IRS posttranslational modifications in regulating insulin signaling

Jinghua Peng¹² and Ling He¹
¹Division of Metabolism and Endocrinology, Departments of Pediatrics and Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
²Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, China

Correspondence should be addressed to L He: heling@jhmi.edu

Abstract
Insulin resistance is the hallmark of type 2 diabetes; however, the mechanism underlying the development of insulin resistance is still not completely understood. Previous reports showed that posttranslational modifications of IRS play a critical role in insulin signaling, especially the phosphorylation of IRS by distinct kinases. While it is known that increasing Sirtuin1 deacetylase activity improves insulin sensitivity in the liver, the identity of its counterpart, an acetyl-transferase, remains unknown. Our recent study shows that elevated endotoxin (LPS) levels in the liver of obese mice lead to the induction of the acetyl-transferase P300 through the IRE1-XBP1s pathway. Subsequently, induced P300 impairs insulin signaling by acetylating IRS1 and IRS2 in the insulin signaling pathway. Therefore, the P300 acetyl-transferase activity appears to be a promising therapeutic target for the treatment of diabetes.

Activation of the insulin signaling pathway regulates nutrient metabolism
Insulin, secreted by the β-cells of the islets of Langerhans in the pancreas, is the most powerful anabolic hormone known to be involved in regulating glucose, lipid and amino acid metabolism. The regulation of anabolic metabolism by insulin is through the activation of the insulin signaling pathway (Avruch 1998, Taniguchi et al. 2006). The insulin receptor (IR) is a tetrameric complex consisting of two extracellular α-subunits and two transmembrane β-subunits that are covalently linked through disulfide bonds (Sweet et al. 1987, Cheatham & Kahn 1995). Both α- and β-subunits are generated by proteolytic cleavage from a single precursor. Insulin binding to the α-subunits leads to a conformational change, the activation of tyrosine kinase activity in the β-subunits and the transphosphorylation of β-subunits. Phosphorylation at Y972 of the β-subunit generates an NPXpY motif that is critical for the recognition and binding of insulin receptor substrate (IRS) proteins (White et al. 1988). Several IRS proteins have been identified, including IRS1–6, Grb-2-associated protein (GAB1) and Shc1–3 (White 2003, Taniguchi et al. 2006). IRS binds to the IR through its pleckstrin-homology domains (PH domains) and phosphorylase-binding domains (PTB domains). The tyrosine phosphorylation of IRS by the IRβ results in the recruitment of intracellular molecules that contain Src-homology-2 domains (SH2 domains) and the activation of the PI3K-AKT signaling cascade (Guo 2014). The initiation of this cascade of phosphorylation events results in the suppression of gluconeogenic enzyme gene expression along with the augmentation of lipogenic enzyme gene expression and protein synthesis and glycogen storage.
IRS1 and IRS2 play a key role in mediating insulin’s effect on the regulation of glucose metabolism

Both IRS1 and IRS2 are ubiquitously expressed and are the primary mediators of IRS proteins in insulin-dependent regulation of glucose metabolism in most cells (White 2002). Mice lacking both IRS1 and IRS2 are embryonically lethal and die before implantation (Withers et al. 1999). Mice with IRS1 knockout exhibit mild insulin resistance without diabetes (Araki et al. 1994, Tamemoto et al. 1994). Inactivation of IRS2 in mice results in the development of diabetes due to insulin resistance and decreases insulin secretion from pancreatic β-cells (Tamemoto et al. 1994, Kido et al. 2000). Since defective hepatic insulin sensitivity in type 2 diabetes results in increased glucose production, which is the major cause of hyperglycemia in diabetic patients (Magnusson et al. 1992, Kunert et al. 2003), mice with liver-specific double IRS1 and IRS2 knockout display severe hyperglycemia and hyperinsulinemia (Dong et al. 2006). These data suggest that IRS1 and IRS2 are critical mediators of insulin’s regulation of glucose metabolism.

Phosphorylation of IRS1 and IRS2 proteins affects their functions

Following the landmark discoveries that insulin mediates the tyrosine phosphorylation of IRS1 (White et al. 1985), and that this phosphorylation event is critical for the recruitment of downstream mediators to the plasma membrane (Guo 2014), multiple tyrosine residues were found to be phosphorylated in these two proteins after insulin treatment. The phosphorylation of tyrosine residues at 460, 546, 628, 658, 727, 935, 983 and 1006 in IRS1 (mouse) and tyrosine residues at 536, 594, 649, 734, 814 and 1061 in IRS2 (mouse) leads to the generation of the YXXM motif (Sun et al. 1991). Subsequently, an SH2 domain containing downstream mediator, such as the adaptor protein p85 in PI3K, binds to these YXXM motifs, resulting in the activation of PI3K (Myers et al. 1992).

On the other hand, it was proposed that there are more than 40 potential serine/threonine phosphorylation sites, and phosphorylation of these sites serves to negatively regulate insulin signaling (Cheatham & Kahn 1995, Rui et al. 2001). To define the posttranslational modification sites in IRS1 and IRS2, we generated adenoviral FLAG-tagged IRS1 and IRS2 (mouse) expression vectors and transduced these adenoviral vectors into Hepa1–6 cells. After the purification of these proteins (Cao et al. 2017), we subjected these proteins to mass spectrometry to identify the modification sites. In good agreement with previous findings that tyrosine residues are not phosphorylated in untreated cells (White et al. 1985), we could not detect any tyrosine phosphorylation sites in these two proteins, as Hepa1–6 cells were harvested without insulin treatment. However, we found that 40 serine/threonine residues are phosphorylated in IRS1 protein and 19 serine/threonine residues are phosphorylated in IRS2 protein (Table 1).

Endoplasmic reticulum stress impairs insulin signaling via JNK phosphorylation of IRS

The endoplasmic reticulum (ER) is the primary site of protein synthesis, maturation, folding and transport. Disturbance of these processes triggers the unfolded protein response and leads to the activation of three canonical pathways: inositol-requiring enzyme 1 (IRE1)-XBP1s, PKR-like ER-regulating kinase and activating transcription factor 6 (Ron & Walter 2007). The activation...
of these pathways can alleviate ER stress by placing a brake on protein synthesis, and/or by increasing the production of protein chaperones needed for protein folding, or by degrading unfolded proteins. Excess nutrient intake causes ER stress and insulin resistance in obese animal models and obese human subjects. ER stress results in the formation of the IRE1α–TRAF 2 complex, which leads to the phosphorylation and activation of JNK. Subsequently, activated JNK induces insulin resistance through serine phosphorylation of IRSs 1 and 2 (Hirosumi et al. 2002) (Fig. 2). In an elegant study, Dr White’s group showed that the activation of ER stress by thapsigargin- or anisomycin-mediated activation of JNK caused broad IRS serine/threonine phosphorylation in CHO IRS1 cells (Hancer et al. 2014).

Hyperglycemia and abnormal intracellular accumulation of lipids result in IRS serine/threonine phosphorylation by protein kinase C

In diabetic and obese patients, hyperglycemia is the consequence of insufficient insulin secretion from β-cells and insulin resistance in target tissues/organisms; however, hyperglycemia can in turn aggravate insulin resistance. In cultured hepatocytes, treatment with high concentrations of glucose augmented IRS serine phosphorylation and impaired insulin-stimulated AKT phosphorylation (Nakajima et al. 2000). Inhibition of protein kinase C (PKC) activity abolished the serine phosphorylation of IRS resulting from high glucose treatment, suggesting that PKC can phosphorylate IRS. Moreover, intracellular lipid accumulation was correlated with insulin resistance, and infusion of lipids into patients caused insulin resistance (Pan et al. 1997, Boden et al. 2001). The cumulative evidence has shown that lipids such as diacylglycerols and ceramides can activate conventional and novel PKC isoforms and impair insulin signaling by inducing multiple serine phosphorylation of IRS (Itani et al. 2002) (Fig. 2). Corroborating these findings, deletion of PKC family members decreased IRS serine phosphorylation and ameliorated insulin resistance in skeletal muscle and liver (Kim et al. 2004, Samuel et al. 2007).

Activation of the IKK/NF-κB pathway causes insulin resistance via increasing IRS serine/threonine phosphorylation

Obesity is associated with a low-grade chronic inflammation in different metabolic tissues, including adipose tissue and the liver. Low-grade chronic inflammation triggers the pathogenesis of insulin resistance. We found that mice fed a high-fat diet (HFD) for 2 weeks exhibited insulin resistance and elevated glucose production in the liver. Since low-grade chronic inflammation can activate TLR/CD14 signaling, we tested the importance of this signaling pathway in glucose metabolism. Our studies showed that HFD feeding did not increase liver glucose production in CD14 knockout mice (Cao et al. 2017). Furthermore, studies in IKKβ transgenic mice indicate that NF-κB may be linked to the pathogenesis of insulin resistance (Cai et al. 2005), which was proven in NF-κB p50 knockout mice, as this mouse model exhibited improved insulin sensitivity and produced significantly less glucose in the liver (Gao et al. 2009). Further studies have revealed that IKKβ can phosphorylate multiple serine/threonine sites in IRS (Cai et al. 2005, Gao et al. 2009). In addition, IKKβ activation could augment serine phosphorylation of IRS via activation of the mTORC1/P70S6K pathway (Cai et al. 2005, Gao et al. 2009) (Fig. 2).

Table 1 Serine/threonine phosphorylation sites in IRS1 and IRS2.

<table>
<thead>
<tr>
<th></th>
<th>Serine residue</th>
<th>Threonine residue</th>
</tr>
</thead>
</table>

Figure 2

Phosphorylation of IRS at serine and threonine residues by Ser/Thr kinases impairs insulin signaling. Activation of Ser/Thr kinases by hyperglycemia, lipids, inflammation and ER stress leads to the inhibitory serine and threonine phosphorylation of IRS, causing insulin resistance. O-GlcNac modification of IRS can affect its phosphorylation.
Overall, due to the fact that IRS1/2 are heavily phosphorylated at serine and threonine residues (Table 1), it has been proposed that serine and threonine phosphorylation in IRS is a general mechanism to negatively modulate insulin signaling and contribute to pathological insulin resistance. In this respect, mice harboring muscle-specific mutated IRS1, in which three serine residues were mutated to alanine at 302, 307 and 612, were protected from fat-induced insulin resistance and exhibited improved insulin sensitivity in muscle (Morino et al. 2008). Of note, the activation of downstream Ser/Thr kinases ERK1/2 and P70S6K by insulin can also modulate IRS serine and threonine phosphorylation (Copps & White 2012), thus causing the signal to be self-limited. Analogous to phosphorylation, O-GlcNAc molecule can be introduced to IRS serine and threonine residues by O-GlcNAc transferase; this O-GlcNAc modification can block IRS phosphorylation and may affect the association of downstream mediators with IRS (Klein et al. 2009) (Fig. 2).

**Acetyl-transferase P300 is a negative regulator of insulin signaling**

The deacetylase SirT1 is able to restore insulin sensitivity in cells or tissue with insulin resistance (Sun et al. 2007, Schug et al. 2010). Moreover, activation of SirT1 by resveratrol improved insulin sensitivity (Kang et al. 2012). This evidence indicates that acetylation of mediators in the insulin signaling pathway downregulates insulin signaling. Several mechanisms have been proposed to explain the upregulation of insulin signaling by the deacetylase SirT1, including deacetylation of the IRS (Zhang 2007, Frojdo et al. 2011). While it is known that increasing SirT1 deacetylation activity improves insulin sensitivity, the identity of its counterpart, an acetyltransferase, remains unknown.

It has been known for nearly a decade that curcumin, a specific inhibitor of P300 and CBP acetyltransferase activity (Balasubramanyam et al. 2004, Marcu et al. 2006, Morimoto et al. 2008), can improve hyperglycemia in diabetic patients and in animal models (Shehzad et al. 2011, Chuengsamarn et al. 2012, Perez-Torres et al. 2013). We found that curcumin treatment increased the phosphorylation of AKT and GSK3 in a concentration-dependent manner in Hepa1–6 cells (Cao et al. 2017). Given that both P300 and CBP are acetyltransferases and critical co-activators in the regulation of hepatic glucose production (He et al. 2009, 2012, 2014), we examined the protein levels of these co-activators in a diet-induced obese mouse model and found that P300 protein levels were dramatically induced by HFD feeding (Cao et al. 2017). Furthermore, we found that ob/ob mice have elevated P300 protein levels in their liver when compared to heterozygous lean control mice. In comparison, the protein levels of CBP remained unchanged in the liver of HFD feeding mice and ob/ob mice. We tested whether the acetylation of mediators in the insulin signaling pathway by P300 acetyltransferase led to downregulation of insulin signaling. First, we found that overexpression of P300 reduced insulin-mediated AKT and GSK3 phosphorylation. Second, depletion of P300, but not its closely related protein CBP, improved insulin sensitivity. Third, treatment with C646, a chemical P300-specific acetyltransferase inhibitor (Bowers et al. 2010, Wondisford et al. 2014), improved insulin signaling/sensitivity in HFD feeding mice and ob/ob mice. These data suggest that P300 is an acetyl-transferase that functions as a negative regulator of insulin signaling.

**Acetylation of IRS1 and IRS2 by P300 impairs insulin signaling**

Treatment with the P300 acetyl-transferase specific inhibitor C646 led to a significant increase in the phosphorylation levels of AKT and GSK3 and increased PI3K activity by 6-fold. These data indicate that the IRS or IR might be the targets of P300. Indeed, we found that IRS1 and IRS2, but not the IR, were heavily acetylated in the liver of ob/ob mice. To mimic the effect of P300 induction on IRS acetylation, we co-transfected P300 and IRS1 and IRS2 expression vectors into Hepa1–6 cells. IRS1 and IRS2 proteins were purified and used to map acetylation sites by mass spectrometry. Seven lysine residues in IRS1 and fifteen in IRS2 can be acetylated (Fig. 3).

Interestingly, the blockade of a single acetylation site in either IRS1 or IRS2 by the substitution of the lysine residue with arginine as a mimic of non-acetylated lysine (K to R mutation) had a mild effect on AKT and GSK phosphorylation. However, combined mutations (K to R mutation) at the 315/767/862, 1017/1080 and 1017/1080/1131 lysine residues in IRS1, and the 683/802, 683/802/1081, 1173/1264 and 683/802/1081/1173/1264 lysine residues in IRS2 significantly increased insulin signaling. In particular, combined triple KR mutations at the 1017/1080/1131 lysine residues in IRS1 and combined double KR mutations at the 1173/1264 lysine residues in IRS2 had the strongest effect on the augmentation of insulin signaling (Cao et al. 2017). We further assessed the effects of IRS1/2 KR mutations on insulin signaling...
in HFD-fed mice using adenoviral expression vectors to express similar amounts of wild-type IRS1 and IRS2, or IRS1 and IRS2 KR mutants in the liver. Mice with injections of IRS1 and IRS2 KR mutants exhibited improved insulin sensitivity, suggesting that the acetylation of IRS1 and IRS2 impairs insulin signaling. Of note, combined KR mutation in either IRS1 or IRS2 dramatically decreased P300-mediated IRS1 and IRS2 acetylation, indicating that these identified acetylation sites in IRS1 and IRS2 are P300 target sites.

### Acetylation of IRS1 and IRS2 by P300 results in the reduction of their tyrosine phosphorylation

Our studies showed that inhibition of P300 acetyltransferase activity by C646 led to increased tyrosine phosphorylation of IRS1 and IRS2 in cultured Hepa1–6 cells and in the liver of ob/ob mice. To validate these data, we overexpressed IRS1 or IRS2 to similar levels as their corresponding IRS1-panKR or IRS2-panKR (all identified acetylation sites in IRS1 or IRS2 lysine residues were substituted with arginine residues) in Hepa1–6 cells. We found that KR mutations in IRS1 or IRS2 resulted in elevated IRS tyrosine phosphorylation. Intriguingly, we observed increased association of IRS1/2-panKR with IRβ when compared to IRS1/2 (wild-type), suggesting that acetylation of IRS by P300 blocks the association of IRS with IRβ, such that IRS cannot be phosphorylated at tyrosine residues. To test this hypothesis, Hepa1–6 cells were treated with C646, and specific antibodies of IRS1, IRS2 and IRβ were used to immunoprecipitate these proteins and their associated proteins. Inhibition of P300 acetyltransferase activity by C646 drastically increased the tyrosine phosphorylation of IRS1 and IRS2 and increased association of IRS1 and IRS2 with IRβ. However, C646 had a minimal effect on IRβ’s tyrosine phosphorylation. Our data suggest that the acetylation of IRS may function as a ‘gatekeeper’ to prevent the association of IRS with IRβ in the unstimulated state. In accordance with our findings, inhibition of deacetylase activity increased IRS2 acetylation, resulting in a reduction of IRS2 tyrosine phosphorylation (Kawada et al. 2017). However, it has been reported that IRS1 acetylation had a permissive effect on its tyrosine phosphorylation (Kaiser & James 2004, Tan et al. 2015), suggesting that IRS can be acetylated by other acetyl-transferases at different sites; these acetylation sites might have distinct functions in regulating insulin signaling and controlling cell growth.

### Endotoxemia-mediated P300 induction through the activation of the IRE1-XBP1s pathway

A high-fat, western-style diet is an important predisposing factor for the onset of diabetes and obesity. HFD feeding results in changes in gut microbiota that initiate endotoxin (LPS)-induced inflammation and ER stress (Cani et al. 2007, 2008, Li et al. 2012). We found that LPS levels were significantly increased in the liver of mice fed an HFD, and treatment with LPS triggered the activation of the IRE1-XBP1s pathway in the ER stress response and increased P300 protein levels in the liver and cultured hepatocytes. Although P300 is a nuclear protein, we found substantial amounts of P300 relocated into the cytoplasm in the liver cells of mice fed an HFD. Moreover, LPS treatment or overexpression of XBP1s resulted in P300 induction and cytoplasmic localization in hepatocytes, disrupting insulin signaling by acetylating IRS1/2 in the cytoplasm (Cao et al. 2017). Since LPS treatment significantly decreased ubiquitin-conjugated P300 and increased P300 protein levels, and XBP1s can bind directly to P300, it could be speculated that the binding of XBP1s to P300 prevents P300 ubiquitination and nuclear localization. Collectively, our study proposes a mechanism underlying the disruption of insulin signaling by elevated LPS in obese animal models through the acetylation of IRS1 and IRS2 by induced P300.

Figure 3
Identified acetylation sites in IRS1 and IRS2. Seven lysine residues in IRS1 and fifteen lysine residues in IRS2 can be acetylated. The tyrosine residues in IRS1 and IRS2, which are important for the generation of YXXM motif after phosphorylation, are also shown.
P300 acetyl-transferase activity is a potential therapeutic target

Previous studies showed that the inhibition of P300 and CBP acetyl-transferase activity by curcumin alleviated hyperglycemia in diabetic patients and animal models (Balasubramanyam et al. 2004, Marcu et al. 2006, Morimoto et al. 2008). We found that the inhibition of P300 acetyl-transferase activity by C646 significantly alleviated hyperglycemia in obese ob/ob mice and improved insulin sensitivity in HFD-fed mice (Cao et al. 2017). The improvement of insulin signaling by the inhibition of P300 acetyl-transferase activity leads to an increase in AKT-mediated phosphorylation and degradation of FoxO1 and CRTC2 (Accili & Arden 2004, Koo et al. 2005), two important transcription factors for gluconeogenic gene expression, which should lead to the suppression of hepatic glucose production. However, the complex of CREB-CBP/P300 is critical for glucagon stimulation of hepatic glucose production (He et al. 2009). Therefore, inhibition of P300 acetyl-transferase activity by C646 or curcumin can affect the function of the CREB-CBP/P300 complex, resulting in the suppression of hepatic glucose production as well. Furthermore, inhibition of histone acetyltransferase activity of P300 by C646 significantly decreased hepatic Foxo1 mRNA through the regulation of Foxo1 gene transcription (Wondisford et al. 2014). This line of evidence suggests that the P300 acetyl-transferase activity is a therapeutic target for the treatment of diabetes and obesity. Since P300 is an important co-activator in the activation of gene transcription and participates in many critical developmental processes, a key priority is undoubtedly the development of a drug targeting P300 acetyl-transferase activity in the liver. Previously, C646 was found to be a promising agent for cancer treatment (Santer et al. 2011, Yang et al. 2013), and we have found that this agent can be used to treat diabetes, with its primary target being hepatic P300 (Cao et al. 2017). However, it is conceivable that more work needs to be done before this agent can be used in clinic.

Perspective

Insulin resistance is the hallmark of type 2 diabetes, and defective hepatic insulin sensitivity in type 2 diabetes results in increased glucose production, which is the major cause of hyperglycemia in diabetic patients (Magnusson et al. 1992, Wajngot et al. 2001, Doria et al. 2008). Elevated serum LPS levels from changes in gut microbiota cause low-grade inflammation and ER stress, which play a critical role in the development of insulin resistance. We believe that the induction of P300 links inflammation and metabolic diseases. Even though we found that acetylation of IRS1 and IRS2 by P300 led to the impairment of insulin signaling by decreasing the association of IRS with IRβ, it is also possible that the acetylation of IRS might impede its tyrosine phosphorylation by receptor kinase IRβ, therefore resulting in insulin resistance. Because protein tyrosine phosphatase 1B is the protein phosphatase responsible for the dephosphorylation of tyrosine residues in mediators of insulin signaling (Kenner et al. 1996, Elchebly et al. 1999), it is also possible that acetylation of IRS1 and IRS2 will facilitate the dephosphorylation of tyrosine residues in these proteins. Since the cardinal feature of diabetes and obesity is insulin resistance, improving insulin resistance is a critical step in combating these metabolic diseases. Thus, the P300 acetyl-transferase activity appears to be a promising therapeutic target for the treatment of diabetic patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

This work was supported in part by grant from the National Institute of Diabetes and Digestive and Kidney Diseases R01DK107641.

References


Kanner KA, Ananyewu E, Olefsky JM & Kuri J 1996 Protein-tyrosine phosphatase 1B is a negative regulator of insulin- and insulin-like growth factor-I-stimulated signaling. Journal of Biological Chemistry 271 19810–19816. (https://doi.org/10.1074/jbc.271.33.19810)


Klein AL, Berkow BN, Nuse MG & Ball LE 2009 O-linked N-acetylglucosamine modification of insulin receptor substrate-1 occurs in close proximity to multiple SH2 domain binding motifs. Journal of Biological Chemistry 284 11454–11458. (https://doi.org/10.1074/jbc.M108.700435)


White MF, Maron R & Kahn CR 1985 Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. *Nature* 318 183–186. (https://doi.org/10.1038/318183a0)


Witters DJ, Burks DJ, Towery HH, Altamuro SL, Flint CL & White MF 1999 IRS-2 coordinates Igf-1 receptor-mediated beta-cell development and peripheral insulin signalling. *Nature Genetics* 31 34356–34364. (https://doi.org/10.1074/ng.318.118340)

