Diabetologia (2004) 47:170–184
DOI 10.1007/s00125-003-1313-3

Diabetologia

Modulation of insulin action

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Abstract

Insulin is a key hormone regulating the control of metabolism and the maintenance of normoglycaemia and normolipidaemia. Insulin acts by binding to its cell surface receptor, thus activating the receptor's intrinsic tyrosine kinase activity, resulting in receptor autophosphorylation and phosphorylation of several substrates. Tyrosine phosphorylated residues on the receptor itself and on subsequently bound receptor substrates provide docking sites for downstream signalling molecules, including adapters, protein serine/threonine kinases, phosphoinositide kinases and exchange factors. Collectively, those molecules orchestrate the numerous insulin-mediated physiological responses.

A clear picture is emerging of the way in which insulin elicits several intracellular signalling pathways to mediate its physiologic functions. A further challenge, being pursued by several laboratories, is to understand the molecular mechanisms that underlie insulin action at the peripheral level, deregulation of which ultimately leads to hyperglycaemia and Type 2 diabetes.

We review how circulating factors such as insulin itself, TNF-α, interleukins, fatty acids and glycation products influence insulin action through insulin signalling molecules themselves or through other pathways ultimately impinging on the insulin-signalling pathway. Understanding how the mechanism by which molecular insulin action is modulated by these factors will potentially provide new targets for pharmacological agents, to enable the control of altered glucose and lipid metabolism and diabetes. [Diabetologia (2004) 47:170–184]

Keywords Insulin signalling · Insulin resistance · IRS proteins · SOCS proteins · Hyperglycaemia · Hyperinsulinaemia · Serine/threonine phosphorylation

Received: 6 August 2003 / Revised: 4 December 2003
Published online: 13 January 2004
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Abbreviations: AMPK, Adenosine 5'-monophosphate-activated protein kinase · Erk1/2, extracellular regulated kinase 1 and 2 · GSK-3, glycogen synthase kinase 3 · IKK, inhibitor kappa B kinase · IR, insulin receptor · JAKs, janus kinases · JNK, jun amino terminal kinase · MAPK, mitogen activated protein kinase · PI3K, phosphoinositide 3-kinase · PKB, protein kinase B · PKC, protein kinase C · S/T, serine/threonine · SOCS, suppressor of cytokine signalling · TOR, target of rapamycin

Introduction

Studies from numerous laboratories in the past 20 years have elucidated the principal features of insulin action at the molecular level [1]. Binding of insulin to its heterotetrameric receptor activates its intracellular tyrosine kinase activity. The cytoplasmic kinase domains of the activated receptor transphosphorylates tyrosines 1158/62/63 in the catalytic loop of the kinase domain and subsequently C-terminal tyrosines 1328/34 and the juxtamembrane region tyrosine 972. Tyrosine 972 is the major docking site for downstream interacting proteins [2]. Insulin receptor interacting proteins (including IRS-1/4, GAB and the recently identified IRS5/DOK4 and IRS6/DOK5 [3]) are in turn phosphorylated on tyrosines providing docking

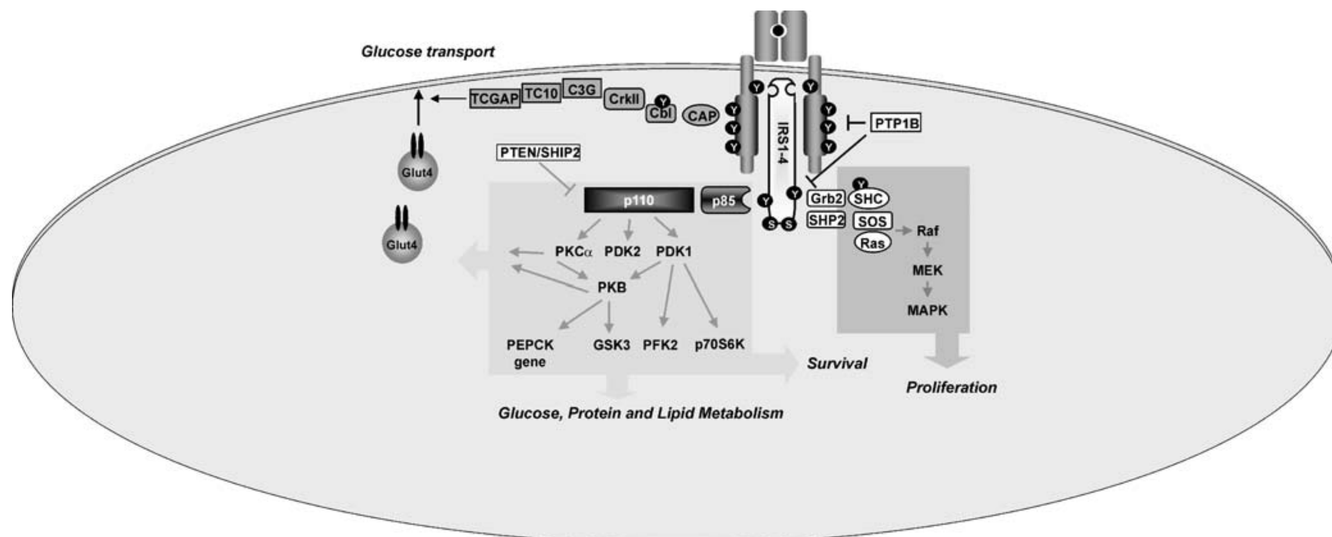

Fig. 1. Principal components of the insulin signalling pathway. Activation of the insulin receptor by its ligand induces autophosphorylation of the receptor, creating docking sites for downstream interacting proteins, such as IRS1 and IRS2. These are themselves phosphorylated and provide further docking sites for SH2 domain-containing proteins. These events result in the activation of three major pathways, the PI3K, CAP/Cbl/Tc10 and the ERK pathway, allowing the transmission of the signals that result ultimately in the diverse biological effects of insulin

sites for SH2 domain containing proteins [4]. The three major pathways emanating from the activated IRS are the PI3K, the CAP/Cbl/Tc10 and the MAPK pathway [5], with the two former pathways mainly involved in the positive control of insulin action (Fig. 1). Insulin has also been shown to promote the activation of NFκB by regulating its inhibitor IκB, via a PI3K-dependent pathway [6, 7].

Type I PI3Ks are heterodimeric cytosolic proteins composed of a 50–85 M_r adapter subunit and a 110 M_r catalytic subunit. PI3Ks are translocated at the plasma membrane by the interaction of the adapter's SH2 domains with tyrosine phosphorylated YxxM motifs on IRS proteins. This interaction positions PI3K in close contact with its substrate—PtdIns(4,5)P_2—which is then phosphorylated on the 3′ position of the inositol ring yielding the second messenger PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 recruits the PH domain-containing protein serine kinases PDK-1, PKB and atypical PKCs ζ/λ at the plasma membrane. Membrane-located PDK1 is simultaneously activated by PtdIns(3,4,5)P_3 and, by phosphorylating its effectors PKB and PKCs, it relays their full activation [8, 9]. The activation of this pathway mediates several insulin-induced responses, including GLUT4 translocation at the plasma membrane [10], glycogen synthesis—via PKB mediated inhibitory phosphorylation of GSK-3, a kinase that negatively regulates glycogen synthase—[11], lipogenesis via up-regulation of the expression of the fatty acid synthase gene [12], as well as a more general control of gene expression patterns [13] (Fig. 1).

The necessity of the PI3K pathway for insulin-induced GLUT4 translocation and glucose uptake has been shown by the use of pharmacological agents and dominant negative/constitutively active PI3K mutants. However, recent evidence indicates that a parallel pathway acts in concert with the PI3K pathway to fully induce glucose uptake. This pathway is initiated by recruiting the protooncogene Cbl to the activated insulin receptor via the adapter protein CAP. After tyrosine phosphorylation of Cbl, the CAP-Cbl complex localises to lipid rafts by interacting with the caveolar protein flotillin, thereby forming a ternary complex [14]. Here, Cbl acts by recruiting the guanosine exchange factor C3G which specifically activates the small GTP binding protein Tc10 [15] and ultimately the Tc10 downstream effector TCGAP [16] (Fig. 1).

The MAPK pathway is also activated by insulin, via both SHC association with the insulin receptor and Grb2 association with both the insulin receptor and IRS molecules. The classic Erk1/2 MAPKs do not play a major role in mediating insulin's metabolic responses, but increased basal MAPK activity seems to contribute to the development of insulin resistance. On the contrary, the p38 MAPK has been proposed as a positive regulator of insulin action because of its capability to increase, in an as yet unknown manner, the uptake of glucose by the plasma membrane-localised GLUT4 transporter [17].

Type 2 diabetes results from a complex interplay between genetic and environmental conditions. At the onset of the disease, the major classic peripheral insulin-responsive tissues, muscle and adipose tissue, display a diminished response to insulin resulting in decreased disposal of excess circulating glucose and fatty acids. Concomitantly, due to reduced insulin action on the liver, hepatic gluconeogenesis increases, further exacerbating hyperglycaemia. In the initial stages of the disease, the pancreatic beta cell compensates by

secreting an increased amount of insulin. However, during this compensatory hyperinsulinaemic phase existing before overt diabetes develops, progressive beta-cell dysfunction is also observed [18] emphasising the fact that Type 2 diabetes is dependent on insults occurring both at peripheral as well as the beta-cell level [19]. Thus, when insulin secretory reserves cannot compensate for the defects in peripheral insulin action, overt diabetes results. Evidence obtained from knockout studies in mice indicates that insulin resistance at the level of the beta cell could also be an initial causative factor in diabetes development [19]. Also, it has been shown in human islets that chronic hyperglycaemia, by affecting both insulin receptor gene expression and signalling, impairs insulin secretion [20]. The dysfunctions occurring at the beta-cell level during the progression towards the disease have been reviewed [21, 22]. In addition to these events, the intracellular insulin-signalling machinery in peripheral tissues is deregulated, as a result of a combination of several factors. We review the recent progress towards understanding the modulation of the insulin signalling cascade by circulating factors as well as the understanding of how deregulation of these interactions might contribute to the development of Type 2 diabetes.

Insulin receptor down-regulation

Insulin resistance is characterised by a diminished responsiveness to the action of insulin at its multiple target organs. The insulin receptor itself is therefore a primary candidate molecule, the down-regulation of which might contribute to the decreased hormone action. Cases of insulin resistance or diabetes have been linked to mutations of the insulin receptor gene [23]. Besides these rare genetically determined cases, early studies showed that the IR tyrosine kinase activity was reduced in insulin-resistant obese mice [24] and in Type 2 diabetic patients [25] independent of insulin receptor genetic variants. This functional down-regulation seemed to depend, in part, on diminished receptor content at the cell surface consequential to increased degradation of the insulin receptor [26, 27]. However, the decreased IR content at the cell surface is not sufficient to account for the loss in IR activity. Thus, a S/T phosphorylation on the cytoplasmic side of the receptor by several PKC isoforms has been proposed as an additional factor contributing to the down-regulation of the IR kinase activity. A thorough mutagenesis study of the intracellular part of the IR β-subunit showed that the serine residues 1189/90/94 located C-terminally to the kinase domain, although not affecting IR autophosphorylation, are required to achieve phosphorylation of the substrates IRS-1, IRS-2 and SHC [28]. A second possible mechanism leading to decreased insulin action in obese subjects and patients with chronic primary hyperinsulinaemia might involve the increased abundance of insulin/insulin-like growth factor-I hybrids in insulin target tissues, a consequence of hyperinsulinaemia-induced IR down-regulation and/or IGF-1R up-regulation [29, 30].

Restoring IR responsiveness to its natural ligand or mimicking insulin by using pharmacological agents has been a route hotly pursued in pharmaceutical research and has resulted in the discovery of a small insulin-mimicking molecule endowed with antidiabetic activity which, in mice, activates the IR kinase activity [31].

Modulation of the activity and expression of IRS molecules

IRS molecules become readily tyrosine phosphorylated by the activated insulin receptor. To investigate whether a defective activity of the insulin receptor could influence the responsiveness of downstream IRS molecules, the tyrosine phosphorylation state and protein amount of IRS-1 in hyperinsulinaemic *ob/ob* mice were evaluated, showing that both were reduced in liver and skeletal muscle [32]. Whereas decreased tyrosine phosphorylation might be explained by the defective receptor's activity, novel mechanism(s) had to be invoked to explain the decrease in IRS-1 protein content.

Treatment of isolated soleus muscle and 3T3-L1 adipocytes with okadaic acid, a general inhibitor of serine/threonine phosphatases, diminished IRS protein signalling capability, indicating that S/T phosphorylation of IRS might play an inhibitory role [33]. This hypothesis was biochemically confirmed in a IR-expressing CHO cell system, in which over-expression of the phospholipid- and calcium-activated protein kinase C had an inhibitory effect on insulin action [34]. Furthermore, an increased serine kinase activity directed towards IRS-1 has been reported in liver and muscles of obese JCR:LA-cp rats [35].

In addition to the IRS-1 down-regulation by serine/threonine phosphorylation mechanisms, a decreased content of IRS proteins has been found in insulin-resistant states in animals, humans and cultured cells as a consequence of hyperinsulinaemia or TNF-α treatment. This decrease is not dependent on defective transcription of the IRS-1 mRNA nor on impaired protein synthesis, rather it is reversed by lactacystin treatment, indicating that hyperinsulinaemia-induced IRS-1 degradation occurs through the proteasome pathway [36]. Thus, the two seemingly separate events of S/T phosphorylation and degradation are consequential, as PI3K-mTOR mediated S/T phosphorylation on IRS-1 appears to be necessary to drive IRS-1 to proteasomal degradation [37].

Another less intensively investigated mechanism, which could modulate the signalling potential of

S/T phosphorylated IRS is interaction with 14-3-3 proteins. The 14-3-3 proteins are widely expressed 28–33-M_r acidic proteins which spontaneously assemble into dimers. Several 14-3-3 isoforms have been identified to function in several biological processes by binding in a sequence-specific manner to phosphoserine-containing motifs of various signalling proteins, including Raf, Bcr and Foxo transcription factors [38]. Using the two hybrid system and in vitro assays for protein interaction, a study showed the interaction between the ε isoform of 14-3-3 and IRS-1 [39]. Similarly, by screening a heart cDNA library with ^{32}P-labelled IRS-1 and a co-immunoprecitation approach, the β, ε and ζ 14-3-3 isoforms were shown to interact with IRS proteins and work as negative regulators of insulin-induced PI3K activity in 3T3-L1 adipocytes [40, 41]. Then how do the 14-3-3 proteins contribute to the desensitisation of insulin action? Rather than a step leading to proteasomal degradation, a study suggests that 14-3-3, by binding to IRS-1, displaces the IRS-1-PI3K complex from the plasma membrane to the cytosol, thus terminating the PtdIns(3,4,5)P_3-producing activity of PI3K and eventually leading to decreased downstream signalling [42].

Protein kinases acting upon IRS-1

PI3K-dependent protein kinases. Upon insulin stimulation, class IA PI3K readily associates with IRS molecules at the plasma membrane and catalyses the formation of PtdIns(3,4,5)P_3. Class IA PI3Ks are dual-specificity kinases endowed with a protein kinase activity, initially shown towards the serine 608 residue of the p85α regulatory subunit [43]. In the search for other putative substrates, a study showed that PI3K also phosphorylates IRS-1 in vitro, a wortmannin-reversible process [44]. Nevertheless, it is not clear whether PI3K phosphorylates IRS proteins in vivo and, if so, the possible physiological relevance of this event is still unkown. Towards this end, a study induced cellular insulin resistant state in adipocytes by expressing a constitutively active version of the p110α PI3K catalytic subunit [45]. However, PI3K inhibition-directed blockade of IRS-1 S/T phosphorylation and amelioration of insulin action also occurs via a number of PI3K-downstream kinases implicated in the phosphorylation and negative regulation of IRS-1, including GSK3, mTOR, and the atypical PKCζ [46, 47, 48, 49]. It has yet to be precisely defined which S/T residues are targeted by each kinase and the quantitative contribution of each kinase towards the determination of the overall IRS-1 S/T phosphorylation state. A better understanding of the action of each kinase might permit the pharmacological alteration of the S/T phosphorylation status of IRS-1, thus reversing the associated insulin-resistant state [50].

MAP kinases. Neither of the three major MAPK signalling cascades—namely Erk1/2, p38 and jun amino terminal kinase (JNK)—although activated by insulin, promote insulin-mediated metabolic responses. Rather, they seem to exert feedback regulatory functions. Early observations indicated that, after activation by TNF-α, a cytokine known to induce insulin resistance, JNK1 induces phosphorylation of the IRS-1 residue S307 (corresponding to human S312) [51]. S307 is located in the IRS-1 PTB domain, and its phosphorylation is thought to hinder the PTB binding to the activated receptor [52].

In addition to these biochemical studies linking JNK-mediated S307 phosphorylation on IRS-1 to insulin resistance, more physiological evidence has been provided by a JNK-1 KO mouse model that showed ameliorated insulin sensitivity associated with decreased phosphorylation at the JNK-target residue [53]. However, other pathways converge on S307 phosphorylation. Notably, insulin/IGF-1 also promotes phosphorylation at the inhibitory S307 via a PI3K-dependent pathway leading to feedback inhibition of insulin action [54, 55]. A report using PDK1-deficient cells and a pharmacological approach defined a PI3K-PDK-mTOR pathway leading to human S312 phosphorylation and development of cellular insulin resistance, which was reversed in cells expressing a S312A IRS1 mutant [56].

As discussed above, several lines of evidence place S307 as a central phosphorylation target that allows the negative modulation of IRS-1. However, IRS-1 possesses a number of other potential S/T phosphorylation sites, thus action on these sites—especially on S/T placed close to SH2-domain interacting sequences—might just as well interfere with the action of IRS. Accordingly, a report shows that in primary cultures derived from skeletal muscle of Type 2 diabetic patients, the basal phosphorylation of S636 (S632 in mouse)—a residue in a MAPK consensus sequence—is twofold higher than controls and is decreased by pharmacological inhibition of MAPK [57]. The contribution of Erk1/2 MAPKs to the modulation of the IRS-1 function has also been observed in human embryonic kidney cells treated with an activator of protein kinase C, phorbol 12-myristate 13-acetate [58]. Therefore, increased activity of the Erk1/2 MAPKs could contribute to insulin resistance. Confirmative data have been obtained from TNF-α-treated adipocytes, in which the TNF-α induced insulin resistance was reversed by inhibiting Erk1/2 [59] and from studies on muscle biopsies from obese and diabetic patients in which down-regulation of the PI3K, but not MAPK, pathway was observed [60].

The contribution of p38 to the induction of insulin-resistance has been studied by adenoviral-mediated overexpression of the upstream kinase MKK6 in 3T3-L1 adipocytes. Although increased S/T phosphoryla-

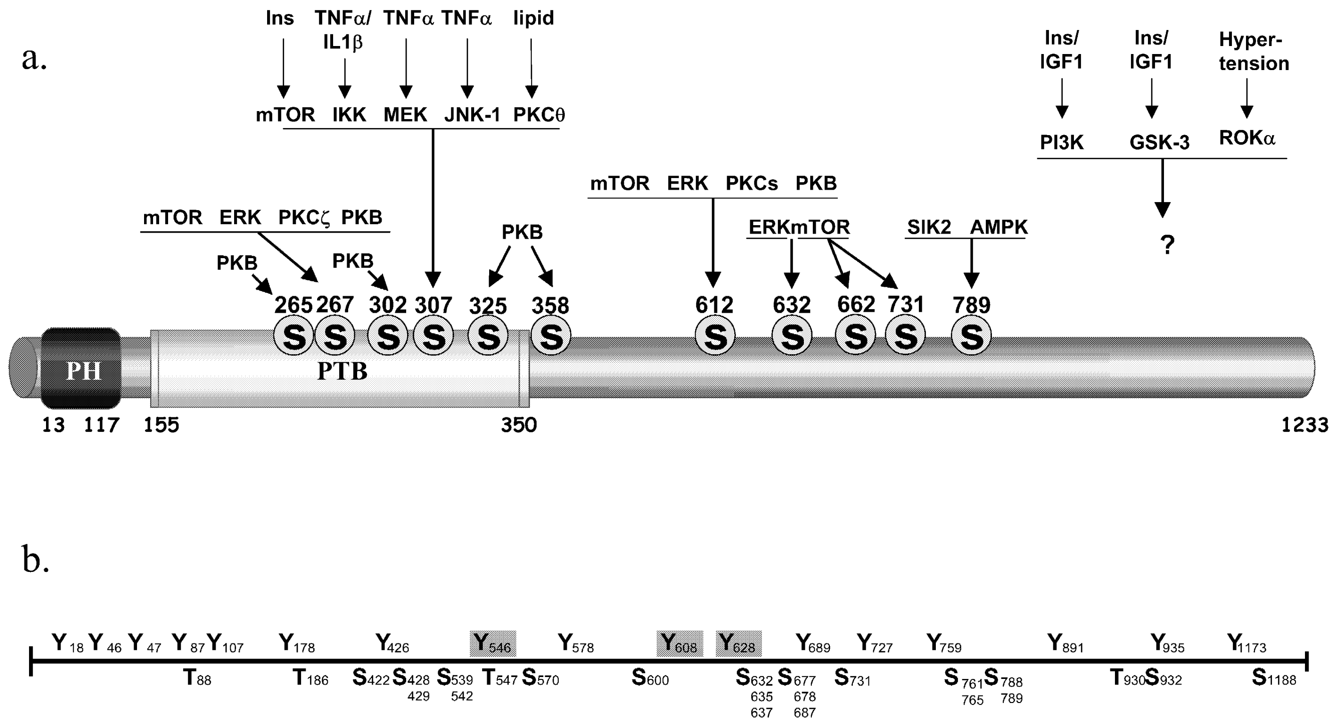

Fig. 2. (**a**) Schematic of serine phosphorylated residues of mouse IRS1. Shown are the serine residues implicated in the regulation of insulin signalling and the factors that have been shown to induce their phosphorylation. Numbering denotes residue position in mouse IRS1, although some of these sites were first shown in the rat or human homologues. For a description of the various phosphorylation events and their physiological context refer to the text. (**b**) Schematic of tyrosine residues conserved between IRS1 and IRS2 and the conserved serine and threonine residues found in their vicinity. Numbering denotes residue position according to the sequence of mouse IRS1. Highlighted are tyrosines contained within SH2 consensus motifs conserved between IRS1 and IRS2

tion was not formally demonstrated, activation of p38 moderately inhibited the expression of IRS-1/2 as well as associated PI3K activity, without significantly affecting IR expression and activity [61]. Moreover, in adipocytes of Type 2 diabetic patients, inhibition of p38 reversed the insulin-induced decrease of GLUT4 protein expression but had no effect on the insulin-stimulated decrease of IRS-1 [62]. Thus, p38, in contrast to Erk1/2 and JNK, does not appear to negatively regulate insulin action to a major extent. On the contrary, p38 activation—although not impinging in the insulin signalling cascade—directly increases the glucose transport capability of the plasma membrane-located GLUT4 transporter [63].

AMPK and other protein kinases. AMPK is a central metabolic switch that, by phosphorylating key target proteins controlling metabolic pathways, increases skeletal muscle fatty-acid oxidation and represses triglyceride synthesis and lipogenesis. In addition, AMPK mediates the stimulation of glucose uptake induced by muscle contraction by a PI3K-independent mechanism. An increased recruitment of the AMPK signalling system could thus correct insulin resistance and Type 2 diabetes resulting from defects in the insulin signalling cascade [64]. Although chiefly acting on a parallel pathway, another report also provides evidence for an interaction between AMPK and the insulin signalling cascade at the level of IRS-1. Murine IRS-1 was phosphorylated by AMPK on S789 both in vitro and in C_2C_{12} myotubes incubated with the AMPK activator AICAR. This phosphorylation correlated with increased IRS-1-associated PI3K activity after insulin stimulation [65]. Whether AMPK also phosphorylates IRS-2, which also has a serine residue at position 819 that aligns with IRS-1 S789 (Fig. 2b), remains to be determined. More recently, the novel adipocyte-specific AMPK-related protein kinase SIK2 (salt-inducible kinase) has been shown to phosphorylates mouse S789 of IRS-1 (Human S794), thus impairing its action. Of note, both the expression and the catalytic activity of SIK2 is increased in white adipose tissue of *db/db* mice, providing in vivo evidence that SIK2 might negatively modulate insulin action [66].

Further investigation of the roles of AMPK and SIK2 is needed to define whether phosphorylation on the same IRS-1 serine residue might have positive or negative consequences depending on the kinase performing the reaction, or whether the differences seen between AMPK and SIK2 are due to the different cell types studied.

Insulin resistance has circumstantially been associated with activation of the inhibitor kappa B kinase (IKK) based on the following experimental evidence: (i) treatment of obese rodent models with salicylate (an IKK inhibitor when administered at high doses)

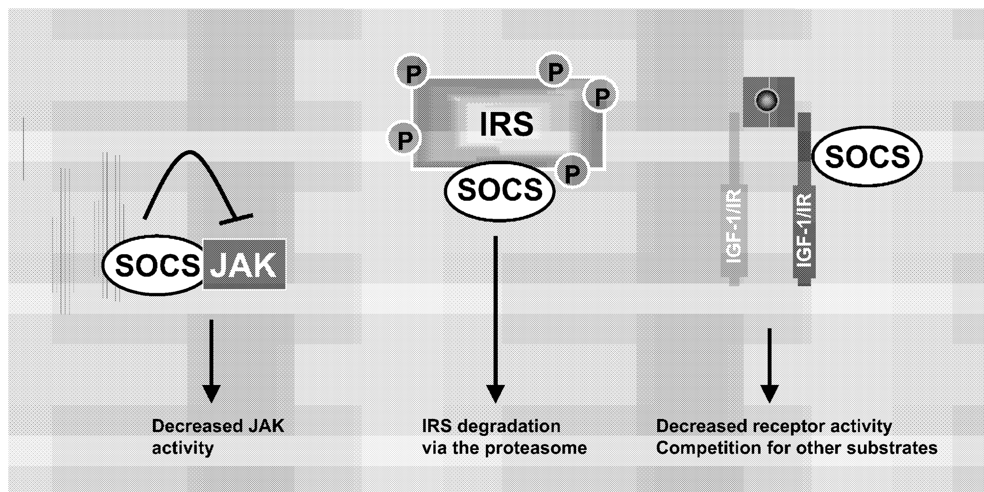

Fig. 3. Modulation by SOCS of the signalling potential of IRS molecules. SOCS modulates IRS signalling potential via at least three mechanisms: via competition for receptor binding sites, inhibition of JAK activity and induction of proteosomal degradation of IRS

sensitised insulin signalling and (ii) genetically obese mice and high-fat fed mice bearing an heterozygous deletion in the gene for the *IKK*-β subunit were protected against the development of insulin resistance [67]. To further evaluate the involvement of IKK in conferring insulin resistance, insulin signalling was measured in rats subjected to hyperinsulinaemic-euglycaemic clamps after lipid infusion following a pre-treatment with high doses of salicylate. Lipid infusion decreased insulin action, and salicylate pre-treatment prevented these lipid-induced effects. Moreover, the decreased insulin action in the same experimental set-up was not observed in mice lacking IKK-β [68]. Mechanistically, IKK targets S307 as assessed by decreased S307 phosphorylation in cells derived from IKK knockout mice or in aspirin-treated cells [69]. However, aspirin seems to exert an additional protective effect by inhibiting the action of other TNF-α-activated kinases, including PKB and mTOR [70]. The above experimental evidence suggests that inhibition of the IKK complex by pharmacological means might be efficacious to reverse the conditions of insulin resistance [71]. However, although salicylate treatment and investigation of IKK-β heterozygous mice clearly point to a role of IKK-β in insulin resistance, direct experimental evidence showing increased IKK activity in insulin resistance and obesity is still missing.

In a two-hybrid study using the PH-PTB domains of IRS-1, Rho kinase α (ROKα) was identified as a potential IRS-1 binding protein [72]. Further studies showed that hypertension (in spontaneously hypertensive rats), or overexpression of active RhoA(V14), up-regulates ROKα activity, leading to increased ROKα-IRS-1 association, increased IRS-1 serine phosphorylation and subsequent inhibition of insulin signalling. These results demonstrate the existence of negative crosstalk between Rho/ROKα and insulin signalling [73].

Involvement of SOCS proteins in IRS-1/2 modulation

SOCS (suppressor of cytokine signalling) proteins define a family of eight proteins including CIS and SOCS-1 to 7 [74]. CIS, the first member of the SOCS family to be identified, was discovered as an immediate-early gene induced by cytokine treatment which bound activated IL-3 and erythropoietin receptors [75]. More compelling evidence defining SOCS proteins as negative regulators of cytokine signalling came subsequently with the identification of three genes, SOCS-1 to 3, capable of inhibiting the IL-6-induced differentiation of M1 cells [76].

Whereas SOCS proteins possess unrelated amino terminal domains of variable length, they share, as similar functional components, a central SH2 domain and a carboxy-terminal “SOCS box”. Mechanistically, SOCS proteins act at three different levels to perform their inhibitory function: (i) via their SH2 domain they bind and inactivate tyrosine-phosphorylated Janus kinases (JAKs); (ii) SOCS SH2 domains compete with other signal transduction proteins, such as STATs and SHP2, for the access to tyrosine phosphorylated receptor binding sites and (iii) more recent observations indicate that the SOCS box functions as an adaptor that facilitates the ubiquitination and subsequent degradation of various signalling proteins [77] (Fig. 3).

Although SOCS proteins were initially shown to function as negative modulators of cytokine action, recent studies suggest that they are also involved in the negative regulation of insulin signalling. Firstly, SOCS-3 is induced by insulin treatment in insulin-responsive 3T3-L1 adipocytes [78] as well as in insulin-responsive tissues [79]. Induction of SOCS-3 by insulin is directed by STAT5B. A STAT5B binding site is present in the SOCS-3 promoter and cells lacking STAT5B are refractory to SOCS-3 induction by

insulin [78, 80]. So how do SOCS proteins act to modulate insulin signalling? Besides being induced by insulin, SOCS-3, as well as SOCS-1, -2 and 6, interact both with the phosphorylated IR and the IGF-1 receptor thus reducing their signalling capabilities [78, 81, 82]. In addition, SOCS-3 acts as a negative regulator by binding to phosphorylated tyrosine 972 of the IR [78], the major IRS-1 binding residue [83]. However, some controversies have emerged over the fact that the IR might be the sole target for the action of SOCS proteins [84], and evidence has been provided, upon ectopic expression of SOCS proteins, for an action also directed towards IRS-1 with all the three mechanisms mentioned above possibly being involved to different extents [78, 85, 86]. Concerning the suppression of insulin signalling via binding to JAKs, although SOCS binds to JAK with high affinity and overexpression of either SOCS-1 or -3 inhibits insulin-induced JAKs activation [85], it is likely to be a secondary mechanism, due to the small contribution of JAK towards the phosphorylation of IRS molecules. Of probably more physiological significance are the mechanisms acting on the competition for crucial phosphorylated tyrosines on the receptor [78] and on the proteasomal targeting of SOCS-associated proteins after formation of a complex with Elongins B and C mediated by the carboxy-terminal portion of the SOCS protein. The occurrence of this last mechanism is neatly demonstrated in a study showing that degradation of IRS-1 and 2 is consequential to the binding to SOCS-1 or 3 and is mediated by the latter's "SOCS box" [86].

The above mentioned studies, although providing a framework for understanding the mode of action of SOCS proteins, often involved in vitro experiments and ectopic expression of SOCS protein at supraphysiological amounts. Nevertheless, physiological approaches have subsequently validated those studies. For example, the implication of SOCS-3 in the development of TNF-α-mediated insulin resistance has been directly confirmed by the observations that (i) SOCS-3 is induced in adipose tissue of TNF-α injected mice, (ii) SOCS-3 is overexpressed in obese mice and (iii) obese mice lacking the TNF-α receptor have a reduced SOCS-3 content [79]. In keeping with the negative control mediated by SOCS proteins is the observation that ablation of the SOCS-1 gene yields mice which are hypersensitive to the action of insulin [85].

Whereas most studies linking the expression of SOCS proteins to down-regulation of insulin action mainly focussed on SOCS-1 and -3, recent observations showed that SOCS-6 also plays a similar role, via its binding to IRS-2 and IRS-4 [87] as well as association with the IR [84]. However, since mice deficient in SOCS-6 do not have defects in glucose homeostasis, it is possible that this isoform plays a minor role in conferring decreased insulin responsiveness.

A deeper understanding is clearly needed to understand how each SOCS protein contributes to the deregulation of the insulin signal, given that these molecules provide a molecular link between cytokine action and their negative consequences on insulin signalling as well as play a direct role in desensitisation of insulin action [88].

Modulation of IRS-downstream proteins

In parallel to the extensive work which has led to an understanding of how insulin action is regulated by modulation of IRS proteins and their interplay with SOCS proteins, evidence also exists linking the impairment of insulin signalling to events downstream of IRS proteins.

Transcription of the adapter subunit p85α of PI3K is positively regulated by insulin. In muscle biopsies from Type 2 diabetic patients it has been observed that, although basal levels of p85α mRNA after an overnight fast are not significantly different from control subjects, insulin-stimulated expression of p85α is decreased, indicating a role for PI3K expression in the pathogenesis of Type 2 diabetes [89]. A defect of PI3K activity as the first impaired step of the insulin signalling pathway has also been shown upon high-fat feeding in rats, which associated with diminished activities of both PKB and atypical PKCs [90]. However, these observations differ from data generated from a mouse model bearing a heterozygous p85α gene deletion, in which the decreased amount of p85α protein (and thus an higher proportion of p85α/p110 heterodimeric active PI3K versus the free p85 adapter) leads to a higher sensitivity to insulin [91], which has also been hypothesised to occur owing to an isoform switch from p85α to p50α in the heterozygous animals [92]. These studies highlight the important role of PI3K and its isoforms in glucose homeostasis. Further work will be necessary to clarify whether decreased p85α gene dosage leads to improvement or desensitisation towards insulin action.

Finally, decreased insulin action has also been associated with alterations of glucose transporters. Decreased GLUT4 expression was observed in insulin-resistant and diabetic subjects [93], which is in part compensated by an higher basal GLUT1 content at the plasma membrane [94].

Controversies

It is now well established that S/T phosphorylation mediated by several protein kinases and subsequent proteasomal degradation can modulate the signalling capability of IRS molecules. However, some controversies have yet to be resolved. The first question is whether proteasomal degradation is specific for the

IRS-1 isoforms or whether this also occurs with the other IRS isoforms. In CHO cells expressing the IR and IRS-1, -2 or chimeras thereof, insulin-induced proteasomal degradation was specific to the IRS-1 isoform, with the N-terminal region including the PH and PTB domain essential for targeting to the ubiquitin-proteasome degradation pathway [95]. However, contrasting reports show that, in 3T3-L1 fibroblasts and adipocytes, FAO cells and mouse embryo fibroblasts, prolonged insulin treatment induces proteasomal degradation of IRS-2 as well [96]. Similarly, evidence for insulin-induced IRS-1 and 2 degradation has been provided in primary rat adipocytes [97] and L6 muscle cells in which IRS protein degradation occurs via different pathways—a PI3K but mTOR-independent pathway for IRS-1, and a PI3K-mTOR pathway for IRS-2 [98].

Considering the mode of regulation of IRS proteins, it is also controversial whether S/T phosphrylation acts only as a negative regulatory mechanism. In fact, PKB-mediated phosphorylation of mouse IRS-1 on serine residues 265, 302, 325 and 358 enhances its action in 293 cells [99] and in vitro a S/T phosphorylation threshold on IRS-1 and 2 must be achieved for full tyrosine phosphorylation by the activated IR to occur [56]. Genetic evidence for a positive role of S/T phosphorylation is provided by the human IRS-1 T608R polymorphism recently identified in a diabetic patient, which impaired metabolic insulin signalling, possibly due to the removal of a potential positive threonine phosphorylation site [100].

While a lot of effort has been directed towards the understanding of the modulatory mechanisms acting upon IRS-1, less is known about the regulation of IRS-2. Given that (i) ablation of IRS-2 leads to overt diabetes [101] and (ii) a number of S/T phosphorylation sites are conserved between IRS-1 and IRS-2, (Fig. 2b) future efforts directed at understanding the molecular events modulating IRS-2 action are foreseeable.

Insulin resistance inducing factors: molecular mechanisms

Hyperinsulinaemia and hyperglycaemia

Along with hyperlipidaemia, the most obvious clinical parameters modified in the insulin-resistant state are increased glycaemia and the subsequent compensatory hyperinsulinaemia. Both hyperinsulinaemia and hyperglycaemia per se are factors that exacerbate the insulin-resistant state. Whether hyperinsulinaemia-induced peripheral insulin resistance acts by impairing the tyrosine kinase activity of the IR is still a matter of controversy, with studies showing a decreased receptor activity [25, 102, 103], and others showing a normal IR function, in spite of clear insulin resistance downstream of the receptor and at a metabolic level [104, 105, 106]. In keeping with this second hypothesis is the observation that IRS-1 protein content is reduced in 3T3-L1 adipocytes chronically exposed to insulin [107] as well as in adipocytes from insulin-resistant subjects [108] and patients with overt Type 2 diabetes [109]. Likewise, insulin- and IGF-1-induced degradation of IRS-2 has also been proposed to contribute to the insulin-resistant state [96]. Downstream of IRS-1 and -2, other insulin signalling molecules are deregulated by hyperinsulinaemia. PKB activation was reduced in adipose tissue and skeletal muscle from diabetic *db/db* mice [110] and GLUT4 expression is lower in insulin-resistant individuals [93]. Thus, hyperinsulinaemia-induced deterioration of insulin action occurs at several sites along the insulin-signalling pathway.

Hyperglycaemia also has adverse consequences on the efficacy of insulin action. In the liver of the Zucker diabetic rat—during the hypoinsulinaemic and hyperglycaemic phase—tyrosine phosphorylation of IR and IRS-1/2 were significantly enhanced, together with the associated PI3K activity, while activation of PKB was severely suppressed [111]. Similar findings were obtained in a model of neonatally streptozotocin-treated Type 1 diabetic rats, in which the resulting hyperglycaemia severely impaired PKB activation and GLUT4 translocation in skeletal muscle, while IRS-1 and -2 associated PI3K activity was enhanced [112]. These observations suggest that hyperglycaemia, either resulting from Type 1 or Type 2 diabetes, does not affect the proximal part of the insulin signalling pathway (IR, IRS and PI3K) but reduces the efficiency of the activation step from PI3K and PKB.

Fatty acids

High concentrations of circulating fatty acids contribute to the induction of insulin resistance by decreasing insulin-induced PI3K activation. Infusion of a lipid emulsion in rats resulted in a time-dependent intracellular accumulation of acyl-CoA and diacylglycerol which activated PKCθ, leading to increased PKCθ-mediated IRS-1 phosphorylation on rat S307 [113]. A thorough study addressing the ability of insulin to stimulate PI3K and downstream targets in lipid-infused rats showed fatty acid-mediated impairment of IRS-1/IRS-2 and phosphotyrosine-associated PI3K activity, as well as of the activation state of the kinases PKBα, PKCλ/ζ and GSK-3α and β, while PKBβ and $p70^{S6k}$ were not impaired [114]. The definition of the deregulated protein kinase cascades in animal or cell-culture models might translate into a pharmacological application, as a similar mechanism, notably the fatty acid-induced decrease in IRS-1-associated PI3K activity, has been observed in biopsies from healthy human subjects infused with fatty acids [115].

As discussed above, increased fatty acid concentrations inhibit insulin-mediated glucose metabolism by affecting a number of protein kinases. A further molecule with deregulated expression has been found in transgenic mice overexpressing the lipoprotein lipase (LPL) in skeletal muscle. Munc18c is a negative regulator of insulin-induced GLUT4 translocation and was increased in LPL transgenics, implicating it as contributing to fatty acid-induced insulin resistance [116].

Studies in the INS-1 pancreatic beta-cell line indicate that increased concentrations of fatty acids also inhibit the compensatory mass expansion of beta cells that occurs during insulin resistance. This inhibition of beta-cell mitogenesis originates from fatty acid-induced activation of several PKC isoforms and a subsequent decreased activation of PKB by insulin or IGF-1 [117].

Although high concentrations of fatty acids have negative consequences on insulin action, it should be emphasised that unsaturated fatty acids might have, at least a partially beneficial action [118]. A recent study on high-fat diet fed rats indicated that the presence of 3-polyunsaturated fatty acids in the diet, although unable to prevent the development of hyperinsulinaema, hyperglycaemia and liver insulin resistance, allowed the maintainance of normal insulin sensitivity in muscle [119].

TNF-α and other cytokines

Interleukin 1 (IL-1), IL-6 and TNF-α are pro-inflammatory cytokines essential for mediating the immune response to pathogens. In addition, they are also predisposing factors towards the development of obesity and insulin resistance [120]. Moreover, subclinical inflammatory reactions dependent on increased circulating levels of IL-1β and IL-6 are associated with an increased risk of developing Type 2 diabetes [121].

Among the various pro-inflammatory cytokines, adipose tissue-produced TNF-α was first recognised as an insulin resistance-inducing agent and its expression is increased in the adipose tissue of several animal models of obesity [122]. TNF-α decreases insulin signalling by impairing the insulin-stimulated tyrosine phosphorylation of IRS molecules, both via phosphorylation of IRS-1 S307 and the induction of SOCS proteins. In addition, new observations of the mechanism of TNF-α-induced insulin resistance indicate that the induction of cellular stress by TNF-α leads to activation of ErbB2/ErbB3 and recruitment of PI3K. This ternary complex is able to cross-talk at the level of the insulin pathway by inducing serine phosphorylation on IRS proteins, ultimately leading to insulin resistance [123].

Another pro-inflammatory cytokine implicated in insulin resistance is IL-6, which inhibits insulin signalling in hepatocytes [124]. Similar to TNF-α, IL-6-induced insulin resistance occurs via the induction of SOCS-3 [125].

Besides being released to regulate inflammatory events, multiple cytokines that contribute to the development of insulin resistance are induced in monocytes after exposure to a hyperglycaemic milieu, these include the monocyte chemoattractant protein 1 (MCP-1), TNF-α and IL-1β [126]. Thus, inflammatory states can be viewed as predisposing factors for the development of insulin resistance [120].

Since increased pro-inflammatory cytokine levels are triggers for inducing insulin resistance, anti-inflammatory cytokines such as IL-10 might have the opposite effect. In fact, an epidemiological study showed that a low IL-10 production capacity, leading to a pro-inflammatory response, was associated with Type 2 diabetes [127].

Glycated proteins and advanced glycated end products

Glucose and other reducing sugars slowly react non-enzymatically with free amino groups of both intra- and extracellular proteins forming early glycation products (Schiff bases) and intermediate glycation products (Amadori products). These glycated proteins are further degraded to AGE, a heterogeneous class of compounds which includes glyoxal, methylglyoxal, carboxymethyl-lysine and others [128]. Increased AGE have been associated with various diabetic complications, including diabetic retinopathy, neuropathy and nephropathy as well as cataract formation and diabetic atherosclerotic disease. However, it is not yet known whether accumulation of AGE is directly responsible for the above complications or a mere consequence of them. Likewise, although glycated haemoglobin is used as an analytical index to monitor long-term blood glucose control, little is known about the role that glycated proteins can play in the pathophysiology of diabetes.

As glycation is a non-enzymatic reaction, it can occur on any protein. Insulin itself has been shown to be glycated within beta cells, resulting in impaired biological activity [129]. In a recent report, by using a specific radioimmunoassay, it was shown that glycated insulin circulates at higher concentration in Type 2 diabetic patients [130].

Increased circulating amounts of glycated albumin—a major long-lived protein component of serum—and AGE have been associated with the development of diabetic retinopathy [131, 132] via induction of vascular endothelial growth factor expression [133] and diabetic nephropathy [134].

Glycated albumin also acts intracellularly by down-regulating the metabolic effects of insulin in L6 skeletal muscle cells without affecting the mitogenic action of the hormone. Thus, pre-treatment of L6 cells with

glycated albumin leads to decreased insulin-induced PI3K, PKB and GSK-3 activation—resulting in impaired glucose uptake and glycogen synthesis—without affecting the insulin-induced activation of the MAPK Erk1/2 and its associated mitogenic action. This differential effect on insulin signalling seems to be mediated by specific PKCα-mediated serine phosphorylation of IRS-1 that differentially affects the two major insulin-signalling cascades [135].

Glucosamine

The glucosamine biosynthetic pathway, although quantitatively utilising a small fraction of glucose, is yet another contributor to the insulin-resistant state. Glucose, once imported into the cell, is converted to fructose-6-phosphate (Fru-6-P). While the majority of Fru-6-P enters the glycolytic pathway, a small fraction (1–3%) is used by the hexosamine biosynthetic pathway to produce UDP-N-acetyl-glucosamine, a major substrate for glycosylation reactions [136]. Earlier studies showed that an increased metabolic flux through the hexosamine pathway led to the desensitisation of the glucose transport system in adipocytes [137]. Likewise, several studies showed that chronic exposure to glucosamine—a precursor of the hexosamine pathway—impairs insulin responsiveness, thus contributing to the formation of an insulin-resistant state in cultured human skeletal muscle cells [138], and rat adipocytes [139]. At the molecular level, the insulin resistance induced by the hexosamine pathway correlates with increased glycosylation—and subsequent deregulation—of various proteins involved in insulin action, including IRS-1, which leads to diminished insulin-stimulated phosphorylation of T308 of PKB and S9 of GSK-3 [140]; and the syntaxin 4 binding protein Munc18c, resulting in a reduced insulin-stimulated GLUT4 translocation at the plasma membrane [141]. Similarly, increased glycosylation of glycogen synthase contributes to its reduced activation by insulin [142].

Further evidence for the role of the hexosamine pathway in conferring insulin resistance came from two transgenic mouse models overexpressing glutamine:fructose-6-phosphate amidotransferase (GFAT), the pathway's rate-limiting enzyme, in the liver and skeletal muscle. Transgenics expressing GTAF in the liver developed hyperlipidaemia and became obese and insulin resistant [143] and skeletal muscle GTAF overexpression led to decreased GLUT4 translocation [144]. On the contrary, contrasting reports exist as to whether the hexosamine pathway reduces insulin sensitivity upon short-term exposure to glucosamine [145, 146].

In addition to its negative action at the peripheral level, activation of the hexosamine pathway also deteriorates beta-cell function. Adenoviral-mediated overexpression of GFAT in isolated rat islets impaired glucose-stimulated insulin secretion and decreased the expression of beta-cell-specific genes including GLUT2, insulin and glucokinase. Treatment of beta cells with an anti-oxidant, however, reversed the effects of overexpressed GFAT, suggesting that the deterioration of beta-cell function occurs via the induction of oxidative stress [147].

Conclusions

As briefly discussed, it is clear that regulation of insulin signalling takes place at several levels. In addition to the "classic" action of protein phosphatases, which restore the dephosphorylated/inactive state of the IR, IRS proteins and downstream kinases (for reviews see [148, 149]), more recently described molecular events such as protein S/T phosphorylation and degradation mainly acting on IRS proteins, impair insulin action.

Of particular interest is the link between these molecular events and the onset of insulin resistance and diabetes, with deregulated concentrations of circulating factors (cytokines, insulin itself, etc.), metabolites (glucose, glucosamine, fatty acids) or glycated proteins and AGE, which often contribute to the aggravation of the insulin-resistant or diabetic condition by acting via overlapping mechanisms.

The current picture and future improvements in our understanding of the S/T phosphorylation pathways on IRS-1 (and IRS-2) and action of SOCS proteins, will define a number of putative targets, the inhibition of which might relieve the negative effects caused by the insulin-resistant state. If proteins which act by down-regulating insulin action can be identified and made the target of novel bioactive molecules, this could eventually lead to the development of new drugs to be used for the prevention and treatment of the insulin-resistant state, and hence diabetes.

Sources. This review is mainly based on the relevant literature published in the English language from 2000 and available through PubMed. Major prior contributions have been nevertheless cited. Cited papers were identified through PubMed searches for "insulin", "IRS", "SOCS" and "insulin resistance" and through reading of the recent literature.

Acknowledgements. We apologise to all the researchers working in the field of modulation of insulin action whose work, due to space limitations, could not be cited. L. Pirola was supported by FRM (Fondation pour la Recherche Médicale) and by a "Poste Vert" postdoctoral fellowship, A.M. Johnston was supported by an INSERM "Poste Vert" postdoctoral fellowship. Our work discussed in this review was supported by INSERM, Université de Nice—Sophia-Antipolis, la Région PACA, Aventis (Frankfurt, Germany), Lipha-Merck (Lyon, France) and the European Community (grants QLGI-CT-1999-00674 and QLK3-CT-2000-01038).

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