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THE REGULATION OF IRS-1 VIA P70 S6K-1 AND P300 ACTIVITY

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by

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ABSTRACT

The study of IRS-1 has increased over the years due to the prevalence of cardiometabolic diseases. An increase in IRS-1 serine phosphorylation is often associated with the development of insulin resistance due to the downregulation bestowed upon it by different kinases such as p70 S6K-1. Recently, p300 has been shown to acetylate IRS-1 thus leading to its downregulation. However, studies have not looked into the relationship that exists between serine phosphorylation and lysine acetylation of IRS-1. In this study, insulin stimulated HepG2 cells were treated with either PF4708671, a selective p70 S6K-1 inhibitor, or C646, a selective p300 inhibitor. PF4708671 treatment led to the successful dose dependent decrease in the phosphorylation of S312, S636/639, and S1101 on IRS-1. Treatment with 10μM of C646 did successfully decrease p300 activity. By inhibiting p300 activity there was a decrease in S312 and S1101 phosphorylation. The inhibition of p300 also led to an exponential increase in the activation of insulin stimulated RPS6, thus meaning that there was an increase in IRS-1 insulin signaling. Thus, p300 inhibition prevents the phosphorylation of the negative regulatory sites S312 and S1101. In addition, p300 activity enhances the phosphorylation of S1101, while the overall phosphorylation by p70 S6K-1 enhances the overall acetylation of IRS-1. There is a connection that exists between the acetylation and serine phosphorylation of IRS-1 and further research will need to be done to narrow down the exact mechanism. Together we elucidate that the inability of p300 to acetylate IRS-1 leads to a decrease in the phosphorylation of IRS-1 S312 and S1101 in insulin stimulated HepG2.

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LIST OF ABBREVIATIONS

AKT	protein kinase B
BARK1	β-adrenergic receptor kinase-1
CamKIV	Calcium/calmodulin-dependent protein kinase IV
CBP	cyclic AMP response element-binding protein
CREB	cyclic AMP response element-binding
ERK	extracellular signal-regulated kinase
ET-1	Endothelin-1
GNC5	general control non-repressed protein
GRK2	G protein-coupled receptor kinase 2
GSK3	glycogen synthase kinase-3
HAT	histone acetyltransferase
HFD	High fat diet
IKK	IkappaB kinase
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
JNK	c-Jun N-terminal Kinase
KAT	lysine acetyltransferase
mTROC1	mammalian/mechanistic target of rapamycin complex 1
NLS	Nuclear localization signal
P 70 S6K-1	Ribosomal protein S6 kinase beta-1
P300	E1A binding protein p300
PDK	phosphoinositide-dependent protein kinase -1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase c
PP1	protein phosphatase 1

PTB	Phosphotyrosine binding
RING	really interesting new gene
ROCK1	rho-associated coiled-coil containing protein kinase 1
Snf1	sucrose non-fermenting
TCS	Tuberous sclerosis complex
TNF-α	tumor necrosis factor-alpha

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The regulation of IRS1 via S6K-1 and p300 activity

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Introduciton

1.1 Overview of IRS-1

1.1

Discovery and history

The discovery of insulin in 1921 paved thea way for the discovery of the insulin receptor substrate-1 (IRS-1), a 185kD protein related to insulin signaling in 1985 [1]. This initial discovery led to the understanding that IRS-1 is directly phosphorylated on some of its tyrosines by the insulin receptor (IR), which is a heterotetrameric-membrane-bound tyrosine kinase receptor, in 1987 [1,2]. Following this, an important discovery was made which revealed that IRS-1 obtained maximal tyrosine phosphorylation merely 30 seconds after insulin stimulation. However, this maximal effect iswas diminished in half after 4hrs of continuous stimulation, while the phosphorylation status of IR remains unaffected [3]. While this discovery may have not seemed significant at the time, it pointed to further intracellular components that were responsible for indirectly regulating insulin signaling via IRS-1 posttranslational modifications, further revealing pertinent components in the understanding of metabolic diseases such as diabetes. Between 1989 and 1997, IRS-1 negative regulation by serine/threonine phosphorylation was discovered, and later in 1997 the elevation in serine/threonine phosphorylation was linked to insulin resistance, an important indicator of Type 2 diabetes [4].

Normal physiology of insulin action

Insulin is a peptide hormone that binds to membrane-bound receptors that are typically distant from the site of its synthesis, the pancreas. The pancreas is an organ located in the left hypochondriac region of the abdomen together with the spleen, colon, and superior region of the left kidney.[7] The pancreas has both endocrine and exocrine functions that are not equally distributed throughout the entire pancreas. While most of the pancreas is dedicated to exocrine function, a very small section composed of a cluster of islets of Langerhans makes up the endocrine portion of the pancreas. The islets of Langerhans consist of five distinct cells types that secrete five different hormones. The cell types are nameds after the first, second, fourth, fifth, and twentieth letters of the Greek alphabet: alpha, beta, delta, epsilon, and upsilon. The hormones produced by these cell types are glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, respectively.[8] Addtionally, tThe beta cells are also known to produce amylin. [8]

While there are relatively fewAs few as the islets of Langerhans are, the beta cells of the pancreas can initiate the production of at least 6000 preproinsulin molecules from mRNA in a second. Preproinsulin is later modified to proinsulin upon the removal of a signal peptide (it is suggested to be the signal that translocates the entire molecule to the endoplasmic reticulum (ER)) on its N terminus, which allows for the effective folding of proinsulin at the ER.[8,9] Further post-translational modifications occur in the ER including the cleavage of the C-peptide domain by endopeptidases, which leaves 2 fragments of proinsulin: the α-chain and the β-chain.

Both fragments are bonded parallel to each other by disulfide bond formation from two cysteine pairs thus making what we now call mature insulin. [9,10] Mature insulin and C-peptide are

transported to the Golgi apparatus where they are packaged in secretory granules [10] awaiting calcium-dependent intracellular influx which will initiate the exocytosis of the secretory vesicles. The mature insulin is secreted into the blood simultaneously with C-peptide and transported through the blood to membrane-bound receptors to elicit an anabolic response. [8-10] Its secretion into the blood is stimulated mainly by increased blood sugar. However, free fatty acids, amino acids, and other nutrients can stimulate its release too. Once released insulin regulates metabolic mitogenic processes that affect tissues such as the liver, adipose tissue, and muscles . (Figure 1).

The structure of IRS-1

The structure and function of IRS-1 can be understood by comparing its relationship with insulin and the IR. Insulin is secreted by the pancreas throughout the bloodstream to various tissue types. After insulin is secreted into the blood it is transported to the target site where it binds to the IR. As mentioned before, the IR is a heterotetrameric-membrane-bound tyrosine kinase receptor and contains two α and two β subunits. Insulin binds to the α subunits that are exposed on the extracellular portion of the cell membrane, while the membrane-spanning β subunits undergo a conformational change that transmits signals intracellularly [5,6]. (Figure 2) Consequently, the activation loop of IR contained within the β subunits undergoes trans-autophosphorylation on tyrosine residues Y1162, Y1158, and Y1163 [5,6]. These residues are NXPY motifs, tyrosine phosphorylation recognition elements, that bind to the phospho-tyrosine binding (PTB) domains of other proteins. When these sites are transphosphorylated it leads to the auto-phosphorylation of Y972 [7,8]. The structure of IRS-1 allows for its recruitment to the plasma

membrane, anchoring, and activation via the IR. IRS-1 contains a pleckstrin-homology (PH) domain on its N-terminus followed by a PTB domain and a C-terminus rich in tyrosine phosphorylation sites . (Figure 2). Initially, IRS-1 is recruited due to its recognition of the phosphorylated IR NXPY motifs, [9,10] then it is bound to the auto-phosphorylated Y972 of IR via its PTB domain. [11] IRS-1 is then anchored to the cellular membrane by the PH domain due to the production of phosphoinositide 3,4,5-trisphosphate (PIP3) by phosphoinositide-3-kinase (PI3K) [11]. Lastly, the YXXM motifs contained within the C-terminus, specifically Y465, Y612, Y632, Y941, and Y989 [12] are phosphorylated by IR creating a docking site for the SH2 domain found on the regulatory subunit (p85) of PI3K. PI3K also contains a catalytic subunit (p110) that propagates further downstream insulin signaling. [11]

1.2 The metabolic and mitogenic effects of IRS-1 signaling

The metabolic and mitogenic effects of IRS-1 signaling: AKT signaling

The outcomes of IRS-1 signaling are either metabolic or mitogenic. Most of these outcomes occur due to the activation of PI3K. When activated, PI3K adds a phosphate to phosphatidylinositol 4,5-bisphosphate (PIP2) to produce phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) [13]. Subsequently, PIP3 recruits protein kinase B (PKB/AKT), an important indirect regulator of IRS-1, and phosphoinositide-dependent protein kinase -1 (PDK-1) to the inner leaflet of the plasma membrane due to the PH domain on both AKT and PDK-1. Upon their recruitment, PDK-1 phosphorylates AKT at T308 on the activation loop, while mechanistic target of rapamycin complex 2 (mTORC2) phosphorylates AKT at S473 on the hydrophobic

motif [14-16]. AKT then directly initiates mitogenic responses such as cell growth and differentiation by inhibiting proteins involved in the inhibition of the cell cycle [17]. Akt specifically inhibits glycogen synthase kinase (GSK) 3β via phosphorylation to prevent apoptosis [18]. The metabolic effects of AKT activation include inhibition of gluconeogenesis via direct phosphorylation of FOXO1, promotion of glycogen and fatty acid synthesis via the inhibition of GSK-3, promotion of fatty acid synthesis via the direction activation of SREBP-1 [19,20], and promotion of glucose uptake by glucose transporter type 4 (GLUT4) via the direct phosphorylation of AKT substrate of 160 kDa (AS160), a Rab-GTPase activating protein, and TBC1 domain family, member 1 (TBC1D1) [17,21]. The activation of TBC1D1 also further elicits the mitogenic response initiated by IRS-1 activation such as cell growth and differentiation [21] (Figure 3) . The aforementioned mitogenic and metabolic downstream targets of AKT effects are heavily affected by the dysregulation of IRS.

The metabolic and mitogenic effects of IRS-1 signaling: mTORC1 signaling

AKT phosphorylation leads to the activation of mTORC1 (mechanistic target of rapamycin complex 1), a complex with kinase activity that not only promotes further metabolic and mitogenic pathways but is also an important direct regulator of IRS-1. To activate mTORC1, AKT initially phosphorylates tuberous sclerosis complex 2 (TSC2), also known as tuberin, which results in its dissociation from hamartin (TSC1). This action activates mTORC1 since the TSC1-TSC2 complex inhibits mTORC1 indirectly by functioning as a GTPase activating protein (GAP) to catalyze the conversion of Rheb-GTP to Rheb-GDP [22,23]. Rheb-GTP serves to directly stimulate mTORC1 activity (Figure 4)

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mTORC1 is a serine/threonine-protein kinase complex that functions as a nutrient/energy sensor that promotes and regulates translation and protein synthesis. It exists as a complex composed of 3 main components: mTOR, Raptor, and GβL (also known as mLSTB8 - mammalian lethal with Sec13 protein 8). Raptor is the regulatory protein associated with mTOR. It facilitates substrate recruitment of mTORC1 by binding the TOR signaling (TOS) motif found on several canonical mTORC1 substrates. Additionally, it is required for correct subcellular localization of mTORC1. GβL (G protein beta subunit-like) is known to associate with the catalytic domain of mTORC1 and may stabilize the kinase activation loop. mTORC1 has two 2 inhibitory subunits: Proline-rich AKT substrate of 40kDa (PRAS40) and DEP domain-containing mTOR interacting protein (DEPTOR). AKT does not only inhibit the association of TSC1 and TSC2, which prevents the formation of the inhibitory TSC-TSC2 complex, but it also inhibits PRAS40 thus leading to the activation of mTORC1. Although Rheb is an essential activator of mTORC1, exactly how Rheb stimulates mTORC1 is not completely understood. Some have postulated that mTORC1 can be phosphorylated at 2448 by AKT, which is indicative of its catalytic activity, however, this view is not supported as it has been shown to be an inadequate measurement of activity [24].

mTORC1 signaling is mainly associated with cell growth and proliferation via the following processes: lipogenesis, nucleotide synthesis, ribosome biogenesis, mRNA translation, proteo-genesis, and the inhibition of autophagy [25]. Once active, mTORC1 prepares eukaryotic translation initiation factor 4E - binding protein 1, also called 4E-BP1, for inactivation by directly phosphorylating T37 and T46. S65 and T70 are subsequently phosphorylated by mTORC1 to promote the dissociation of 4E-BP1 from eukaryotic translation initiation factor 4E (eIF4E). As a result, eIF4E is released and allowed to assemble with eIF4G1, eIF4A, and eIF4B1

at the 5’ cap of mRNA to initiate translation which subsequently leads to protein synthesis [26,27]. mTORC1 inhibits autophagy via phosphorylation of Unc-51 like kinase-1 (ULK1) at S758 which leads to the inability of adenosine monophosphate-activated protein kinase (AMPK) to activate ULK1 via phosphorylation at S317. mTORC1 also inhibits autophagy via phosphorylation of autophagy-related 14 (ATG14). mTORC1 indirectly activates sterol regulatory element-binding proteins (SREPB) [27], a protein that is known to promote the synthesis of endogenous cholesterol, fatty acids, triacylglycerol, and phospholipid synthesis [28], by directly phosphorylating Lipin-1 . (Figure 54). Furthermore, mTORC1 promotes nucleotide synthesis, ribosome biogenesis, and elongation during translation by directly phosphorylating S6K-1 at T389 [27].

The metabolic and mitogenic effects of IRS-1 signaling: S6K-1 signaling

p70 S6K-1 is transcribed from a gene called RPS6KB1 on chromosome 17, not to be confused with RPS6KB2 found on chromosome 11, which encodes for S6K-2. RPS6KB1 codes for 3 isoforms of S6K-1, S6KαI, S6KαII, and p31 S6K-1, each with distinct functions. S6KαII is infamously known as p70 S6K-1 due to its size, while S6KαI is known as p85 S6K-1. Both these isoforms are identical in sequence, yet S6KαI does not contain the first 23 amino acids seen in S6KαII [29]. p31 S6K-1 contains 31 amino acids and is only similar to p85 S6K-1 as it pertains to function. Even though p85 S6K-1 has a nuclear localization signal (NLS) and p70 S6K-1 does not, p85 S6K-1 and p70 S6K-1 are often confused with each other due to their involvement in the mTORC1 signaling pathway. They contain an 80-90 amino-acid-long PDZ domain located on the C terminus (given its acronym from the first 3 proteins known to have this domain; postsynaptic density protein of 95 kDa (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (Zo-1))) [30]. The PDZ domain is well known to associate with

actin within the cytoskeleton close to the membrane, making it ideal for Raptor to recruit and promote the interaction of p85 S6K-1 and p70 S6K-1 to the mTORC1 complex by binding to their TOS motif. Subsequently, the mTORC1 complex phosphorylates the hydrophobic motifs, one at T389 (p70 S6K-1) and another at T412 (p85 S6K-1) [29]. While both p85 S6K-1 and p70 S6K-1 can be turned on by the same kinase, p85 S6K-1 promotes tumorigenesis and cell migration making it an oncogenic kinase [31] with no direct relationship to insulin signaling. On the other hand, p70 S6K-1 phosphorylation by mTORC1 is directly related to increased nutrient availability, due to insulin stimulation or amino acid availability. The phosphorylation of p70 S6K-1 at T389 primes it for phosphorylation on its activation loop by PDK1 at T229, rendering p70 S6K-1 fully active.

When p70 S6K-1 is active it phosphorylates ribosomal protein S6 at the following sites in the following order: S236, S235, S240, S244, and S247 [27], [32] leading to ribosomal biogenesis. In addition, active p70 S6K-1 activates GIn-dependent carbamoyl phosphate synthase, Asp carbamoyl transferase, dihydroorotase (CAD) by phosphorylating it at S1859 which results in pyrimidine synthesis. Both mTORC1 and p70 S6K-1 pathways promote nucleotide and protein synthesis, as well as lipid synthesis by activating SREBPs (Figure 6). [27].

1.2 1.3 IRS-1 regulation

1.3

IRS-1 is regulated by both insulin stimulated dependent and insulin independent kinases. Generally, tyrosine phosphorylation is depicted as the positive regulation while negative

regulation is attributed to serine phosphorylation (Figure 7). Recently, acetylation has also being associated with negative regulation of IRS-1 . (Figure 76). However, determining the role serine phosphorylation is complicated, as there are conflicting reports regarding its contribution towards the inhibition of IRS-1 (Figure 7). Whether the regulation is negative or positive is dependent on the kinase, the serine sites phosphorylated on IRS-1, the order in which the sites are phosphorylated, and when the phosphorylation takes place. [33].

Kinase dependent positive regulation of IRS-1

While it is already established that phosphorylation of IRS-1 on its tyrosine residues leads to its anchoring on the plasma membrane and subsequently its activation, the phosphorylation of some serine sites on IRS-1 can enhance its activation. For example, the phosphorylation of IRS-1 at S1223 due to insulin stimulation promotes prolonged IRS-1 tyrosine phosphorylation by diminishing IRS-1-SHP2 (SH protein tyrosine phosphatase -2) interaction. SHP2 usually binds to IRS-1 after IRS-1 is activated by IR. The IRS-1 and SHP2 interaction may beutlilize negative feedback since it leads to the dephosphorylation of the tyrosine residues in which PI3K binds (YMXM motif). [34,35].

When IRS-1 is activated it eventually leads to the activation of AKT, a downstream effector kinase of the insulin signaling pathway. AKT leads to positive feedback that involves the phosphorylation of IRS-1 on S629. This positive regulation of IRS-1 is further understood to avert the inhibitory phosphorylation of IRS-1 at S636 by the extracellular signal-regulated kinase

(ERK). Studies have shown that introducing a mutation on IRS-1 that substitutes the serine on 629 to an alanine, in order to prevent its subsequent phosphorylation, leads to the increased phosphorylation of S636. However, this effect is only seen when Chinese hamster ovary (CHO) cells are treated with 100nM of insulin for 15min after being serum starved for 4hrs [59].

The timing in which a site is phosphorylated can determine whether the regulation will be positive or negative. Such is the case with the phosphorylation of IRS-1 at S307 by p70 S6K-1 and mTORC1, S323 by protein kinase c (PKC) λ/ς, and S636 by rho-associated coiled-coil containing protein kinase 1 (ROCK1) [33]. For example, rapid stimulation by glucose and amino acids enhanced the phosphorylation of S307 at the basal state thus promoting the positive regulation of IRS-1. In this study,Studies have demonstrated that cells were serum starved for 4 hrs. and then treated with 100nM of insulin for varying lengths of time: 0 min, 1 min, 5 min, 15 min, 30 min, 60 min, 120 min, and 240 min. 30 min of treatment with 100nM of insulin yielded the highest phosphorylation of S307, while after 1 hr the phosphorylation was progressively diminished. In addition, studies have also shown that during the 30 min stimulation, AKT concentration increased but decreased at the 1 hr mark; thus, showing a positive regulation [36,37].

On the other hand, hyperinsulinemia which leads to excessive activation of both mTORC1 and S6K-1 led to a pathogenic negative regulation of IRS-1 at S307, thus denoting the phosphorylation of S307 to be either positive or negative. These temporally based regulations can also either be positive or negative depending on the type of kinase that phosphorylates them [33]. However, another study refutes any involvement of p70 S6K-1 or mTORC1 in the

phosphorylation of S307. This argument is based on the view that p70 S6K-1 and mTORC1 may affect S307 phosphorylation buty make not be the kinases responsible for directly phosphorylating S307.[33,38]

Kinase dependent negative regulation of IRS-1

The dysregulation of IRS-1, as it pertains to insulin resistance, can be studied by looking at kinases and the phosphorylation sites they target. An exhaustive list of kinases are responsible for the negative regulation of IRS-1 on at least 19 serine/threonine sites. However, these kinases are usually stimulated by stimulants such as cytokines, angiotensin II, endothelin-1, free fatty acids, amino acids cellular stress and insulin [39].

Protein Kinase C enzymes are commonly known for being stimulated by diacylglycerol (DAG) and/or Calcium (Ca^{2+}). They function as threonine and serine kinases. Of the 15 human PKC isoenzymes, which are categorized as either atypical, novel, or conventional, 6 are known to play a part in the regulation of IRS-1 [40,41].

Conventional protein kinase C alpha and beta (PKCα and PKCβ) require DAG and Ca^{2+} for activation, while novel protein kinase C theta and delta (PKCθ and PKCδ) only require Ca^{2+}. Atypical protein kinase C zeta (PKCζ) does not function as typical PKC enzymes function as, their activation is dependent on lipids as well as PIP3 and PDK1. Among their many functions, PKCα, PKCβ, PKCθ, and PKCδ negatively regulate IRS-1 [40,42].

The first negative regulation of IRS-1 involves the phosphorylation of S24 by PKCα after stimulation by phorbol 12-myristate 13-acetate (PMA) [43]. S24 is located within the PH domain and when it is phosphorylated, the ability for IRS-1 to associate with the membrane is negatively affected. Consequently, there is reduced IRS-1 and IR interaction as well as reduced phosphorylation of the docking sites for PI3K [44], which ultimately reduces mTORC1 activity. Interestingly, activation of PKCα via insulin or ceramide (a common activator of PKCα), did not lead to the phosphorylation of S24 [45,46]. PMA mediated phosphorylation of IRS-1 S24 by PKCα ultimately leads to the degradation of the insulin peptide hormone [47]. The rest of the serine sites are not found within any of the domains but are instead proximal to the PRB domain or further along the C-terminal tail.

Treatment with PMA was later discovered to increase the phosphorylation of IRS-1 S337 and S341, while inhibition of PKCα and PKCβ attenuated the phosphorylation. Furthermore, overexpressing PKCα and PKCβ increased the phosphorylation of IRS-1 S337 and S341. However, a kinase assay revealed that only PKCβ directly phosphorylated S341. An *in vivo* experiment in mice later revealed that PKCβ directly phosphorylated S341 thus priming S337 to be phosphorylated by glycogen synthase kinase-3 (GSK-3). Taken together, S337 phosphorylation by GSK-3 is dependent on the phosphorylation of S341 by PKCβ [48].

Muscles of obese diabetic patients presented with increased PKCθ activity. The increase in fatty acids activates PKCθ, which leads to the phosphorylation of IRS-1 S1101, resulting in insulin resistance. [49].

A study showed that PKCδ has 18 different phosphorylation sites on IRS-1. Of those sites, only 3 were determined as direct targets of PKCδ. An in vitro study revealed that when PKCδ phosphorylates IRS-1 at S307, S323, and S574 that IRS-1 is inhibited from interacting with the IR [50].

Previous studies have found that the overexpression of PKCζ led to reduced IRS-1 stimulated PI3K activity. In fact, it was later determined that insulin stimulated PKCζ resulted in the inhibition of insulin stimulated tyrosine phosphorylation of IRS-1 [51].

PKCζ phosphorylates IRS-1 on S323, S503, and S570. While it has already been discussed that the phosphorylation of S323 by PKCζ after prolonged insulin stimulation leads to a positive regulation of IRS-1, PKCζ does not exhibit that same effect on the other three serine sites [51-54]. When PKCζ phosphorylates S503 and S570, there is reduced IRS-1 and PI3K interaction, especially when S570 is phosphorylated [54].

IRS-1 S270, a site that is most proximal to the PTB domain, is believed to be phosphorylated by S6K-1. This negative regulation of IRS-1 is mediated by tumor necrosis factor-alpha (TNF-α) induced insulin resistance. T389 S6K-1 can be phosphorylated by mTORC1 as a downstream effector for the TNF-α pathway and studies have shown that mutating the S270 to A270 prevented S6K-1 from interacting with IRS-1 which prevented three other S6K-1 target sites from being phosphorylated: S307, S636, and S1101. This suggests that the phosphorylation of S270 by S6K-1 may prime the phosphorylation of S307, S636, and S1101 [33,55]. In addition, p70 S6K-1 has been known to phosphorylate IRS-1 S527 in vitro in a kinase assay [56]. Similarly, AKT can phosphorylate S527 in vitro in HEK293 cells. A study has shown that tyrosine

phosphorylation of IRS-1 in insulin stimulated HEK293 cells is inhibited when AKT phosphorylates S527 [33,57]. However, this serine phosphorylation by AKT is not enough to completely stop all tyrosine phosphorylation of IRS-1 [57].

In addition, the insulin stimulated phosphorylation of S527, whether by S6K-1 or AKT, has been shown *in vivo* in patients with lean, insulin sensitive muscles given a hyperinsulinaemic clamp [58].

One study showed that the phosphorylation of IRS-1 S1101 is nutrient dependent and is induced by p70 S6K-1 in insulin and amino acid stimulated L6 cells immunoprecipitants. The insulin induced phosphorylation of S1101 is further increased in obese diabetic mice. These obese diabetic mice also display hyperphosphorylation of p70 S6K-1 when stimulated with insulin alone. Furthermore, muscle biopsies of patients stimulated with both amino acids and insulin revealed that the hyperactivation of p70 S6K-1 was linked both to insulin resistance and S1101 phosphorylation. Insulin alone increases S1101 phosphorylation less than when combined with the infusion of amino acids [59].

In addition, when observing the phosphorylation status of S1101 in type 2 diabetic patients, there is increased S1101 phosphorylation before any insulin is administered. Both 1 hr and 2 hrs after insulin is given there is no change in the phosphorylation of S1101 [58].

JNK and IKK have been shown to phosphorylate S312 in vitroin vitro, thus decreasing IR and IRS-1 tyrosine phosphorylation. When both JNK and IKK were inhibited there was a decrease in the phosphorylation of IRS-1 S312 [60-64]. This regulation is not stimulated by insulin but rather by (tumor necrosis factor-alpha) TNF-α via inflammation [65].

Studies have also shown that an increase in a high fat diet increases the phosphorylation of S312 and that this phosphorylation has been seen to be decreased in JNK1 knockout mice. The knockout JNK1 mice showed a reduction in adiposity and an improvement in insulin sensitivity. Even obese mice with a mutant JNK1 were protectiveshowed lower incidence of against high blood sugar and hyperinsulinemia [39]. The involvement of S6K-1 and mTORC1 in phosphorylating S312 is only implied [33]. It seems that the phosphorylation of S312 has been used as a way to identify of the progression or commencement of insulin resistance. It would be good to study the phosphorylation status of S312 in non-insulin resistant models [33].

G protein coupled receptor kinase 2 (GRK2) negatively regulates IRS-1 upon the phosphorylation of S616 which leads to the suppression of GLUT4 translocation. When GRK2, also known as β-adrenergic receptor kinase-1 (BARK1) is knocked down in 3TL1 adipocytes treated with endothelin-1 (ET-1), there is a decrease in the phosphorylation of S616 IRS-1. which led to the restoration of the GLUT4 translocation [33,66].

As previously mentioned, phosphorylation of S323 can lead to positive regulation of IRS-1 only when PKCλ/ζ is involved, however, when mTORC1 or PKCδ phosphorylate S323 it leads to negative regulation of IRS-1. In other words, while one kinase can phosphorylate one site to positively regulate IRS-1, another kinase can phosphorylate the same site to negatively regulate IRS-1. Similarly, phosphorylation by ROCK1 at IRS-1 S636/639 can lead to positive regulation, however, the opposite occurs when it is phosphorylated by mTORC1 and ERK1/2 [33].

1.4 1.4 Dysregulation of IRS-1 and the progression of insulin resistance

During normal regulation, insulin binds to the alpha subunits of IR. Insulin is produced and secreted from the pancreas via calcium dependent exocytosis upon the presence of glucose in the blood. Blood glucose is not the only stimulant that promotes insulin secretion, as fatty acids, amino acids, and other nutrients are also known to stimulate its release. Nutrient supply plays a crucial role in the initiation and progression of insulin resistance. In other words, excess nutrients are detrimental to the insulin signaling pathway primarily due to the eventual desensitization of IRS-1. Therefore, the next signal transductionactivation of the insulin pathway will require an even greater amount of insulin to induce signaling as ist depicted in Figure 87. The dysregulation of IRS-1 eventually leads to insulin resistance, rendering insulin ineffective in sending out further signals. In addition, IRS-1 degradation consequently occurs (Figure 8). Studies have shown that an increase in the consumption of amino acids in the form of meat or glucose in the form of simple sugars exponentially increases incidences of type 2 diabetes, a disease notably characterized by insulin resistance [67].

1.5 1.5 mTORC1 and S6K-1 signaling in relation to IRS

As previously mentioned, insulin binds to the IR which initiates the signal transduction pathway that leads to the activation of activates mTORC1 which then phosphorylates p70 S6K-1. The initiation of this pathway can be negatively regulated by both mTORC1 and S6K-1. Both these kinases will phosphorylate the serine residues mentioned previously to desensitize IRS-1 from

any more incoming signals from the binding of insulin to the IR. Studies have already shown that tyrosine phosphorylation on IRS-1 leads to its activation while serine phosphorylation usually leads to negative regulation of IRS-1. An increase in dietary or caloric intake is detrimental because it leads to excess nutrient availability such as sugars, lipids, and amino acids which is why obesity is a risk factor for insulin resistance and many other metabolic disorders. Consequently, when there is prolonged insulin stimulation due to excess nutrients, p70 S6K-1 is hyperphosphorylated by mTORC1. As a result, the outcome is a greater than normal suppression of IRS-1 by p70 S6K-1. In this case, S6K-1 is hyperphosphorylatedion due to prolonged insulin stimulation consequently leadings to the proteasomal degradation of IRS-1. However, proteasomal degradation alone is not a determinant for the development of insulin resistance. Also, hyper-aminoacidemia does not lead to the proteasomal degradation of IRS-1; however, it primes IRS-1 for inhibitory serine phosphorylation which still leads to insulin resistance.

Studies have shown that p70 S6K-1 can impact the phosphorylation of IRS-1 at S312, S636, and S639. Hyper-aminoacidemia leads to increased phosphorylation of IRS-1 S312, while hyperinsulinemia increases the phosphorylation of IRS-1 S636 and S639. Interestingly, mTORC1 is also capable of negatively regulating IRS-1 by phosphorylating S307, S636, S662, and S731 [33].

Serine phosphorylation of IRS-1 is not the only inhibitory post-translation modification that occurs. Recent studies have revealed another post-translation modification that regulates IRS-1 signaling, namely acetylation [68]. It is suggested that p300, an acetyltransferase, may also contribute to this IRS-1 downregulation [69], while other studies have suggested that p300 may be

protective against insulin resistance [70]. Protein acetylation involves an acetyltransferase transferring an acetyl group from acetyl coenzyme A onto a lysine on a substrate protein.

1.6 p300 Overview

History and structure

p300 is an acetyltransferase that was first identified in 1985 as a protein that binds to adenovirus early region 1A (E1A), an adenoviral oncogenic transcription factor [71-75].

As a result, p300 is also known as E1A associated protein p300 (EP300). p300 is encoded by EP300 located at 22q13.2, contains 31 exons, and is 2414 amino acids long [76].

Named for its 300KD size [72], p300 is always mentioned in concert with cyclic AMP response element-binding (CREB) protein (CBP), a protein made up of 2441 amino acids found on the chromosome 8 on locus 16p3.3. CBP was discovered in 1993 [77] and was also found to be able to bind to E1A just as p300 was found to be able to bind to CREB[72].

Despite both CBP and p300 being paralogous and having a high sequence homology of 63% [74,75,78], in 1996, both were discovered to have histone acetyltransferase activity [79,80]. As similar as p300 and CBP may be, they still display differences in their specificity and selectivity for lysines that are dependent on the availability of either their substrate or cofactor, acetyl CoA [81].

P300 contains the following domains shown in Figure 98: NRID (1-100) , TAZ1 (347-414) [76], KIX (566-646) [76], bromodomain (1047-1161), RING (1144-1216) [76], HAT (1281-1664), ZZ zinc finger (1700-1751) and TAZ2 (1723-1836), NCBD or IBID. Additionally, p300 has a lysine rich regulatory loop contained within the HAT domain (1523-1554) [76,82,83].

The RING domain interacts with the HAT domain, and this interaction appears regulatory as studies have shown that when the RING domain is deleted there is an increase in the catalytic activity of p300. Furthermore, studies have shown that the bromodomain though essential for the nuclear localization of p300, does not contribute to the catalytic activity of p300 [84,85].

Regulation of p300

P300 has the ability to activate itself by autoacetylation on the lysine rich regulatory loop found on its HAT domain, although it is not the only HAT capable of this phenomenon. The residues that are autoacetylated are K1499, K1549, K1554, and K1560 [85]. When these residues are acetylated, p300 is activated. Studies have revealed that when this loop is deleted, p300 becomes constitutively active while decreasing the Michaelis constant (Km) of Acetyl-CoA and the target protein instead of increasing the rate of enzymatic activity. In other words, before acetylation, the loop, which is inhibitory, covers the site for substrate binding while acetylation exposes the substrate binding site. [86,87].

Found also within the HAT domain are two catalytic residues: Y1467, which positions the Acetyl-CoA and adds a proton to the leaving group, and Y1436, that positions the substrates lysine. [88].

p300 is well known for its involvement in histone acetylation in relation to gene transcription regulation, which is why it is commonly known as a histone acetyltransferase (HAT). However, it was later discovered that p300 can localize in the cytoplasm and acetylate cytoplasmic proteins. Consequently, all HATs were renamed lysine acetyltransferases (KAT). [89]. p300 is a highly promiscuous protein, even compared to other HATs, [85], acetylating more than 80 known substrates. [72,90]. In addition, p300 interacts with more than 411 proteins. Therefore, it is not surprising that p300 is involved in so many cellular mechanisms including but not limited to NFκB [72], Notch [91], cAMP signaling [92], estrogen [93], stress response [94-96], and DNA damage response [97-99] pathways [72].

PKC is known to phosphorylate p300 at S89 resulting in the repression of p300 regulated transcription. [100]. It has been demonstrated that mTORC1-dependent phosphorylation removes the intra-molecular inhibition of p300. This inhibition is achieved because it prevents the catalytic HAT domain from binding to the RING domain. When p300 is inactive, the RING domain is tightly associated with the HAT domain active site. [101]. When mTORC1 phosphorylates p300 it causes the dissociation of the RING domain from the HAT domain on p300. It is hypothesized that the phosphorylation leads to a conformational change that leads to the displacement of the RING domain from the catalytic site of the HAT domain. [101,102].

mTORC1 phosphorylates p300 at 4 serine residues in the C-terminal domain, namely S2271, S2279, S2291, and S2315 (Figure 10). It was previouslyhas been demonstrated that the deletion of the C-terminal domain resulted in a failed interaction between mTORC1 and p300. It was discovered that p300 is associatesd with Flag-Raptor using Co-IP. Treatment with the mTOR inhibitor, Torin1, which inhibits both mTORC1 and mTORC2, paired with amino acid starvation decreased the interaction while the overexpression of Rheb or the replenishing of amino acids after starvation immensely enhanced the association of p300 with Raptor. [102] of p300 with Raptor.

AKT phosphorylates p300 on S1834 (Figure 10) leading to its recruitment to intercellular adhesion molecule- 1 (ICAM-1) promoter, resulting in ICAM-1 gene expression upon histone acetylation by p300 [103,104]. ERK2 has been discovered to increase p300 HAT activity by phosphorylating the C-terminal serine residues S2279, S2315, and S2366, (Figure 10) resulting in the acetylation of H3 by p300 to promote the expression of keratin 16 gene [94]. Other potential ERK2 phosphorylation sites include T317, T938, and T1960, (Figure 10) but their phosphorylation was not as effective compared to the phosphorylation of the serine residues located on the C-terminus [94]. Other post-translational modifications that take place include methylation and SUMOylation [72].

Acetyltransferase Activity on IRS-1

While it is fully understood and well documented that p70 S6K-1 and mTORC1 downregulate insulin signaling, the involvement of p300 raises questions. According to a study conducted in

2004, the acetylation of IRS-1 is suggested to be permissive for tyrosine phosphorylation while any deacetylation promotes the downregulation of IRS-1 [70]. However, this is contradictory to a study done in 2017 that discovered that p300 does indeed acetylate IRS-1 on specific sites. The result of this is the development and progression of insulin resistance [69]. When IRS-1 sites K315, K623, K767, K862, K1017, K1080, and K1131 are acetylated by p300, insulin signaling is impaired [69], although not all these sites need to be acetylated for insulin resistance, as a combination of acetylating K315-K767-K862, K1017-K1080, and K1017-K1080-K1131 is sufficient to mimic insulin resistance. Of the combinations, K1017-K1080-K1131 shows the highest downregulation of IRS-1. Both these studies cannot be true at the same time unless there is a different site or KAT that leads to the re-establishment or attenuation of insulin sensitivity [69]. One study suggested that Lysine acetyltransferase 5 (Tip60) may be the KAT that acetylates IRS-1 to restore its sensitivity, however, no further research has validated this hypothesis [68].

1.,7 Introduction to Dissertation

Purpose of Study

While most studies have studied the effects of p300 on IRS-1 or p70 S6K-1 on IRS-1 have been studied individually, they have not looked into the crosstalk between each of these interactionspathways have not been studied. Specifically, they have not looked into the crosstalk between the phosphorylation and acetylation or IRS-1 warrants investigation. For example, are both the phosphorylation and acetylation occurring at the same time; is one permissive for the

action of the other; do they both produce simultaneously elicit the same regulation on IRS-1; does the phosphorylation of p300 prime it to acetylate IRS-1; without mTORC1 phosphorylation is p300 still able to acetylate IRS-1?. The curiosityinterest behind this crosstalk is inspired by the proximity of the acetylation sites to the phosphorylation sites . (Figure 9). Due toBecause of the implications that persist due to the hyperphosphorylation of negative regulatory sites on IRS-1, it is pertinent that further research needs to be done to fully understand the dysregulation of IRS-1 that leads to insulin resistance. It is also important to understand the negative regulation elicited by p300 on IRS-1 since its action is dependent on its presence in the cytoplasm. P300 is generally viewed as a nuclear protein due to its work in transcription regulation. Its translocation to the cytoplasm in relation to IRS-1 has been linked to an increase in a high fat diet (HFD) and a progression to insulin resistance via IRS-1 acetylation.

It is already known that one posttranslational modification can affect another. An example of this is the phosphorylation and acetylation of H3. H3 can be phosphorylated on S10 by sucrose non-fermenting (Snf1) thus increasing the binding affinity of general control non-repressed protein (GNC5) an acetyltransferase. GNC5 can then acetylate K14 . It is also understood that the overexpression of protein phosphatase 1 (PP1) diminishes K14 acetylation, displaying that Snf1 activity primes the action of GNC5 action on H3. (Figure 120).

Aims

The goal of this study is to learn if there is a relationship between the phosphorylation and acetylation of IRS-1. This question can be answered by a series of methods that include kinase assays, HAT assays, site-directed mutagenesis, protein and plasmid purification, and cell culture. This study will reveal if there is any possible crosstalk between the acetylation and phosphorylation of IRS-1 by p300 and p70 S6K-1, respectively.

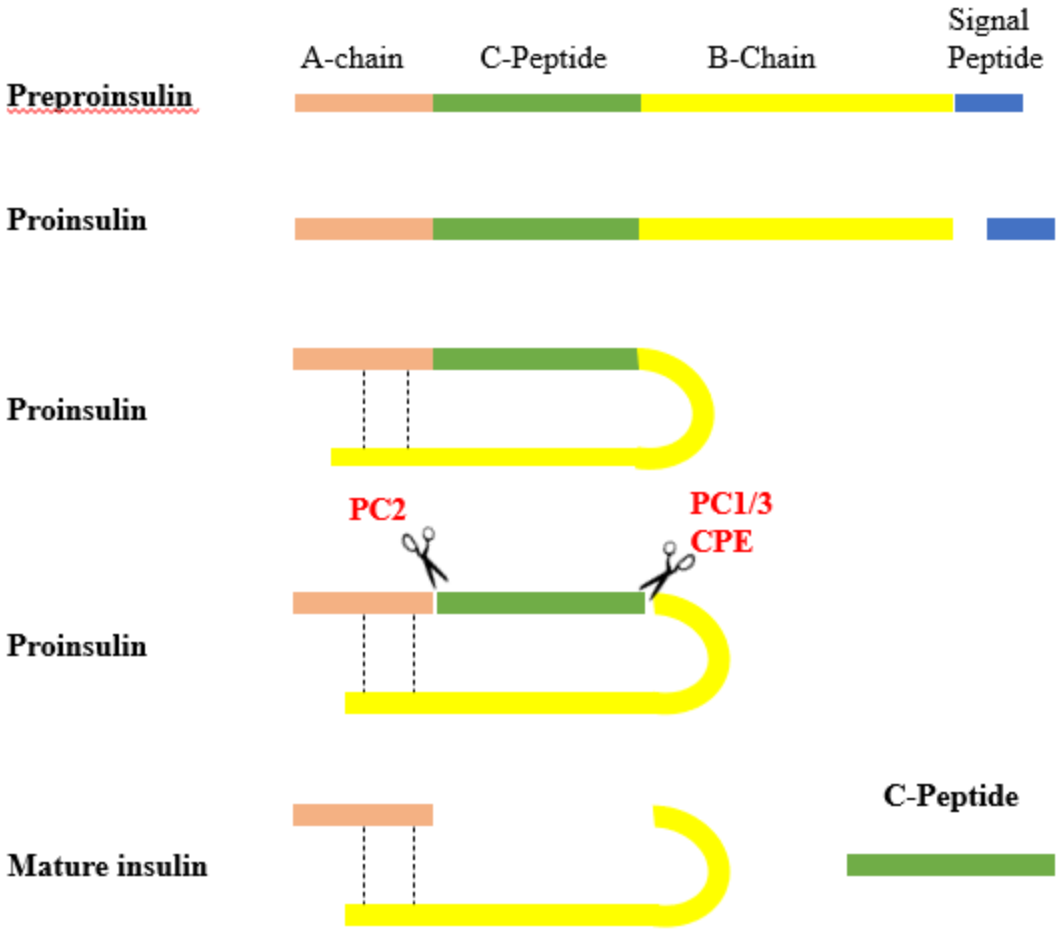


Figure 1. Insulin Posttranslational modifications

Insulin is a peptide hormone made from the pancreas. Insulin is translated into preproinsulin which is made up of an A-chain, C-peptide, B-chain, and signal peptide. Preproinsulin becomes proinsulin when the signal peptide is later removed thus also leading to its translocation from the ER to the Golgi apparatus. Cleavage by prohormone convertases (PC) 1/3, proprotein convertase 2 (PC2), and carboxypeptidase E (CPE) leads to the formation of mature insulin and C-peptide hormone which are both secreted into blood stream from secretory vesicles. Mature insulin is made up of the A-chain and B-chain which are bound by disulfide bonds. from cysteines.

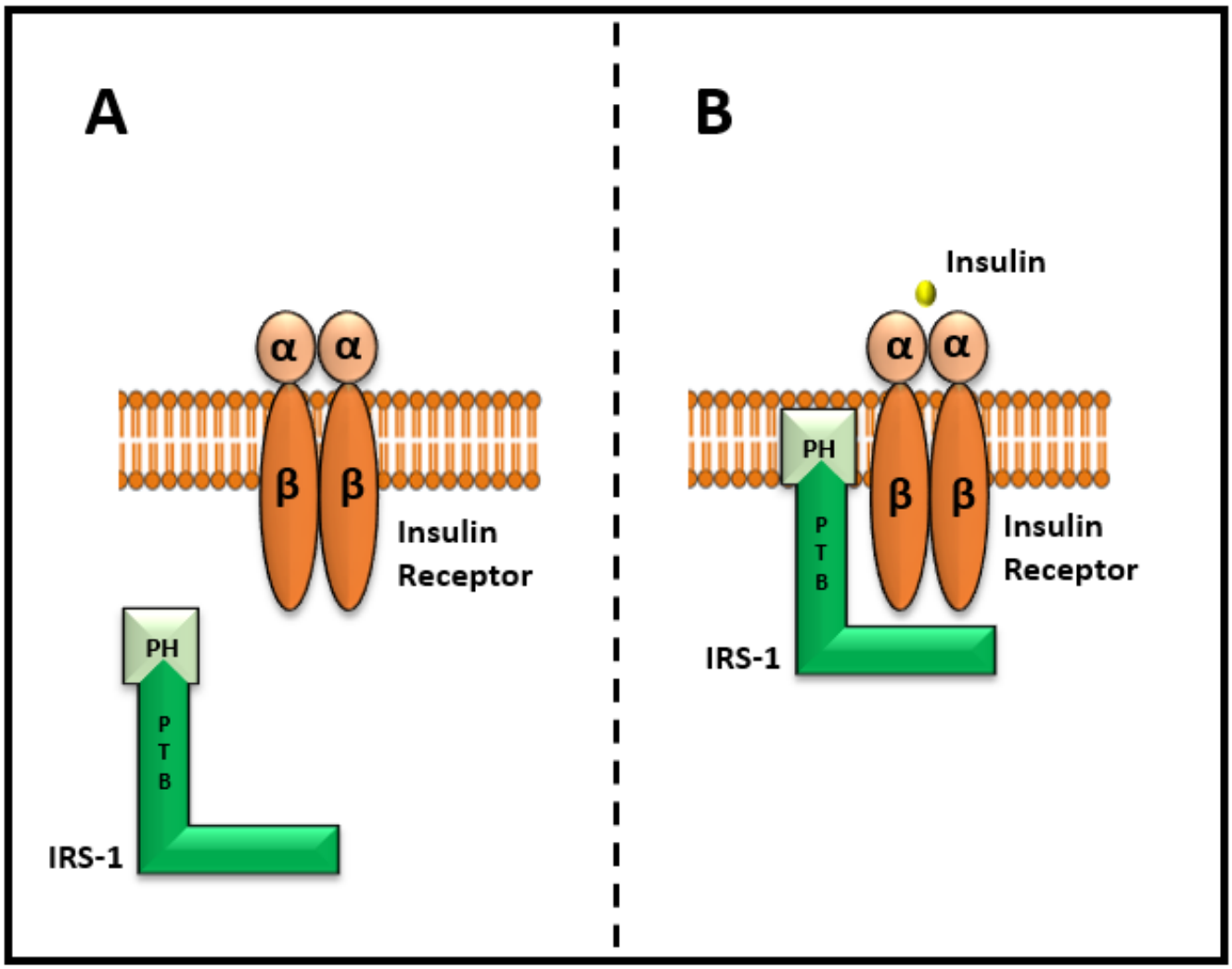


Figure 2. The interaction of IRS-1 and the IR.

The recruitment of IRS-1 to the IR is dependent on the insulin signaling. When insulin binds the α subunit of the IR, a signal is transmitted that leads to the autophosphorylation of the β subunit of the IR. Consequently, IRS-1 is recruited to the plasma membrane where the IR associates with the phospho-tyrosine binding (PTB) domain of IRS-1. Lastly, IRS-1 gets anchored to the plasma membrane by its pleckstrin homology (PH) domain.

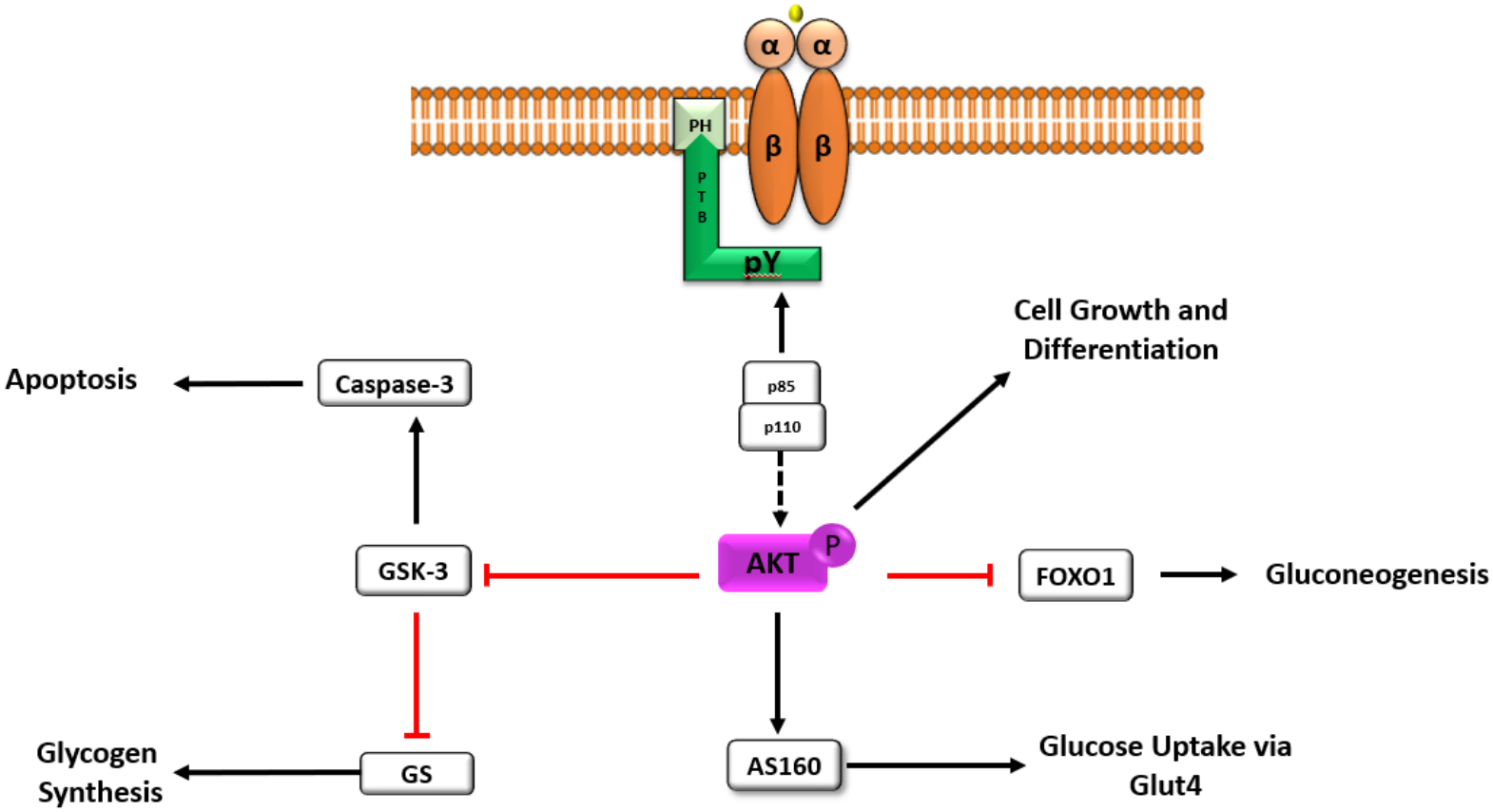


Figure 3. The metabolic and mitogenic effects of IRS-1 signaling: AKT signaling

AKT signaling via IRS-1 leads to mitogenic effects such as cell growth, differentiation, and the inhibition of apoptosis. AKT does so by the inhibition of GSK-3 and directly promoting cell growth and differentiation. Moreover, there are metabolic effects such as gluconeogenesis, glycogen synthesis, and glucose uptake from the bloodstream. The metabolic effects take place when AKT inhibits FOXO1 and GSK-3, as well as activation AS160.

AKT signaling via IRS-1 leads to mitogenic effects such as cell growth, differentiation, and the inhibition of apoptosis. Moreover, there are metabolic effects such as glycogen synthesis, and glucose uptake from the bloodstream that also occur.

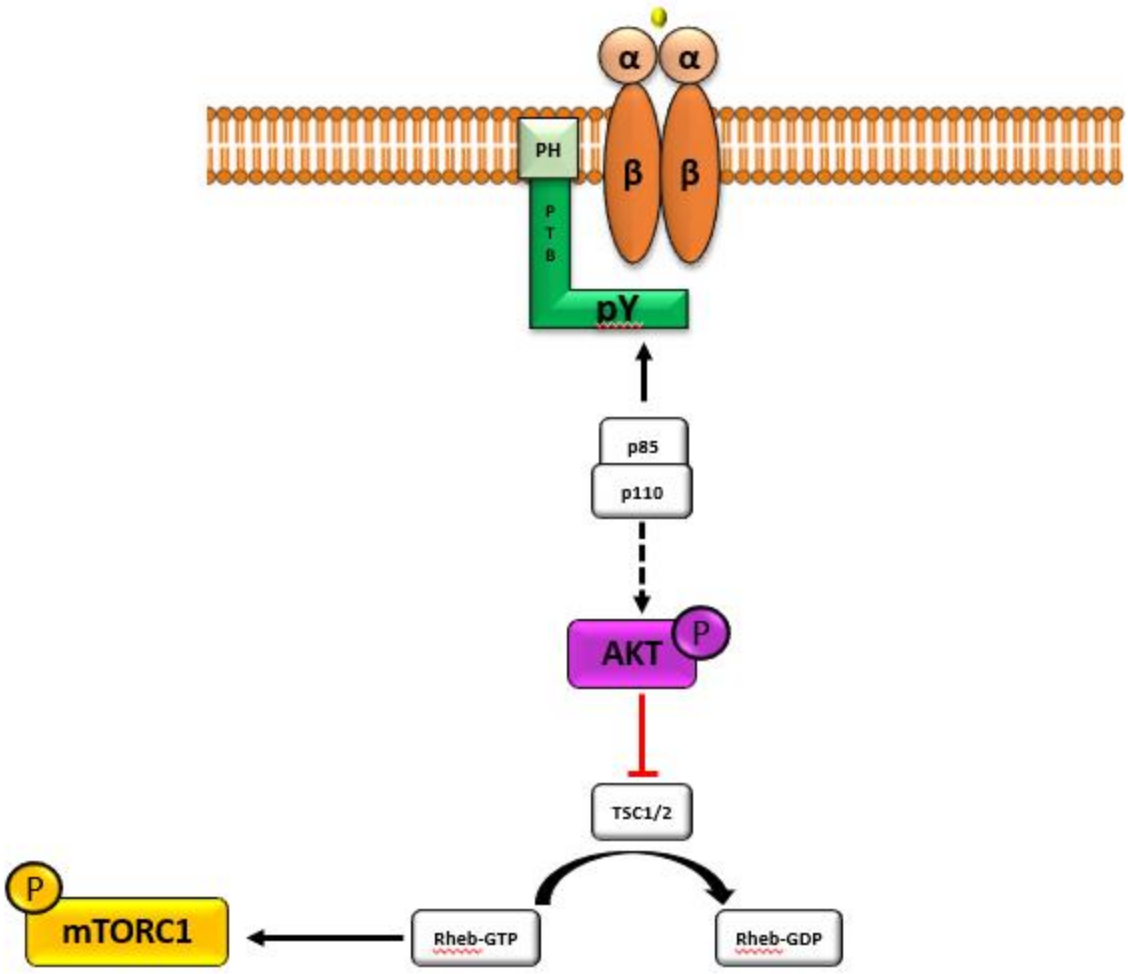
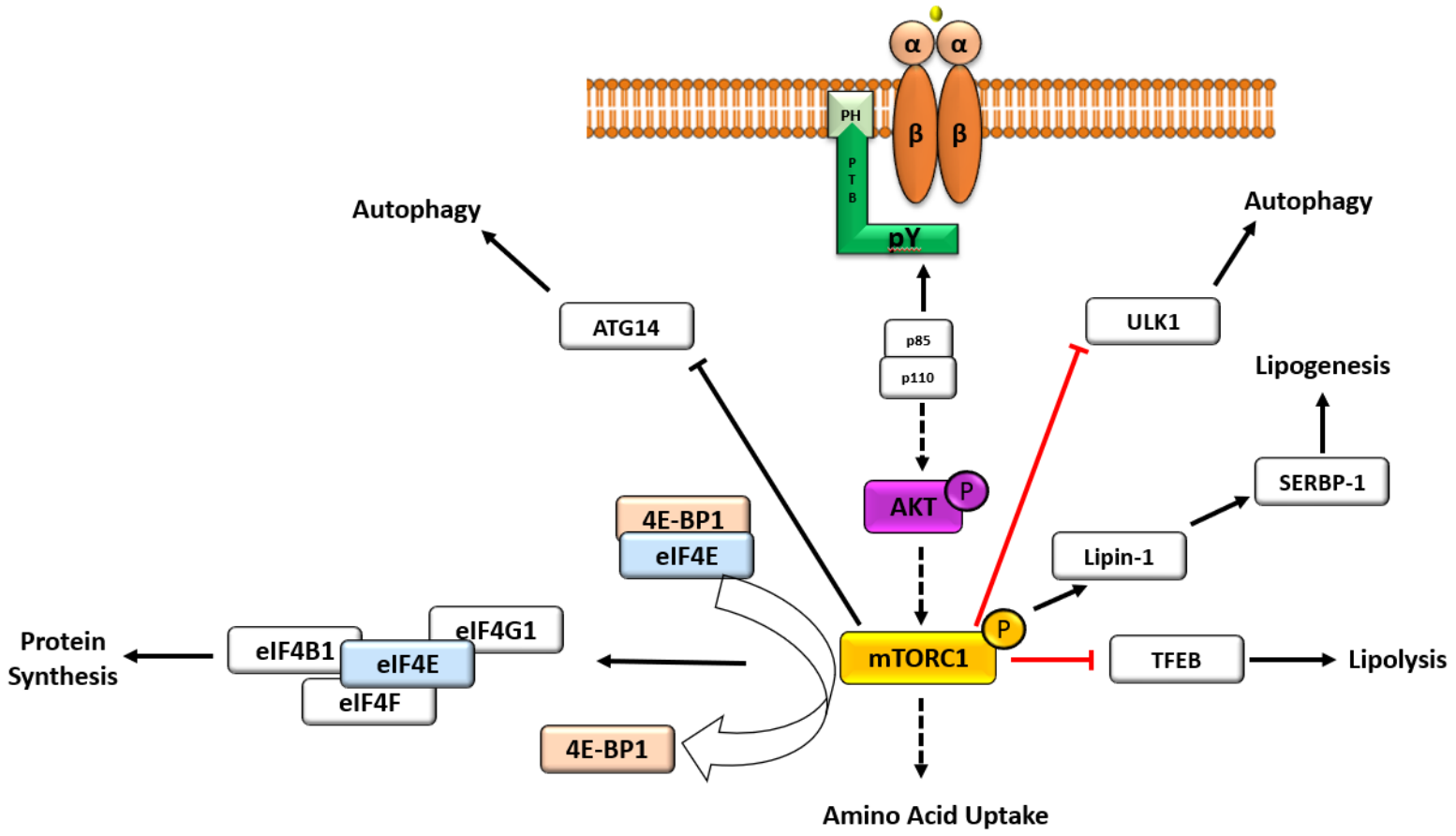


Figure 4. The activation of mTORC1

mTORC1 is activated when Rheb-GTP binds to it. This process is usually inhibited by TSC2, a GTPase-activating protein, when it is bound to TSC1. When AKT is activated upon insulin stimulation, it phosphorylates TSC2, which leads to the dissociation of TSC1 and TSC2 complex. This dissociation prevents the conversion of Rheb-GTP to Rheb-GDP, thus allowing the activation of mTORC1.


α
α
β
β
PH
PTB
pY
Autophagy
ATG14
p85
p110
ULK1
Autophagy
Lipogenesis
SERBP-1
AKT
P
4E-BP1
eIF4E
Lipin-1
Protein
Synthesis
eIF4B1
eIF4G1
eIF4E
eIF4F
mTORC1
P
TFEB
Lipolysis
4E-BP1
Amino Acid Uptake

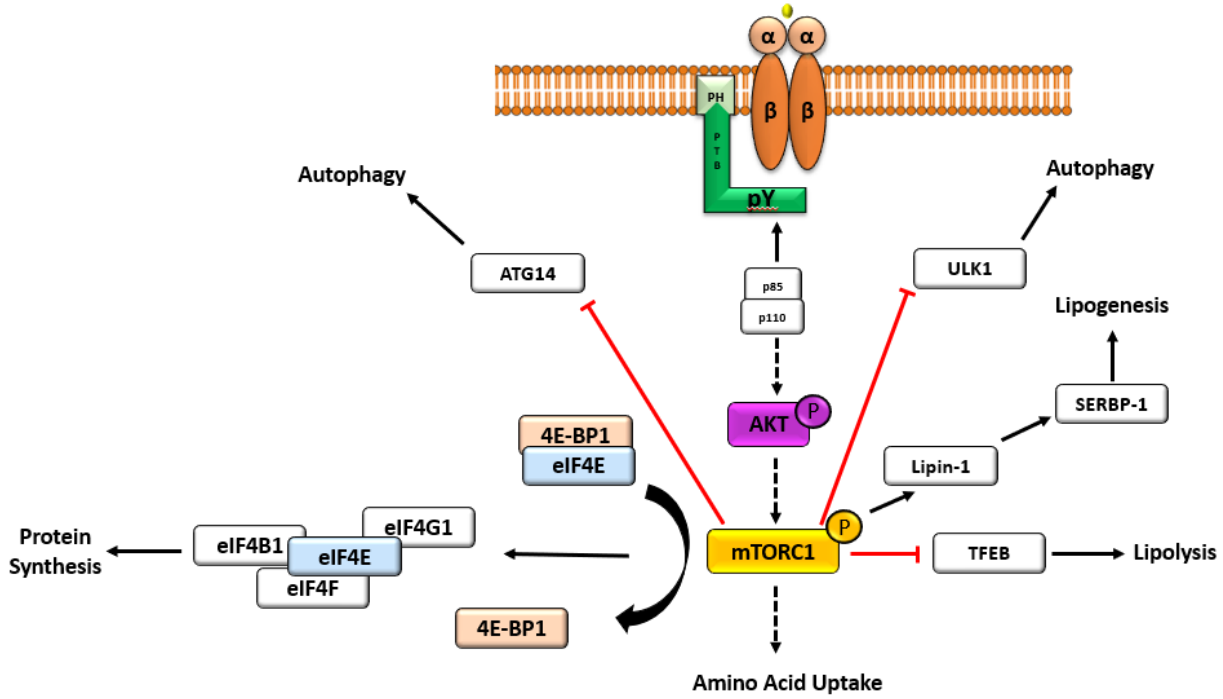


Figure 54. The metabolic and mitogenic effects of IRS-1 signaling: mTORC1 signaling

mTORC1 signaling via IRS-1 leads to protein synthesis by catalyzing the disscoicaiton of 4E-BP1 from eIF4E while also promoting amino acid uptake. mTORC1 both promotes lipogenesis by directly activation lipin-1, while also inhibiting lipolysis by directly phosphorylating TFEB. mTORC1 can also directly phosphorylate ATG14 and ULK1 leading to their inhibition. This inhibition leads to autophagy.

mTORC1 signaling via IRS-1 leads to protein synthesis, amino acid uptake, lipolysis, autophagy, and lipogenesis.

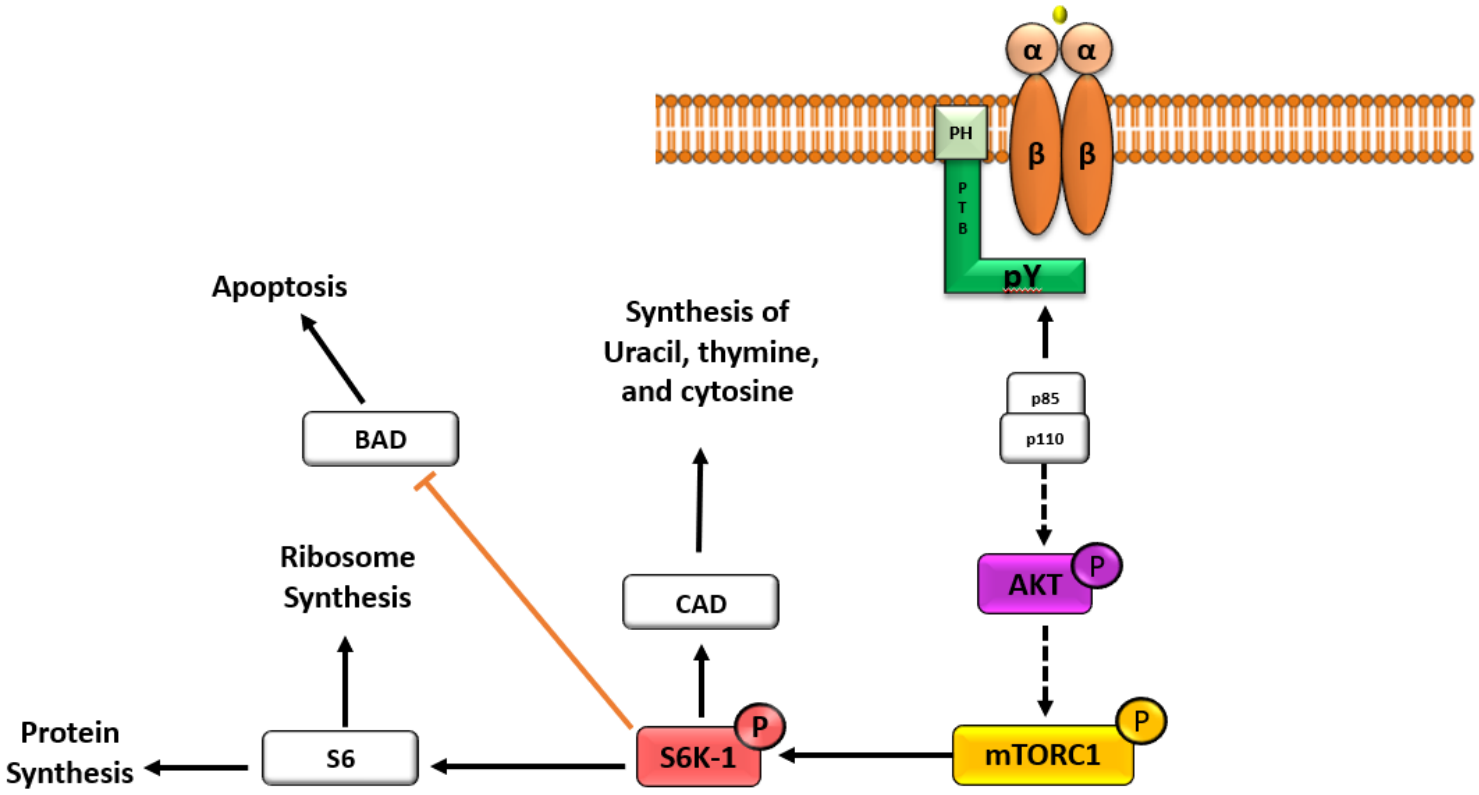


Figure 6. The metabolic and mitogenic effects of IRS-1 signaling: S6K-1 signaling

p70 S6K-1 signaling via IRS-1 leads to the inhibition of apoptosis when BAD is inhibited. p70 S7K-1 directly phosphorylates RPS6 to promote the synthesis of proteins. p70 S6K-1 also promotes the synthesis of uracil, thymine, cytosine, and ribosomes via the phosphorylation of CAD.

Figure 5. The metabolic and mitogenic effects of IRS-1 signaling: S6K-1 signaling

S6K-1 signaling via IRS-1 leads to the inhibition of apoptosis and the synthesis of proteins, uracil, thymine, cytosine, and ribosomes.

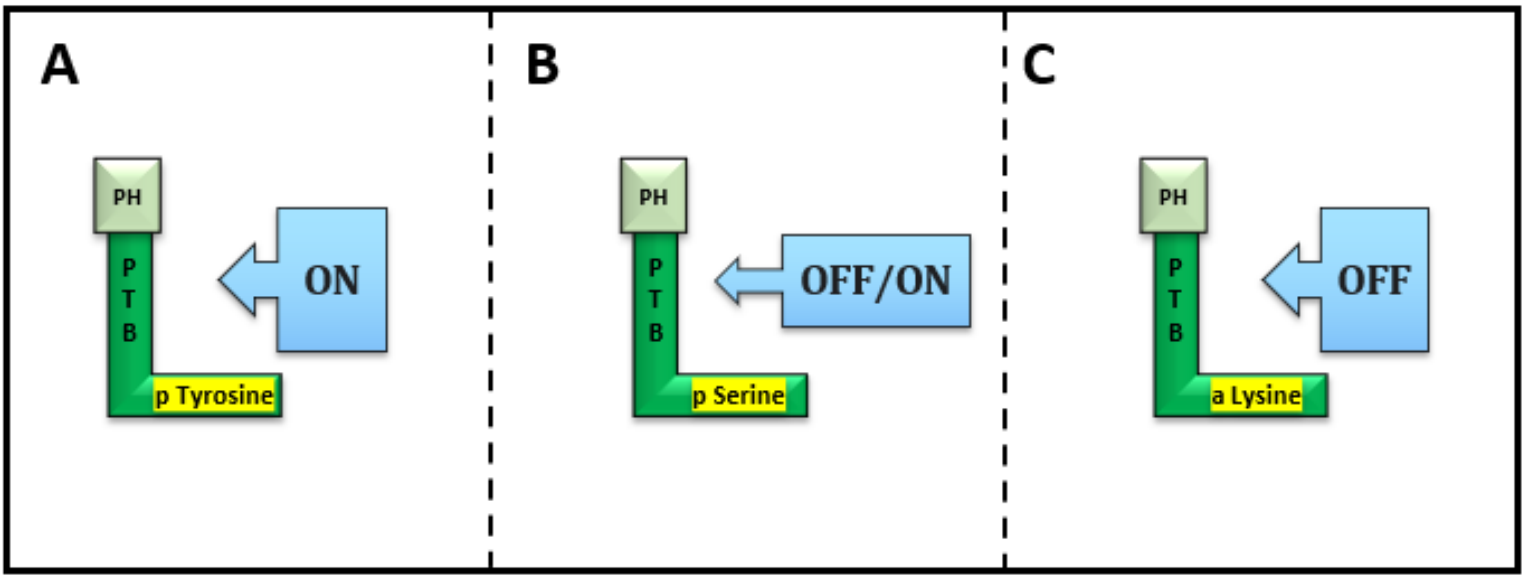


Figure 7. IRS-1 Regulation

IRS-1 is activated due to the phosphorylation of tyrosine residues (A). The phosphorylation of its serine residues can either enhance IRS-1 activity or inhibit it (B). This posttranslational modification outcome is dependent on the kinase and the target site. The acetylation of IRS-1 on its lysine residues located on the C terminus leads to its inhibitions (C)

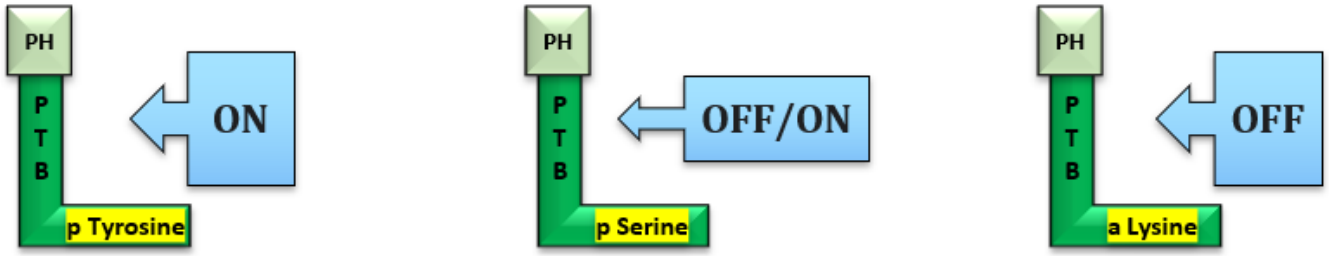


Figure 6. IRS-1 Regulation

IRS-1 is activated due to the phosphorylation of tyrosine residues. The phosphorylation of its serine residues can either enhance IRS-1 activity or inhibit it. Acetylation of IRS-1 leads to its inhibitions.

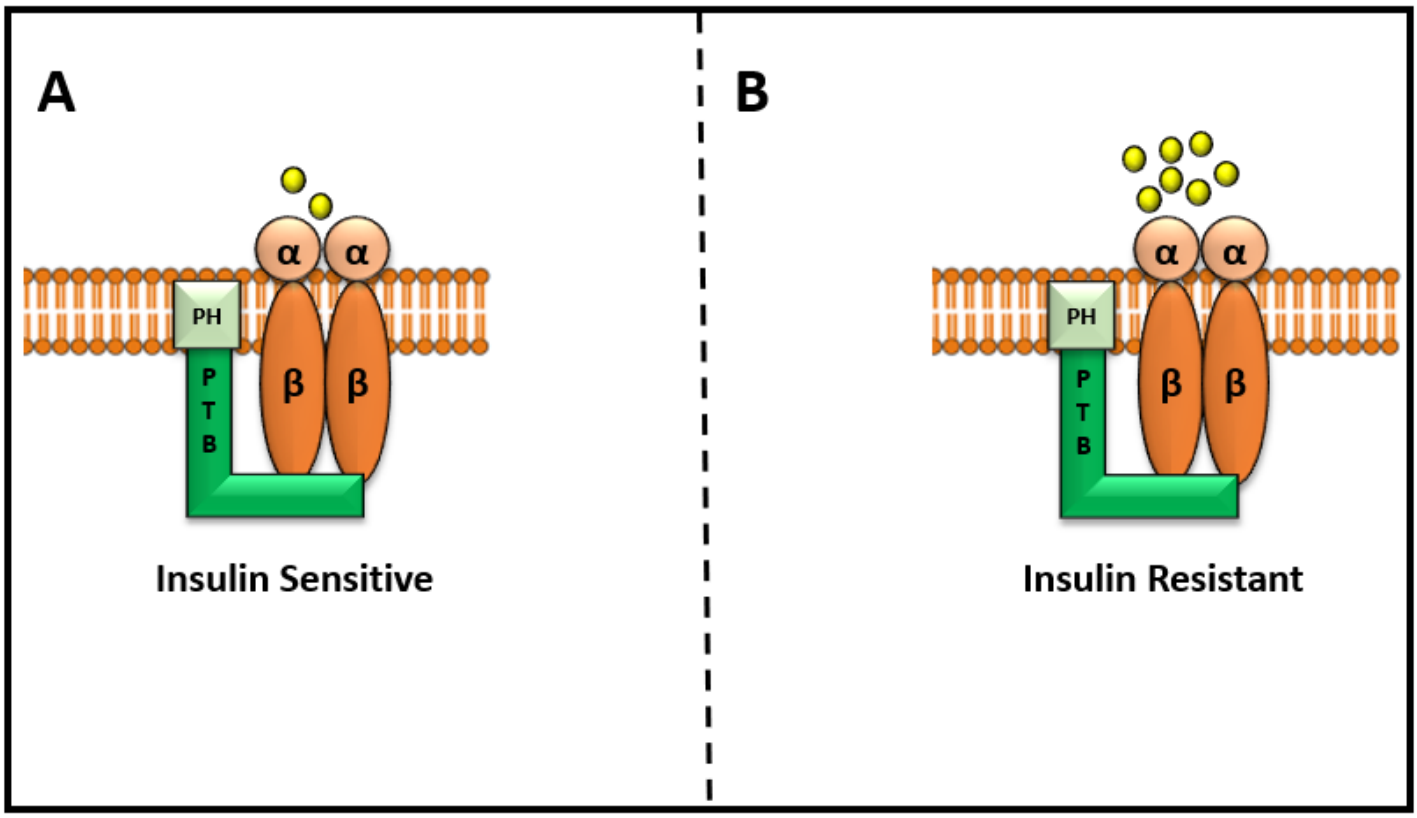


Figure 7. The Dysregulation of IRS-1

Excess caloric intake leads to increased insulin signaling which can lead to the desensitization of IRS-1. Once desensitized, the next signal is going to require more insulin to elicit a response similar to the initial one.

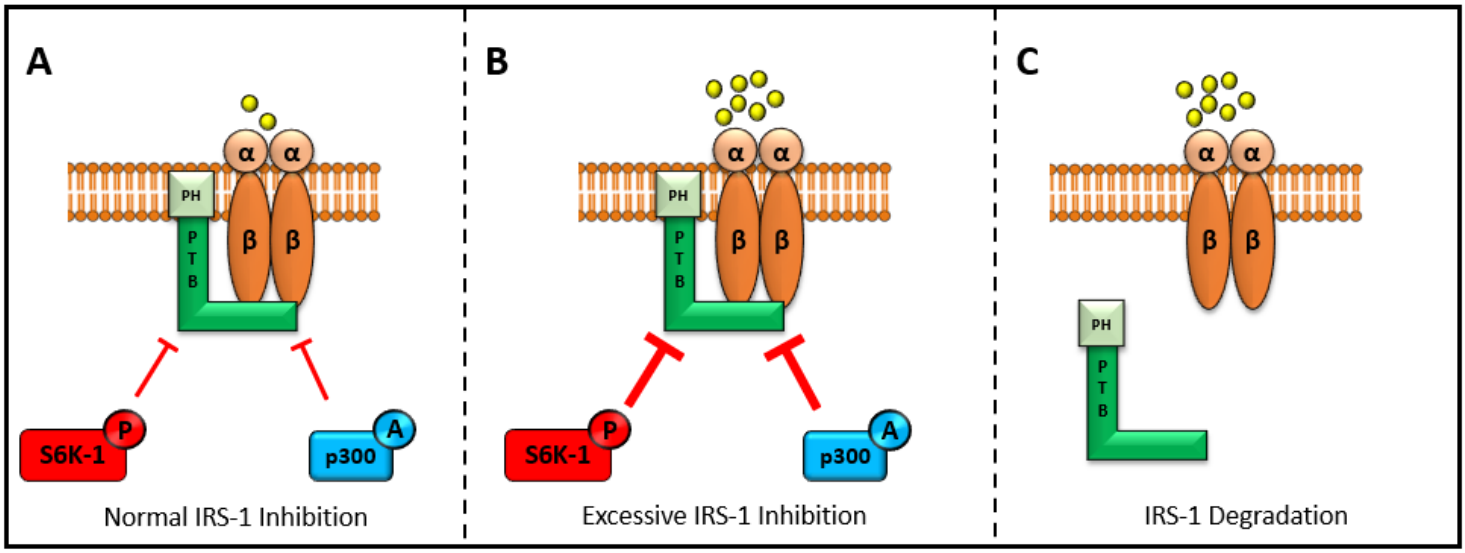


Figure 8. The Dysregulation of IRS-1

Normal insulin signaling is achieved with physiological levels of insulin (A), while excess caloric intake leads to increased insulin signaling which can lead to the excessive phosphorylation and acetylation of IRS-1 due to S6K-1 hyperphosphorylation and p300 acetylation (B). Excessive IRS-1 inhibition leads to its degradation (C). Once insulin resistance occurs the next signal is going to require more insulin to elicit a response similar to when a physiological concentration of insulin is administered to an insulin sensitive model.

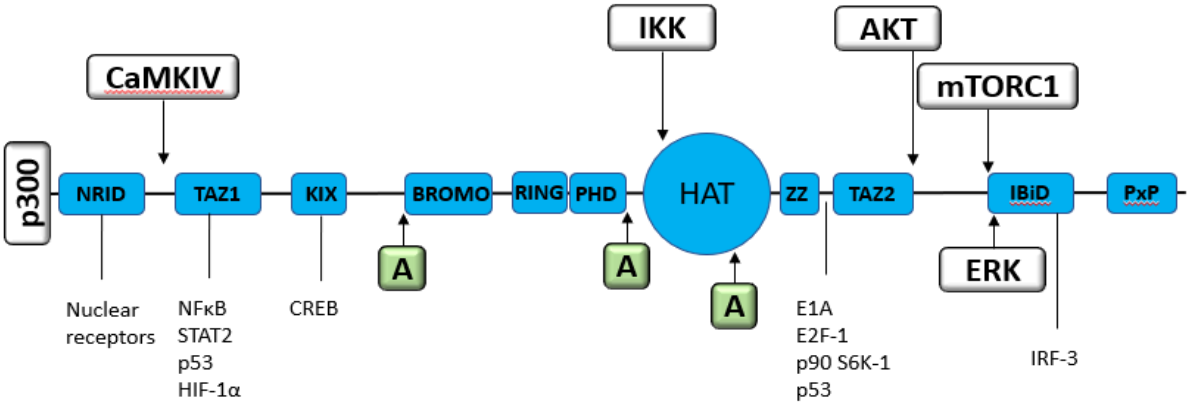


Figure 98. p300 Domain

P300 contains different domains with autoregulatory HAT domain. Nuclear receptor binds to the nuclear receptor interaction domain (NRID). NFκB, STAT2, P53, and HIF-1α bind to the transcriptional adaptor zinc-finger 1 (TAZ1) domain. CREB binds to the kinase-inducible domain interacting (KIX) domain. E1A, E2F-1, p90 S6K-1, and p53 bind to the ZZ-type zinc finger (ZZ) domain. P300 can be phosphorylated by CaMKIV, IKK, AKT, mTORC1, and ERK.

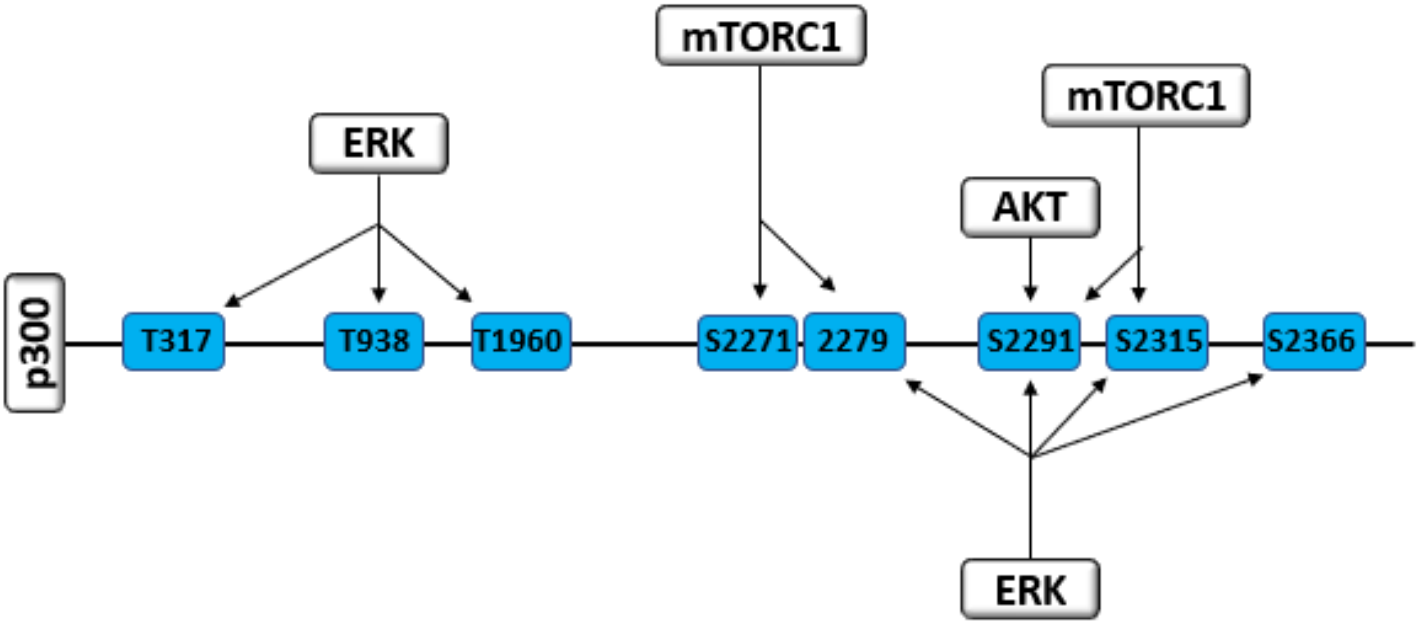


Figure 10. p300 phosphorylation sites

P300 can.be phosphorylated by, AKT, mTORC1, and ERK. AKT phosphorylates p300 on S1834 leading to its recruitment to intercellular adhesion molecule- 1 (ICAM-1) promoter. ERK2 can increase p300 HAT activity by phosphorylating the C-terminal serine residues S2279, S2315, and S2366. ERK2 can also phosphorylate T317, T938, and T1960. mTORC1 phosphorylates p300 at S2271, S2279, S2291, and S2315. This the outcome of this posttranslational modification is not understood.

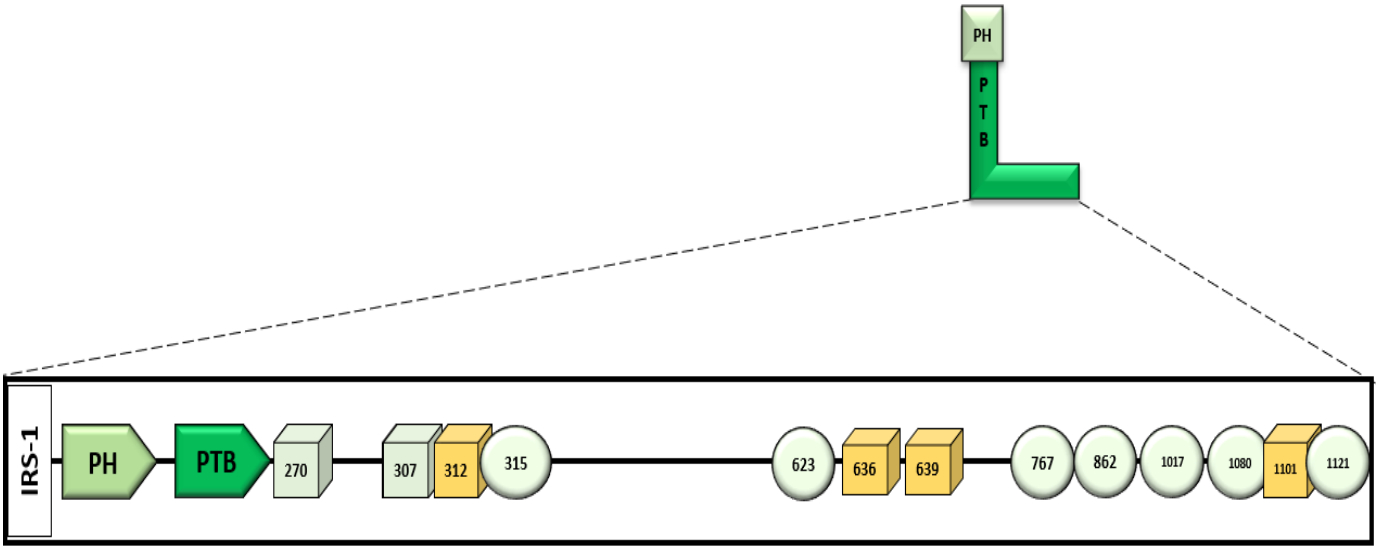


Figure 119. IRS-1 model structure

IRS-1 has multiple sites for posttranslational modifications located on the C termina end. IRS-1 contains pleckstrin homolog (PH) and a phosphotryrosine-binding (PTB) domains, which are useful in the activation and function of IRS-1. The serine phosphorylation sites are represented by boxes while the acetylation sites are represented by circles.

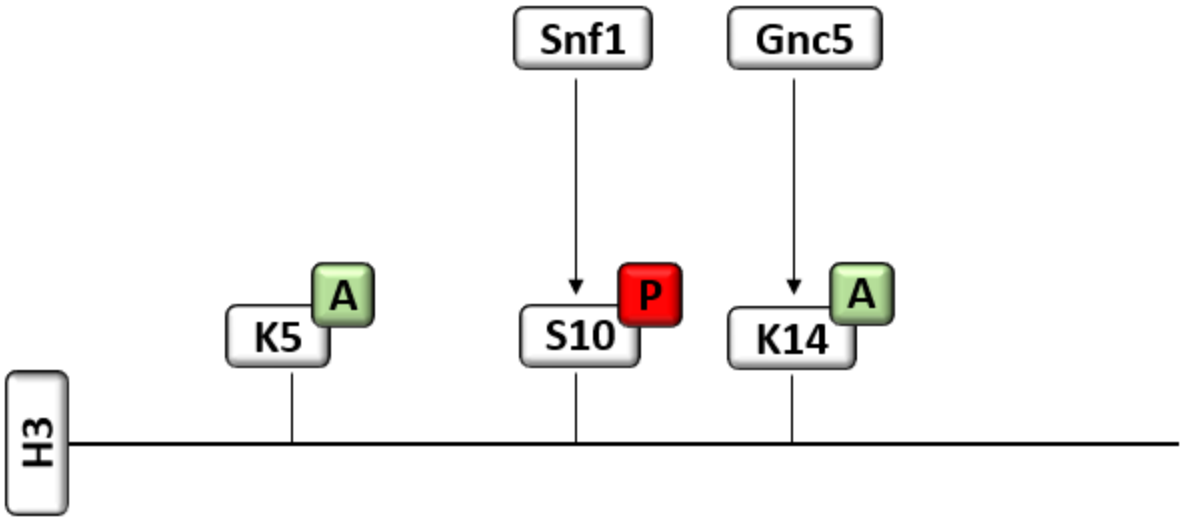


Figure 120. H3 phosphorylation and acetylation

H3 is phosphorylated by sucrose non-fermenting 1 (Snf1) at S10 to promote the acetylation of K14 by general control non-repressed protein 5 (GNC5).

Item	Company	Catalog #
Acetylated Lysine Ab	Cell Signaling Tech.	9814S
IRDye 680RD Goat anti-Rabbit IgG	LI-COR	926-68071
	ATCC	30-2003
HepG2	ATCC	HB-8065
T175 Flasks	Genesee	25-211
Hemacytometer	Weber Scientific	3048-11
PBS	Millipore Sigma	P4417-100TAB
Magnesium/ATP Cocktail	Millipore Sigma	20-113
Acetyl-CoA Sodium Salt	Millipore Sigma	A2056-5MG
BL21 DE3 Cells	Thermo Fisher	EC0114
EMEM	ATCC	30-2003
PVDF	Fisher	IPVH00005
HisPur Resin	Fisher	PI88221
rProtein A Agarose	Genessee	20-525
Mini-PROTEAN TGX Precast Gels (12 Well)	BioRad	4-20% Gradient
S6 Ribosomal Protein (5G10) Rabbit mAb	Cell Signaling Tech.	2217S
Phospho-S6 Ribosomal Protein (Ser235/236) (91B2) Rabbit mAb	Cell Signaling Tech.	4857S
Anti-Actin (20-33) Ab produced in rabbit	Millipore Sigma	A5060-200UL
Trichostatin A	Millipore Sigma	T8552-5MG
ReadyShield Protease and Phosphatase Inhibitor Cocktail	Millipore Sigma	PPC2020-5ML
1 x Kinase Assay Buffer	Abcam	ab189135
Recombinant S6K1	Abcam	ab268834
IBM SPSS Statistics Grad Pack 28.0 STANDARD	Software Package	
4-20% Mini-PROTEAN TGX Precast Gels	BioRad	4561095
Phospho-IRS-1 (Ser312) Polyclonal Antibody	ThermoFisher Scientific	500-8984
Phospho-IRS-1 (Ser636) Polyclonal Antibody	ThermoFisher Scientific	PA1-1055
Phospho-IRS-1 (Ser1101) Polyclonal Antibody	ThermoFisher Scientific	PA5-36719
IRS1 Recombinant Protein	Bio Source	MBS515143
IRS1 Recombinant Protein	Bio Source	MBS515371
IRS-1 Antibody	Cell Signaling Tech.	2382S
PF-4708671	Apex Bio	B2228
C646	Apex Bio	B1577
2x Laemmli buffer	Bio Rad	1610737

Cell Culture

HepG2 (ATCC, HB-8065) hepatocellular carcinoma cells were maintained in EMEM (Eagle's minimum essential medium) (ATCC, 30-2003) supplied with EquaFETAL Bovine Serum (Atlas Biologicals, EF-0050-A), and 1% Pen Strep (penicillin and streptomycin). The cells were grown in a 37˚C incubator with 5% CO_2. Cells were split and plated into 6 well plates with 10% EquaFETAL and 1% Pen Strep before each experiment. Once cells reached about 85% confluency, the cells were starved of their full serum media via aspiration and were then washed with sterile PBS (Sigma Millipore, A2056-5MG). Next, the cells were supplied with serum free EMEM supplemented only with 1% Pen Strep.

Dose Responses

S6K-1 Inhibition

Cells were split and plated in 6 well plates with 10% EquaFETAL and 1% Pen Strep. Once cells reached approximately 85% confluency, medium was removed, the cells were washed with sterile PBS , and the media was replaced with EMEM containing 1% Pen Strep. After 24hrs of being serum starved, the cells were treated with a p70 S6K-1 inhibitor, PF4708671 (APExBIO, B2228), for 16hrs at the following concentrations diluted in DMSO: 1 µM, 5 µM, 10 µM, 20 µM, and 40 µM. 3hrs before the 16hrs elapsed cells were treated with 10nM of insulin.

p300 Inhibition

After 24hrs of being serum starved, the cells were treated with thea p300 inhibitor, C646 (APExBio, B1577), for 16hrs at the following concentrations diluted in DMSO: 1 µM, 5 µM, 10 µM, 20 µM, and 40 µM. 3hrs before the 16hrs elapsed cells were treated with 10nM of insulin for the rest of theremaining 3hrs.

Western Blot

After the treatment of HepG2 cells with insulin and either PF4708671 or C646, the cells were washed with sterile PBS and collected with 2x Laemmli buffer (Bio-Rad, 1610737). Samples were boiled at 95˚C for 5min and briefly centrifuged. Each treatment was assessed using gradient 4-20% precast polyacrylamide gels (Bio-Rad, 4561093EDU) . Each gel was then transferred on to a PVDF membrane (Fisher, IPVH00005), then blocked in 5% nonfat dry milk in tris buffered saline tween-20 (TBST) for an hour at room temperature or overnight at 4˚C on a rocker. Following this, the blots were washed with TBST followed by the addition of the appropriate primary antibody. The antibodies used are listed ion Table 1. The blots were then incubated at 4˚C overnight with rocking. Subsequently, the blots were washed with TBST and then incubated for 1hr in LI-COR goat anti-rabbit secondary antibody in 5% milk with TBST (LI-COR, 926-68071). Once the blots were washed with TBST, the blots were scanned on the LI-COR CLX imager and quantified using the LI-COR software Image Studio.

Proteins Assays

In vitro Kinase Assay

In vitro *Kinase Assay* - The activity of rA protein assay of recombinant p70 S6K-1 kinase (Abcam, ab268834) on on two recombinant constructs of IRS-1, IRS-1 1-355 (1μug) and IRS-1 600-1245 (1μgug) (MyBioSource, MBS515143 and MBS515371) was observedperformed. p70 S6K-1 kinase (0.25uμg) activity was first measured in a 10X kinase buffer (45mM MOPS, 37.5mM β-glycerol phosphate, 100mM $MgCl_2$, 10mM EGTA, 2mM EDTA, 1mM Na_3VO_4, and 0.5mM ATP). The buffer was supplemented with 1.25mM DTT at the time of use. Each reaction contained either IRS-1 1-355 or IRS-1 600-1245 substrate and incubated at 37˚C for 30min. The target sites for p70 S6K-1 were S312 on IRS-1 1-355, S636/639 and S1101 on IRS-1 600-1245. The samples were prepared as follows: Negative control – kinase buffer, ATP, HAT buffer, and acetyl-CoA; Positive control – kinase buffer, ATP, p70 S6K-1, HAT Buffer, and acetyl-CoA; Experimental – kinase buffer, ATP, p70 S6K-1, HAT Buffer, acetyl-CoA, and p300.

The negative control was made without p70 S6K-1, while the positive control was made with p70 S6K-1. The products were resolved on gradient pre-cast gels (4%-20%), transferred on to a PVDF membrane, then immunoblotted by the following phosphor-specific IRS-1 antibodies: S312, S636/639, and s1101.

***In vitro* HAT Assay**

In vitro *HAT Assay* - P300 activity assays were performed. Each reaction contained a 5X HAT buffer (250mM Tris-HCl pH 7.9, 0.5mM EDTA, and 750mM NaCl) and was supplemented with 0.2mM PMSF and 0.5mM DTT immediately before starting the HAT assay. Each reaction was initiated by adding Acetyl-CoA (Millipore Sigma, A2056-5MG) then incubated at 30˚C for

30min. The samples were prepared as follows: Negative control – HAT buffer, acetyl-Co-A, kinase buffer and ATP; Positive control –HAT Buffer, acetyl-CoA, p300, kinase buffer, and ATP; Experimental –HAT Buffer, acetyl-CoA, p300, kinase buffer, ATP, and p70 S6K-1.

The negative control was made without p300, while the positive control contained p300. The products were resolved on gradient pre-cast gels (4%-20%), transferred on to a PVDF membrane, then immunoblotted to access the acetylation status of IRS-1 before and after the addition of p300.

In vitro Mixed Assay

In vitro *Mixed Assay* - After performing a kinase assay to phosphorylate IRS-1, a HAT assay was set up by adding HAT buffer, acetyl-CoA, and p300. The samples were prepared as follows: Negative control – kinase buffer, ATP, HAT buffer, and acetyl-CoA; Positive control – kinase buffer, ATP, p70 S6K-1, HAT Buffer, and acetyl-CoA; Experimental – kinase buffer, ATP, p70 S6K-1, HAT Buffer, acetyl-CoA, and p300.

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After IRS-1 had been acetylated, a kinase assay was performed with all the necessary reagents – kinase buffer, ATP, and p70 S6K-1. The samples were prepared as follows: Negative control – HAT buffer, acetyl-Co-A, kinase buffer and ATP; Positive control –HAT Buffer, acetyl-CoA, p300, kinase buffer, and ATP; Experimental –HAT Buffer, acetyl-CoA, p300, kinase buffer, ATP, and p70 S6K-1.

SDS-PAGE was performed on each of the above samples on an 4-20% gradient pre-cast gel transferred on to a PVDF membrane, and then immunoblotted by either a pan acetyl antibody or IRS-1 phospho-specific antibodies.

5 Immunoprecipitation of IRS-1 acetylation

On the first day of the experiment, cells were plated on a 10cm^2 plate with 10% EquaFETAL and 1% Pen Strep. Once cells reached about 85% confluency, the cells were starved of their full serum media via aspiration and were washed with sterile PBS. The cells were then supplied with serum free EMEM supplemented with just 1% Pen Strep. After 24hrs of being serum starved, the cells were treated.

To adequately collect the cells and stop the treatments, the cells were washed with cold sterile PBS then treated with 500µl of IP Lysis/Wash Buffer (Fisher Scientific, PI26146) supplemented with a protease and phosphatase inhibitor cocktail (Sigma, PPC2020-5ML). Each plate was placed on ice for 5min before scraping the cells into pre-chilled 1.7ml Eppendorf tubes. Afterwards, cells were sonicated for 5sec each while on ice before they were centrifuged at 4˚ C for 10min at speed of 13,000 x g. The supernatant was transferred into new pre-chilled 1.7ml Eppendorf tubes and incubated with an IRS-1 antibody overnight at 4˚ C. Afterwards, Sepharose A/G beads (Abcam 206996) were added to each tube and incubated for 2hrs at 4˚C. The bead, antibody, and protein aggregates were collected and washed in a wash buffer containing (10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 150mM NaCl, 1% Triton X-100, and 0.2mM sodium orthovanadate). The samples were eluted with an acidic elution buffer containing

primary amine (Fisher Scientific, 26146). The eluents were then neutralized with 1M Tris pH 9.5 in order to perform functional assays.

Methods performed for future research

Dose Response

AKT1 Inhibition

Serum starved cells were treated with a AKT inhibitor, MK2206 (APExBIO, A3010), for 24hrs hrs at the following concentrations diluted in DMSO: 0.67µM, 1.17µM, 1.67µM, 2.17µM, and 2.67µM. 10min before the 24hrs elapsed cells were treated with 10nM of insulin. A second dose

response was performed using the following concentrations: 4nM, 8nM, 16nM, 32nM, and 64nM. 1hr before the 24hrs elapsed cells were treated with 10nM of insulin. The products were resolved on gradient pre-cast gels (4%-20%), transferred on to a PVDF membrane, then immunoblotted to access the phosphorylation status of AKT.

p70 S6K-1 stimulation by insulin

Following 24hrs of being serum starved, the cells were treated, for 1hr at the following concentrations of insulin diluted in sterile PBS; 10nM, 50nM, and 100nM. The samples were collected in sample buffer (0.128M Tris Base, 26% glycerol, 2.6% SDS, 0.32% 2-Mercaptoethanol). An SDS PAGE was performed using 6%, 7.5%, or 10.5% resolving gel (6% Acryl/0.04%BIS, 0.37M Tris Base pH 8.8, 0.55mM ammonium persulfate, and 0.1% TEMED) and stacking gel (4.3% Acryl/0.12% BIS, 0.11M Tris Base, and 0.09% SDS, 0.274mM ammonium persulfate, 0.05% TEMED). The gel was then transferred using transfer bufferTBST on to a PVDF membrane, and then immunoblotted by total p70 S6K-1 or phospho p70 S6K-1.

p70 S6K1 Site Directed Mutagenesis

Site directed mutagenesis was performed on a wild type p70 S6K-1 construct in order to create a constitutively active and constitutively inactive p70 S6K-1. Initially exponential amplification PCR was performed with the following setup: 0.5µM forward primer, 0.5µM reverse primer, 5ng

of wildtype p70 S6K-1 construct, and Q5 Hot Start High Fidelity Master mix (product numbNEB, E0554er). The PCR scheme involved an initial first denaturation for 30sec at 98˚C followed by 25 cycles of denaturing (for 10sec at 98˚C), annealing (for 20sec at 60 ˚C), and extension (for 3.4min at 72 ˚C). After the 25 cycles each sample underwent final extension for 2min at 72 ˚C. A kinase ligase and DpnI (KLD) treatment was added to each sample with DH5α cells followed by at 30min incubation on ice. (Figure 11AB) The samples were heat shocked at 42 ˚C for 30sec each then placed on ice for 5min. SOC media was added and samples were put in a gentle shaker for 1hr. Lastly, the samples were plated inon to petri dishes and grown overnight. The plasmid DNA was purified from the colonies and sent for sequencing to verify the mutations. (Figure 111BA).

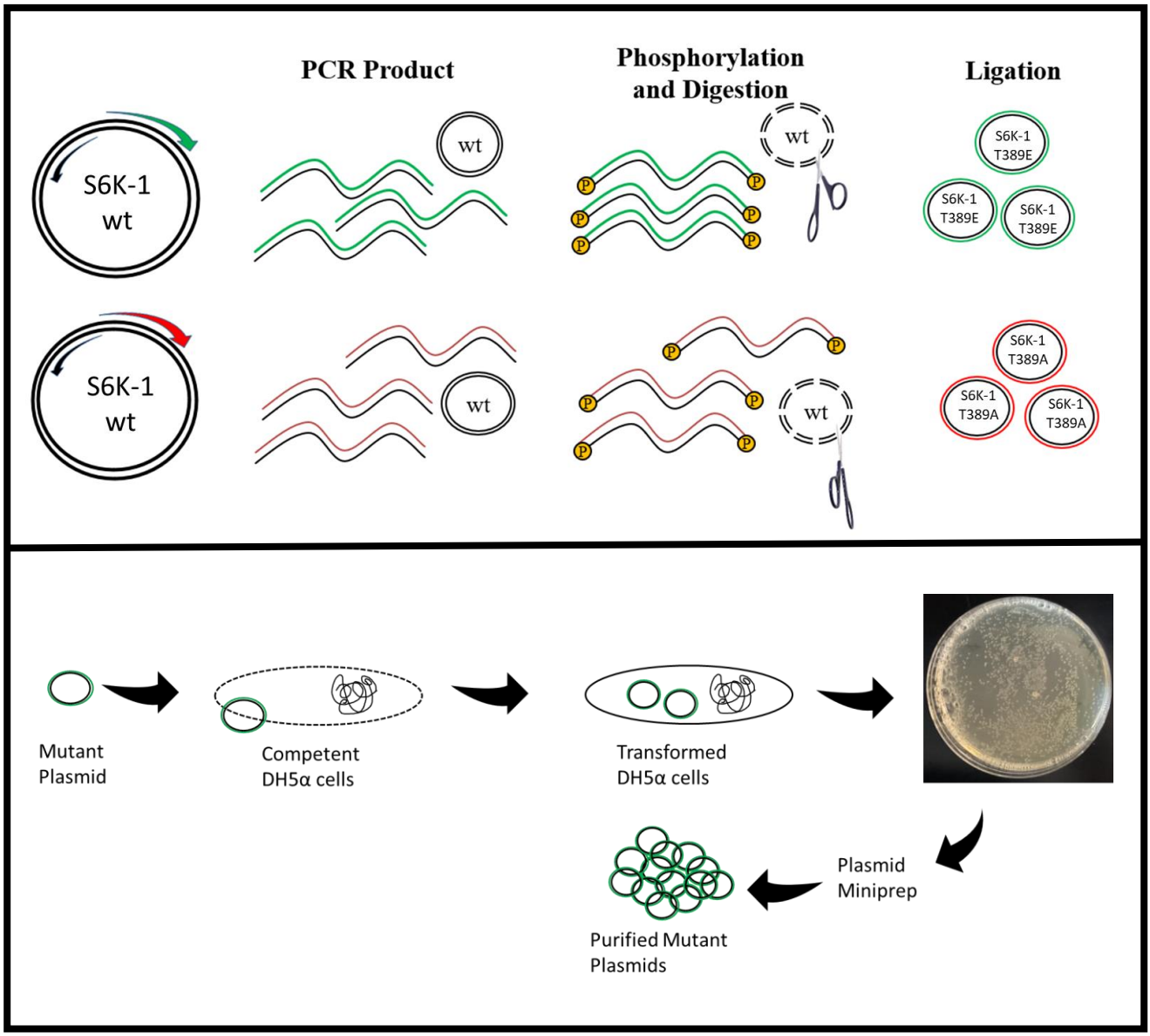

Figure 11. Site Directed Mutagenesis.

p300 enhances the phosphorylation of IRS-1 on specific sites by p70 S6K-1

Introduction

The study of insulin resistance and the resulting impacts on IRS-1 regulation has been popular widely studied over the past decade. A common theme has been shown that a high caloric intake can lead to insulin resistance among other factors, such as obesity, thus rendering IRS-1 insensitive to stimulation by physiological concentrations of insulin. Risk factors that lead to insulin resistance can lead to cardiometabolic complication such as coronary heart disease or type 2 diabetes. Therefore, the study of the dysregulation of IRS-1 remains relevant for further development of potential rehabilitation of IRS-1 homeostatic function. Multiple posttranslational modifications have been revealed to be responsible for the dysregulation of IRS-1 caused by hyperinsulinemia, hyperaminoacidemia, or the administration of a high fat diet (HFD).

p300, a well-known acetyltransferase, has been shown to be greatly elevated in mice liver that had been fed a high fat diet (HFD) compared to those fed a regular diet, before the development of insulin resistance. Administering a HFD led to an increase in lipopolysaccharides which prevented the degradation of p300 thus increasing protein levels of p300. Importantly, in these experiments the transcription of the p300 gene was not stimulated. This increase is not due to the stimulation of p300 transcription. After the development of insulin resistance due to a continual HFD, p300 protein levels remained elevated. This study revealed that although p300 levels increase, CREB binding protein (CBP), a p300 homologue and its transcriptional co-activator, did not display any change its in protein levels meaning that p300 was solely affected.

Furthermore, an increase in lipopolysaccharide treated in Hep1-6 cells, led to the localization of p300 when from a preferably localizing in the nucleus toin the cytoplasmic localization rather than the nucleus. Further research revealed that as a result p300 led to a decreased in insulin signaling while localized in the cytoplasm. This decrease in insulin signaling was revealed be due to acetylation of IRS-1 on various lysine site. Selectively inhibiting p300 using C646 has been shown to improve insulin resistance by both preventing the acetylation of IRS-1 and increasing the interaction between IRS-1 and IR. Acetylation is not the only posttranslational modification that negatively regulates IRS-1, as. sSpecific serine phosphorylations can negatively regulate IRS-1 as welltoo. While different kinases target different sites, p70 S6K-1 is among the few kinases that can phosphorylate IRS-1 on multiple sites based on stimuli such as hyperinsulinemia or hyperaminoacidemia. PF4708671 is a well-known selective inhibitor of p70 S6K-1. Both PF4708671 and C646 are a great tool to study the crosstalk between the acetylation and serine phosphorylation of IRS-1.

Results

PF4708671 selectively inhibits p70S6K-1, which leads to negative regulation of RPS6 is a dose dependent manner

To determine the involvement of S6K1 in the phosphorylation of specific sites on IRS-1, PF4708671, a selective inhibitor of p70 S6K-1, was used. A dose response was conducted to determine the appropriate concentration necessary to inhibit p70 S6K-1. HepG2 cells were treated with insulin, after which the inhibitor was added, and the phosphorylation of RPS6, a direct target protein for p70 S6K-1, was examined. Cells deprived of serum showed little to no phosphorylation of RPS6, while cells with serum exhibited a small amount of phosphorylation. When 10nM insulin was added to the serum starved cells for 3hrs, the phosphorylation of RPS6 increased 10-fold. The effect insulin had on the phosphorylation of RPS6 was attenuated by PF4708671 in a dose dependent manner. The concentrations used were 1µM, 5µM, 10µM, 20µM and 40µM for 16hrs. At 5µM, RPS6 phosphorylation was reduced by more than half (Figure 12).

PF4708671 inhibits p70S6K-1, which leads to reduced phosphorylation of S1101 on IRS-1 in a dose dependent manner

While p70 S6K-1 is known to phosphorylate RPS6, it is also known to phosphorylate specific serine residues on IRS-1. The inhibition of S6K-1 by PF4708671 led to a statistically significant decrease in IRS-1 S1101 phosphorylation in insulin treated Hep G2 cells. After treatment with 10µM of PF4708671, the phosphorylation of S1101 showed a statistically significant decrease of 10% , however at 20µM there was a 70% decrease (Figure 2?). In figure 12, there was an increase in RPS6 S235/236 phosphorylation from insulin treated HepG2 cells compared to serum starved only cells. In contrast, IRS-1 S1101 phosphorylation did not change when treated with 10nM of insulin (Figure 132).

PF4708671 inhibits p70S6K-1, which leads to reduced phosphorylation of S312 on IRS-1 in a dose dependent manner

Just as S1101 phosphorylation did not increase from insulin stimulation (Figure 13), IRS-1 S312 phosphorylation did not either in response to 10nM insulin treatment. (Figure 2). However, the addition of PF4708671 reduces the phosphorylation of S312 in a dose dependent manner. At 10µM of PF4708671, the phosphorylation of S312 decreased by 50%, however at 20µM there was a 70% decrease (Figure 14)3).

PF4708671 inhibits p70S6K-1, which leads to reduced phosphorylation of S636/S639 on IRS-1 in a dose dependent manner

The phosphorylation status of S636/639 in serum starved cells was compared to serum starved cells treated with insulin. There was no significant difference between the two. Treatment of HepG2 cells with 10 µM of PF4708671 led to a 30% decrease in the phosphorylation of S636/639. However, when p70 S6K-1 is inhibited by 20µM PF4708671, the phosphorylation of S636/639 is decreased by 50%. (Figure 154).

C646 inhibits p300, thus reducing the acetylation of H3 at K9 and K14 in a dose dependent manner

The role of that p300 plays ion in the regulation of IRS-1 activation is a subject that groups are has been studied and revealed to be negative. actively researching. However, how the effect of that p300 effects affect to indirectly enhance has on the phosphorylation status of IRS-1 through acetylation is not currently understood. To assess the acetyltransferase activity of p300, the optimal dose of inhibitor that is required to successfully decrease the acetyltransferase activity of p300 was assessed by treating cells with C646 for 16hrs, a selective p300 inhibitor. The acetylation status of H3, a direct target of p300, was assessed in serum starved cells and serum starved cells treated with 10nM insulin for 3hrs. The acetylation status did not change, showing that insulin has no role in regulating histone acetylation via p300. However, once cells were treated with C646 the H3 acetylation gradually decreased. At a concentration of 5µM H3 acetylation was decreased by more than half, while at 10µM there was a 10-fold decrease; thus, demonstrating the effectiveness of C646 at specifically inhibiting p300 (Figure 165).

Inhibition of p300 leads to reduced phosphorylation of IRS-1 S1101 and S312 in a dose dependent manner

Cells were treated with C646 were used to assess the change in the phosphorylation status of S312 and S1101 on IRS-1. Treatment with 5µM of C646 led to a 40% decrease in S1101 phosphorylation compared to HepG2 cells treated with insulin. In addition, insulin stimulated HepG2 cells treated with 10µM of C646, significantly which reduced the ability of p300 to acetylate IRS-1, leading led to an 80% decrease in IRS-1 S1101 phosphorylation (Figure 176). On the other hand, inhibiting p300 with 5µM of C646 led to a 62% decrease in IRS-1 S312 phosphorylation, while the 10µM treatment of C646 led to a 75% decrease (Figure 187).

The absence of p300 activity did not change the phosphorylation status of IRS-1 S636/639

Insulin stimulated HepG2 cells treated with C646 showed no statistically significant change in the phosphorylation of IRS-1 S636/639. Inhibition of p300 did not reduce the phosphorylation of IRS-1 S636/S639 in a dose dependent manner, and neither is there a change in the phosphorylation status of S636/639 when comparing serum starved cells to insulin stimulated cells (Figure 198). This suggests that the basal phosphorylation of S636 cannot be changed by insulin stimulation and that C646 has no effect on this phosphorylation.

Inhibiting p300 via C646 increases p70 S6K-1 activity but reduces phosphorylation of S6K1 specific sites on IRS-1

After being serum starved for 24 hrs, HepG2 cells were treated with C646 for 16hrs and with insulin for 3hrs. RPS6 phosphorylation of site 235/236 increased after treatment with insulin without C646 treatment. When the insulin stimulated HepG2 cells are treated with 5µM and 10µM of C646 there is a statistically significant increase in S235/236 RPS6 phosphorylation. At 5µM RPS6 S235/236 phosphorylation increases 120-fold, while at 10µM it increased by 250-fold (Figure 209).

p300 enhances the phosphorylation of IRS-1 S1101 by p70 S6K1, but not S636/639

An *in vitro* kinase assay was performed to assess p70 S6K-1 activity on IRS-1. The control lacked the presence of S6K1 while the experimental group had both p70 S6K-1 as well as ATP. There was a 100-fold increase in the phosphorylation of IRS-1 S1101 due to S6K1 activity. The addition of p300 with acetyl CoA to assess the effect of acetylation of IRS on the phosphorylation of IRS-1 S1101 led to an even greater increase in S1101 phosphorylation (Figure 2110). On the other hand, not only did p300 have no effect on the phosphorylation of S636/639, but p70 S6K-1 was insufficient in changing the phosphorylation status of S636/639 when compared to the ATP-depleted negative control (Figure 2121). It is imperative to note thatT the overall acetylation of IRS-1 was not affected by when the presence of p70 S6K-1 was added (Figures 2132A and 2132B). Therefore, it is clear that the acetylation status of IRS-1

enhances the phosphorylation of S1101, while the phosphorylation of IRS-1 has not effect on the its acetylation.

Preliminary Results for Future Research

AKT1 Dose Response

After being serum starved for 24 hrs., HepG2 cells were treated with MK2206 for 24hrs and with insulin for 1hr. Serum starved cells hads very little S473 AKT phosphorylation. Cells that had serum had double the amount of phosphorylation. Insulin stimulated cells displaced displayed an exponential increase phosphorylation of S473. When the insulin stimulated HepG2 cells were treated with 4nM to 64nM of MK2206 there was a dose dependent decrease in S473 phosphorylation. At 4nM, there was no change in phosphorylation, however at 8nM AKT phosphorylation dropped by half. Eventually treatment with 64nM led to a decrease that emulated cells that had not been treated with insulin . (Figure 2413).

p70 S6K-1 stimulation by insulin

Insulin treated cells were immunoblotted to verify if 10nM of insulin for 1hr is enough to stimulate the phosphorylation of p70 S6K-1. A gel shift assay revealed 4 separate bands that are known as alpha, beta, gamma and delta forms of p70 S6K-1. The presence of the beta gamma, and delta band represents the phosphorylated state of p70 S6K-1 while the presence of the alpha band represent an unphosphorylated p70 S6K-1 (Figure 25). a shift in iInsulin stimulated cells had phosphorylated p70S6K-1 compared to those cells only treated with sterile PBS insteadwhich hd no phosphorylation. .

p70 S6K-1 Site Directed Mutagenesis

Site directed mutagenesis was successfully conducted performed to design an active construct of S6K1. The wild type T389 was mutated into a glutamic acid to represent a constitutively active p70 S6K-1 construct. Additional mutations were needed to activate it, as (put here what else is needed). Additionally, a constitutively inactive p70 S6K-1 construct was made by mutating the threonine T389 into A389an alanine (Figure 26).

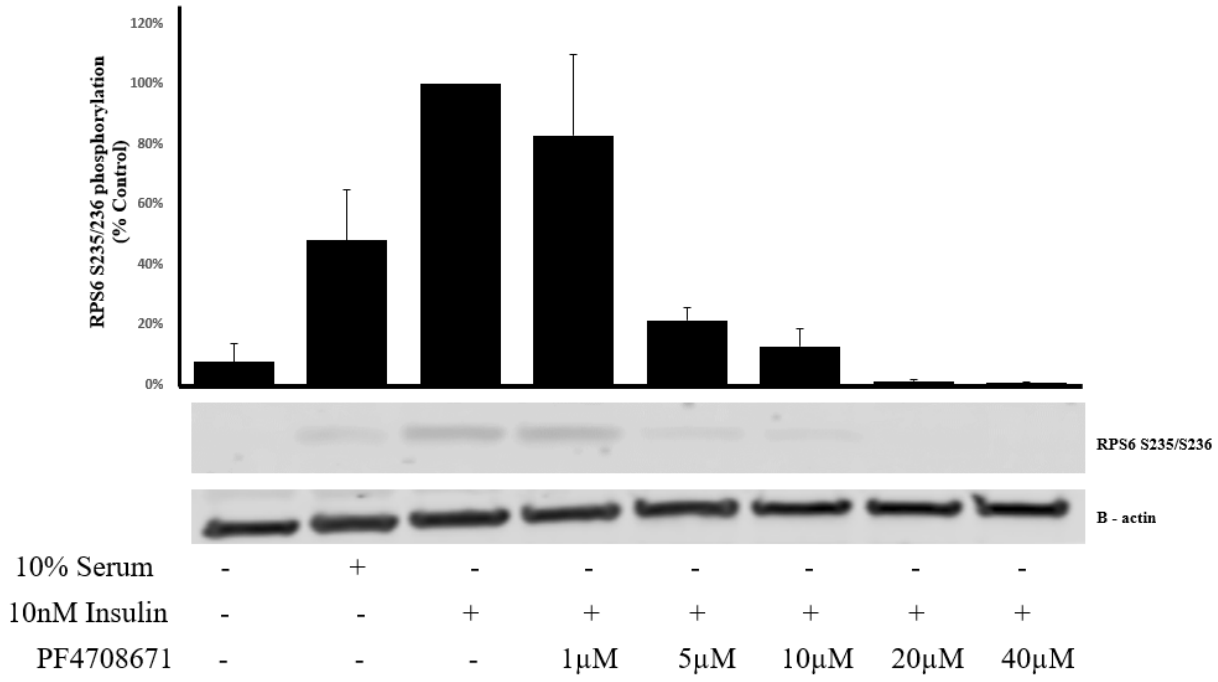

Figure 12. PF4708671 selectively inhibits insulin stimulated p70S6K-1, negatively regulating RPS6 phosphorylation in a dose dependent manner. To confirm the ability of PF4708671 to inhibit S6K-1, HepG2 cells were serum starved for 24hrs then treated with 10nM insulin and P4708671 (a selective p70 S6K-1 inhibitor). The negative control was treated with DMSO for 16hrs. The positive control had 10nM insulin for 3hrs within the 16hrs treatment of DMSO, and no serum. In the negative control there is minimal phosphorylation of RPS6 S235/S236 , while the positive control gives a 10-fold increase in RPS6 S235/236 phosphorylation. After adding 1μM of PF4708671, there is little to no change in the phosphorylation levels of RPS6. However, after 5μM, RPS6 phosphorylation is reduced by 80%. At 20μM and 40μM there is almost a 100% reduction in phosphorylation compared to when cells are treated with insulin. SEM was generated from an n of 3.

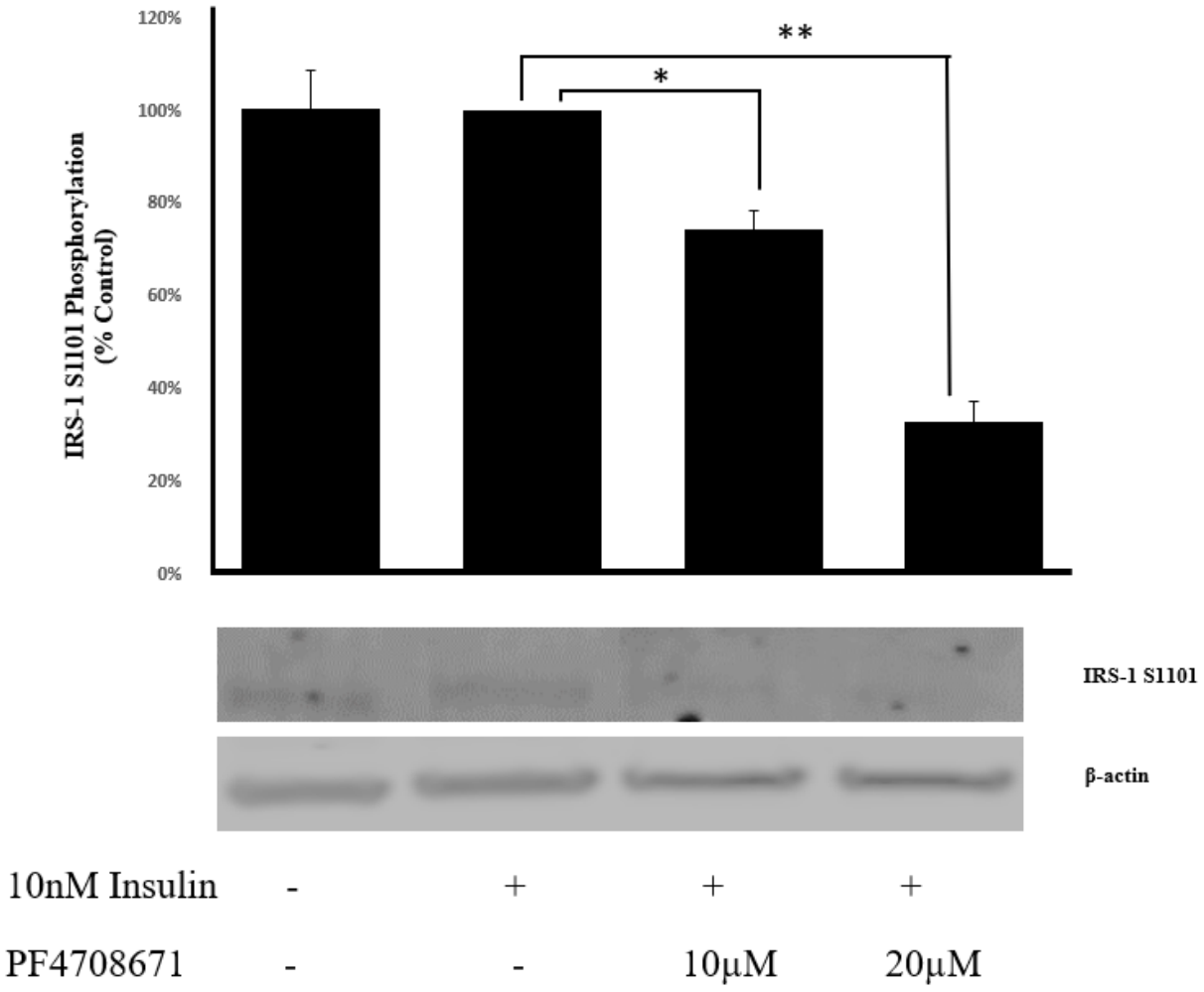

Figure 132. PF4708671 inhibits p70S6K-1, which leads to reduced phosphorylation of S1101 on IRS-1 in a dose dependent manner. The concentrations of P4708671 chosen to inhibit p70 S6K-1 based on figure 12 were 10 μM and 20μM . Treatment of HepG2 cells with 10μM and 20μM of PF4708671 for 16hrs followed and by 3hrs of treatment with 10nM of insulin before the end of the 16hr PF4708671 treatment was enough to show a statistically significant decrease in IRS-1 S1101 phosphorylation by p70 S6K-1. While insulin does increase p70 S6K-1 activity in HepG2 cells (see Figure 12), this does not lead to an increase in the phosphorylation of IRS-1 S1101, but once p70 S6K-1 is inhibited in insulin stimulated HepG2

cells, the phosphorylation of IRS-1 S1101 is decreased considerably. The graph represents the mean ± SEM of 3 separate experiments that were performed in triplicate and were independently analyzed using a one-way ANOVA. Representative blots are shown beneath the graphs. * = $p < 0.05$, ** = $p < 0.005$.

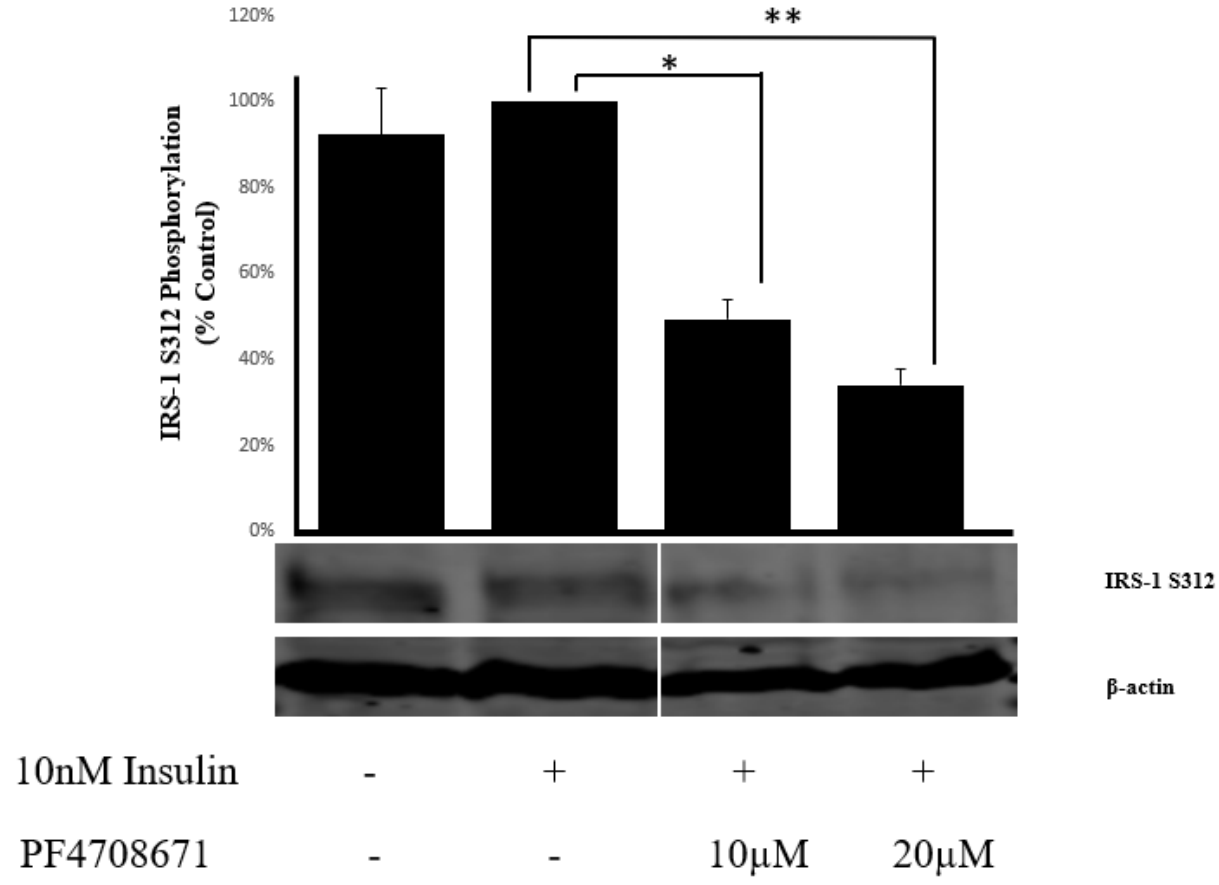

Figure 3. PF4708671 inhibits p70S6K-1, which leads to reduced phosphorylation of S312 on IRS-1 in a dose dependent manner. HepG2 cells were treated with 10μM and 20μM of PF4708671 for a total of 16hrs with the last 3hrs of containing 10nM of insulin. followed by3hrs of treatment with 10nM of insulin before the end of the 16hr PF4708671 treatment. Each treatment exhibited a statistically significant decrease in IRS-1 S312 phosphorylation by p70 S6K-1 compared to cells treated with just insulin. Inhibiting p70 S6K-1 in insulin stimulated HepG2 cells with 20μM of PF4708671, greatly decreases the phosphorylation of IRS-1 S312 by 70%. The graph represents the mean ± SEM of 3 separate experiments that were performed in triplicate and were independently analyzed using a one-way ANOVA. Representative blots are

shown beneath the graphs. * = p < 0.05, ** = p < 0.00

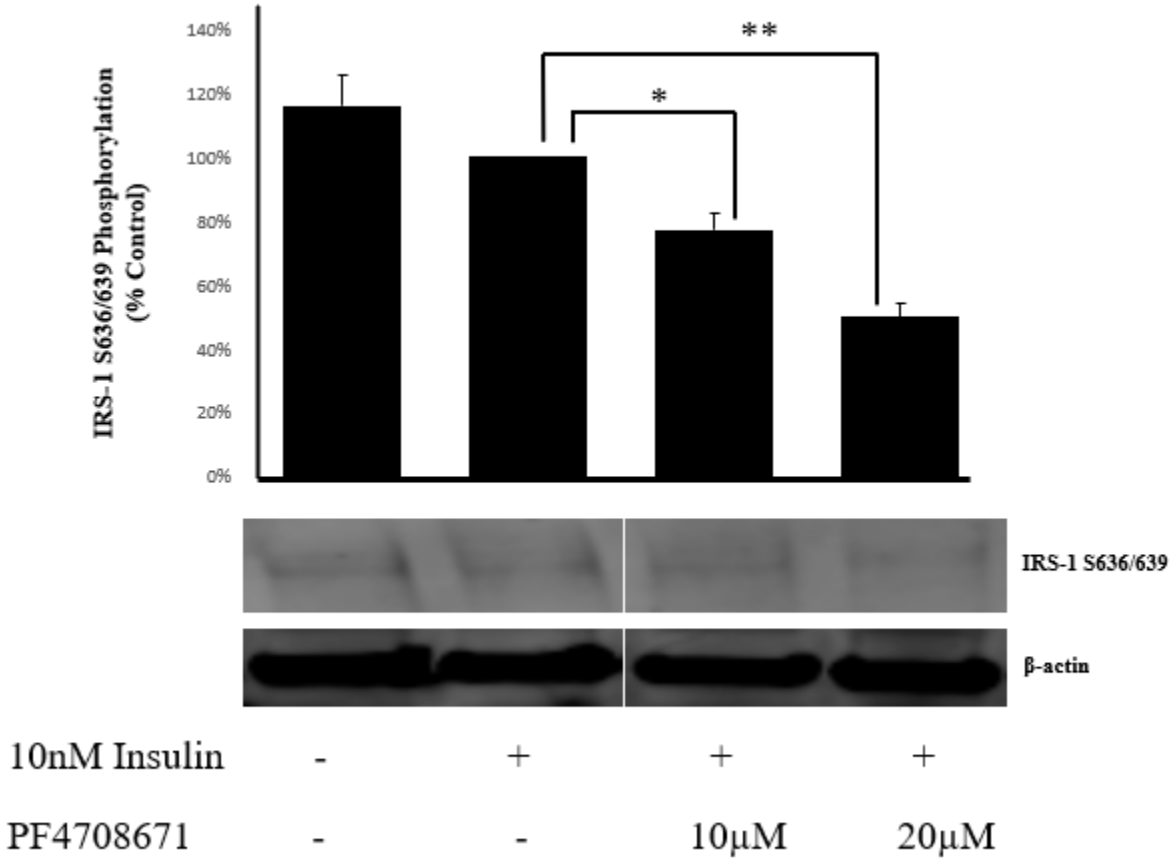

Figure 4. PF4708671 inhibits p70S6K-1, which leads to reduced phosphorylation of 636/S639 on IRS-1 in a dose dependent manner. HepG2 cells were treated for a total of 16hrs with 10μM and 20μM of PF4708671 for 16hrswith the last followed by 3hrs of treatment containing with 10nM of insulin before the end of the 16hr PF4708671. Both treatments led to a decrease in IRS-1 636/S639 phosphorylation by p70 S6K-1. Inhibiting p70 S6K-1 with 10μM led to a 30% decrease, while 20μM of PF4708671 led a 50% reduction in 636/S639 phosphorylation. The graph represents the mean ± SEM of 3 separate experiments that were

performed in triplicate and were independently analyzed using a one-way ANOVA. Representative blots are shown beneath the graphs. * = $p < 0.05$, ** = $p < 0.007$.

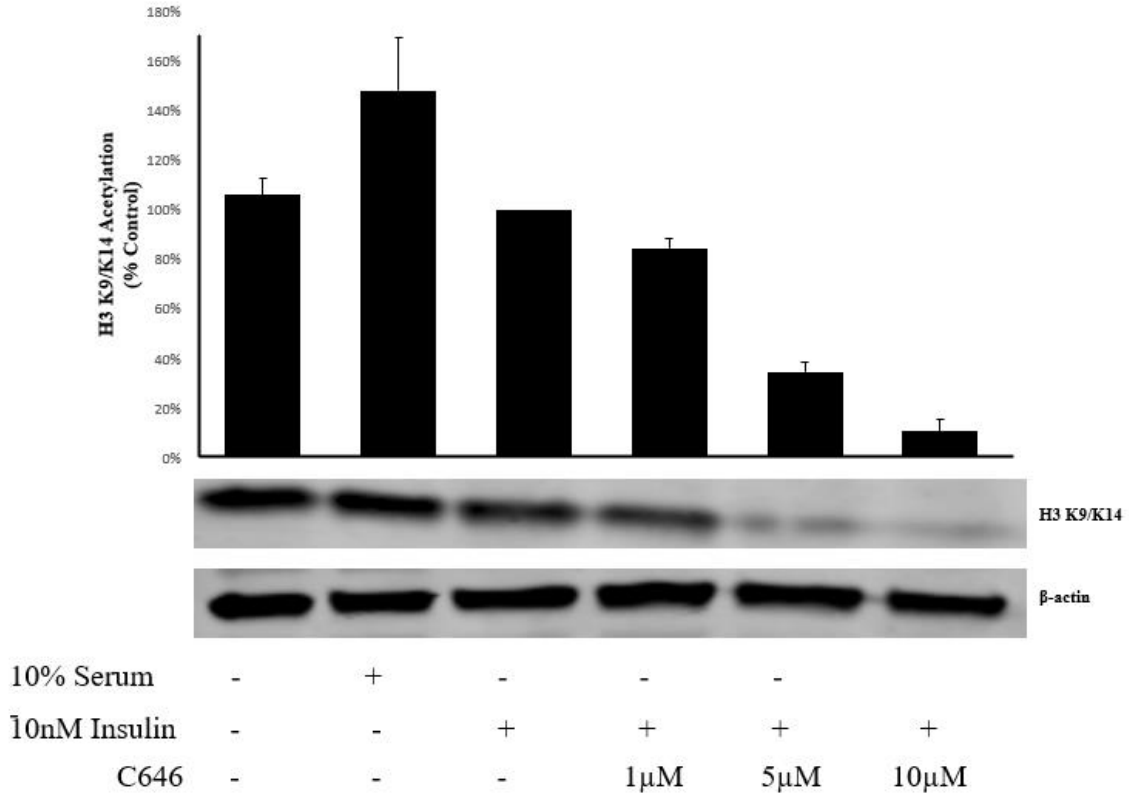

Figure 5. C646 inhibits p300, thus reducing the acetylation of H3 at K9 and K14 in a dose dependent manner. To confirm the ability of C464 C646to inhibit p300, HepG2 cells were serum starved for 24hrs then treated with 10nM insulin and C646 (a selective p300 inhibitor). The negative control was treated for a total of 16hrs with DMSO for 16hrs followedand including sterile PBS for the last 3hrs. by 3hrs of sterile PBS before the end of the 16hr C646. The positive control either had 10nM insulin for 3hrs with 16hrs treatment of DMSO, and no serum. or no insulin, 16hrs treatment of DMSO, and 10% serum. After treatment with C646 for 16hrs there is no statistically significant decrease in H3 K9/K14 acetylation. However, after when treated with 5μM of C646 there is a 70% decrease compared to HepG2 cells treated with

insulin. Treatment with 10μM of C646 reduced the ability for p300 to acetylate H3 by almost 100%. SEM shown was generated from an n of 3.

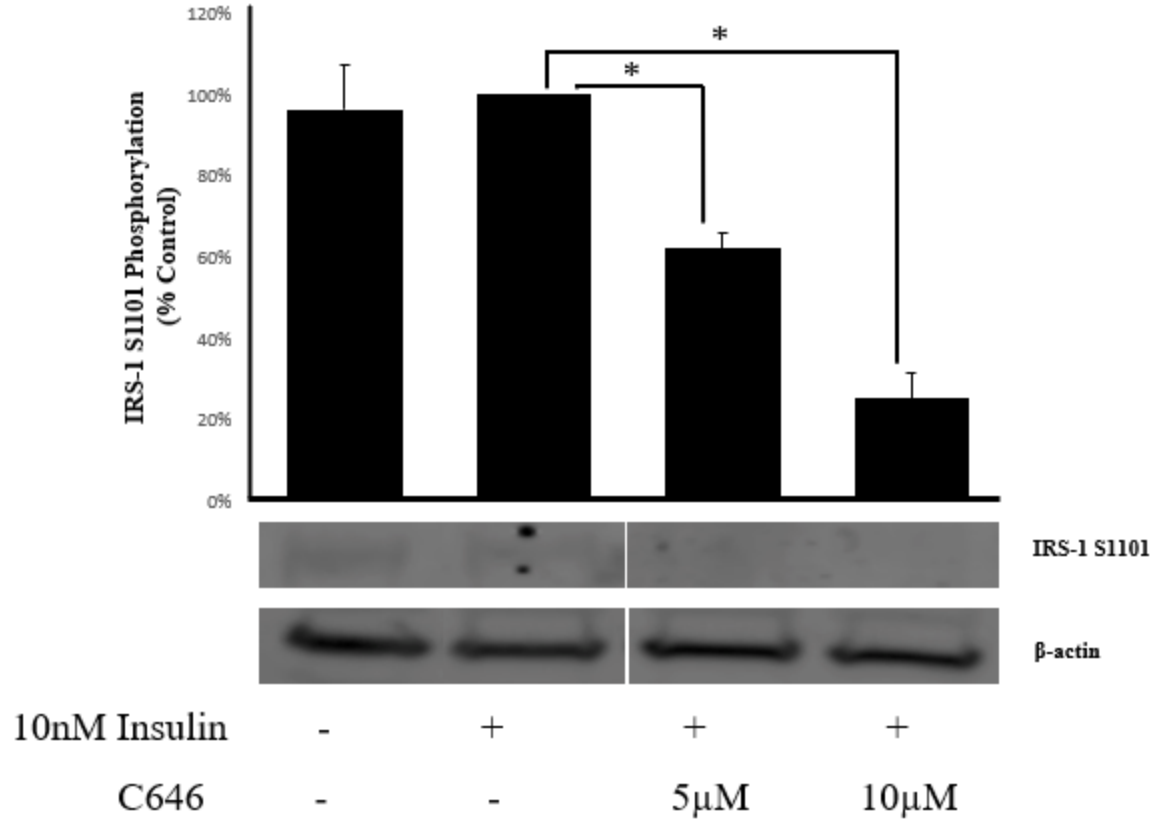

Figure 6. Inhibition of p300 leads to reduced phosphorylation of IRS-1 S1101 in a dose dependent manner. After being serum starved for 24hrs, HepG2 cells were treated with C646 for a total of 16hrs, with insulin included for the last 3hrs. and insulin for 3hrs before the end of the 16hr C646 treatment . IRS-1 S1101 phosphorylation decreased in a dose dependent manner. 5μM of C646 caused a 40% decrease compared to HepG2 cells treated with insulin alone. Treatment with 10μM of C646 led to an 80% decrease in IRS-1 S1101 phosphorylation. The graph represents the mean ± SEM of 3 separate experiments that were performed in triplicate and were independently analyzed using a one-way ANOVA. Representative blots are shown beneath the graphs. * = $p < 0.01$.

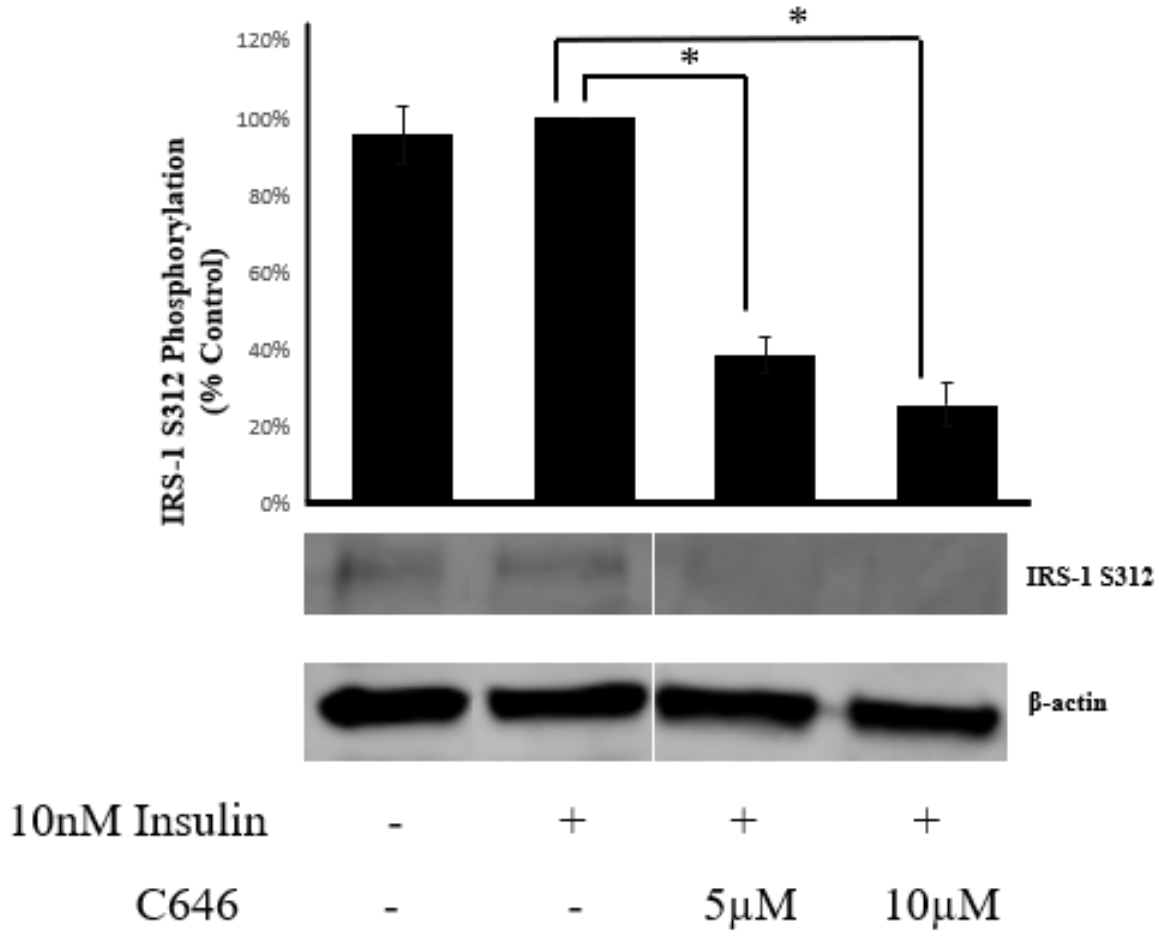

Figure 7. Inhibition of p300 leads to reduced phosphorylation of IRS-1 S312 in a dose dependent manner. HepG2 cells were starved of serum for 24hrs before treatment with C646 for a total of 16hrs with 3hrs of insulin treatment directly before sample collection. and insulin for 3hrs before the end of the 16hr C646 treatment Each increase in C646 concentration led to a statistically significant decrease in IRS-1 S312 phosphorylation by p70 S6K-1 compared to cells treated with just 10nM insulin. Inhibiting p300 with 5μM of C646 led to a 62% decrease in IRS-1 S312 phosphorylation, while 10μM of C646 led to a 75% decrease. The graph represents the mean ± SEM of 3 separate experiments that were performed in triplicate and were independently

analyzed using a one-way ANOVA. Representative blots are shown beneath the graphs. * = p < 0.005.

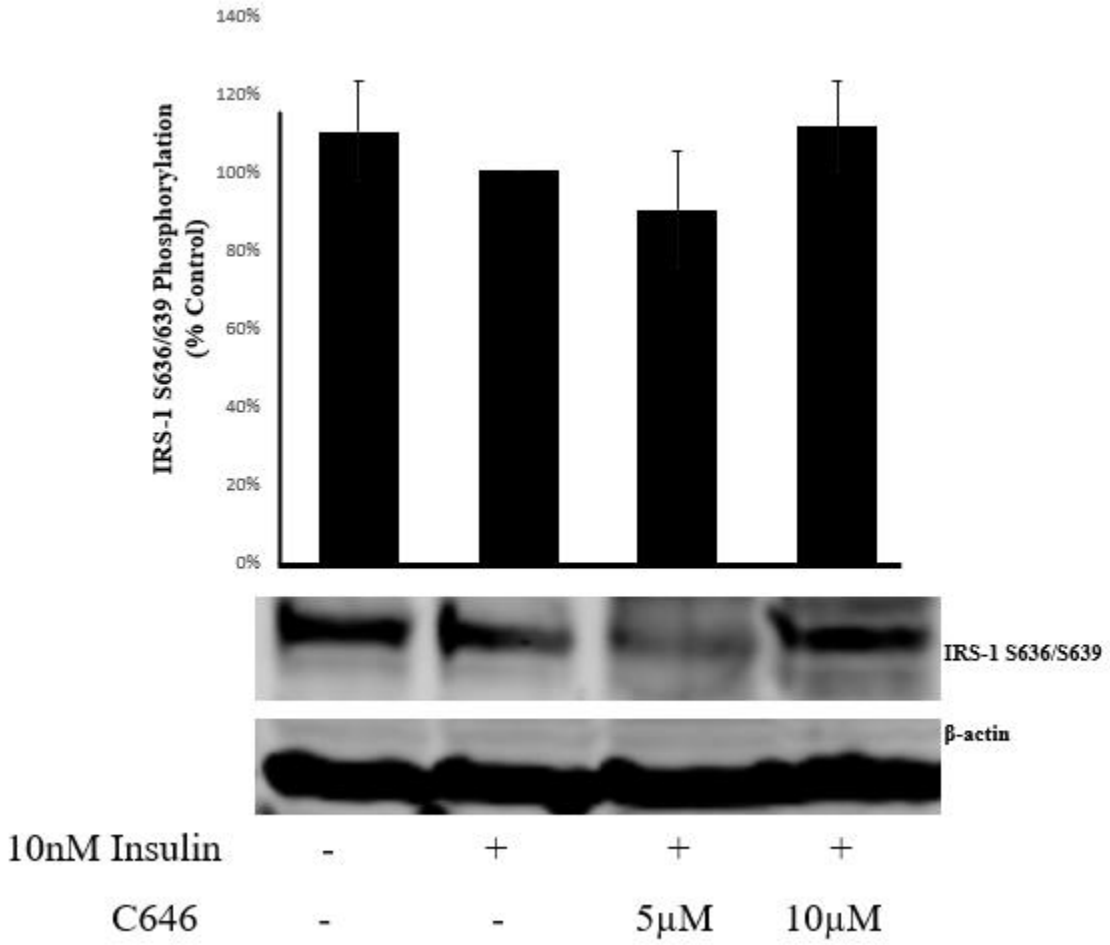

Figure 8. Inhibition of p300 does not reduce the phosphorylation of IRS-1 S636/S639. There was no statistically significant change in the phosphorylation status of IRS-1 S636/S639 when HepG2 cells were treated with C646 for 16hrs with 10nM insulin compared to cells treated with only 10nM insulin. Each treatment of insulin was for 3hrs within the 16hr treatment of C646 or DMSO for the negative control. The graph represents the mean ± SEM of 3 separate experiments that were performed in triplicate and were independently analyzed using a one-way ANOVA. Representative blots are shown beneath the graphs.

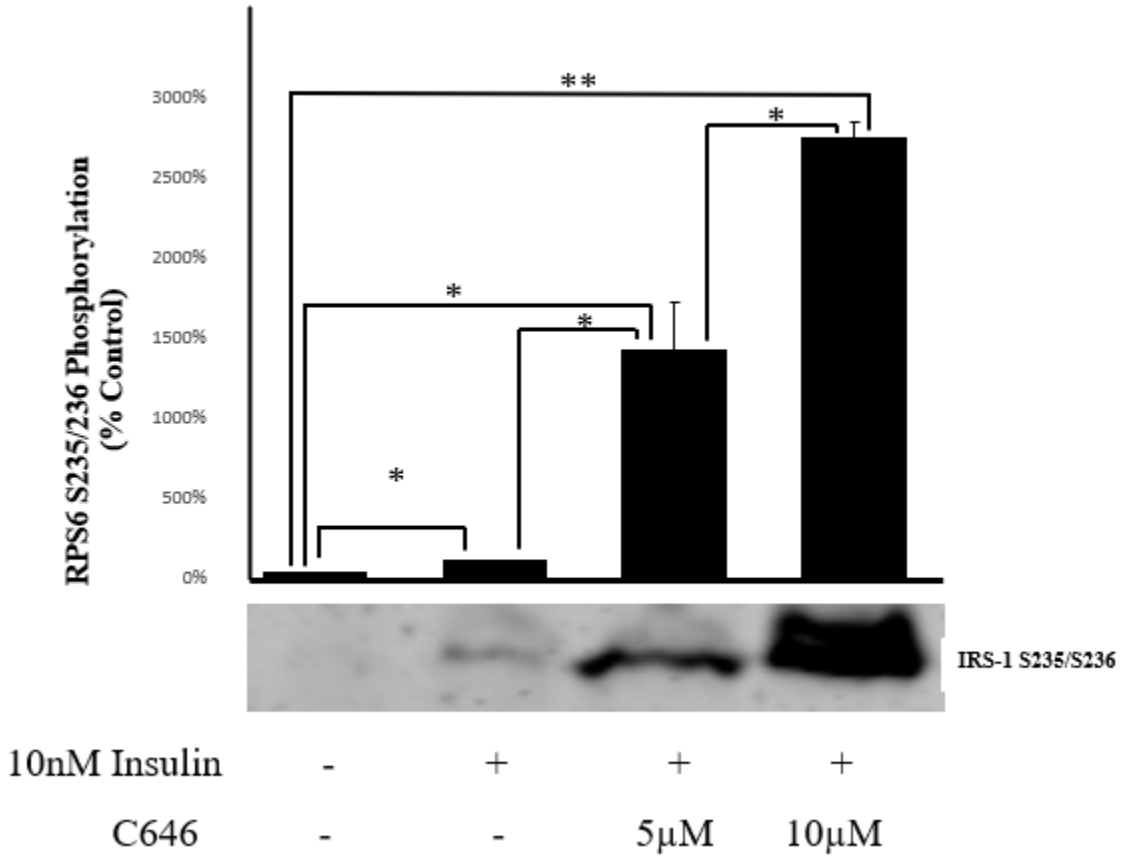

Figure 9. C646 treatment leads to the positive regulation of RPS6. After being serum starved for 24 hrs., HepGg2 cells were treated with C646 for 16rs and with insulin for 3hrs before the end of the 16hr C646 treatment. RPS6 phosphorylation of site 235/236 increased after treatment with insulin without C646 treatment. When the insulin stimulated HepG2 cells are treated with 5μM and 10μM of C646 there is a statistically significant increase in S235/236 RPS6 phosphorylation. At 5μM RPS6 S235/236 phosphorylation increases 120-fold compared to the treatment with only insulin. While at 10μM it increased by 250-fold, compared to with inulin treatment alone. The graph represents the mean ± SEM of 3 separate experiments that were performed in triplicate and were independently analyzed using a one-way ANOVA.

Representative blots are shown beneath the graphs. * = p < 0.05, ** = p < 0.01

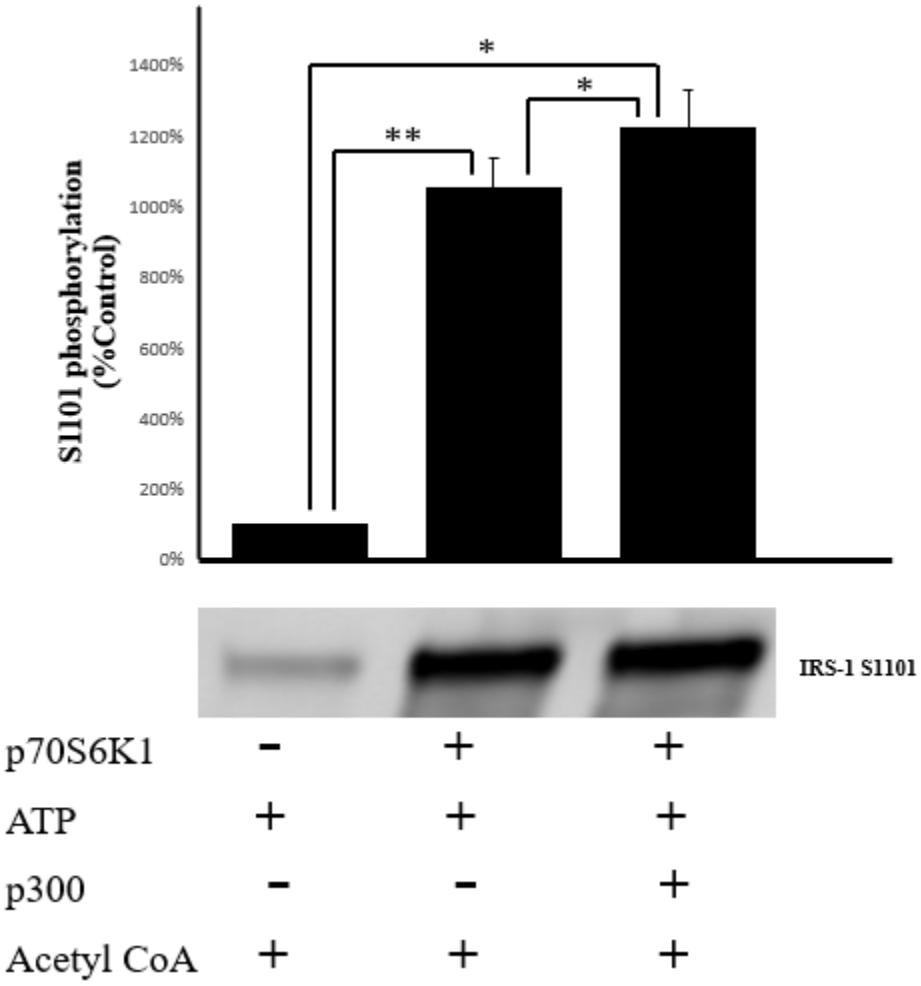

Figure 10. p300 activity does enhance the phosphorylation of IRS-1 by p70 S6K1 in vitro.

IRS1 S1101 is phosphorylated by p70 S6K1 on the 600-1245 IRS1 fragment (0.5µg) in the presence of ATP. This phosphorylation is enhanced by the addition of p300. The graph represents the mean ± SEM of 3 separate experiments that were independently analyzed using a one-way ANOVA. Representative blots are shown beneath the graphs. * = p < 0.05, ** = p < 0.01

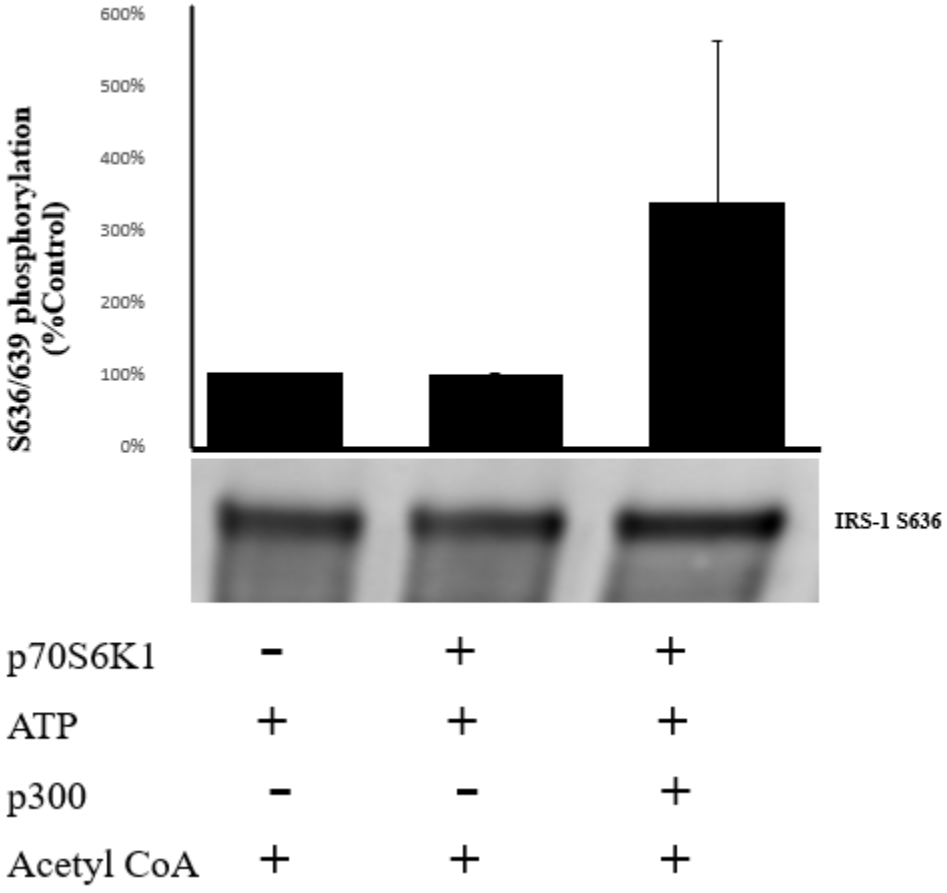

Figure 11. S6K-1 does not directly phosphorylate S636.

IRS-1 S636 does not get phosphorylated by p70 S6K1 in the presence of ATP nor does the presence or activity of p300 change that as is shown by both a kinase and mixed protein assay. The graph represents the mean ± SEM of 3 separate experiments that were independently analyzed using a one-way ANOVA. Representative blots are shown beneath the graphs.

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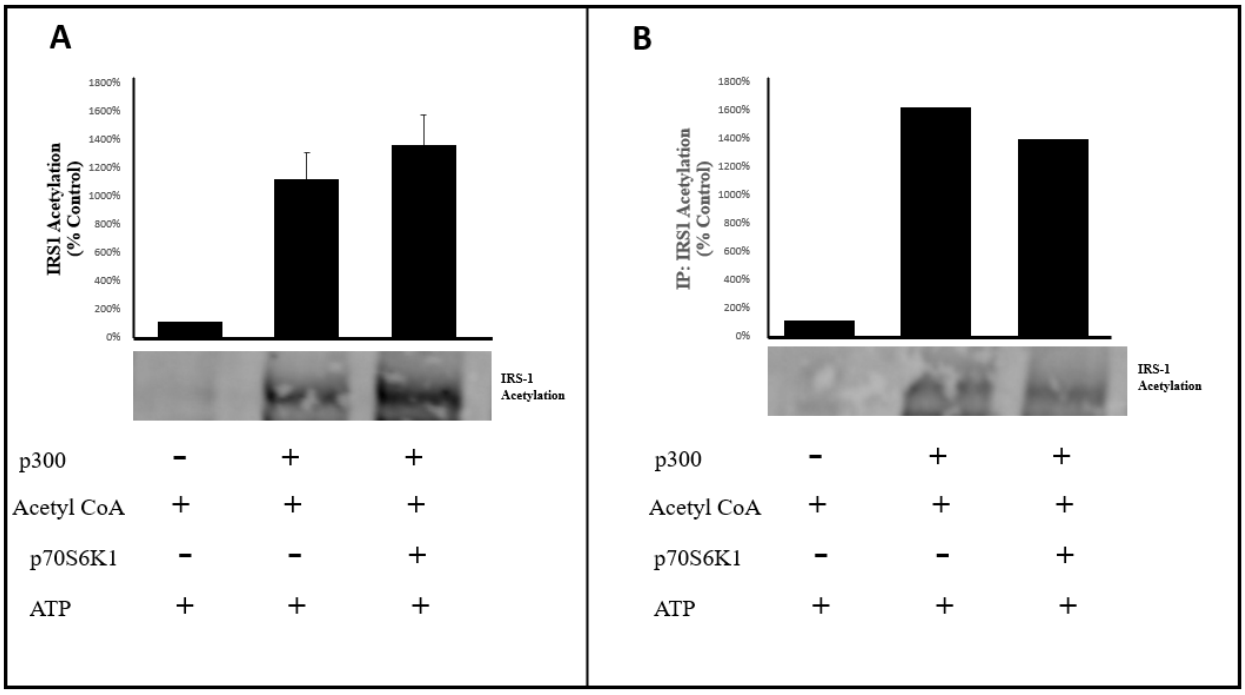

Figure 12. p70 S6K1 activity has no effect on the acetylation status of IRS-1 A) IRS-1 600-1245 fragment is acetylated by p300 in the presence of Acetyl CoA (A). This acetylation does not change in statistically significant manner upon the addition of S6K-1. The graph represents the mean ± SEM of 3 separate experiments HAT assays that were independently analyzed using a one-way ANOVA. Representative blots are shown beneath the graphs. B) Hep G2 immunoprecipitated IRS-1 is acetylated by p300 in the presence of Acetyl CoA (B). This acetylation does not change in a statistically significant manner upon the addition of S6K-1.

Preliminary results to future research

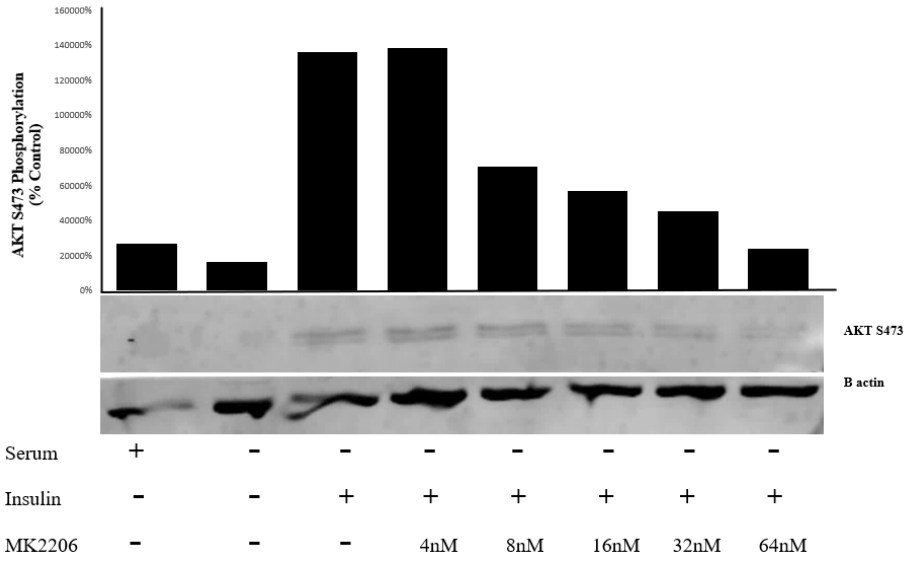

Figure 13. MK2206 inhibition AKT S473 in a dose dependent manner.

Treatment with MK2206 for 24hrs led to a dose dependent decrease in AKT phosphorylation. Insulin stimulated HepGg2 that were treated with MK2206 exhibited dose dependent decrease in AKT S473 phosphorylation compared cells with no treatment.

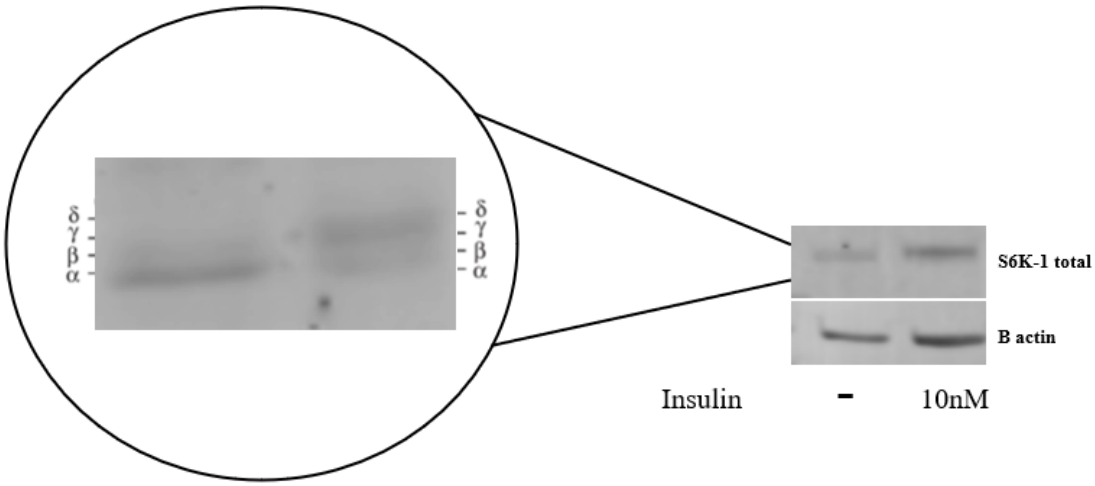

Figure 14. p70 S6K-1 band shift as a representation of phosphorylation. HepG2 cells were treated with 10nM insulin for 1hr which was adequate enough to stimulate the phosphorylation of p70 S6K-1.

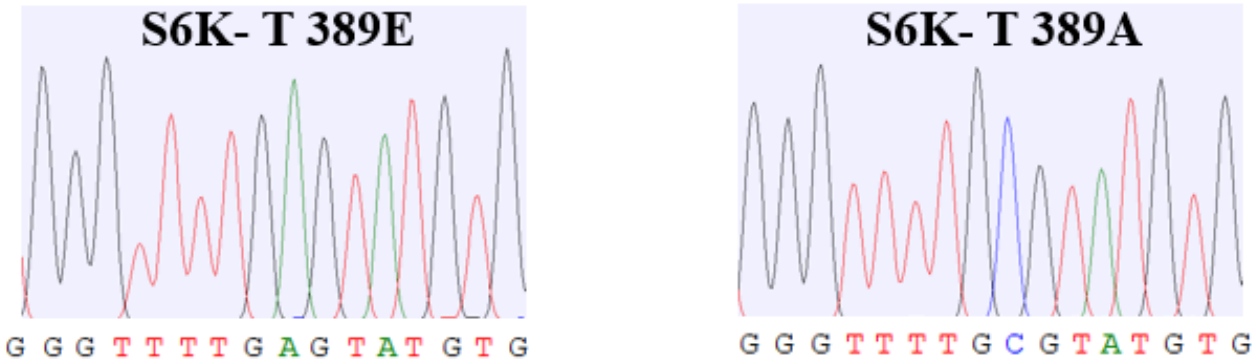

Figure 15. p70 S6K-1 mutant constructs

Site directed mutagenesis was successful conducted to construct a constitutively inactive and constitutively active p70 S6K-1. The wild type T389 was mutated into either a glutamic acid (GAG) to depict a p70 S6K-1 that cannot be turned off. orThe wild type T389 was also mutated to an alanine (GCG) to depict a p70 S6K-1 construct that cannot be turned on. Sequencing results reveal that the constructs were adequately mutated.

Discussion

p70 S6K-1 directly phosphorylates S1101 but only promotes the phosphorylation of S636/639 and S312

Previous studies and our current study how shown that PF4708671 can successfully decreased RPS6 phosphorylation by selectively inhibiting p70 S6K-1. This was done to acquire the required dosage to effectively inhibit p70 S6K-1 for the assessment of IRS phosphorylation sites S312, S1101, and S636/639. The expectation was that PF4708671 would also lead to the reduction in S312, 636/639 and S1101 would have reduced phosphorylation upon treatment with PF4708671in insulin stimulated HepG2 cells. Our The present cell culture findings corroborate what previous studies have shown, that p70 S6K-1 is required for the phosphorylation of IRS-1 S312, S636/639, and S1101. While the inhibition of p70 S6K-1 with 20μM of PF4708671 for 16hrs led to a decrease in the phosphorylation of S1101, S312, and S636, a 3hr treatment with 10nM insulin stimulation did not affect the phosphorylation status of S1101, S312, and S636, thus questioning whether p70 S6K-1 directly phosphorylates these serine sites.

p70 S6K-1 indirectly regulates S312 phosphorylation.

Studies have shown that stimulation by both amino acids and insulin leads to the phosphorylation of S312 (Rui et al., 2001; Greene et al., 2003; Carlson et al., 2004). In addition, amino acid stimulation seems to be the most potent stimulant for S312 phosphorylation. This points back to the problem with excess nutrient intake and why individuals with obesity, type 2 diabetes, and insulin resistance are the most commonly found to have phosphorylation of this site.

Interestingly, nutrient availability is not the only way catalyst for S312 can be phosphorylationed. Inflammation triggered by TNF-α can lead to S312 phosphorylation by JNK alone (Aguirre et al., 2000; Hirosumi et al., 2002). The results displayed have shown that apart from insulin and inflammation stimulated S312 phosphorylation, there is basal S312 phosphorylation that is decreased with the treatment of PF4708671. As previously mentionedThis is different from what previous studies have shown which is that the, phosphorylation of S312 is usually the determinant for the development and progression of insulin resistance and consequently degradation in an insulin resistant model. Also, studies have shown that the measurement of IRS-1 (S312) phosphorylation alone does not necessarily imply causality for IRS-1 degradation. S312 phosphorylation alone was shown to be enough to display the down regulation of IRS-1 in insulin stimulated cells (Greene et al., 2003). In these aforementioned studies, the phosphorylation of S312 in an non-insulin resistant model was not taken into consideration. However, Our current data reveals that it appears, that there is basal phosphorylation that can be decreased by the down regulation of p70 S6K-1 and that 10nM of insulin is not sufficient to create an insulin resistant model as was observed by the lack of increase in the phosphorylation of S1101, S312, and S636/639 with insulin stimulation (Figure 13-15). While it is evident that p70 S6K-1 activity can be significantly increased by 10nM of insulin as observed by an increase in RPS6 S235/236 phosphorylation, such a concentration of insulin is not enough to mimic insulin resistance in HepG2 cells. Taken together it is the increase in the phosphorylation of S312 from basal levels rather than merely identifying the presence of S312 phosphorylation in a basal state that could be is indicative of the down regulationdevelopment and progression of insulin resistance leading up to the degradation of IRS-1. mTORC1 may be the kinase responsible for insulin stimulated S312 phosphorylation in

an insulin resistant model, while S6K-1 is the key kinase responsible for basal phosphorylation of IRS-1 S312.-As a result, S312 should not be regarded as the sole site that determines whether a model is insulin resistant or not. , Additionally, and while JNK is the responsible for inflammation stimulated S312 phosphorylation. ~~As a result, S312 should not be regarded as the sole site that determines whether a model is insulin resistant or not.~~

One Some studies study implyied that p70 S6K-1 phosphorylates S312, while anothers denyied its direct involvement using a kinase assay. This makes sense as other studies have already shown that other kinases such as mTORC1 and JNK (inflammation stimulated phosphorylation) phosphorylate S312. Our results do not show any direct relationship between S312 phosphorylation and p70 S6K-1 but rather they show an indirect influence in the regulation of S312 by observing the absence of p70 S6K-1, which leads to a decrease in basal S312 phosphorylation. All things considered; it could be that p70 S6Kk-1 may phosphorylate a kinase that is responsible for directly phosphorylating IRS-1 S312. This is corroborated by one study that established that p70 S6K-1 prefers to phosphorylates a serine or threonine preceded by arginine at positions -5 and -3 (RXRXX S/T sites), such as IRS-1 S307, S527, and S1101, in target proteins as opposed to serine or threonine sites followed by proline residues, such as IRS-1 S312, S616, and S636/639.[turnover paper]. All in all, further research will need to be conducted to reveal exactly how S6K-1 influences S312 phosphorylation.

p70 S6K-1 indirectly regulates S636/639 phosphorylation.

Increased amino acid availability inhibits insulin stimulated glucose regulation in human skeletal muscles via the phosphorylation of IRS-1 S312 and S636/639. As a result, there is decreased

activation of PI3K (Tremblay et al., 2005). In addition, other studies have previously shown that hyperinsulinemia alone can increase the phosphorylation of IRS-1 S636/639. However, in most studies, S6K-1 is observed in supraphysiological insulin concentrations as high as 100nM or as low as 20nM in 3T3-L1 mouse embryo. In this study, the concentration of insulin used to observe p70 S6K-1 and its phosphorylation of IRS-1 is 10nM in human liver HepG2 cells meaning that this phosphorylation may be dose, tissue, and organism dependent. Not only is insulin stimulated S636/639 phosphorylation dependent on the aforementioned factors, but it also is kinase dependent. For example, when mTORC1, or ERK1 phosphorylate S636/639, IRS-1 is negatively regulated, whereas phosphorylation by ROCK1 leads to a positive regulation of IRS-1, which promotes insulin sensitivity. Therefore, the phosphorylation of S312 does not necessary mean that IRS-1 will be down regulated since its phosphorylation is dependent on specific conditions and treatment with specific kinases. For example, ERK1 will only phosphorylate IRS-1 S636/639 in an insulin resistant model .[50, 51] . It appears that p70 S6K-1 is similar to mTORC1 and ERK1 in that it negatively regulates IRS-1, however the difference is that it does not directly phosphorylate S636/639 as is revealed by the kinase assay performed in the current research. This is not unusual as JNK is another kinase that also negatively regulates IRS-1 by indirectly promoting S636/639 phosphorylation. Since selectively inhibiting p70 S6K-1 does prevent the basal phosphorylation of S636/639, perhaps p70 S6K-1 regulates another kinase that is responsible for the phosphorylation of IRS-1 S636/639.

However, understanding the phosphorylation of this site is already complex enough especially since the site can be phosphorylated to either negatively or positively regulate IRS-1. Look for an example of a completely different site in a different pathway that can be negative and positively regulated on the same site. What makes IRS-1 S636/639 regulation even more complex is that it

has been shown that the phosphorylation of S270, a site most proximal to the PTB domain, by p70 S6K-1 can prime the phosphorylation of S636/639 among two other sites. However, what the study elucidated is that after the phosphorylation of S270, p70 S6K-1 directly phosphorylates S636/639, which our kinase assay revealed does not happen. This makes sense as p70 S6K-1 does not usually phosphorylate serine or threonine sites followed by proline residues.[Turnover paper] We suggest that p70 S6K-1 may prime the phosphorylation of S636/639 by a different kinase. Therefore, perhaps the p70 S6K-1 dependent negative regulation of IRS-1 via S636/639 phosphorylation is a multistep process that is dependent on both stimulus and targeting kinase. Further research will need to be conducted to fully understand the process by which S636/639 is phosphorylated.

p70 S6K-1 directly regulates S1101 phosphorylation.

Unlike S312 and S636/639, S1101 has been shown to be directly phosphorylated by p70 S6K-1. While studies have shown this direct relationship, we have yet to see if there is any basal phosphorylation that is diminished by the inhibition of insulin stimulated p70 S6K-1 by PF4708671. A kinase assay corroborated that p70 S6K-1 directly phosphorylates S1101, but not S636. Treatment with 20 μM of PF4708671 in insulin stimulated HepG2 diminished the phosphorylation of S1101, S312, and S636/639. Taken together this is indicative of the ability of p70 S6K-1 to directly impact S1101 and indirectly affect the phosphorylation of S636 and S312 which is consistent with previously published data. In addition, S1101 is one of the preferred RxRxxS/T sites that p70 S6K-1 phosphorylates, thus validating the reason why S1101 is directly phosphorylated unlike S312 and S636/639.

Inhibition of p70 S6K-1 decreases basal phosphorylation of S1101

One study has shown that not only does p70 S6K-1 directly phosphorylate S1101, but that S1101 phosphorylation is also increased in a 100nM insulin stimulated L6 rat myoblasts, Fao rat hepatocytes, and 3T3-L1 mouse embryos. Interestingly, in both the L6 and 3T3-L1 cells there was baseline phosphorylation of S1101 before stimulation with insulin. Then, after treatment with 100nM of insulin there was a slight increase in S1101 phosphorylation in L6 and 3T3-L1 cells. Moreover, supplementing with amino acids to insulin stimulated cells led to a significant increase in S1101 phosphorylation . (58). On the other hand, another study revealed that there is no change in the phosphorylation status of S1101 before and after insulin treatment in insulin sensitive human lean skeletal muscles. In fact, S636/639, is also among the sites that also displays no change in phosphorylation in an insulin sensitive model. (59). Our data corroborates this finding, as there was no change in S1101 phosphorylation in insulin stimulated human HepG2 cells. This is quite interesting considering p70 S6K-1 activity is increased with insulin stimulation especially when simultaneously supplemented with amino acids. We The current work elucidate suggests that the amount of insulin added, the organism, the supplementation of amino acids, and whether the model is insulin resistant or insulin sensitive impacts whether or not S1101 will be phosphorylated. Therefore, insulin can lead to an increase in S1101 from its basal phosphorylation. Moreover, whether p70 S6K-1 was responsible for the increase in S1101 phosphorylation or not, its absence negatively impacts S1101 phosphorylation.

It appears that S1101 may be part of a multistep process similar to what was discussed in S636/639 phosphorylation. It has been shown that mutating S270 to A270 prevented p70 S6K-1 from interacting with IRS-1 thus preventing S1101 from being phosphorylated by p70 S6K-1

itself. It would be interesting to see if there is no change in the phosphorylation of S270 in an insulin sensitive model after treatment with insulin since previous studies focus mostly on insulin resistant models. It was only in 2017 that the inhibition of p300 was tied to improved insulin signaling. p300 was shown to acetylate specific sites on IRS-1, leading to insulin resistance.

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Inhibiting p300 from acetylating IRS-1 leads to a decreased in the phosphorylation of IRS-1 S312 and S1101 without a decrease in p70 S6K-1., but not S636.

It was only in 2017 that the inhibition of p300 was tied to improved insulin signaling. p300 was shown to acetylate specific sites on IRS-1, leading to insulin resistance. A recent study revealed that inhibition of p300 by C646 increased insulin sensitivity by promoting the interaction between the IR and IRS-1. Interestingly, out results reveal that C646 also reduces the negative regulation of IRS-1 elicited by the phosphorylation of IRS-1 at S312 and S1101, but not S636. Thus, not only is C646 promoting the tyrosine phosphorylation of IRS-1 by increasing the IR and IRS-1 interaction, but the inhibition of the negative regulation of IRS-1 also promote the tyrosine phosphorylation. Previous studies indicated that C646 also led to glucose tolerance in mice that were fed a HFD and increased PI3K activity in Hepa 1-6 cells. The effect p300 has on IRS-1 may be tissue specific. A HFD induces p300 activity in liver tissue but not adipose and muscle tissue. In addition, C646 augmented AKT phosphorylation exclusively in liver tissue but not muscle or adipose tissue by increasing the association of IRS-1 to the β subunit of IR. It is not known why the activity of p300 on IRS-1 is tissue specific.

Therefore, the inability of p300 to acetylate IRS-1 has a negative effect on the phosphorylation of S312 and S1101, but the same cannot be said for S636/639 phosphorylation. The lack of an effect on S636/639 phosphorylation by p300 inhibition may be because when these serine residues are phosphorylated, it can lead to either a positive or negative regulation of IRS-1 different kinases.[50] . For example, when mTORC1or ERK1 phosphorylate S636/639, IRS-1 is negatively regulated, whereas phosphorylation by ROCK1, a suppressor or inflammatory cell migration, leads to a positive regulation of IRS-1, thus promoting insulin sensitivity.[50] F.Further investigation is necessary to understand S636/639 phosphorylation by these different kinases specifically when cells have been treated with C646, especially because p70 S6K-1 is not the direct target kinase as was shown by the kinas assay. Taken together, it appears that S636/639 phosphorylation is promoted by but not performed by p70 S6K-1, while the in ability of p300 to acetylate IRS-1 does not affect S636/639 phosphorylation status.

The Acetylation of IRS-1 by p300 enhances IRS-1 S1101 phosphorylation by p70 S6K-1.

On the other hand, not only Our current study has revealed that does the inhibition nability of p300, towhich prevents phosphorylate IRS-1 acetyla,tion leads to decreased phosphorylation of S1101., In addition, the presence of p300 during the phosphorylation enhances the S1101 phosphorylation by S6K-1. While it is not known exactly how this enhancement occurs, such a regulation has been seen before where one posttranslational modification influences another. Studies have shown that the phosphorylation of H3 on S10 can prime its acetylation at K14 by KAT Gnc5. When protein phosphatase 1 (PP1) is overexpressed, there is a decrease in the acetylation of K14. While the phosphorylation of S1101 is not dependent on acetylation, acetylation does augment S1101 phosphorylation in a kinase assay.

Studies have shown that p300 does acetylate p70 S6K-1 at K516, the outcome of this acetylation has not been explored. To ensure that the Therefore, an assumption could be made that the effects of C646 on IRS-1 serine sites are not due to a negative regulation of p70 S6K-1 by p300., However, in our study, we were able to look at the effect C646 has on RPS6 activity was assessed. Our study shows that the inhibition of p300 via C646 dramatically increases RPS6 phosphorylation compared to only with insulin stimulation. Consequently, this means an increase in the activity of its upstream activator p70 S6K-1. This suggests that p300 activity usually diminishes the phosphorylation of RPS6. Therefore, C646 increases RPS6 phosphorylation while effectively decreasing IRS-1 S312 and S1101 phosphorylation in insulin stimulated HepG2 cells. As previously stated, C646 increases insulin sensitivity by increasing IR and IRS-1 interaction, while the current study demonstrates that the negative regulation of IRS-1 is also inhibited while allowing the insulin signaling to still occur. Further research will need to be conducted to understand the exact mechanism that leads to decreased S312 and S1101 phosphorylation but not S636/639 when cells are treated with C646.

Conclusions and Future perspectives

Insulin resistance is commonly characterized as a precursor to type 2 diabetes. However, insulin resistance is a risk factor to other metabolic diseases such as metabolic syndrome, a multifactorial condition described by a cluster of cardiometabolic risk factors: abdominal obesity, high blood pressure, high triglyceride levels, low high density lipoprotein (HDL) cholesterol, and high blood sugar. Metabolic syndrome is also called insulin resistance syndrome or syndrome x. Previous studies have studied examined the dysregulation of IRS-1 in models that assess either obesity, HFD, or insulin resistance. All these aforementioned outcomes are the main components of the development of metabolic syndrome. The dysregulation of IRS-1 may mostly be studied in insulin resistant models; however, it can also be seen in obesity and HFD. And in these studies, C646 has been shown as a possible treatment for all 3. The mechanism by which these three risk factors lead to the dysregulation of IRS-1 is the same. It involves but is not limited to increased acetylation and serine phosphorylation on IRS-1. Since metabolic syndrome affects 100 million Americans, and since its development leads to complications such as coronary heart disease (CHD), stroke, and type 2 diabetes, research in new treatment plans and interventions is important. So far one of the most cost-effective treatment is reduced caloric intake which directly impacts blood sugar, amino acid, and triglyceride levels. However, in America, treatment that involves change in lifestyle choices is usually less preferred than taking medications. Therefore, C646 could be a potential treatment for metabolic syndrome, however, p300 acetylates more than 80 known substrates and interacts with more than 411 proteins, thus its inhibition by C646 may be more detrimental than helpful. P300 has been identified as pertinent for the regulation of many genes, it is also known to be pivotal not only in the regulation of DNA transcription, but also in DNA repair, development, apoptosis, and innate

immune response. [pdf file on iPad] p300 can either promote or repress transcription depending on the target gene.

There needs to be a balance between the acetylation and deacetylation of proteins. For example, p300 activates transcription and decondenses chromatin by acetylating H3K14 and Sirtuin (Sirt) 2, a NAD-dependent deacetylase, removes this acetylation. In this case, the absence of p300 would prevent this acetylation. There are many acetyltransferases and one might think they could pick up the slackcompensate for p300 in its absence and in the acetylateion of H3K14 in the absence of p300. However, whilech there are other acetyltransferases, the absence of p300 has been shown to be detrimental causing issues such as neurodegenerative diseases, cancers, disorder of sex development (DSD) or even death in embryos lacking p300.[pdffromipad] Therefore, the complete absence of p300 is not ideal. In order for C646 treatment to effective, the treatment could either be dose dependent or p300 could be regulated in order to prevent its localization to the cell membrane where IRS-1 and the IR are expressed found during insulin signaling. So far at a dose of 10-20µM, C646 leads to increased IRS-1 and IRS interaction, increased tyrosine phosphorylation, and decreased serine phosphorylation. However, a dose of 10-20µM is still way is too high and thus not safeto remain be physiologically relevant. It is not fully understood how p300 shuttles from the nucleus to the cytoplasm, but it is presumed that p300 interacts with a protein that is going to be localized in the cytoplasm. On the other hand, its return to the nucleus is due to its nuclear localization signal (NLS), which also allows it to shuttle other proteins with it into the nucleus.

One study revealed that administering a HFD led to an increase in LPS which led to an increase in p300. This induction did not change p300 mRNA levels but increased p300 protein levels by reducing the ubiquitination and degradation. Interestingly, p300 is mostly a nuclear protein,

however, after LPS induction, p300 cytoplasmic protein levels increase greatly. LPS induction leads to increase in active X-box binding protein 1 (XBP1), a transcription factor responsible for the regulation of genes that are involved in cellular stress response and immune system function. XBP1 is usually activated with endoplasmic reticulum (ER) stress that can be triggered by a variety of stimuli, including LPSof which LPS induces. Therefore, when a HFD is administered there is ER stress which then leads to the activation of XBP1 which in turn results in increased cytoplasmic p300. Interestingly, the absence of XPB1 in mice that are fed a HFD does not lead to an increase in cytoplasmic p300. Exactly how XBP1 is important for the expression of p300 in the cytoplasm is unknown. However, we elucidate thatit is possible that either XBP1 is responsible for keeping p300 in the cytoplasm whereas and a different, as yet unidentified protein, shuttles p300 into cytoplasm is unknown., Alternatively, or XBP1 both could brings p300 into the cytoplasm and keeps its there thus leading to the acetylation of cytoplasmic proteins. Either way the presence of p300 in the cytoplasm allows it to be available to acetylate IRS-1. And since the presence of p300 enhances the phosphorylation of IRS-1 S1101, disrupting its transport to the cytoplasm could be a potential treatment.

Interestingly, XBP1 mRNA make two proteins, XBP1U and XBP1. While XBP1 only has an NLS, XBP1U has both an NLS and a nuclear export signal (NES). Thus, XBP1U is responsible for the cytoplasmic localization of XBP1. This complex maybe exactly how p300 makes its way to the cytoplasm.

Another, perspective involves the phosphorylation of p300. P300 may go through a posttranslational modification that leads to its export from the nucleus to the cytoplasm. There are some posttranslational modifications of p300 whose end result is not fully understood. Such an example includes the phosphorylation of p300 at S2271, S2279, S2291, and S2315 by

mTORC1. Future research will look into the phosphorylation of p300 by mTORROC1 and how that is connected to the acetylation of IRS-1 by p300. We need to look into whether phosphorylation of p300 by We elucidateThe current work suggests that when that insulin signaling is taking place that mTORC1 has any effect on the ability of p300 to acetylate IRS-1. phosphorylates p300 which will probably have already been shuttled to the cytoplasm probably by XBP1-XBP1U complex, as a negative feedback in instances where the stimulation began due to insulin resistance or LPS induction. Thus, we concluded that the dysregulation of IRS-1 that is stimulated by a high caloric intake or HFD, whether insulin, aminnio acid, or LPS stimulated, leads to down regulation of IRS-1 as has been shown by previous studies can be prevented through the inhibition of both acetylation and serine phosphorylation of IRS-1, which can both be reversed by the administration of C646. PF4708671 still remains as a potential treatment for the dysregulation of IRS-1 caused by insulin resistance, however, further research will need to be done to confirm whether it inhibition serine phosphorylation will also haveas any effect on the acetylation of IRS-1. Our HAT assay from immunoprecipitated IRS-1 revealed that the presence of active p70 S6K-1 does not change the acetylation status of IRS-1 even though the presence of active p300 enhances the phosphorylation of IRS-1.

The regulation of IRS-1 through acetylation is fairly new, thus further research in this area will be valuable in the are of metabolic diseases. The crosstalk the phosphorylation and acetylation of IRS has been revealed in this paper and further research is necessary to further understand exactly how p300 acetylation of IRS-1 enhances the phosphorylation of IRS-1 by p70 S6K-1. Known this fact may be exactly what is needed to specifically target p300 and IRS-1 interaction without disrupting the other pathways in which p300 is involved.

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