TrkA and Insulin Receptor in Streptozotocin Induced Diabetes Rat Brain

by

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A thesis submitted to the Graduate Faculty of Auburn University
Auburn University
in partial fulfillment of the
requirement of the Degree of
Master of Science

Auburn, Alabama
May 8, 2016

Keywords: Insulin receptor (IR), insulin receptor substrate-1 (IRS-1), Tropomysoin receptor kinase A (TrkA), nerve growth factor (NGF), and streptozotocin (STZ)

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Abstract

Abnormal blood glucose homeostasis and subsequent hyperglycemia, due to insufficient insulin production, is characteristic of type 1 diabetes mellitus. Neuronal cells are classified as insulin insensitive, thereby insulin is incapable of increasing glucose uptake in neurons. Tropomyosin receptor kinase A (TrkA) is a transmembrane receptor for nerve growth factor (NGF), which is responsible for regulating neuronal survival and differentiation. We have previously shown in our lab that NGF or insulin elicits TrkA to complex with insulin receptor (IR) and insulin receptor substrate -1 (IRS-1), and phosphorylation of these proteins requires a functional TrkA kinase in PC12 cells. It was also shown that a functional TrkA kinase is necessary for Akt activation in PC12 cells. Following these findings, investigation into the activity of TrkA in the diabetic rat brain, created by streptozotocin (STZ) administration, have shown a decrease in its phosphorylation and increase in nitrosylation as compared to control rat brain samples. Further experimentation showed the interaction of TrkA with IR and IRS-1 as well as the tyrosine phosphorylation of these signaling proteins is decreased in STZ rat brain samples. Lastly, STZ rat brain samples had decreased phosphorylation of Akt as compared to control rat brain samples. Therefore, functional TrkA is necessary for proper functioning of the insulin signaling

proteins IR, IRS-1, and Akt in neuronal cells, and disruption of its functioning can be seen in neuronal cells of the type 1 diabetic rat model.

Acknowledgements

Foremost, I would like to extend my deep gratitude to Dr. Ramesh Jeganathan for his unending patience, motivation, and knowing guidance as I completed my research. I am thankful for his direction, which has helped me gain confidence in my work, within and outside of the research lab. I would like to thank Dr. Kevin Huggins and Dr. Robert Judd for their support of my research and future through sitting on my committee. I would also like to show my sincere appreciation for Dr. Geetha Thangiah for her valuable contributions to my research project. I am grateful to have conducted my research surrounded by the professors in the Department of Nutrition whose passion for nutrition and students' success goes far beyond the classroom walls. I would like to thank my lab group members, Chen, Shraddha, and Talia, for their constant motivation and friendship throughout my time in the lab. Lastly, I am eternally grateful for my parents constant, loving support of my endeavors.

Reference Style

This document is referenced using the citation style of the journal Nature.

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CHAPTER 1: INTRODUCTION

Chronic diseases are increasing dramatically on a global scale with the largest increase in developed countries.[1,23] Worldwide, there has been a transition in nutritional state for many countries due to economic growth and revolutionized practices for food production.[2] Globally, there is an increase in nutritional goods and services based on Western practices. This results in increased intake of processed foods, typical to the Western diet, and a decreased consumption of traditional meals.[4] The expansion of industrialization and increasing prevalence of the Western diet globally have together led to ever-increasing rates of obesity and diabetes world-wide.[5] As reported by the International Diabetes Federation, 382 million people are living with diabetes and the prevalence is projected to increase to 592 million by the year 2035.[6]

This increasing prevalence of diabetes and the complications associated with the disease has gained the attention of many individuals internationally.[3,7] It is estimated that 6.8% of global deaths in individuals 20-79 years of age were due to diabetes in 2010.[8] The risk of death of an individual from any age group doubles with a diagnosis of diabetes when compared with healthy, age-matched individuals.[9] There are numerous side effects of unmanaged diabetes, making early diagnosis critical. Some of the

more serious side effects include retinopathy, lower limb amputation, cardiovascular disease, and renal failure.[10,11] These complications associated with un-controlled diabetes result in disability, impaired quality of life, and are potentially life-threatening.[11] The damaging complications of diabetes and ever-increasing rates of prevalence support the need to advance understanding of diabetes to mitigate the disease and its complexities.

Contrary to previous association with an affluent population, diabetes is now prevalent in low-income populations.[6] It is estimated that 80% of patients diagnosed with diabetes live in low to middle income countries and 84% are estimated to remain undiagnosed.[6] Campaigns to raise awareness about diabetes are essential to combat the growing global prevalence of the disease due to its indiscriminate symptoms and inadequate healthcare systems in most countries.[1,4]

The seventh leading cause of death in the United States is type 2 diabetes mellitus (T2DM).[12] As reported by the American Diabetes Association, 8.3% of Americans have been diagnosed with diabetes while 35% age 20 or older have pre-diabetes.[13] Estimates of future diabetes prevalence indicate an expansion of those diseased by 25-58% by the year 2050.[14] In the United States, the medical cost of diabetes reached an estimated $245 billion in 2012 according to the American Diabetes

Association.[15] Apart from weight and lifestyle, ethnicity has been shown to play a role in diabetes prevalence. The National Health Interview Survey found that diabetes prevalence for Hispanics and non-Hispanic black ethnicities is 12% as compared to only 7.4% among non-Hispanic whites.[16] In the United States, diabetes is the leading cause of kidney failure, neuropathy, non-traumatic lower limb amputation, and blindness.[13]

Alabama ranked 5th in nation in terms of diabetes prevalence in 2010, with an 11.1% prevalence rate.[17] In the state of Alabama, the challenging health disparities have been primary factors impeding the process of reducing diabetes prevalence rates.[17] Diabetes interventions are further obstructed in rural areas of Alabama due to a reported lack of local healthcare facilities and an adequate amount of healthcare professionals to perform the necessary diabetes checks.[17]

Type 1 diabetes mellitus (T1DM) accounts for 5-10% of diabetes diagnoses in the U.S.[16] Destruction of the insulin producing pancreatic β-cells, lack of insulin in the bloodstream, and subsequent hyperglycemia are characteristic of a T1DM diagnosis.[18,19] When left untreated, T1DM can result in ketouria, polydipsia, polyphagia, wasting, and ketoacidosis.[10,20] The treatment of T1DM is though exogenous insulin therapy, commonly through an insulin pump.[20,21]

T2DM makes up the remaining 90-95% of diabetes cases in the U.S. and is a multi-factorial disease.[12] The defining characteristics of T2DM are dysfunctional pancreatic β-cells and insulin resistance.[22] The hallmark of T2DM is elevated blood glucose levels (hyperglycemia).[23] Hyperglycemia is a condition that has its own set of symptoms including: polyuria, blurred vision, polyphagia, polydipsia, fatigue, impaired wound healing, and chronic infections.[24] There are two major risk factors that are associated with T2DM: obesity and insulin resistance.[25,26] Several therapies have become available to mitigate T2DM; however, diet and exercise are the first line of therapy and have been shown to prevent and even reverse T2DM.[27-29]

Insulin acts as a growth hormone to signal glucose uptake in skeletal, adipose, and hepatic tissue.[30] Insulin action is impaired in patients diagnosed with T2DM causing abnormalities in metabolism.[31,32] It has been proposed that insulin impairment, known as insulin resistance, is the underlying cause of obesity and cardiovascular disease.[33] What exactly causes insulin resistance has not been clearly defined, although evidence suggests some individuals may have defects in the insulin-signaling pathway.[32,34] An intra-cellular cascade is initiated when insulin docks to the insulin receptor on the plasma membrane causing a conformational change.[35] Insulin acts on cellular insulin receptors to initiate the translocation of glucose transporter GLUT4

to the cellular membrane.[36] The function of GLUT4 is to transport glucose into hepatic, skeletal, and adipose tissue for cellular energy homeostasis.[37] Although extensive research has been done, there still exist several gaps in understanding molecular interactions in the insulin-signaling pathway.[38]

Akt/Protein Kinase B is a cellular modulator protein in the insulin-signaling pathway.[39] Akt, along with being critical for cellular survival, serves in protein transcription, cell proliferation, nutrient metabolism, and anti-apoptotic pathways.[39,40] Akt is a kinase and modulates cell activity through the highly conserved PI3K/Akt/mTOR pathway.[40] Insulin activates Akt, which is necessary for the translocation of GLUT4 to the cell membrane.[32] Akt knockout (KO) mice have shown the importance of Akt in diabetes pathology.[41] Akt2 KO mice show severe glucose intolerance preceding β-cell dysfunction and subsequent diabetic complications.[41]

The insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) are two key players in the insulin signaling cascade. IR consists of two α subunits and two β subunits and is a highly conserved transmembrane glycoprotein.[42,43] Upon insulin binding, the IR undergoes dimerization and autophosphorylation, which activates two intracellular tyrosine kinase subunits.[42,44] These tyrosine kinases then elicit phosphorylation and

subsequent activation of IRS-1, which is required for the recruitment of GLUT4 to the cell membrane and subsequent glucose uptake.[45]

TrkA is a cell surface transmembrane receptor tyrosine kinase for nerve growth factor (NGF).[46] There are three types of Trk receptors: TrkA, TrkB, and TrkC. TrkA binds specifically to NGF while TrkB to BDNF and TrkC to neurotrophin-3.[47] Binding of NGF to TrkA causes TrkA to dimerize and autophosphorylate, which recruits downstream signaling proteins, including phospholipase C-γ1, Shc, FRS-2, and PI3K.[48-51] TrkA is polyubiquitinated, mediating its internalization into the signaling vesicles. Inside these vesicles it activates the mitogen-activated protein kinase (ERK/MAPK) that promotes cell differentiation.[52-55] Our lab recently discovered that NGF or insulin induces TrkA to form a molecular complex with IR and IRS-1.[56] The tyrosine phosphorylation of the INSR and IRS-1 requires functional TrkA kinase in PC12 cells.[56] Lastly, our lab discovered that TrkA influences insulin signaling thorough activation of Akt and Erk5, revealing an overlapping signaling mechanism between NGF/TrkA and insulin/INSR.[56]

Streptozotocin (STZ) is a widely used alkylating agent to induce pancreatic β-cell destruction and subsequent T1DM in rodents.[57] A single injection of STZ into the tail vein of the rodent can confer T1DM while injecting directly into the brain confers only insulin resistance.[58,59] STZ is

produced by the bacterium *Streptomyces achromogens* and recognizes the GLUT2 receptor abundant on the cell membrane of pancreatic β-cells.[57,60]

The goal of this study was to determine whether TrkA activity and associations with IR and IRS-1 were impaired in STZ rat brain samples. Results of this research will enhance understanding of the insulin-signaling cascade and may be implemented in future therapeutic diabetes research.

Objective and Hypothesis

Our first objective was to determine the levels of Pro-NGF, MMP-7, and NGF in the T1DM rat brain. Second, we assessed if TrkA is phosphorylated and activated in the T1DM rat brain. Third, we analyzed if TrkA interacts with the insulin receptor and IRS-1 in the T1DM rat brain. Fourth, we investigated if the phosphorylation of the insulin receptor, IRS-1, and Akt was affected in the T1DM rat brain. We hypothesized that the interaction of TrkA with key insulin signaling proteins, namely the insulin receptor and IRS-1, as well as phosphorylation of the insulin receptor, IRS-1, and Akt is impaired in the T1DM rat brain.

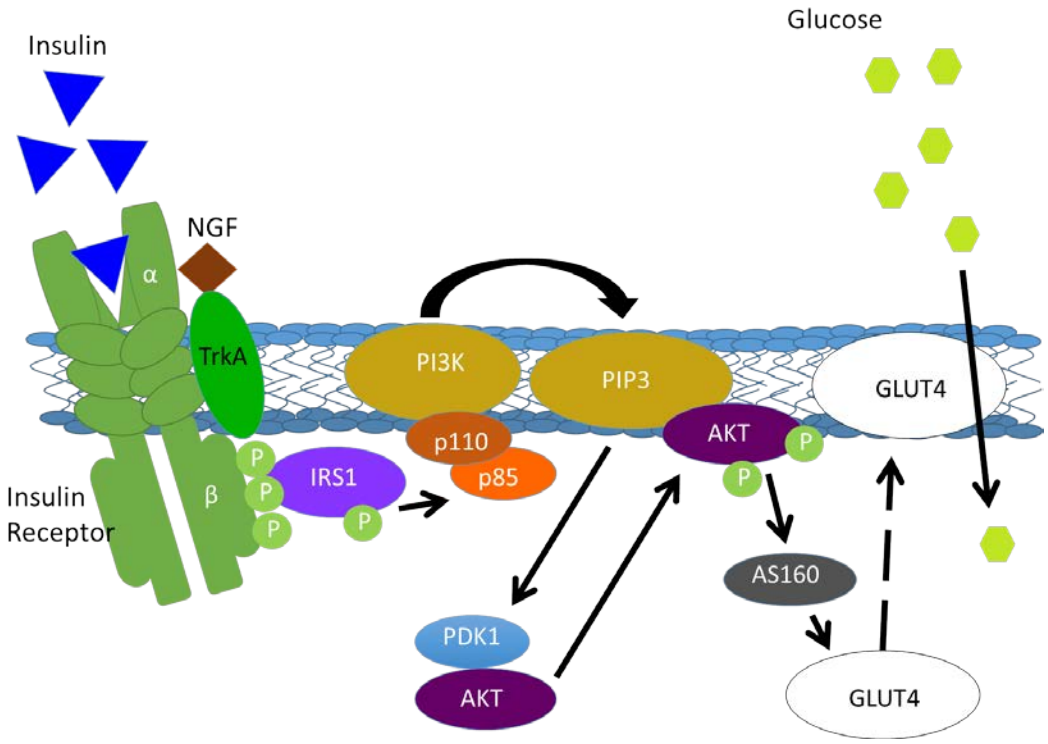

Figure 1: Schematic representation of hypothesized insulin signaling in neuronal tissue. Upon insulin and NGF stimulation TrkA forms a heterodimer with the insulin receptor and IRS-1. This complex drives phosphorylation of the insulin receptor and IRS-1, as well as leading to Akt phosphorylation.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Diabetes Mellitus

Diabetes mellitus is an array of disorders characterized by high glucose levels that can lead to kidney, eye, and nerve complications as well as an increased risk for cardiovascular disease.[61] Inadequate insulin production, nonresponsive cells to insulin, or both may cause the characteristic high glucose levels. Chronic exposure to elevated glucose could result in microvascular complications in the retina, kidney, or peripheral nerves.[62] Diabetes is categorized into four major types: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes, and few other specific forms.[63] In T1DM, pancreatic beta cells are disintegrated by an autoimmune inflammatory mechanism.[64] T2DM is associated with beta cell dysfunction and multiple levels of insulin resistance.[65] Maturity-onset diabetes of the young (MODY), is a genetic form of diabetes inherited in an autosomal dominant fashion.[63] There is also a form of diabetes associated with disease of the pancreas, often known as "secondary diabetes". Gestational diabetes occurs or is first recognized during pregnancy and is classified as a medical complication of pregnancy.[63] Diabetes is most commonly diagnosed by hemoglobin A_{1c} levels (HbA_{1c}). The HbA_{1c} gives an

average blood glucose reading from the past 2-3 months, with a reading of ≥48 mmol/mol or 6.5% being the diagnostic cut-off.[63]

2.2 Diabetes Epidemiology

As of 2013, 382 million people worldwide had been diagnosed with diabetes. It has been estimated that by the year 2035, this number will have risen to 592 million.[66] Leading the world in terms of diabetes prevalence is China with an estimated 90 million people diagnosed, and India is a close second with 61 million people diagnosed.[67] Adoption of the Western diet by many countries contributes to the prevalence of diabetes. It has also been shown that countries with the highest prevalence of diabetes also have a high prevalence of obesity.[66]

As of 2011, 28.5 million people in the U.S. have been diagnosed with diabetes, and 70,000 individuals die from the disorder annually. The disease costs the U.S. $174 billion and a direct cost range of $150-$14,060 per patient per year, numbers that have only risen in years since.[68] Diabetes affects more men than women in all of the states (13.7% men, 11.7% women). The region of the U.S. with the highest prevalence of diabetes is the Southeast, with the Northeast and Midwest being the lowest areas of prevalence.[69] Alabama ranks #6 in the U.S. for diabetes death per year with a ratio of 32 diabetes-

related deaths per 100,000 deaths each year. Alabama also has the highest population of diabetics with 8.9 out of 100 people diagnosed.

2.3 Type 1 Diabetes Mellitus

There are two types of diabetes mellitus: Type 1 (T1DM) and Type 2 (T2DM). These two variations differ by insulin production or reception. T1DM, previously known as Juvenile Diabetes due to its commonality in children and adolescents, occurs when the body's immune system attacks and destroys the β-cells in the pancreas responsible for producing insulin.[64] It is a T-lymphocyte mediated autoimmune inflammatory response in the islets (insulitis) and is associated with humoral (B-lymphocyte) production of autoantibodies to beta cell antigens.[64] It is the CD4+ and CD8 + T-lymphocytes in particular that are associated with the destruction of the pancreatic beta cells.[64] In a healthy immune system, the self-reactive T-cells are recognized and eliminated by regulatory T cells, thereby controlling any autoimmune behavior.[70] The rate of beta cell destruction varies between individuals, giving logic to its presentation at any age.[64] The onset of T1DM is associated with environmental exposures and genetics, particularly three T1DM-associated loci combined with chromosome 6p21.[64] Hyperglycemia, characteristic of T1DM, can lead to increased thirst, polyuria, shock, coma, and in extreme cases, death[64]. Children typically present T1DM with polyuria,

weight loss, polydipsia, and occasionally diabetic ketoacidosis.[62] T1DM accounts for only 5% of diagnosed cases of diabetes, as could be predicted due to its autoimmunity source.[64] Individuals at risk for developing T1DM can be screened for the genetic markers and with the use of autoantibodies.[64] Individual treatment of T1DM varies from patient to patient depending on time of onset and severity. Some patients may be treated with diet alone, diet and oral hypoglycemic agents, or insulin treatments. Rarely, patients can remain insulin independent for up to 10 years after diagnosis.[64]

2.4 Type 2 Diabetes Mellitus

T2DM is far more common, accounting for 90-95% of all adult cases in the U.S. The prevalence of T2DM in the United States has doubled over the previous decade in concurrence to the prevalence of obesity.[65] The ever increasing diagnoses of T2DM in the United States can be attributed to the modern lifestyle including high calorie foods, decreased energy expenditure, and modern luxuries such as cars and fast food.[71] An elevated glucose level characterizes T2DM, as it does in T1DM. Much of the morbidity and mortality of T2DM is due to atherosclerosis complications.[65] Atherosclerosis develops because of the increased plaque deposits in the arteries caused by high cholesterol and blood triglycerides.[72] T2DM has been shown to have a genetic predisposition. The mutation in the insulin gene can do one of two

things: inhibit the conversion of proinsulin to insulin or reduce the affinity of the insulin receptor for insulin.[73] Both scenarios are characteristic of T2DM as insulin resistant or decreased insulin production by the beta cells of the pancreas.[71] Diagnosed T2DM is also characterized abnormal inhibition of glucagon after the consumption of a meal, leading to hyperglycemia via glucose secretion from the liver.[65] Although a gene has been identified, it merely shows the individual is at a predisposition for glucose intolerance. Environmental factors determine if the genotype manifests to T2DM phenotype.[71] The environmental factors are ones mentioned previously that are associated with the modern lifestyle.[71] Although insulin resistance is an important contributor to the pathogenesis or T2DM, it is the consensus that it is the beta cell health that determines the glycemic level in individuals genetically predisposed to T2DM.[71] Despite numerous studies, there is no physiological evidence of how T2DM, insulin resistance, and obesity are linked, although there is a trend showing positive correlations between all factors.[74] The first suggested therapy for T2DM is lifestyle changes, such as exercise and decreased caloric intake. Both of these interventions can help decrease the risk of impaired glucose tolerance and development of prediabetes status.[71] A 7% weight loss lead to a 58% decreased incidence of T2DM after three years as established by the Diabetes Prevention Program

(DPP). For those who qualify for bariatric weight loss surgery, a remission rate between 40 and 90% can be expected, furthering that weight loss is a large contributor to remission of the condition.[75]

2.5 Role of Insulin

Insulin is a hormone essential for tissue growth, development, and glucose homeostasis.[76] The hormone is secreted by the β-cells of pancreatic islets of Langerhans upon stimulation by increased circulating blood glucose levels or amino acid levels.[77] Insulin down-regulates the secretion of hepatic glucose by inhibiting gluconeogenesis and glycogenolysis at the level of the liver.[78] It also acts on muscle and adipose tissue by stimulating the uptake of the circulating glucose.[30] These actions, in combination, maintain the levels of circulating glucose in the body.[79] Insulin plays a role in the metabolism of dietary carbohydrates, lipids, and fats.[30]

Elevated glucose or amino acid levels in the blood initiate insulin secretion from the pancreas.[19] Glucose stimulates a biphasic system of insulin release. In the first phase, insulin is released within the first few minutes of exposure to elevated glucose followed by a permanent second release.[80] Insulin is synthesized as preproinsulin in the ribosomes of the rough endoplasmic reticulum in the beta cells of the pancreas.[80] This preproinsulin is cleaved to yield proinsulin, which is then transported to the

Golgi apparatus.[80] The Golgi serves to package the proinsulin into granules near the cell membrane.[80] Within the granules, the proinsulin is cleaved to yield the active form of insulin, which is secreted upon fusion of the granule with the cell membrane.[80] When insulin enters the blood stream, it binds and activates the cellular insulin receptor (IR) causing downstream activation of many cellular transduction pathways.[78] Glucose uptake, protein synthesis, fatty acid synthesis, and production of glycogen are among the pathways stimulated upon this binding of insulin to IR.[78] Insulin's mechanistic action is as a growth-promoting hormone thereby inhibiting cellular apoptosis and stimulating cellular proliferation.[78] Insulin promotes the sequestering of glucose into cells by stimulating translocation of glucose transporters to the cell membrane thereby enhancing glucose uptake.[78]

The hormone whose action is antagonistic to insulin is glucagon.[81] Glucagon acts as a co-regulator of plasma glucose homeostasis in conjunction with insulin.[81] When there is a decrease in blood glucose below the threshold level (70 mg/dL), the α-cells of the pancreas secrete glucagon to restore the glucose level back to normal.[82] Although the action of glucagon is predominately seen under lower blood glucose conditions, growth, illness, and stress can also cause a rise in glucagon secretion.[82]

2.6 Insulin Resistance

The pathogenesis of insulin resistance is attributable to multiple factors, with the most common ones being a diet high in fat or sugar, physical inactivity, and overweight BMI.[83] Insulin resistance is defined as a state of reduced insulin sensitivity leading to the decreased effectiveness of insulin in lowering plasma glucose levels. This ineffectiveness is attributable to a decreased stimulation of skeletal and adipose tissue in glucose uptake and a decreased inhibition of hepatic gluconeogenesis and glycogenolysis.[84] Insulin resistance, closely linked with development of T2DM, affects 79 million Americans along with millions around the globe.[85] There are some diseases where the common underlying cause is hypothesized to be insulin resistance. These diseases include T2DM, dyslipidemia, hypertension, obesity, and coronary artery disease and subsequently atherosclerosis.[86] Insulin plays a pivotal role in the management of many lipid levels in the body along with inhibition of gluconeogenesis.[87] The inability of insulin to regulate these levels during insulin resistance leads to increased LDL cholesterol production, free fatty acids, triglycerides, and blood pressure.[88,89]

The primary cause of insulin resistance has been largely attributed to the secretion of pro-inflammatory cytokines due to fat accumulation in cells.[62] These pro-inflammatory cytokines, namely IL-6 and TNF-α, are secreted by the white adipose tissue in obese individuals, which causes

sequestration of macrophage to the tissue and systemic inflammation.[90] Systemic inflammation underlies many disease states, but has largely been associated with obesity and insulin resistance.[91] The accumulation of diacylglycerol (DAG) in skeletal muscle tissue along with hepatic tissue furthers the severity of insulin resistance, suggesting that DAG may contribute to the pathogenesis of insulin resistance.[92] Prolonged insulin resistance can be reversed through diet and exercise.[93] There has been a positive correlation shown between the intensity of the exercise performed and increasing insulin sensitivity.[94] A loss of 10% body weight was directly correlated with restoration of increased sensitivity, affirming that diet and exercise can be a primary therapeutic target for insulin resistance.[95]

2.7 Impaired Insulin Secretion and Signaling

T2DM symptoms cause impairment in protein-protein interactions within the insulin-signaling cascade. This impairment, or insulin resistance, perturbs insulin function as a homeostatic cellular modulator and growth hormone.[83] Akt is a primary modulator activated by insulin interaction with the insulin receptor.[96] Research has shown that T2DM pathology disturbs the normal function of Akt as a cellular modulator.[97] Skeletal muscle in mature, insulin resistant ob/ob mice exhibited significantly reduced Akt phosphorylation (70%) and glucose uptake under the stimulation of

insulin.[98] Akt protein expression was lessened in ob/ob mice liver (25%), adipose (60%), and soleus muscle (25%).[98] Declined Akt activation hinders Akt downstream effector inhibition or activation leading to metabolic dysfunction.[32] Insulin resistance in T2DM patients impairs the regulatory feedback system of gluconeogenesis.[83] The gluconeogenic pathway is uninhibited in the T2DM patient causing unregulated production of endogenous glucose resulting in increased serum glucose levels.[83]

Insulin resistance of the skeletal muscle and adipose tissue inhibits glucose uptake into peripheral tissues, and has been proposed as the beginning stage of hyperglycemia.[99] β-cells undergo hyperplasia and hypertrophy facilitating the secretion of abnormally high amounts of insulin, known as hyperinsulinemia, to maintain normal glucose tolerance.[83] Insulin resistance and hyperinsulinemia precede diabetes and lead to further complications if left untreated.[86] As β-cell dysfunction progresses, defects in the first phase insulin response occur followed by death of the β-cells.[100]

T2DM pathology induces chronic elevated fasting glucose levels and free fatty acid levels.[99,101] Elevated levels of glucose and lipids generate toxic conditions atypical to normal cellular function.[102] Glucotoxicity and lipotoxicity foster sustained reactive oxygen species (ROS) levels in β-cells, leading to β-cell apoptosis.[103-105] The term “glucolipotoxicity” describes the

presence of both glucotoxic and lipotoxic conditions.[106] Cultured mouse islet cells exposed to glucotoxic conditions, defined as 30mM glucose for 72 hours, exhibited significantly reduced glucose-stimulated insulin secretion.[107] Lipotoxicity suppresses glucose-stimulated insulin secretion and normal insulin function.[108] Increased DAG concentration stimulates serine phosphorylation on insulin receptor substrate 1 (IRS-1), inhibiting PI-3 activation and subsequent Akt stimulation.[108]

A select few patients may have insulin resistance due to defects in the insulin signaling pathway or defects in the insulin receptor (IR).[109] Hyperinsulinemia leads to prolonged insulin stimulation, which induces IRS and IR degradation, leading to reduced IR concentration, and further impairing normal insulin response.[76,110] Rats insulin resistance induced via soybean oil showed decreased expression of GLUT4 and impaired GLUT4 translocation to the plasma membrane suggesting a defect in upstream signaling events.[111]

The Insulin Signaling Cascade

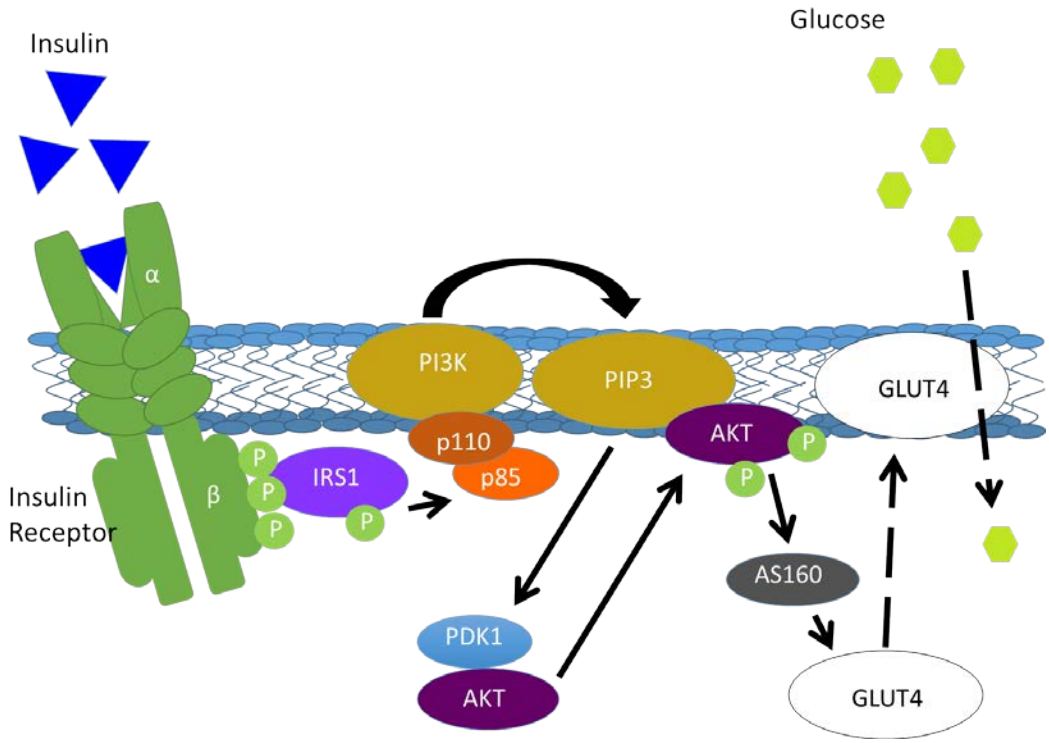

Figure 2: The insulin signaling cascade. Multiple factors are included in the model of insulin action to enhance cellular glucose uptake. Insulin is initially secreted from the pancreatic β-cells located in the islets of Langerhans.[112] Insulin stimulates a signaling cascade leading ultimately to the translocation of GLUT4 from the cytosol to the plasma membrane when acting on muscle and adipose tissue.[113] Insulin binds the glycoprotein IR on the cell membrane at one of the two extracellular α-subunits.[44] The insulin receptor undergoes a conformational change, which induces autophosphorylation of the β subunit.[78]

The transmembrane β-subunit of the IR is autophosphorylated at Tyr^{1158}, Tyr^{1162}, and Tyr^{1163} and tyrosine kinase is activated upon insulin stimulation.[42,44] Tyrosine kinase then phosphorylates IRS-1 at tyrosine residues.[113] IRS-1 recruits p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K) to the cell membrane.[36] Two Src Homology 2 (SH2) domains of the p85 subunit on the PI3K bind with tyrosine receptor domains of IRS-1.[114] Binding of IRS-1 to PI3K activates PI3K's 110 catalytic subunit.[113] PI3K activation generates a conformational change in the catalytic domain of PIP2.[40] PIP2 conforms to PIP3, which then activates PDK1.[115] Akt is recruited to the membrane and docks with PIP3 through the pleckstrin Homology (PH) domain.[113,116] The act of docking with PIP3 alters Akt conformation stimulating T^{308} and S^{473} phosphorylation.[44] Activated Akt continues the downstream signaling cascade resulting in the recruitment of GLUT4 to the plasma membrane.[113] GLUT4 then transports extracellular glucose into the cell for subsequent energy production.[117]

2.8 Insulin Receptor

Insulin receptors (IR) are tyrosine kinase receptors located within the membranes of mammalian cells.[43] IR are stimulated by insulin-like growth factors and insulin itself, and function to relay hormonal stimulation by a molecular signaling cascade.[42] IR are highly conserved transmembrane

glycoproteins consisting of two α subunits and two β subunits.[42,43] Extracellular α subunits function as insulin and insulin-like growth factor binding sites along with functioning as cellular mediators.[42] Two intra-membrane β-subunits extend into the cytosol and function as an intracellular signal.[118] Upon insulin binding, insulin receptors dimerize into an inter-membrane $\alpha_2\beta_2$ complex.[44] Dimerization initiates autophosphorylation of both β subunits at Tyr^{1158}, Tyr^{1162}, and Tyr^{1163}.[35] Phosphorylation of all three tyrosine sites initiates activation of two intracellular tyrosine kinase subunits.[42,44] Tyrosine kinases initiate phosphorylation of IRS-1 at YMXM motifs stimulating the generation of docking sites for SH2 domains.[45] IRS-1 activation is required in the recruitment of GLUT4 to the cell membrane.[45]

Insulin receptors are essential in modulating cellular energy homeostasis.[119] IR knockout mice have rapid onset of insulin resistance, hyperinsulinemia, hyperglycemia, ketoacidosis, and death.[119] Patients with mutations in the IR gene exhibit symptoms of severe hyperglycemia and hyperinsulinemia.[120] Tyrosine sites must undergo phosphorylation for signaling, as mutations in the phosphorylation site Lys-1030 inhibit kinase phosphorylation and IR signaling.[121] Chinese hamster ovary cells (CHO) overexpressing insulin receptors (CHO-IR) or IRS-1 (CHO-IRS-1) are more

responsive to insulin stimulation.[114] CHO-IR cells are a primary model used in the investigation of the insulin-signaling cascade.[114]

2.8 Akt/Protein Kinase B

Akt (Protein Kinase B) functions in cellular metabolism as a kinase within the AGC kinase family.[113,122] There exist three Akt isoforms within the body: Akt1, Akt2, and Akt3.[122] Akt1 is ubiquitously expressed, Akt2 is found in liver, heart, muscle, and kidney tissue, and Akt3 is found exclusively in the brain and testes.[122] Akt2 is recruited to the plasma membrane upon insulin stimulation and is involved in the regulation of GLUT4.[123]

The N-terminal of Akt is composed of highly conserved pleckstrin Homology (PH) domain of approximately 100 amino acids.[124] The kinase domain resides in the central stretch of Akt with the regulatory domain found in the C-terminal end.[96] The C-terminal has a hydrophobic motif composed of approximately 40 amino acid residues.[96] The regulatory Thr^{308} residue is located in the C-lobe of the kinase domain, and Ser^{473} is located within the hydrophobic section of the C-terminal end.[96]

2.8.1 Functions of Akt

The predominant function of Akt is modulation of cell survival and growth.[39] Akt modulates several cellular functions through phosphorylation of target receptor tyrosine kinases.[44]Akt activation initiates cellular signal

cascades involved in a multitude of activities including nutrient metabolism, cell growth, transcription, angiogenesis, and cell survival.[122] The activating stimuli of Akt determine Akt's downstream signaling target.[122] Activating stimuli of Akt are diverse and include: basic fibroblast growth factor, insulin, insulin-like growth factor, nerve growth factor, endothelin, interleukins, and TNF-α.[122] Twenty-five upstream Akt binding proteins have been identified.[125]

Over 170 proteins have been documented as downstream targets of Akt kinase activity.[125] Akt modulates several pathways in nutrient metabolism thorough kinase-activity of downstream substrates.[96] Akt activation inhibits glycogen synthase kinase 3β by phosphorylation of S^{9}, initiating the de-phosphorylation and activation of glycogen synthase.[44,126] Akt modulates activation of protein synthesis through inhibition of Tsc2.[127] Tsc2 inhibition elicits activation of the mRNA translator protein mammalian target of rapamycin 1 (mTOR1).[127] Akt phosphorylates Foxo1 at S^{253} inhibiting hepatic gluconeogenesis.[44] Besides nutrient metabolism, Akt also serves in the apoptotic pathway through the phosphorylation of BAD, inhibiting apoptosis.[39,128]

2.8.2 Akt in Insulin Signaling

Upon insulin stimulation, Akt is recruited to the plasma membrane and undergoes phosphorylation at two active sites.[113] The translocation of

Akt to the membrane is ambiguous; however, evidence implies PI3K activates PDK1, which recruits Akt to the membrane through binding with Akt's PH domain.[129,130] Akt docks to PtdIns(3,4,5),P3 (PIP3), and PtsInd(3,4)P2 (PIP2) at the membrane via Akt's PH domain.[131] Lysine 63 (K63) ubiquitin chains associate with Akt facilitating translocation to the plasma membrane.[132] At the membrane, Akt is activated through phosphorylation of the Thr^{308} by PDK-1 and Ser^{473} by the mTORC2 complex.[133,134] Phosphorylation initiates Akt signal transduction through kinase activity.[113] Akt phosphorylates the Rab-GTPase activating protein AS160 (TBC1D4) causing the stimulation of GLUT4 recruitment to the plasma membrane.[113,135]

2.8.3 Akt Inhibition

Insulin stimulated Akt activation maintains metabolic homeostasis.[32] The absence or impairment of Akt is clearly associated with the progression of T2DM.[44] Insulin resistance can impair Akt activation and metabolic regulation, thereby advancing diabetes etiology.[44] Akt knockout (KO) mice rapidly develop T2DM symptoms in the absence of active Akt.[44] It has been shown that inhibition of Akt2 in mature muscle tissue decreased glucose uptake by 70-90%.[98] Akt's upstream signaling protein, PI3K, is impaired in T2DM skeletal muscle abrogating Akt activity.[32]

2.9 IRS-1

The insulin receptor substrate-1 (IRS-1) is a multi-domain transmembrane protein.[173] IRS-1 has a molecular size of approximately 131,000 kDa with an apparent weight of 185,000 kDa on SDS-polyacrylamide gels.[174] While it is not intrinsically catalytic, it mediates the interaction of the insulin receptor (IR) with downstream effectors.[173] The domains from N-terminal to C-terminal are as follows: pleckstrin homology (PH) domain, phosphotyrosine binding (PTB) domain, and finally several tyrosine and serine residues prone to phosphorylation.[175-177] There are over 20 tyrosine phosphorylation residues that function as docking sites for downstream signaling proteins.[173] Some of the proteins that dock on the tyrosine residues include: phosphatidylinositol 3-kinase (PI3 kinase), 14-3-3 protein, and phosphotyrosine phosphatase SHP-2.[178]

2.9.1 IRS-1 in Insulin Signaling

Insulin signals the phosphorylation and subsequent activation of IRS-1. Insulin upon binding IR activates its tyrosine kinase activity leading to phosphorylation of IRS-1 on its tyrosine residues.[178,179] Phosphorylation of IRS-1 provides sites for Grb2 to bind.[180] Binding of Grb2 leads to cell proliferation through activation of MAPK.[180] Phosphorylated IRS-1 also recruits phosphatidylinositol 3-kinase to the plasma membrane, generating phosphatidylinositol 3,4,5-triphosphate thereby recruiting 3-

phosphoinositide-dependent protein kinase-1 (PDK-1). PDK-1 subsequently phosphorylates and activates Akt causing translocation of GLUT4 to the cell membrane leading to increased glucose uptake in fat and muscle tissue.[178-180]

2.10 proNGF/NGF/MMP7

Nerve growth factor (NGF) is a protein that affects the central and peripheral nervous system. The NGF gene is located on human chromosome 1 and has two variants.[181,182] NGF is a dimer of polypeptide chains and appears similar in all tissues.[183] The primary function of this protein is to stimulate the survival and differentiation of neurons.[184] NGF is the best characterized member of a family of structurally and functionally similar neurotrophins, which can be found in mammals and lower vertebrates.[185] The neurotrophin family of molecules includes brain-derived neurotrophic factors (BDNF), neurotrophin-3,(NT-3) and neurotrophin-4 (NT-4).[186-188] ProNGF is the precursor to the mature and biologically active β-NGF. In tissues, proNGF undergoes post-translational processing to generate the mature β-NGF form.[189,190]

Matrix metalloproteinases (MMPs) play a key role in embryogenesis, proliferation, cell motility, remodeling, wound healing, angiogenesis, and key reproductive events.[191] MMPs are a family of zinc-dependent, proteolytic enzymes with 26 different forms currently described.[191] MMPs have been

identified in modulating the processing, activation, and deactivation of several soluble factors.[191] Macrophage-derived matrix metalloproteinase 7 (MMP7) cleaves proNGF to produce the mature β-NGF form. [192] NGF is 118 amino acids in length and consists of three subunits: α_2, β, and γ_2.[185] Its molecular weight is approximately 130 kDa.[185] The α_2 subunit appears inactive, the β subunit is responsible for the protein's biological activity, and the γ_2 subunit is a highly specific protease capable of converting premature NGF to the mature form.[185] NGF is not utilized exclusively by neuronal cells. It has been shown to be produced and utilized by epithelial cells, immune inflammatory cells, smooth muscle cells, and keratocytes.[193-195] This evidence of use outside of the nervous system exclusively has deemed NGF a pleiotropic factor.[196]

The submandibular gland is responsible for secreting mature NGF, but in other tissues (prostate, hair follicles, thyroid gland, retina, skin, colon, dorsal root ganglia as well as central nervous system) NGF is mainly secreted as proNGF or a mixture of the two forms.[185,190] ProNGF is chemically different from NGF in a number of ways: it is less basic, has a different isoelectric point, and is more long-lived than mature NGF.[190] Two receptors for NGF have been identified: TrkA and $p75^{NTR}$.[49,197]

2.11 Trk Receptor

Trk describes a transmembrane glycoprotein belonging to the tyrosine kinase receptor family. The three Trk variants (TrkA, TrkB, and TrkC) are encoded by a gene on human chromosome 1.[185] TrkB serves as a receptor for BDNF and NT-4, while TrkC functions as a receptor for NT-3, but not exclusively as NT-3 can also bind $TrkA_{II}$.[49,198] Signaling of TrkA through NGF results in the hallmark actions of NGF, differentiation and survival.[199] TrkA has two isoforms, one of which is deemed $TrkA_{II}$ due to inclusion of six amino acids near the transmembrane domain leading to differences in their extracellular domains.[198] This inclusion confers a relaxation leading to enhanced TrkA activation through NT-3.[198] The second immunoglobulin (Ig)-like domain of TrkA is engaged by NGF at NGF's two distinct patches.[200] The second patch is formed by NGF's unique amino terminus serving to specify NGF binding to TrkA.[200]

2.11.1 TrkA-NGF Signaling

It was discovered that binding of NGF to TrkA elicited a rapid tyrosine phosphorylation of endogenous TrkA in PC12 cells and exogenous TrkA in transfected fibroblasts.[201-203] Binding of NGF to TrkA elicits a series of events characteristic to receptor tyrosine kinase signaling. These characteristic events include dimerization and transphosphorylation of activation loop tyrosines causing activation of kinase activity and subsequent

autophosphorylation of tyrosine residues outside the activation loop.[204] These autophosphorylation sites serve as binding sites for specific proteins, and the subsequent phosphorylation and activation of these accessory proteins generates a signaling cascade of receptor-independent pathways.[205] The cascade includes the following pathways: MAPK-Ras-Erk pathway, SNT proteins (suc-associated neurotrophic factor-induced tyrosine-phosphorylated target), phospholipase Cy1, and P3I kinase.[185,206] It has been shown that PC12 cells with up-regulated $TrkA^{NGFR}$ showed NGF-induced cell death merely through $TrkA^{NGFR}$. This opposing action of NGF between cell survival and death is not shared with other neurotransmitters.[46]

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CHAPTER 3: TrkA and Insulin Receptor in Streptozotocin Induced Diabetes Rat Brain

3.1 Introduction

TrkA is a tyrosine receptor kinase that specifically binds to nerve growth factor (NGF).[1] NGF causes the dimerization and autophosphorylation of TrkA, which recruits downstream signaling proteins, including phospholipase C-γ1, Shc, FRS2, and PI3K.[1-4] NGF regulates the survival and differentiation of neurons in the central and peripheral nervous system.[5,6] Insulin receptor (IR) and insulin receptor substrate (IRS-1) are prominent proteins in the insulin-signaling pathway and are necessary for the cell to properly respond to insulin.[7] Neurons, however, are insulin insensitive, thereby making insulin incapable of increasing their glucose uptake as it does in muscle and fat cells.[8,9]

Insulin plays a crucial role in brain functions such as memory improvement and energy metabolism.[10-14] Insulin binds with the insulin receptor on the plasma membrane causing phosphorylation of tyrosine residues and protein dimerization.[15] IRS-1 docked to the insulin receptor is phosphorylated upon dimerization of the insulin receptor.[16,17] PI3K docks with IRS-1 inducing a conformational change from PIP2 to PIP3. [16,17] PIP3 recruits phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (Akt) to the membrane where PDK1 phosphorylates and activates Akt.[18] Akt

activation leads to increased glucose uptake. In addition to PI3K, IRS-1 also interacts with growth factor receptor binding protein 2, leading to mitogen activated protein kinase (MAPK) activation, which mediates mitogenesis and cell survival.[19,20]

Previous research in our lab indicates that NGF or insulin induces TrkA to form a molecular complex with IR and IRS-1.[21] We also discovered that the tyrosine phosphorylation of IR and IRS-1 requires a functional TrkA kinase in PC12 cells.[21] In addition, TrkA influences insulin signaling through activation of Akt and MAPK. This revealed a novel overlapping signaling mechanism between NGF/TrkA and insulin/IR.[21] In this study we have expanded in diabetic animal model and report that TrkA activation, interaction with IRS-1 and signaling was reduced in STZ (Type 1 diabetic model) rat brain compared to control brain samples.

3.2 Materials and Methods

3.2.1 Antibodies and Reagents

Anti-TrkA (C-14), pIRS-1 (Tyr-632), insulin receptor, and nitrotyrosine antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-IRS-1 was purchased from Millipore (Temecula, CA). Antibodies against pAkt (Ser-473), pINSR (Tyr-1146), pINSR (Tyr-1150/1151), total Akt were purchased from Cell Signaling Technology

(Danvers, MA). Anti-phosphotyrosine (PY99) was from BD Transduction Laboratories. Anti-rabbit IgG and anti-mouse IgG-HRP-linked secondary antibody were from GE Healthcare, and ECL was from Thermo Scientific. Protein A-Sepharose beads, and all other reagents were obtained from Sigma.

3.2.2 Experimental model

Male 8-wk-old Wistar rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were housed in pairs, allowed *ad libitum* access to standard lab chow and water, and maintained on a 12:12-h light-dark cycle. After 1 week of acclimation, rats were divided into the following two groups: nondiabetic (control), STZ induced diabetes (type 1 diabetes). Diabetes was induced by an intravenous injection of STZ (60 mg/kg in citrate buffer, pH 4.5) via the penile vein under isoflourane anesthesia. The high dose of STZ used in the investigation is a well-established approach to model the typical phenotype of Type 1 diabetes in a rat. STZ induces necrosis and death of pancreatic β-cells, leading to low plasma insulin and hyperglycemia.[22] Control animals were given a vehicle injection of only citrate buffer. Hyperglycemia was confirmed by testing urine glucose (Diastix, Bayer) and by plasma glucose measurements. Rats were euthanized by decapitation after CO_2 inhalation. Brain tissues were excised immediately and stored in liquid nitrogen. All experimental

protocols were approved by the Institutional Animal Care and Use Committee of Midwestern University.

3.2.3 Immunoprecipitation and Western Blotting Analysis

Frozen whole rat brain was homogenized in Triton lysis buffer (50 mM HEPES (pH 7.6), 150 mM NaCl, 20 mM sodium pyrophosphate, 10 mM NaF, 20 mM 13-glycerophosphate, 1% Triton, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin) using a Potter- Elvehjem homogenizer. Bradford procedure (Bio-Rad) was used to estimate the protein with bovine serum albumin (Sigma- Aldrich) as a standard. The brain homogenates (500 □g)were incubated with 2 μg of the primary antibody. The immunoprecipitates were collected overnight with protein A-Sepharose beads at 4 °C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the samples and boiled. The proteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane (Millipore) and western blotted using appropriate antibodies such that the immune complex was detected by enhanced chemiluminescence.

3.3. Results

3.3.1 Active NGF was reduced in STZ rat brain

Our lab has shown previously that NGF binding to TrkA allows TrkA to participate in the insulin signaling cascade of PC12 cells.[13] First we want to determine the expression of NGF, its precursor proNGF and matrix metalloproteinase 7 (MMP-7) in whole brain samples from STZ and control rat brains by western blot analysis. ProNGF functions in a biologically opposite manner, inducing apoptosis by binding with the p75 neurotrophin receptor.[23] ProNGF is extracellularly processed to NGF by the enzyme MMP-7.[24] Results indicate that levels of MMP-7 and NGF were reduced in STZ rat brain as compared to control (Fig. 3). Conversely, Pro-NGF level was elevated in the STZ rat brain as compared to the control rat brain samples (Fig. 3).

3.3.2 TrkA tyrosine phosphorylation was reduced but more nitrosylated in STZ rat brain.

As the expression of NGF is lower in diabetic rat brain, we investigated whether TrkA activity is altered in STZ rat brain samples. Control and STZ rat brain homogenates were immunoprecipitated with TrkA antibody or IgG, with IgG serving as a negative control. The immunoprecipitates were Western blotted for TrkA phosphorylation (PY99), TrkA nitrosylation, and total TrkA. We included the control and STZ rat brain homogenates as input in the Western blot analysis to serve as positive

control. Results indicated that phosphorylation of TrkA was reduced, whereas TrkA nitrosylation was increased in STZ rat brain in comparison to control brain (Fig. 4).

3.3.3 Interaction of TrkA with IR and IRS-1 is impaired in STZ rat brain.

TrkA is known to associate and form a complex with IR and IRS-1 upon insulin or NGF stimulation in PC12 cells.[21] We investigated whether this interaction was impaired in STZ rat brain samples. Control and STZ rat brain samples were homogenized with lysis buffer. These brain homogenates were immunoprecipitated with TrkA andibody or IgG, with IgG serving as a negative control. The samples were then Western blotted for IR and TrkA antibody. The control and STZ rat brain homogenates were included in the Western blot to serve as a positive control. Results indicate that the interaction between TrkA and IR is markedly reduced in STZ rat brain samples as compared to control rat brain homogenates (Fig. 5A).

Control and STZ rat brain homogenates were also immunoprecipitated with TrkA antibody or IgG. The samples were then Western blotted for IRS-1 and TrkA antibody. The control and STZ homogenates were included in the Western blot to serve as a positive control. The results indicate that interaction between TrkA and IRS-1 is

reduced in STZ rat brain samples as compared to control rat brain homogenates (Fig. 5B).

3.3.4 IR tyrosine phosphorylation was reduced in STZ rat brain

Our lab previously showed that an active TrkA kinase is required for IR phosphorylation in PC12 cells.[21] In STZ rat brain, the tyrosine phosphorylation of TrkA was impaired hence we wanted to determine the phosphorylation of IR as well. Brain homogenate from control and STZ rat brains were Western blotted for phospho-IR antibody that recognizes the tyrosine 1150/1151 or 1146 of IR and total IR. Results indicate that phosphorylation of IR at tyrosine residues 1150/1151 and 1146 was reduced in STZ rat brain samples (Fig. 6A). We further quantified these blots and found thatthe difference in tyrosine phosphorylation at residue 1150/1151 and 1146 of control and STZ rat brain samples was significant with $p<0.01$ (Fig. 6B) and $p<0.05$ (Fig. 6C) respectively.

3.3.5 IRS-1 tyrosine phosphorylation was reduced in STZ rat brain

In addition we also investigated whether the tyrosine phosphorylation of IRS-1 was impaired in STZ rat brain samples as compared to control. Brain homogenate from control and STZ rat brains were Western blotted for phospho-IRS-1 antibody that recognizes the tyrosine 632 amino acid of IRS-1 and total IRS-1. Results indicate that tyrosine phosphorylation

of IRS-1 is significantly reduced in STZ rat brain samples (Fig. 7A). We further quantified the data and showed that the phosphorylation was significantly different between the control and STZ rat brain samples with $p<0.05$ (Fig. 7B).

3.3.6 Phosphorylation of Akt was reduced in STZ rat brain

TrkA signaling leads to Akt phosphorylation and activation, we investigated whether Akt phosphorylation was impaired in STZ rat brain samples as compared to control samples. Control and STZ rat brain homogenate were Western blotted for phospho-Akt antibody that recognizes serine residue 473 and non-phospho antibodies. Results indicate that phosphorylation of Akt is reduced in STZ rat brain samples as compared to control samples (Fig. 8A). Quantification of the blots revealed that there was a statistically significant difference between phosphorylation of Akt at serine residue 473 in STZ rat brain samples and control samples with $p<0.05$ (Fig. 8B).

3.4 Discussion

Diabetes impairs insulin action on the insulin receptor, resulting in declined glucose uptake and abnormal glucose homeostasis. Neuronal cells are insulin insensitive, as insulin is incapable of eliciting glucose uptake into

these tissues.[5,6] Previous research from our lab has reported that TrkA acts as a new receptor in the insulin signaling cascade of PC12 cells.[13]

Our intention was to expand on these previous findings by furthering the investigation of TrkA's role in the insulin signaling cascade of the T1DM rat brain. In the neuronal cell model, TrkA interacts with the insulin receptor upon insulin and nerve growth factor (NGF) stimulation.[13] A functional TrkA kinase is necessary for the phosphorylation and activation of the insulin receptor, insulin receptor substrate-1 (IRS-1), as well as Akt.[13] In this study, we report similar findings in the T1DM rat brain.

T1DM rat brain samples exhibited decreased NGF and MMP-7 levels but greater Pro-NGF levels as compared to control samples. Decreased level of MMP-7 have impaired the cleavage of proNGF into mature NGF, thereby leading to its accumulation in brain of STZ rat. Phosphorylation of TrkA was decreased, but its nitrosylation was increased which may be due to increase in proNGF levels in the T1DM rat brain. Interaction of TrkA with the insulin receptor and IRS-1 was also impaired in the T1DM rat brain. In addition, phosphorylation and activation of the insulin receptor, IRS-1, and Akt were all markedly decreased in the T1DM rat brain. These results suggests that in T1DM, the insulin signaling in brain is impaired due to the reduction of expression of mature NGF, which in turn impairs the heterodimerization of

TrkA with insulin receptor and affects the downstream signaling. Studies have shown that NGF is an important regulator for the synthesis and secretion of insulin from pancreatic β cells. Withdrawal of NGF leads to β cell apoptosis and decreases the secretion of insulin.[25] Our findings that β cell destruction in STZ rats causes reduction in the expression of NGF in brain. This suggests that NGF-TrkA is involved in the insulin signaling cascade in the rat brain, and its activity is impaired in the T1DM rat brain.

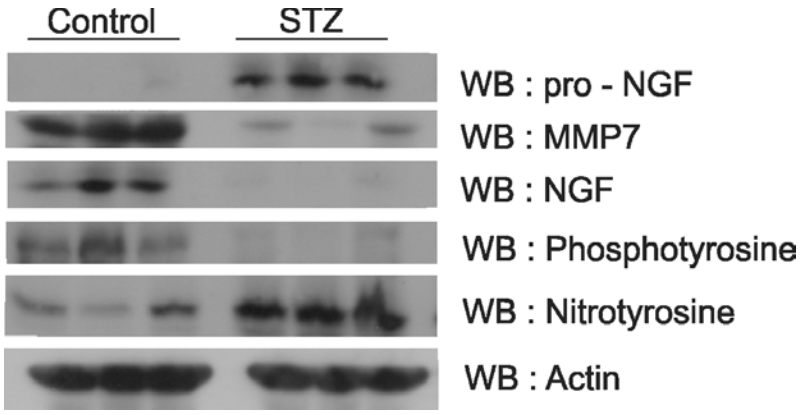

Figure 3: Active NGF and phosphorylation was reduced in STZ rat brain. Homogenates from 3 control and 3 STZ rat brain were Western blotted for pro-NGF, NGF, MMP7, phosphotryrosine, nitrosylation and actin antibodies.

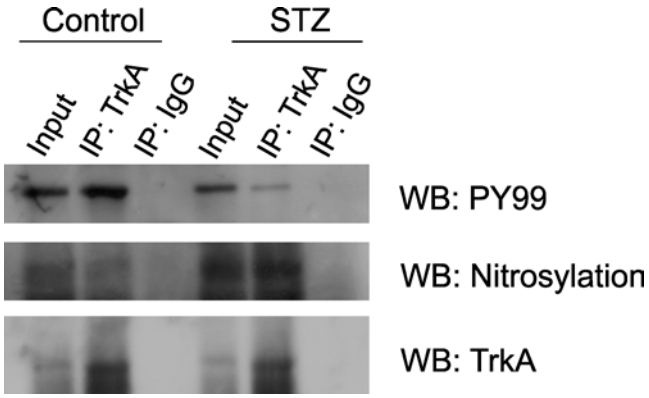

Figure 4: TrkA tyrosine phosphorylation was reduced but more nitrosylated in STZ rat brain. Brain homogenate from control and STZ rat were immunoprecipitated with TrkA antibody or IgG (negative control) and Western blotted for tyrosine phosphorylation (PY99), nitrosylation and TrkA antibody. The control and STZ rat brain homogenate (input) were also included in the Western blot for positive control.

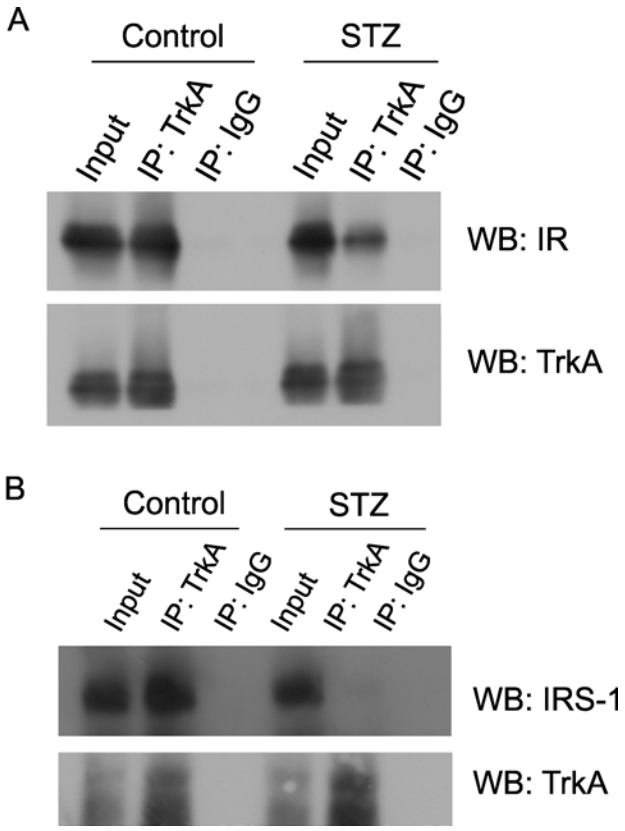

Figure 5: Interaction of TrkA with IR and IRS-1 is impaired in STZ rat brain. (A) Control and STZ rat brain homogenate were immunoprecipitated with TrkA antibody or IgG and Western blotted for IR and TrkA antibody. (B) TrkA immunoprecipitates were also western blotted for IRS-1 and TrkA antibody. The control and STZ rat brain homogenate (input) were also included in the Western blot.

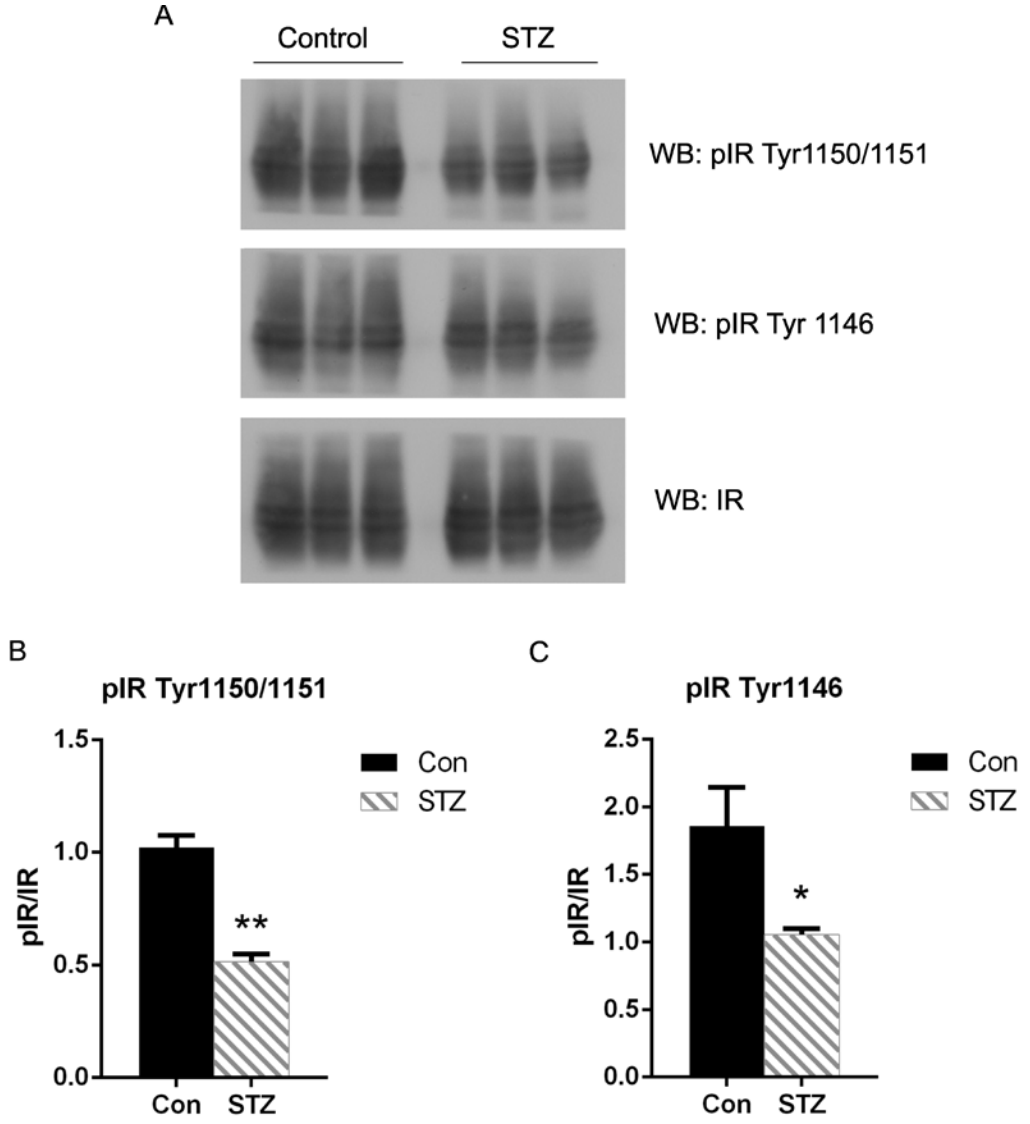

Figure 6: IR tyrosine phosphorylation was reduced in STZ rat brain. (A) Brain homogenate from control and STZ rat were Western blotted for phospho-IR antibody that recognizes the tyrosine 1150/1151 or 1146 of IR and total IR. (B) Bar graph quantifying the western blot of Phospho IR 1150/1151 shown in A. Control was compared to STZ rat brain homogenates ($p< 0.01$). (C) Bar graph quantifying the western blot of Phospho IR 1146 shown in A. Control was compared to STZ rat brain homogenates ($p<0.05$).

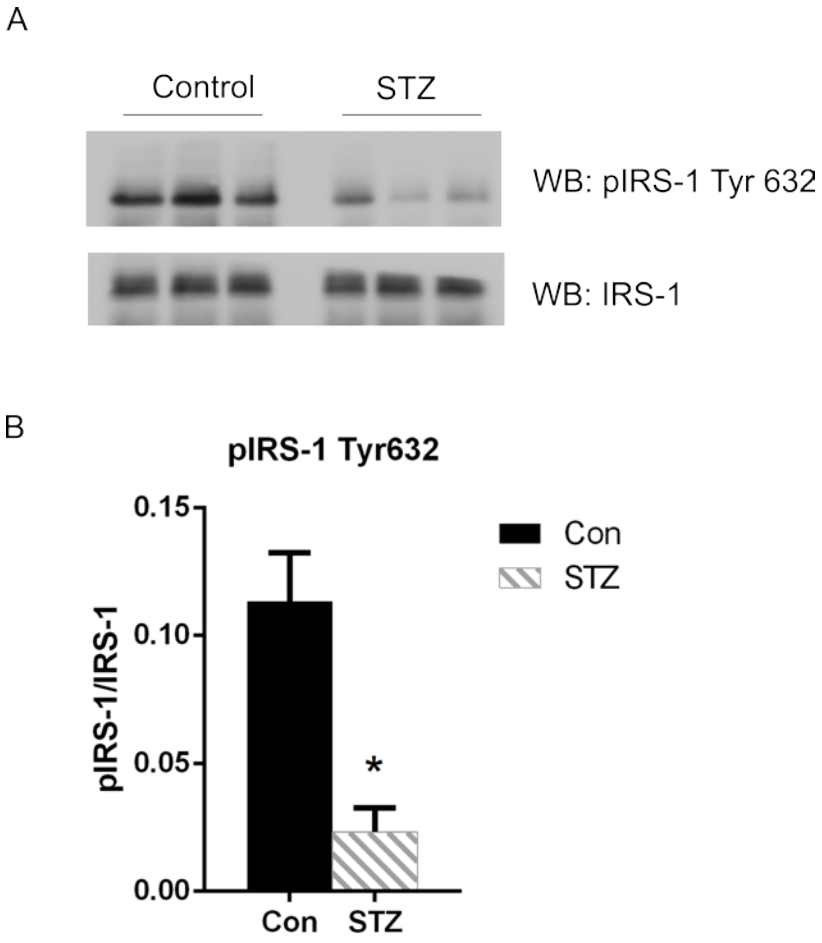

Figure 7: IRS-1 tyrosine phosphorylation was reduced in STZ rat brain. (A) Brain homogenate from control and STZ rat were Western blotted for phospho-IRS-1 antibody that recognizes the tyrosine 632 amino acid of IRS-1 and total IRS-1. (B) Bar graph quantifying the western blot of Phospho IRS-1 632 shown in A. Control was compared to STZ rat brain homogenates ($p<0.05$).

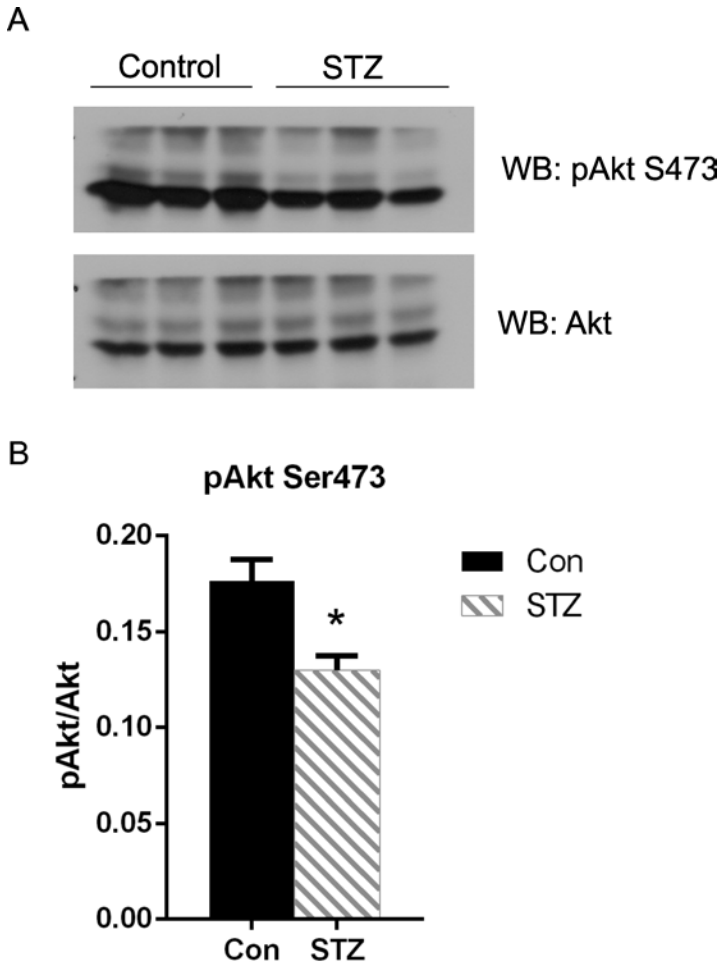

Figure 8: Phosphorylation of Akt was reduced in STZ rat brain. (A) Control and STZ rat brain homogenate were Western blotted for phospho-Akt (S473) and non-phospho antibodies. (B) Bar graph quantifying the western blot of Phospho Akt shown in A. Control was compared to STZ rat brain homogenates ($p<0.05$)

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