Insulin resistance: a phosphorylation-based uncoupling of insulin signaling

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Insulin resistance refers to a decreased capacity of circulating insulin to regulate nutrient metabolism. It is associated with the development of type 2 diabetes – an ever-increasing epidemic of the 21st century. Recent studies reveal that agents that induce insulin resistance exploit phosphorylation-based negative-feedback control mechanisms, otherwise utilized by insulin itself, to uncouple the insulin receptor from its downstream effectors and thereby terminate insulin signal transduction. This article describes recent findings that present novel viewpoints of the molecular basis of insulin resistance, focusing on the cardinal role of Ser/Thr protein kinases as emerging key players in this arena.

Insulin, produced by the pancreas, is the major anabolic hormone whose action is essential for growth, development and homeostasis of glucose, fat and protein metabolism. At the molecular level, insulin binding to its transmembrane receptor (IR) stimulates the intrinsic Tyr kinase activity of the receptor (IRK), which then phosphorylates target proteins such as Shc and the family of insulin receptor substrate (IRS) proteins (IRS-1 to IRS-4) on selective Tyr residues that serve as docking sites for downstream effectors molecules. This triggers two major kinase cascades, the phosphoinositide 3-kinase (PI3K) and the mitogen-activated protein (MAP) kinase pathways, which mediate the metabolic and growth-promoting functions of insulin.

Insulin resistance is a common pathological state in which target cells fail to respond to ordinary levels of circulating insulin. Individuals with insulin resistance are predisposed to developing type 2 diabetes, and insulin resistance is frequently associated with a number of other health disorders, including obesity, hypertension, chronic infection and cardiovascular diseases.

Recent studies have focused on Ser/Thr phosphorylation of the IRS proteins as a key negative-feedback control mechanism that uncouples the IRS proteins from their upstream and downstream effectors and terminates signal transduction in response to insulin, under physiological conditions. Emerging data further suggest that agents such as tumor necrosis factor α (TNFα), free fatty acids (FFA) and cellular stress, which inhibit insulin signaling and induce insulin resistance, take advantage of this mechanism by activating unidentified Ser/Thr kinases known as IRS kinases that phosphorylate the IRS proteins and inhibit their function. Thus, while the underlying molecular pathophysiology of insulin resistance is still not well understood, Ser phosphorylation of IRS proteins, which is the focus of this review, represents a new and possibly unifying mechanistic theme. Other potential mechanisms for the induction of insulin resistance, such as increased activity of lipid- or protein-Tyr phosphatases (PTPs), or the genetics of insulin resistance, are discussed elsewhere.

Fig. 1. Insulin signal transduction. Circulating insulin interacts with its cognate receptor, which is a transmembrane tyrosine kinase, having an α2β2 configuration. Insulin binding to the α subunits leads to a conformational change and stimulation of the receptor kinase activity through autophosphorylation of Tyr residues in the β subunits. The activated insulin receptor kinase (IRK) then phosphorylates substrate proteins, such as Shc, Gab-1, Cbl/CAP and the family of insulin receptor substrate (IRS) proteins, on selective Tyr residues that serve as docking sites for downstream effector molecules. This triggers two major kinase signaling cascades—the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways. Recruitment of the proteins Grb-2 and Sos to Tyr-phosphorylated Shc activates the MAPK cascade, whereas association of PI3K with the IRS proteins (IRS-1 to IRS-4) results in production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that activates PDK1 (PI3K-dependent kinase 1) and its downstream effector kinases PKB (protein Ser/Thr kinase B, also named Akt), mTOR, p70S6 kinase and the atypical isoforms of PKC (PKCε/PKCη). Collectively, these kinase cascades mediate the metabolic and growth-promoting functions of insulin, such as translocation of vesicles containing GLUT4 glucose transporters from intracellular pools to the plasma membrane (PM), stimulation of glycogen and protein synthesis, and initiation of specific gene transcription. Phosphorylation of Cbl mediates glucose transport in a PI3K-independent manner.
How can the same molecular mechanisms be utilized to terminate insulin signal transduction under physiological or pathological conditions? Which kinases are involved? How does Ser/Thr phosphorylation impact upon IRS protein function and insulin signaling? This article reviews recent experimental data that have begun to shed light upon these questions.

**Feedback regulation of insulin signaling cascades**

Control mechanisms are essential for cellular signaling. Control can be achieved by autoregulation, whereby downstream enzymes inhibit upstream elements (homologous desensitization). Alternatively, signals from apparently unrelated receptor pathways can inhibit the signal (heterologous desensitization). Tyr-phosphorylated IRS proteins, which are key players in propagating insulin signaling, are the targets of such feedback regulatory systems. Regulation involves proteasome-mediated degradation\(^7,8\), phosphatase-mediated dephosphorylation\(^8\) and Ser/Thr phosphorylation. The latter is an attractive regulatory mechanism because it enables multilevel control of the activity of IRS kinases, and of the specific targets among nearly 100 potential Ser/Thr-phosphorylation sites in IRS proteins.

It is becoming apparent that Ser/Thr phosphorylation of IRS proteins has a dual function in serving either as a positive or a negative modulator of insulin signaling. Phosphorylation of Ser residues within the P-Tyr-binding (PTB) domain of IRS-1 (Fig. 2), by insulin-stimulated protein kinase B (PKB, also known as Akt), protects IRS proteins from the rapid action of PTPs and enables the IRS proteins to maintain their Tyr-phosphorylated active conformation, thus implicating PKB as a positive regulator of IRS-1 functions\(^10\). By contrast, Ser/Thr phosphorylation of IRS proteins by other insulin-stimulated Ser/Thr kinases serves as a negative-feedback control mechanism that inhibits further Tyr phosphorylation of IRS proteins. Ser/Thr phosphorylation can inducise the dissociation of IRS proteins from the insulin receptor (IR)\(^11,12\), hinder Tyr-phosphorylation sites\(^13\), release the IRS proteins from intracellular complexes that maintain them in close proximity to the receptor\(^14,15\), induce degradation of IRS proteins\(^7,16\), or turn IRS proteins into inhibitors of the IRK\(^17\) (Fig. 2). These observations raise the question: which insulin-stimulated kinases act as negative modulators of IRS protein function?

A clue was provided when the activity of the insulin-stimulated inhibitory kinases was blocked by inhibitors of the PI3K pathway, implicating downstream effectors of PI3K (Fig. 1) as negative regulators of IRS protein function\(^16,12\). A potential candidate is the mammalian target of rapamycin (mTOR), which enhances phosphorylation of Ser residues at the C-terminus of IRS-1. This phosphorylation inhibits insulin-stimulated Tyr phosphorylation of IRS-1 and its ability to bind to PI3K\(^18-20\). Recent studies provide evidence that PKC\(_\varepsilon\), which is activated by insulin\(^21\), mediates phosphorylation of IRS protein\(^12,22\). This leads to the dissociation of the IR–IRS complexes\(^12\) (Fig. 2), inhibits the ability of IRS proteins to undergo insulin-stimulated Tyr phosphorylation and terminates insulin signaling. IRS-1 serves as a substrate for PKC\(_\varepsilon\) in vitro, and
endogenous IRS-1 forms complexes with PKCζ in an insulin-dependent manner^{22}. These findings suggest that PKCζ can function as an insulin-stimulated IRS kinase, although downstream effectors of PKCζ could also fulfill this role. A potential candidate effector is the IκB kinase β (IKKβ). Although there is no evidence that IKKβ is activated by insulin, IKKβ binds to PKCζ both in vitro and in vivo, serves as an in vitro substrate for PKCζ and is activated by a functional PKCζ^{23}.

PKB, mTOR and PKCζ are downstream effectors of P13K in the insulin signaling pathway. This suggests that their action should be orchestrated to allow phosphorylation by PKB and sustained activation of IRS-1, prior to the activation of mTOR or PKCζ the actions of which are expected to terminate insulin signal transduction. Of note, the negative-feedback mechanism induced by PKCζ (or mTOR) includes a self-attenuation mode, whereby P13K-mediated activation of PKCζ inhibits IRS-1 function, reduces complex formation between IRS-1 and P13K and thereby inhibits further activation of PKCζ itself.

Other aspects of insulin signaling are also subjected to homologous desensitization. Chronic stimulation with insulin results in persistent phosphorylation of the GDP–GTP exchange factor mSOS, which keeps it dissociated from the adaptor Grb2 and allows the GTPase Ras to return to its GDP-bound, inactive, phase^{24}. This process is apparently mediated by a MAPK that phosphorylates mSOS^{25}. Hence, two major insulin signaling pathways, mediated by IRS proteins and Shc, are subjected to homologous desensitization in the form of insulin-induced Ser/Thr phosphorylation. This conclusion suggests that there might be value in pharmacological interventions aimed at disease states in which this mechanism is the underlying cause of insulin resistance.

Ser/Thr phosphorylation of IRS proteins and insulin resistance

A contributing role to the induction of insulin resistance is attributed to agents such as phosphor esters and TNFα^{26}, whose common feature is their ability to enhance the Ser/Thr phosphorylation that inhibits insulin-stimulated Tyr phosphorylation of IRS proteins^{27}. Since Ser/Thr phosphorylation of IRS proteins is stimulated by insulin treatment and by inducers of insulin resistance, the question arises as to whether the same kinases and signaling pathways are being activated under both physiological and pathological conditions. Recent studies have attempted to address this question.

Role of PKCζ and IKKβ

The idea that TNFα and insulin might stimulate the same IRS kinases emerged when it was realized that TNFα activates PKCζ and its downstream target IKKβ^{23,28}. Potential mechanisms could involve TNFα-mediated activation of sphingomyelinase^{29} and production of ceramide, which stimulates PKCζ activity^{30}. Indeed, the effects of TNFα are mimicked by sphingomyelinase and ceramide analogs^{31,32}, suggesting that TNFα triggers a ceramide-activated kinase such as PKCζ. Alternatively, TNFα can induce complex formation between PKCζ, p62 and RIP proteins that serve as adaptors of the TNF receptor and link PKCζ to TNFα signaling^{32}.

More recent studies shifted the spotlight to IKKβ, a downstream target of PKCζ. Activation or overexpression of IKKβ attenuated insulin signaling, whereas IKKβ inhibition by high doses of salicylates^{33} or by a reduction in IKKβ gene dosage reversed obesity- and diet-induced insulin resistance^{34}, implicating IKKβ as a potential mediator of insulin resistance. At the molecular level, inhibition of IKKβ prevented Ser/Thr phosphorylation of IRS proteins induced by high-fat diet, TNFα or phosphatase inhibitors, whereas it improved insulin-stimulated Tyr phosphorylation of IRS proteins, indicating that IKKβ or its downstream effectors serve as IRS kinases (Fig. 3). The effects of salicylates on IRS protein function were in part secondary to the enhanced IRK activity induced by salicylate treatment of insulin-resistant animals^{34}. These results suggest that IKKβ can negatively regulate the activity of both IR and IRS proteins^{34}, making it a target for insulin sensitization.

c-Jun N-terminal kinase (JNK)

The c-Jun N-terminal kinase (JNK) promotes insulin resistance by associating with IRS-1 and phosphorylating Ser307, which inhibits insulin-stimulated Tyr-phosphorylation of IRS-1^{35}. Since Ser307...
is adjacent to the PTB domain of IRS-1, its phosphorylation might disrupt the interaction between the juxtamembrane domain of the IR and the PTB domain of IRS-1 (Fig. 2). Interestingly, insulin and TNFα stimulate phosphorylation of IRS-1 at Ser307 through distinct pathways. While insulin stimulates Ser/Thr kinases downstream of PI3K, TNFα effects are mediated by members of the MAPK pathway. Of note, JNK itself is unlikely to serve as an insulin- or TNFα-stimulated IRS kinase because its activity is insensitive to inhibitors that block phosphorylation of Ser307 in response to these stimuli. Ser307, phosphorylated by currently unknown kinases, might therefore integrate feedback and heterologous signals to attenuate IRS-1-mediated signals and contribute to insulin resistance.

**Conventional PKCs and MAP kinases**

The kinases described above—PKCζ, IKKβ and JNK—are newcomers to the game and join a respected list of Ser/Thr kinases already implicated in phosphorylating IRS proteins when triggered by agents that induce insulin resistance. These include ‘conventional’ members of the PKC family, such as PKCα, activated by phorbol esters or endothelin-1, the activity of which is mediated, at least partially, by members of the MAPK pathway. These kinases phosphorylate IRS-1 at Ser612 (located in a consensus MAPK phosphorylation site) and at additional sites in its C-terminal tail. Such phosphorylation prevents the association of IRS-1 with the juxtamembrane domain of IR, impairs the ability of IRS-1 to undergo insulin-stimulated Tyr phosphorylation and inhibits recruitment of downstream effectors such as PI3K.

**Obesity, insulin resistance and the kinase connection**

Elevated levels of FFA are characteristics of obesity, insulin resistance and type 2 diabetes, and increasing evidence supports the contention that FFA inhibit insulin action at peripheral target tissues. A recent study combined an observation made 120 years ago, indicating that high doses of salicylates lower blood glucose concentrations in diabetic patients, with contemporary knowledge regarding obesity and activation of IKKβ induced by high-fat diets, to show that salicylates prevent fat-induced muscle insulin resistance by inhibiting the activity of IKKβ and its ability to mediate phosphorylation and inactivation of IRS-1 function. Lipid infusion failed to alter insulin signaling in skeletal muscle of IKKβ knockout mice, further implicating a protective role for IKKβ inactivation in fat-induced development of insulin resistance. Although the key data in this study are correlational, they place IKKβ as a potential mediator of Ser phosphorylation of IRS proteins. The mechanism by which lipids might activate IKKβ presumably involves an increase in FFA-derived metabolites, such as diacylglycerol and ceramide, which are potent activators of PKCζ and PKCε, both known to activate IKKβ. Obesity-induced insulin resistance is not limited to the effects of increased levels of FFA or TNFα. Other ‘adipokines’ secreted by fat cells, such as resistin, might also contribute to the development of insulin resistance through Ser/Thr phosphorylation of IRS proteins, but further studies are required to address this possibility.

**Conclusions and future directions**

Our understanding at the molecular level of insulin signal transduction, insulin resistance, and the connection between the two, is evolving extremely rapidly. Current findings implicate IRS proteins as major targets for insulin-induced, phosphorylation-based, negative-feedback control mechanisms that uncouple the insulin receptor from its downstream effectors and terminate insulin signaling under physiological conditions. The kinases involved are still under investigation, with current focus on PKCζ, IKKβ and mTOR as potential candidates. Recent studies further strengthen the concept that the varied agents and conditions that induce insulin resistance, such as TNFα, FFA and obesity, also activate IRS kinases, with IKKβ and its downstream effectors being key candidates. Still other inducers of insulin resistance, such as endothelin-1, presumably utilize additional kinases to phosphorylate IRS proteins (Fig. 3).

These findings raise several pertinent questions: which kinases are indeed the IRS kinases? At present, PKB, PKCζ, IKKβ, MAPK, JNK and mTOR appear as potential candidates, but additional kinases are likely to emerge. Even in the case of IKKβ, its role as an IRS kinase is presently unresolved. The facts that inducers of insulin resistance activate IKKβ, while salicylates, which selectively inhibit IKKβ activity, prevent Ser/Thr phosphorylation of IRS proteins and insulin resistance, implicate IKKβ as a potential IRS kinase. Still, there is no direct evidence to indicate that IKKβ indeed phosphorylates IRS proteins, and it might well be that downstream effectors of IKKβ play this role. The rapid phosphorylation of IRS proteins, which occurs upon activation of IKKβ, argues against the possibility that IKKβ-mediated activation of NFκB leads to de novo synthesis of inducers of insulin resistance. Nevertheless, further studies are required to address this possibility.

As each of the potential IRS kinases has a unique substrate specificity, the question remains as to which Ser sites are being modified by each kinase and what the consequences are of such phosphorylation. Present studies indicate that negative-regulatory sites are found both in close proximity to the PTB domain and at the C-terminal end of IRS-1. How does each phosphorylation site affect the structure and consequently the function of the IRS proteins? Ser phosphorylation might impair IR–IRS interactions, or might induce degradation of IRS proteins, and, in so doing, inhibit altogether subsequent IRS-mediated signaling. Alternatively, phosphorylation of a Ser site might selectively interfere with binding of a specific effector of IRS proteins (Fig. 2), thus impeding only selected aspects of insulin signaling.

Are the same or different mechanisms being utilized by insulin or agents that induce resistance to regulate the activity of IRS kinases? While recent studies indicate that the same kinases (PKCζ, IKKβ) are being activated (e.g. by insulin, FFA and TNFα), there is no evidence that these kinases are being activated along the same pathways. Indeed, kinases localized along different pathways are activated by insulin and TNFα to induce phosphorylation of the inhibitory Ser307 site. Given the large number of stimuli, pathways, kinases and potential sites involved, it appears that Ser/Thr phosphorylation of IRS proteins represents a combinatorial consequence of several kinases, activated by different pathways, acting in concert to phosphorylate multiple sites.
While many questions await answers, the new paradigms and emerging target kinases described above give a novel viewpoint of the molecular basis for insulin resistance. This should enable rational drug design to selectively inhibit the activity of the relevant enzymes and generate a novel class of therapeutic agents for type 2 diabetes.

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