L1 preadipocytes were preincubated for 30 minutes with or without 100 μM PD98059 before stimulation for an additional 15 minutes with 20 ng/ml TNF-α. JNK-1 was immunoprecipitated with αJNK-1, and subjected to an in vitro kinase assay using GST-cJun as a substrate (top). Cell lysates were immunoblotted with anti–phospho-MAPK (bottom). (**b**) The 3T3-L1 preadipocytes were preincubated for 30 minutes with or without 20 μM LY294002 before stimulation for an additional 10 minutes with 100 nM insulin. JNK-1 was immunoprecipitated with αJNK1, and subjected to an in vitro kinase assay using GST-cJun as a substrate.

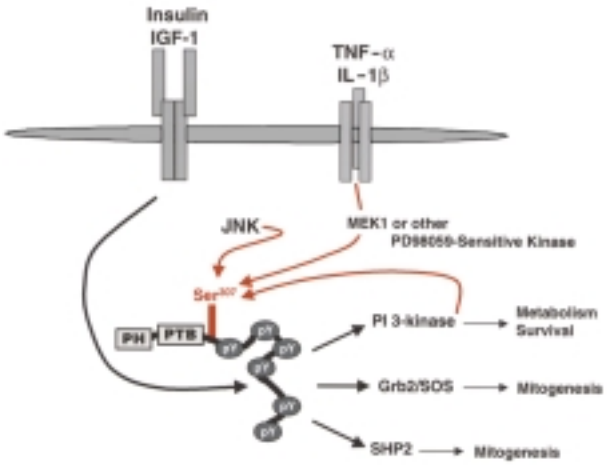


Figure 9
Models for the function of Ser^{307} phosphorylation. Upon insulin stimulation, IRS-1 is tyrosyl-phosphorylated by the IR, resulting in the activation of PI 3-kinase that mediates Ser^{307} phosphorylation. Ser^{307} phosphorylation subsequently inhibits the ability of IRS-1 to be further tyrosyl phosphorylated by the IR and to propagate insulin signaling. An increase in Ser^{307} phosphorylation, such as that possibly trigged by TNF-α in chronic obesity, also induces insulin resistance. As β cells compensate for insulin insensitivity with compensatory hyperinsulinemia, chronic insulin stimulation induces more Ser^{307} phosphorylation through the PI 3-kinase pathway, thus further increasing the pool of inactive, Ser^{307} phosphorylated IRS-1. This vicious cycle might continue until hyperinsulinemia fails to compensate for insulin resistance.

MEK/ERK pathway activated by both insulin/IGF-1 and TNF-α, it only inhibits TNF-α-induced Ser^{307} phosphorylation. These observations suggest that the MEK/ERK cascade might be required, but is not sufficient for Ser^{307} phosphorylation. Perhaps other signals that are specific to TNF-α but not insulin/IGF-1 act together with the MEK/ERK pathway to induce Ser^{307} phosphorylation. In this scenario, the MEK/ERK pathway is not involved in phosphorylation of Ser^{307} in response to insulin and IGF-1. Alternatively, an unidentified, PD98059-sensitive kinase(s) that is activated by TNF-α but not insulin/IGF-1 mediate TNF-α-stimulated Ser^{307} phosphorylation.

Our results suggest that phosphorylation of Ser^{307} in IRS-1 might be a common site integrating heterologous inhibition of the insulin signal by certain cytokines with feedback inhibition during chronic hyperinsulinemia (Figure 9). Consistent with this conclusion, insulin and IGF-1 act synergistically with TNF-α to promote phosphorylation of Ser^{307} to a much higher extent. A potential mechanism for peripheral insulin resistance emerges from these results. During the early stages of glucose intolerance, moderate hyperinsulinemia compensates for peripheral insulin resistance. Although this strategy promotes adequate metabolic regulation in moderate cases, chronic hyperinsulinemia might increase the steady-state level of Ser^{307} phosphorylation, eventually reducing the available pool of active IRS-1 molecules. Compensation might continue to adjust to this dynamic interaction until heterologous mechanisms, such as cytokine-stimulated phosphorylation of Ser^{307}, exacerbate the situation and diabetes ensues because β cells fail to produce sufficient insulin (Figure 9).

At the molecular level, the mechanism of inhibition of IRS-1 by Ser^{307} phosphorylation is an important question, but the answer is unclear. Previous reports suggest that serine or threonine phosphorylation of IRS-1 impairs its association with the insulin receptor (23, 35, 36). This inhibition might result from dysregulation of PTB domain. Previous reports indicate that TNF-α might inhibit the ability of the PTB domain in IRS-1 to interact with the phosphorylated NPEY-motif in the activated insulin receptor (23). Given that Ser^{307} is adjacent to the PTB domain in IRS-1, this mechanism must be directly tested. Alternatively, phosphorylation of Ser^{307} might create a binding site for inhibitory molecules, including the binding of protein tyrosine phosphatases.

In summary, TNF-α, insulin, and IGF-1 stimulate phosphorylation of Ser^{307} in IRS-1 in cultured cells. Insulin also stimulates phosphorylation of Ser^{307} in skeletal muscle of mice, rats and humans. Insulin– and IGF-1–induced phosphorylation of Ser^{307} depends on PI 3-kinase, whereas phosphorylation of Ser^{307} induced by TNF-α requires the activation of MEK1 or an unknown PD98059-sensitive kinase. Phosphorylation of Ser^{307} inhibits subsequent tyrosyl phosphorylation of IRS-1 and activation of IRS-1–mediated signaling pathways in response to insulin, which might contribute to insulin resistance.

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