# REGULATION OF AKT ISOFORMS-AS160-GLUT4 AXIS BY PHLPP AND PP1 ISOFORMS AFFECTING NEURONAL INSULIN SIGNALING AND RESISTANCE.

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Insulin signaling is a circuitous network of molecules that brings about contradistinctive ramifications in the body. Any hindrance in this pathway paves the way for insulin resistance like phenotype leading to a variety of diseases like diabetes, obesity etc. Out of all potential targets, one of the most significant, considering its pleiotropic roles in metabolism and disease, is Akt. Akt's multifarious role in insulin signaling is very well known and has long been established, the latest aspect being isoform specificity. All three isoforms have been extensively studied in all insulin responsive tissues, their relative tissue distribution investigated (Akt1 expression varies across tissues, Akt2 is highly expressed in insulin-responsive tissues and Akt3 is expressed in brain and testis), and their specific and non-specific roles analyzed (Akt1 plays a predominant role in cellular growth and angiogenesis, Akt2 plays a role in glucose homeostasis, and Akt3 plays a role in neuronal development). However, keeping in mind that any deregulation of Akt and its isoforms is the hallmark of insulin resistance paving way for many diseases, further studies are needed. Apart from insulin sensitive tissues, Akt isoforms have also been reported to regulate neuronal development, neuronal maturation and outgrowth, polarization and axon branching, synapse formation and neuronal survival. Thus, many *in vitro* and *in vivo* studies have been conducted in human/rodents to understand their relationship. Even following years of extensive studies, there remains a window about their individual roles, possible interplay and the molecular mechanism underlying differential effects amongst the isoforms. Furthermore, the isoforms have shown potential compensation, and thus designation of differential roles has been difficult. Thus, in order to explicate further on role of Akt isoforms, in depth studies have to be conducted. These may assist in simplifying complexity of insulin signaling and resistance in neuronal cells.

Therefore, in the present study, attempts were made to study the role of Akt isoforms in neuronal insulin signaling and resistance.

To attain this, we utilized neuronal cells like Neuro-2a and HT22, their insulin-resistant versions, generated in our laboratory and insulin-resistant diabetic mice whole brain tissues to study Akt isoform interplay. We elucidated this by isoform specific silencing [single (silencing of one isoform at a time) and double (silencing of two isoforms at a time) as well as isoform specific over-expression, and elucidated how Akt isoforms regulate AS160 and ultimately glucose uptake in insulin sensitive and -resistant condition.

Our study is the first to report isoform specific role of all Akt isoforms in regulating neuronal insulin-signaling and -resistance. We find that in neuronal cells (a) all Akt isoforms regulate AS160 activation and glucose uptake; Akt2 play a predominant role, with Akt1 and Akt3 playing significant role as well; (b) Activation of all isoforms decreased differentially under insulin-resistance with Akt2 being affected most, followed by Akt3 and Akt1; (c) Insulin-resistance is reversed by over-expression of any isoform of Akt, but predominantly by Akt2; (d) Insulin-dependent translocation on plasma membrane determines isoform specificity with Akt2 translocating the most, followed by Akt3 and then Akt1; (e) Insulin-resistance hampered this insulin-dependent translocation of all Akt isoforms to plasma membrane, irrespective of isoform; (f) Akt3, despite being neuron specific isoform contributed substantially to AS160 regulation, neuronal glucose uptake, and insulin-resistance. However, Akt2 was still the predominant isoform in regulating all the above functions. This points to a novel, differential yet compensatory interplay of all Akt isoforms in neuronal insulin signaling and insulin-resistance.

Having established the differential role of Akt isoforms in regulating neuronal insulin signaling and insulin resistance, our next step was to study possible upstream regulation by phosphatases. PHLPP (PH domain Leucine-rich repeat Protein Phosphatase) has previously been studied as primary regulators of Akt isoforms. Majority of the studies establish that PHLPP1 regulates Akt2

and Akt3, and PHLPP2 regulates Akt1 and Akt3 in cancer cells. However, there are paradoxical data regarding PHLPP isoform specific regulation of Akt isoforms as well, with PHLPP1 regulating Akt1 and Akt2, with no contribution of PHLPP2 in cardiomyocytes. Thus, PHLPP isoform mediated regulation of Akt isoforms is complex and could be tissue specific. Defects in PHLPP like single-nucleotide polymorphism (SNP), perturbed mRNA and protein expression under normal versus pathological conditions in insulin sensitive tissues have also been reported, adding another dimension to the PHLPP isoform specific function. In brain, PHLPP has been reported to various roles like, but the role of PHLPP in neuronal insulin signaling and insulin resistance is largely missing. Thus, role of individual PHLPP isoforms, their regulation of Akt isoforms and possibly other downstream processes was an imminent question.

In attain this, we used neuronal cells, insulin sensitive and insulin resistant, like Neuro-2A and SH-SY5Y and insulin resistant diabetic mice whole brain lysates to study role of PHLPP in regulating neuronal insulin signaling and insulin resistance. We test this by PHLPP isoform specific silencing as well as over-expression and study its effect on Akt isoforms, AS160 and neuronal glucose uptake under insulin sensitive and insulin resistant condition to elucidate isoform specific functionality. We probed further to study the role of scaffold protein, Scribble, in determining the reason behind the isoform specificity.

Our study is the first to report isoform specific role of both PHLPP isoforms in regulating neuronal insulin signaling and resistance. We find the following: (a) elevated expression of both PHLPP1 and PHLPP2 in insulin resistant neuronal cells and high-fat-diet mice whole brain lysate; (b) PHLPP1 regulates serine phosphorylation of Akt2 and Akt3, and PHLPP2 regulates serine phosphorylation of Akt1 and Akt3 in neuronal cell insulin signaling; (c) PHLPP1 regulates serine phosphorylation of Akt2 and Akt3, and PHLPP2 regulates serine phosphorylation of Akt1 and

Akt3 in insulin resistant neuronal cells; however, first time observed in any resistant cell; (d) both PHLPP isoforms regulate three Akt isoforms, extending the regulation to AS160, reported for the first time in any tissue system; (e) both the isoforms of PHLPP regulate glucose uptake in insulin sensitive and insulin resistant neuronal cells (f) PHLPP isoform specificity is mediated by Scribble, which mediates cellular localization of isoforms, regulating neuronal insulin signaling; (g) a novel role of Scribble in regulating GLUT4 translocation and glucose uptake in neuronal cells is being reported for the first time in any cellular system. All data points to a differential and independent role of PHLPP isoforms in neuronal insulin signaling and insulin resistance.

Having established role of one prominent phosphatase in regulating Akt isoforms and having found interesting results, we proceeded to test role of PP1 phosphatase in neuronal insulin signaling and resistance as well. PP1 phosphatases are composed of heterodimers of catalytic and regulatory subunits which provide them specific cellular compartmentalization and substrate specificity. The mammalian genome encodes three catalytic subunits of PP1 namely PP1α, PP1β, PP1γ and > 200 regulatory subunits. Previously, PP1 specific inhibition studies have established Akt as its direct target in insulin signaling. However, these studies are tissue specific, with interaction of different catalytic and regulatory subunits defining downstream signaling. In brain, PP1α and PP1γ are expressed predominantly, with varying expression in different regions of the brain. Even with high expression and differential expression pattern, and established an established role in Akt regulation, possible role of PP1 isoforms in neuronal insulin signaling has not been studied. Furthermore, how PP1 isoforms regulate Akt isoforms in any tissue system has not been addressed yet. Thus, role of individual PP1 isoforms, their regulation of Akt isoforms and possibly other downstream processes was an impending question.

In attain this, we used neuronal cells, insulin sensitive and insulin resistant, like Neuro-2A and SH-SY5Y and insulin resistant diabetic mice whole brain lysates to study role of PP1 in regulating neuronal insulin signaling and insulin resistance. We test this by PP1 isoform specific silencing as and study its effect on Akt isoforms, AS160 and neuronal glucose uptake under insulin sensitive and insulin resistant condition to elucidate isoform specific functionality.

Our study is the first to report isoform specific role of both PP1 isoforms in regulating neuronal insulin signaling and resistance. We find the following: (a) no change in expression of either PP1α or PP1γ in insulin resistant neuronal cells and high-fat-diet mice whole brain lysate; (b) PP1α does not regulate Akt isoforms in neuronal insulin signaling and resistant condition; (c) PP1γ regulates only Akt2 (but not Akt1 or Akt3) in neuronal insulin signaling and resistant condition.

These observations are significant and insightful in understanding insulin signaling and insulin resistance. Further, this study in neuronal insulin resistance, which is one of the hallmarks of several neurodegenerative diseases, may help in taking a step forward in solving problems associated with a Type 3 diabetes, diabetes complications and neurodegenerative disorders.