Role of Insulin in the Pathogenesis of Free Fatty Acid-Induced Insulin Resistance in Skeletal Muscle

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Abstract: Insulin resistance is a pathophysiological link of obesity to type 2 diabetes. The initial cause of insulin resistance is critical for prevention and treatment of type 2 diabetes. Lipotoxicity is a well-known concept in the explanation of initiation of insulin resistance. Although there are several prevailing hypotheses about the cellular/molecular mechanisms of lipotoxicity, such as inflammation, oxidative stress, hyperinsulinemia, and ER stress, the relative importance of these hypothesized events remains to be determined. The role of hyperinsulinemia is relatively under documented in the literature for the initiation of insulin resistance. In this review, an interaction of fatty acid and beta-cells, and a synergy between free fatty acids (FFAs) and insulin are emphasized for the role of hyperinsulinemia. This article presents the evidence about FFA-induced insulin secretion *in vitro* and *in vivo*, recent advances in the molecular mechanism of FFA action in beta-cells, a role of GPR40 in the development of insulin resistance, and the negative feedback loop of the insulin receptor signal pathway. The negative feedback loop is discussed in detail with a focus on IRS-1 serine kinases. This article provides a substantial support for the role of insulin in the early stages of FFA-associated insulin resistance. The hypothesis of insulin's role in lipotoxicity is referred to as the "**insulin hypothesis"** in this review. According to this hypothesis, prevention of increased beta-cell response to glucose may be a potential approach for early intervention of metabolic syndrome.

I. HISTORY OF INSULIN RESISTANCE THEORIES

Insulin resistance is a hallmark feature of type 2 diabetes. Insulin resistance is defined as an impaired ability of the hormone to suppress hepatic glucose output and to promote peripheral glucose disposal. It is believed that insulin resistance is a result of lipotoxicity, a well-accepted concept in the understanding of the pathogenesis of obesity-associated insulin resistance [1,2]. Although there are several prevailing hypotheses about the cellular/molecular mechanisms of lipotoxicity (Fig. **1**), such as inflammation [3,4], DAG-PKC [5], hyperinsulinemia [2], ER stress [4,6], and oxidative stress [7,8], the relative importance of these hypothesized events in the initiation of insulin resistance remains to be determined. Hyperinsulinemia is a common feature of obesity. Hyperinsulinemia and insulin resistance coexist in obesity and metabolic syndrome [1]. Regarding the relationship of insulin resistance and hyperinsulinemia, there has been confusion about which occurs first in the obese condition. Insulin resistance is known to induce hyperinsulinemia, but a high level of insulin is also known to induce insulin resistance. Since hyperinsulinemia can be detected easily, it was believed that the high level of insulin leads to insulin resistance in obesity. This concept is the core of "insulin hypothesis". However, in recent years, the insulin hypothesis has been replaced by the compensation hypothesis [9-11], which suggests that hyperinsulinemia is a result of the beta-cell's compensation for insulin resistance. This hypothesis led to a huge campaign to search for the cause of insulin resistance. In this regard, lipotoxicity was proposed as a major cause of insulin resistance. Under this concept, several hypotheses, including inflammation [3,4], DAG-PKC [5], ER stress [4,6], and oxidative stress [7], have been developed to specify the cellular and molecular mechanisms of lipotoxicity. These hypotheses are supported by evidence in mainstream publications. Although these hypotheses have provided promising leads to the mechanism of lipotoxicity, they also raise some questions, such as: (a) What mechanism is used by FFA to activate inflammation, DAG-PKC, ER stress, and oxidative stress? (b) What is the relative importance of these hypothesized events in insulin resistance? (c) Is there a common pathway used by FFA in the induction of insulin resistance? An increasing body of evidence consistently suggests that the insulin hypothesis may provide an answer to these questions. This is reflected by recent findings about the role of the FFA receptor GPR40 in beta-cells [12,13].

II. INSULIN HYPOTHESIS

A high level of insulin is known to contribute to insulin resistance. This is well-established in both cellular and animal models. *In vivo*, insulin injection induces insulin resistance in humans, dogs, and rats [14-18]. *In vitro*, insulin at physiological (<5 nM) or pharmacological (100 nM) concentrations induces insulin resistance in adipocytes and in muscle cells [19-23]. At the molecular level, insulin leads to a reduction in activity of signaling components, such as IR, IRS-1, PI3K, Akt, and GLUT4 proteins, in the insulin receptor signaling pathway in 3T3-L1 adipocytes [24-28]. In human and rodents, lipid infusion leads to an increase in plasma insulin and this occurs within hours after elevation of FFA in the circulation. The increase in insulin precedes the reduction in insulin sensitivity [29,30]. These observations suggest that hyperinsulinemia contributes to insulin resistance.

FFA induction of insulin secretion in beta-cells was briefly discussed in a review by McGarry [31]. Here, a history of relevant studies is reviewed in light of the insulin hypothesis. It has been known for almost 40 years that FFA

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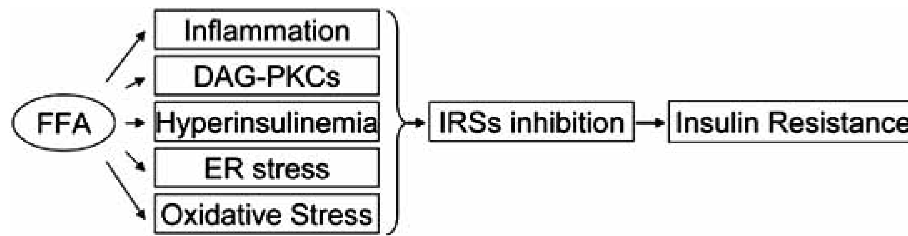

Fig. (1). Mechanisms of FFA-associated insulin resistance. FFA is able to induce many kinds of responses as listed in the figure to induce insulin resistance. These responses lead to the defect in post-receptor signal transduction by inhibition of IRSs function, which happens after over phosphorylation of Ser/Thr residues in IRSs protein. Abbreviation: FFA, Free Fatty Acid; DAG, Diacylglyerols; PKC, Protein Kinase C; ER stress, Endoplasmic Reticulum stress; IRSs, Insulin Receptor Substrates.

are able to induce insulin secretion [32,33]. In 1967, two independent groups reported that plasma FFAs were able to increase insulin levels in the blood [32,33]. In these studies, plasma FFAs were raised by infusion of triglyceride emulsion. Later, similar observations were reported by other labs [29,34]. One of these studies demonstrated that heparin was able to increase plasma FFA in lipid infusion [35]. A combination of heparin and a triglyceride emulsion had a much stronger effect in the induction of FFA (more about heparin below). As a result, the increase in plasma insulin was enhanced by heparin in lipid infusion. In anesthetized dogs with chronic portacaval shunts, the FFA-induced elevation in insulin was associated with a fall in blood glucose [34]. The cellular mechanism of FFA-induced insulin elevation is related to stimulation of beta-cells by long chain fatty acids. FFAs were reported to stimulate beta-cells for insulin secretion [36-38]. Although these studies demonstrate that in beta-cells, acute effect of FFAs is to stimulate insulin secretion, other studies show that chronic exposure of beta-cells to high levels of FFAs results in impairment of insulin production, a phenomenon recognized as lipotoxicity [31].

Dietary FFAs also promote insulin secretion in beta-cells. However, the insulin elevation in plasma is much smaller than that induced by intravenous lipid infusion. Intravenous lipid infusion and fat ingestion were compared for induction of plasma insulin [39]. Infusion of sodium oleate (FFA source) plus heparin induced a rapid increase (2- to 12-fold) in plasma insulin in conscious dogs. The change in insulin was followed by a marked fall in blood glucose. The glucose reduction was so strong that it occurred even under intravenous glucose infusion. The magnitude of changes in insulin and glucose was positively correlated with the mean value of plasma FFAs during the lipid infusion. A large increase in plasma insulin and fall in glucose also occurred when oleate was infused together with glycerol, which was used to simulate endogenous lipolysis. The insulin level in the pancreaticoduodenal vein blood increased markedly during oleate infusion, while plasma ketone level changed only slightly. With oleate infusion at 36.5 uEq/kg/min, the plasma FFA was increased at 45 mins and the elevation remained for 90 mins. In contrast, FFA elevation from triolein ingestion was observed 8 hrs later. Although insulin change matched the FFA increase in both cases, lipid infusion had a much stronger effect with 2-12 fold increase in insulin. Fat ingestion produced a smaller increase (10%) in plasma insulin. Infusion of heparin without lipid also led to insulin elevation in the control group, but on a much smaller scale (<10%).

Heparin synergizes with lipid in the induction of insulin secretion. The synergistic effect of heparin and lipid was first reported when the lipid-heparin protocol (fat meal-heparin regimen) was invented [35]. In this study, heparin was found to regulate plasma FFAs in healthy subjects. The volunteers were fed with a fat-meal that contained 60 g corn oil. Plasma FFAs (NEFA) increased 50-60% (from 488 + 45 to 767 + 11 uEq/L) in three hours. At this time point, intravenous injection of 50 mg of heparin sodium led to a more than 200% increase in plasma FFAs (from to 767 + 11 to 1,929 + 274 uEq/L) within 15 minutes. The plasma FFAs were maintained at over 1,300 uEq/L for more than an hour. This study established the role of heparin in the regulation of plasma FFAs. Heparin provided an answer to the stronger effect of lipid infusion than lipid ingestion in the induction of insulin secretion. Heparin was able to increase the hydrolysis of triglyceride into FFAs and the hydrolysis was increased by three fold [40]. These studies provided a rationale to include heparin in the standard protocol of lipid infusion, which is widely used in the study of glucose metabolism in response to FFA. Therefore, the action mechanism of heparin may be related to hydrolysis of triglyceride that leads to a increase in plasma FFAs. FFA elevation was shown to impair glucose tolerance in 1965 [35]. This may represent early evidence that FFA induces insulin resistance.

Chronic exposure of beta-cells to high levels of FFAs may lead to hyperinsulinemia. Plasma FFAs are required for beta-cells to maintain their responsiveness to glucose [41]. This is important in the fasting condition as an elevation in FFAs leads to an enhanced insulin secretion in response to glucose [41]. Chronic exposure of beta-cells to elevated FFAs may lead to hyperinsulinemia through an increase in beta-cell insulin secretion, which occurs in response to the basal level of glucose in fasting condition. This possibility is supported by several *in vitro* studies using pancreatic islets [42-44]. Beta-cell response to glucose was investigated after FFA-treatment [43]. After exposure to FFA for 48 hrs, beta-cells exhibited a seven-fold increase in insulin secretion in response to the basal level of glucose (3.3 mM) [43]. A 30-40% decrease in insulin secretion was observed in the beta-cells at a high level (27 mM) of glucose. After a 7-day exposure to FFA, beta-cells exhibited a two-fold increase in insulin secretion in response to the basal level of glucose (3 - 5.6 mM). In the same study, pancreatic islets were compared between lean Wistar rats and obese Zucker rats. The pancreatic islets of Zucker rats produced 1.5 fold more insulin in response to the basal level of glucose. The fold induction

was normalized with the beta cell volume in each rat. Since hyperlipidemia is associated with hyperinsulinemia in the obese Zucker rats, these data support that the hyperlipidemia- or FFA-induced insulin production under the low level of glucose may contribute to hyperinsulinemia.

III. MECHANISM OF FFA-INDUCED INSULIN SECRETION

The molecular events underlying the FFA-induction of insulin secretion have been identified recently. Three mechanisms have been proposed to explain the activity of FFAs in beta-cells: (a) FFAs induce insulin secretion through the cell membrane receptor GPR40 [45]; (b) FFA derivatives, such as LC-CoA or malonyl-CoA, promote beta-cell response to glucose or activators of serine kinases PKC and PKA [46-49]; (c) FFAs activate GPR120 in the intestine to stimulate secretion of insulin-inducing peptides, such as GLP-1 [50]. The three mechanisms are discussed below.

GPR40 (G-protein-coupled receptor 40) is abundantly expressed in the pancreatic beta-cells, where it serves as a cell membrane receptor for long-chain FFAs [45]. Activation of GPR40 by long-chain FFAs leads to amplification of the glucose signal in beta-cells. GPR40 was an orphan (whose ligands were unidentified) G-protein-coupled receptor (GPCR) originally isolated from a human genomic DNA fragment. GPR40 is highly conserved cross species and its cDNA has been cloned from human, monkey, mouse, rat and hamster. Expression of GPR40 mRNA was examined in variety of tissues and cell lines with quantitative RT-PCR [45]. The highest expression level of GPR40 was found in the pancreatic islet beta-cells or beta-cell lines, such as: MIN6, BetaTC-3, and HIT-T15. GPR40 mRNA was not found in liver, adipose tissue, skeletal muscle, and non-beta cell lines that include 3T3-L1, C2C12, PANC-1 and NIH3T3. Long chain fatty acids, including saturated (C12 to C16) and unsaturated (C18 to C20) fatty acids, were all able to activate GPR40 as determined by Ca^{2+} mobilization. FFAs only activate GPR40 in the absence of BSA. FFAs bound to BSA can not activate GPR40.

GPR40 activates beta-cells through the PLC and cAMP pathways (Fig. **2**). It was believed that FFAs must be metabolized into long-chain fatty acyl-coenzyme A in beta-cells to stimulate insulin secretion [51,52]. Long-chain acyl-CoA esters (LC-CoA) were believed to be the mediators of FFA signals [53,54]. However, this concept has recently been challenged by several studies about the signal transduction pathway of GPR40 [45,55-57]. In the signaling pathway of GPR40, FFA induce activation of phospholipase C (PLC), which induces hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate (IP3). Then, IP3 mobilizes intracellular Ca^{2+} from the endoplasmic reticulum [58,59]. In one study, oleic acid (OA) was examined in rat primary beta-cells in a single cell system [58]. OA has a high affinity for rat GPR40. At 1-10 uM, OA increased Ca^{2+} in a dose-dependent manner in the presence of glucose (5.6, 8.3, and 11.2 mM). GPR40 is required for the OA activity, as knockdown of GRP40 led to inhibition of OA activity. Phospholipase C (PLC) is also required, as PLC inhibitors blocked OA activity. A similar observation was made in INS-1E beta-cells [59]. Ca^{2+} influx and voltage-gated Ca^{2+} channels are involved in FFA-induced intracellular Ca^{2+} mobilization in beta-cells [58,59]. In addition to the PLC-dependent Ca^{2+} pathway, the cAMP/PKA pathway may also be involved. One study suggests that the unsaturated FFA linoleic acid (C18:2) leads to the Ca^{2+} increase through inhibition of the voltage-gated K^+ current in rat pancreatic beta-cells, and that this event is dependent on activation of the cAMP/PKA pathway downstream of GPR40 [60].

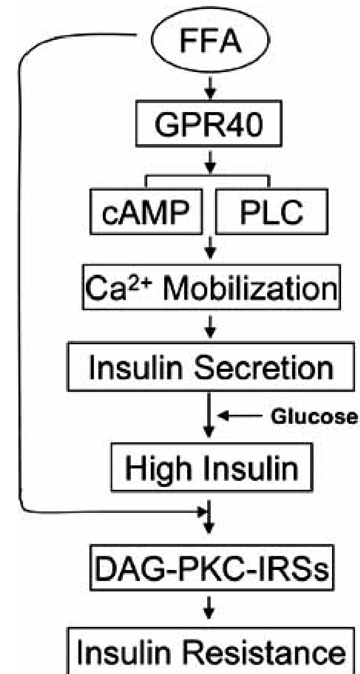

Fig. (2). Signaling pathway of GRP40 for insulin secretion. FFA binds to GPR40 receptor in beta-cells, and enhances signaling activity of cAMP, which induces calcium mobilization in beta-cells. This change enhances beta-cell response to glucose in the secretion of insulin. High level of plasma insulin and FFA may promote insulin resistance through DAG-PKC-IRSs pathway. Abbreviation: GPR40, G-Protein-coupled Receptor 40; cAMP, Adenosine 3′,5′-cyclic Monophosphate; DAG, Diacylglyerols; PKC, Protein Kinase C; IRSs, Insulin Receptor Substrates.

Beta-cells are primed by GPR40 signal for a stronger response to the low level of glucose [45,55-57]. A new study suggests that GPR40 is required for the pathogenesis of hyperinsulinemia in obese mice [57]. This was demonstrated in gene knockout mice with dietary obesity. In the GPR40 knockout (GPR40-/-) mice, body weight was increased on a high fat diet and the weight gain rate was identical to the wild type (WT) mice. Unlike the WT mice, the KO mice did not develop hyperinsulinemia in the obese condition. Consistently, peripheral insulin sensitivity, which was determined with glucose tolerance, insulin tolerance, plasma glucose, and hepatic glucose production, was impaired in the WT mice, but not in the KO mice. Given the tissue-specific role of GPR40 in beta-cells, the study suggests that inactivation of the FFA-GPR40 signaling pathway is sufficient to prevent hyperinsulinemia in the obese condition. Since the KO mice did not develop insulin resistance, the study suggests that in the WT mice, insulin resistance might be a consequence of

hyperinsulinemia. This study provides a piece of key evidence that beta-cells are major targets of FFAs for hyperinsulinemia, and that insulin resistance might be a result of hyperinsulinemia.

FFA derivatives, such as LC-CoA or malonyl-CoA, may be involved in beta-cell responses to glucose [46-49]. Exposure of islets to palmitate in tissue culture increased beta-cell responses to glucose. This effect of FFA was blocked by Triacsin C, an inhibitor of fatty acyl-CoA synthase [46-48], implying that an increase in LC-CoA was required in beta-cells for the FFA-associated super response to glucose. FFA may also act through induction of glucagon-like peptide-1 (GLP-1) [50]. G-protein-coupled receptor 120 (GPR120), which is abundantly expressed in the intestine, was shown to function as a receptor for unsaturated long-chain FFAs [50]. Activation of GPR120 by FFAs promoted the secretion of GLP-1 *in vitro* and *in vivo*, and this was associated with an increase in circulating insulin. The significance of the GPR120-GLP1 pathway in the body is not clear. In GPR40 KO mice, this pathway is intact. Since the KO mice were protected from hyperinsulinemia in the presence of GPR120-GLP-1 activity, the phenotype suggests that GPR40 is more important than GPR120 in the pathogenesis of hyperinsulinemia and insulin resistance.

FFA-induced insulin secretion in beta-cells is dependent on glucose [45,55-57]. Although many studies show that plasma insulin is increased after lipid infusion, the increase was not observed in all the studies [35]. This discrepancy might be a result of difference in blood glucose. In the fasting condition, the amount of insulin production is minimal in beta-cells as a result of low level of glucose. With a higher level of glucose, the amount of insulin produced in response to FFAs is maximized. The increase in plasma insulin is easy to be detected. This possibility was supported by a study that FFA-associated insulin secretion was enhanced by glucose under hyperglycemic conditions in humans [29].

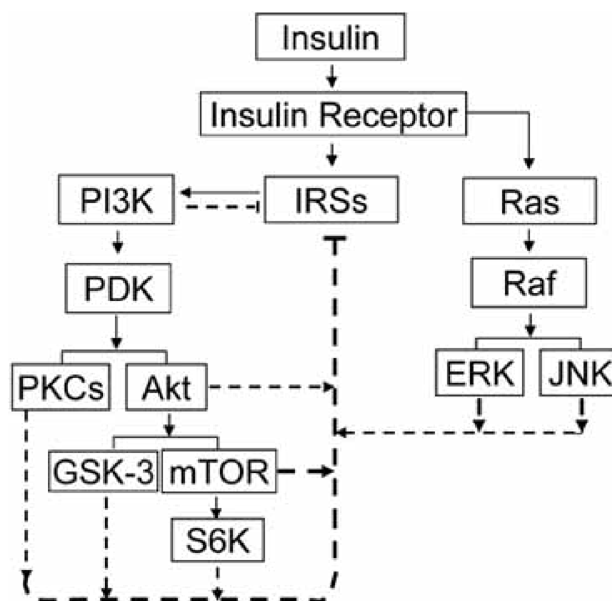

Fig. (3). The negative feedback loop of insulin signaling pathway. Serine kinases in the insulin signaling pathway mediate metabolic and growth signals of insulin. Many of them also are able to inhibit IRSs function through serine/threonine phosphorylation. These kinases include PI3K, Akt, PKCs, mTOR, S6K, and GSK-3 in the metabolic branch of insulin signaling pathway. JNK and ERK are two kinases in the growth-control branch of insulin signaling pathway. The negative feedback loop is indicated by the broken lines. Abbreviation: IRSs, Insulin Receptor Substrates; PI3K, Phosphatidylinositol-3 Kinase; PDK, Phosphoinositide-Dependent Kinase; Akt (PKB), Protein kinas B; PKC, Protein Kinase C; mTOR, Mammalian Target Of Rapamycin; GSK-3, Glycogen Synthase Kinase 3; S6K, S6 Kinase; JNK, c-JUN N-terminal Kinase; ERK, Extracellular signal-Regulated Kinases.

IV. MECHANISMS OF INSULIN RESISTANCE BY INSULIN

The insulin signaling pathway has a negative feedback loop (Fig. **3**), which is activated by insulin and involved in the inhibition or termination of the metabolic branch of the insulin receptor pathway. In the negative feedback loop, insulin receptor and insulin receptor substrates (IRSs) are the targets of feedback regulation. Here, insulin receptor substrate 1 (IRS-1) is used as an example for negative feedback since there is abundant information about IRS-1 activity in the literature.

IRS-1 links the insulin receptor to downstream serine kinases in the insulin signaling pathway [61,62]. IRS-1 (160-185kDa) was cloned in 1991 as a tyrosine kinase substrate of the insulin receptor from rat liver cells [63]. IRS-1 is a conserved and ubiquitous protein in all types of tissue cross species. Human IRS-1 is encoded by a single exon on human chromosome 2q36-37 (mouse chromosome 1) and is 88% identical with rat IRS-1 [64]. Human IRS-1 mRNA is rare and consists of two species of 6.9 and 6 kilobases. IRS-1 contains 1242 amino acids with a calculated molecular mass of 132 kDa, but migrates between 160 and 185 kDa in SDS-PAGE due to extensive serine phosphorylation from post-translational modification [63-65]. There are more than 50 Ser/Thr phosphorylation sites in human IRS-1 [64] and more than 30 potential Ser/Thr phosphorylation sites in rat IRS-1 [63]. Phosphorylation of some of these sites is inducible and is responsible for the inhibition of IRS-1 function. In the basal condition, IRS-1 stays in the internal membrane or perinuclear membrane [66,67]. After insulin stimulation, IRS-1 is translocated to the plasma membrane [66] and becomes activated to bind 85-kDa subunit of PI3K [68]. After 30 minutes of insulin treatment, IRS-1 spreads in the cytosol [67]. This redistribution is dependent on the serine phosphorylation of IRS-1.

IRS-1 activity is regulated by tyrosine and serine phosphorylation. IRS-1 contains over ten potential tyrosine phosphorylation sites, six of which are in Tyr-Met-X-Met motifs, a recognition site of the Src homology (SH) 2 domains. IRS-1 acts as a multisite 'docking' protein to bind signal-transducing molecules containing Src-homology 2 and Src-homology-3 domains. Tyrosine phosphorylation is required for the function of IRS-1. Tyrosine phosphorylation is catalyzed by the insulin receptor β subunit, the catalytic subunit of the insulin receptor. The tyrosine-phosphorylated IRS-1 activates downstream PI3K by interaction with the regula-

tory p85 subunit of PI3K. Tyrosine phosphorylation of IRS-1 is decreased in cells expressing mutant insulin receptors, treated with PKC activator phorbol esters, [69] or the inflammatory cytokine TNF-alpha [70].

Serine phosphorylation of IRS-1 may have double effects in the regulation of IRS-1 function. Non-inducible Ser/Thr phosphorylation (also called basal phosphorylation) is a result of posttranslational modification and seems to be required for maturation of IRS-1 [71]. A reduction in basal Ser/Thr phosphorylation sites by mutation impaired subsequent tyrosine phosphorylation, suggesting that basal Ser/Thr phosphorylation of IRS-1 plays a positive role in IRS-1 activity. Inducible Ser/Thr phosphorylation serves as a negative regulatory mechanism for IRS-1, and contributes to the post-receptor signaling deficiency in the insulin receptor pathway [69,72-80]. Induced serine phosphorylation is triggered by many stimuli, including: TNF-alpha [70,73,74,81], insulin [25,82], insulin like growth factor (IGF) [82], serine phosphatase PP1 or PP2A inhibitors (Calyculin A or Okadaic acid) [74,79,83, 84], and PKC activators [75]. Induced serine phosphorylation has several negative effects on IRS-1: (a) leads to inhibition of tyrosine phosphorylation of IRS-1 [70,81,85]; (b) reduces the protein level of IRS-1 [27,86-88]; (c) reduces IRS-1 association with PI3K [74]. All of these events contribute to inhibition of IRS-1 function.

Several inducible serine phosphorylation sites have been identified in IRS-1. These include Ser307 in rodent IRS-1 (Ser312 in human IRS-1) [82,85,89], Ser612 (Ser616 in human IRS-1) [75,76,85], Ser265 (Ser270 in human IRS-1) [90,91], Ser636/639 [92], and Ser632/662/731 [93]. Ser307/Ser312 phosphorylation is induced by several factors including TNF-alpha [82,89,94], insulin [82], IGF [82], IL-6 [95], and FFA [96,97]. Phosphorylation of Ser307/Ser312 contributes to insulin resistance induced by the above factors. The mechanisms include impaired IRS-1 interaction with the insulin receptor [85] and PI3K [97], and accelerated IRS-1 degradation [28,97]. In addition to Ser307/312, other serine residues including Ser612/616 [85] and Ser636/639 [92] are also phosphorylated after TNF stimulation. It has been reported recently that in mouse IRS-1, insulin or phorbol ester induced phosphorylation at seven serine residues (S265, S302, S325, S336, S358, S407, and S408) [98]. Phosphorylation of these serine residues contributes to inhibition of IRS-1 function in response to insulin or phorbol ester [98]. Mutation of these serines into alanines (cannot be phosphorylated) led to enhanced IRS-1 interaction with the insulin receptor after insulin treatment. Of the seven serine residues, S408 was further characterized and was believed to be a major inhibitory site. Studies also suggest that inducible serine phosphorylation can promote signaling activity of IRS-1. For example, S318 phosphorylation was shown to promote IRS-1/Akt interaction in muscle treated with IL-6, but not in liver [95]. Phosphorylation of S302 [99] and S270 [91] in IRS-1 were also shown to promote IRS-1/Akt interaction.

The search for IRS serine/threonine kinases has led to the identification of several candidates for IRS-1 kinases, based on their abilities to phosphorylate IRS-1 *in vitro* and *in vivo* (Fig. **4**). These serine kinases are mainly components of the MAPK (Mitogen-Activated Protein Kinase) and PI3K signaling pathways.

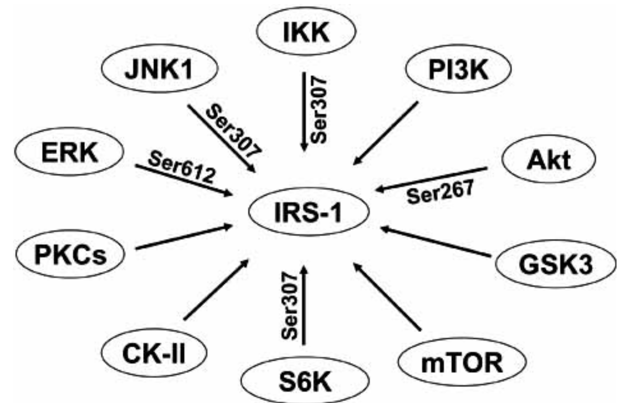

Fig. (4). Serine kinases of IRS-1. These kinases include JNK, ERK, IKK, PI3K, Akt, PKC, mTOR, S6K, GSK-3 and CKII. The phosphorylation sites in rodent IRS-1 are indicated for some of the kinases. Abbreviation: IRSs, Insulin Receptor Substrates; JNK, c-JUN N-terminal Kinase; IKK, Inhibitor KappaB Kinase; ERK, Extracellular signal-Regulated Kinases. PI3K, Phosphatidylinositol-3 kinase; PDK, phosphoinositide-dependent kinase; Akt (PKB), Protein kinas B; PKC, Protein Kinase C; mTOR, Mammalian Target Of Rapamycin; GSK-3, Glycogen Synthase Kinase 3; S6K, S6 kinase; CK-II, Casein Kinase II.

(1) **JNK** (c-JUN N-terminal Kinase): JNK was identified as a serine kinase for mouse S307 (S312 in human) phosphorylation in IRS-1 [82,89]. JNK was cloned in 1994 as a serine kinase activated by UV-light, EGF, TNF-alpha, and stress [100-103]. JNK has several isoforms, including: JNK1, JNK2, and JNK3. Since JNK and its isoforms are activated by stress signals, they are also called Stress-Activated Protein Kinases (SAPKs) [103]. Activation of JNK is marked by transient phosphorylation at two amino residues (Thr183 and Tyr185) by its upstream kinase MKK4 or MKK7 (MAPK kinase 4 or 7). Many studies suggest that JNK is a major kinase for insulin resistance induced by TNF-alpha, FFA, insulin, PKC activators (DAG), and ER stress [82,85,94,97,104-106]. JNK was shown to phosphorylate IRS-1 at S307/312 and S302 [85,107]. The role of JNK in obesity-associated insulin resistance is supported by data from several lines of transgenic mice [108-110].

(2) **ERK** (Extracellular signal-Regulated Kinases): Several studies indicate that ERK can phosphorylate IRS-1 at a couple of serine residues [75,76,82,111]. ERK is a serine kinase that can be activated by many extracellular signals. ERK is a member of the MAPK family that includes ERK, JNK and p38. ERK has two major isoforms: ERK1 (44 kDa) and ERK2 (42 kDa). Activation of ERK is marked by rapid phosphorylation of threonine and tyrosine (Thr202/Tyr204) residues by its upstream kinase MEK1 (Map/Erk kinase-1). It was shown that PKC induces S612 phosphorylation of mouse IRS-1 [75]. ERK2 may mediate PKC signal for the S612 phosphorylation [76]. ERK may also mediate TNF-alpha and EGF signals for S612 phosphorylation [74,94].

(3) **PI3K** (Phosphatidylinositol-3 Kinase): It was reported that PI3K directly phosphorylated IRS-1 on serine residues in the response to insulin [78,112,113] or by overexpression of the active form of PI3K [114]. PI3K is a dual

kinase that phosphorylates the phospholipid PtdIns at the 3-hydroxyl group of the inositol ring and proteins at serine residues. PI3K is a key kinase to mediate the insulin signal at a step next to IRS-1. PI3K has two subunits, the regulatory subunit p85 that interacts with IRS-1 or IRS-2, and catalytic subunit p110 that induces phosphorylation of substrates, such as the phospholipid PtdIns. Under insulin stimulation, PI3K induced IRS-1 phosphorylation at serine residues [78]. This serine phosphorylation was blocked by PI3K inhibitors (wortmannin) [112]. However, the position of the phosphorylated serine residue remains unknown. In addition to this direct phosphorylation by PI3K, IRS-1 might also be phosphorylated by kinases downstream of PI3K.

(4) **Akt** (Protein Kinase B, PKB): Akt is a downstream kinase of PI3K in the insulin signaling pathway. PI3K activates Akt through phosphorylation of Thr308 in the Akt protein by phosphoinositide-dependent kinase (PDK). Activation of the catalytic activity of Akt is marked by phosphorylation at both Thr308 and Ser473 in the Akt protein. The role of Akt in the regulation of IRSs was studied with mouse IRS-1 [91]. There are four consensus Akt phosphorylation sites (RXRXXS/T) in IRS-1 and IRS-2 proteins [91]. These four sites are located in the PTB domain of IRS-1 (amino acids 155-309) that interacts with the NPEY motif within the juxtamembrane (JM) region of the insulin receptor. The four serine residues in rodents IRS-1 are S265 (S270 in humans), S302 (S307 in humans), S325 (S330 in humans) and S358 (no equivalent in humans). Akt is able to phosphorylate IRS-1 at these residues *in vitro* and *in vivo*. Mutation analysis of the Akt consensus sites suggests that phosphorylation of these sites by Akt might protect IRS-1 from tyrosine dephosphorylation by tyrosine phosphatase. This is the first study suggesting that serine phosphorylation of IRS-1 enhances IRS-1 function [91].

(5) **mTOR** (Mammalian Target of Rapamycin): mTOR is one of the serine kinases downstream of PI3K/Akt in the insulin signaling pathway [115]. Several studies suggested that mTOR could result in IRS-1 serine phosphorylation in response to several extracellular signals including insulin [116,117], TNF-α (at Ser636/639) [92], amino acids, [118] and PDGF (at Ser 632/662/731) [93]. mTOR-induced phosphorylation occurs at several serine residues including S636/S639 [117], S632/662/731 [93], S307/312 [119] and S302 [99]. Activation of mTOR may lead to a decrease in tyrosine phosphorylation of IRS-1, and at the same time increase proteasome-dependent degradation of IRS-1 [116]. S307/312 was indicated as a target of mTOR since its phosphorylation was blocked by mTOR inhibitors [28,120-122]. Knockout of the mTOR downstream kinase S6K1 (S6 kinase 1) led to less S307 phosphorylation [119], suggesting that mTOR may also act through S6K1. Consistently, mTOR activity is increased in liver and muscle in the obese condition, and the increase is associated with IRS-1 phosphorylation at other serine residues like S636/S639 [117]. mTOR/S6K1 was also reported to induce S302 phosphorylation in rodent IRS-1, leading to an enhanced signaling activity of the insulin receptor [99].

(6) **GSK-3** (Glycogen Synthase Kinase 3): GSK-3 is another serine kinase downstream of PI3K/Akt. GSK-3 was shown to phosphorylate IRS-1 at serine residues *in vivo* and *in vitro* [77]. The phosphorylation occurred at Thr502/Ser99 and this modification converted IRS-1 into an inhibitor of insulin receptor tyrosine kinase *in vitro*. Expression of wild-type GSK-3 in CHO cells that overexpress IRS-1 and IR increased serine phosphorylation of IRS-1 and decreased tyrosine phosphorylation of IRS-1, suggesting that IRS-1 is a molecular target of GSK-3. In a recent study, S332 was identified as the GSK-3 target in the IRS-1 protein [123].

(7) **PKC** (Protein Kinase C): PKC is closely involved in lipid-induced insulin resistance. PKC has multiple isoforms that are generally divided into three groups: classical PKC (α, βI, βII, γ), novel PKC (σ, ε, η, θ, μ), and atypical PKC (ζ, λ, ι). PKCzeta (PKCζ) is a serine/threonine kinase downstream of PI3K in the insulin signaling pathway. *In vitro*, wild-type PKCζ (but not kinase-deficient mutant PKCζ) phosphorylated IRS-1 [124,125]. Overexpression of PKCzeta in NIH-3T3 [124] and Fao [125] cells significantly impaired tyrosine phosphorylation of IRS-1 and reduced IRS/PI3K interaction in response to insulin. It was shown that PKCzeta phosphorylated rat IRS-1 at S318 (S323 in human) and prevented IRS-1 interaction with insulin receptor [126]. It was shown that activation of PKCζ, not Akt, by insulin is normal in diabetic animals [127]. This suggests that PKCζ may mediate insulin resistance in obesity. Other isoforms of PKC, including PKCalpha, PKCbeta1/2, PKCepsilon, and PKCtheta were also suggested to directly phosphorylate IRS-1 at serine residues, such as S318 [107,122]. PKCdelta and PKCepsilon were shown to phosphorylate IRS-1 at multiple sites including S307 [128,129]. PKCtheta was shown to mediate FFA-induced insulin resistance [5,97] and induce IRS-1 serine phosphorylation at S307 and S1101 [96,130].

(8) **IKK** (Inhibitor Kappa B Kinase): IKK was shown to phosphorylate IRS-1 at S307/312 leading to a reduced IRS-1 interaction with insulin receptors [131]. Data from IKK2 (IKKbeta) knockout or over-expression mice suggested that IKK activity contributed to obesity-associated insulin resistance [132-134]. Activation of IKK2 might be a result of the increased expression of TNF-alpha and elevated FFA levels in the obese condition. Both TNF-alpha and FFA are able to activate IKK2 for insulin resistance in cellular models [94,97]. Consistently, TNF-alpha was shown to induce insulin resistance in muscle through IKK activation, and p38 was required for IKK activation [135].

(9) **CK-II** (Casein Kinase II): Though casein kinase 2 mainly phosphorylates rat IRS-1 on Thr502, and weakly on S99, the major site (Thr502) is not conserved between human and rat [136].

Above information suggests that of the nine IRS-1 serine kinases, seven (JNK, ERK, PI3K, Akt, mTOR/S6K, GSK-3, PKC) are activated by insulin. Activation of these kinases may lead to inhibition of IRS-1 function through serine phosphorylation (Fig. **3**). These serine kinases form a negative feedback loop in the insulin receptor signaling pathway to warrant accurate and dynamic control of the insulin signal. Additionally, these serine kinases are also activated by other signals, such as: the inflammatory cytokine TNF-alpha, FFA, oxidative stress, or ER stress. Therefore, this negative feedback loop might be used by these factors to inhibit IRS-1 function, causing insulin resistance. In this negative feed-

back loop, the IRS-1 protein may be a direct substrate of the kinases. It can also be an indirect target of many kinases, such as PI3K, Akt, and PKC.

V. INSULIN SYNERGIZES WITH FFAs IN THE INDUCTION OF INSULIN RESISTANCE

It is rare to find insulin resistance in insulinoma patients. This suggests that a high level of insulin alone may not be sufficient to induce insulin resistance. The synergy between FFA and insulin may provide an alternative approach for insulin to induce insulin resistance. This possibility was supported by study in which the effects of FFA were investigated in a test of euglycemic-hyperinsulinemia clamp in human volunteers [137]. In the study, an intravenous infusion of lipid/heparin was used to increase plasma FFA. The FFA effect was tested on carbohydrate and fat oxidation (by indirect calorimetry), and on glucose disappearance rate during euglycemic-hyperinsulinemia clamp test in healthy men. It was concluded that under hyperinsulinemia, FFA promptly replaced carbohydrate as fuel for oxidation in muscle, and inhibited glucose uptake by peripheral tissues.

The mechanism of FFA+insulin synergy might be related to activation of the PKC signaling pathway in skeletal muscle (Fig. **2**). In human [137-139] and animal studies [5,96], intravenous infusion of lipid/heparin was shown to induce systemic insulin resistance 3 hours after initiation of the infusion. In muscle, activation of PKCtheta was detected when insulin resistance occurred [5,96,140]. Activation of PKCtheta is associated with an increase in intracellular DAG [96,140], a metabolic activator of PKCs. Insulin can promote PKC activation through several approaches. First, insulin induces biosynthesis of DAG. This is related to an increase in uptake of glucose, FFA, and expression of related enzymes. Increased GLUT4 translocation and glucose uptake can stimulate DAG biosynthesis [141]. In addition to GLUT4, insulin also stimulates membrane translocation of fatty acid transporters, such as CD36 [142] and FATP [143], which promote uptake of FFA by muscle and adipose cells. Insulin activates expression of enzymes for DAG biosynthesis, such as acetyl CoA carboxylase and fatty acid synthase, through activation of the transcription factor SREBP [144]. DAG can activate classical (cPKC) and novel PKC (nPKC), but not atypical PKC (aPKC), because aPKC lacks one of the C1 domains found in cPKC and nPKC. Secondly, insulin may induce phosphorylation of PKCs at the activation loop, and this is mediated by PDK1. PDK1 in the PI3K-dependent pathway was shown to phosphorylate all of the PKC isoforms [145]. This phosphorylation is required for the activation of PKCs by DAG. Under euglycemic-hyperinsu-linemic clamp testing, an increase in plasma FFA by lipid/ heparin infusion was associated with an increase in DAG mass in muscle in healthy volunteers [140]. A similar observation was also made in healthy rats [96]. The increase in DAG was associated with the activation of PKCtheta and S307 phosphorylation in IRS-1 for muscle insulin resistance.

The role of insulin in the FFA+insulin synergy suggests that insulin sensitivity is required for the synergistic effect to occur. The synergistic effect should be stronger in tissues with higher insulin sensitivity. In tissues with insulin resistance, the activity of insulin in the stimulation of DAG biosynthesis and PKC phosphorylation at the activation loop will be reduced. As a result, activation of PKC will be limited. In this condition, the high insulin in euglycemic-hyperinsulinemia clamp test will lead to less DAG production in muscle that already has insulin resistance. This pre-existing insulin resistance may protect the mice from the secondary insulin resistance induced by FFA+insulin. This possibility may explain the discrepancy in the metabolic phenotype of PKCtheta KO mice. Insulin sensitivity of the PKCtheta KO mice has been independently studied using euglycemic-hyperinsulinemic clamp test in our and Kim's studies [146]. According to our study (AJP in press), the PKCtheta mice suffer muscular insulin resistance as a result of loss PKCtheta in muscle. The clamp results show that the KO mice have severe insulin resistance after eight weeks on a high fat diet, suggesting that the KO mice have a higher risk for insulin resistance. In Kim's study, the clamp testing was performed on mice fed a chow diet. The results show that the KO mice are protected from insulin resistance induced by lipid infusion, suggesting that the KO mice are at a lower risk for insulin resistance. The discrepancy might be a result of the pre-existing muscular insulin resistance in the KO mice that reduced the muscle's response to FFA+insulin in the experimental setting in Kim's study. In this setting, the PKCtheta KO mice might be protected by the pre-existing insulin resistance from the secondary insulin resistance, which was induced by the FFA+insulin treatment. Such protection was not available in the WT mice since there was not pre-existing insulin resistance in the WT mice. When the secondary insulin resistance is stronger than the pre-existing insulin resistance, insulin resistance in the WT mice may be more severe than that observed in the KO mice. This might have led to the conclusion that the KO mice had better peripheral insulin sensitivity than the WT mice. In our study, the clamp testing was done in the absence of lipid infusion. The synergistic effect of FFA+insulin was reduced. Our study may provide information about the pre-existing insulin resistance in the KO mice.

Insulin may promote insulin resistance through obesity. Insulin promotes energy storage in adipocytes. This may contribute to obesity through several approaches, such as increasing food intake, enhancing efficiency of TG biosynthesis, and inhibiting lipolysis. The importance of insulin signaling in the growth and development of adipose tissues was elegantly demonstrated *in vivo* using fat-specific insulin receptor knockout (FIRKO) mice [147]. Fat-specific knockout of the insulin receptor prevented age- and hypothalamic lesion-related obesity in FIRKO mice [147]. Importantly, insulin resistance was also prevented in the mice. In humans, it is well known that insulin accelerates body weight gain in patients with type 1 diabetes. The rate of such weight gain is positively associated with the dosage of insulin administrated. Accumulation of fat significantly contributes to the insulin-induced body weight gain as indicated by the FIRKO mice. Although the combination of FFA and insulin contributes to the initiation of insulin resistance, such insulin resistance might be transient and reversible in healthy subjects [29]. After reduction of FFA in the plasma, the insulin resistance will disappear. In obese subjects, the transient insulin resistance may become permanent, since plasma FFA are permanently elevated.

In summary, strong evidence supports that insulin may play a critical role in the pathogenesis of FFA-associated insulin resistance. This hypothesis is supported by the well-established role of FFA in the promotion of insulin secretion in beta-cells, and new findings about GPR40 in the signal translation of FFA in beta-cells. Although a high level of insulin is able to induce insulin resistance through the negative feedback loop, this response is transient and may not be sufficient by itself for the permanent defect in the post-receptor signal transduction in obesity. FFA may have a direct (insulin-independent) effect on the insulin target tissues in insulin resistance. However, this effect may not be strong enough to induce chronic or permanent insulin resistance. A combination of insulin and FFA represents a promising mechanism for the initiation of obesity-associated insulin resistance. Although insulin resistance is able to lead to hyperinsulinemia, hyperinsulinemia may occur before insulin resistance. Control of FFA-priming effect in beta-cells may be a potential approach for early intervention in metabolic syndrome.

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