

Insulin Receptor Signaling in Normal and Insulin-Resistant States

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In the wake of the worldwide increase in type-2 diabetes, a major focus of research is understanding the signaling pathways impacting this disease. Insulin signaling regulates glucose, lipid, and energy homeostasis, predominantly via action on liver, skeletal muscle, and adipose tissue. Precise modulation of this pathway is vital for adaptation as the individual moves from the fed to the fasted state. The positive and negative modulators acting on different steps of the signaling pathway, as well as the diversity of protein isoform interaction, ensure a proper and coordinated biological response to insulin in different tissues. Whereas genetic mutations are causes of rare and severe insulin resistance, obesity can lead to insulin resistance through a variety of mechanisms. Understanding these pathways is essential for development of new drugs to treat diabetes, metabolic syndrome, and their complications.

Insulin and IGF-1 control a wide variety of biological processes by acting on two closely related tyrosine kinase receptors. Receptor activation initiates a cascade of phosphorylation events that leads to the activation of enzymes that control many aspects of metabolism and growth. Insulin/IGF-1 signaling contains many different points of regulation or critical nodes, controlled both positively and negatively, to ensure proper signal duration and intensity (see the schematic in Fig. 1). Perturbations in these signaling pathways can lead to insulin resistance. Here we review the insulin-signaling network, its critical nodes, and how these are perturbed in insulin-resistant states.

INSULIN AND IGF-1 RECEPTORS

Insulin and IGF-1 mediate their biological effects via the insulin and IGF-1 receptors (IR and IGF-1R). These highly homologous tyrosine kinase receptors are members of a family that also includes the orphan insulin receptor-related receptor (IRR), which has been suggested to play a role in testis determination (Nef et al. 2003) and act as an extracellular alkali sensor (Deyev et al. 2011). Although insulin and IGF-1 preferentially bind to their own receptors, both ligands can also bind to the alternate receptor with reduced affinity (Belfiore et al. 2009).

The IR, IGF-1R, and IRR are tetrameric proteins that consist of two extracellular α subunits

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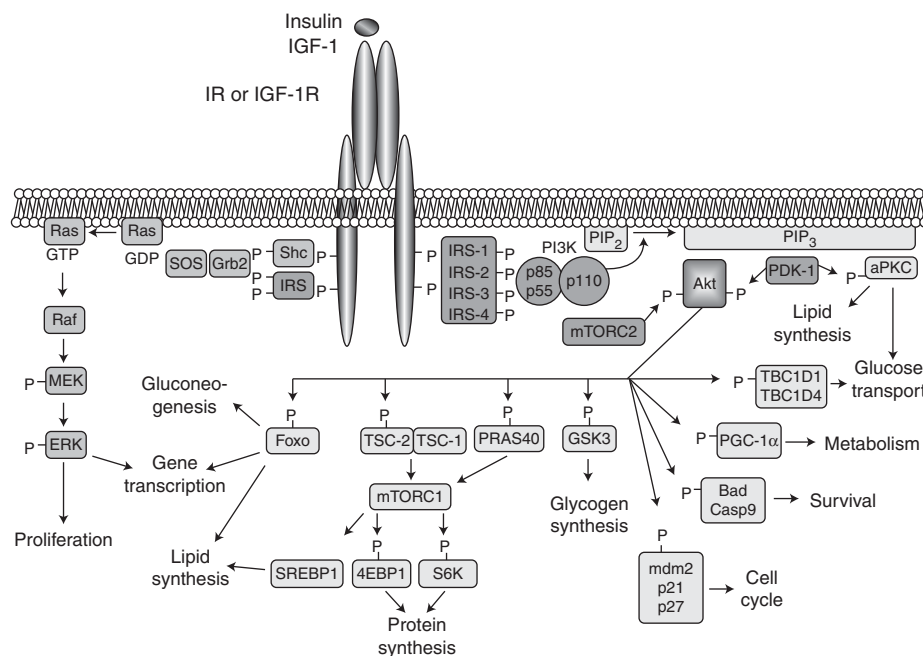


Figure 1. Insulin- and IGF-1-signaling pathways. Activation of insulin and IGF-1 receptors by their ligands initiates a cascade of phosphorylation events. A conformational change and autophosphorylation of the receptors occur at the time of ligand binding, leading to the recruitment and phosphorylation of receptor substrates such as IRS and Shc proteins. Shc activates the Ras-MAPK pathway, whereas IRS proteins mostly activate the PI3K-Akt pathway by recruiting and activating PI3K, leading to the generation of second messenger PIP₃. Membrane-bound PIP₃ recruits and activates PDK-1, which phosphorylates and activates Akt and atypical PKCs. Akt mediates most of insulin's metabolic effects, regulating glucose transport, lipid synthesis, gluconeogenesis, and glycogen synthesis. Akt also plays a role in the control of cell cycle and survival. The Shc-Grb2-Sos-Ras-Raf-MAPK pathway controls cellular proliferation and gene transcription.

and two transmembrane β subunits that are joined by disulfide bonds. Both subunits are generated from a single large precursor by proteolytic cleavage. The IR messenger RNA (mRNA) undergoes alternative splicing of exon 11 to yield two isoforms that differ by exclusion (isoform A) or inclusion (isoform B) of a 12-amino-acid sequence in the carboxy-terminal part of the α subunit (Mosthaf et al. 1990). IR-A is predominantly expressed in fetal tissues and in the brain, has higher affinity for both insulin and IGF-2, has a higher rate of internalization than the type-B isoform, and tends to be up-regulated in cancer (Frasca et al. 1999), whereas IR-B expression is highest in the liver. Heterotetramers composed of an α/β dimer of IR and an α/β dimer of IGF-1R can form hybrid receptor complexes that bind preferentially

IGF-1 and IGF-2 over insulin (Benyoucef et al. 2007). Their formation appears to occur randomly in cells expressing both receptors and depends on the relative expression level of each type of receptor (Bailey et al. 1997; Pandini et al. 1999). Insulin and IGF-1 differential effects *in vivo* reflect mostly the hormone concentration and relative expression level of receptors in different tissues rather than the capacity of IR and IGF-1R to convey different signaling (Boucher et al. 2010; Siddle 2012).

INSULIN RECEPTOR SUBSTRATES

At the time of ligand binding to the α subunits, IR and IGF-1R undergo a conformational change-inducing activation of the kinase activity in the β subunits. This results in transphos-



phorylation among β subunits, further activating the kinase and allowing the recruitment of receptor substrates. The best characterized substrates are members of the insulin receptor substrate (IRS) family of proteins, simply referred to as IRS-1 through IRS-6, which act as scaffolds to organize and mediate signaling complexes (Sun et al. 1991, 1995; Lavan et al. 1997a,b; Cai et al. 2003; White 2006; Shaw 2011). IRS proteins are recruited to the membrane and the activated receptors through both pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains in their amino terminus (Voliovitch et al. 1995). They are subsequently phosphorylated by the activated receptors on multiple tyrosine residues that form binding sites for intracellular molecules that contain Src-homology 2 (SH2) domains (Sun et al. 1993).

Although these substrates have similar tyrosine phosphorylation motifs, they clearly have different functions *in vivo*. IRS-1 knockout (KO) mice show growth retardation and impaired insulin action, especially in muscle (Araki et al. 1994), but have normal glucose tolerance. IRS-2 KO mice display growth reduction in only selective tissues, such as certain neurons and islet cells, but also have defective insulin signaling in the liver, which when combined with the loss of β cells results in the development of diabetes (Withers et al. 1998). At the cellular level, IRS-1 preadipocytes show defects in differentiation, whereas IRS-2 KO preadipocytes differentiate normally, but have impaired insulin-stimulated glucose transport (Miki et al. 2001; Tseng et al. 2005). In skeletal muscle cells, IRS-1, but not IRS-2, is required for myoblast differentiation and glucose metabolism, whereas IRS-2 is important for lipid metabolism and ERK activation (Huang et al. 2005; Bouzakri et al. 2006).

IRS-3 and IRS-4 show a more restricted tissue distribution pattern. In rodents, IRS-3 is most abundant in adipocytes, liver, and lung (Sciacchitano and Taylor 1997), whereas in humans, the *IRS-3* gene is a pseudogene, so no protein is produced at all (Bjornholm et al. 2002). In mice, disruption of the gene for *IRS-3* alone does not result in abnormalities, but leads to a severe defect in adipogenesis when

combined with deletion of IRS-1 (Laustsen et al. 2002). IRS-4 mRNA is present in skeletal muscle, liver, heart, brain, and kidney (Fantin et al. 1999), and IRS-4 KO mice show only very minimal growth retardation and glucose intolerance (Fantin et al. 2000). IRS-5 (also called DOK4) and IRS-6 (DOK5) have limited tissue expression (Cai et al. 2003) and are relatively poor IR substrates (Versteyhe et al. 2010).

In addition to the IRS proteins, the insulin and IGF-1 receptors can phosphorylate several other substrates (Siddle 2012). Shc proteins are tyrosine phosphorylated by IR and IGF-1R, and participate in the activation of the Ras/ERK pathway. Grb2-associated binder (GAB) proteins are also substrates for a variety of receptors, including IR and IGF-1R. GAB proteins resemble IRS proteins, but lack a protein tyrosine phosphatase (PTP) domain, and could play a role in insulin/IGF-1 signaling in cells expressing low IRS protein levels. APS (SHB2) and Cbl are IR/IGF-1R substrates that recruit other proteins, such as the Cbl-associated protein (CAP), to the insulin-signaling complex. The latter participates in the control of insulin-stimulated glucose uptake (Baumann et al. 2000). SH2B1 directly binds to insulin receptors and IRS proteins and enhances insulin sensitivity by promoting insulin receptor catalytic activity and by inhibiting tyrosine dephosphorylation of IRS proteins.

PHOSPHATIDYLINOSITOL (3,4,5)-TRIPHOSPHATE AND PHOSPHOINOSITIDE 3-KINASE

The critical pathway linking IRS proteins to the metabolic actions of insulin is the PI3-kinase (PI3K) and Akt pathway. The class Ia PI3-kinases are heterodimers consisting of a regulatory and catalytic subunit, each of which occurs in several isoforms (Vadas et al. 2011). Recruitment and activation of the PI3K depends on the binding of the two SH2 domains in the regulatory subunits to tyrosine-phosphorylated IRS proteins (Myers et al. 1992; Shaw 2011). This results in activation of the catalytic subunit, which rapidly phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate

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the lipid second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP₃). The latter recruits Akt to the plasma membrane, where it is activated by phosphorylation and induces downstream signaling.

The different isoforms of the regulatory subunit of PI3K are encoded by three distinct genes. *Pik3r1* encodes 65%–75% of all regulatory subunits, mostly in the form of p85 α , but also the splice variants p55 α and p50 α . *Pik3r2* encodes p85 β and accounts for ~20% of the regulatory subunits. *Pik3r3* encodes p55 γ , which is similar in structure to p55 α , but expressed at low levels in most tissues.

The three different catalytic subunits—p110 α , β , and δ —are derived from three different genes. Binding of a regulatory to a catalytic subunit increases the catalytic subunit stability and maintains it in an inhibited state. This is relieved by binding of the regulatory subunit to specific phosphotyrosine motifs in IRS proteins, resulting in its activation (Yu et al. 1998; Burke et al. 2011; Zhang et al. 2011). Liver-specific ablation of p110 α , and to a lesser extent p110 β , in mice results in glucose intolerance and insulin resistance (Jia et al. 2008; Sopasakis et al. 2010). Surprisingly, knockouts of the regulatory subunits of PI3K, including a heterozygous deletion of p85 α , p85 β KO, or p50 α /p55 α double KO, all display increased insulin sensitivity (Terauchi et al. 1999; Ueki et al. 2002). Different mechanisms by which reducing concentration of regulatory subunits can increase insulin action have been identified. Regulatory subunits typically are in excess concentration to catalytic subunits and thus compete with the enzymatically competent p85/p110 heterodimer for binding to IRS proteins. The p85 α monomer has also been linked to regulation of the phosphatase and tensin homolog (PTEN) (Taniguchi et al. 2010). More recently, p85 α has been shown to bind to the transcription factor XBP-1 and to modify the unfolded protein response, which contributes to insulin resistance (Park et al. 2010; Winnay et al. 2010).

In addition to PI3K, IRS proteins recruit other proteins potentially contributing to insulin and IGF-1 action. Proteomics analysis of the phosphotyrosine interactome of IRS-1 and

IRS-2 indicates that most interacting proteins bind to both substrates, such as adaptor proteins Grb2 or Crk, or tyrosine phosphatase SHP2. However, other interaction partners seem to bind exclusively to IRS-1 (Csk) or IRS-2 (Shc, DOCK-6, and DOCK-7) (Hanke and Mann 2009).

ACTIVATION OF DOWNSTREAM KINASES

Most of the physiological effects of PI3K-generated PIP₃ are mediated by a subset of AGC protein kinase family members, which include isoforms of Akt/protein kinase B (PKB), p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK), as well as several isoforms of protein kinase C (PKC), particularly the atypical PKCs. AGC kinase family members share similar structure and mechanisms of activation via phosphorylation of two serine and threonine residues (Pearce et al. 2010). PDK-1 (3-phosphoinositide-dependent protein kinase 1) is the major upstream kinase responsible for the phosphorylation and activation of the AGC kinase members regulated by PI3K (Bayascas 2010). PDK-1 contains a PH domain that binds to membrane-bound PIP₃, triggering PDK-1 activation. PDK-1 phosphorylates and activates AGC protein kinases at serine/threonine residues, such as Thr-308 for Akt (Alessi et al. 1997). However, Akt phosphorylation at Ser-473 is required for full activation, and this is accomplished by the mammalian target of rapamycin complex 2 (mTORC2) (Sarbasov et al. 2005; Oh and Jacinto 2011). DNA-dependent protein kinase (DNA PK) has also been described to phosphorylate and activate Akt in response to DNA damage (Bozulic et al. 2008), and is involved in insulin regulation of metabolic genes such as fatty acid synthase (Wong et al. 2009).

The Akt/PKB family of proteins consists of three different isoforms of serine/threonine protein kinases encoded by different genes (Schultze et al. 2011). All isoforms possess a PH domain, allowing interaction with PIP₃ and recruitment to the plasma membrane. Akt2 is most abundant in insulin-sensitive tissues and seems to play a predominant role in

mediating insulin action on metabolism. Thus, Akt2 KO mice are insulin resistant and develop diabetes (Cho et al. 2001), whereas Akt1 and Akt3 KO mice do not.

ACTIONS OF INSULIN DOWNSTREAM FROM AKT

Activation of Akt by PDK-1 and mTORC2 allows the phosphorylation and activation of many downstream targets. Akt phosphorylates tuberous sclerosis complex protein 2 (TSC-2), inducing the degradation of the tumor suppressor complex that consists of TSC-2 and TSC-1, which activates the mTORC1 complex. Akt-induced activation of mTORC1 can also be achieved by phosphorylation of proline-rich Akt substrate 40 kDa (PRAS40), an inhibitor of mTORC1, thereby relieving the inhibition. The mTORC1 complex then phosphorylates and inhibits 4E-binding protein 1 (4E-BP1), activates ribosomal protein S6 kinases S6K1 and S6K2 and SREBP1, and leads to the regulation of a network of genes controlling metabolism, protein synthesis, and cell growth (Duvel et al. 2010).

Transcription factors of the Forkhead box O (Foxo) family control the expression of lipogenic and gluconeogenic genes. Akt phosphorylates Foxos at several sites which provides docking sites for binding proteins of the 14-3-3 family. This interaction leads to the exclusion of Foxo from the nucleus, thus blocking its transcriptional activity (Tzivion et al. 2011). Interestingly, although mice lacking Akt1 and Akt2 show severe hepatic insulin resistance and high levels of hepatic glucose production, these defects are normalized when Foxo1 is concomitantly ablated in the liver. This indicates that an additional pathway exists in the control of hepatic glucose metabolism beyond the Akt/Foxo1 axis, which allows for insulin-mediated regulation of hepatic glucose production (Lu et al. 2012).

There are multiple other substrates of Akt involved in insulin action. The GTPase-activating protein Akt substrate of 160 kDa (AS160), also called TBC1D4, and its homolog TBC1D1, are phosphorylated by Akt and are involved in insulin- and contraction-mediated glucose

uptake (Sano et al. 2003; Sakamoto and Holman 2008; Taylor et al. 2008; An et al. 2010). Akt also phosphorylates and inactivates glycogen synthase kinase 3, resulting in glycogen synthase activation and glycogen accumulation in liver (Kim et al. 2004b). Akt-dependent phosphorylation of PGC-1 α impairs the ability of PGC-1 α to promote gluconeogenesis and fatty acid oxidation (Li et al. 2007). Phosphorylation of phosphodiesterase 3B (PDE3B) by Akt results in its activation and in a decrease in cyclic AMP levels (Kitamura et al. 1999), which plays important roles in the effect of insulin to inhibit lipolysis in adipocytes and insulin secretion in β cells (Degerman et al. 2011).

OTHER ACTIONS OF INSULIN DOWNSTREAM FROM PI3K

Akt plays a central role in mediating many other insulin actions by regulating the expression and activity of a wide range of proteins, including enzymes, transcription factors, cell cycle regulating proteins, or apoptosis and survival proteins (Manning and Cantley 2007). Murine double minute 2 (Mdm2) is phosphorylated by Akt, which inhibits p53-mediated apoptosis and contributes to tumorigenesis (Cheng et al. 2010). Akt phosphorylates cell cycle inhibitors p21Cip1/WAF1 and p27Kip1, resulting in cytoplasmic localization, cell growth, and inhibition of apoptosis (Zhou et al. 2001; Motti et al. 2004). Akt also phosphorylates and inhibits Bax, Bad, and caspase-9, which promotes cell survival (Datta et al. 1997; Cardone et al. 1998; Yamaguchi and Wang 2001; Gardai et al. 2004). Akt can phosphorylate and activate I κ B kinase (IKK), leading to NF- κ B activation (Bai et al. 2009). Akt phosphorylates and activates endothelial nitric oxide synthase (eNOS), which catalyzes the production of the vasodilator and anti-inflammatory molecule nitric oxide (NO), providing a potential link between insulin resistance and cardiovascular disease (Dimmeler et al. 1999; Fulton et al. 1999; Yu et al. 2011a). Although less well studied in insulin action, the serum- and glucocorticoid-induced protein family of kinases (SGK) are highly homologous to Akt, are also activated by dual phosphoryla-

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tion by PDK-1 and mTORC2 in a PI3K dependent manner, and have many downstream substrates in common with Akt (Bruhn et al. 2010).

PROTEIN KINASES C

PKC isoforms are both mediators and modifiers of insulin's metabolic action. Of the three major classes of PKC, the atypical PKCs (aPKCs), PKC- ζ and PKC- λ/ι , are activated via phosphorylation by PDK-1. aPKCs play an important role in insulin-stimulated glucose transport and regulation of lipid synthesis, and their expression and/or activation is decreased in muscle from obese and diabetic humans (Farese and Sajan 2010). Both PKC- λ and PKC- ζ have been shown to function interchangeably in mediating insulin-stimulated glucose transport (Sajan et al. 2006). Muscle-specific deletion of PKC- λ in mice leads to impairment in insulin-induced glucose uptake and insulin resistance (Farese et al. 2007). Mice with liver-specific deletion of PKC- λ display decreased insulin-induced expression of SREBP1c and triglyceride content in the liver, resulting in increased insulin sensitivity in these mice (Matsumoto et al. 2003).

THE GRB2-SOS-RAS-MAPK PATHWAY

A second essential branch of the insulin/IGF-1-signaling pathway is the Grb2-SOS-Ras-MAPK pathway, which is activated independently of PI3K/Akt. Activated receptors and IRS proteins both possess docking sites for adaptor molecules that contain SH2 domains such as Grb2 and Shc. The carboxy-terminal SH3 domain of Grb2 binds to proteins such as Gab-1, whereas the amino-terminal SH3 domain binds to proline-rich regions of proteins such as son-of-sevenless (SOS). SOS is a guanine nucleotide exchange factor (GEF) for Ras, catalyzing the switch of membrane-bound Ras from an inactive, GDP-bound form (Ras-GDP) to an active, GTP-bound form (Ras-GTP). Ras-GTP then interacts with and stimulates downstream effectors, such as the Ser/Thr kinase Raf, which stimulates its downstream target MEK1 and 2 that phosphorylate and activate the MAP kinases ERK1 and 2. Stimulated ERK1/2 play

a direct role in cell proliferation or differentiation, regulating gene expression or extra-nuclear events, such as cytoskeletal reorganization, through phosphorylation and activation of targets in the cytosol and nucleus.

NEGATIVE REGULATORS OF INSULIN SIGNALING

Insulin and IGF-1 signaling are tightly controlled because uncontrolled activity of the downstream pathways could lead to severe perturbations in metabolism and tumorigenesis. Intensity and duration of the signal play an important role in determining the specificity of the response to their pleiotropic effects. Therefore, the ability to turn off the insulin signal in a rapid manner at different levels is critical (Fig. 2). On the other hand, some of these inhibitory mechanisms can be altered in pathophysiological conditions and participate in the development of insulin resistance.

Phosphoprotein Phosphatases as Negative Regulators of Insulin Action

Both cytoplasmic protein tyrosine phosphatases, such as PTP1B, and transmembrane phosphatases, such as LAR, have been shown to dephosphorylate the tyrosine residues on activated IR and IGF-1R, as well as IRS proteins, thereby reducing their activity (Goldstein et al. 1998). Although the role of LAR in the control of insulin signaling in vivo remains controversial, PTP1B is an essential component of insulin action. PTP1B KO mice show enhanced insulin sensitivity, increased IR phosphorylation in muscle and liver, and are also resistant to high-fat-diet-induced obesity and associated insulin resistance (Elchebly et al. 1999; Klamann et al. 2000).

The serine/threonine phosphatase protein phosphatase 1 (PP1) has been implicated in the regulation of several rate-limiting enzymes in both glucose and lipid metabolism, including glycogen synthase, hormone-sensitive lipase, or acetyl CoA carboxylase (Brady and Saltiel 2001). Protein phosphatase 2A (PP2A), which accounts for ~80% of serine/threonine phos-

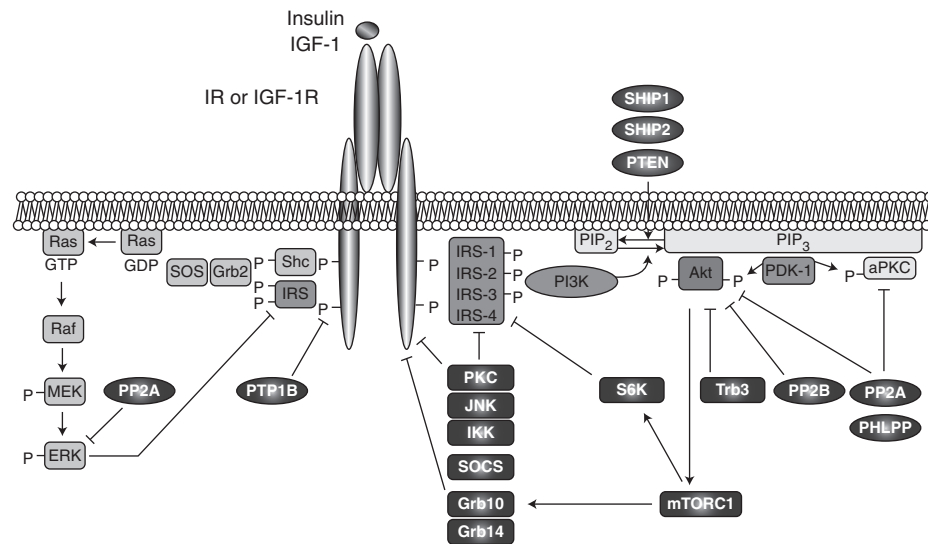


Figure 2. Negative modulators of insulin and IGF-1 signaling. Intensity and duration of insulin and IGF-1 signaling play an important role in determining the specificity and the nature of the response to these hormones. Signaling is attenuated by action of several phosphatases, which dephosphorylate the receptors, IRS proteins, PKCs, and ERK or PIP₃. In addition, stress kinases such as JNK, IKK, and ERK, as well as PKCs or S6K, inhibit insulin/IGF-1 signaling by inducing inhibitory serine/threonine phosphorylation of IR/IGFR and IRS proteins. Trb3 inhibits Akt, and adaptor proteins such as SOCS and Grb bind to the receptors and IRS proteins and inhibit signaling by competition.

phatase activity in cells, also regulates the activities of many protein kinases involved in insulin action, including Akt, PKC, S6K, ERK, cyclin-dependent kinases, and IKK (Millward et al. 1999). Several studies indicate that PP2A is hyperactivated in diabetic states (Kowluru and Matti 2012).

Other serine/threonine phosphatases have been implicated in insulin action. Protein phosphatases 2B (PP2B), also known as calcineurin, has been shown to dephosphorylate Akt (Ni et al. 2007). Two novel members of the PP2C family involved in regulation of insulin action are the PH domain leucine-rich repeat protein phosphatases PHLPP-1 and -2, which dephosphorylate both Akt and PKCs (Brognard and Newton 2008). Overexpression of PHLPP1 in cells impairs Akt and glycogen synthase kinase 3 activity, resulting in decreased glycogen synthesis and glucose transport (Andreozzi et al. 2011). Elevated levels of PHLPP1 have been found in adipose tissue and skeletal muscle of obese and/or diabetic patients and correlate

with decreased Akt2 phosphorylation (Cozzone et al. 2008; Andreozzi et al. 2011).

Lipid Phosphatases as Negative Regulators of Insulin Action

Lipid phosphatases can regulate insulin signaling by modulating PIP₃ levels. PTEN dephosphorylates PIP₃, thus antagonizing PI3K signaling in cells (Cantley and Neel 1999; Carcacedo and Pandolfi 2008). Muscle, adipose tissue, or liver-specific deletion of PTEN in mice increases insulin sensitivity (Stiles et al. 2004; Kurlawalla-Martinez et al. 2005; Wijesekara et al. 2005), and mice with whole-body PTEN haploinsufficiency show improved glucose tolerance and increased insulin sensitivity (Wong et al. 2007). Interestingly, the p85 α regulatory subunit of PI3K has recently been shown to bind directly to and enhance PTEN activity, creating a unique interface between the generation and degradation of PIP₃ (Taniguchi et al. 2006b; Chagpar et al. 2010).

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SH2 domain-containing inositol 5-phosphatases (SHIP) 1 and 2 also dephosphorylate PIP₃. SHIP1 expression is restricted to hematopoietic cells, whereas SHIP2 is ubiquitously expressed and plays a role in insulin signaling (Suwa et al. 2010). SHIP2 deficiency in mice results in hypoglycemia, enhanced insulin-induced Akt activation, and resistance to high-fat-diet-induced obesity, indicating that SHIP2 is a key regulator of glucose and energy homeostasis in vivo (Clement et al. 2001; Sleeman et al. 2005). Conversely, SHIP2-overexpressing mice show reduced insulin-induced Akt activation in the liver, fat, and skeletal muscle (Kagawa et al. 2008).

Other Negative Modulators (Grb, SOCS, Trb3, IP7)

Grb10 and Grb14 are cytoplasmic adaptor proteins that decrease IR and to a lesser extent IGF-1R activity, and prevent access of substrates to the activated receptors (Holt and Siddle 2005). Deletion of the *Grb10* gene in mice leads to increased growth, enhanced insulin signaling, and increased glucose tolerance (Smith et al. 2007; Wang et al. 2007). Grb10 overexpression, on the other hand, results in impaired growth, glucose intolerance, and insulin resistance (Shiura et al. 2005). Grb14 expression is increased in adipose tissue of insulin-resistant animal models and type-2 diabetic patients (Cariou et al. 2004), and Grb14 KO mice display increased glucose tolerance and insulin sensitivity, consistent with an inhibitory role of Grb14 on insulin signaling (Cooney et al. 2004). Grb10 and Grb14 share similar mechanisms as insulin signaling is not further increased in mice with deletion of both proteins (Holt et al. 2009).

Proteins of the suppressor of cytokine signaling (SOCS) family are adaptor proteins that act as negative regulators of cytokine and growth factor signaling. In addition, SOCS proteins, in particular SOCS1 and SOCS3, negatively regulate insulin signaling and thus link cytokine signaling to insulin resistance. Their expression is increased in obesity, and they induce insulin resistance via either inhibition of the tyrosine kinase activity of the IR, competi-

tion for binding of the IRS proteins to the receptor, or targeting the IRS proteins to degradation (Emanuelli et al. 2000, 2001; Rui et al. 2002; Ueki et al. 2004a,b; Sachithanandan et al. 2010).

Tribbles homolog 3 (Trb3) is a member of the family of pseudokinases that is thought to function as adaptor proteins. Trb3 expression is induced in liver in fasting and diabetes, and disrupts insulin signaling by binding to Akt and blocking its activation. Trb3 knockdown in mice improves glucose tolerance (Du et al. 2003; Koo et al. 2004). In cultured cells, insulin-stimulated S6K activation is decreased when Trb3 is overexpressed, and increased when Trb3 levels are reduced (Matsushima et al. 2006). Trb3 action in adipose tissue seems to be independent of Akt. Thus, whereas insulin promotes lipogenesis, Trb3 stimulates lipolysis by triggering the ubiquitination and degradation of acetyl-CoA carboxylase. Transgenic mice overexpressing Trb3 in adipose tissue are protected from diet-induced obesity because of enhanced fatty acid oxidation and display increased insulin sensitivity (Qi et al. 2006).

A novel negative regulator of insulin signaling is the inositol phosphate IP7. It was recently shown that insulin and IGF-1 increase IP7 levels, which in turn inhibits Akt translocation to the plasma membrane and subsequent activation, creating a potential feedback mechanism that attenuates insulin signaling (Chakraborty et al. 2010). Deletion of the enzyme that catalyzes IP7 formation in mice causes increased insulin responsiveness. Further studies will be needed to elucidate the contribution of this pathway in normal or pathological conditions.

Regulation by Inhibitory Serine and Threonine Phosphorylation

Tyrosine phosphorylation is essential for IR/IGF-1R and IRS activation. On the other hand, serine and threonine phosphorylation of the receptors or IRS proteins is primarily involved in turning the insulin signal down (Fig. 3). Increased inhibitory Ser/Thr phosphorylation of IR and especially IRS-1 and -2 occurs in response to cytokines, fatty acids, hyperglycemia,



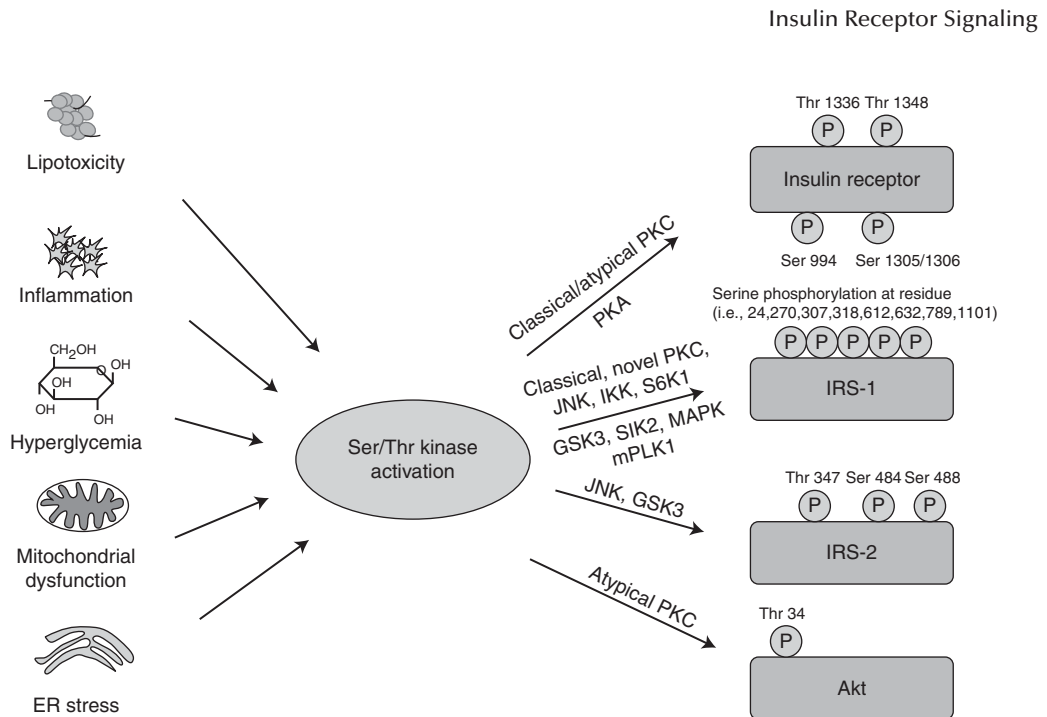


Figure 3. Activation of Ser/Thr kinases causes inhibitory phosphorylation on insulin-signaling molecules. Lipotoxicity, inflammation, hyperglycemia, and subsequently oxidative stress, as well as mitochondrial dysfunction and ER stress, all converge on activation of Ser/Thr kinases, inducing inhibitory Ser/Thr phosphorylation of IR, IRS proteins, and Akt on multiple residues, causing insulin resistance.

mitochondrial dysfunction, and ER stress, and insulin itself via activation of multiple kinases, predominantly by c-Jun amino-terminal kinase (JNK), IKK, conventional and novel PKCs, but also mTORC1/S6K and MAPK (De Fea and Roth 1997a; Aguirre et al. 2000; Gao et al. 2002; Gual et al. 2003; Li et al. 2004; Zhang et al. 2008a; Boura-Halfon and Zick 2009). Increased IR serine phosphorylation associated with decreased tyrosine kinase activity has been observed in insulin-resistant states, both in rodents and in humans (Karasik et al. 1990; Dunaif et al. 1995; Zhou et al. 1999; Shao et al. 2000). An increase in cAMP concentration also induces inhibitory serine phosphorylation of IR in a PKA-dependent manner (Stadtmauer and Rosen 1986; Roth and Beaudoin 1987).

Although inhibitory IRS-1 serine phosphorylation occurs at many different sites (Boura-Halfon and Zick 2009), the best studied of these modifications occurs at Ser-307 (Aguirre et al. 2002). IRS-1 Ser-307 phosphorylation is increased in obese and diabetic mice (Hirosumi

et al. 2002; Um et al. 2004). Although this is widely believed to contribute to insulin resistance by inhibiting insulin receptor kinase activity, recent studies have made this association less clear. Thus, insulin itself can stimulate phosphorylation of IRS-1 on Ser-307 in humans (Yi et al. 2007), and mice with a knock-in of IRS-1 Ser307Ala mutant developed more severe insulin resistance than control mice when fed a high-fat diet, indicating that Ser-307 is required to maintain normal insulin signaling (Copps et al. 2010). Thus, increased IRS-1 Ser-307 phosphorylation observed in insulin-resistance states may be associated with, but not cause, insulin resistance.

Lipids, through their metabolic product diacylglycerols, can activate classical (α , β , γ) and novel PKC members (δ , θ , ϵ) and impair insulin signaling by inducing multiple serine phosphorylation of IRS proteins and IR specifically at Thr-1336, Thr-1348, and Ser-1305/1306 (Bollag et al. 1986; Karasik et al. 1990; Lewis et al. 1990; Chin et al. 1993; De Fea and Roth

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1997b; Turban and Hajdich 2011). Thus, deletion of any member of the novel PKC family prevents the development of insulin resistance in skeletal muscle and liver by decreasing IRS-1 Ser-307 phosphorylation (Kim et al. 2004a; Samuel et al. 2007; Mack et al. 2008; Bezy et al. 2011). Atypical PKC- ζ also inhibits insulin signaling by inducing serine phosphorylation of IRS-1 (Ravichandran et al. 2001) and Thr-34 phosphorylation of Akt, thereby inhibiting its recruitment to the plasma membrane (Powell et al. 2003, 2004).

Another component of the negative feedback loops in insulin signaling is mTORC1. Activation of mTOR and S6K is not only downstream from insulin signaling, but also inhibits it by increasing serine phosphorylation and reducing IRS tyrosine phosphorylation. This is illustrated by the phenotype of S6K null mice, which are lean and display enhanced insulin sensitivity (Um et al. 2004). In addition, IRS-1 is hyperphosphorylated and degraded in TSC-2 KO fibroblasts, which show constitutive S6K activation (Harrington et al. 2004; Shah et al. 2004). mTORC1 also mediates phosphorylation and stabilization of Grb10, leading to feedback inhibition of insulin signaling (Hsu et al. 2011; Yu et al. 2011b).

MECHANISMS OF INSULIN RESISTANCE

A central feature of type-2 diabetes is insulin resistance, a condition in which cells cannot respond properly to insulin. This occurs primarily at the level of so-called insulin-sensitive tissues, such as liver, muscle, and fat, and can be caused by multiple mechanisms (Fig. 3 and Table 1).

Genetic Causes of Insulin Resistance

Insulin Receptor

Mutations in the insulin receptor gene have been identified in several rare forms of severe insulin resistance, including leprechaunism, Rabson-Mendenhall syndrome, or the type-A syndrome of insulin resistance. These patients often require a hundredfold or more insulin

than a typical diabetic patient (Kahn et al. 1976; Cochran et al. 2005). Most of these patients have nonsense or missense mutations in the extracellular ligand-binding domain or intracellular tyrosine kinase domain of the receptor, which leads to severely reduced insulin binding, altered kinetics of insulin binding, or reduced tyrosine kinase activity, but some also have presumed promoter defects leading to reduced receptor mRNA expression (Taylor et al. 1991; Haruta et al. 1995). Insulin receptor mutations have not been observed in patients with routine type-2 diabetes (T2D).

Insulin Receptor Substrate Proteins

The G972R polymorphism of IRS-1 is observed with higher frequency in patients with T2D and leads to decreased insulin signaling, mostly decreasing PI3K activity (Almind et al. 1996; Hribal et al. 2008). Although this finding has not been confirmed in all large-scale population analyses (Florez et al. 2004; van Dam et al. 2004), recent studies have continued to show an association between a single-nucleotide polymorphism (SNP) in IRS-1 and T2D (Burguete-Garcia et al. 2010; Martinez-Gomez et al. 2011). AT608R missense mutation in IRS-1 resulting in decreased insulin signaling has been reported in a patient with T2D, but appears to be very rare (Esposito et al. 2003). Numerous polymorphisms have been identified in the human *IRS-2* gene, but a clear association between these polymorphisms and T2D has not been found (Bernal et al. 1998).

Phosphoinositide 3-Kinase

An M326I polymorphism in the p85 α regulatory subunit of the PI3K was identified in Pima Indian women and is associated with decreased prevalence for T2D (Baier et al. 1998). However, this M326I mutation only has modest effects on insulin signaling in vitro by decreasing p85 α binding to IRS-1 and increasing p85 α degradation (Almind et al. 2002). Another polymorphism in p85 α (SNP42) is associated with fasting hyperglycemia, but its molecular mechanism so far remains elusive (Barroso et al. 2003).

Table 1. Molecular mechanisms of insulin resistance

Cause	Mechanism	Effect	References
Lipotoxicity Inflammation Hyperglycemia Mitochondrial dysfunction ER stress	Activation of Ser/Thr kinases	Inhibitory phosphorylation of insulin-signaling molecules	Bollag et al. 1986; Karasik et al. 1990; Lewis et al. 1990; Chin et al. 1993; Powell et al. 2003; Boura-Halfon and Zick, 2009
Genetic mutations	Point mutations in IR and insulin-signaling molecules	Increased protein turnover Reduced expression and ligand affinity Decreased signaling capacities	Kahn et al. 1976; Taylor et al. 1991; Haruta et al. 1995; Almind et al. 1996; George et al. 2004; Prudente et al. 2005; Hribal et al. 2008; Dash et al. 2009; Prudente et al. 2009
	SNP causing increased gene expression	Increased PTEN action leading to reduced PIP ₃ levels	Ishihara et al. 2003
Lipotoxicity	Hyperactivation of protein phosphatase PP2A	Reduced phosphorylation of IR and insulin-signaling molecules	Kowluru and Matti 2012
Inflammation	Cytokine-induced SOCS3 activation	Inhibition of IR tyrosine kinase activity Competition for IRS binding to IR Increased IRS degradation	Emanuelli et al. 2000, 2001; Rui et al. 2002; Ueki et al. 2004a,b; Steppan et al. 2005
	Cytokine-induced reduction in gene expression	Decreased expression of insulin-signaling molecules	Rotter et al. 2003; Jager et al. 2007
Hyperglycemia	Glycation of insulin-signaling molecules	Reduced affinity for IR Decreased DNA-binding capacities of transcription factors	Federici et al. 1999; Riboulet-Chavey et al. 2006; Housley et al. 2008
	Hyperactivation of protein phosphatase PP2A	Reduced phosphorylation of IR and insulin-signaling molecules	Kowluru and Matti 2012
Hyperinsulinemia	Hyperactivation of PHLPP1 and Grb14	Decreased AKT Ser 473 phosphorylation Competition for IRS binding to IR	Cariou et al. 2004; Cozzone et al. 2008; Andreozzi et al. 2011

Multiple molecular mechanisms of insulin resistance have been described, in addition to inhibitory Ser/Thr phosphorylation on insulin-signaling molecules (Fig. 3). Genetic mutations, dephosphorylation events, posttranslational modifications, and formation of inhibitory complexes have all been shown to cause insulin resistance.

Phosphatase and Tensin Homolog

In diabetes, mutations of PTEN have not been reported yet. However, three Japanese type-2 diabetic subjects have been identified with polymorphisms in the *PTEN* gene, one of which was associated with T2D. This SNP caused a higher expression rate of PTEN and reduced insulin-

induced Akt activation in cells (Ishihara et al. 2003). Very recently, it has been found that individuals with PTEN haploinsufficiency are both obese and insulin sensitive, with a decreased risk of T2D but increased risk of cancer (Pal et al. 2012). The impact of this in the general population is unknown.

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AKT and Related Targets

A rare missense mutation (R274H) in Akt2 leading to loss of kinase activity has been identified in a patient with diabetes (George et al. 2004). Two other missense mutations (R208 K and R467W) have also been identified in diabetic patients, but surprisingly, these mutant forms display unaltered insulin-stimulated kinase activities in vitro (Tan et al. 2007). In type-2 diabetic patients, a gain-of-function mutation (Q84R) in Trb3 has been associated with insulin resistance and decreased insulin-stimulated Akt phosphorylation (Prudente et al. 2005, 2009). A mutation in AS160 at position 363, resulting in a premature stop codon, was identified in a patient with severe postprandial hyperinsulinemia, and acts in a dominant-negative manner to reduce glucose transport (Dash et al. 2009).

Lipotoxicity

One feature of metabolic syndrome is ectopic accumulation of lipids, especially fatty acids (FA), which is believed to cause insulin resistance via multiple mechanisms. Tissue-specific increase in lipid content in nonadipose tissues provides direct evidence of lipotoxicity. Increased hydrolysis of circulating triglycerides owing to muscle-specific overexpression of lipoprotein lipase leads to skeletal muscle insulin resistance (Ferreira et al. 2001), whereas increased lipid transport in heart or liver leads to lipotoxic cardiomyopathy and nonalcoholic fatty liver disease, respectively (Chiu et al. 2005; Koonen et al. 2007). Besides the effect of increased lipid flux on insulin sensitivity, multiple lipid intermediates have been shown to promote insulin resistance.

Elevated circulating free fatty acids (FFA) are observed in obesity and induce activation of JNK, IKK, and PKC and IRS-1 Ser-307 phosphorylation (Schenk et al. 2008). The fatty acid palmitate plays a particular role in promoting insulin resistance as it induces endoplasmic reticulum (ER) stress, cytokine production, and activates JNK (Ozcan et al. 2004; Shi et al. 2006). In addition, palmitate activates NF- κ B

signaling while inhibition of this pathway reverses lipid-induced insulin resistance (Kim et al. 2001a; Sinha et al. 2004). Interestingly, the detrimental effect of palmitate on skeletal muscle insulin resistance can be reversed by coinfusion with oleate, thereby changing its conversion from phospholipids and diacylglycerol (DAG) to triglycerides (Peng et al. 2011). This indicates that FFA induces insulin resistance through multiple mechanisms, and combinations of FA can influence insulin signaling and highlight the crucial interplay of lipids with respect to dietary interventions.

The lipid metabolite DAG has also been shown to induce insulin resistance. Increased muscle DAG (intramyocellular lipid) leads to muscle insulin resistance by activating PKC- θ and inducing IRS-1 Ser-307 phosphorylation (Yu et al. 2002). Conversely, reducing DAG levels in skeletal muscle and liver protects mice against high-fat-diet-induced insulin resistance (Liu et al. 2007; Ahmadian et al. 2009; Samuel et al. 2010).

Increased plasma concentration of the sphingolipid ceramide is observed in obese and diabetic patients and is associated with severe insulin resistance (Haus et al. 2009). Ceramide has been shown to induce insulin resistance via PKC and JNK activation (Westwick et al. 1995; Schenk et al. 2008) and, thus, inhibition of ceramide synthesis ameliorates insulin resistance (Holland et al. 2007). Ceramides also inhibit Akt activation by increasing the interaction of PP2A with Akt, and phosphorylation of Akt at Thr-34 by PKC ζ , resulting in reduced binding of PIP₃ to Akt (Teruel et al. 2001; Powell et al. 2003; Blouin et al. 2010).

In addition to effects on kinases, alteration of membrane-lipid composition affects insulin signaling. An increase in the saturated-to-unsaturated FA ratio is observed in type-2 diabetic patients and is thought to reduce membrane fluidity and insulin sensitivity (Field et al. 1990; Bakan et al. 2006). Moreover, an increase in the phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio in endoplasmic reticulum leads to the activation of ER stress and is associated with insulin resistance (Fu et al. 2011).



Inflammation

Obesity is characterized by the development of a chronic low-grade inflammatory state, which is considered a key component in promoting obesity-associated insulin resistance (Osborn and Olefsky 2012). Adipose tissue expansion occurs in response to caloric overload, and is associated with an increase in immune cell infiltration and a subsequent proinflammatory response (Sun et al. 2011). Two cell types are especially important in this scenario: adipocytes and macrophages, both of them capable of secreting proinflammatory cytokines and inducing insulin resistance. Increased secretion of the chemokine MCP-1 by adipocytes drives macrophage accumulation into adipose tissues and induces insulin resistance (Kamei et al. 2006). Deletion of MCP-1 or its receptor CCR2 improves insulin sensitivity and ameliorates inflammation in mice (Kanda et al. 2006; Weisberg et al. 2006). Increased secretion of cytokines, such as TNF- α , IL1 β , or IL-6, by both immune cells and adipocytes is observed with obesity and induces insulin resistance via multiple mechanisms, including activation of Ser/Thr kinases (Ozes et al. 2001; Yuan et al. 2001; Hirosumi et al. 2002; Zhang et al. 2008a; Fan et al. 2010), decreasing IRS-1, GLUT4, and PPAR γ expression (Rotter et al. 2003; Jager et al. 2007), or activation of SOCS3 in adipocytes (Steppan et al. 2005).

Another driving factor in obesity-associated inflammation is caused by activation of Toll-like receptor (TLR), especially activation of TLR-2 and -4. TLRs belong to the innate immune system and are generally activated by pathogen-associated molecular patterns such as LPS, and induce inflammation via activation of the NF- κ B pathway (Akira and Takeda 2004). TLRs are ubiquitously expressed and TLR-4 is elevated in skeletal muscle (Reyna et al. 2008) and adipose tissue (Shi et al. 2006) with obesity. Interestingly, saturated FA can also activate this pathway (Lee et al. 2001; Shi et al. 2006), indicating a potential role for these receptors in obesity-driven inflammation. Thus, mice with reduced TLR-2- or TLR-4-signaling proteins (Shi et al. 2006; Kleinridders et al. 2009; Himes

and Smith 2010) are protected from obesity and obesity-associated insulin resistance.

Negative Regulation by Hyperglycemia

Glucose itself, at supraphysiological concentrations, is able to alter insulin sensitivity in muscle and fat, as well as decrease insulin secretion from β cells (Leahy et al. 1986; Hager et al. 1991). Hyperglycemia induced by decreased glucose transport in skeletal muscle impairs adipose and hepatic insulin action (Zisman et al. 2000; Kim et al. 2001b) and induces insulin resistance through several pathways, which are all believed to be linked to oxidative stress (Evans et al. 2005). Advanced glycosylation end products (AGE) inhibit insulin signaling by increasing Ser-307 phosphorylation of IRS-1 and forming methylglyoxal-IRS-1 adducts (Riboulet-Chavey et al. 2006).

Hyperglycemia increases the flux through the polyol pathway, which causes JNK activation and increases the hexosamine-biosynthetic pathway. This has been shown to promote insulin resistance in adipose tissue, skeletal muscle, liver, and pancreas in part by O-GlcNAcylation of IRS proteins (Marshall et al. 1991; Patti et al. 1999; McClain 2002; McClain et al. 2002). Furthermore, hyperglycemia also leads to O-GlcNAcylation of IR, which impairs receptor dimerization (Federici et al. 1999), and of Foxo1 leading to increased gluconeogenic gene expression (Housley et al. 2008).

Hyperglycemia also activates the PKC pathway by inducing de novo synthesis of DAG (Xia et al. 1994) and causes insulin resistance by forming a multimolecular complex, including receptor of AGE/IRS-1/Src, thereby activating PKC- α and increasing IRS-1 Ser-307 phosphorylation (Miele et al. 2003; Cassese et al. 2008).

Mitochondrial Dysfunction and ROS Formation

Although low levels of reactive oxygen species (ROS) can enhance insulin action (Krieger-Brauer et al. 1992; Mahadev et al. 2001), high concentration of ROS causes oxidative stress when unresolved. ROS formation occurs as a

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by-product of the electron transport chain and is a major consequence of mitochondrial dysfunction (Chang and Chuang 2010). Increased ROS levels have been observed in obese and diabetic states and can be caused by an increased metabolite flux into mitochondria, alterations in mitochondrial proteins, and reduced expression of antioxidant enzymes (West 2000; Rosen et al. 2001; Evans et al. 2005; Fridlyand and Philipson 2006). Increased oxidative stress leads to the activation of stress kinases that induce insulin resistance by serine phosphorylation of IRS proteins (Rudich et al. 1998; Evans et al. 2005; Dokken et al. 2008). Besides the aspect of ROS-mediated insulin resistance, altered mitochondrial dynamics in the form of increased mitochondrial fission leads to insulin resistance and can be rescued by inhibiting fission, which decreases the activity of p38 MAP kinase and increases IRS-1 and Akt activation (Jheng et al. 2012). Impairment of mitochondrial FA oxidation in liver can also lead to elevated DAG content, resulting in PKC- ϵ activation and decreased IRS-2 phosphorylation and PI3-kinase activity (Koh et al. 2005; Zhang et al. 2007).

ER Stress

The ER stress response, also known as unfolded protein response (UPR), is an adaptive process to ensure proper protein folding, maturation, and quality control in the ER. The three crucial pathways of the UPR (PERK, IRE1 α , and ATF6) are all activated with obesity and act together to reduce the burden of unfolded proteins (Hotamisligil 2010). Obese mice display enhanced PERK and IRE1 α activity in adipose tissue and liver, causing JNK and IKK activation and insulin resistance by phosphorylation of IRS-1 on Ser-307 (Ozcan et al. 2004, 2009; Hu et al. 2006; Zhang et al. 2008b). The transcription factor XBP-1 is activated by splicing during ER stress and increases gene expression of molecular chaperones to restore ER homeostasis. Overexpression of spliced XBP-1 reduces ER stress response, decreases activation of JNK, and increases insulin signaling by decreasing IRS-1 serine phosphorylation (Ozcan et al. 2004).

CONCLUDING REMARKS

Insulin and IGF-1 acting via specific tyrosine kinase receptors propagate signals via two main branches: the PI3K-PDK-1-Akt and the Grb2-SOS-Ras-MAPK pathways that control proliferation, differentiation, and survival at the cellular level, and growth and metabolism in organisms. These signaling pathways contain several points of regulation, signal divergence, and cross talk with other signaling cascades that define critical nodes (Taniguchi et al. 2006a). The complexity of this signaling system is essential to mediate the variety of insulin and IGF-1 biological responses. Many steps are negatively regulated by action of phosphatases or inhibitory proteins. One of the great challenges remaining is deciphering the complexity of insulin-resistance pathogenesis. Causes of insulin resistance are numerous and the mechanisms are multifactorial. In rare cases, the cause is genetic, but in most others, insulin resistance is triggered by cellular perturbations, such as lipotoxicity, inflammation, glucotoxicity, mitochondrial dysfunction, and ER stress, which lead to deregulation of genes and inhibitory protein modifications, resulting in impaired insulin and IGF-1 action. Identifying new molecules that impact insulin signaling and new levels of control, as well as better understanding the causes and mechanisms leading to insulin resistance, will be essential for a more effective treatment of type-2 diabetes and associated diseases.

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