Serine Phosphorylation of Insulin Receptor Substrate-1: A Novel Target for the Reversal of Insulin Resistance

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Insulin resistance, the failure to respond to normal circulating concentrations of insulin, is a common state associated with obesity, aging, and a sedentary lifestyle. Compelling evidence implicates TNFα as the cause and link between obesity and insulin resistance. Serine phosphorylation of insulin receptor substrate-1 seems prominent among the mechanisms of TNFα-induced insulin resistance. Recent advances indicate that serine kinases may phosphorylate and thus inhibit the tyrosine phosphorylation of insulin receptor substrate-1, revealing an integration point of TNFα and insulin signaling pathways. Selective targeting of the molecular scenery whereby this key phosphorylation occurs/operates represents a rich area for the development of rationally designed new antidiabetic drugs. In relation to efficacy and side effects, this prospect should permit a more precise and perhaps individualized approach to therapeutic intervention, allowing clinicians to focus the attack where the problem lies. (Molecular Endocrinology 15: 1864–1869, 2001)

TYPE 2 DIABETES mellitus (T2DM) is a prevalent cause of morbidity and mortality, mainly through its long-term cardiovascular complications. Despite decades of intense investigation, its pathogenesis remains incompletely understood. Clinical T2DM is thought to ensue when insulin production by the pancreas fails to compensate for the insulin resistance manifested by the target tissues of insulin. In diabetic patients, skeletal muscle, adipose tissue, and the liver display a lower than normal response to insulin action. Insulin resistance is believed to be the earliest and dominant metabolic defect in T2DM and is central to the pathogenesis of hypertension, hyperlipidemia, atherosclerosis, and other constituents of the metabolic syndrome. T2DM is a multifactorial disease with a strong genetic component. The major predisposing environmental factor is obesity, which is virtually always associated with insulin resistance (1).

During the past decade TNFα, a cytokine, has been implicated as a link between obesity and insulin resistance. Data have come from three lines of evidence (2): 1) association studies, in which TNFα production by animal and human adipose and muscle tissues has been correlated with obesity and indices of insulin resistance; 2) in vivo experiments, which utilize TNFα infusion and neutralization in animals and humans; and 3) transgenic mice, in which the cytokine or its receptors have been knocked out. TNFα is not the sole mediator of insulin resistance in obesity; other candidates include FFAs, leptin, hyperinsulinemia, and the newly discovered hormone, resistin (3). TNFα is expressed as a transmembrane protein that is cleaved to release a soluble cytokine. Muscle- and fat-derived TNFα may act on target cells in an endocrine, paracrine, or autocrine manner, and both soluble and transmembrane forms can associate with TNFα receptors (4). Receptors are devoid of enzymatic activity but serve as docking molecules for other signaling proteins that induce both apoptotic and antiapoptotic signals.

Multiple, not mutually exclusive, mechanisms have been proposed to account for TNFα-induced insulin resistance in obesity (4). These include elevation of plasma FFAs due to its lipolytic action, down-regulation of insulin-sensitive glucose transporter (GLUT4) translocation to the plasma membrane, antagonism of the PPARγ pathway, interplay with leptin and resistin, and, most interesting, direct interference with insulin’s signal transduction pathway.

Insulin binding to the α-subunits of its heterotetrameric membrane receptor triggers the tyrosine kinase (insulin receptor kinase, IRK) activity of the β-subunits, which transphosphorylate themselves and tyrosine-phosphorylate endogenous substrates, including Shc and insulin receptor substrates (IRSs).
IRSs are related protein substrates of IRK, with a highly conserved N terminus containing a PH domain and a phospho-Tyr-binding (PTB) domain, and a poorly conserved C terminus with several Tyr phosphorylation motifs (5). At least four IRS isoforms occur in mammals: IRS-1 and IRS-2 are widely expressed, whereas IRS-3 is restricted to adipose tissue and IRS-4 is expressed in the thymus, brain, and kidney. To delineate the functional roles of IRS isoforms, IRS and insulin receptor (IR) genes have been disrupted to create homozygous null-mice and various combinations of homozygous-null and/or compound heterozygous (5, 6). Moreover, the in vivo impact of IRS-1 vs. IRS-2 gene disruption on carbohydrate and lipid metabolism has been assessed by euglycemic hyperinsulinemic clamps (7). These studies suggest that IRS isoforms have tissue-specific roles: IRS-3 and IRS-4 appear to have minor or redundant roles in insulin signaling. IRS-1 is the most important isoform in muscle, whereas IRS-2 has an impact on liver, muscle, and adipose tissue. Because of exciting new insights into the molecular machinery that fine tunes IRS-1 function, the scope of this presentation will be restricted to the latter.

After Tyr phosphorylation IRS-1 serves as a docking protein for a number of effector molecules bearing the SH2 domain, such as Grb2, Syp, Nck, and the regulatory subunit of PI3K, p85. p85 binding to IRS-1 stimulates the PI-3K activity of the p110 catalytic subunit, as well as downstream signaling proteins. Phosphorylated Shc and IRS-1 bind to Grb2 and mediate p21ras GTP-loading via the guanyl nucleotide exchange factor, SOS. Active p21ras associates with and activates the Raf-1 kinase (a MAPK kinase kinase, MAPKKK), which phosphorylates and activates MEK-1 (a MAPK kinase, MAPKK), which in turn phosphorylates and activates ERK, a member of the MAPK family of signaling enzymes. Activation of the MAPK cascade is associated mostly with transcriptional and mitogenic effects of insulin, while the PI3K pathway is generally engaged with the hormone’s metabolic effects (Fig. 1).

TNFα has been shown to interfere with early steps of insulin signaling, and this is arguably the dominant mechanism by which it induces insulin resistance (2, 7).

Fig. 1. Insulin and TNFα Signaling Pathways Converge on IRS-1 Ser307 Phosphorylation

After insulin binding, Tyr-phosphorylated (orange spheres) insulin receptor activates Ras through Shc/Grb2/SOS (green arrow) or via IRS-1/Grb2/SOS (pink arrow). Ligand-bound TNFα receptor activates Raf-1 and has mixed effects on MEKK-1 through the sphingomyelinase pathway (yellow arrows), and activates MEKK-1 via the TRADD/RIP/TRAF2 complex (blue arrow). Tyr-phosphorylated IRS-1 binds to regulatory p85 to stimulate p110 PI-3K activity. Kinases downstream of PI3K and MEK-1 phosphorylate IRS-1 Ser307 (red sphere) in response to insulin and TNFα, respectively. Ser307 is adjacent to the PTB domain of IRS-1, which is crucial for functional interaction with the JM domain of insulin receptor. PhosphoSer307 may impair this interaction by steric hindrance, or by recruitment of unknown inhibitory proteins, hence leading to insulin resistance.
Increased serine phosphorylation of IRS-1 is the proposed molecular mechanism by which TNFα inhibits insulin signaling (4). In cultured fat cells TNFα induces Ser phosphorylation of IRS-1, which subsequently inhibits IRK activity in vitro. The presence of IRS-1 is required for TNFα-induced inhibition of insulin signaling in intact cells. Furthermore, an inhibitory form of IRS-1 is also present in adipose tissue and skeletal muscle of obese fa/fa rats. In cultured hepatoma cells, TNFα also induces Ser phosphorylation of IRS-1 and decreases its association with PI3K, but does not affect IRK activity. Thus, TNFα-induced Ser phosphorylation of IRS-1 is central to inhibition of insulin signaling, but the precise inhibitory function of Ser-phosphorylated IRS-1 may depend on cell type. In hepatoma cells it has been shown that TNFα-induced Ser phosphorylation of IRS-1 impairs its interaction with the juxtamembrane (JM) domain of IR and may thus render IRS-1 a poorer substrate for IRK (8). Alternatively, Ser-phosphorylated IRS-1 may recruit unknown signaling molecules that sterically hamper its interaction with IR and/or inhibit IRK activity.

Increased serine/threonine phosphorylation of IRS-1 is a common finding in insulin resistance and T2DM. Numerous agents that induce insulin resistance, such as TNFα and other cytokines, okadaic acid, platelet-derived growth factor, angiotensin II, hyperglycemia, and hyperinsulinemia, all increase IRS-1 phospho-Ser/phospho-Thr content. Some, including TNFα, are activators of Ser/Thr kinases, while others are inhibitors of Ser/Thr phosphatases. Interestingly, IRS-1 is Ser phosphorylated at the basal level, and this phosphorylation is augmented after insulin stimulation. Therefore, Ser/Thr phosphorylation of IRS-1 emerges as a negative feedback mechanism in normal insulin signaling, which is also employed by various factors that counteract insulin action (9). The kinetics of insulin-induced IRS-1 Ser/Thr phosphorylation, as compared with signal-propagating Tyr phosphorylation, is compatible with this notion. In a parallel way, Ser/Thr phosphorylation of SOS after prolonged insulin stimulation leads to dissociation of the Grb2/SOS complex. The MAPK pathway is subsequently inactivated as GTP-p21ras is hydrolyzed to the inactive GDP-bound form (10).

However, not all IRS-1 Ser phosphorylation is inhibitory: Protein kinase B (PKB/Akt) lies downstream of PI3K and is phosphorylated and activated by PI3K-dependent kinase (PDK1). After insulin stimulation, PKB has been shown to phosphorylate Ser residues within and adjacent to the PTB domain of IRS-1 (Ser265, Ser302, Ser325, and Ser358 in mouse IRS-1) (11). Phosphorylation at these sites protects IRS-1 from the action of Tyr phosphatases and maintains the substrate in its signal-propagating, Tyr-phosphorylated form. Therefore, Ser phosphorylation of IRS-1 at specific residues could be part of a positive feedback loop, in which PKB is the key switch. Insulin-activated kinases involved in negative feedback loops are expected to lie downstream or independent from PKB. Theoretically, however, a kinase upstream of PKB might also be responsible, at least in part, as it may well be that it is the vigor and rhythm of protein-to-protein cross-talk that determines the resultant outcome on a target molecule rather than the sequence of the molecules in the cascade. The action of the positive and negative circuits must nonetheless be temporally coordinated in a way that permits the propagation and amplification of the insulin cascade by the positive loop, before the negative loop predominates to attenuate the signal.

The identification of the insulin-dependent kinase or kinases that mediate the inhibitory Ser/Thr phosphorylation of IRS-1 and of their target residues is an area of intensive investigation (reviewed in Ref. 12). This identification is important because it may permit the development of rationally designed pharmaceutical agents that will interfere with the molecular events involved in insulin resistance. It is, however, a fierce task, given that IRS-1 contains more than 30 potential Ser/Thr phosphorylation sites nested within sequences that conform to various kinase motifs, including PKA, PKC, MAPK, casein kinase II, Cdc2 kinase, and PKB/Akt. At least some of the unknown kinases, should there be more than one, are downstream effectors of PI3K, because their action is sensitive to wortmannin, a specific PI3K inhibitor (11). Therefore, the search is for a kinase downstream of PI3K other than positively acting PKB. MEK-1 and p38 MAPK are unlikely to be involved, because they are not wortmannin sensitive and their inhibitors do not interrupt the feedback loop. Inhibitors of PKC isoforms α, β, γ, δ, ε, and μ were also ineffective (11). Glycogen synthase kinase-3 is capable of phosphorylating IRS-1 and impairing the insulin signal, but is not likely to be the kinase in question because insulin stimulation inhibits glycogen synthase kinase-3 (13). PI3K-dependent kinases (PDKs) are also potential candidates, but they
are located upstream of PKB. The same holds true for PI3K itself, which has been shown to stimulate IRS-1 Ser phosphorylation and inhibit IRS-1-associated PI3K activity when a membrane-targeted form is expressed in 3T3-L1 adipocytes (14). PKB isoforms β and γ cannot be ruled out, but are also unlikely candidates because their substrate selectivity is similar to that of PKBα.

The chase for the insulin-induced kinase that inhibits IRS-1 has not, in fact, been totally unfruitful. Tetradecanoylphorbol acetate, a potent activator of several PKC isoforms, inhibits the interaction of IRS-1 with the JM domain of IR and the ability of insulin to phosphorylate IRSs (8). PKC modulation of IRS-1 Tyr phosphorylation requires Ser612 (of rat IRS-1) (15), and this effect may be mediated by MAPK (16). Among PKC isoforms, atypical members such as PKCζ, η, and θ, are prominent candidates. Indeed, PKCζ is a downstream effector of PI3K that Ser phosphorylates IRS-1 and impairs its Tyr phosphorylation at specific residues (Tyr612 and Tyr632 in human IRS-1) and its ability to activate PI3K in response to insulin (17). The specific Ser-phosphorylation sites of PKCζ have not yet been identified. Other likely candidates are protein kinases p70 kDa S6 and mTOR (mammalian target of rapamycin), which are downstream of PDK1 and PKB. The PI3K/PKB pathway phosphorylates IRS-1 at residues Ser632, Ser662, and Ser731 (in rat IRS-1) and inhibits its ability to bind PI3K and activate PKB; this effect is sensitive to rapamycin, an inhibitor of mTOR (18, 19). Interestingly, a rapamycin-dependent pathway also leads to IRS-1 degradation by the proteasome (20). This effect is presumably regulated by the balance of Ser vs. Tyr phosphorylation of IRS-1 (21). However, the Ser phosphorylation sites that mediate the degradation are different to those that functionally inhibit IRS-1, although both pathways are mediated by mTOR. This implies a divergence of the two pathways downstream of mTOR with the possible recruitment of different kinases.

Previous evidence suggested that TNFα-induced activation of the c-Jun N-terminal kinase (JNK) leads to phosphorylation of Ser307 in IRS-1, and that this may mediate, at least in part, the inhibitory effect of TNFα on insulin signaling (22). JNK is a member of the MAPK family, activated in the general MAPKKK→MAPKK→MAPK scheme. JNK is also termed stress-activated protein kinase because it responds to various stress signals, whereas the ERK pathway is responsive to growth and differentiation stimuli. Using CHO cells expressing IR and IRS-1, it has been demonstrated that JNK associates with the C terminus of IRS-1 in quiescent cells and phosphorylates it when activated (22). Phosphopeptide mapping and mutational analyses identified Ser307, a residue conserved in all IRS-1 homologs, as a major site of JNK phosphorylation. Moreover, it was shown that in myeloid 32D cells and in 293 cells, a Ser307→Ala mutation abolishes the inhibitory effect of TNFα on insulin-induced IRS-1 Tyr phosphorylation. There was no proof that JNK is the endogenous kinase that mediates obesity-linked, TNFα-associated insulin resistance in muscle and adipose tissue. However, JNK is a downstream effector of TNFα (Fig. 1) and has also been implicated as both a mediator and negative modulator of insulin signaling. Thus, the identification of JNK as a kinase that phosphorylates IRS-1, and of the phosphorylated residue, Ser307, as a TNFα-induced phosphorylation target revealed JNK as a converging point in insulin signaling, which mediates both downstream propagation of signals as well as desensitization of the pathway by TNFα and insulin itself. As such, JNK would qualify as a target for insulin resistance-directed drug development. However, this inhibition might not be without undesirable consequences, because JNK is ubiquitously expressed and is involved in a variety of housekeeping functions including apoptosis, as well as some of the metabolic and transcriptional effects of insulin.

Very recently, the same research group has studied IRS-1 Ser307 phosphorylation in response to insulin/IGF-1 and TNFα in 3T3 L1 preadipocytes and adipocytes, as well as Ser307 phosphorylation in response to insulin in skeletal muscle of mice, rats, and humans (23). They reverified that Ser307 is phosphorylated by these stimuli, leading to impairment of insulin signaling. Importantly, using selective inhibitors, they identified distinct pathways that are activated in response to insulin and TNFα and converge on IRS-1 Ser307. Phosphorylation in response to insulin is sensitive to Wortmannin or LY294002, which implies that it is mediated by the PI3K pathway, whereas phosphorylation in response to TNFα is sensitive to PD98059 and thus appears to be MEK-1 dependent (Fig. 1). Surprisingly, JNK was not found to participate in Ser307 phosphorylation. These new data have two important implications. First, Ser307 emerges as a possible hallmark of insulin resistance in biologically important cells and tissues. Second, because different kinases are activated by distinct signals and converge on a single residue to promote insulin resistance, therapeutic targeting can be directed away from the unrealistic goal of kinase inhibition and toward the specific prevention or reversal of Ser307 phosphorylation, or against its effect on the insulin signaling cascade.

The demonstration that TNFα Ser-phosphorylates IRS-1 via a PI3K-independent pathway contrasts somewhat with the findings of Ozes et al. (24). Using NIH 3T3 cells, 293 embryonic kidney cells, and C3H 10T1/2 C18 myoblasts, these investigators demonstrate that TNFα Ser-phosphorylates IRS-1 through the PI3K/PKA/mTOR pathway and inhibits insulin-induced Tyr phosphorylation of IRS-1. PTEN, a human tumor suppressor gene and phospholipid phosphatase that inhibits PI3K and PKB pathways, is shown to antagonize TNFα insulin resistance. Their analysis identified residues other than Ser307 as the important phosphorylation sites, i.e., Ser636 and Ser639. It is conceivable that TNFα may activate both the PI3K and MAPK pathways and each pathway may phosphory-
late different serines on IRS-1. Thus, an important question regarding the candidate kinases and residues that inhibit IRS-1 function or promote its degradation in response to insulin or external stimuli, is that of the relative (and hierarchical?) contribution of specific kinase/residue phosphorylation to the overall insulin resistance. To this end, we note that studies employing tissues, animals, and human subjects (23) may be preferential to in vitro (cell-line) experiments (24), because they are more likely to identify pathways and processes that closely resemble the in vivo situation and thereby unveil clinically promising pharmaceutical targets.

The precise molecular mechanism of insulin signaling inhibition by IRS-1 Ser\(^{307}\) phosphorylation is presently unknown. A prominent candidate mechanism is the inhibition of functional interaction between phosphorylated IRS-1 and IR. Ser\(^{307}\) is adjacent to the PTB domain of IRS-1, which lies C-terminally to the PH domain in the N terminus of IRS-1. The PH domain targets IRS-1 to the plasma membrane and allows the PTB domain to bind to a phosphorylated Tyr in the JM domain of IR and form a functional contact between the two proteins (Fig. 1) (5). It is possible that Ser\(^{307}\) phosphorylation impairs this functional interaction and thus inhibits insulin signaling (8). An alternative mechanism may involve the recruitment of inhibitory molecules by phospho-Ser\(^{307}\), such as Tyr phosphatases or other proteins that interfere with insulin signaling.

The crystal structure of IRS-1 PTB, alone and complexed with the JM region of IR, is known (25). Moreover, the three-dimensional structure of the PH-PTB targeting region of IRS-1 has recently been solved (26). We propose that this knowledge may suffice for the purpose of drug development, through high-throughput screening of available large-compound libraries or through structure-based (de novo) ligand design methodologies (27). The aim would be to search for or design small-molecule drugs that will bind to the N terminus of IRS-1 with high affinity and extreme selectivity and stabilize (trap) the three-dimensional conformation of the PH-PTB region in its IR-bound form, so that it will not be influenced by the effects of Ser\(^{307}\) phosphorylation. A different strategy would be to discover organic ligands that bind to or in the vicinity of Ser\(^{307}\) and prevent its phosphorylation by masking it from cognate kinases, or minimize its deleterious effects. A major challenge of these rational approaches will entail incorporating IRS-1 tissue distribution, cellular topography, and isotype selectivity, as well as changes in macromolecular interaction profile elicited by Ser\(^{307}\) phosphorylation.

Undoubtedly, the molecular events that lead to insulin resistance are only partly understood, and it is well recognized that multiple mechanisms are involved. However, development of drugs need not and cannot await detailed elucidation of signaling pathways. Currently available insulin-sensitizing drugs are the thiazolidinediones, activators of the PPAR\(^{\gamma}\) transcription factors, which increase insulin sensitivity possibly through down-regulation of the adipocyte-derived resistin (3). However, thiazolidinediones may have untoward side effects in tissues other than fat expressing PPAR\(^{\gamma}\) (e.g. colorectal carcinoma and polyps, atherosclerosis), and it remains unclear whether they truly tackle the underlying metabolic defects. The identification of IRS-1 Ser\(^{307}\) as an in vivo phosphorylation site with pivotal importance may provide a promising pharmaceutical target in the regimen of treatment of T2DM and its related syndromes. Similar insights into the IRS-2 molecule, which is crucial also for \(\beta\)-cell compensation in the face of insulin resistance (7), might lead to the development of rationally designed restorers of both the peripheral insulin responsiveness and \(\beta\)-cell inadequacy that characterize this devastating disease.

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