The Novel Role of Interleukin-1 Receptor-Associated Kinase 1 in the Signaling Process Controlling Innate Immunity and Inflammation

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Abstract

Obesity-induced chronic inflammation plays a key role in the pathogenesis of insulin resistance and the metabolic syndrome. Proinflammatory cytokines can cause insulin resistance in adipose tissue, skeletal muscle and liver by inhibiting insulin signaling transduction. Interleukin-1 receptor-associated kinase-1 (IRAK-1) is a serine/threonine kinase functioning in Toll-like Receptor signaling pathways, and plays an important role in inflammation and immune response. In our studies, we demonstrated that IRAK-1 is involved with the negative regulation of PI3K-Akt dependent signaling pathway induced by insulin and TLR 2&4 agonists. Out data also indicate that IRAK-1 can interact with IRS-1 protein both in vivo and in vitro. The binding sites for the IRAK1-IRS1 biochemical interaction are IRS-1's PH domain and IRAK-1's proline-rich LWPPPP motif. Our studies also indicate that IRAK-1 is involved with the negative regulation of glycogen synthesis through inhibiting PI3K-Akt signaling pathway and thus releasing GSK3β's inhibitory effect on glycogen synthase. Moreover, our studies also suggest that IRAK-1 is involved in the activation of transcription factors CREB and ATF-1 by stimulating CREB-Ser133 and ATF-1 phosphorylation. CREB transcription factor family induces genes involved in cellular metabolism, gene transcription, cell cycle regulation, cell survival, as well as growth factor and cytokine genes. That may partially explain our finding that IRAK-1 may be also involved with cell proliferation and survival pathway.

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1. Introduction

Obesity-induced chronic inflammation plays a key role in the pathogenesis of insulin resistance and metabolic syndrome. Interleukin-1 receptor-associated kinase-1 (IRAK-1) is a serine/threonine kinase that is functioning in Toll-like Receptor signaling pathways, and plays an important role in inflammation and immune response. It participates in various cell signaling networks, ranging from inflammatory response, gene transcription regulation, to cell survival and metabolic activities. In this project, we will define IRAK-1's novel roles in the regulation of PI3K-Akt dependent insulin signaling pathway and reveal the biochemical natures behind them. We will also explore IRAK-1's functions in the regulation of glucose metabolism, pro- and anti-inflammatory gene expression, as will as cell proliferation and cell survival processes.

2. Review of Literature

2.1 Toll-like-receptor (TLR) signaling and the regulation of innate immunity

Here we review TLR signaling and its regulation mechanism of human innate immunity, as IRAK-1 is a key mediator in Toll-like receptor signaling processes controlling human innate immunity.

2.1.1 Toll-like-receptors (TLRs) signaling stimulates human innate immunity

Innate immunity is the first line of defense in the body and senses abnormalities both outside and inside of host cells. It was originally termed in contrast with the adaptive immunity and was thought to be non-specific host response to microbial pathogens. However, after the discovery and further study of Toll-like-receptors (TLR) and their recognition of distinct pathogen associated molecular patterns, it is now realized that innate immunity is as specific and complex as the adaptive immunity.

Through TLR signaling pathways, human host can respond specifically to diverse microbial pathogens and other substances, and trigger highly specific innate immune response. So far, the study of human genomic database has led to the discovery of 10 different Toll-like-receptors [1].

With conventional biochemical approaches and transgenic mice studies, the ligand specificities of distinct TLRs have gradually come to light. For example, TLR4 primarily mediates gram negative bacterial product-induced immune responses [2]; TLR2 mainly responds to gram positive bacterial and fungal products [3]; viral RNAs specifically triggers the innate immune response through TLR3 [4]; bacterial DNAs trigger the immune response through TLR9 [5].

The intracellular domains of TLRs share sequence and structural similarity with the IL-1 receptor intracellular domain, and are therefore termed as Toll-IL-1-Receptor (TIR) domain [6]. Through the TIR domains, TLRs can recruit several adaptor molecules including MyD88, TIRAP/MAL, and TRIF/TICAM, which subsequently lead to diverse downstream signaling. These adaptor molecules can subsequently recruit a family of Interleukin-1 receptor associated kinases (IRAKs), named IRAK-1, 2, M and 4. IRAK 1, 2 and 4 are involved in positively regulating innate and adaptive immune response by facilitating the expression of various inflammatory mediators and by enhancing activation and proliferation of various immune cells [7-9]. In contrast, IRAK-M is a negative regulator that counteracts these effects [10, 11] (Figure 2-1).

In addition of recruiting TIR-domain containing adaptors and activating IRAKs, some TLRs can also cause Phosphotidylinositol-3 Kinase (PI3K) activation. For example, TLR2 intracellular domain can bind directly with the p85 adaptor subunit of PI3 kinase and leads to PI3K activation [12]. Although the PI3K binding motif (YXXM) is only present in a subset of TLRs (1, 2, and 6) [12], several studies indicate that TLR4 ligand LPS can also induce PI3K activation [13, 14]. PI3K pathway is involved in activating diverse downstream signaling leading to various gene transcriptions and translations. The precise mechanism of how PI3K pathway specifically mediates distinct TLR responses is not fully defined.

Although human innate immune response is essential for host defense, excessive TLR signaling caused by microbial infection or other challenges often leads to detrimental inflammation, tissue damage, and even fatality. To prevent the excessive adverse effects of prolonged immune responses, human host possesses various down-regulating mechanisms

negatively regulating TLR signaling. These include down regulation of the TLRs, decreased IRAK level and activity, as well as decreased activation of NF-kB. Besides TLRs down regulation, several studies have reported that IRAK-1 is no longer responsive upon prolonged challenges with various TLR ligands including TLR2, 4, and 5 [15, 16]. It is very likely that different cells and tissues use one or several of these negative regulatory mechanisms to prevent excessive TLR-mediate immune response.

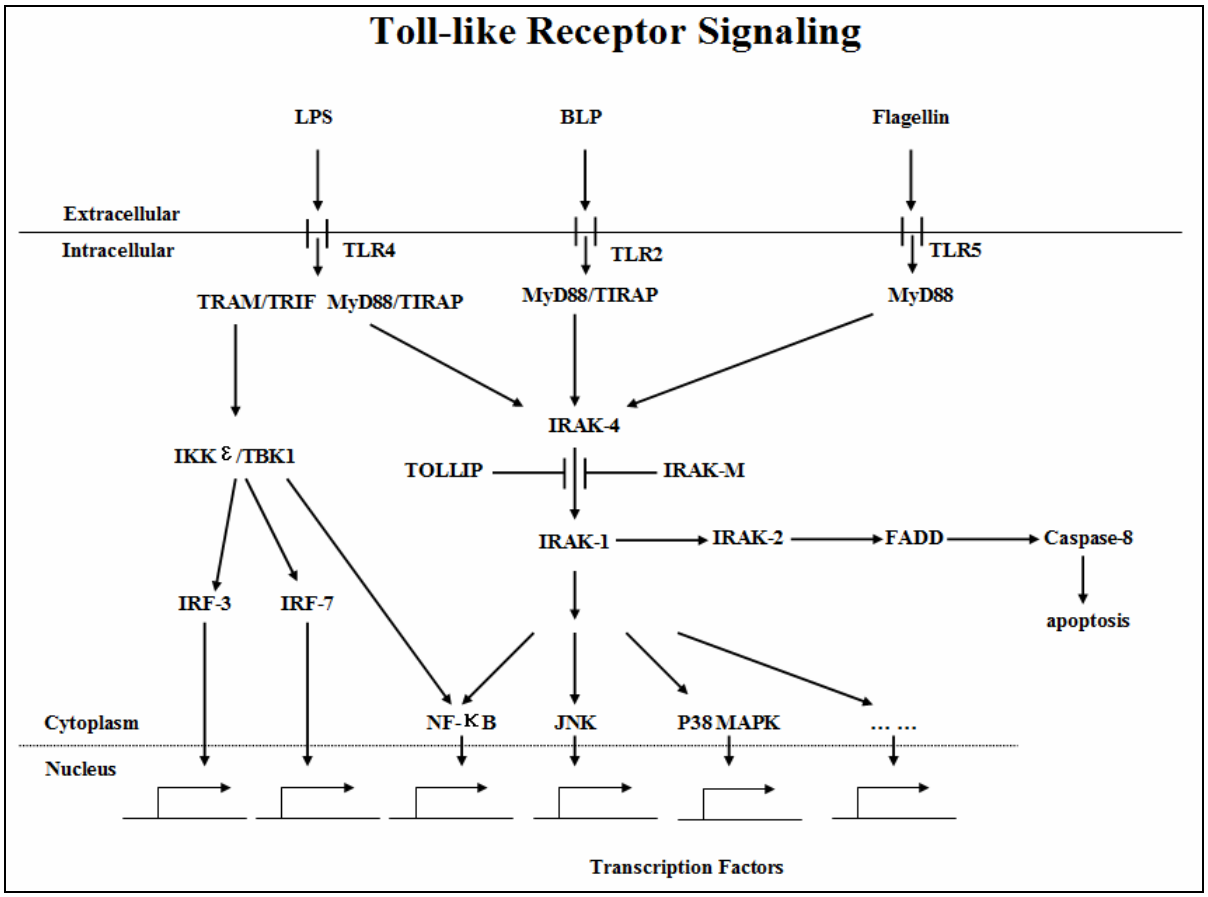


Figure 2-1. Toll-like Receptor signaling pathways. TLR signaling processes are critical for human innate immune response. TLRs can recognize diverse substances from microbial and non-microbial substances, and relay signals downstream to activate the expression of various pro- and anti-inflammatory mediators.

2.1.2 The role IRAK-1 in immunity and inflammatory response

IRAK-1 was firstly identified through purification of the IL-1 dependent kinase that co-immunoprecipitates with the IL-1 type 1 receptor [17]. Micropeptide sequencing and cDNA

library screening led to a full-length cDNA clone encoding a protein with 712 amino acids and a predicted molecular size of approximate 76 kDa. IRAK-1 protein is expressed ubiquitously in diverse human tissues. It contains an N-terminus death domain, a central serine/threonine kinase domain, and a C-terminus serine/threonine rich region. Using human THP-1 cells, primary blood mononuclear cells, as well as mice splenocytes, our lab have demonstrated that there are two signature forms of IRAK-1: the unmodified 80 kDa form, and the modified 100 kDa form [15]. The modification of IRAK-1 protein includes phosphorylation, ubiquitination, and sumoylation [18-20] . Through different kind of modification, IRAK-1 may perform distinct functions, including the activation of IRF5/7 [21-23], NFκB [24-26] , and Stat1/3 [27, 28], all of which are critically involved with the expression of pro- and anti-inflammatory mediators, cell proliferation, differentiation, apoptosis, tumor defense, and other crucial cell responses.

2.2 The regulation of insulin signaling pathway

Here we review the regulation of insulin signaling pathways, as insulin signaling processes are critical for glucose metabolism and contribute to the pathogenesis of obesity-induced systemic insulin resistance, type-2 diabetes and metabolic syndrome.

2.2.1 Inflammatory complication of insulin resistance

Insulin resistance is a characteristic feature of human type II diabetes. In Type II diabetes, the body produces insulin, the insulin target tissues are not responsive to insulin. Compared with that, Type I diabetes is associated with the loss of insulin-producing beta cells in pancreatic island, leading to insulin deficiency. Insulin resistance is also one of the clinical features of metabolic syndrome. Metabolic syndrome is a collection of physiological dysregulations, which may include obesity, diabetes and hypertension. 2000 US census data has shown that about 47 million Americans suffer from metabolic syndrome. Since the obesity and metabolic syndrome are becoming more and more epidemic, this number is very likely to rise rapidly in near future [29].

It is becoming more and more evident that obesity and obesity-induced chronic inflammation

play major roles of insulin resistance. Studies in these areas have shown a strong association between the chronic pro-inflammatory state and reduced insulin sensitivity in the body (Figure 2-2). Tumor necrosis factor-a (TNF-a), IL-6, IL-8, as well as the acute inflammatory phase market C reactive protein, have been shown to increase in insulin resistant states [30-34].

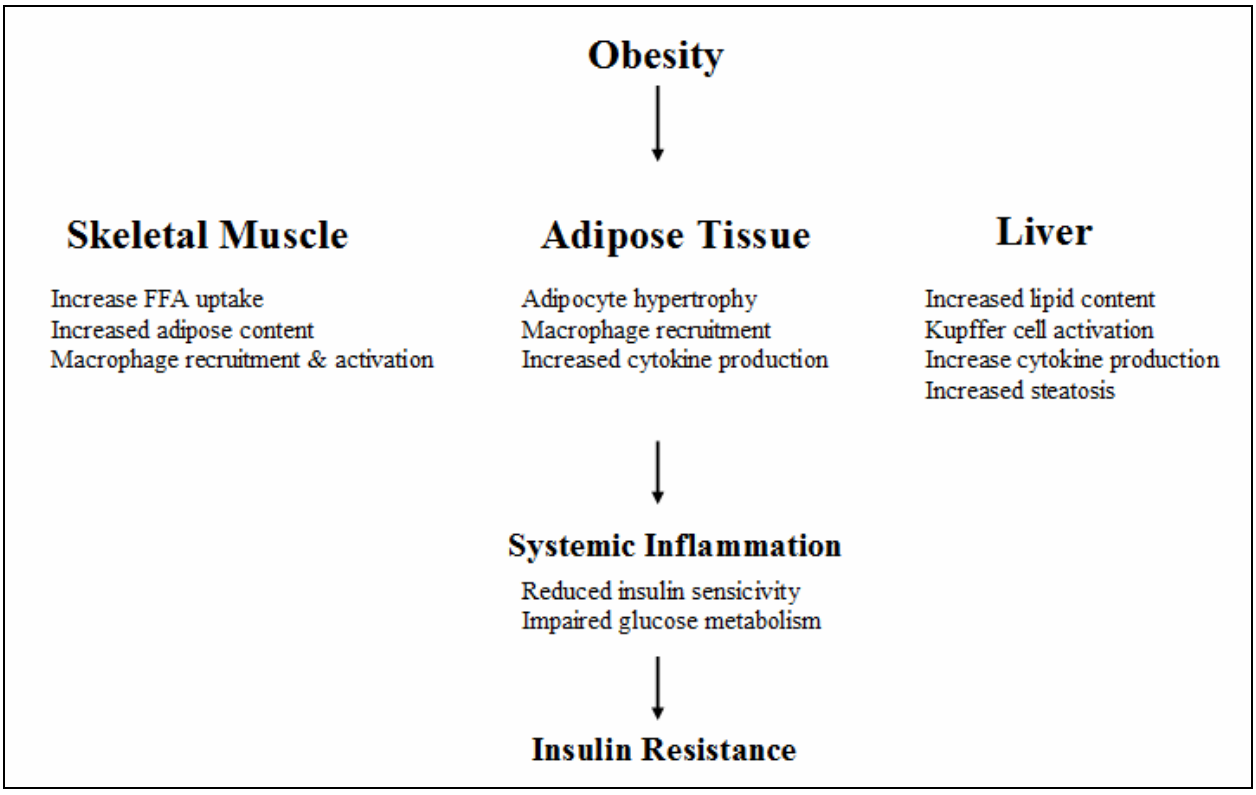


Figure 2-2. The links among obesity, inflammation and insulin resistance. Obesity-induced physiological changes in insulin target tissues: skeletal muscle, adipose tissue and the liver, lead to localized inflammation and insulin resistance. Endocrine cross-talk between these insulin target tissues lead to systemic insulin resistant state.

2.2.1.1 Obesity induces systemic inflammation and insulin resistance

The precise mechanism of how obesity induces systemic inflammatory response is not fully defined. One current theory explains that the enlarged adipose tissue leads to adipocyte hypertrophy, and the large adipocytes exceed local oxygen supply which then results in the activation of various cell stress signaling pathways. Cells with autonomous inflammation release pro-inflammatory cytokines and other signals to the nearby tissues.

In adipose tissues, adipokines secreted by adipocytes can also exaggerate inflammation. As

part of the chronic inflammatory process, locally secreted cytokines attract pro-inflammatory macrophages into the adipose tissue where they form crown-like structures around dead or dying adipocytes. These macrophages also release cytokines in neighboring tissues, exacerbating inflammation. In the liver, the steatosis and increased hepatocyte stress can lead to cell autonomous inflammation. Kupffer cells, the liver-resident macrophage-like cells, can be activated and release pro-inflammatory cytokines which further exacerbates inflammation. In the circulation system, obesity are often coupled with increased levels of Free Fatty Acids (FFAs). Saturated FFAs can directly activate pro-inflammatory signaling in vascular endothelial cells, adipocytes, as well as bone marrow-derived cells. All of these factors directly or indirectly impair insulin-mediated signaling pathways, and thus damage the glucose metabolism and energy homeostasis. The consequence is the systemic inflammation and insulin resistance in whole body.

2.2.1.2 Glucose metabolism in the insulin target tissues

The hormone insulin is produced in pancreatic island and has diverse functions in human body, including stimulation of glucose transport into cells, regulation of gene expression, modification of enzyme activity, and regulations of energy storage and expenditure [35, 36]. These functions are accomplished through three major insulin target tissues: the liver, the skeletal muscles, and the adipose tissue. In the liver, insulin inhibits the expression of key glucose-producing enzymes, and thus insulin resistance in liver leads to increased glucose production. In skeletal muscles, insulin promotes glucose uptake into the cells by stimulating the translocation of GLUT4 glucose transporter from cytoplasm to cell membrane, and thus insulin resistance in skeletal muscles results in impaired glucose consumption. In adipose tissue, insulin signaling suppresses lipase activity, which inhibits free fatty acid (FFA) transport outside the fat cells, and thus insulin resistance in fat tissues increases FFA levels in circulation system, which can lead to decreased insulin sensitivity in skeletal muscles [37-39].

Liver is the main organ in charge of the glycogen synthesis and storage. Hepatocytes make up

70~80% of the mass of the liver. They are functioning on the synthesis, transformation and storage of proteins and carbohydrates, as well as many other critical physiological processes. Hepatocytes are the primary location for glycogen synthesis process. They have high levels of glucose transporters, insulin receptors and glycogen synthase, and are very physiologically responsive. Therefore, Hepatocytes are a valuable primary cell model for studying insulin-mediated metabolic signaling pathways. We are interested in using wild type and IRAK-$1^{-/-}$ mouse hepatocytes to study the involvement of IRAK-1 in the regulation of glycogen synthesis.

2.2.1.3 The role of macrophage in the development of insulin resistance

A major advance in the field of obesity-induced systemic inflammation and insulin resistance was made by the discovery and further study of the bone marrow-derived macrophages (BMDMs) in adipose tissue of obese mice and humans [40, 41]. Weisberg et al. have demonstrated that there is a subset of genes consistently expressed in obese mice; interestingly, these genes are not expressed in adipocytes, but they are macrophage-derived. Immuno-histochemical study has further confirmed that the expression of these genes is primarily from adipose tissue-resident macrophages. This study shows that the percentage of macrophages in a given adipose tissue is positively correlated with adiposity and the size of fat cells. Weisberg et al. indicated that as high as 50% of the total tissue mass is contributed by such macrophages, compared to only 10% in lean control [40]. They also found that the adipose tissue-resident macrophages contribute to almost all adipose tissue TNF expression and a significant part of NOS2 and IL-6 expression. All of these discoveries have shed a new light on studying the mechanisms and therapies for obesity-induced insulin resistance.

Because of the important roles macrophages play in inflammation and insulin resistance, we are interested in using wild type and IRAK-$1^{-/-}$ mouse Bone-Marrow Derived Macrophages (BMDMs) to study the role of IRAK-1 in macrophage physiological activities ranging from insulin signaling to inflammatory responses.

2.2.2 PI3K-Akt dependent insulin signaling pathway

Due to goals of our project, it is necessary to understand the mechanisms of the finely regulated insulin signaling pathway. Insulin signaling involves several complex signaling cascades downstream the insulin receptor. These signaling cascades can be divided into two main pathways. The first and the major one is the phosphatidylinositol3-kinase (PI3K)-Akt pathway which is largely responsible for insulin action on glucose uptake, metabolic activities and regulation of gene transcription. The second pathway is the Ras-mitogen activated protein kinase (MAPK) pathway which regulates gene expression, but also interacts with the PI3K-Akt pathway to control cell growth and differentiation [42]. In this review part, we will focus on the mechanism of PI3K-Akt pathway which is most relevant to our project.

Insulin signaling cascades is initiated through the insulin receptor (IR). Insulin receptor is a heterotetrameric bifunctional complex, with 2 extracellular α subunits that bind insulin and 2 transmembrane β subunits with tyrosine kinase activity. Insulin binds to α subunits and induces the transphosphorylation of one β subunit by another one on specific tyrosine residues in an activation loop, resulting in the increased tyrosine kinase activity of β subunit [43, 44]. Insulin receptor also performs autophosphorylation at other tyrosine residues in the juxtamembrane regions and intracellular tail to further activate its activity.

The activated insulin receptor phosphorylates the tyrosine residues of its intracellular substrates, including the insulin receptor substrate (IRS) family (IRS1 through 4) [43, 44]. Upon phosphorylation, the IRS proteins will interact with a variety of adapter molecules containing Src homology 2 (SH2) domains. Although IRS family members (IRS-1, 2, 3, and 4) share similar structures, IRS isoforms have both overlapping and distinct functions [45]. For example, IRS-1 knockout mice have a phenotype of growth retardation and insulin resistance [46], whereas IRS-2 knockout mice have a phenotype of frank diabetes due to β-cell destruction [47]. IRS-1 seems to be the most important IRS isoform for mediating the metabolic effects of insulin in skeletal muscle and fat tissue.

Upon tyrosine phosphorylation, IRS proteins will interact with the p85 regulatory subunit of PI3-Kinase, resulting in the activation of PI3K and its localization to the cell membrane. PI3K is directly associated with the generation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), a lipid product that modulates the localization and activity of a large variety of downstream proteins [48]. PI3-Kinase is critical and required for the translocation of Glut4 glucose transporter and glucose uptake process. Inhibition of PI3-Kinase using pharmacological inhibitors, e.g. wortmannin, will block the stimulation of glucose uptake by insulin [49].

The increased PIP3 product recruits and activates a large variety of downstream proteins containing pleckstrin homology (PH) domains. These target proteins include enzymes, their substrates, adapter molecules, as well as cytoskeleton proteins. An important enzyme in PI3K-Akt pathway is the serine/threonine kinase PDK1, which phosphorylates and activates several downstream kinases including protein kinase C(PKC) ζ/Λ and Akt 1-3 [50]. PIP3 can mediate the translocation of Akt from cytoplasm to the cell membrane via Akt's PH domain [51].

The serine/threonine kinase Akt, also known as protein kinase B (PKB), is the central mediator in the PI3K-Akt pathway. It is activated by the phosphorylation on its Thr308 and Ser473 sites. Activated Akt can phosphorylate diverse downstream targets of the PI3K pathway, which mediate a large variety of cell activities, including glycogen synthesis, fatty acid synthesis, protein synthesis, glucose uptake, gene transcription, apoptosis, proliferation, etc. (Figure 2-3). So far, Akt has been considered as a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli. It has emerged as one of the most important and versatile kinases at the core of human physiology and diseases.

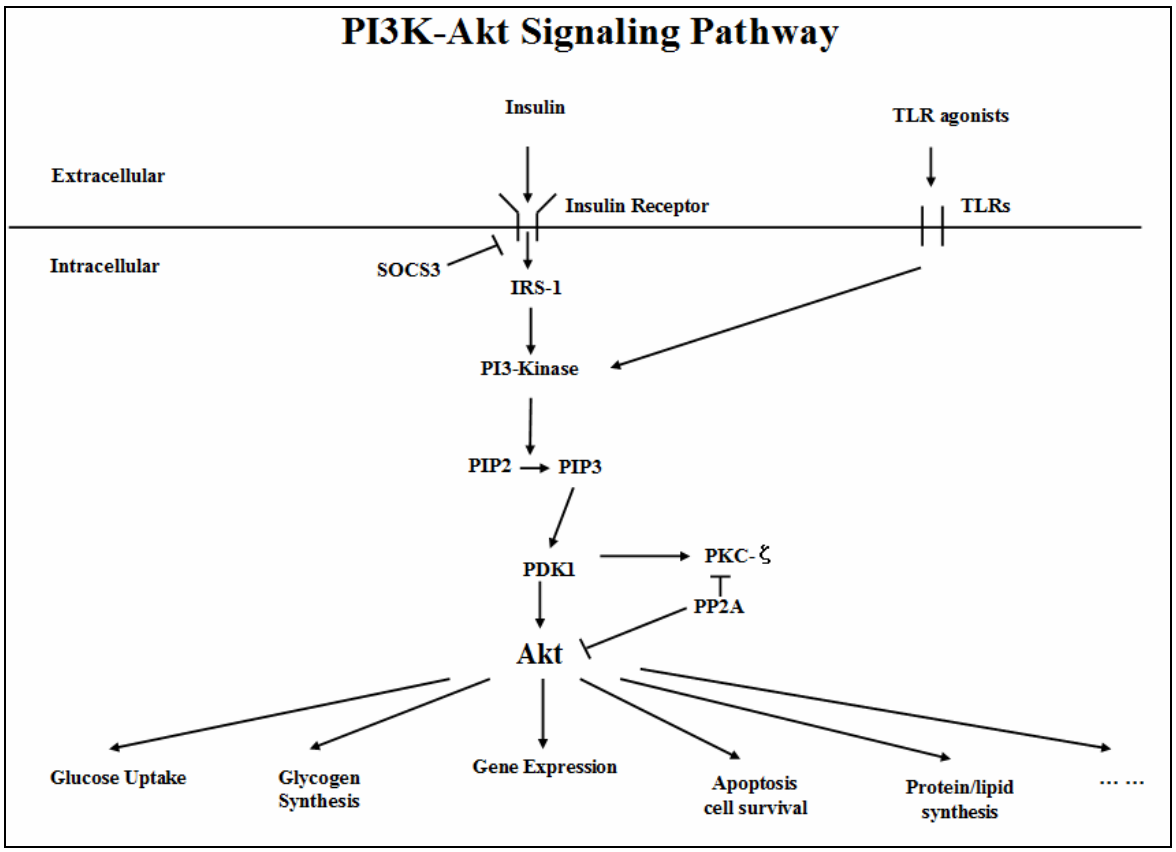


Figure 2-3. PI3K-Akt dependent insulin signaling pathway. Insulin is the most potent physiological anabolic agent ever known. It stimulates the synthesis and storage of lipids, protein and carbohydrates, and inhibits their breakdown and release into the circulation. Meanwhile, inflammation contributes to insulin resistance in diabetes and obesity, indicating that insulin signaling may play a role in the processes of inflammation and immune response.

2.2.3 IRAK-1 may contribute to linking inflammation with insulin resistance via a novel substrate IRS-1

So far, the physiological substrates for IRAK-1 have not been completely identified. IRAK-1 critically participates in the activation of various inflammatory mediators, including IKKβ, JNK, and NF-κB. Meanwhile, studies have suggested a potential crosstalk between inflammation and insulin-mediated metabolism. For example, IKKβ, a critical mediator of inflammatory signaling pathway which activates NF-κB, has been shown to be a suppressor in insulin signaling pathway [52-55]. Moreover, another inflammatory signaling molecule JNK may also contribute to insulin resistance of obesity [56]. Together with other studies, researchers hypothesized that IRS-1 may be a novel substrate for IRAK-1, and IRAK-1 may mediate cross-talk between pro-inflammatory

signaling and insulin signaling pathways [57]. Michael J. Quon's group has identified that Ser24 in the pleckstrin homology (PH) domain of IRS-1 is a phosphorylation site for IRAK-1 that may contribute to insulin resistance [57]. Their study demonstrated that IRAK-1 can phosphorylate IRS-1 at Ser24 site; upon phosphorylation, IRS-1 kinase activity is impaired [57]. Therefore, IRAK-1 may have a novel function to mediate crosstalk between inflammatory signaling and insulin signaling via serine-24 phosphorylation of IRS-1 [57].

It has also been reported that, a proline-rich F/L/W/YPPPP motif present many proteins is a novel ligand for the EVH1 domain, a protein module in the Ena/VASP family [58]. Interestingly, IRAK-1 has a proline-rich LWPPPP motif (167-172 sites). Comparing the 3D structures of EVH-1 domain in VASP protein and pleckstrin homology (PH) domain in IRS-1 protein, our lab found that these two domains have high structural similarity: seven β-sheets and one big α-helix [59] (Figure 2-4). Our lab hypothesized that IRAK-1 may interact with IRS-1 protein via the protein-protein interaction between IRAK-1's LWPPPP motif and IRS-1's PH domain; upon the biochemical interaction, IRAK-1 may phosphorylate the serine-24 site in PH domain (N-terminal) of IRS-1 protein.

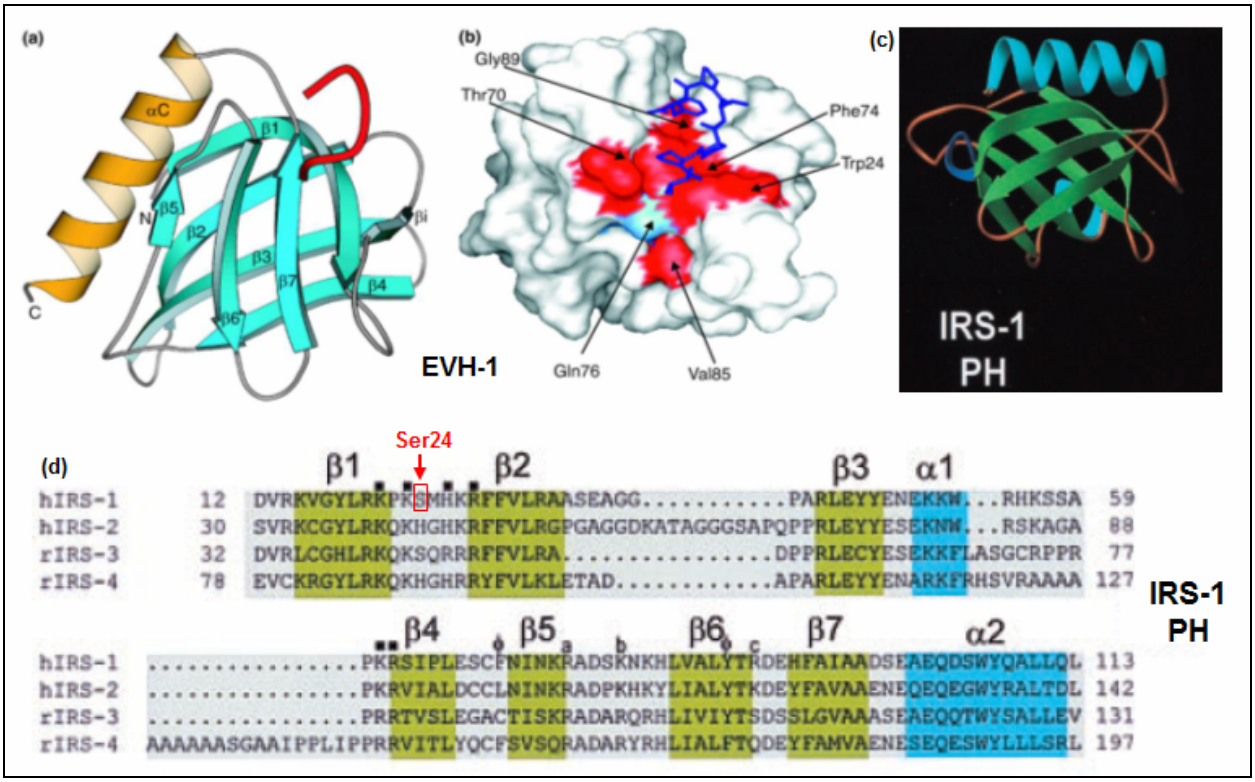


Figure 2-4. Structures of VASP EVH-1 domain and IRS-1 PH domain. (a) Secondary structure of VASP EVH-1 domain (figure shown on online website). (b)White portion is EVH-1 domain, blue portion is proline-rich motif. Structural modeling shows that the proline-rich F/L/W/YPPPP motif can be embedded in to EVH-1 domain, indicating a high potential of domain-domain interaction between this proline-rich motif and PH domain which resemble EVH-1 domain structure (figure shown on online website). (c) Secondary structure of IRS-1 pleckstrin homology (PH) domain (figure from [59]). (d) Amino acid sequence of IRS-1 PH domain, with seven β–sheets and one classic α–helix (α2) (figure from [59]). Interestingly, the IRS-1 Ser24 site is located in the PH domain, suggesting that this protein-protein biochemical interaction may be critical for IRS-1 Ser24 phosphorylation by IRAK-1.

2.3 GSK3β regulates glycogen synthesis and inflammatory gene expression

Here we review the nature and functions of GSK3. GSK3 is downstream of PI3K-Akt signaling, and plays key roles in the regulation of glycogen synthesis as well as gene expression. We are interested in whether IRAK-1 could affect this aspect of PI3K-Akt downstream signaling.

2.3.1 GSK3 negatively regulate glycogen synthesis by inhibiting glycogen synthase (GS)

In mammalian, carbohydrate is primarily store in the form of glycogen, and the major storage sites are the liver and skeletal muscle. The glycogen concentration is higher in the liver than in

the skeletal muscle. But due to the much greater mass, skeletal muscle stores more glycogen. Stimulation of glycogen synthesis is one of the major roles of insulin. Insulin mediates the glycogen synthesis through two steps: by controlling the glucose uptake, and by regulating the enzymes that involve in the synthesis and degradation of glycogen [60]. Glycogen synthase is the enzyme directly for glycogen production. It catalyzes the addition of UDP-glucose to the existing glycogen chain. Glycogen synthase is regulated by both covalent and allosteric mechanisms [61]. It is phosphorylated at multiple serine residues, resulting in its progressive inactivation. Insulin stimulates glycogen synthase activity by prompting its net dephosphorylation, through the phosphatase activation including protein phosphatase-1 (PP-1), and the kinase inhibition including glycogen synthase kinase-3β (GSK3β).

Insulin can inhibit the activity of GSK3, a key kinase in the negative regulation of glycogen synthesis. GSK3 is a serine/threonine kinase that can phosphorylate a series of serine residues at the C terminus of glycogen synthase and lead its inactivation. GSK3 is has two isoforms: α and β, with 51 and 47 kDa in molecular weight respectively [62]. Both of these two isoforms are constitutively active in their basal states, and are transiently inhibited via Ser21/Ser9 phosphorylation near their N-terminus in induced by insulin and some growth factors [63-65]. Insulin can trigger PI3K-Akt dependent signaling pathway, and activated Akt phosphorylates serine-9 site of GSK3β, leading to its inhibition [66] (Figure 2-5). Although these two isoforms share high similarity, especially at the kinase domain with 97% homology, they are shown not to be functionally redundant based on the knockout mice studies [67, 68]. Most studies so far have mainly focused on GSK3β isoform.

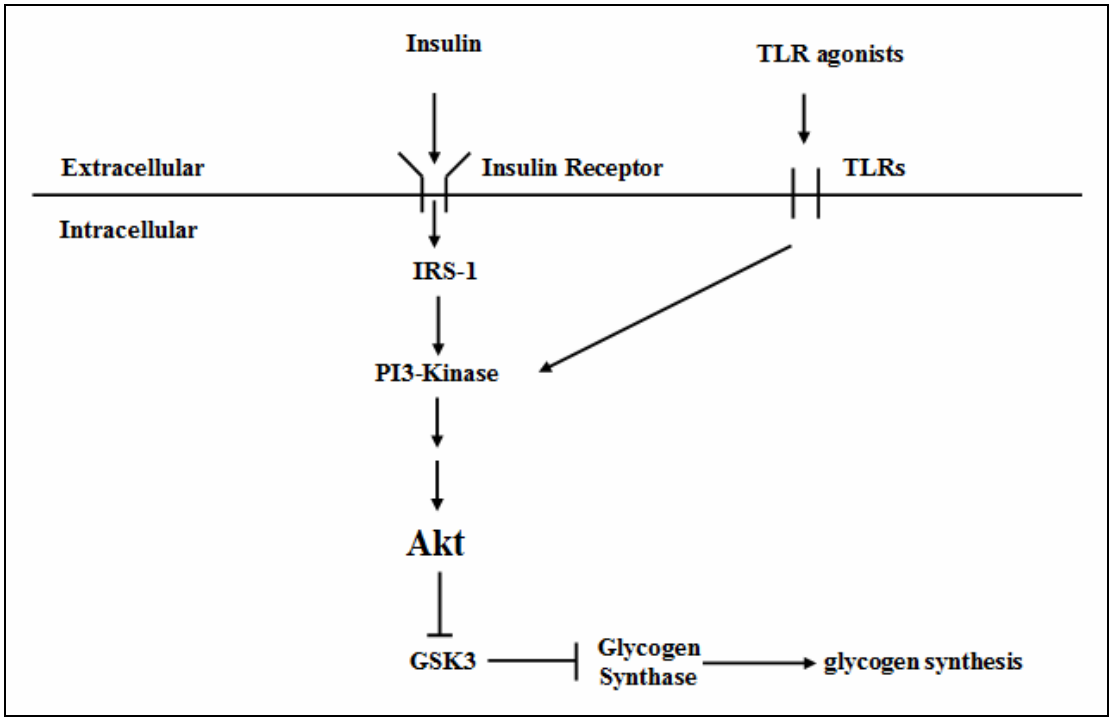


Figure 2-5. GSK3β negatively regulates insulin-induced glycogen synthesis. Insulin binds to insulin receptor (IR) and triggers PI3K-Akt signaling pathway. Akt phosphorylates Ser9 in GSK3 and inactivates it. Inactive GSK3 relieves the inhibition to glycogen synthase (GS), leading to active form of GS and increasing glycogen synthesis production.

2.3.2 GSK3β is critically involved with regulation of pro- and anti-inflammatory gene expression

Now, It is known that GSK3 is not only a critical mediator in glycogen synthesis process, but also plays an important role in regulating many critical transcription factors. For example, it has been reported that GSK3β regulates a variety of transcription factors involved with cell survival and apoptosis signaling, including β catenin [69, 70], AP-1 [71], nuclear factor kappa-B (NF-κB) [72], and heat shock factor-1 (HSF-1) [73, 74]. Moreover, it has been demonstrated that GSK3β could differentially regulate TLR-mediated pro- and anti-inflammatory cytokine expression [75]. Researchers have shown that TLRs can activate PI3K-Akt signaling [76] which then phosphorylates GSK3β and lead to its inhibition. Stimulation of monocytes or peripheral blood mononuclear cells with different TLR agonists could induce large increase in anti-inflammatory cytokine IL-10 production, but suppress the pro-inflammatory cytokines IL-12 production,

through the GSK3β inhibition [75]. So far, GSK3β has been shown to be critically involved in diverse physiological pathways ranging from metabolism, cell cycle, gene expression, development and other cell activities [77]. It is also considered as a potential target for anti-diabetes therapy [77].

2.4 The role CREB and ATF-1 in the regulation of gene transcription and cell survival

Here we review the natures and functions of CREB and ATF-1. The transcription factors CREB/ATF-1 are in the downstream of various signaling pathways which may be regulated by IRAK-1. CREB/ATF transcription factor family is a set of transcription mediators regulating a large variety of genes controlling metabolism and inflammatory response.

2.4.1 CREB and ATF-1 in CREB family

cAMP response element (CRE)-binding protein (CREB) is one of the best characterized stimulus-inducible transcription factors. It activates transcription of various target genes in response to a large variety of stimuli, including peptide hormones and growth factors which activate a variety of protein kinases. These kinases can phosphorylate CREB at a serine-133, resulting in its activation. The phosphorylation of CREB-Ser133 is required for CREB-mediated gene transcription.

So far, several CRE binding proteins have been identified within the CREB family. The best characterized ones include CREB, CREM, and ATF 1-3. These proteins share highly similar C-terminus leucine zipper dimerization and basic DNA binding domains (bZIP domains), but differ in their N-terminus. Although each of the ATF/CREB family members is capable of binding to CRE DNA binding motif in its homodimeric form, certain members also bind as heterodimers, both within the CREB family and even with members of the AP-1 transcription factor family [78]. CREB/ATF transcription factors induce gene expression involved in cell metabolism, cell cycle, cell survival, as well as growth factor and cytokine genes [79]. The transcriptional activity of CREB protein dimers is controlled by phosphorylation of its serine-133

site, which then promotes recruitment of the transcriptional co-activator CREB-binding protein (CBP) and the binding to CRE DNA binding region [80, 81]. Many stimuli, including increased cAMP levels, growth factors, and stress signals, could induce CREB phosphorylation through several critical protein kinases, including protein kinase A (PKA), protein kinase C (PKC), Akt, calcium-calmodulin kinases (CaMKs), and p38 mitogen-activated protein kinase (p38 MAPK) [80, 82-84] (Figure 2-6).

It has been reported that CREB and ATF-1 play essential roles in regulating the expression of matrix metalloproteinase 2, a key enzyme in melanoma invasion and metastasis [85]. Meanwhile, CREB and ATF-1 act as survival factors that promote melanoma survival both in vivo and in vitro [86, 87]. Moreover, ATF-1 has been reported to play an essential role in the induction of NOX1, a catalytic subunit of NDAPH oxidase. Elevated NOX1 could cause hypertrophy of vascular smooth muscle cells [88].

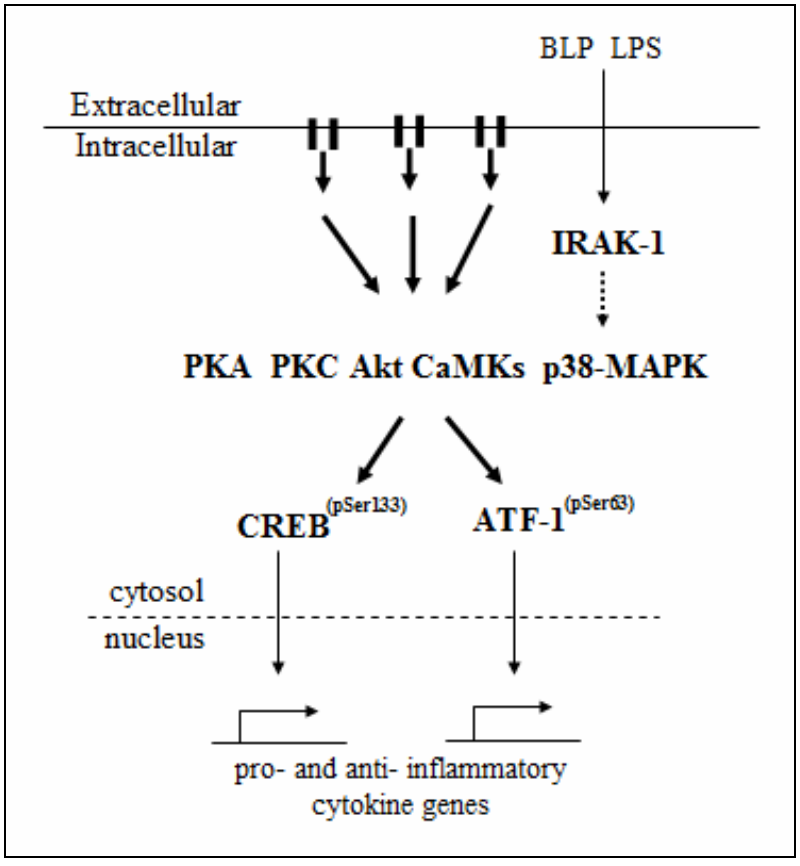


Figure 2-6. CREB and ATF-1 regulate diverse inflammatory gene expression.

The transcription factors are responsive to via various cell stimuli through diverse signaling pathways. There are activated via phosphorylation at Ser-133/Ser-63 sites by various key kinases, including p38-MAPK which is downstream of IRAK-1.

2.4.2 The activity of CREB is regulated by complex phosphorylation mechanisms

The complex phosphorylation mechanism for CREB activation/inactivation is not completely defined. Serine-133 site is not the only phosphorylation site for control. Phosphorylation of CREB serine-133 site could create a consensus sequence, SXXXS(p), for hierarchical phosphorylation of CREB serine-129 site by the kinase GSK3β [89-91]. So far, researchers have demonstrated the functional consequences of CREB-Ser 129 phosphorylation by GSK3β; however, they researched two opposite conclusions. One possible consequence would be that phosphorylation of CREB serine-129 by GSK3β synergizes with serine-133 phosphorylation to further activate CREB [89]. The other function would be that phosphorylation by GSK3β to act as a signal for termination of CREB activity [91]. Some other research groups have reported to support the second possibility that phosphourlation of CREB serine-129 by GSK3β provides inhibitory regulation of CREB DNA binding activity [92].

2.5 Unanswered questions and their relevance to this project

Although researchers have been highly focused on IRAK-1's functions in inflammation and innate immunity, IRAK-1's roles in regulating PI3K-Akt signaling and glucose metabolism are not well understood. Some of these questions have drawn into our interest and are the major aims of this project (Figure 2-7).

Firstly, we hypothesize that IRAK-1 may negatively regulate PI3K-Akt dependent signaling through certain feedback inhibition mechanism (Aim 1). Secondly, the detailed mechanism of interaction between IRAK-1 and IRS-1 remains not clear (Aim 2). Thirdly, we hypothesize that IRAK-1 may involved with glycogen synthesis process by inhibiting PI3K-Akt signaling (Aim3). Fourthly, we are interested in further exploring IRAK-1's role in TLR downstream signaling controlling inflammatory gene expression.

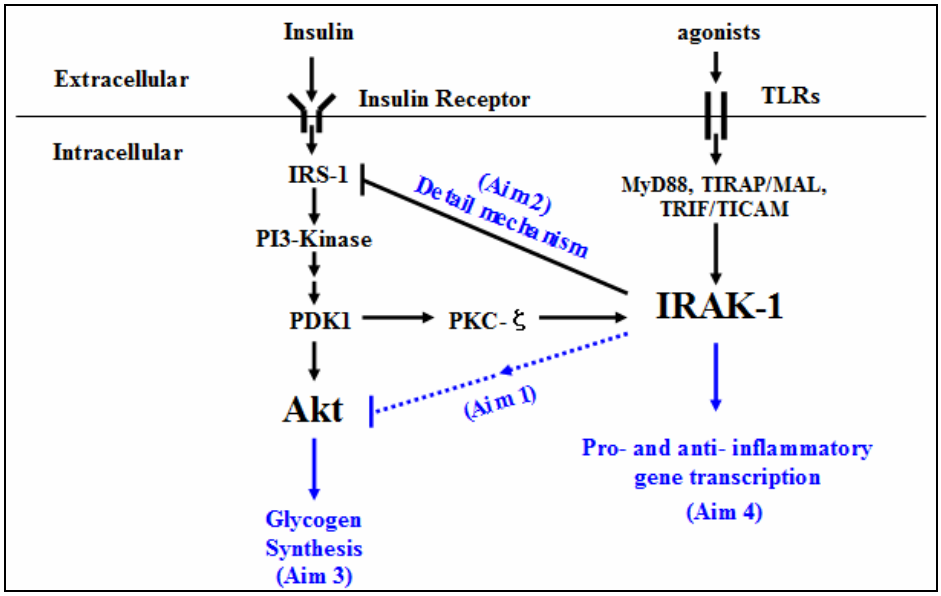


Figure 2-7. Unanswered questions and aims of this project. Aim 1: IRAK-1 may potentially regulation PI3K-Akt signaling pathway. Aim 2: the biochemical nature behind the interaction of IRAK-1 and IRS-1. Aim 3: IRAK-1 may potentially regulate glycogen synthesis via inhibit Akt signaling. Aim 4: The role of IRAK-1 in TLR downstream signaling controlling inflammatory gene expression remained further explored.

3. Materials and Methods

3.1 Cell culture

Bacteria cell culture is made with standard LB broth and LB agrose plates (Invitrogen) with the addition of Ampicillin antibiotics. MAT-2 cells and bone-marrow derived Macrophages (BMDMs) are cultured with standard DMEM medium (Invitrogen), with the addition of 10% FBS and 1% p/s antibiotics. For phosphorylation-detecting purpose, BMDMs are changed with serum free medium and incubate overnight. Mouse embryonic fibroblasts (MEFs) are cultured with standard DMEM medium (Invitrogen), with the addition of 10% FBS, 1% NEAA, 1% L-Glutamine and 1% p/s antibiotics. For phosphorylation-detecting purpose, MEFs are changed with 1% FBS medium and incubate overnight. THP-1 cells are cultured with RPMI-1640 medium (Invitrogen), with the addition of 10% FBS and 1% p/s antibiotics.

3.2 Plasmids and Cells

GST-IRS-1-PH plasmid is constructed based on pFN2A(GST) flexi vector (Promega). The flag-IRAK1 wild type and site mutated plasmids are from our lab. Plasmid amplification uses JM109 competent cells (Promega). The expression of GST-fusion proteins uses BL21-DE3 competent cells (Invitrogen). MAT-2 cells, THP-1 cells, and wt and IRAK-1 $^{-/-}$ MEFs are from lab liquid nitrogen stock. Wt and IRAK-1-/- BMDMs are kindly harvested, cultured and provided by Sarah Davis in our lab. Wt and IRAK-1$^{-/-}$ mouse hepatocytes are kindly harvested, cultured and provided by Samantha Baglin in our lab.

3.3 Immonoblotting and antibodies

Immunoblotting follows standard protocol of SDS-PAGE and Western Blotting. Antibodies are largely from Santa Cruz, Cell Signaling. Anti-total protein antibodies typically use 1:1000 dilution in 5% milk. Anti-phosphorylated protein antibodies are typically from Cell Signaling, and use 1:1000 dilution in 5% BSA. Anti-IRAK-1 antibody is from upstate. Anti-flag antibody is from Sigma Aldrich. Non-specific SDS-PAGE gel staining uses Instant Blue stain solution (Fisher Scientific) which is faster, easier and has higher resolution than standard commassie blue staining.

3.4 Transfection and immunoprecipitation (IP)

Transfection of pFlag-IRAK plasmids uses Lipofectamine-2000 Transfection Reagent (Invitrogen). Transfection follows standard protocol described in the reagent kit. Immunoprecipitation (IP) use relevant antibodies and Protein A/G-PLUS Agarose Immunoprecipitation Reagent (Santa Cruz). Immunoprecipitation follows standard IP protocol described in the reagent kit and modified by our lab. The immunoprecipitation is kindly helped by Lu Gan in our lab.

3.5 GST fusion protein in vitro binding assay

GST-fusion proteins are expression via IPTG induction, following expression protocol in pFN2A flexi vector manual (Promega). GST-fusion protein purification and in vitro binding assay use Immobilized Glutathione Agarose Resin (Pierce), following standard protocol as described in the resin kit.

3.6 Glycogen synthesis assay

Hepatocytes are pre-treated with insulin, and harvested after 3 hours. Glycogen productions are measured using Glycogen Assay Kit (BioVision). The glycogen synthesis assay is performed with the kind help of Neeraj Singh in our lab.

3.7 Cell proliferation assay

Wt and IRAK-1$^{-/-}$ MEFs are used in cell proliferation assay. The cell proliferation/survival rate is measured using CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) (Promega). The assay follows standard protocol described in the kit, and $5x10^3$ MEF cells are set in each well of a 96-well plate.

The CellTiter 96 Assay is a collection of qualified reagents that provide a convenient method of determining viable cell number. It is a modification of the MTT assay method described by Mosmann [93] and incorporates several improvements to the method that address previous technical problems. The assay is performed by adding a premixed, optimized Dye Solution to culture wells of a 96-well plate. During 4-hour incubation, living cells convert the MTT tetrazolium component of the Dye Solution into a formazan product. The Solubilization/Stop Solution is then added to the culture wells to solubilize the formazan product, and the absorbance at 570nm is recorded using a 96-well plate reader. The quantity of formazan product as measured by the amount of 570nm absorbance is directly proportional to the number of living cells in culture.

4. Results

4.1 IRAK-1 is involved with negative regulation of Akt phosphorylation in response to insulin and TLR agonists in BMDMs.

In has been reported that IRAK-1 may inhibit IRS-1 activity [57]. To examine precisely how IRAK-1 is involved with regulation of Akt phosphorylation, we stimulate mouse bone-marrow derived macrophages (BMDMs) with insulin and BLP and detect Akt phosphorylation levels. After insulin stimulation, Akt serine-473 phosphorylation level is kept increasing in IRAK-1$^{-/-}$ BMDMs; whereas, in WT BMDMs, Akt phosphorylation reached a peak in 5 minutes and decrease afterward (Figure 4-1). TLR-2 agonist BLP stimulation has similar inhibitory effect on wild type BMDMs, but not in IRAK-1$^{-/-}$ cells (Figure 4-2). Since IRAK-1 can also be activated by BLP-activated TLR pathway [94], the BLP data also support IRAK-1 inhibitory function on Akt phosphorylation. These data suggest that, IRAK-1 is involved with negative regulation of Akt phosphorylation. Akt serine-473 phosphorylation is a critical step to activate Akt kinase activity in PI3K-Akt signaling pathway. Although activation mechanism of Akt phosphorylation is widely studies, the attenuation step, which is as critical as initiation, is still not well unkown. Therefore, our finding may reveal a novel inhibitory regulation of Akt signaling via IRAK-1.

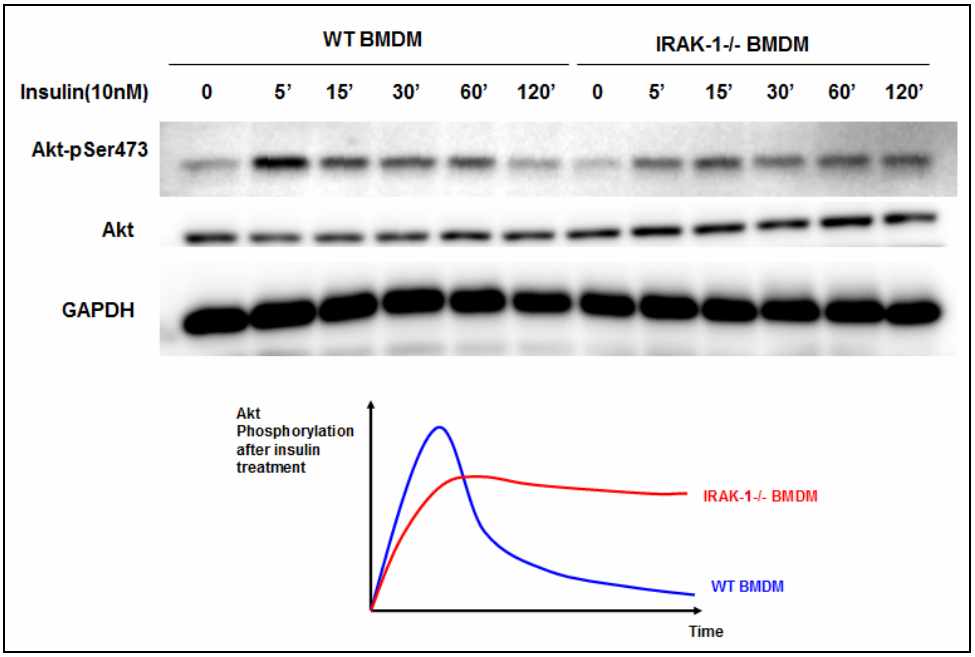


Figure 4-1. IRAK-1 is involved with the regulation of Akt phosphorylation in response to insulin. In wild type BMDMs, Akt Serine473 phosphoryation is activate by insulin, and then decrease resulting from certain negative regulation. In contrast, in IRAK-1 deficient BMDMs, the level of negative regulation is much reduced.

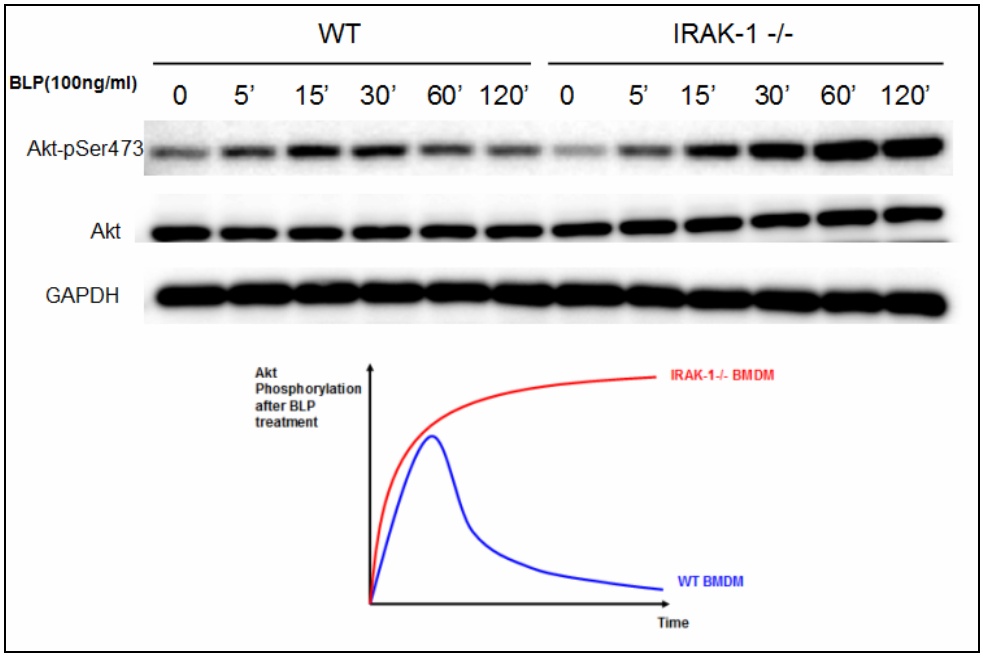


Figure 4-2. IRAK-1 is involved with the regulation of Akt phosphorylation in response to BLP. The pattern is similar to the one in response insulin stimulation. In wild type BMDMs, Akt Serine473 phosphoryation and decrease rapidly in response to BLP stimulation, suggesting certain negative regulation on Akt. In contrast, in IRAK-1 deficient BMDMs, the Akt phosphorylation

keeps increasing with little inhibitory control.

4.2 IRAK-1 interacts with IRS-1 in vivo in THP-1 cells

It has been reported that the Serine24 in the pleckstrin homology (PH) domain of IRS-1 is a specific phosphorylation site for IRAK-1 [57]. Because the protein-protein interaction is essential for subsequent phosphorylation, we want to confirm whether IRAK-1 can bind to IRS-1 in vivo. THP-1 cell is a human acute monocytic leukemia cell line. It produces abundant endogenous proteins which can be used for analysis of protein-protein interaction both in vivo and in vitro. We immunoprecipted IRAK-1 followed by Western blotting probed with IRS-1. Our data confirm that IRAK-1 interacts with IRS-1 in vivo in THP-1 cells (Figure 4-3).

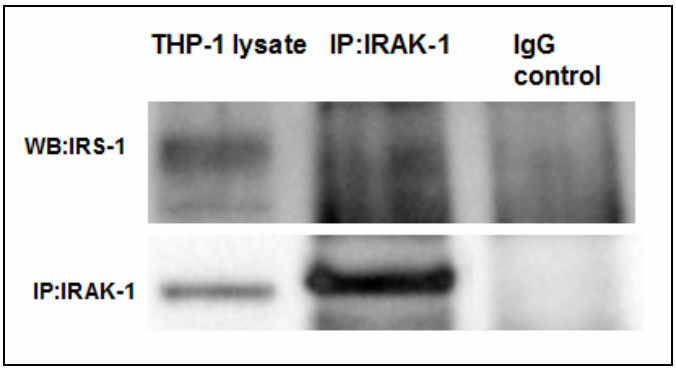


Figure 4-3. IRAK-1 interacts with IRS-1 in vivo. IRS-1 is co-immunoprecitated with IRAK-1 and detected by Western blotting, indicating an in vivo interaction of IRAK-1 and IRS-1.

4.3 IRAK-1 directly binds to IRS-1-PH domain via its LWPPPP motif in vitro.

Based on the studies we and other researchers have done so far (details in Review part 2.3), we hypothesized that IRAK-1 may interact with IRS-1 protein via the protein-protein interaction between IRAK-1 LWPPPP motif and IRS-1 PH domain; this binding may be critical for subsequent phosphorylation of the Serine24 in PH domain (N-terminal) of IRS-1 protein by IRAK-1.

To testify our hypothesis that the IRS-1 may interact with IRAK-1 via its PH domain, we construct the GST-IRS-1-PH domain expression plasmid. Using BL21DE3 E.coli expression

system and Immobilized Glutathione Agarose Resin, we expressed and purified the GST-IRS-1-PH domain protein as well as GST protein (Figure 4-4). Instead of eluting the GST fusion protein off the resin, we incubated the protein-bound resin with THP-1 cell lysate containing endogenous IRAK-1 protein, which is called the in vitro binding assay.

Our data clearly showed that IRAK-1 bind to GST-IRS-1-PH domain protein in vitro; in contrast, IRAK-1 did not bind to GST protein bound resin or empty resin (Figure 4-5). This data strongly support our hypothesis that IRAK-1 may directly bind to IRS-1, and the IRS-1 binding site is its PH domain.

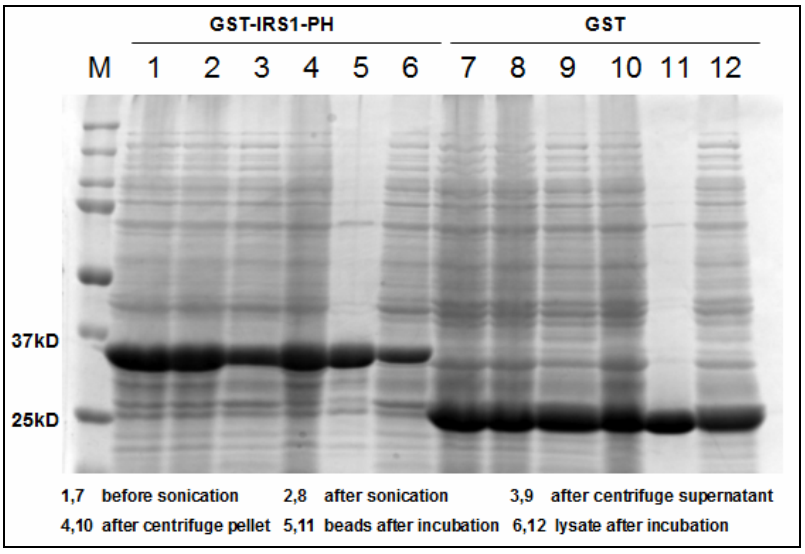


Figure 4-4. GST fusion protein expression and purification. pFN2A(GST) Flexi$^{(R)}$ Vector was used to construct GST fusion protein plasmid. IRS-1-PH domain coding sequence was sub-clone into the vector. After IPTG induction and Immobilized Glutathione Agarose Resin purification, purified GST-IRS1-PH protein and GST protein are obtained and bound to the resin (lane 5 and 11).

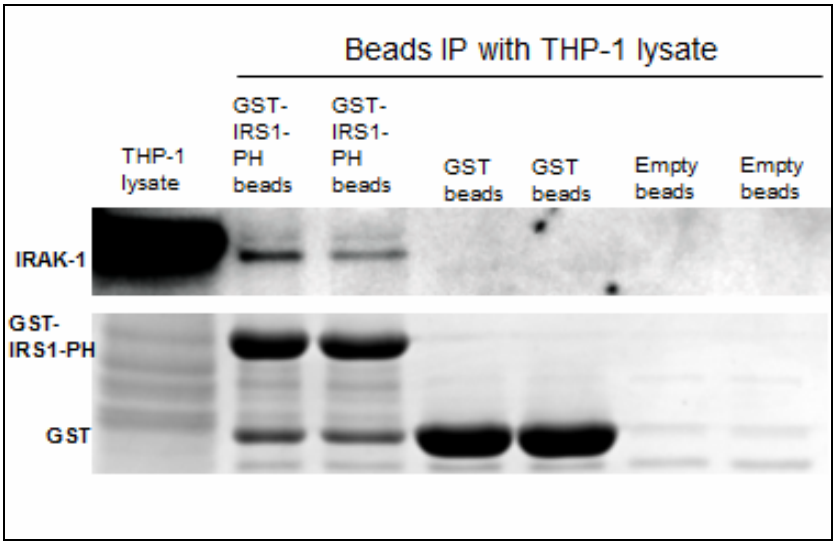


Figure 4-5. IRAK-1 binds IRS-1-PH domain in vitro. The upper part is Western Blotting probed with IRAK-1. The lower part SDS-PAGE gel stained with Instant Blue stain. Duplicate samples are set in this experiment. It is clearly shown that IRAK-1 strongly binds to GST-IRS-1-PH domain protein, but not GST protein control or empty resin. THP-1 cell lysate sample confirmed the location of IRAK-1.

To further testify our hypothesis that the IRAK-1 binding site is its proline-rich LWPPPP motif, we transfected pFlag-IRAK1-wt plasmid and pFlag-IRAK1-(L167A,W168A) double mutant plasmid into MAT-2 cells. MAT-2 cell lysates were then incubated with GST fusion protein-bound resin. Our data indicate that IRS-1 PH domain strongly binds to exogenous endogenous wild type IRAK; whereas, the binding is dramatically decreased when LWPPPP motif in IRAK-1 is dot-mutated to AAPPPP (Figure 4-6). This data strongly agree with our hypothesis that the proline-rich LWPPPP motif in IRAK-1 protein is critical for the interaction between IRAK-1 and IRS-1.

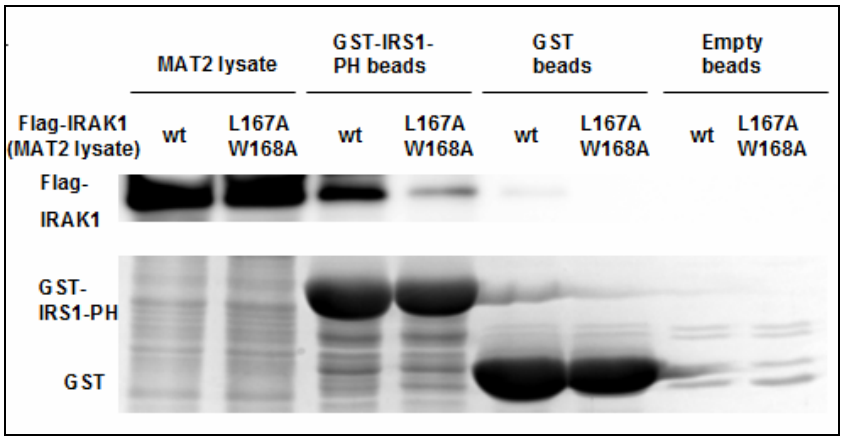


Figure 4-6. IRAK-1 LWPPPP motif is the binding site to IRS-1. Exogenous flag-IRAK-wt and flag-IRAK1-(L167A,W168A) were incubated with GST fusion protein. It is clearly shown that IRS-1-PH domain strongly binds to wt-IRAK-1; however, the binding affinity is significantly decreased when LWPPPP motif integrity is destroyed. Anti-flag antibody is probed in Western blotting to detect exogenous IRAK-1 protein.

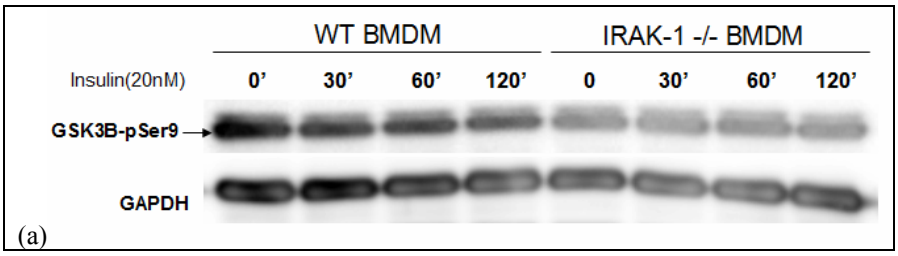
Collectively, our data support our hypothesis that IRAK-1 can interact with IRS-1 protein. Both the IRS-1 PH domain and the IRAK-1 proline-rich LWPPPP motif are critical binding sites for the biochemical interaction between IRS-1 and IRAK-1. This conclusion also agrees with the finding by other group that IRAK-1 can inhibit IRS-1 activity by phosphorylating Serine24 site in IRS-1 PH domain [57].

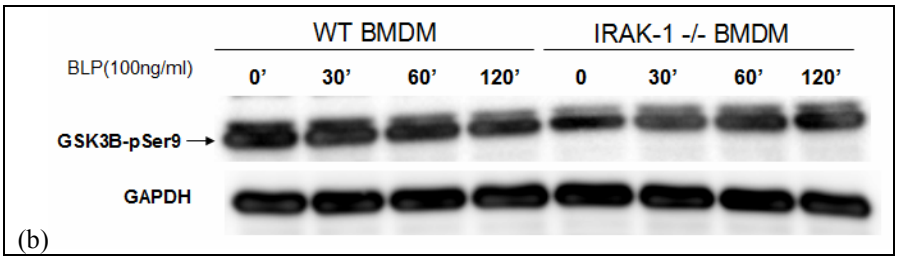
4.4 IRAK-1 positively regulates GSK3β activity and glycogen synthesis

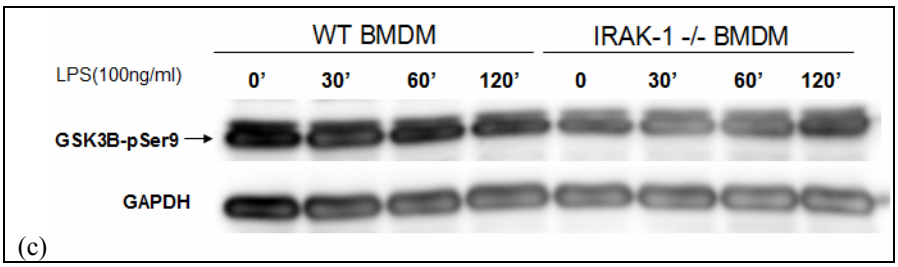
Since we have shown that IRAK-1 may negatively regulate Akt, which is a major kinase that phosphorylates and inhibit GSK3β, we want to further testify whether IRAK-1 can regulate GSK3β activity. PI3k-Akt dependent signaling pathway can be induced via both insulin receptors and TLRs. So we stimulate mouse MEFs and BMDMs with insulin, TLR2 agonist bacterial lipoprotein (BLP), and TLR4 agonist lipopolysaccharide (LPS) to detect GSK3β Serine 9 phosphorylation level under the effect of IRAK-1.

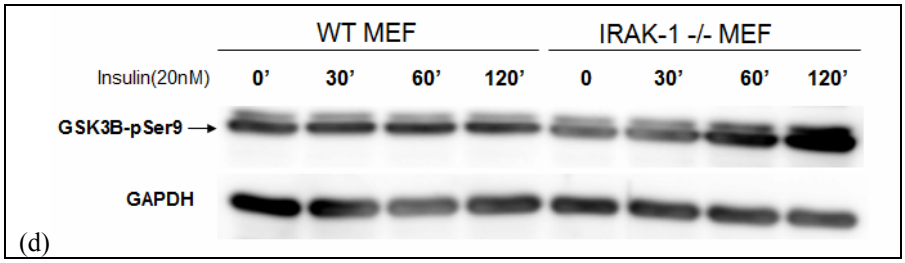
Our data clear show that in both BMDMs and MEFs, GSK3β Serine-9 phosphorylation levels

keep increasing in IRAK-1 $^{-/-}$ genotype after insulin, BLP and LPS stimulation. In contrast, there is not significant increase of pSer9 in wild type cells (Figure 4-7). These results strongly support our findings that IRAK-1 can negatively regulate Akt activity. In wild type cells, Akt activity is inhibited by IRAK-1 so GSK3β is less phosphorylated by Akt and thus more active; however, Akt activity keeps increasing without inhibitory effect of IRAK-1, resulting in GSK3 Ser9 phosphorylation and its inhibition. Taken together, our data show that IRAK-1 is involved with the positive regulation of GSK3βactivity.









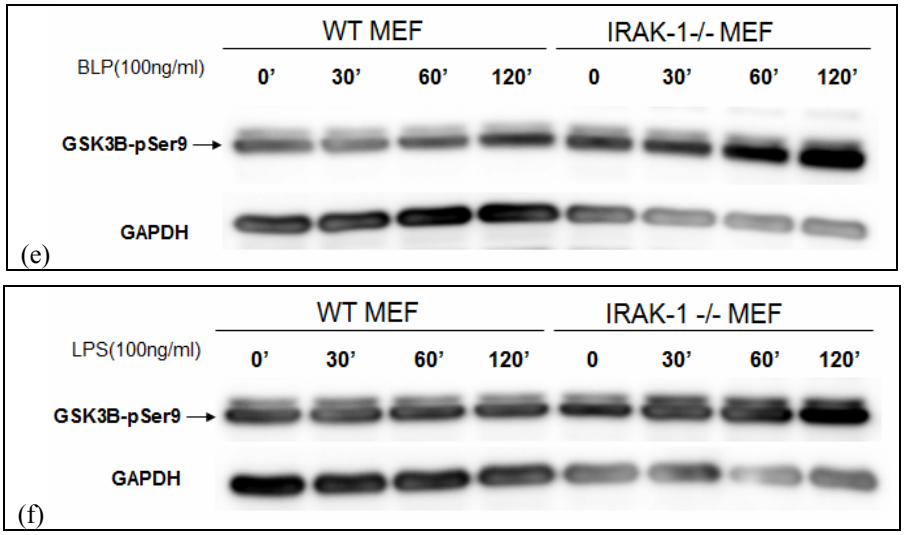


Figure 4-7. IRAK-1 positively regulates GSK3β activity in mouse BMDMs and MEFs. In both BMDMs (a-c) and MEFs (d-f), GSK3β Ser9 phosphorylation levels keep increasing in IRAK-$1^{-/-}$ genotypes, but not in wild type, after insulin, BLP and LPS stimulations. GSK3β kinase activity is inhibited after Ser9 phosphorylation. These data suggest that IRAK-1 may positively regulate GSK3β activity.

GSK3 is a key kinase that regulates glycogen synthesis by inhibiting glycogen synthase (GS) activity. In order to examine whether the effect of IRAK-1on GSK3β can further affect glycogen synthesis, we performed glycogen synthesis assay. Wt and IRAK-$1^{-/-}$ mouse hepatocytes were harvested, cultured, and treated with insulin for 3 hours. Cells were then harvested and glycogen productions were measured by Glycogen Assay Kit (BioVision), following the protocol described in the assay kit.

Our data clearly show that, IRAK-1-/- hepatocytes, glycogen synthesis production significantly increase after insulin induction within only 3 hours ($p<0.01$); whereas, the induction level is less in wild type cells ($p<0.05$) (Figure 4-8). This result suggests that IRAK-1 may negatively regulate glycogen synthesis process.

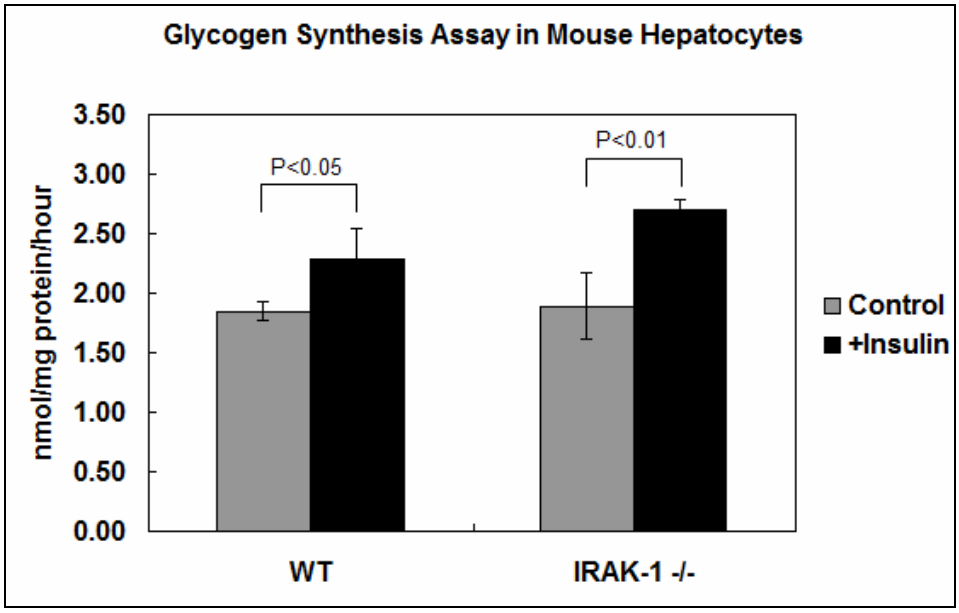


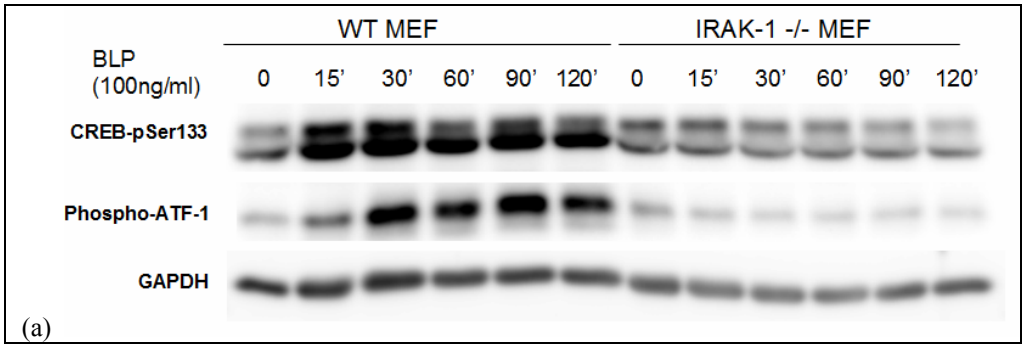
Figure 4-8. IRAK-1 negatively regulates glycogen synthesis in mouse hepatocytes. After insulin stimulation for 3 hours, glycogen synthesis productions increase more significantly in IRAK-$1^{-/-}$ hepatocytes ($p<0.01$) than in wild type cells ($p<0.05$). This result suggests that IRAK-1 may negatively regulate glycogen synthesis.

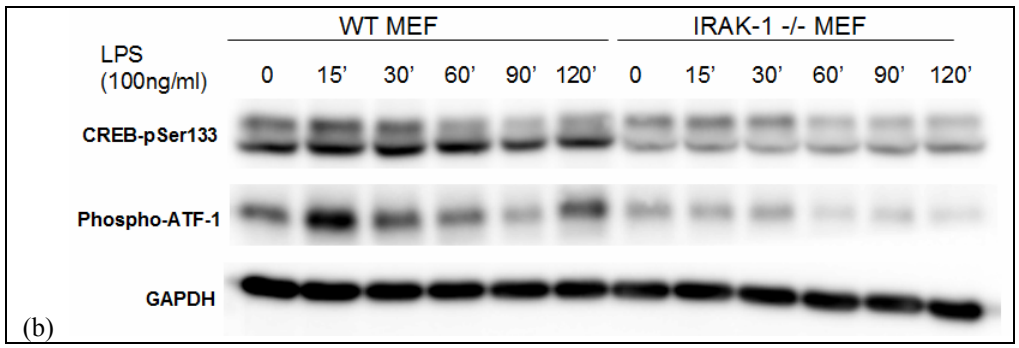
Collectively, our data show that IRAK-1 may positively regulate GSK3β activity and inhibit glycogen synthesis process. It is highly likely that these effects of IRAK-1 are due to the inhibitory effect of IRAK-1 on PI3K-Akt dependent signaling pathway, which is strongly agreed with our previous findings.

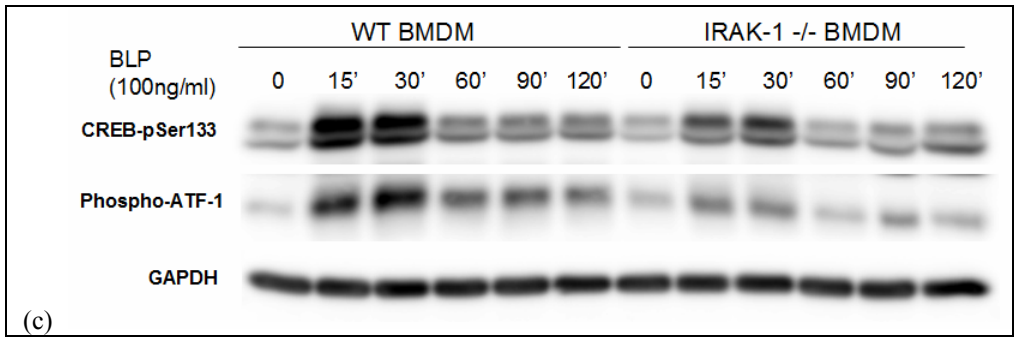
4.5 IRAK-1 positively regulates CREB and ATF-1 activities by stimulating CREB-Ser133 and ATF-1 phosphorylation

It has been reported that the transcription factors CREB and ATF-1 can be activated by various signaling pathways including p38-MAPK pathway, and that IRAK-1 is recruited by TLRs and plays an important role in the upstream of several of these pathways (details in Review part 2.1 and 2.4). Moreover, recently researchers in our lab have found that the NADPH oxidase catalytic subunit NOX-1 expression level is higher in wild type cells compared with in IRAK-1-/- genotype. Interesting, NOX-1 can be actively by transcription factor ATF-1 [88]. Therefore, we hypothesized that IRAK-1 may be involved the activation of CREB and ATF-1.

To examine these connections, we stimulated mouse MEFs and BMDMs with TLR2 agonist BLP and TLR4 agonist LPS, and detect CREB-Ser133 and ATF-1 phosphorylation levels. Our data clearly show that, in both MEF and DMDM cells, CREB-Ser133 and ATF-1 phosphorylation levels are rapidly and dramatically increased after BLP and LPS stimulation in wild type cells. In contrast, CREB-Ser133 and ATF-1 phosphorylation levels only have a much less increase in IRAK-1$^{-/-}$ cells (Figure 4-9). Since CREB-Ser133 and ATF-1 phosphorylation are essential steps for their transcription factor DNA binding activity and their activation, these results strongly agree with our hypothesis that IRAK-1 may be critically involved with CREB and ATF-1 activity upon TLR2 and TLR4 agonist stimulation.







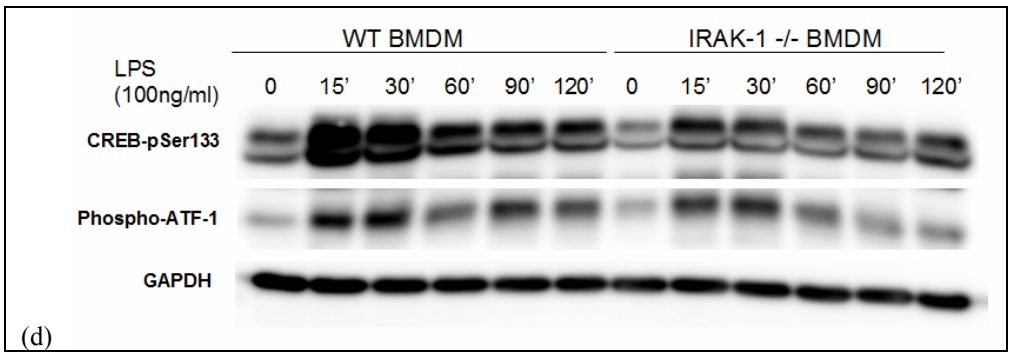


Figure 4-9. IRAK-1 is involved with CREB and ATF-1 activation in MEFs and BMDMs. In both MEFs (a, b) and BMDMs (c, d), CREB-Ser133 and ATF-1 phosphorylation levels are rapidly and dramatically increased in wild type cells after BLP and LPS stimulations. In contrast, the induction effect is much reduced in IRAK-$1^{-/-}$ cells.

It has been reported that the transcription factor CREB and ATF-1 are critically involved with cell survival pathways [87]. Interestingly, in the daily cell culture of MEFs and BMDMs, we noticed that wild type cells always grow faster than IRAK-$1^{-/-}$ cells, even IRAK-$1^{-/-}$ cells are larger in size. We want to quantitatively measure this difference which may be involved with cell proliferation, apoptosis and survival processes.

To quantify the cell proliferation / survival rate in mouse MEFs without stimulation, we used the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) (Promega). Our data show that, the cell proliferation/survival rate is higher in wild type cells than in IRAK-$1^{-/-}$ cells after 48 hours culture, but not after 24 hours (Figure 4-10). Since initial cells numbers are set the same in two genotypes, after 24 hours they may grow similarly; whereas, after a long-term 48 hours, cell proliferation, survival, apoptosis process may start to work. Our data suggest that IRAK-1 may be involved with cell proliferation and survival. However, the complex mechanisms behind them remain unclear.

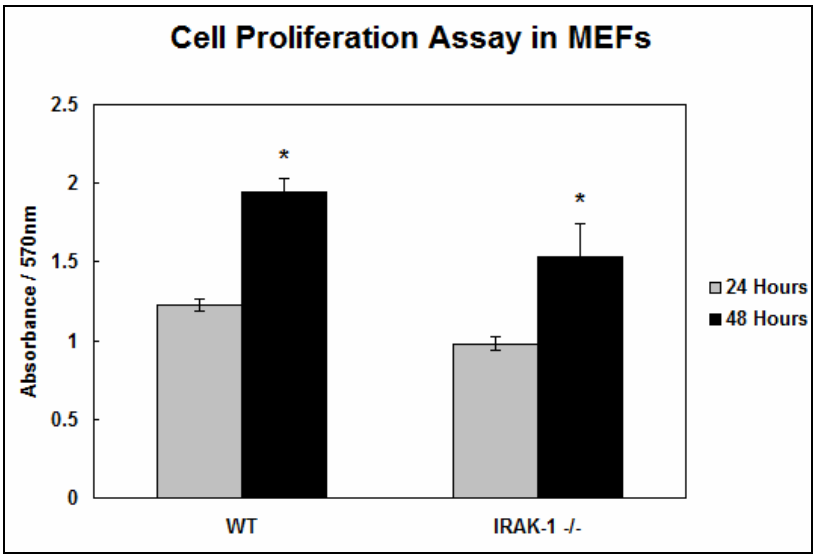


Figure 4-10. IRAK-1 may be involved with cell proliferation and cell survival in MEFs. The initial cell numbers are set the same at 5000 cells for wild type and IRAK-$1^{-/-}$ MEFs. The cell growth rates are similar between wt and IRAK-$1^{-/-}$ cells after 24 hours. In contrast, the cell proliferation/survival rate is much higher in wt than in IRAK-$1^{-/-}$ cells after 48 hours ($p<0.05$). This difference may due to the cell proliferation, apoptosis and survival processes.

Collectively, our data show that IRAK-1 activates CREB and ATF-1 activities by stimulating CREB-Ser133 and ATF-1 phosphorylation in mouse MEFs and BMDMs. These results are strongly agreed with recent findings by our lab that NOX-1 expression level is higher in wild type than in IRAK-$1^{-/-}$ cells (data not shown). Moreover, these data also suggest that IRAK-1 may be involved with cell proliferation and survival process.

5. Discussion

Obesity-induced chronic inflammation is a critical component in the pathogenesis of insulin resistance and the metabolic syndrome. Pro-inflammatory cytokines can cause insulin resistance in adipose tissue, skeletal muscle and liver by inhibiting insulin signal transduction. Interleukin-1 receptor-associated kinase-1 (IRAK-1) is a serine/threonine kinase is functioning in Toll-like Receptor signaling pathways, and plays an important role in inflammation and immune response. It participates in various cell signaling networks, ranging from inflammatory response, gene transcription regulation, cell survival and metabolic activities.

While researchers are highly focused on IRAK-1's functions in these areas, IRAK-1's role in regulating PI3K-Akt signaling and glucose metabolism is not well understood. In our studies, we demonstrated that IRAK-1 is involved with the negative regulation of PI3K-Akt dependent signaling induced by insulin and TLR-2 and TLR-4 agonists (Figure 4-1, 4-2). Out data also indicate that IRAK-1 can interact with IRS-1 protein both in vivo (Figure 4-3) and in vitro (Figure 4-5, 4-6). Moreover, we have identified that binding site on IRS-1 is its PH domain, and the binding site on IRAK-1 is its proline-rich LWPPPP motif. Other researchers have reported that IRAK-1 can phosphorylate IRS-1 on its Ser-24 site in PH domain and inhibit IRS-1 activity [57]. Our data strongly agree with their findings and further suggest that the biochemical interaction between IRAK-1 LWPPPP motif and IRS-1 PH domain is critical for kinase recruitment and subsequent phosphorylation. Since IRS-1 is in the upstream in the PI3K-Akt dependent signaling, our data provide a possible explanation of feedback inhibition mechanism in the regulation of PI3K-Akt signaling. Furthermore, our studies suggest that IRAK-1 may contribute to linking inflammation with insulin resistance via the novel substrate IRS-1.

Our studies also indicate that IRAK-1 is involved with the negative regulation of glucose metabolism through inhibiting PI3K-Akt signaling pathway. Akt is one of the most important kinases plays central rules in a large variety of cell activities. Activated Akt phosphorylates GSK3β on its Ser-9 site and inhibit GSK3β activity. GSK3β is a key kinase that negatively regulates glycogen synthesis by phosphorylating glycogen synthase and thus inhibiting its activity. Our data indicate that GSK3β Ser-9 phosphorylation level is higher in IRAK-$1^{-/-}$ cells, and thus it has a lower kinase activity (Figure 4-7). This may be due to higher Akt activity without the inhibitory effect of IRAK-1. In consequence, glycogen synthase is more active in $IRAK^{-/-}$ hepatocytes, and there is higher glycogen production in IRAK-$1^{-/-}$ mouse hepatocytes after insulin stimulation (Figure 4-8). Collectively, our studies suggest that IRAK-1 is involved with the negative regulation of glucose metabolism. Moreover, because GSK3β is not only a key regulator in glycogen synthesis process, but also critically involved with the regulation of various pro- and anti-inflammatory cytokine expression. Further study on IRAK-1's role on

GSK3β-related gene expression may be promising.

Moreover, our studies also suggest that IRAK-1 is involved with the activation of transcription factors CREB and ATF-1, two members in CREB transcription factor family. CREB family transcription factors induce genes involved in cellular metabolism, gene transcription, cell cycle regulation, cell survival, as well as growth factor and cytokine genes [79]. The CREB-Ser133 and ATF-1 phosphorylation are critical for their DNA binding activity. Our studies show that CREB-Ser133 and ATF-1 phosphorylation levels are higher in wild type cells than in IRAK-$1^{-/-}$ cells in response to TLR2 and TLR4 agonists (Figure 4-9). A possible reason is that IRAK-1 stimulates CREB and ATF-1 phosphorylation through p38 MAPK pathway downstream of TLR signaling. Moreover, we also found that wild type cells has higher proliferation/survival rate compared with IRAK-$1^{-/-}$ cells (Figure 4-10), suggesting that IRAK-1 may be also involved with cell proliferation and survival pathway. But the detail mechanism of this finding remains to be defined. Further study on IRAK-1's role in CREB/ATF-1-involved gene expression is very intriguing.

6. Conclusion

Taken together, our studies indicate that IRAK-1 protein is involved with negative regulation of PI3K-Akt dependent signaling pathway. A feedback mechanism is fulfilled by IRAK-1's novel substrate, IRS-1, which is in the upstream of PI3K-Akt pathway. We have also identified that IRAK-1 can bind to IRS-1 both in vivo and vitro, and the biochemical interaction binding sites may be IRS-1's PH domain and IRAK-1's proline-rich LWPPPP motif. Meanwhile, our studies indicate that IRAK-1 may negatively regulate glycogen synthesis process by stimulating GSK3β activity through inhibition of PI3K-Akt pathway. Moreover, our data also show that IRAK-1 may activate the transcription factors CREB and ATF-1's activity, which may provide a clue to explain IRAK-1's positive role in cell proliferation and survival process. Collectively, our studies demonstrate that IRAK-1 protein is involved with several critical signaling pathways that regulate ranging from glucose metabolism, gene transcription, to cell proliferation and survival.

Further study on both the downstream signaling and functional outcomes is promising.

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