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The Role of Vascular Endothelial Growth Factor at the Blood-Brain Barrier in Diabetes

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THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR AT THE BLOOD-BRAIN BARRIER IN DIABETES

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by
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Dedication

This work is dedicated to my first teachers and parents, Gualberto and Jocelyn Marcelo. Their guidance and support in my education have been enormous, and they have shown me that hard work and perseverance are important. They have shaped me into who I am today, and I thank them for always being there for me.

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List of Symbols/Nomenclature

Aβ----Amyloid-β peptide
Akt---Protein kinase B (PKB)
ALS----Amyotrophic lateral sclerosis
ANOVA----Analysis of variance
BBB---Blood-brain barrier
BCRP----Breast cancer resistance protein
CAD---Coronary artery disease
CNS----Central nervous system
DAG----Diacylglycerol
ELISA----Enzyme-linked immunosorbent assay
eNOS----Endothelial nitric oxide synthase
Erk 1/2----Extracellular signal regulated kinase 1/2
Flt-1----fms-like tyrosine kinase-1
Flk-1----Fetal liver kinase-1
GAPDH----Glyceraldehyde-3-phosphate dehydrogenase
HGF----Hepatocyte growth factor
Hsp27----Heat shock protein 27
IP_3----Inositol (1,4,5)-triphosphate
JAM---Junctional adhesion molecules
L-NAME----N^G-nitro-L-arginine-methyl ester
LRP-1----Low density lipoprotein related protein 1
MAPK---Mitogen activated protein kinase
MIN----Minocycline
MiRNA----MicroRNA
MMP----Matrix metalloproteinase
MRP----Multidrug resistant protein
NAD----Nicotine adenine dinucleotide
NO----Nitric oxide
NP-1----Neuropilin-1
NP-2----Neuropilin-2
NVU----Neurovascular unit
OAT----organic anion transporter
OATP----organic anion transporting polypeptide
PARP----Poly(ADP-ribose) polymerase
PCR----Polymerase chain reaction
PDGF----Platelet derived growth factor
Pgp---P-glycoprotein
PI3K---Phosphoinositide-3-kinase
PKC----Protein kinase C
PLCγ----Phospholipase C gamma
PLGF----Placental growth factor
PPARγ----Peroxisome-proliferator-activated receptor
RAGE----Receptor for advanced glycation end products
R_{br}----Distribution ratio to brain

SEMA 3A----Semaphorin 3A
sFlt-1----soluble Flt-1
STZ----Streptozotocin
T1D----Type 1 diabetes
T2D----Type 2 diabetes
VEGF----Vascular endothelial growth factor
VEGFR----Vascular endothelial growth factor receptor
ZO-1----Zona occludens-1
ZO-2----Zona olccludens-2

Abstract

Diabetes is a risk factor for stroke and vascular dementia. Clinical studies using gadolinium-magnetic resonance imaging techniques have shown diabetic patients exhibit a permeability increase at the blood-brain barrier (BBB) (Starr et al, 2003). The BBB, a vascular interface at the level of brain microvascular vessels, functions to provide nutrients and oxygen from the peripheral circulation, mediates waste efflux from the brain, and protects the brain from toxins. These functions are due to the presence of tight junction proteins. Animal studies have shown increased BBB permeability is due to a decrease in these proteins (Hawkins et al, 2007). Based on these studies and using the streptozotocin (STZ) model of diabetes, I hypothesized that the permeability and molecular changes can be attenuated by minocycline, a tetracycline known to cross the BBB. STZ animals treated with minocycline exhibited a decrease in permeability and an increase in the tight junction protein, claudin-5. Previous studies have shown that the pro-angiogenic and permeability cytokine, vascular endothelial growth factor (VEGF), is able to influence claudin-5 expression (Argaw et al, 2009). Therefore, VEGF may be involved in a minocycline-induced increase in claudin-5 expression. VEGF significantly increased in STZ animals with minocycline treatment. Additionally, there was a significant increase in expression of the VEGF receptor, Flt-1, of control animals treated with minocycline, and a similar trend in minocycline-treated STZ animals. However, there were no changes in the other VEGF receptors. Based on these results, I hypothesized that VEGF might exert its effects on the BBB in diabetes. Although there were no changes in VEGF, there was a glucose-dependent change in PLCγ, a downstream signaling molecule involved in proliferation, which might help explain the permeability change observed in diabetes.

Chapter 1: Introduction

Diabetes

Diabetes mellitus is a metabolic disorder that is characterized by hyperglycemia. It is generally classified into two types: Type I (T1D) and Type II (T2D). T1D is typically diagnosed in children and young adults and is due to auto-immune destruction of the beta cells of the pancreas. Thus, no insulin is produced, and patients with this disease are insulin-dependent via injections or a pump. The other pancreatic islets that secrete glucagon, somatostatin, and pancreatic polypeptide remain intact. The symptoms of T1D are increased thirst, urination, hunger and weight loss. Additionally, ketoacidosis and dyslipidemia may be present. Ketoacidosis is due to reduced insulin levels, decreased glucose use, and an increase in gluconeogenesis (Trachtenbarg, 2005). Insulin deficiency leads to uncontrolled beta-oxidation of free fatty acids, which are converted to ketone bodies by the liver. Poorly controlled T1D can lead to hypertriglyceridemia and reduced HDL, but insulin therapy can alleviate these abnormalities (Goldberg, 2001). T1D has a genetic component; however, environmental factors could be involved in the pathogenesis of the disease. Others have proposed that T1D is triggered by a virus, perhaps Coxsackie or rubella viruses (Alba et al., 2005).

T2D is characterized by insulin resistance and over time an insufficient production of insulin. Patients with T2D exhibit similar symptoms to those with type I diabetes. The development of T2D is likely a combination of genetic factors and lifestyle habits. T2D is associated with poor lifestyle habits such as lack of exercise and poor diet. Patients develop insulin resistance, which is the inability to utilize insulin at normal levels. Additionally, the liver, which normally stores glucose as glycogen, will

release abnormal amounts of glucose into the blood. Elevated triglycerides, low HDL-C, and LDL-C are evident in T2D (Kumar and Singh, 2010). Skeletal muscle, a target tissue for glucose uptake, becomes insulin resistant (Olefsky and Nolan, 1995). Management of T2D has been focused on lifestyle changes such as incorporating diet and exercise into routine, lowering cardiovascular risks, and managing glucose levels within normal range. Treatment for T2D typically involves anti-diabetic medications such as metformin, sulfonylureas, and thiazolidinediones. The glucose-lowering effect of metformin is due to its ability to reduce hepatic glucose production and increase peripheral glucose uptake (Bailey et al., 1996). Sulfonylureas bind to a receptor that is a component of the ATP-sensitive K+ channel in the beta cell. Upon binding, the ATP-sensitive K+ channels close, which will lead to increased insulin secretion. Thiazolidinediones are ligands for peroxisome-proliferator-activated receptor γ (PPARγ), a nuclear transcription factor found in adipose tissue. PPARγ is important for adipocyte differentiation, proliferation, fatty acid uptake, and storage. Thiazolidinediones act by promoting the fatty acid uptake and storage (Yki-Järvinen, 2004). Insulin treatment may be required as well.

Worldwide, diabetes affects approximately 171 million people, and this number is likely to increase to about 336 million by 2030 (Wild et al., 2004). According to the National Diabetes Data Sheet compiled by the Centers of Disease Control and Prevention (2011), 25.8 million people, or 8.3% of the population in the United States, is affected by diabetes. The American Diabetes Association reports that diabetes is the seventh leading cause of death but is likely to be underreported as a cause of death. The cost of diabetes is staggering with a total of $174 billion, of which $116 billion is due

to direct costs and $58 billion due to indirect costs such as disability, work loss, and premature mortality (Centers of Disease Control and Prevention, 2011). Moreover, the American Diabetes Association estimates that the total cost increases to $218 billion when factoring undiagnosed diabetes, prediabetes, and gestational diabetes.

Diabetic complications are characterized by changes in the macro- and microvasculature. Macrovascular changes in diabetes result in cardiovascular disease. An estimated 80% of diabetic patients die from cardiovascular disease. Of those deaths about 75% result from coronary artery disease (CAD) with the remaining being due to cerebrovascular disease, peripheral or other macrovascular disease (Cocherri, 2007). Separately, CAD and diabetes are associated with endothelial dysfunction; however, diabetes leads to further endothelial dysfunction in patients with CAD (Keymel et al 2011). Diabetic patients have a greater number of coronary arteries with diffuse distribution of atherosclerotic lesions compared to non-diabetics, and similar characteristics are seen in the peripheral vasculature (Milicevic et al., 2008). There is a one-to-four fold increase in stroke and stroke-related events due to diabetes (Cocherri, 2007).

Microvascular changes in diabetes can lead to poor wound healing, nephropathy, retinopathy, and neuropathy. Hyperglycemia leads to endothelial dysfunction by altering endothelial production of vasoconstrictor prostaglandins and affects endothelial cell matrix production leading to the thickening of the basement membrane (Hsueh and Anderson, 1992). Poor wound healing in diabetes is due to a host of factors, including impaired or decreased growth factor production and angiogenic response (Brem and Tomic-Canic, 2007). The angiogenic response is decreased because of reduced

vascular endothelial growth factor (VEGF) and stromal-derived growth factor-1α (SDF-1α). VEGF induces activation of endothelial nitric oxide synthase (eNOS), resulting in increased NO levels. NO triggers the mobilization of bone marrow endothelial progenitor cells into circulation. SDF-1α causes these cells to move to the site of injury (Brem and Tomic-Canic, 2007). Changes in the renal vasculature occur in diabetes with increased glomerular capillary growth in the early stages of the disease. Hyperglycermia is believed to induce protein kinase C and increase advanced glycation end-products that can lead to microvascular changes (Guo et al., 2005). Diabetic retinopathy constitutes a host of symptoms, including microaneurysms that will eventually lead to macular edema, ischemic changes, microvascular abnormalities, and proliferative changes (Antonetti et al., 2012). Diabetes can contribute to various neuropathies, such as abnormal sensation and pain (Obrosova, 2009). Cognitive impairment occurs because of inflammation in the brain, affecting the blood vessels and causing atrophy of brain tissue (Novak et al., 2011). Additionally, diabetes has also been linked to the development of Alzheimer's disease with both diseases having overlapping features such as poor glycemic control and impaired insulin function (Moreira et al., 2007). Insulin signaling is believed to be involved in generation of Aβ peptides as well as limiting its degradation. Furthermore, decreased glucose metabolism has been observed, which includes decreased enzyme activity (Moreira et al., 2007). Diabetes is also an independent risk factor for stroke and vascular dementias (Abbott et al., 1987), and changes in the blood-brain barrier may play a part in these disease processes.

Blood-brain barrier (BBB)

The brain is at the center of the central nervous system. It coordinates and integrates all the signals for the body to function properly. Therefore, the brain requires protection from the rest of the body without compromising its requirement for nutrients, such as oxygen and glucose. The BBB functions to protect the brain by inhibiting toxins in the peripheral circulation from entering the brain, while allowing for nutrients to enter through the barrier. Furthermore, it mediates waste efflux from brain to blood and maintains a constant environment in the brain.

The concept of the BBB originated with the studies of Paul Ehrlich in 1885 who observed that water-soluble dyes injected into the circulatory system stained all organs except for the brain and spinal cord (Hawkins and Davis, 2005). His student, Edwin Goldmann, injected dyes into the cerebral spinal fluid, which stained the brain but not the rest of the body (Hawkins and Davis, 2005). These observations suggested that a barrier must exist separating the brain and central nervous system from the peripheral circulation. Studies with silver nitrate demonstrated differences in the nervous system compared to the periphery (Dempsey and Wislocki, 1954). However, it was not until the 1960s when scientists were able to confirm the existence of a barrier at the structural level. Electron microscopy showed that high doses of exogenous peroxidase failed to penetrate the brain vascular endothelium and remained in the lumina of blood vessels and in a few micropinocytotic vesicles within the endothelium. This finding led to the conclusion that tight junctions probably were responsible for preventing intercellular passage of peroxidase (Reese and Karnovsky, 1967). Brightman and Reese (1969) demonstrated in separate experiments using intravascular injections of peroxidase and

lanthanum hydroxide that tight junctions were present, separating the vascular lumina from the underlying spaces. Furthermore, they confirmed that the tight junctions (as described below) in the vascular endothelium have a pentalaminar configuration, which is distinct from gap junctions that have a seven-layer configuration (Brightman and Reese, 1969).

The BBB is comprised of endothelial cells that line cerebral microvessels. These endothelial cells are characterized by few pinocytic vesicles, the absence of fenestrations, and a high number of mitochondria (Bernacki et al., 2008). They originate from permeable capillaries of the perineural vascular plexus and invade the embryonic neuroectoderm to form intraneural capillaries (Wolburg et al., 1994). The ability of the BBB to perform its functions is due to the presence of tight junctions, which hold the endothelial cells close together. The tight junction complex is composed of tight junction and adherens junction proteins (Figure 1). Tight junction proteins include junctional adhesion molecules (JAMs), occludin, claudins, and membrane associated guanylate kinase-like proteins. JAMs are single membrane-spanning proteins are involved in the maintenance and formation of the barrier (Abbott et al., 2006). Occludin is a transmembrane protein that functions to increase electrical resistance, and can interact with zona occludens protein 1 (ZO-1) in the cytoplasmic side (Hawkins and Davis, 2005; Abbott et al., 2006). The claudin family consists of four transmembrane domains, and claudin-3 and -5 are expressed in endothelial cells of the BBB. They seem to be the primary building protein of the tight junctions (Bernacki et al., 2008). The membrane associated guanylate kinase-like proteins are accessory proteins found in the cytoplasm that enable protein-protein interactions. Two of these proteins have

been identified at the tight junction: zona occludens-1 (ZO-1) and zona occludens-2 (ZO-2). ZO-1 links occludin to the actin cytoskeleton, and ZO-2 binds to structural constituents of the tight junction (Hawkins and Davis, 2005). Adherens junctions are also “tightening” structures between endothelial cells and are composed of cadherins. These cadherins bind to the cytoskeleton via α-, β-, and γ-catenins. It is thought that ZO-1 and catenins interact with each other, suggesting that tight and adherens junctions cooperate in maintaining the BBB (Bernacki et al., 2008).

The tight junction complex does not allow for paracellular transport of water-soluble agents and polar drugs. However, the endothelial cells have various transport systems to allow for nutrients from the peripheral circulation to enter the brain and for waste to efflux from brain to blood. Gases and lipophilic molecules are capable of transcellular movement. Glucose and amino acids have the transport carriers, GLUT-1 and LAT1 and others, respectively, to cross the BBB (Abbott et al., 2006). Some transport systems are energy-dependent, such as P-glycoprotein (Pgp), multidrug resistant protein (MRP), breast cancer resistance protein (BCRP), organic anion transporting polypeptides (OATP), and organic anion transporters (OAT), which efflux molecules from the brain into the blood and have the potential to reduce the penetration of drugs into the brain (Zlokovic, 2008). Pgp localizes on the luminal membrane of endothelial cells. MRPs are also found in the luminal membrane, and are able to transport organic anions and neutral organic drugs (Bernacki et al.,2008). OATPs are expressed on both luminal and abluminal membranes of endothelial cells, and are involved in the elimination of xenobiotics (Tamai et al., 2000), and amphipathic organic solutes (Bernacki et al, 2008). OATs, particularly OAT3, are expressed in brain

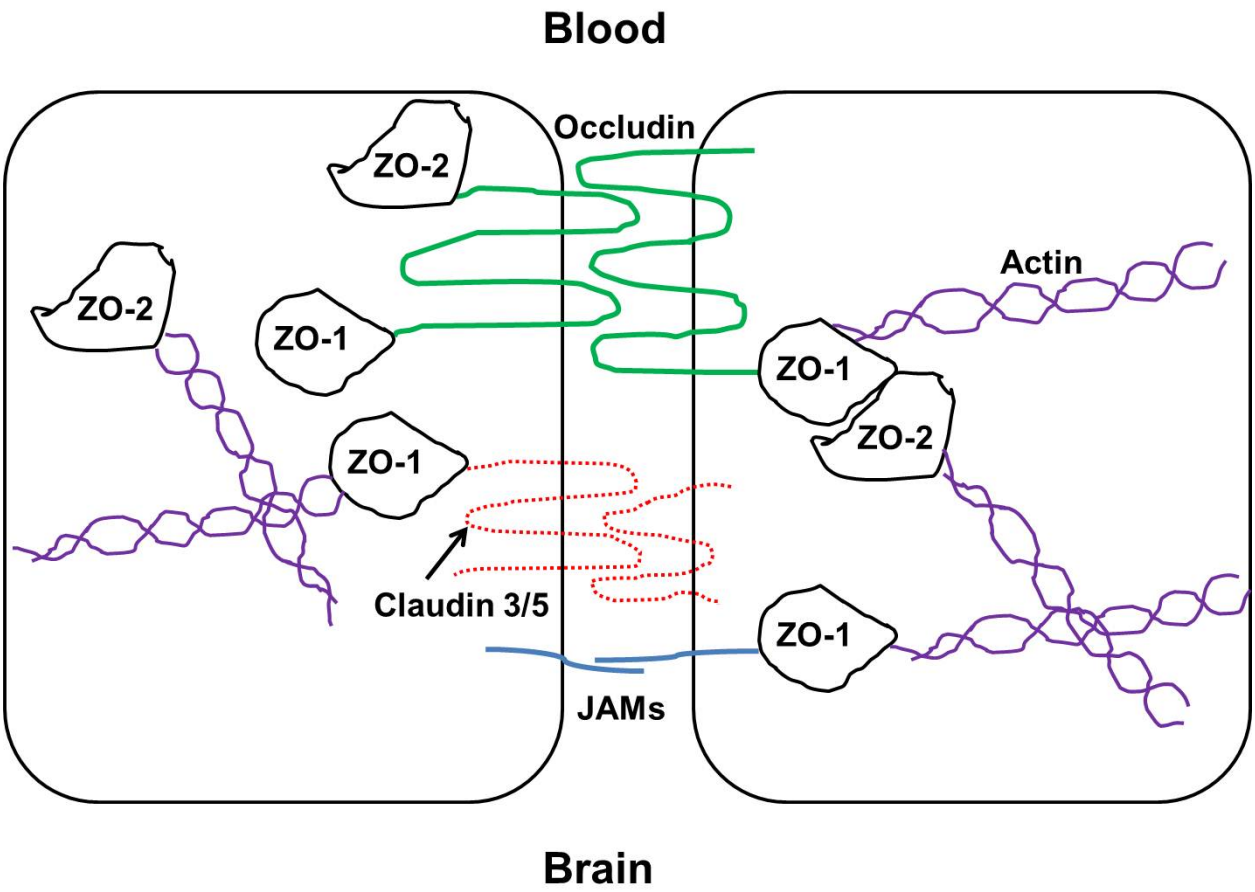


Figure 1.1. The organization of the tight junction. The tight junction is comprised of occludin, claudin 3/5, and JAMs, which form a tight seal between endothelial cells. The tight junction proteins also interact with the membrane associated guanylate kinase-like proteins, such as ZO-1 and ZO-2, that are found in the cytoplasm of the endothelial cells. ZO-1 and -2 link tight junction proteins to the actin cytoskeleton and other accessory proteins in the cytoplasm.

capillaries on the abluminal side, and its substrates include amphipathic organic anions, hydrophilic organic anions, and organic cations (Ohtsuki, 2004). Insulin and transferrin are taken up by receptor-mediated transcytosis while plasma proteins are transferred by absorptive transcytosis if they undergo cationization (Abbott et al., 2006). Ion transporters such as Na^+/K^+-ATPases, Na^+-K^+-$2Cl^-$ cotransporter, and the Na^+/H^+ and Cl^-/HCO_3^- exchangers are also present.

The endothelial cells that line the cerebrovasculature are supported and interact with other structures to form the neurovascular unit (NVU). The NVU comprises astrocytes, neurons, and pericytes interacting with the smooth muscle and endothelial cells of the microvessel (Hawkins & Davis, 2005; Abbott et al., 2006). Anatomically, astrocytes wrap around the microvessels with their endfeet making contact with the vessel wall (Nedergaard et al., 2003). Furthermore, astrocytes secrete various growth factors such vascular endothelial growth factor (VEGF) during retinal angiogenesis, and continue to form and maintain the blood-retinal barrier (Uemura et al, 2008). Astrocytes have a role in inducing BBB properties in vitro (Rubin et al., 1991). It has been reported that with astrocyte loss there is tight junction disruption and loss of BBB integrity (Willis et al., 2004). Neurons are highly metabolic, and therefore have a close relationship with endothelial cells. With astrocytes as the intermediary, neurons and endothelial cells have bi-directional communication. The arrangement of neurons and microvessels varies depending on location within the brain. In grey matter, microvessels grow in hexagonal arrays whereas, in white matter, they are arranged in line with the axons (del Zoppo, 2010). Neurons modulate BBB function, but it is not known if they are involved in BBB development (Hawkins and Davis, 2005). Pericytes encircle the wall of the

microvessel, and their processes can penetrate the basement membrane and make contact with the endothelium (Hirschi and D'Amore, 1996). They regulate microvessel diameter by constricting the vascular wall (Peppiatt et al., 2006; Bell et al., 2010). They also regulate endothelial cell activity and mediate inflammation (Bernacki et al., 2008).

Alterations in the BBB have been implicated in aging and many diseases. Research has focused on the role of BBB dysfunction in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (ALS). In Alzheimer's disease, there is upregulation of GLUT1 and increased glucose transport. Furthermore, there is also a decrease in Pgp, leading to an increase in amyloid-β (Abbott et al., 2006). Low activity of Pgp has been observed in Parkinson's disease (Abbott et al., 2006; Zlokovic, 2008). Leukocyte extravasation across the endothelium is believed to occur in multiple sclerosis, and BBB breakdown is thought to lead to the pathogenesis of ALS (Zlokovic, 2008). BBB dysfunction has also been implicated in inflammation, hypoxia/ischemia, pain, and human immunodeficiency virus-1 (HIV-1) infection (Hawkins and Davis, 2005; Abbott et al., 2006; Zlokovic, 2008).

The BBB in Diabetes

BBB dysfunction has been well established in diabetes. Changes in glucose and amino acid transport, structural and molecular alterations, and permeability have been observed in diabetic animal models. Studies regarding glucose transport during hyperglycemia have yielded mixed results. Gjedde et al. (1981) showed that glucose transport decreased in diabetic rats despite the fact that cerebral blood flow did not change in either the diabetic or control group. These results were supported by a study

by McCall et al. (1982). However, Pelligrino et al. (1990) demonstrated that glucose transport increased in chronically hyperglycemic diabetic rats compared to controls. However, other studies have shown that hypoglycemia increases glucose transport across the BBB (Simpson et al., 1999).

Transport of amino acids into the brain is important for its function. Many amino acids, such as tryptophan and tyrosine, are precursors for neurotransmitters in the brain and central nervous system. It has been shown that transport of the amino acids tryptophan, tyrosine, and lysine does not change in diabetic rats versus control. Ketone-body transport in the form of β-hydroxybutyrate is lower in diabetic animals and is restored or increased upon insulin treatment (McCall et al., 1982). Furthermore, glutamate transport at the BBB is not affected by diabetes in two rat models of diabetes (Hawkins, 2009). Additionally, elevated plasma amino acids leads to a reduced flux of large neutral amino acids across the luminal membrane of the BBB (Mann et al., 2003).

Structural and molecular changes of the BBB are evident in diabetes. Capillary basement membrane thickening occurs, and the density of transcytotic vesicles increases (Tolia et al., 2005). Additionally, there have been reports of changes in tight junction protein expression. It has been reported that diabetic rats not treated with insulin have a reduced occludin expression compared to insulin-treated or control rats whereas ZO-1 remained unaltered (Chehade et al., 2002). Hawkins et al. (2007) saw a similar decrease in occludin content, which together with an increase in MMP resulted in a permeability increase. As diabetes progressed, there was further disruption in the BBB and a region-specific permeability increase (Huber et al., 2006). Tight junction protein expression and permeability changes can be prevented with the administration

of seasmol, a natural antioxidant (VanGilder et al., 2009). Additionally, changes in the transport systems at the BBB have been observed in diabetes. Hawkins et al. (2007) have demonstrated decreased permeability to fluorescein, a marker for paracellular permeability as well as a substrate for OAT3 and MRP2, in diabetic rats. In their study, decreased permeability of fluorescein was in conjunction with an increase in MRP2 protein expression, but not OAT3 expression (Hawkins et al., 2007). Other transport systems and receptors involved in brain efflux are also affected in diabetes. It has been shown that low-density lipoprotein receptor related protein 1 (LRP1), a transporter at the BBB responsible for amyloid-β peptide (Aβ) clearance, is down-regulated in an animal model of diabetes, and clearance of Aβ is significantly decreased (Hong et al., 2009). Additionally, it has been demonstrated that the receptor for advanced glycation end products (RAGE) is up-regulated at the BBB leading to the deposition of Aβ in the brain (Liu et al., 2009).

Changes in permeability to various substances can be dependent on the duration of diabetes. Studies have demonstrated inconsistent results on albumin permeability. In one study, albumin permeability increased (Bouchard et al., 2002); however, in another study albumin permeability remained unaltered (Dai et al., 2002). Interestingly, permeability of sucrose did not change in an experimental model of diabetes as the disease progressed (Bradbury et al., 1991). Clinical studies using gadolinium-magnetic resonance imaging techniques showed a BBB permeability increase in diabetic patients compared to control patients (Starr et al., 2003). Lacunar infarcts, or small microvascular strokes, are common in diabetes, and it has been suggested that BBB failure, that is, leakage of the walls of small cerebral vessels, may be a mechanism

(Wardlaw et al., 2003). The mechanism of the observed permeability changes is not known. Other studies at the blood-retinal barrier showed similar events, and implicated vascular endothelial growth factor (VEGF) as likely candidate for the observed permeability change (Antonetti et al., 1998).

Vascular Endothelial Growth Factor (VEGF)

The VEGF family

The VEGF family consists of several members, VEGF-A, -B, -C, -D, -E, -F and placenta growth factor (PLGF), but the most studied is VEGF-A. They are dimeric glycoproteins that act through three cell surface receptor tyrosine kinases, known as the VEGF receptors (VEGFRs). VEGF-A, -B, and PLGF bind to VEGFR-1 (Flt-1); VEGF-A and –E bind to VEGFR-2 (Flk-1); VEGF-C and –D bind to VEGFR-3; and VEGF-F binds to both Flk-1 and Flt-1 (Olsson et al., 2006). Alternative splicing results in various forms of VEGF-A: VEGF-A_{121}, -A_{145}, -A_{165}, -A_{189}, and –A_{206} (Olsson et al., 2006), with VEGF-A_{165} being the most abundant human isoform (Wirostko et al., 2008) (Figure 1.2). VEGF-A is important for vasculogenesis (blood vessel growth from endothelial progenitor cells) and angiogenesis (blood vessel growth from pre-existing vessels). If there is inactivation of a VEGF allele, embryonic lethality occurs at embryonic day 11-12 in mice and tetraploid embryos, resulting in vascular defects and cardiovascular abnormalities (Carmeliet et al., 1996; Otrock et al., 2007; Lohela et al., 2009). VEGF expression is down-regulated after embryogenesis (Breier et al., 1992), but is up-regulated during physiological and pathological angiogenesis (Ferrera et al., 2003). Additionally, VEGF is important for neurogenesis, and reduced levels of VEGF leads to

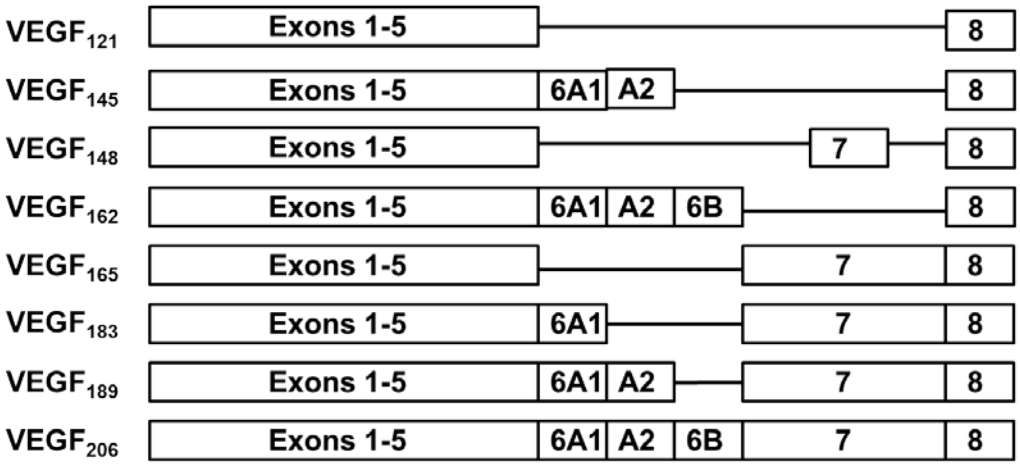


Figure 1.2. The isoforms of VEGF. Alternative splicing of VEGF yields various isoforms, with $VEGF_{165}$ being the most studied isoform.

neurodegeneration by impairing the perfusion of neural tissue (Storkebaum and Carmelier, 2004).

VEGF Receptors

The VEGF receptors are cell surface tyrosine kinase receptors. VEGFR-1, henceforth called Flt-1, acts as a decoy receptor by sequestering VEGF and regulates the interaction between VEGF and VEGFR-2 (Flk-1) by sequestering VEGF (Wirostko et al., 2008). Flt-1 is important for blood vessel development during embryogenesis, and plays an important role in pathological angiogenesis and wound healing by the interaction with Flk-1 (Cebe-Suarez et al., 2006). Flt-1 has poor catalytic activity compared to Flk-1 and the downstream signaling pathways upon its activation are not well known. Flk-1 is involved in permeability, angiogenesis, vasculogenesis, and proliferation (Holmes et al., 2007; Wirostko et al., 2008). VEGFR-3 is important for wound healing, and sFlt-1 (soluble VEGFR-1) scavenges available VEGF (Wirostko et al., 2008). Flk-1 and VEGFR-3 are also involved in lymphatic angiogenesis (Cebe-

Suarez et al., 2006). These receptors have an extracellular domain with immunoglobulin-like folds, followed by a transmembrane region, a tyrosine-kinase domain, and kinase insert, ending with a C-terminal tail (Wirostko et al., 2008). VEGF exerts its effect by binding to Flt-1 and Flk-1 receptors as well as the neuropilin receptors, NP-1 and NP-2. VEGF binding leads to dimerization and subsequent autophosphorylation. This action can lead to either increased permeability or angiogenesis (Figure 1.3).

Flk-1 activation can lead to proliferation, migration, survival, and permeability. Proliferation is due to the phosphorylation of tyrosine 1175, leading to Erk phosphorylation via PLCγ recruitment and a PKC-dependent pathway. Upon activation at tyrosine 1175, PLCγ is phosphorylated and then hydrolyzes phosphatidylinositol (4,5)-bisphosphate into diacyglycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3). IP_3 increases intracellular calcium while DAG activates protein kinase C (PKC), which can then activate the Erk signaling pathway.

Migration of endothelial cells can occur due to the phosphorylation of either tyrosine 1214 or 951. Phosphorylation of tyrosine 1214 allows for the activation of the p38 MAPK signaling pathway and recruitment of Hsp27 for actin remodeling. Tyrosine 951 activation leads to phosphorylation of Src and subsequent actin reorganization. Migration can also occur with the phosphorylation of tyrosine 1175, with the subsequent activation of PI3K and formation of $PIP_{3.}$ PIP_3 leads to activation of Rho, which ultimately leads to actin reorganization and migration. Phosphorylation of tyrosine 1175 can also lead to migration via the recruitment of FAK, followed by focal adhesion turnover. Cell survival occurs with the phosphorylation of tyrosine 1175, leading to

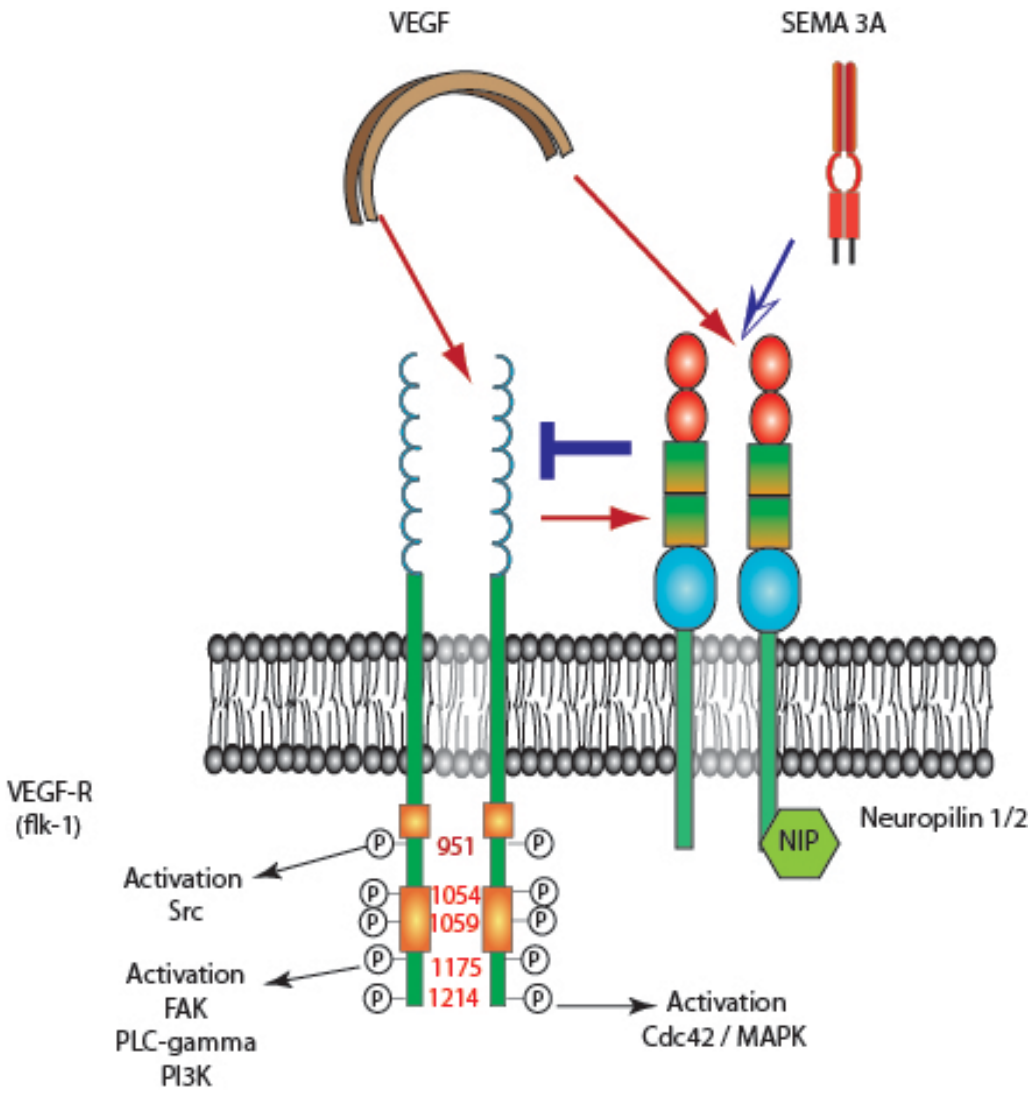


Figure 1.3. Schematic diagram of VEGF signaling. VEGF is able to bind to two classes of receptors: VEGFRs and the neuropilins. The VEGFRs are Flt-1 and Flk-1, a decoy receptor and the active receptor, respectively. Neuropilins enhance VEGF/Flk-1 signaling and are required for full activation of Flk-1. The binding of VEGF to the receptors causes dimerization and autophosphorylation of specific tyrosine residues on the Flk-1 receptor. Phosphorylation of these tyrosine residues leads to signaling pathways involved in permeability, migration, and proliferation. SEMA 3A is an antagonist of VEGF, and exerts its effects by binding to the neuropilin receptors, thereby blocking VEGF from binding to this class of receptors. SEMA 3A is also known as a permeability factor as well, independent of VEGF-induced permeability. (Figure courtesy of Dr. Richard Egleton)

PIP_3 formation via PI3K. PIP_3 causes phosphorylation of Akt/PKB, which inhibits the pro-apoptotic protein, Bad, and caspase-9 activation.

Permeability of the endothelium occurs via activation of tyrosine 951 or 1175. Phosphorylation at tyrosine 951 leads to activation of Src and actin reorganization as seen during migration. At tyrosine 1175, PI3K is activated, followed by PIP_3 formation. As with cell survival, PIP_3 causes phosphorylation of Akt/PKB; however, Akt will lead to the activation of endothelial nitric oxide synthase (eNOS). eNOS will lead to an increase in nitric oxide (NO) production, which will facilitate a permeability increase in endothelial cells.

Neuropilin Receptors

The neuropilin receptors are transmembrane glycoproteins important for axonal guidance, angiogenesis, tumorigenesis, and are involved in the immunologic response (Sulpice et al., 2008). The receptors denoted NP-1 and NP-2 act as co-receptors for class III semaphorins (see next section) and members of the VEGF family. They have large extracellular domains and a short cytoplasmic tail that lacks enzymatic activity (Sulpice et al., 2008). However, the lack of enzymatic activity remains unclear as tumor cells lack the Flk-1 receptor, but express neuropilin receptors (Geretti et al., 2008). Their extracellular domains consist of (1) a signal sequence; (2) two complement factors, a1 and a2; (3) two coagulation factors, b1 and b2; and a (4) c domain, which is homologous to A5. The length of these domains is similar between the two receptors, but they have varying degrees of conservation (Kolodkin et al., 1997). The binding site for VEGF is located in the b1 and b2 domains whereas SEMA 3A requires both a and b

domains (Geretti et al., 2008; Sulpice et al., 2008). Furthermore, NP-1 and NP-2 bind to VEGF-A_{165} with different affinities

NP-1 was initially identified as an antigen to the antibody, A5, which was raised against proteins in the optical tectum of *Xenopus* tadpoles (Geretti et al., 2008; Pellet-Many et al., 2008). NP-1 has a large extracellular domain followed by a short transmembrane domain and an intracellular domain. It is expressed in neural tissues, particularly during development of the central nervous system, but is also present in non-neuronal tissues in adults particularly in the placenta, heart, tumor-derived cells, and endothelial cells (Staton et al., 2007). In the vasculature, it is primarily expressed on the arterial end of endothelial cells (Staton et al., 2007; Sulpice et al., 2007). NP-1 can undergo post-translational modification by the addition of glycosaminoglycan in the linker region between b2 and c domains (Pellet-Many et al., 2008). In endothelial cells, this modification of NP-1 enhances the VEGF/Flk-1 signaling, perhaps by stabilizing the Flk-1 receptor and preventing its internalization and degradation (Shintani et al., 2006). Furthermore, VEGF interaction with NP-1 is required for full activation of the Flk-1 receptor (Sulpice et al., 2008). Mutations in NP-1 results in vascular defects, including impaired neural vascularization, defects in the great vessels, heart outflow tracts, and in the vasculature of yolk sacs (Kawasaki et al., 1999). In addition to VEGF-A_{165}, NP-1 can bind to VEGF-B, -E, and PLGF (Koch et al., 2011).

NP-2 was originally identified as an alternative receptor to the semaphorins involved in axonal guidance (Kolodkin et al., 1997; Staton et al., 2007). It is expressed in endothelial cells, primarily at the venous side as well as in lymphatic endothelial cells (Staton et al., 2007). The receptor binds to VEGF-A_{165}, VEGF-A_{145}, VEGF-C, and

VEGF-D (Soker et al., 1998; Koch et al., 2011; Staton et al., 2007). NP-2 co-localizes and directly interacts with VEGFR-3 in lympathic vessels (Karpanen et al., 2006; Staton et al., 2007). Defects in NP-2 result in abnormalities in lymphatic vessels and capilliaries (Staton et al., 2007). As with NP-1, NP-2 enhances the affinity of VEGF to Flk-1 and increases its phosphorylation (Shraga-Heled et al., 2006; Sulpice et al., 2008).

The neuropilin receptors are involved in both physiological and pathological angiogenesis. Neuropilins are believed to be involved in angiogenesis during the menstrual cycle because both NP-1 and NP-2 are expressed in endothelial cells in response to estrogen and progesterone (Staton et al., 2007). Additionally, it has been shown that NP-1 expression is up-regulated during wound healing (Staton et al., 2007; Matthies et al., 2002). However, it has been documented that mice which express NP-1 without its cytoplasmic domain undergo normal vasculogenesis and angiogenesis (Fantin et al., 2011). On the other hand, the role of neuropilins has been well documented in pathological angiogenesis. Overexpression of NP-1 resulted in increased tumor vascular density (Miao et al., 2000; Bagri et al., 2009). NP-1 is expressed in many human cancer cell lines (Bagri et al., 2009) with varying levels of expression (Staton et al., 2007). In pancreatic adenocarcinoma, NP-1 and NP-2 exhibited higher mRNA expression levels than Flt-1 and Flk-1 (Fukahi et al., 2004).

Semaphorin 3A (SEMA 3A)

The semaphorin family is a group of proteins that is subdivided by class specific domains and domain organization. These proteins are subdivied into eight classes, 1-7 and V. Classes 2, 3, and V semaphorins are secreted, and all other classes of

semaphorins are membrane bound (Tran et al., 2007). Semaphorins are best known in participating in both short and long range axonal cues. They mostly act as a repellent to maintain nerve bundling, process extension, and sculpting arborization patterns (Tran et al., 2007). Semaphorins execute their functions via the plexin receptors, which can be divided into four classes, A-D.

In addition to axonal cues, semaphorins, particularly class 3 semaphorins, regulate vascular development, tumor progression, metastasis, the immune response, and platelet function (Bielenberg and Klagsbrun, 2007). SEMA 3A also modulates blood vessel formation through the inhibition of integrin activity (Soker et al., 1998; Acevedo et al., 2008). SEMA 3A interacts indirectly with the plexin A1 receptor, but it requires NP-1 and NP-2 as a co-receptor to assemble the ligand-receptor complex and cause activation (Tran et al., 2007). Little information is known on the role of plexins in the receptor complex (Casazza et al., 2011). It has been shown that SEMA 3A disrupts VEGF-mediated migration of endothelial cells (Miao et al., 1999; Acevedo et al., 2008). Additionally, SEMA 3A is anti-angiogenic within the tumor vasculature (Maione et al., 2009) and reduces the number of pericyte-coated vessels (Casazza et al., 2011). SEMA 3A deletion leads to excess endothelial cells in the capillaries of the kidney, and its overexpression results in the down-regulation of Flk-1 (Reidy et al., 2009). Because VEGF and SEMA 3A share a common receptor, it is likely that SEMA 3A regulates VEGF function. Indeed Acevedo et al. (2008) showed that SEMA 3A suppresses VEGF-mediated angiogenesis but also acts as a permeability factor. SEMA 3A antagonizes VEGF interaction with the neuropilin receptor and prevents the dimerization and angiogenesis.

The Streptozotocin (STZ) Model of Diabetes

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is a nitrosourea analogue synthesized by *Streptomycetes achromogenes* and has been used clinically as an antimicrobial and chemotherapeutic agent (Figure 1.4). In research settings, it has also been widely used to induce insulin-dependent, or T1D. Alloxan, another drug used to induce T1D, has been used in research settings, but there are some differences between alloxan and STZ: (1) The selectivity of STZ on β-cells is greater than alloxan; (2) the dosage range is broader in alloxan administration than with STZ; (3) a subdiabetic dose of STZ, followed by a second identical dose leads to increased diabetogenic activity, which is not seen with alloxan; and (4) the diabetogenic activity of STZ is not influenced by changes in nutritional state, unlike the activity of alloxan in which changes in sensitivity are induced by fasting or feeding (Junod et al., 1969). Alloxan can also affect the liver by decreasing non-protein and protein-bound sulhydryl groups, which could lead to inactivation of enzymes (Szkudelski et al., 1998). Additionally in comparison with alloxan, STZ produces permanent diabetes, which is ideal for T1D studies, and its use is more reproducible and better for the induction of diabetes that is appropriate for endocrine and metabolic studies (Karunanayake et al., 1974).

Diabetes results from beta cell toxicity and subsequent beta cell death through necrosis (Lenzen, 2008). These events occur because STZ is transported into the beta cells of the pancreas via the GLUT2 transporter and causes methylation of nucleic acids and proteins (Konstantinov and Berger, 2008). The cell attempts to overcome this damage by the activation of poly(ADP-ribose) polymerase (PARP), which then leads to

depletion of pyridine nucleotides, nicotine adenine dinucleotide (NAD) and its reduced form, NADH, and subsequently, ATP (Lenzen, 2008) (Figure 1.5). Additionally, there is speculation that STZ induces diabetes through oxidative stress. STZ-induced diabetes can also lead to an increase in nitric oxide (NO) production (Figure 1.5). NO acts as a modulator of oxidative stress, and is also a free radical in the form of NO·. Studies have shown that pancreatic islets have higher nitrate/nitrite levels in STZ-treated animals versus control, and that NO had deleterious effects on the enzymes that are protective in oxidative stress (Gonzalez et al., 2000). It has also been reported that NO-synthase (NOS) inhibitors, such as N^G-nitro-L-arginine-methyl ester (L-NAME), can lead to attenuation of STZ-induced hyperglycemia in mice (Kolb et al., 1991). Haluzik and Nedvidkova (2000) demonstrated that two NOS inhibitors, L-NAME and methylene blue, were able to partially suppress the development of STZ-induced diabetes.

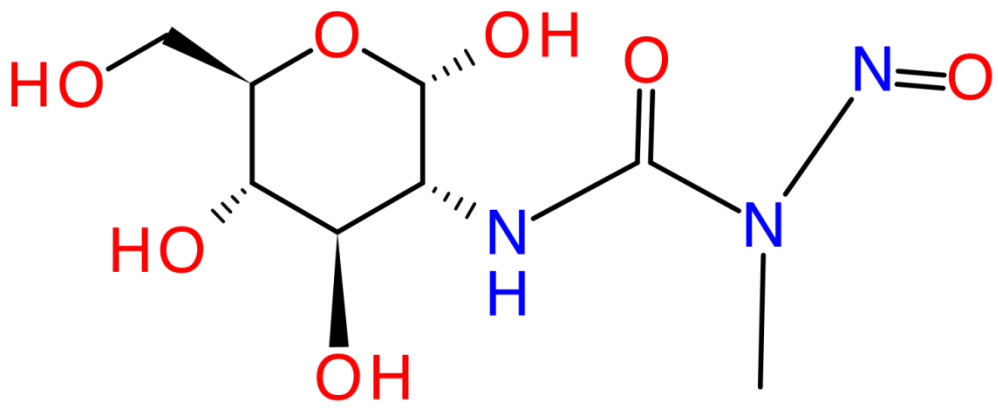


Figure 1.4. Chemical structure of streptozotocin.

Streptozotocin can be injected intravenously in adult rats as well as intraperitoneally at concentrations between 40 to 60 mg/kg or higher (Szkudelski, 2001). There is a triphasic glucose response upon streptozotocin administration (Lenzen, 2008). During the first phase, hyperglycemia with a decrease in blood insulin is evident within two hours of STZ administration. Beta cells exhibit morphological changes such as intracellular vacuolization, dilation of the rough ER, decreased area of the Golgi, reduced secretory granules and insulin content, and swollen mitochondria (Lenzen, 2008). Hypoglycemia, along with high levels of blood insulin, occurs after six hours. The last phase is hyperglycemia and a decrease in blood insulin levels (Szkudelski, 2001).

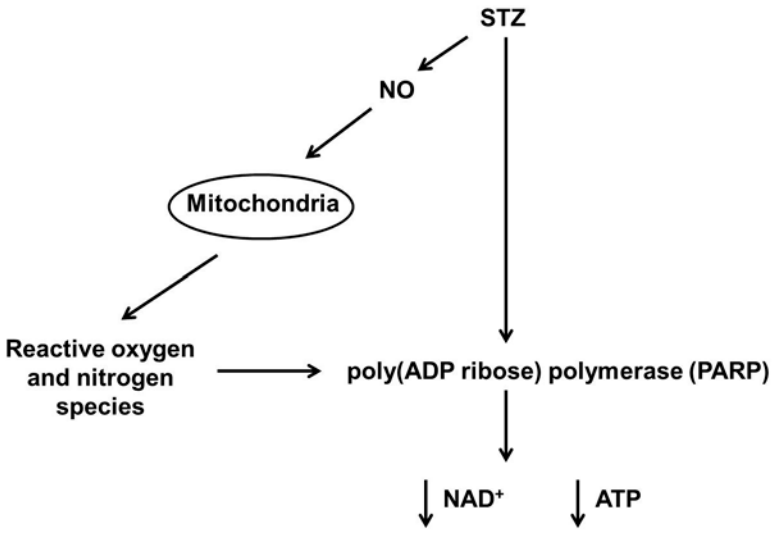


Figure 1.5. Mechanism of STZ-induced diabetes. STZ is able to enter the pancreatic beta-cell via the GLUT2 transporter. It primarily induces DNA alkylation, but can also affect proteins. This leads to activation of PARP and subsequent reduction of NAD^+, NADH, and ATP. Additionally, STZ is also able to lead to an increase in nitric oxide production and development of free radicals, which can result in DNA damage as well.

STZ is primarily administered via single injection; however it can be administered with multiple injections depending on concentration. The half-life of STZ is about 15 minutes. Interestingly, beta cell necrosis begins within 24 hours after injection (Like and Rossini, 1976). Using radiolabelling studies, Karunanayake et al. (1974) demonstrated that after 15 minutes there was intense accumulation of STZ in the kidney and STZ liver uptake reached its maximum at 30 minutes after injection. Thus, it seems likely that only a small amount of STZ is required to reach the pancreas to induce its toxic effects.

Hypothesis and Specific Aims

In diabetes, BBB permeability increases. However, the mechanism of this permeability change is not known. The goal of this project is to determine the mechanism of the permeability increase using the streptozotocin (STZ) model of diabetes, which is a well characterized type I model. I hypothesized that the increase in permeability at the BBB is due to VEGF and its interaction with the Flk-1 receptor, and subsequent activation of a signaling pathway involved in permeability. I also hypothesized that these BBB changes can be attenuated by administration of a possible therapeutic agent, minocycline.

Specific Aim 1: To investigate if minocycline is able to attenuate the observed BBB changes in an STZ model of diabetes.

Specific Aim 2: To investigate the role of the VEGF signaling system as a mechanism for the observed BBB changes.

Chapter 2: Minocycline Decreases Blood-Brain Barrier Permeability Yet Increases Vascular Endothelial Growth Factor in the Streptozotocin Model of Diabetes

Abstract

Diabetes is associated with increased permeability and a decrease in tight junction protein expression at the blood-brain barrier (BBB). There are no clinical treatments to attenuate the BBB changes in diabetes, though several strategies have been used in animal models. Minocycline (MIN), a tetracycline antibiotic, has anti-inflammatory properties and is able to cross the BBB. Minocycline attenuates BBB disruption via a number of proposed mechanisms including inhibition of microglia activation, antioxidant activity, and matrix metalloproteinase inhibition. Minocycline may protect against the previously reported changes in diabetic BBB function. This study tests whether minocycline can attenuate the BBB changes observed in the streptozotocin (STZ) model of diabetes. Minocycline decreased permeability and increased expression of tight junction proteins, especially claudin-5. Furthermore, minocycline increased protein expression of vascular endothelial growth factor (VEGF), a potent angiogenic and permeability factor, in cerebral microvessels. Additionally, minocycline treatment increased in protein expression of Flt-1. These results indicate that minocycline may be a potential therapeutic agent to decrease the permeability changes observed at the BBB in diabetes.

Introduction

The blood-brain barrier (BBB), a semi-permeable vascular interface, is located at the level of brain microvascular endothelial cells. The BBB regulates the CNS extracellular environment by restricting entry from blood into brain. It allows nutrients from the periphery to pass and mediates waste efflux from brain to blood. However, in many pathological conditions, the barrier and its functions are compromised, which can lead to edema and leukocyte transmigration across the barrier. These events can activate microglia, the immune cells of the CNS, which generate immunomodulatory molecules (Yenari et al., 2006).

In neurodegenerative diseases, such as ischemia, Parkinson's disease, spinal injury, multiple sclerosis, and others, treatment with minocycline, a tetracycline antibiotic and anti-inflammatory agent, results in neuroprotection. Minocycline is of particular interest in these diseases because it crosses the BBB and reaches therapeutic concentrations in the brain (Zemke and Majid, 2004). Minocycline inhibits microglial activation, and functions to decrease apoptosis and the production of reactive oxygen species (Plane et al., 2010). Minocycline exerts its anti-inflammatory effects by decreasing proliferation and activation of microglia, which leads to decreased release of cytokines, matrix metalloproteinases, and other inflammatory mediators (Kim and Suh, 2009). It also inhibits transmigration of T lymphocytes (Kim and Suh, 2009). Minocycline also has both caspase-dependent and –independent anti-apoptotic effects. Last, minocycline is involved in signaling pathways, particularly in the inhibition of p38 MAPK activation in microglia (Hau et al., 2005). Minocycline is protective in a rat model of ischemia (Yrjanheikki et al., 1999; Zemke and Majid, 2004). Wasserman and

Schlichter (2007) demonstrated that administration of minocycline protected the BBB and reduced edema after intracerebral hemorrhage. In the retina, minocycline stimulates chemotaxis and decreases proliferation of retinal pigment epithelial cells, and also promotes the expression of platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and MMP-9 (Hollborn et al., 2010).

Diabetes is a risk factor for stroke and other cerebrovascular diseases. Clinical studies using magnetic resonance imaging-gadolinium techniques have shown that the BBB exhibits increased permeability (Starr et al., 2003). This permeability increase has also been shown in the STZ model of diabetes, in conjunction with decreased tight junction protein expression of occludin and zona occludins-1 (ZO-1) as well as an increase in MMP-2, which may also contribute to BBB dysfunction (Hawkins et al., 2007). The increased BBB permeability can be attenuated by therapeutic intervention through statins (Mooradian et al., 2005) and sesamol, a natural antioxidant (VanGilder et al., 2009).

The mechanism of this BBB permeability change, however, has not been elucidated. Other studies using the STZ model have shown similar changes in the blood-retinal barrier and demonstrated that VEGF plays a role in the permeability changes (Antonetti et al., 1998). VEGF functions as a potent pro-angiogenic and permeability factor, and its signaling system is a likely candidate for the permeability changes occurring at the BBB. VEGF signaling occurs via its receptors, Flt-1 and Flk-1, as well as its co-receptors, neuropilin-1 and -2 (NP-1 and NP-2) and endogenous antagonist, semaphorin 3A (SEMA 3A). NP-1 and NP-2 enhances VEGF binding to Flt-

1 and Flk-1 receptors. SEMA 3A binds to the neuropilin receptors, thus inhibiting the ability of VEGF to interact with these co-receptors. Interestingly, SEMA 3A suppresses angiogenesis and also acts as a permeability factor through the neuropilins (Acevedo et al., 2008). In this study, we investigate whether minocycline can attenuate the functional and molecular changes at the BBB in the STZ model of diabetes. Furthermore, we examined the VEGF signaling system in the diabetic brain microvasculature, and whether minocycline has any effect on its expression.

Methods

Animal Care

Animal procedures were performed in strict accordance with the Institutional Animal Care and Use Committee, Marshall University. Male Sprague-Dawley rats (Hilltop Laboratories, Scottdale, PA) were injected with either 65 mg/kg STZ (Sigma, St. Louis, MO) or 0.9% sterile saline. Seven days after STZ injection, animals were injected with 22 mg/kg minocycline or 0.9% sterile saline twice daily for the reminder of the study. The following conditions were maintained: 12h-12h dark-light cycle, with ambient temperature of 22 ± 2º C, food and water given *ad libitum*. Animals were euthanized 14 days after STZ injection via intramuscular injection of a 1 mL/kg cocktail containing ketamine (71.5 mg/mL) and xylazine (5.7 mg/mL). Blood samples were collected from the descending aorta, and tested for glucose, ketones, and lipids with an analyzer (CardioChek PA, Polymer Technology Systems, Indianapolis, IN).

In situ perfusion

In situ perfusion studies were performed in the laboratory of Dr. Richard Egleton at the University of Arizona. Rats were anaesthetized with ketamine and heparinized by intra-peritoneal injection (10,000 U/kg). The common carotid arteries were exposed after a midline incision was made at the neck. Both arteries were cannulated, and the jugular veins were sectioned. The brain was then perfused with an oxygenated mammalian Ringer solution [in mM: 117 NaCl, 4.7 KCl, 0.8 $MgSO_4$, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, and 10 D-glucose, with 39 g/l dextran (mol wt 70,000) and 10 g/l BSA, pH 7.4] with Evans blue at 37°C. When the desired perfusion pressure and rate of ~100 mmHg and 3.1 ml/min was reached, [^{14}C]sucrose (10 µCi/20 ml Ringer) was infused via a slow-drive syringe pump (0.5 ml·min^{-1}·hemisphere^{-1}; model 22, Harvard Apparatus, South Natick, MA). After a 20 minute perfusion (n = 4-6 per treatment), the rat was decapitated and the brain was removed. Samples of the radioactive perfusate were collected from each carotid cannula as a reference. Next, the choroid plexuses and meninges were removed, and the cerebral hemispheres were sectioned and homogenized. Brain tissue and 100 µl samples of perfusate were incubated for two days in 1 ml of tissue solubizer (TS-2, Research Products, Mount Pleasant, IL) in preparation for liquid scintillation counting. Finally, 100 µl of 30% acetic acid and 4 ml of Budget-Solve Liquid Scintillation Cocktail (Research Products) were added, and the samples were measured for radioactivity (model LS 5000 TD Counter; Beckman Instruments, Fullerton, CA). Results are reported as a distribution volume (R_{br}) of radioactivity in the brain to that in the perfusate:

$$R_{br} = \frac{dpm.g^{-1}\ brain\ tissue}{dpm.ml^{-1}perfusate}$$

Cerebral Microvessel Isolation

Cerebral microvessel isolation was performed as described by Hawkins et al., 2007. After anesthetization, brain was removed and placed in a petri dish with a buffer solution A (for 1 L: 6.02 g NaCl, 0.35 g KCl, 0.368 g $CaCl_2$, 0.163 g KH_2PO_4, 0.296g $MgSO_4$, 3.57 g HEPES, pH 7.4, 4^{o}C). Under a dissecting microscope, meninges and choroid plexus were removed. The cortex was dissected from brainstem and cerebellum. The whole brain was minced and homogenized in 5 mL buffer solution B (for 1L: 6.02 g NaCL, 0.35 g KCl, 0.368 g $CaCl_2$, 0.163 g KH_2PO_4, 0.296g $MgSO_4$, 3.57 g HEPES, 2.1 g $NaHCO_3$, 0.11 g sodium pyruvate, 10 g dextran (~64 kDa), 1.8 g glucose, pH 7.4, 4^{o}C). The homogenate was poured into a 50 mL tube through a 100 µm cell strainer. The homogenate was transferred to a 30 mL centrifuge tube (Beckman Ultra-Clear), 20 mL of 26% dextran was added and spun for 10 minutes at 5800 g. After spinning, the thick white layer at the top of tube was removed. A pale white streaky pellet on the side of the tube remained, resuspended in 10 mL buffer solution B, and passed through a 70 µm cell strainer. The suspension was transferred to a 30 mL centrifuge tube and spun at 500 g for 10 minutes. The supernatant was removed, and the final microvessel pellet was resuspended in either tissue extraction buffer (ThermoScientific, Rockford, IL) for protein extraction or TriReagent (Sigma, St. Louis, MO) for RNA isolation. Protein was analyzed using bicinchoninic acid (BCA) protein assay kit (ThermoScientific, Rockford, IL).

Western Blot Analysis of Immunoreactive Protein

Protein samples (20 µg/well) were separated via SDS gel electrophoresis using 4-12% Bis-Tris gel (Criterion, Bio-Rad, Hercules, CA) followed by transfer to

nitrocellulose paper. Occludin, claudin-5, and ZO-1 antibodies were purchased from Invitrogen, Life Technologies (Grand Island, NY). Antibodies directed against VEGF, VEGF receptors (Flt-1, Flk-1, NP-1, and NP-2), and SEMA 3A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (EMD Millipore, Billerica, MA) were used to normalize protein loading. Protein bound to each antibody was detected by horseradish peroxidase conjugated secondary antibody (GE Healthcare Bio-Sciences, Piscataway, NJ), and detected by the enhanced chemiluminescence system (GE Healthcare Bio-Sciences, Piscataway, NJ).

Data Analysis

The data are reported as the mean and standard error of the mean. Statistical analysis for all studies was via ANOVA followed by Student-Newman-Keuls analysis.

Results

Minocycline did not prevent STZ-induced changes in blood chemistry

Table 2.1 lists the selected blood chemistries of the animals in this study. There was a significant increase in blood glucose levels in STZ-treated animals compared to control animals, which was not affected by minocycline treatment. Both STZ groups had high blood triglyceride levels, but animals treated with both STZ and minocycline had a statistically significant increase in triglycerides compared to control. Figure 2.1 shows that STZ significantly reduced weight gain in the animals; however, this was not modulated by minocycline. Additionally, there was no change in brain weights in any of the treatment groups (Figure 2.2).

Minocycline reversed STZ-induced increase in BBB permeability to sucrose

In situ perfusion studies (Figure 2.3) showed that STZ-treated animals had a significant increase in BBB permeability to sucrose compared to control animals. Treatment with minocycline lead to a significant decrease in BBB permeability to sucrose in both control and STZ-treated animals ($p<0.05$, two way ANOVA, followed by Newman-Keuls; n=6 per group).

Minocycline increased claudin-5 expression independently of STZ treatment

Western analysis showed that neither STZ nor minocycline treatment affected ZO-1 (Figure 2.4) or occludin (Figure 2.5). Although STZ did not have an effect on claudin-5 protein expression, treatment with minocycline resulted in a significant increase in claudin-5 in both control and STZ-treated animals (Figure 2.6).

Minocycline increased protein expression of VEGF and Flt-1

Western analysis showed that minocycline significantly increased VEGF protein levels in in STZ-treated animals with minocycline compared to STZ-animals treated with saline (Figure 2.7). There was also a significant increase in the expression of Flt-1 in the control animals treated with minocycline compared to the saline-treated controls. A similar trend was seen in the STZ-treated animals, though this did not reach significance (Figure 2.8). Minocycline did not alter Flk-1 expression in diabetic rats (Figure 2.9). Additionally, minocycline treatment had no significant effect on NP-1 (Figure 2.10) and NP-2 (Figure 2.11) protein levels in diabetic animals. The levels of SEMA 3A protein expression were not significantly decreased with minocycline treatment in either control or STZ-treated rats (Figure 2.12). Interestingly, there was a

	Control + Saline	Control + Minocycline	STZ + Saline	STZ + Minocycline
Glucose (mg/dl)	212 (12.4)	219 (13.5)	453 (35)**	427 (37)**
Ketones (mg/dl)	6.93 (0.80)	6.99 (1.44)	13.07 (2.69)	13.31 (2.20)
Triglycerides (mg/dl)	58.5 (8.5)	53.0 (3.0)	98.3 (21.9)	137 (23.4)**

Table 2.1. Blood chemistries of control and diabetic animals treated with minocycline. STZ treatment significantly increased blood glucose (**$p<0.05$, two-way ANOVA followed by Student-Newman-Keuls). Minocycline had no effect on diabetes since it did not prevent the STZ-induced increase in blood glucose. Ketones and triglycerides were elevated in STZ groups. (n=8 for Control + Saline; n=9 for Control + Minocycline; n=10 for STZ + Saline; n=9 for STZ + Minocycline)

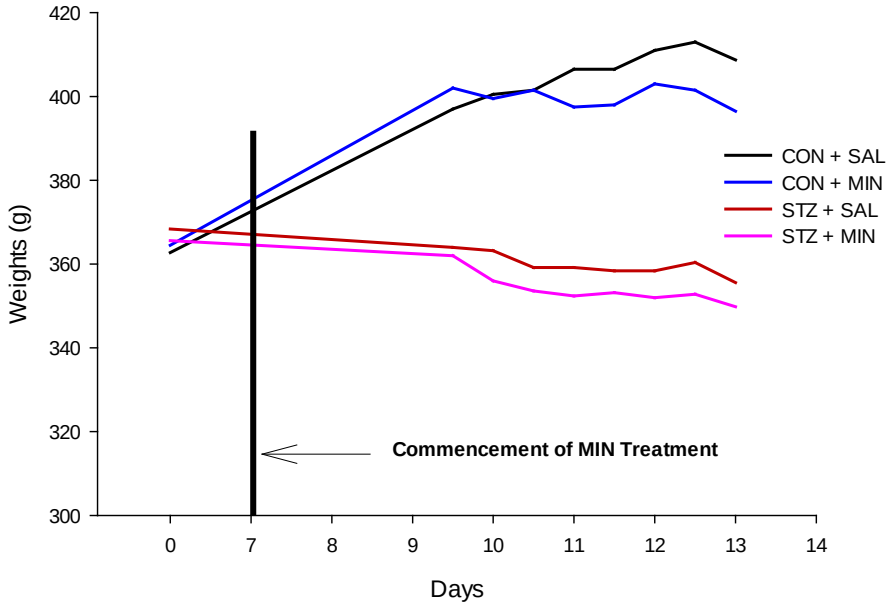


Figure 2.1. Minocycline did not prevent weight loss in STZ-treated animals. STZ treatment significantly reduced body weight in the animals ($p<0.01$ compared to control, two-way ANOVA followed by Student-Newman-Keuls, n=10-14 per group). Treatment with minocycline had no effect on body weight.

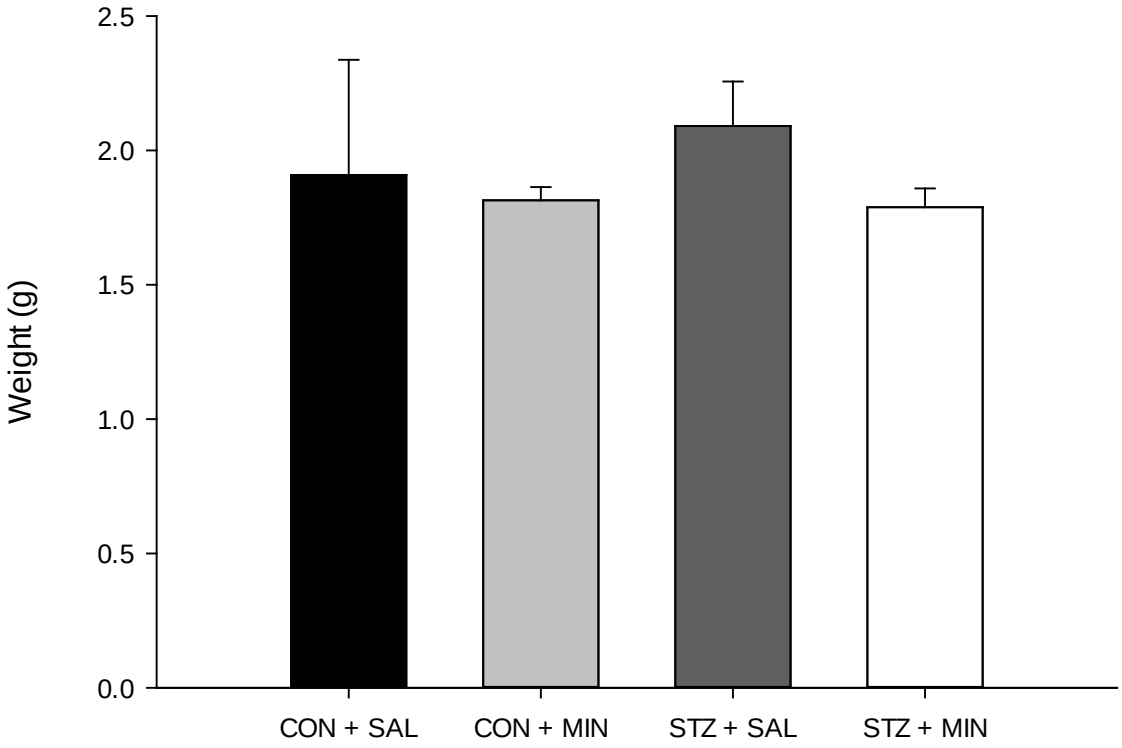


Figure 2.2. No effect of STZ or minocycline on brain weight. Treatment with STZ or minocycline did not affect whole brain weights in the animals. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

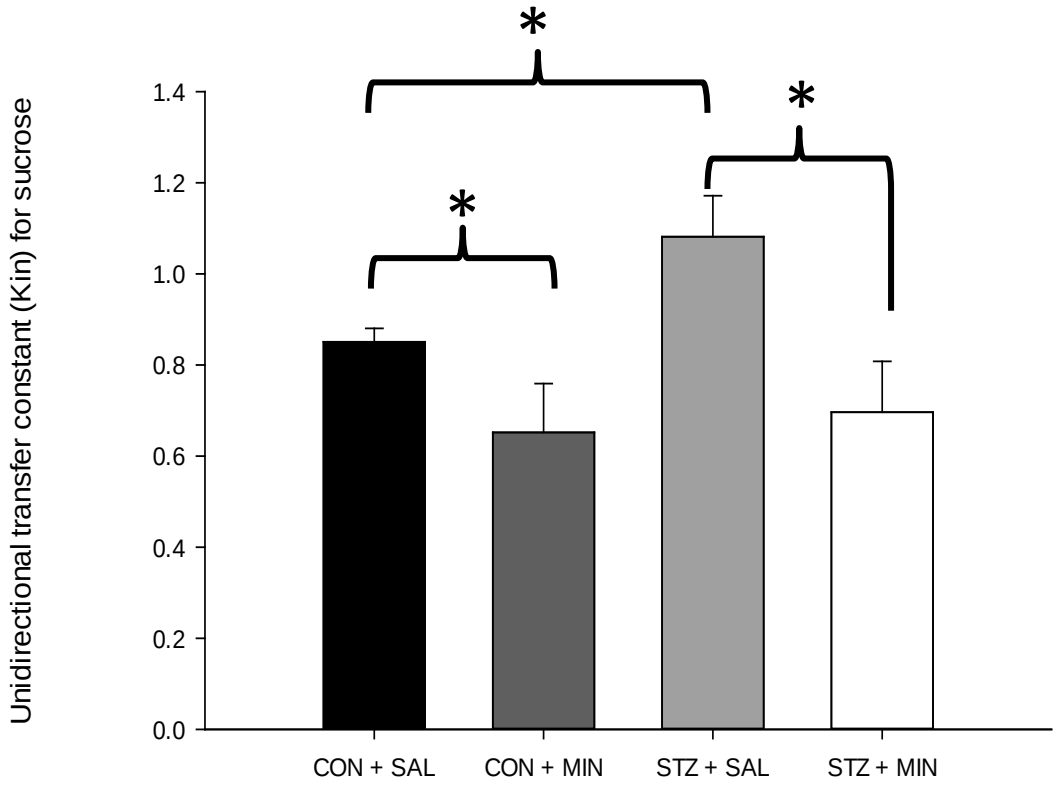


Figure 2.3. Minocycline reversed STZ-induced increase in BBB permeability. STZ significantly increased BBB permeability to sucrose. Treatment with minocycline significantly reduced permeability of the BBB to sucrose in both control and STZ-treated animals ($p<0.5$, two-way ANOVA followed by Newman-Keuls, n=6 per group). Data for this figure were gathered at the University of Arizona by Dr. Egleton under his radiation license for that institution.

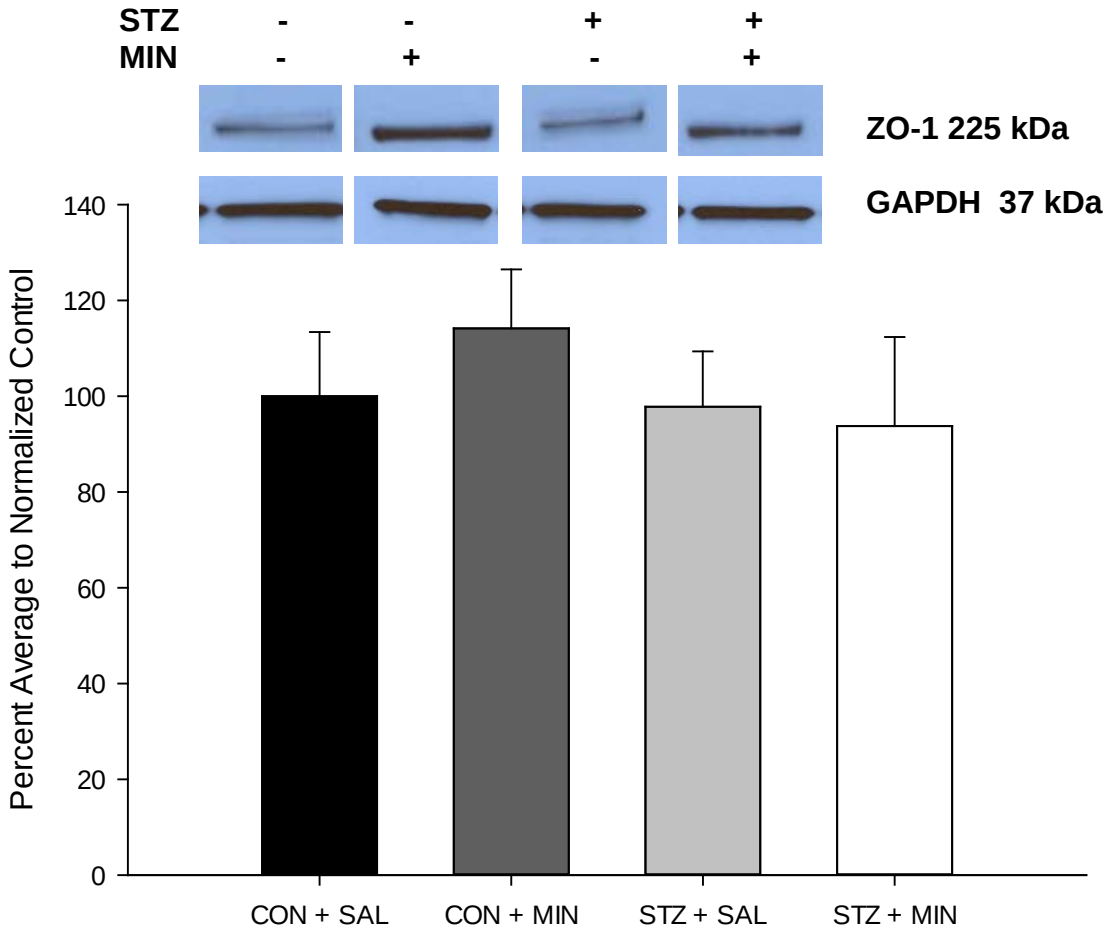


Figure 2.4. No effect of STZ or minocycline on ZO-1. Neither STZ nor minocycline had an effect on ZO-1 protein levels. The positive (+) sign indicates treatment with STZ or minocycline, and the negative (-) indicates no STZ or minocycline treatment. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

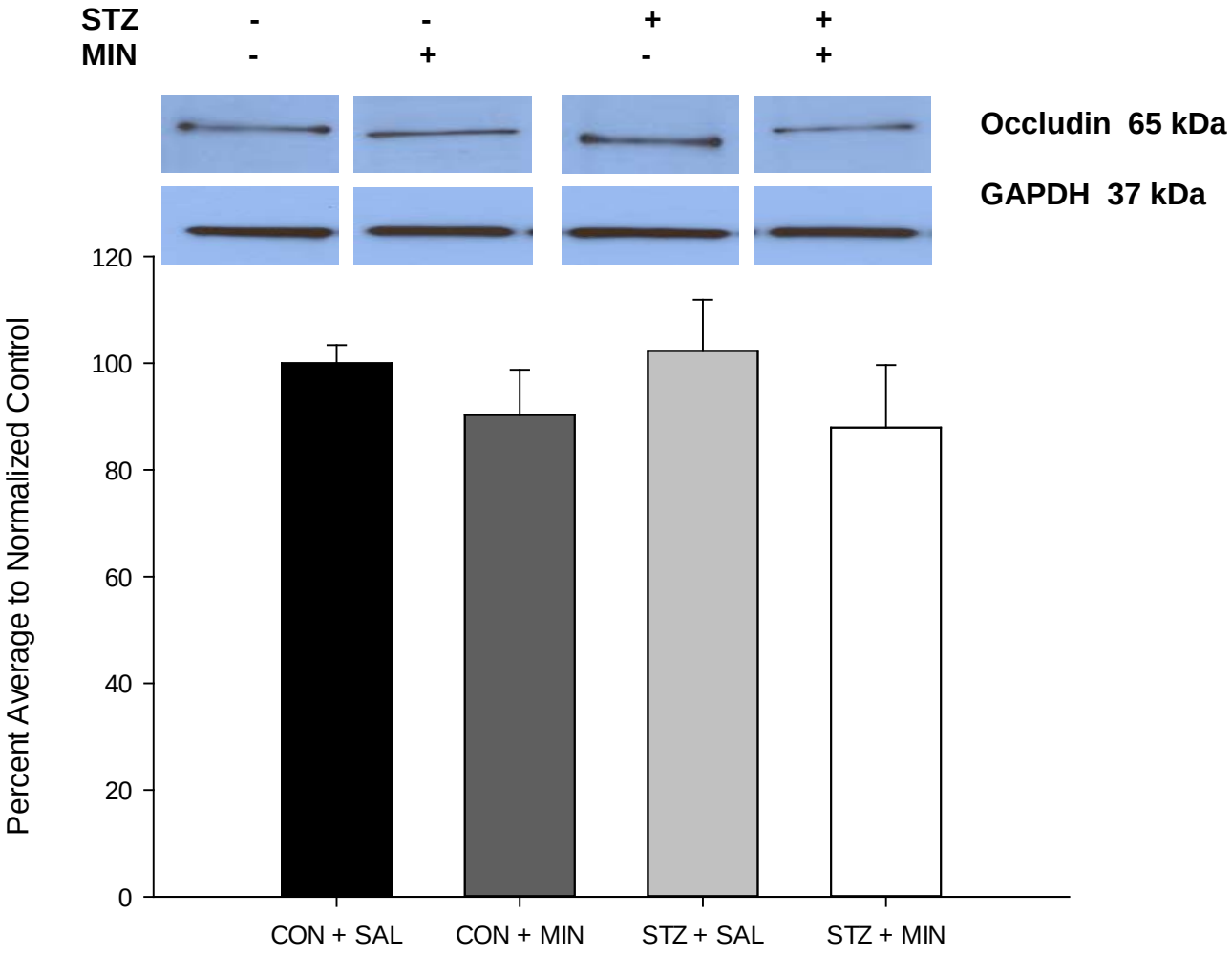


Figure 2.5. No effect of STZ or minocycline on occludin. No change was observed in occludin in any treatment. The positive (+) sign indicates treatment with STZ or minocycline, and the negative (-) indicates no STZ or minocycline treatment. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

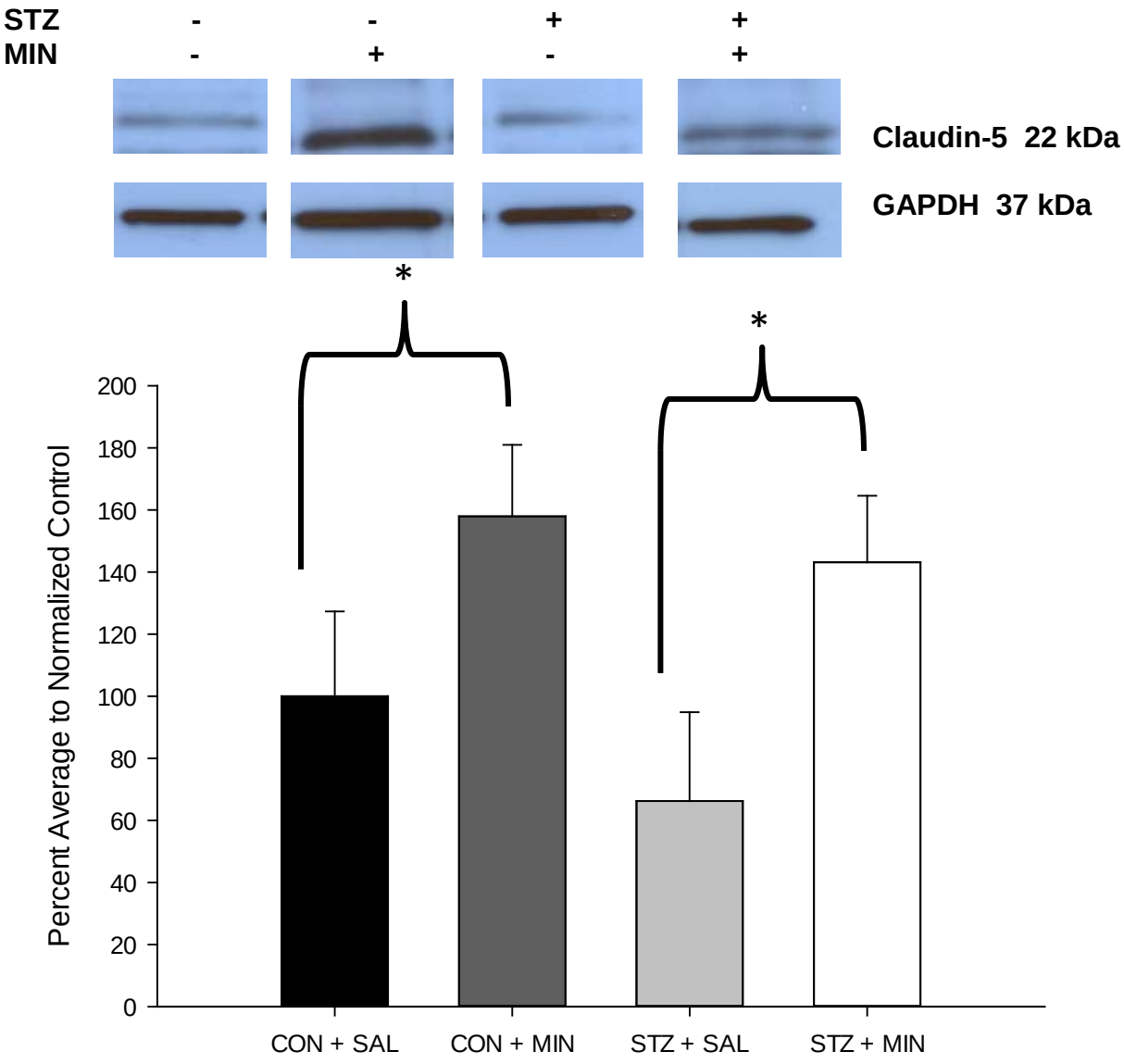


Figure 2.6. Minocycline treatment increased claudin-5. There was a significant increase in claudin-5 protein levels in both control and STZ groups treated with minocycline compared to their corresponding controls (*$p<0.05$, two-way ANOVA followed by Student-Newman Keuls). The positive (+) sign indicates treatment with STZ or minocycline, and the negative (-) indicates no STZ or minocycline treatment. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

shift in the ratio of VEGF to SEMA 3A, indicating that in the minocycline-treated rats there should be an increase in VEGF signaling (Figure 2.13).

Discussion

The goal of this study was to determine whether minocycline could attenuate the previously reported changes in BBB changes permeability to sucrose in the STZ model of diabetes. Additionally, we investigated the effects of minocycline on VEGF and its receptors at the BBB. To our knowledge, this is the first study to show that minocycline is able to decrease BBB permeability to sucrose in a rat model of diabetes and increase protein expression of tight junction proteins. Treatment with minocycline alters the protein expression of VEGF and some components of its signaling pathway, implicating this signaling system in the response to minocycline treatment.

BBB integrity is compromised in diabetes. Clinical studies demonstrated that there is an increase in permeability to gadolinium-DTPA at the BBB in diabetic patients (Starr et al., 2003). This finding has been supported by others using animal models. In the STZ model of diabetes, an increase of various markers for paracellular permeability have been reported including [^{14}C] sucrose (Huber et al., 2006; Hawkins et al., 2007) and dextrans of varying molecular weights (Mooradian et al., 2005). This increase in BBB paracellular permeability has been linked to decreased tight junction protein expression, in particular occludin (Hawkins et al., 2007). In our study we also report that STZ-induced diabetic animals exhibited a significant increase in permeability to sucrose compared to control animals. This result correlates well with the previous studies by our group and others (Huber et al., 2006; Hawkins et al., 2007). In the STZ

animals that were treated with minocycline, the permeability to sucrose was significantly reduced compared to the saline-treated STZ animals (Figure 2.3). BBB permeability in minocycline-treated diabetic animals was not different from control (Figure 2.3). This result complements other studies that used minocycline to inhibit BBB permeability changes (Nagal et al., 2008; Wasserman and Schlichter, 2007). Interestingly however, the permeability of the control animals to sucrose was also reduced, indicating that minocycline effect was not necessarily related to an inhibition of something specific to the STZ model. Further, minocycline did not have a significant effect on the diabetic status of the animals (Table 1).

Changes in sucrose permeability are often linked with changes in tight junction protein expression. Western analysis showed no change in either ZO-1 or occludin protein expression in control and diabetic animals, though the occludin was mildly decreased. This result is different from our previous studies, in which we showed both ZO-1 and occludin decrease at the same time point (Hawkins et al., 2007). Protein expression of claudin-5 did not change in the diabetic animals. However, treatment with minocycline significantly increased protein expression of claudin-5 in both control and STZ-treated animals (Figure 2.6). It has been suggested that claudins act as a primary seal for the tight junctions while occludin is present as a support structure (Hawkins and Davis, 2005). Therefore, the “tighter” junction induced by minocycline may be via an increase in claudin-5. To our knowledge this effect of minocycline has not been reported previously. Most studies only give a small number of doses, and the animals in this study were given minocycline twice a day for seven days. Previous studies have shown that if the levels of claudin-5 are increased, then there will be a decrease in the

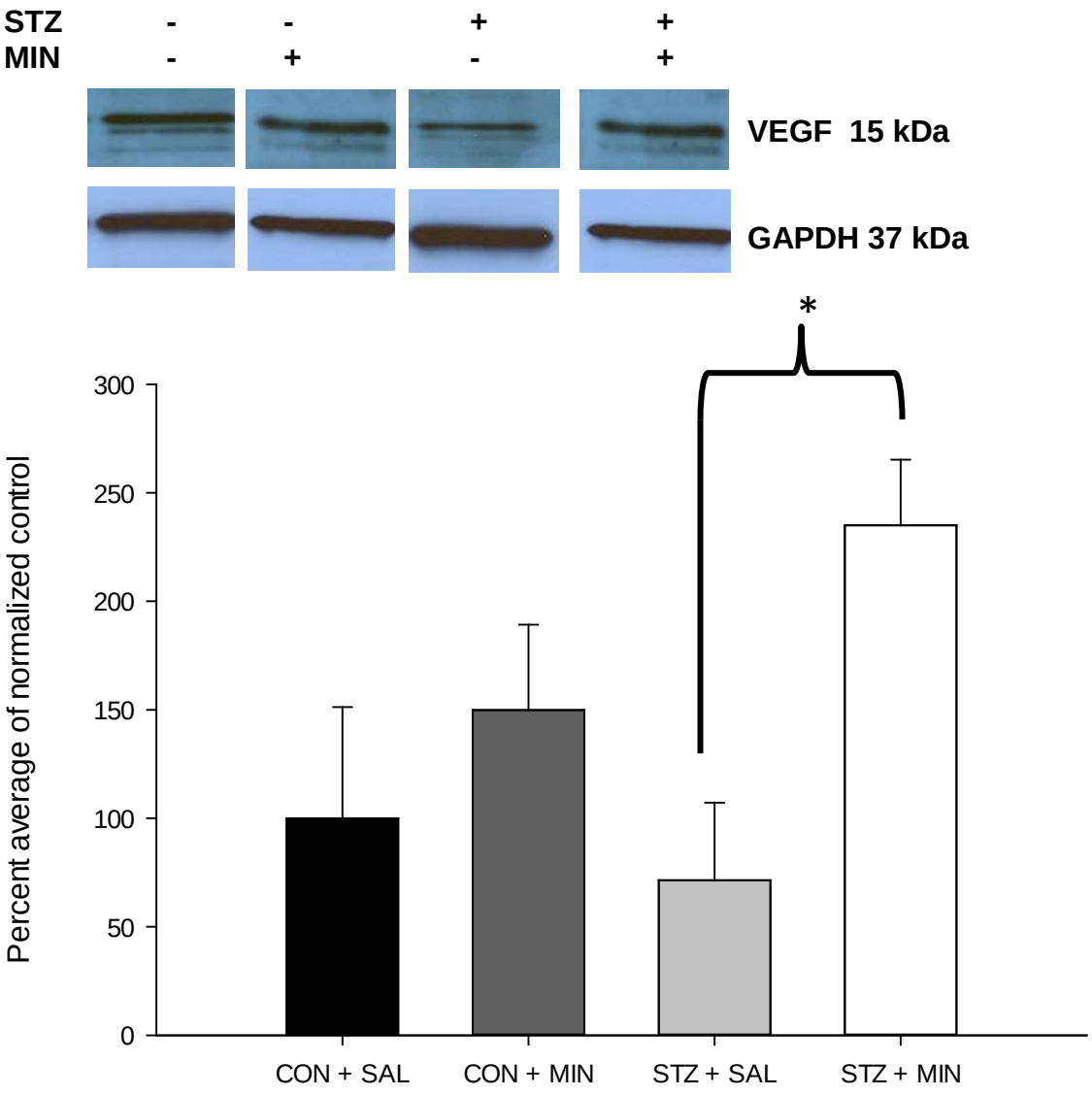


Figure 2.7. Minocycline stimulated VEGF expression in STZ-treated animals. Western analysis showed there was a significant difference between treatment groups (saline vs. minocycline, $p<0.05$, two-way ANOVA followed by Student-Newman-Keuls). There was a significant increase in VEGF protein levels in STZ-induced diabetic animals treated with minocycline (*$p<0.05$, two-way ANOVA followed by Student-Newman-Keuls). A similar trend was observed in control animals treated with minocycline. The positive (+) sign indicates treatment with STZ or minocycline, and the negative (-) indicates no STZ or minocycline treatment. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

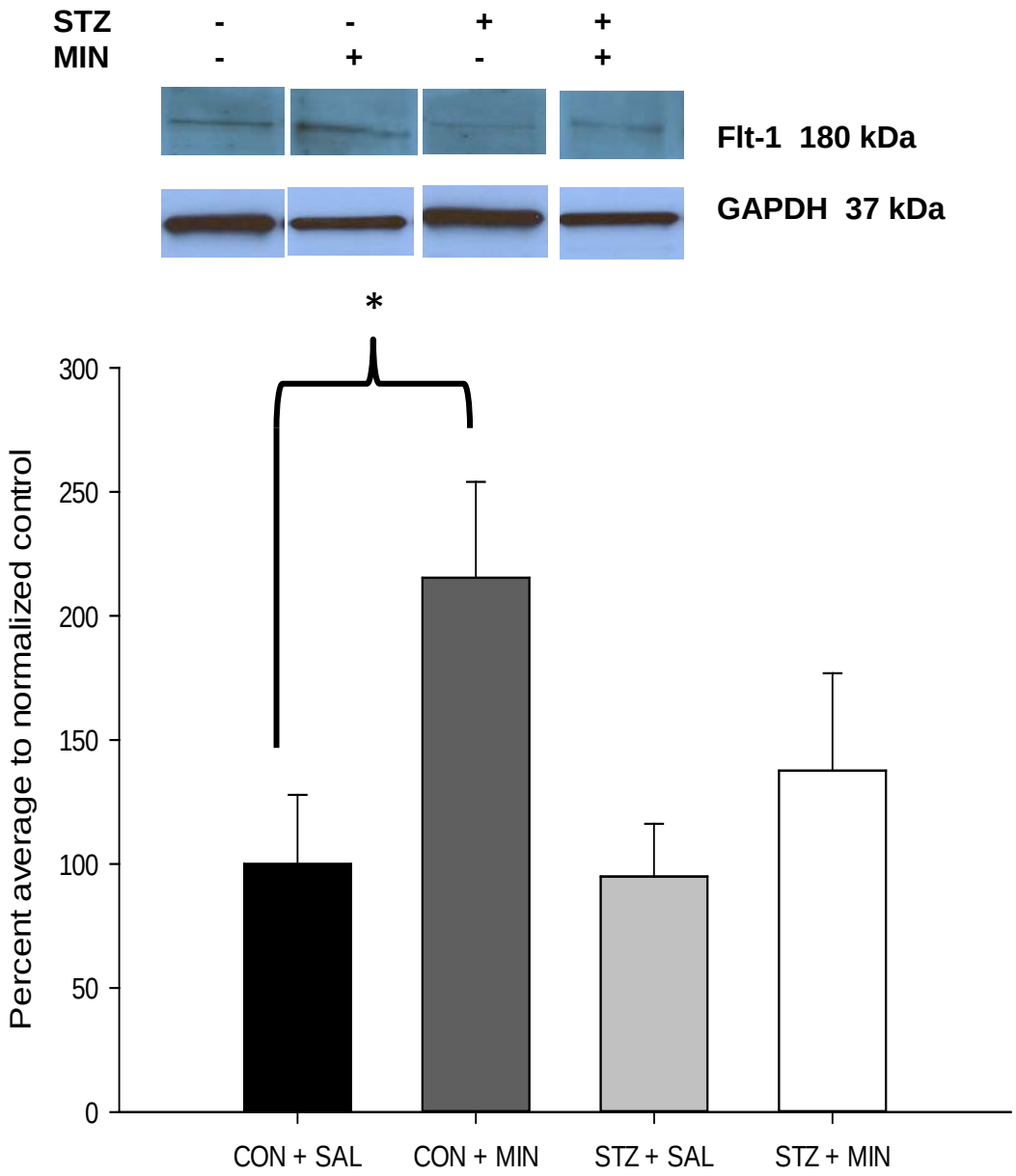


Figure 2.8. Minocycline increased Flt-1 expression in control animals. Western analysis showed that there was a significant difference between treatment groups (saline vs. minocycline, $p<0.05$, two-way ANOVA followed by Student-Newman-Keuls). There was a significant increase in Flt-1 protein expression in control animals treated with minocycline (* $p<0.05$, two-way ANOVA followed by Student-Newman-Keuls). No change was observed in STZ-treated animals upon minocycline treatment. The positive (+) sign indicates treatment with STZ or minocycline, and the negative (-) indicates no STZ or minocycline treatment. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

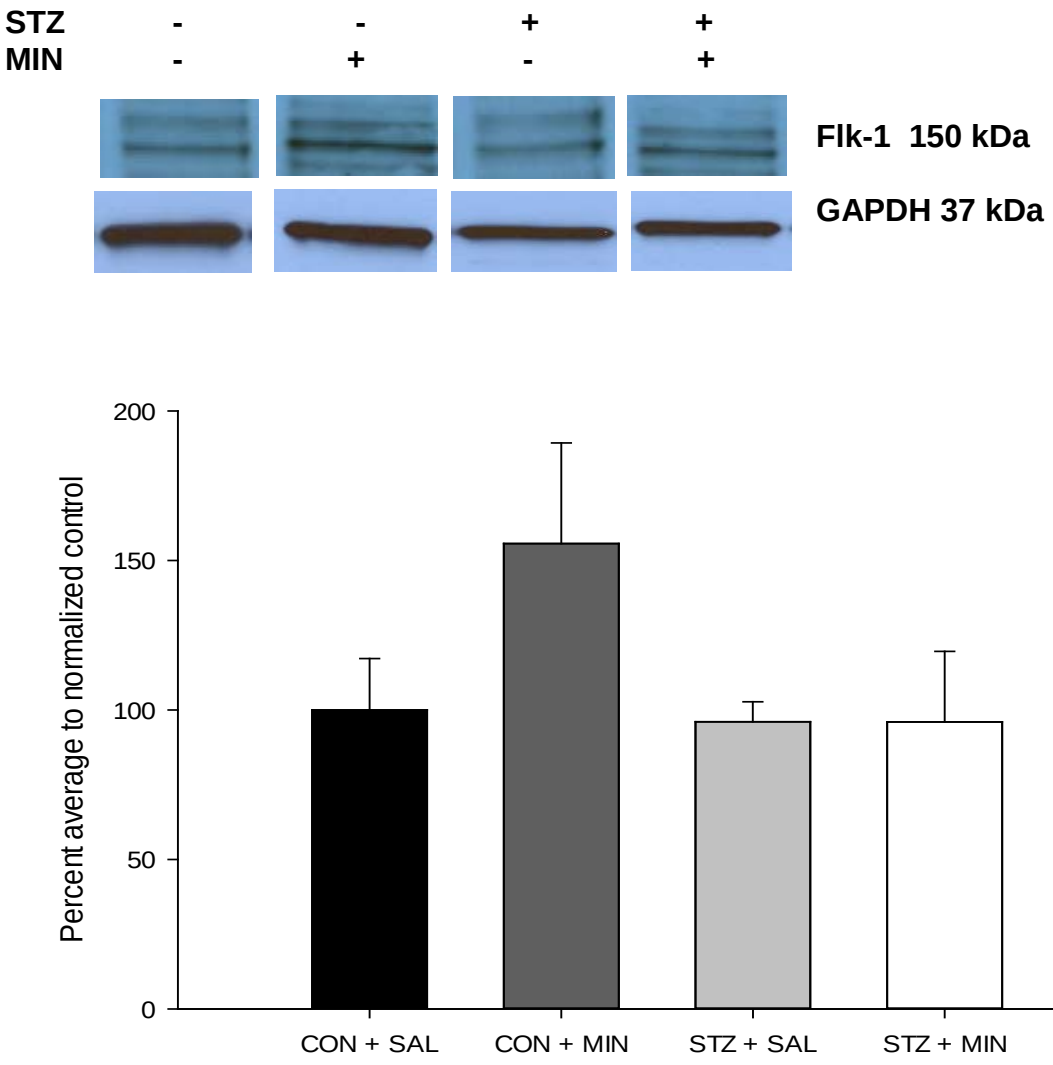


Figure 2.9. Minocycline did not have an effect on Flk-1. Treatment with STZ or minocycline did not have an effect on Flk-1 protein expression (two-way ANOVA followed by Student-Newman Keuls). The positive (+) sign indicates treatment with STZ or minocycline, and the negative (-) indicates no STZ or minocycline treatment. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

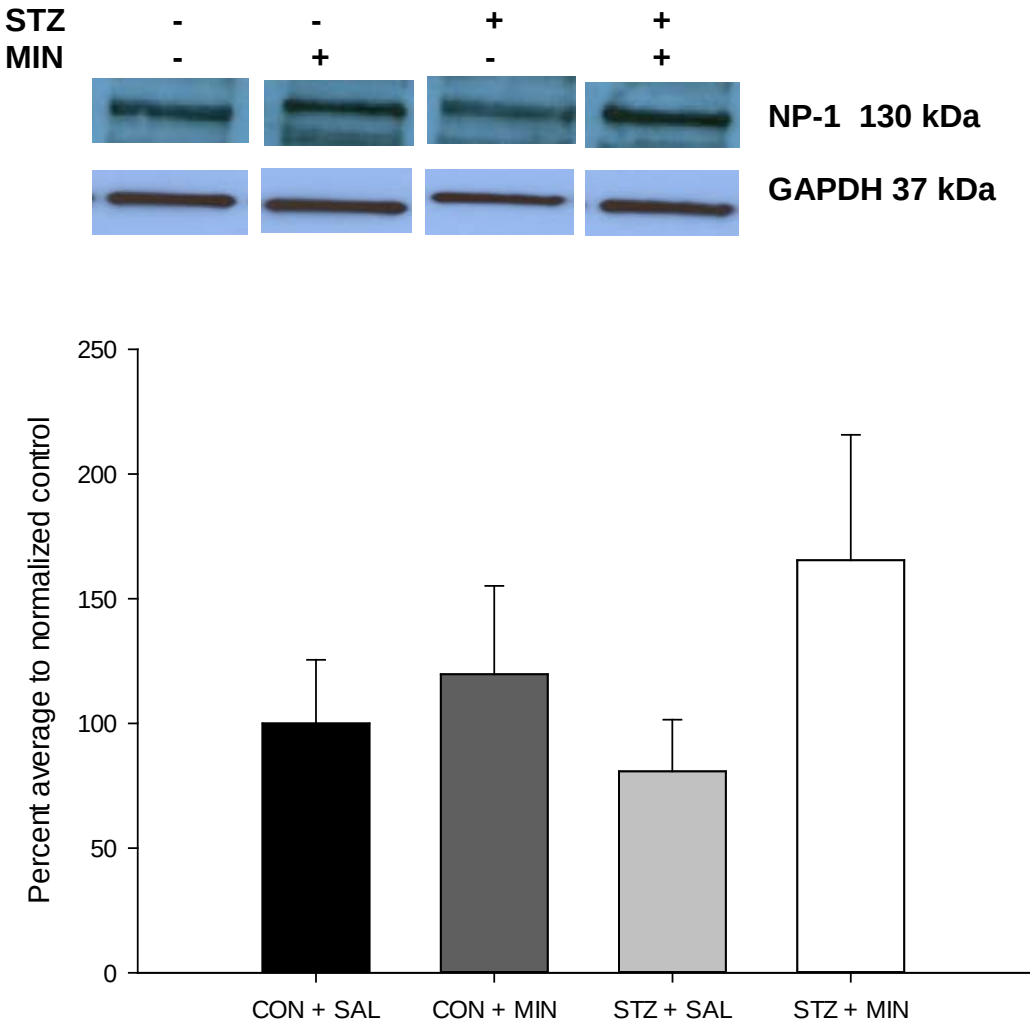


Figure 2.10. Minocycline did not affect NP-1 expression. No significant changes were observed in NP-1 protein levels with STZ or minocycline treatment (two-way ANOVA followed by Student-Newman-Keuls). The positive (+) sign indicates treatment with STZ or minocycline, and the negative (-) indicates no STZ or minocycline treatment. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

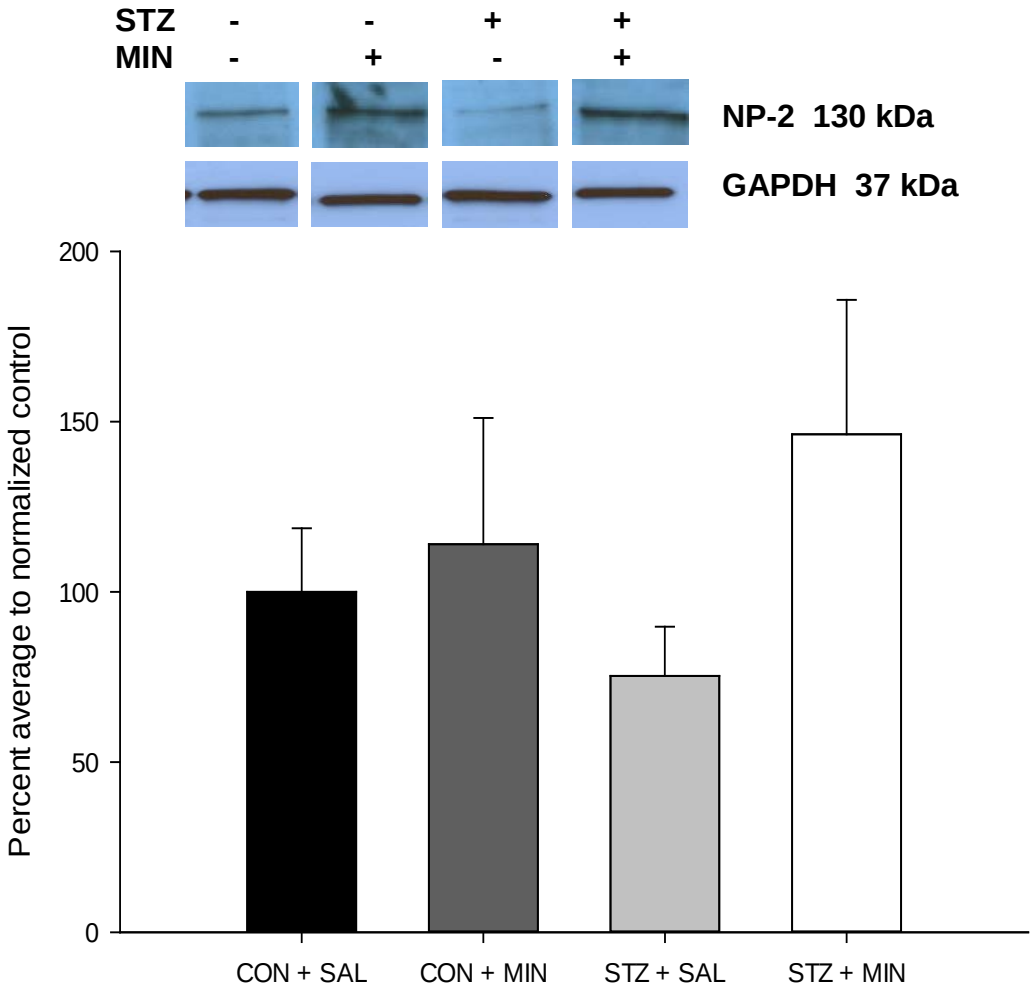


Figure 2.11. No effect of minocycline on NP-2 expression. No significant changes were observed in NP-2 protein levels with either STZ or minocycline treatment (two way ANOVA followed by Student-Newman-Keuls). The positive (+) sign indicates treatment with STZ or minocycline, and the negative (-) indicates no STZ or minocycline treatment. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

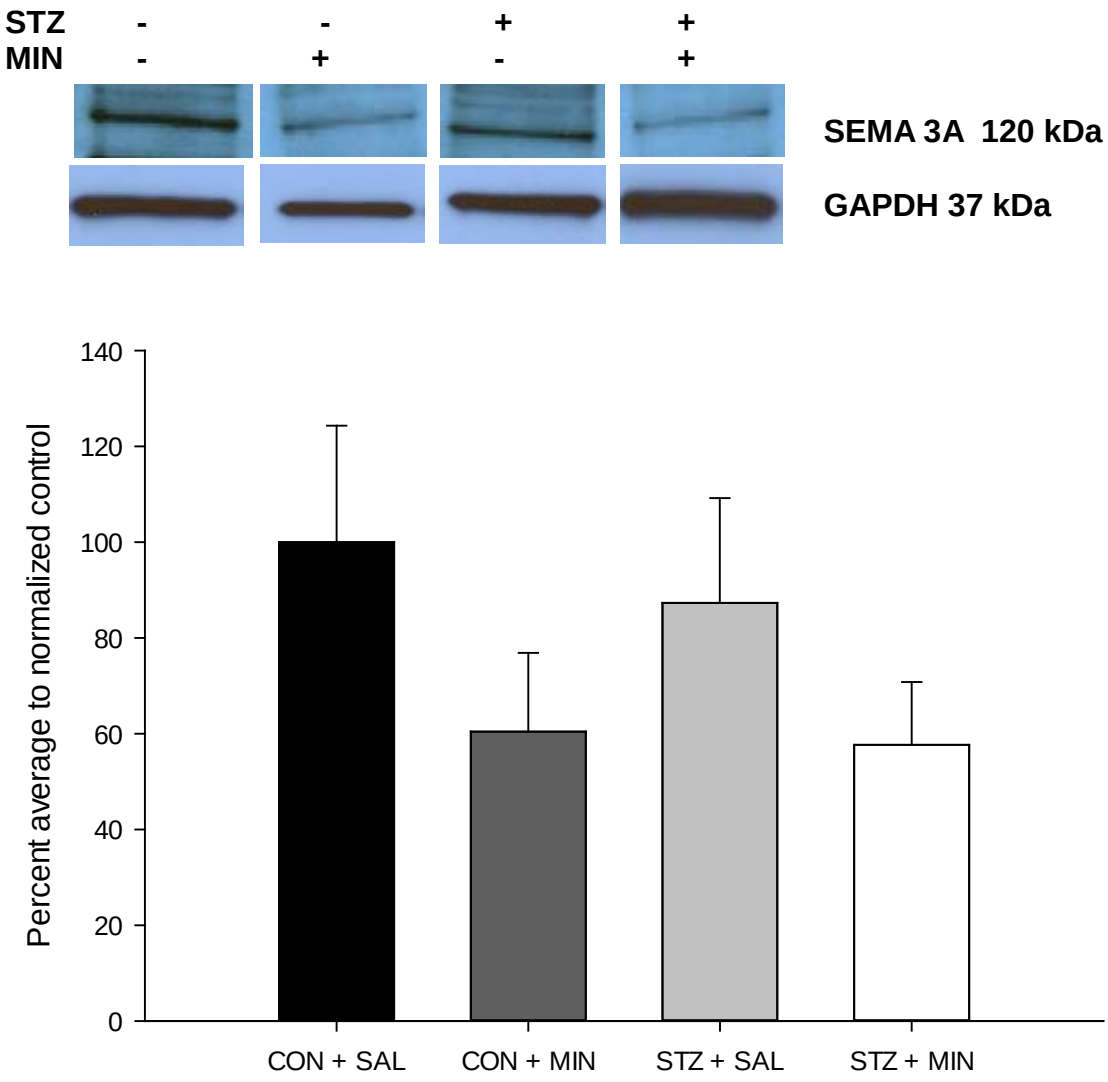


Figure 2.12. Minocycline did not affect SEMA 3A. Western analysis showed that there was no significant change in SEMA 3A protein levels with either STZ or minocycline treatment (two-way ANOVA followed by Student-Newman-Keuls). The positive (+) sign indicates treatment with STZ or minocycline, and the negative (-) indicates no STZ or minocycline treatment. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

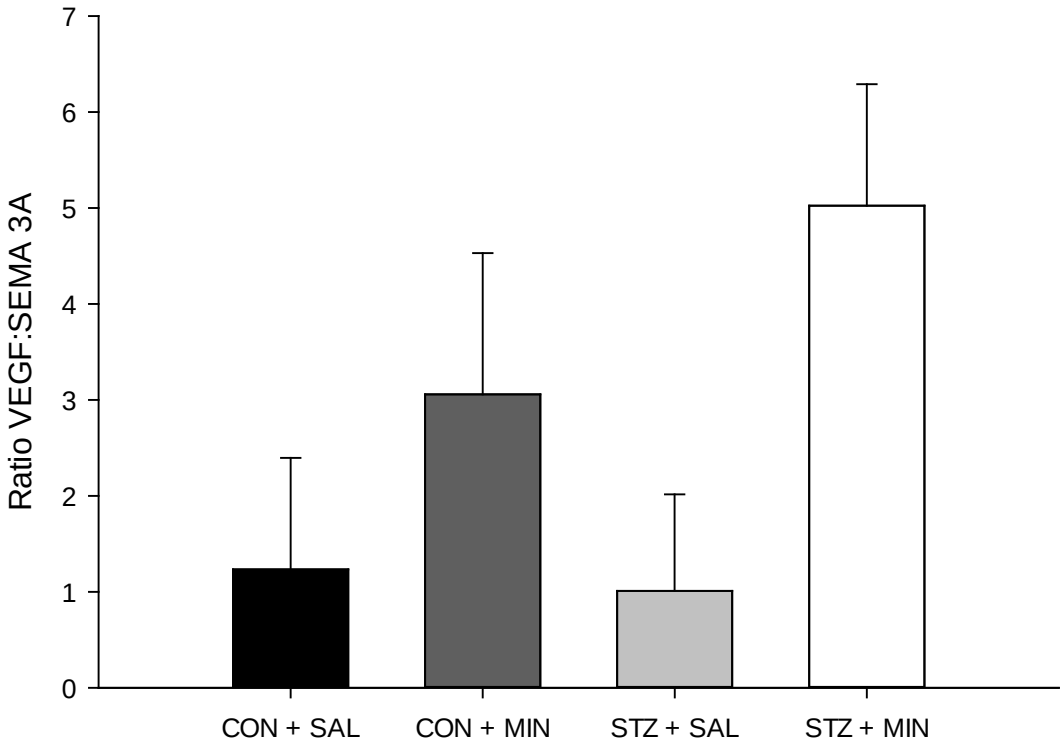


Figure 2.13. Minocycline increased the VEGF:SEMA 3A ratio. Western analysis showed that there was no statistically significant change in VEGF:SEMA 3A ratio. Although, there appears to be a trend in an increase of VEGF:SEMA 3A with minocycline in both control and STZ-treated animals. (n=5 for CON +SAL and STZ+ MIN; n=4 for CON+ MIN and STZ +SAL).

permeability of tight junctions (Feliniski et al., 2008; Kashiwamura et al., 2011). Thus the decrease in permeability seen in this study is consistent with an increased claudin-5 expression. Claudin-5 is known to be regulated via a number of different mechanisms. The tight junctions are highly dynamic structures with a constant recycling of barrier proteins from the junctional domain to either storage or lysosomal components (Utech et al., 2010; Stamatovic et al., 2011; Dukes et al., 2012). Claudin-5 has been reported to be associated with caveolin-1, and its removal from the junctional complex to a caveolin-1-rich microdomain is associated with an opening of tight junctions (Nag et al., 2007; Stamatovic et al., 2011). Multiple post-translational modifications, such as phosphorylation and ubiquitination, of claudin-5 are associated with its recycling. Ubiquitination of claudin-5 appears to trigger the ubiquitin-proteasome system, leading to degradation, partially via a lysosomal-mediated manner (Mandel et al., 2012). Inhibition of this ubiquitination leads to an increase in the levels of claudin-5. Therefore, it is possible that minocycline is inhibiting the recycling of claudin-5 from the membrane and/or its ubiquitination in both the control and STZ-treated animals. The half-life of claudin-5 has been reported to be 70-90 minutes (Mandel et al., 2012). In minocycline-treated control and STZ-treated animals, the inhibition of claudin-5 recycling/degradation could lead to a gradual increase in the levels of claudin-5 and a decrease in BBB permeability.

The role of minocycline in regulating this BBB permeability/claudin-5 is potentially via a combination of mechanisms. Minocycline is also known to inhibit MMPs, which have been reported to degrade several tight junction proteins including claudin-5. In a recent study, doxycycline (a related tetracycline) prevented claudin-5 loss during a

short-term treatment strategy following MCAO, and the mechanism was proposed to be via a reduction in MMP activity (Wang et al., 2012). In our model, we were looking at a long term treatment with minocycline (7 days), and observed an increase in control levels of claudin-5. A previous study reported an increase in MMP activity in our STZ-treated animals (Hawkins et al., 2007). The control animals also had a small, yet measureable, baseline activity of MMP activity (Hawkins et al., 2007). Therefore, it is possible that the MMP is involved in the basal turnover of claudin-5, and the inhibition of this may contribute to the accumulation of claudin-5.

Another mechanism in which minocycline could promote claudin-5 is via inhibition of PKC. PKC activation has been shown in several cell types to alter the distribution of claudins (Sjo et al., 2010). Hence, it is possible that part of the recycling mentioned earlier is PKC-dependent. In fact, PKC-α has been shown to play an important role in caveolin-based endocytosis (Sharma et al., 2004). As a known inhibitor of PKC-α, minocycline could inhibit claudin-5 recycling via this mechanism (Nikodemova et al., 2007), thus promoting a buildup of claudin-5 and reduced sucrose permeability.

VEGF has also been linked to not only BBB function, but also claudin-5 regulation (Argaw et al., 2009). Furthermore, VEGF has been linked to the tight junction dys-regulation in diabetes as well as diabetic-induced endothelial dysfunction (Murakami et al., 2012). VEGF is involved in angiogenesis and increased permeability, and is a likely candidate for involvement in the observed BBB permeability changes in diabetes. Thus, we investigated the role of VEGF in STZ- and minocycline-induced changes in claudin-5.

Western analysis demonstrated that VEGF protein levels decreased in STZ-treated diabetic rats. Minocycline increased VEGF protein expression in both control and STZ-treated rats, with significance in the latter group. This result is opposite to what we expected based on studies in the retina. Harhaj et al. (2006) showed elevated VEGF-induced changes in tight junction proteins leads to increased permeability, and reduced VEGF prevents the permeability change. Isoforms of VEGF can be diffusible or bound to the extracellular matrix. $VEGF_{165}$, the most studied isoform, is secreted but the majority is bound to the extracellular matrix (Ferrera et al, 2003). Hollborn et al. (2010) demonstrated that minocycline promoted the expression of VEGF in retinal pigment epithelial cells. Wasserman and Schlichter (2007) showed that minocycline decreased MMPs at the BBB, likely keeping the extracellular matrix relatively intact. Therefore, the inhibition of MMPs reduced the VEGF release from the basement membrane, perhaps explaining the increased levels of VEGF. It is also important to remember that VEGF response is due to the interaction of a number of receptors and cofactors. Thus, we looked at expression levels of the primary VEGF receptors and its inhibitory cofactor, SEMA 3A.

At the BBB, VEGF has two primary receptors and two co-receptors. The interaction of the VEGF receptors, Flt-1 and Flk-1, with the neuropilin receptors dictates the cellular response induced by VEGF. In our study, STZ did not have an effect on the VEGF receptors, Flt-1 and Flk-1. However, minocycline significantly increased protein expression of Flt-1 in control animals and showed a similar trend in the STZ-treated animals. Minocycline also increased protein expression of Flk-1 in the control group, but it did not have an effect on the receptor in STZ-treated animals. Minocycline had no

significant effect on expression of the neuropilin receptors, NP-1 and NP-2, in either control or STZ groups. Finally, the VEGF regulator, SEMA 3A, was decreased in both control and STZ-treated animals, although not statistically significant.

VEGF-induced angiogenesis and permeability are regulated by a complex interaction of receptors, predominantly Flk-1 and the neuropilin receptors. It has been proposed that the ratio of VEGF to SEMA 3A is a critical factor in driving permeability. Although not significant, we see a decrease in VEGF in the STZ-treated animals. This decrease in VEGF would promote the SEMA 3A portion of the response, which is linked to an increase in permeability (Acevedo et al., 2008; Le Guelte et al., 2012). This observation could explain the change in STZ-induced permeability. Le Guelte et al. (2012) showed that this change is in part due to inhibition of serine phosphatase PP2A activity promoting a dissociation of VE-cadherin from adherens junctions. An increase in Flt-1 and no change in Flk-1 in diabetic rats with minocycline treatment suggests that the "decoy receptor," Flt-1, may be sequestering any VEGF that might be secreted, inhibiting its ability to bind to the Flk-1 receptor, which has the major role in inducing changes at tight junctions. Previous studies have demonstrated that minocycline is able to decrease VEGF-induced smooth muscle cell migration in the vasculature by downregulating the Erk 1/2 and PI3K/Akt signaling pathways (Yao et al., 2004, 2007). VEGF is able to bind to the neuropilin receptors, and its binding enhances its ability to bind to and activate Flk-1 (Sulpice et al., 2008). Last, minocycline decreased protein expression of the VEGF antagonist, SEMA 3A, in both control and STZ-treated animals, although not significant. On its own, SEMA 3A is also a permeability factor (Acevedo et al., 2008). Lower SEMA 3A levels due to minocycline treatment would result in greater

VEGF binding to neuropilin receptors, and consequently, smaller BBB permeability changes than normally in diabetic animals.

In conclusion, our study shows that minocycline is able to decrease permeability to sucrose, a low molecular weight molecule, in diabetic microvessels. This permeability decrease coincides with an increase in protein expression of claudin-5. Minocycline is able to increase protein expression of VEGF in the diabetic group. No change was observed in the Flk-1 receptor, but the Flt-1 receptor increased. This increase in receptor expression suggests that VEGF is sequestered by Flt-1, and is prevented from binding Flk-1. Thus, it is likely that the Flk-1 is not phosphorylated and subsequently, any signaling pathway that would lead to a permeability increase is not activated. Although the protein expression of the neuropilin receptors increased in the diabetic group treated with minocycline, VEGF signaling is unlikely since there was no change in Flk-1. Last, minocycline decreased protein expression of SEMA 3A, but not significantly. It is possible to reduce permeability in diabetic microvessels through this route as well. Therefore, minocycline is a potential therapeutic treatment for decreasing permeability at the BBB in diabetes.

Chapter 3: The Potential Role and Regulation of VEGF in BBB Dysfunction

Abstract

Diabetes is characterized by hyperglycemia, which can lead to various macrovascular and microvascular complications. Microvascular complications are evident in retinal and renal pathologies. Similar complications are evident in the brain microvasculature particularly in the blood-brain barrier (BBB), a specialized microvasculature composed of endothelial cells that interacts with various cell types in the brain. Significant changes to the BBB occur in diabetes; however, the mechanisms leading to these changes are unknown. This study focuses on the vascular endothelial growth factor (VEGF) signaling system, a potent pro-angiogenic and vascular permeability factor, as a potential mechanism for the observed BBB changes. Previously, we have shown a potential role of VEGF-based signaling for the observed BBB changes (Chapter 2). In the present study, results showed changes in mRNA of VEGF and its receptors; however VEGF protein levels remained unchanged in diabetic animals. Interestingly, protein expression of VEGF receptors and co-receptors as well as SEMA 3A, an antagonist of VEGF, did not change. In this study, we investigated the possibility that altered abundance of microRNAs (miRNAs) might explain the discrepancy between our mRNA and protein data. Protein expression of PLC-γ, a downstream signaling target of Flk-1, showed statistically significant changes within the diabetic groups. Taken together, these results indicate that VEGF signaling could be based on the relative diabetic state as a result of the absence of insulin or other changes that occurred as a consequence of STZ toxicity.

Introduction

Diabetes is a risk factor for vascular dementias and cardiovascular events such as stroke. The exact mechanism behind the increased risk is unknown; however it is often linked with hyperglycemia and subsequent cerebral edema. Increased peripheral vascular permeability and the development of vascular complications contribute to the morbidity and mortality of diabetes (Perrin et al., 2009). There is now increasing evidence that the cerebral blood vessels are also compromised, and that this may contribute to the neurological problems associated with diabetes (Huber et al., 2006). Under normal circumstances brain homeostasis is regulated by the blood-brain barrier (BBB). The BBB, a specialized microvascular structure comprised of endothelial cells held together by tight junctions, functions to protect the brain from toxins in the peripheral circulation, to allow nutrients to move from blood to brain, and to mediate waste efflux from the brain (Hawkins and Davis, 2005). Overall, the BBB is important for the maintenance of the central nervous system. Breakdown of the BBB can lead to changes in cognition and poor cardiovascular outcomes (Huber et al., 2006).

Clinical studies have shown that diabetic patients have increased permeability of the BBB (Starr et al., 2003). This permeability increase has been observed in experimental models of diabetes and has been associated with a decreased expression of tight junction proteins, particularly occludin and ZO-1 (Hawkins et al., 2007). The change in permeability can be prevented by insulin acutely (Hawkins et al., 2007; Huber et al., 2006), but not chronically (Huber et al., 2006), and by using seasmol (VanGilder et al., 2009) or minocycline (Chapter 2). Several factors have been linked to these changes including increased matrix metalloproteinase activity (Hawkins et al. 2007),

dyslipidemia (Ginsberg, 1999; Goldberg, 2001), and reactive oxygen species (VanGilder et al., 2009). However, the exact mechanism of these changes is unknown. Similar changes in tight junction expression at the blood-retinal barrier have been shown to be regulated by VEGF (Antonetti et al., 1998).

The VEGF family consists of dimeric glycoproteins VEGF-A, -B, -C, -D, and placental growth factor (Olsson et al., 2006). VEGF-A, the main and most studied, has several isoforms, with $VEGF_{165}$ being the most abundant (Wirostko et al., 2008). VEGF-A is a modulator of both angiogenesis and permeability. It exerts its effects by binding to membrane bound VEGF receptors-1 and -2 (Flt-1 and Flk-1, respectively) as well as the neuropilin receptors-1 and -2 (NP-1 and NP-2). Flt-1 is thought to be a decoy receptor regulating VEGF/Flk-1 interaction, whereas Flk-1 regulates permeability in the kidney and eye as well as directly transmitting signals associated with pathological angiogenesis (Wirostko et al., 2008). The binding of VEGF to the Flk-1 receptor can result in activation of several pathways leading to increased permeability, survival, migration, and proliferation (Holmes et al., 2007). For stimulation of angiogenesis, simultaneous binding of VEGF to both Flk-1 and NP receptors is required (Sulpice et al., 2008). This interaction can be blocked by SEMA 3A, which binds to the NP receptors thereby blocking the VEGF binding site. The presence of SEMA 3A does not however affect VEGF-induced permeability changes, but there is also evidence that SEMA 3A can induce permeability on its own (Acevedo et al., 2008).

Previous studies have shown that the BBB is sensitive to VEGF (Fischer et al, 2004; Argaw et al, 2009). I hypothesized that VEGF signaling is also involved in BBB breakdown in diabetes. We used an experimental model of diabetes to investigate

regulation of the VEGF signaling system at the BBB using real-time PCR, ELISA, and immunoblot techniques, and miRNA studies.

Methods

Animal Care

Animal procedures were performed in strict accordance with the Institutional Animal Care and Use Committee, Marshall University. Male Sprague-Dawley rats (Hilltop Laboratories, Scottdale, PA) were injected with either 65 mg/kg STZ (Sigma, St. Louis, MO) or 0.9% sterile saline. Animals were maintained under a 12h-12h dark-light cycle, at ambient temperature of 22 ± 2^{o} C, with food and water available *ad libitum*. Animals were euthanized 14 days post-injection via an intramuscular injection of 1 mL/kg of a cocktail containing ketamine (71.5 mg/mL) and xylazine (5.7 mg/mL). Blood samples were collected from the descending aorta and tested for glucose, ketones, and lipids with an analyzer (CardioChek PA, Polymer Technology Systems, Indianapolis, IN). The blood samples were centrifuged and plasma samples were stored at -80°C. Diabetic rats were divided into groups based on their blood glucose levels: mild ≥ 300 mg/dl and high ≥ 400 mg/dl.

Cerebral Microvessel Isolation

Microvessels were isolated from rat brains as previously described (Hawkins et al, 2007). The final microvessel pellet was resuspended in either tissue extraction buffer (ThermoScientific, Rockford, IL) for protein extraction or TriReagent (Sigma, St. Louis, MO) for RNA isolation. Protein was analyzed using bicinchoninic acid (BCA) protein assay kit (ThermoScientific, Rockford, IL).

Reverse Transciption and Real-time Polymerase Chain Reaction

RNA was isolated via TriReagent following the manufacturer's protocol (Sigma, St. Louis, MO), and samples were incubated with the complete reaction mixture from the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) to obtain cDNA. Then cDNA was incubated with a reaction mixture containing iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and the appropriate forward and reverse primers of each gene listed in Table 3.1 (Invitrogen, Carlesbad, CA).

ELISA

Rat VEGF ELISA kits were purchased from RayBiotech, Inc (Norcross, GA). All reagents and samples were brought up to room temperature prior to use. Solutions, standards and samples were prepared as per protocol instructions.

Western Blot Analysis of Immunoreactive Protein

The levels of specific proteins were identified by Western blot analysis. Protein samples (20 µg/well) were separated via SDS gel electrophoresis using 4-12% Bis-Tris gel (Criterion, Bio-Rad, Hercules, CA) followed by transfer to nitrocellulose paper. Western blot analysis was performed using primary antibodies directed against VEGF, Flk-1, Flt-1, and p-Flk-1 (Tyr 951,1175, and 1214), NP-1, NP-2, and SEMA 3A (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies directed against p-PI3K, PI3K, p-Akt, Akt, p-PLCγ, PLCγ, p-Erk 1/2, Erk 1/2, p-Src, Src, p-p38, and p38 were purchased from Cell Signaling Technology (Danvers, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were used to normalize protein loading. The presence of protein

bound to each antibody was detected by horseradish peroxidase conjugated secondary antibody (GE Healthcare Bio-Sciences, Piscataway, NJ). Protein bands were detected by the enhanced chemiluminescence system (GE Healthcare Bio-Sciences, Piscataway, NJ).

Extraction and Purification of microRNA (miRNA)

A PreAnalytiX's PAXgene® Blood miRNA Kit was used to extract the miRNA from blood samples from control and STZ animals. Approximately 200 ng of miRNA was reverse transcribed to cDNA using Qiagen RT^2 miRNA First Strand Kit. Because over 300 miRNAs have been identified in rats, Qiagen MAR-3100G miRNA 384 arrays was used to run each assay. BioRAD iQ^{TM} SYBR® Green Supermix and RNAse-free water plus sample were used for the arrays. Ct values were obtained after the data was analyzed on the ROCHE-480 Light Cycler using the Absolute Quantitative/2^{nd} Derivative Max program. Fold change data was analyzed using the SA Bioscience's RT^2 miRNA PCR Array Data Analysis program. Both Rnu6 and miR-16 were used as housekeeping genes. Statistical analyses were performed using student's t-test. Fold change >3.0 and <0.3 was used as cut-off for miRNAs up- or down-regulated respectively, and $P<0.05$ was used to indicate significance.

Primer	Sequence (5'→3')	Forward/Reverse
VEGF	GCCAGCACATAGGAGAGATGAGC	Forward
VEGF	CAAGGCTCACAGTGATTTTCTGG	Reverse
Flt-1	AGGAGAGGACCTGGAACTGTCTT	Forward
Flt-1	ATTCCTGGGCTCTGCAGGCATAG	Reverse
Flk-1	TAGCACGACAGAGACTGTGAGG	Forward
Flk-1	TGAGGTGAGAGAGATGGGTAGG	Reverse
NP-1	GGAGCTACTGGGCTGTGAAG	Forward
NP-1	ATGTCGGGAACTCTGATTGG	Reverse
NP-2	ACACAAGGAGCCATTTCCAG	Forward
NP-2	CGGATCCTGATGAAACGAGT	Reverse
SEMA 3A	AAGGCCGGGCACTCTCAAG	Forward
SEMA 3A	ACTCCTGGGTGCCCTCTCAACT	Reverse
GAPDH	TCCACCACCCTGTGCTGTA	Forward
GAPDH	ACCACAGTCCATGCCATCAC	Reverse

Table 3.1. Primers used for Real-Time PCR experiments

Results

Blood Chemistries

Table 3.2 shows blood characteristics and weights of control and diabetic animals. Blood glucose levels were significantly different in diabetic animals compared to controls. These differences in blood glucose levels within the diabetic group prompted us to separate this group into mild and high glucose groups for subsequent experiments. Ketone levels were significantly different between the diabetic groups as well as between control and high glucose animals. Cholesterol, HDL, and triglyceride levels remained relatively unchanged in both control and diabetic animals. Weight was significantly lower in animals with mild and high glucose levels at 7 and 14 days post-injection compared to control (Table 3.3).

STZ treatment increased VEGF and VEGF receptor mRNA expression

To determine mRNA expression of VEGF and the VEGF receptor system, RNA from both control and STZ-treated rats was isolated (Figure 3.1). Real-time PCR showed that mRNA expression of VEGF increased about 2.5 fold in the microvessels of STZ-treated animals compared to control. Additionally, expression of the VEGF receptors, Flt-1 and Flk-1, as well as the neuropilin receptors, NP-1 and NP-2, increased in diabetic rats. SEMA 3A mRNA expression also increased in diabetic rats compared to control.

VEGF protein levels remain unchanged

To determine whether VEGF levels changed in diabetic versus control rats, protein from microvessels were isolated. ELISA analysis (Figure 3.2A) showed that there was no change in VEGF protein levels in the microvessels of STZ-treated animals. Additionally, plasma levels of VEGF did not change in diabetic animals versus control (Figure 3.2B).

Western analysis showed no appreciable change in VEGF in animals with mild or high glucose compared to control animals (Figure 3.3).

VEGF receptor protein levels did not exhibit glucose-dependent changes

Western analysis showed that protein levels of the VEGF receptors, Flt-1(Figure 3.4) and Flk-1 (Figure 3.5), did not change in the diabetic groups compared to control. Additionally, the co-receptor, NP-1 did not change in all the groups (Figure 3.6). The VEGF modulator SEMA 3A, which competitively binds to the neuropilin receptors, remained relatively unchanged in diabetic animals compared to control (Figure 3.7).

Circulating MicroRNA changes in diabetes

MicroRNA studies showed an up-regulation of miR-330, -26b, -361, -139-5p, and let-7a and let-7c, and a down-regulation in miR-127 and -497. These miRNAs are involved in insulin signaling as well as VEGF and its receptors (Table 3.4).

No change in Flk-1 phosphorylation

To determine whether the Flk-1 receptor was activated, we looked at three phosphorylation sites, tyrosine 951, 1175, and 1214, on the receptor that regulates the downstream signaling of VEGF. Western analysis showed no changes in phosphorylation (Figures 3.8-3.10).

Regulation of downstream targets of Flk-1

To determine if the observed changes at the BBB was due to the activation of Flk-1, we examined downstream signaling targets at the three phosphorylation sites. Phosphorylation of 951 leads to activation of Src, which can subsequently lead to a permeability increase (Holmes et al., 2007). However, in our animal model, expression

	Glucose (mg/dL)	Ketones (mg/dL)	Cholesterol (mg/dL)	HDL	Triglycerides
Control	264	8.8	100	26	87
Mild Glucose	358*	17*	126	44†	177
High Glucose	487*	16*	110	39††	152‡

* Kruskal-Wallis One-Way ANOVA on Ranks p=<0.001 compared to control
† Kruskal-Wallis One-Way ANOVA on Ranks p = 0.021 compared to control
†† Kruskal-Wallis One-Way ANOVA on Ranks p = <0.001 compared to control
‡ Kruskal-Wallis One-Way ANOVA on Ranks p = 0.025 compared to control

Table 3.2. Blood chemistry of control and diabetic animals. (n=15 for control; n=9 for mild glucose; n=6 for high glucose)

	Day 1 (g)	Day 7 (g)	Day 14 (g)
Control	329	368	389
Mild Glucose	324.5	320*	317‡
High Glucose	327	313†	306††

* Kruskal-Wallis One-Way ANOVA on Ranks p = <0.001 compared to control
† One-Way ANOVA p = <0.001 compared to control
‡ Kruskal-Wallis One-Way ANOVA on Ranks p = <0.001 compared to control
†† Kruskal-Wallis One-Way ANOVA on Ranks p = <0.001 compared to control

Table 3.3. Weights of control and STZ-induced diabetic animals during the 14-day time course. (n=15 for control; n=9 for mild glucose; n=6 for high glucose)

of Src did not show any change (Figure 3.11). Phosphorylation at tyrosine 1175 can result in either phosphorylation of PI3K, which leads to increased permeability, or phosphorylation of PLCγ, which results in changes in gene transcription and subsequent proliferation of endothelial cells (Holmes et al., 2007). Our studies showed that total phosphorylation expression of PI3K or its downstream target, Akt, did not exhibit any change (Figures 3.12-3.13). Interestingly, the ratio of p-PLCγ/PLCγ showed a statistically significant decrease between diabetic animals with high glucose (Figure 3.14), but phosphorylation expression of Erk 1/2, the downstream target of PLCγ, did not significantly change (Figure 3.15). Phosphorylation of Flk-1 at tyrosine 1214 can lead to the activation of p38 MAPK to induce actin reorganization and migration of endothelial cells (Holmes et al., 2007); however, our studies did not show any change in p38 protein expression (Figure 3.16).

Discussion

Increased BBB permeability has been observed in both clinical studies and in animal models of diabetes. However, the mechanism of this permeability increase has not been elucidated. Studies have shown that VEGF is a modulator of blood-retinal barrier permeability in a diabetic model of diabetes (Antonetti et al., 1998). The present study focuses on VEGF as a modulator of BBB permeability due to its capability to act as a pro-angiogenic and permeability factor. To determine if VEGF is involved in the observed changes in the BBB in diabetes, we initially looked at gene expression of VEGF and its receptors via real-time quantitative PCR. Our results show that gene expression of VEGF, its receptors, and SEMA 3A increased in diabetic rats compared to control. However, when protein expression was evaluated, there was no change in

VEGF levels in diabetic animals with mild or high glucose compared to the control group. Microvessel-based VEGF can be found in two main locations: 1) within the cell itself or 2) sequestered in the matrix of the basement membrane, where it is bound via its heparin binding domain (Zhang et al., 2009) to glycosaminoglycan chains and other components of the extracellular matrix (Gabhann et al., 2009). Under normal circumstances VEGF-A_{165} is synthesized by the cell and then released into the extracellular matrix. Under conditions which promote basement membrane breakdown, VEGF is rapidly released allowing for repair. Basement membrane disruption is a common feature in BBB breakdown, mediated via an elevation in MMP activity. We have previously reported BBB breakdown in the STZ model paralleled by an increase in MMP activity (Hawkins et al., 2007). It is thus possible that if extra VEGF is being made, it is being released at a rate that equals the rate of production, potentially via MMP-induced basement membrane degradation. In fact, though not significant, there was a ~15% decrease in the levels of microvessel-related VEGF in the STZ animals.

Typically, a pathological event will up-regulate VEGF and its receptors. Protein expression of the VEGF receptors, Flt-1 and Flk-1, and the co-receptor, NP-1, did not change in the diabetic groups compared to control. There are potential mechanisms that might shed light to this observation. VEGF and its receptors have been shown to increase during pathological events. On the other hand, VEGF has also been shown to down-regulate receptor expression, despite an increase in receptor mRNAs (Wang et al., 2000). Additionally, it is possible that receptors are being either degraded or sequestered into the cell. Studies have demonstrated that VEGF stimulation was able

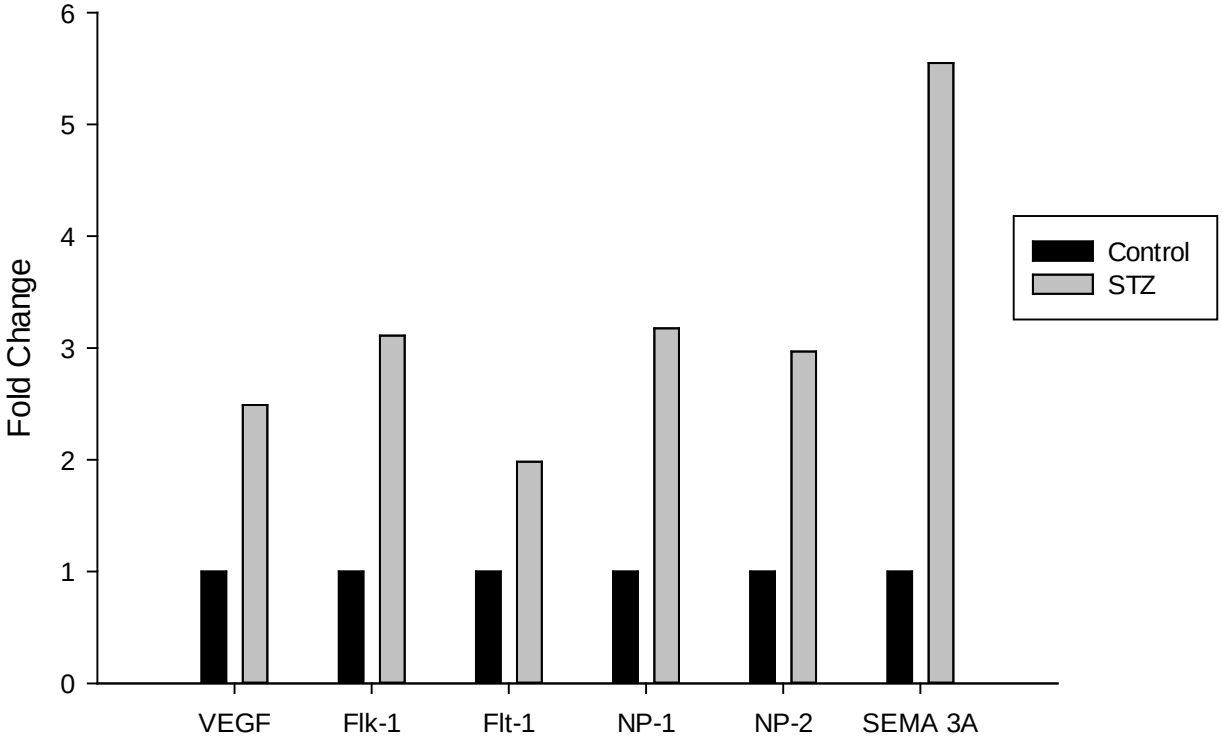


Figure 3.1. Real-time PCR analysis. Gene expression of VEGF, its receptors, co-receptors, and SEMA 3A increased in STZ-induced diabetic animals compared to control (n=5).

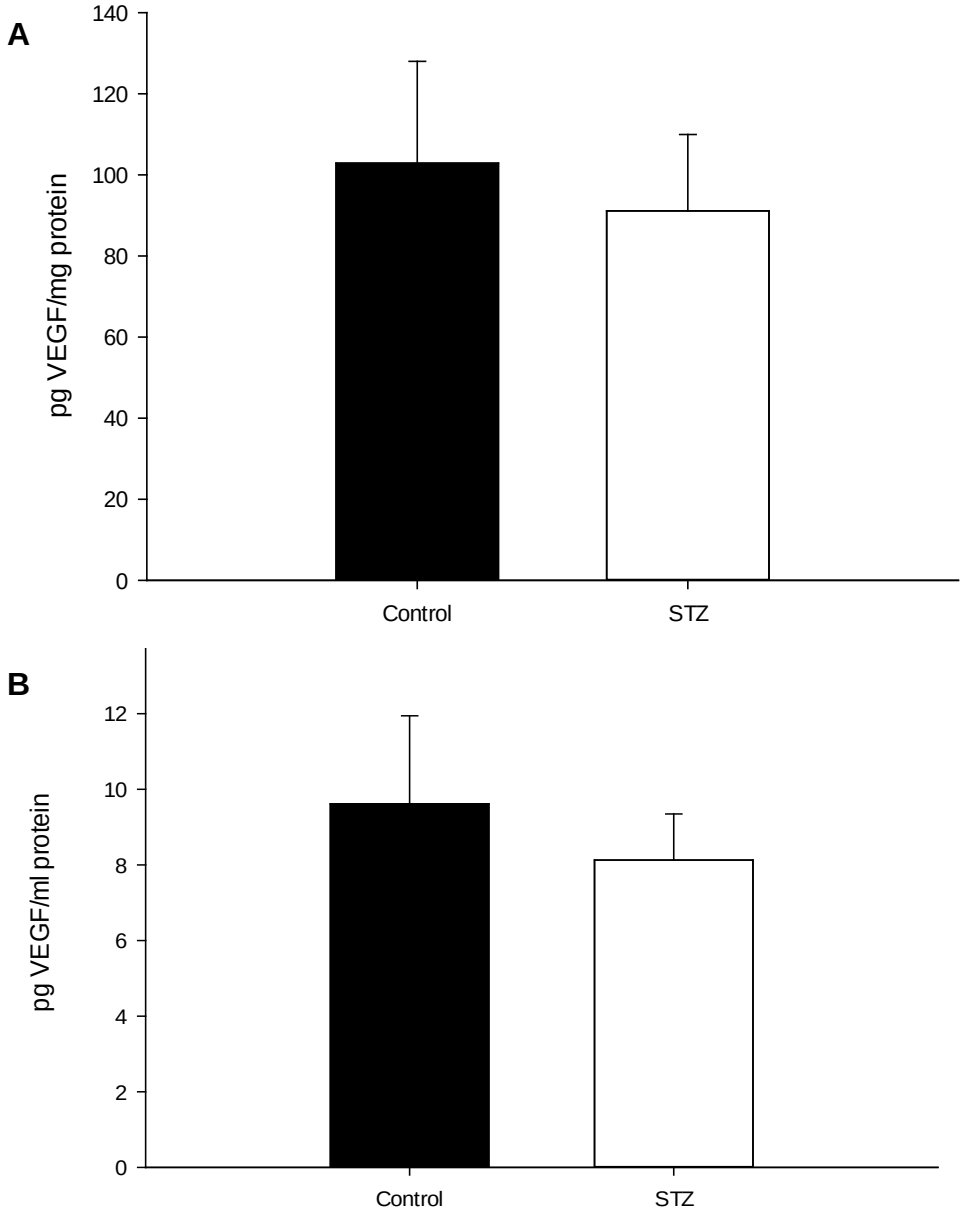


Figure 3.2. ELISA analysis of VEGF protein levels in cerebral microvessels and plasma of control and diabetic animals. VEGF protein levels did not change in (A) microvessels or (B) plasma of either control or diabetic animals. (n=23 for microvessels; n=8 for plasma)

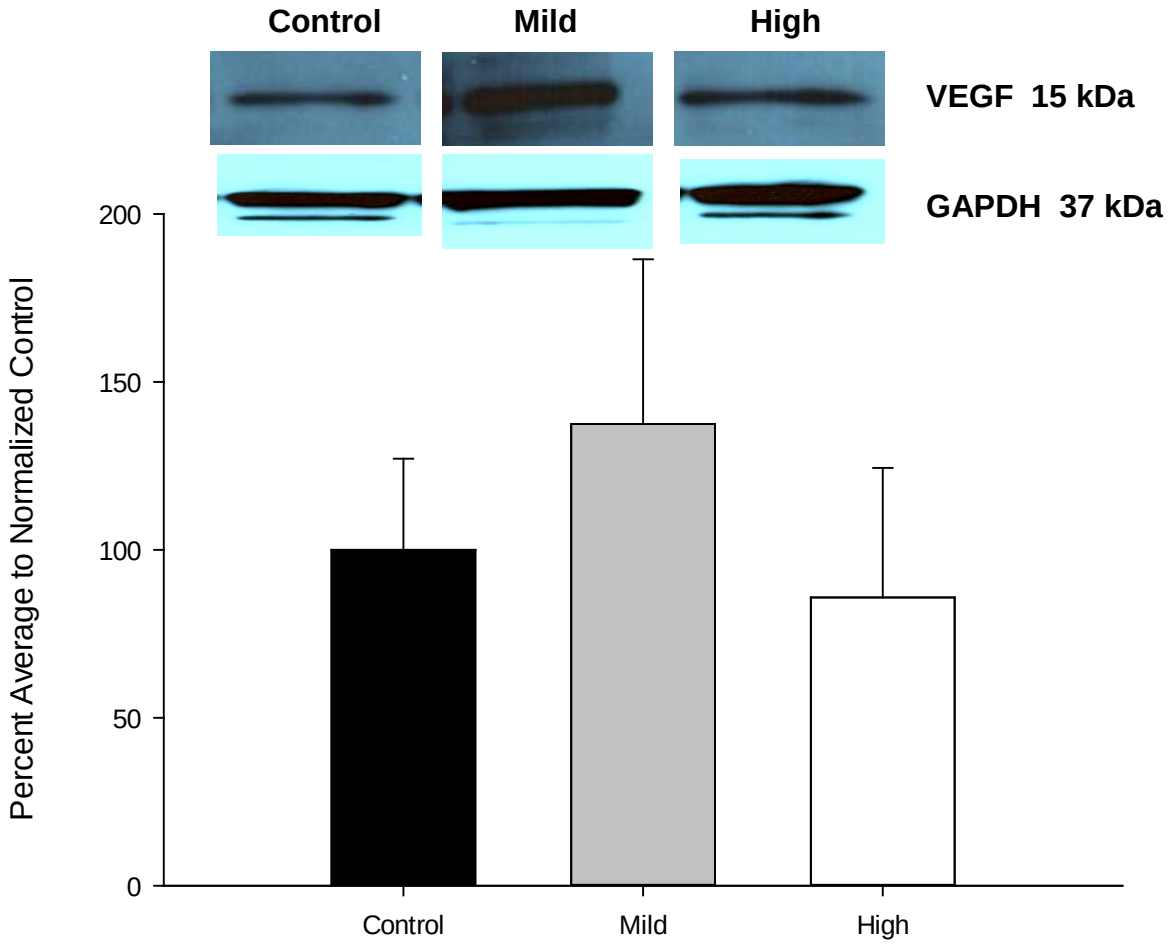


Figure 3.3. VEGF protein levels did not change in diabetic animals. VEGF protein levels did not change in diabetic animals with mild or high glucose levels compared to control. (one-way ANOVA; n=12 for control; n=8 for mild; n=4 high)

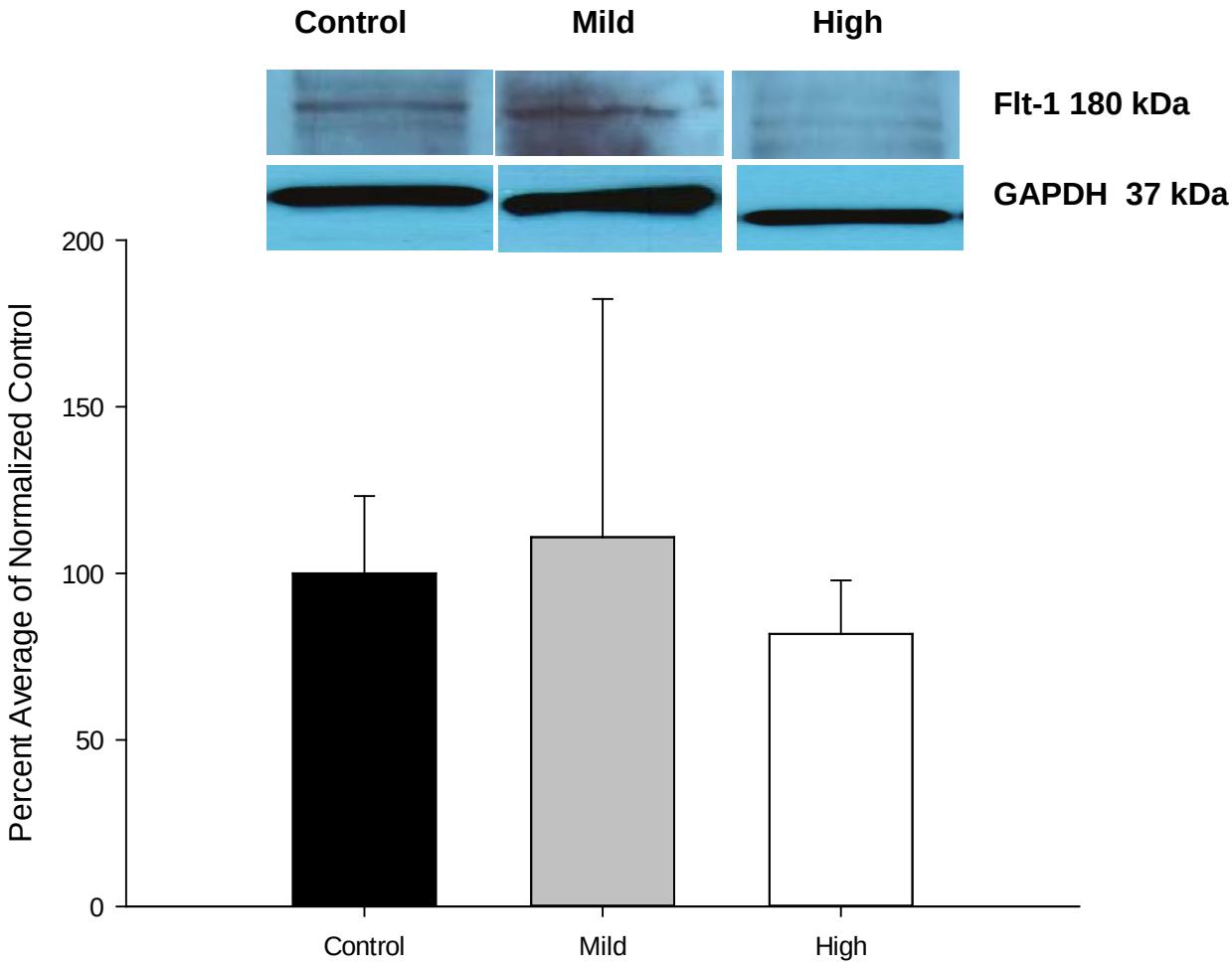


Figure 3.4. Flt-1 protein expression did not change. Western analysis showed that Flt-1 protein expression did not change in diabetic animals with mild or high blood glucose levels compared to control animals. (one-way ANOVA; n=11 for control; n=6 for mild; n=5 for high)

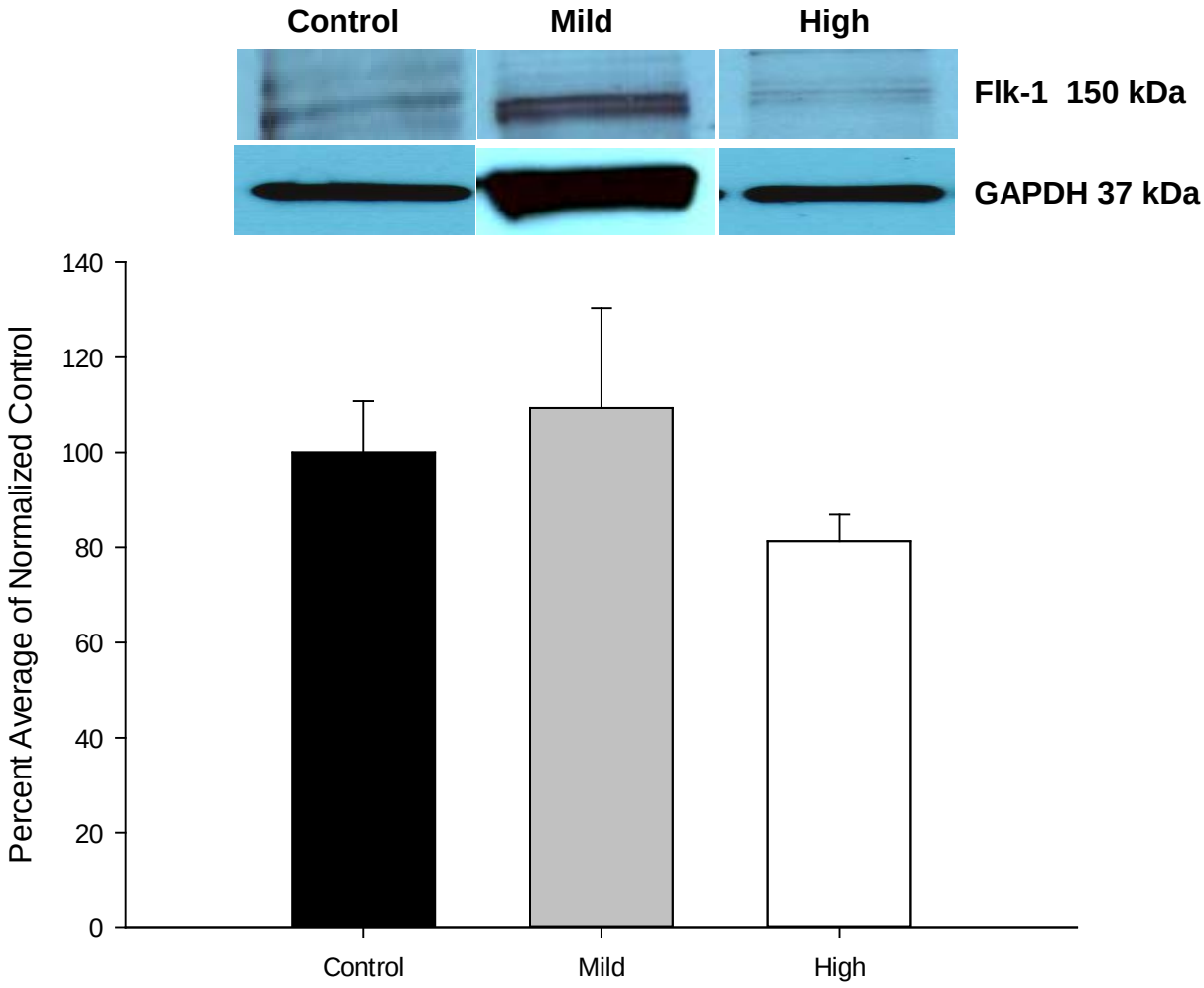


Figure 3.5. Flk-1 protein expression did not change diabetic animals. There was no change in Flk-1 protein levels in diabetic animals with mild or high blood glucose levels compared to control animals. (one-way ANOVA; n=14 for control; n=9 for mild; n=5 for high)

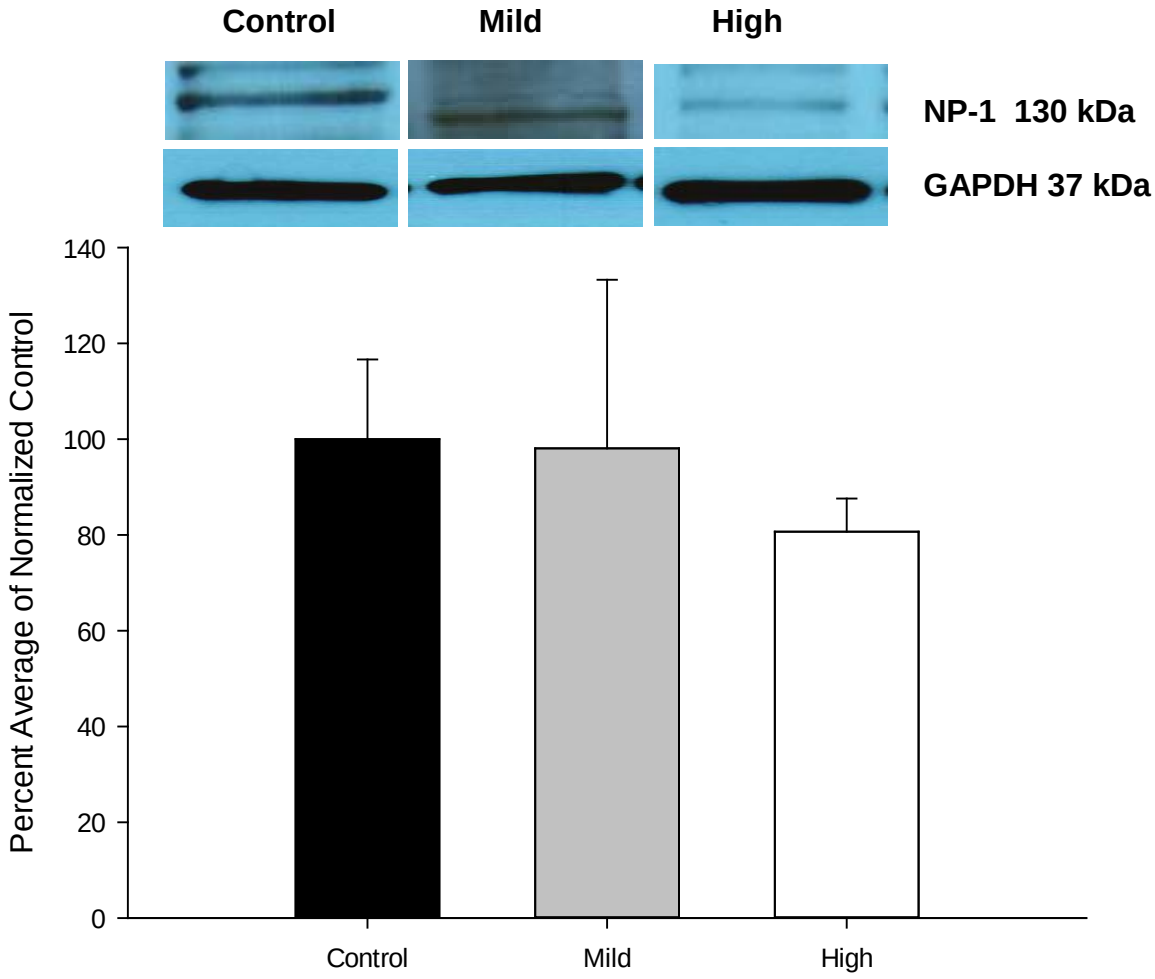


Figure 3.6. No change in NP-1 expression in diabetic animals. No change was observed in NP-1 protein levels in diabetic animals with mild or high blood glucose levels compared to control animals. (one-way ANOVA; n=10 for control; n=5 for mild; n=5 for high)

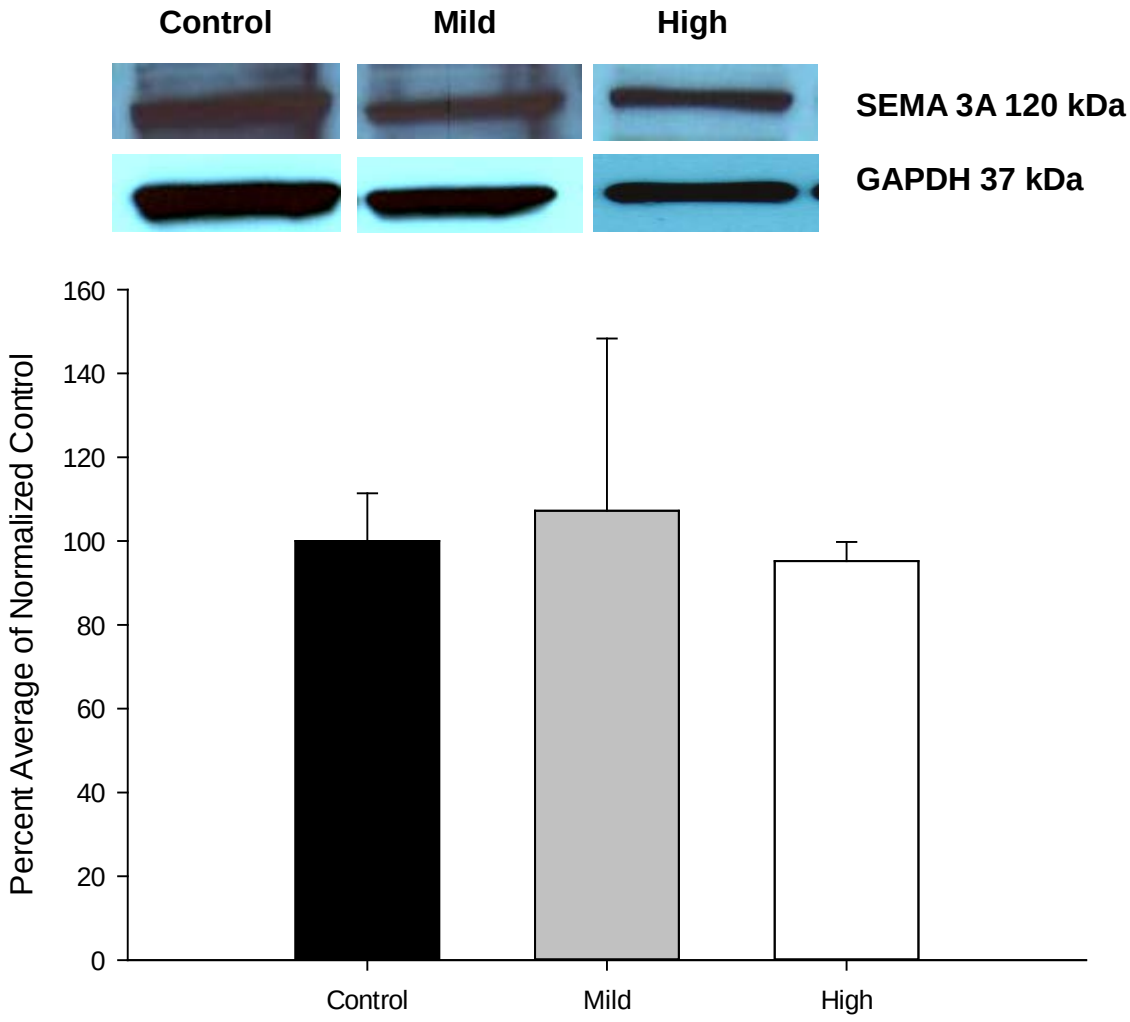


Figure 3.7. No change in SEMA 3A expression. Western analysis showed no change SEMA 3A protein levels in diabetic animals with mild or high blood glucose levels compared to control animals. (one-way ANOVA; n=11 for control; n=6 for mild; n=5 for high)

MiRNA	p-value	Fold Change	Angiogenic Target
miR-330	0.004	27.21 increase	SEMA 3A NP-2
miR-139-5p	0.045	3.01 increase	Rho-kinase 2
miR-497	0.013	4.70 decrease	Flk-1
let-7a	0.030	3.45 increase	TGF-β receptor
let-7c	0.023	3.43 increase	TGF-β receptor
miR-361	0.023	3.99 increase	VEGF-A
miR-127	0.031	5.93 decrease	VEGF-A NP-1

Table 3.4. MicroRNAs involved in VEGF signaling.

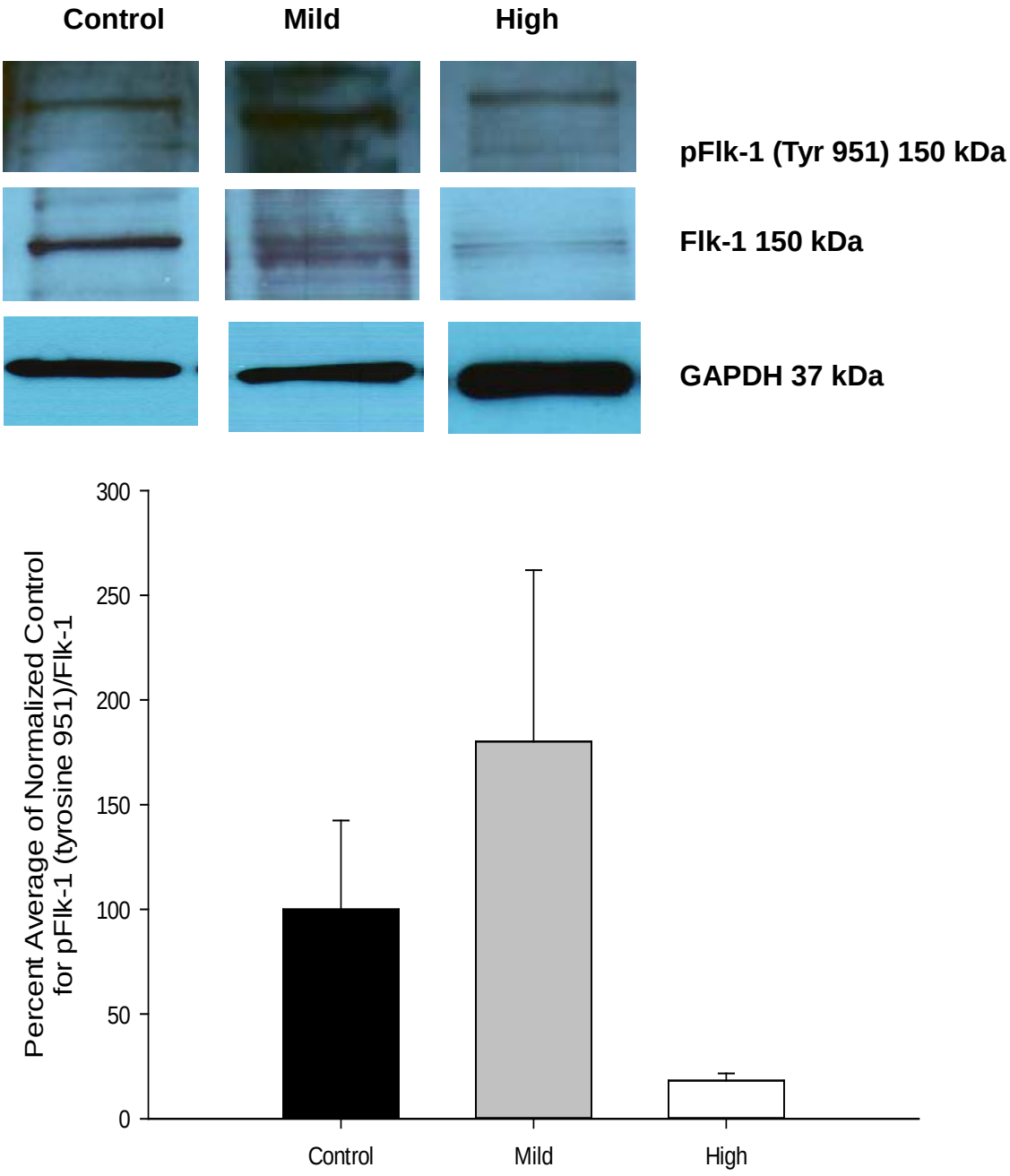


Figure 3.8. No change in p-Flk-1 (tyrosine 951) protein expression in STZ-induced diabetic animals. Western analysis showed that phosphorylation of Flk-1 at tyrosine 951 did not change in diabetic animals compared to control. (one-way ANOVA; n=11 for control; n=6 for mild; n=5 for high)

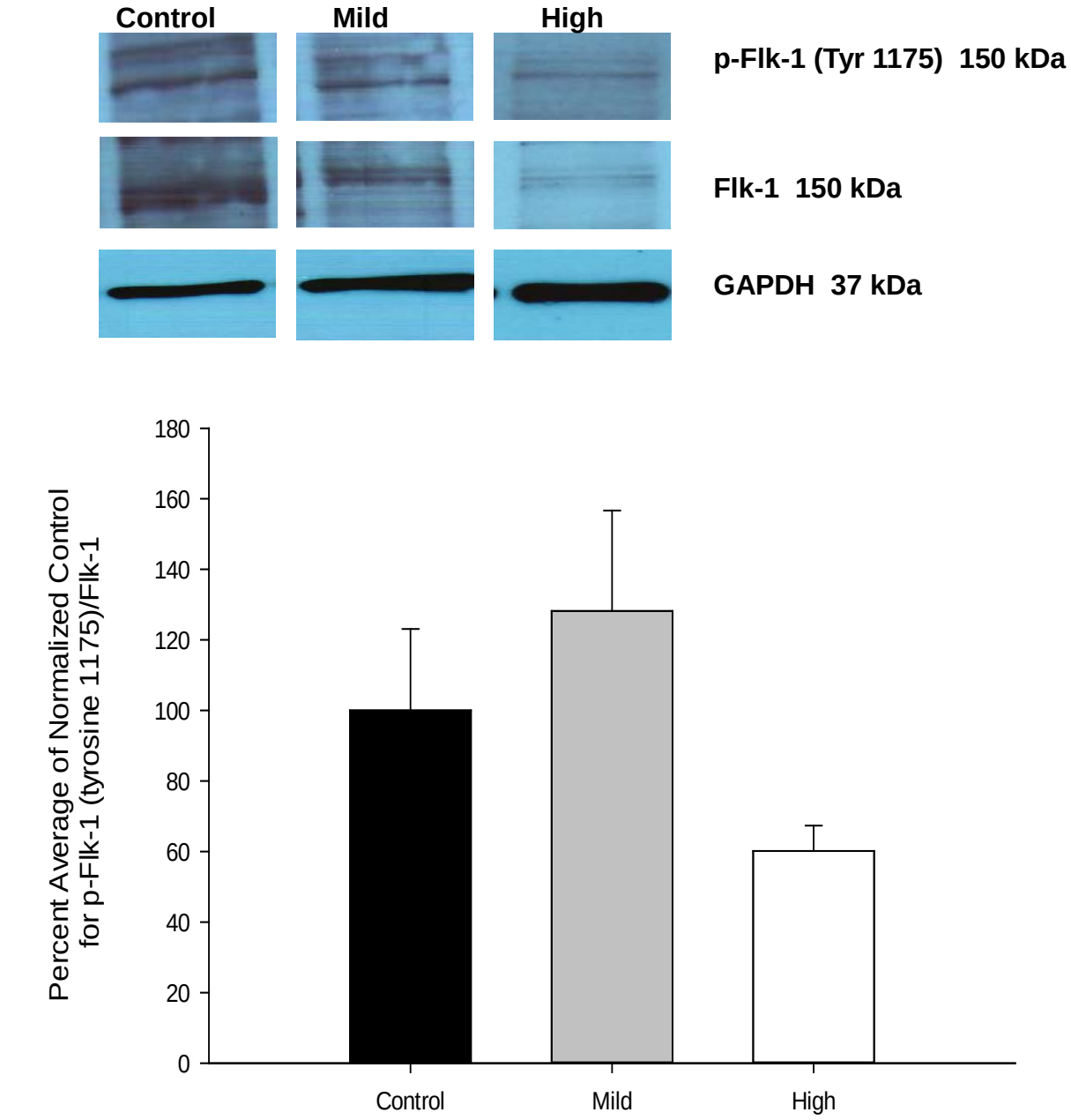


Figure 3.9. No change in p-Flk-1 (tyrosine 1175) protein expression in STZ-induced diabetic animals. Phosphorylation of Flk-1 at tyrosine 1175 did not change in STZ animals compared to control. (one way ANOVA; n=10 for control; n=5 for mild; n=5 for high)

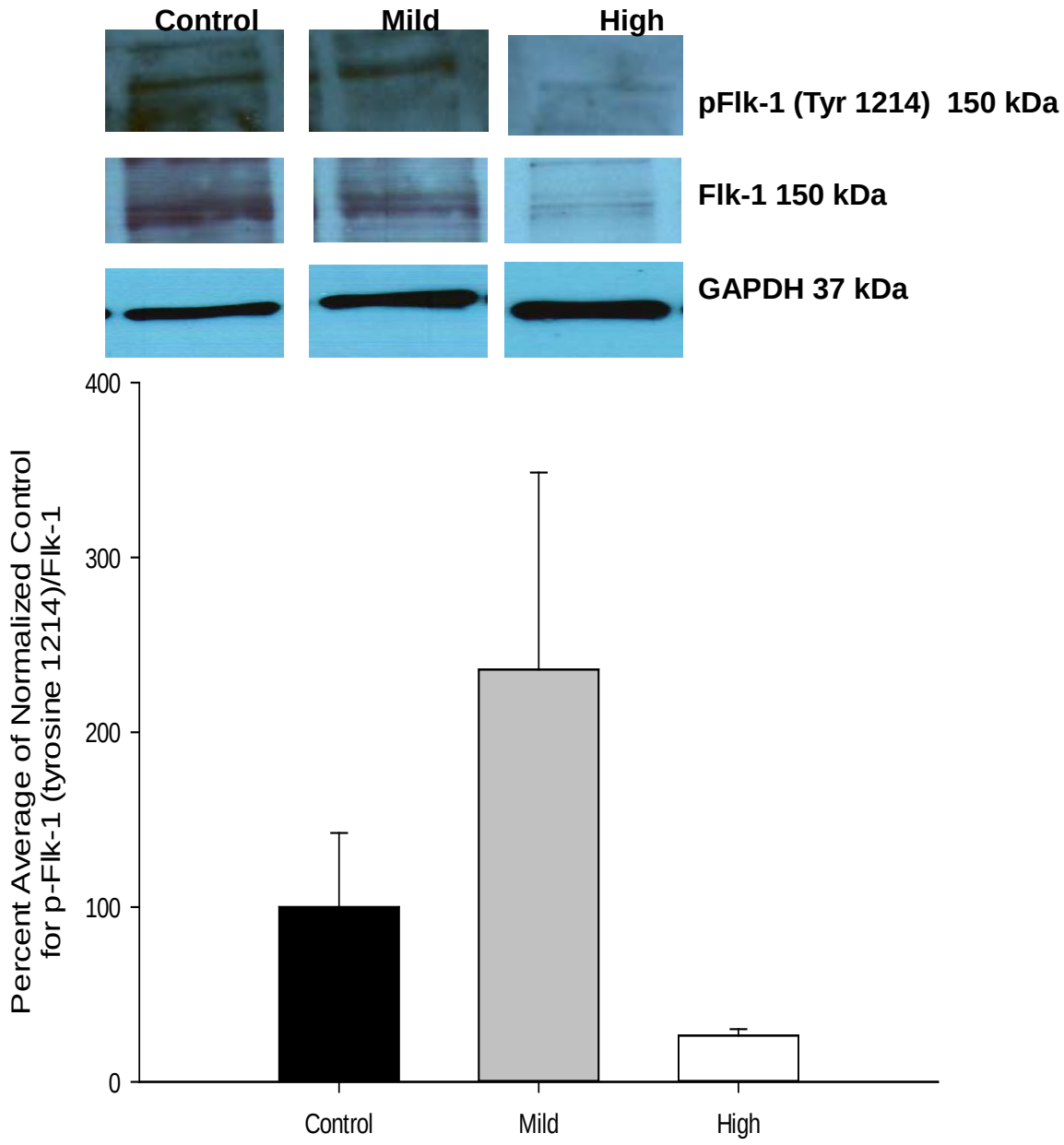


Figure 3.10. No change in p-Flk-1 (tyrosine 1214) protein expression in STZ-induced diabetic animals. Phosphorylation of Flk-1 at tyrosine 1214 did not change in STZ animals compared to control. (one-way ANOVA; n=10 for control; n=5 for mild; n=5 for high)

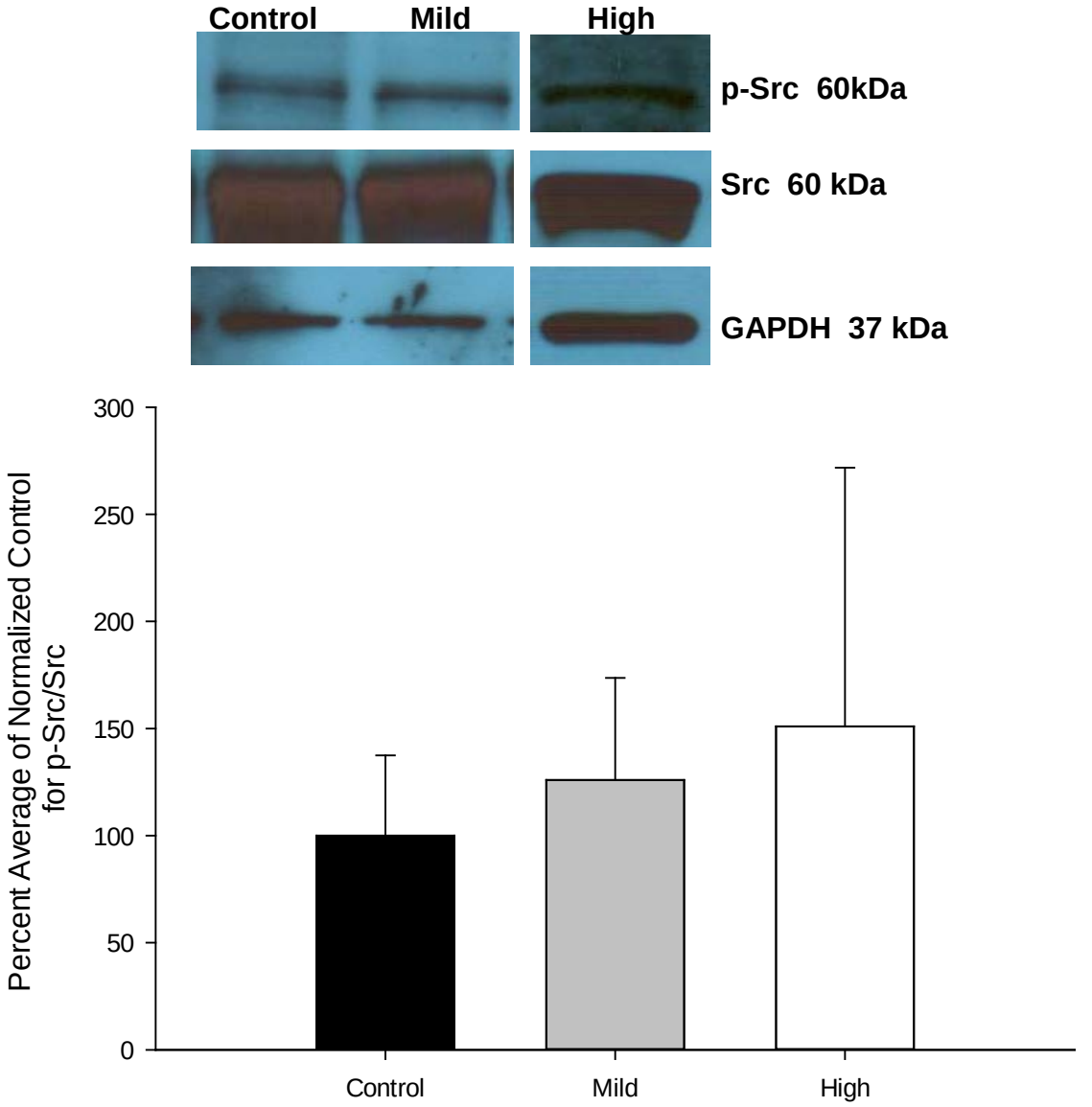
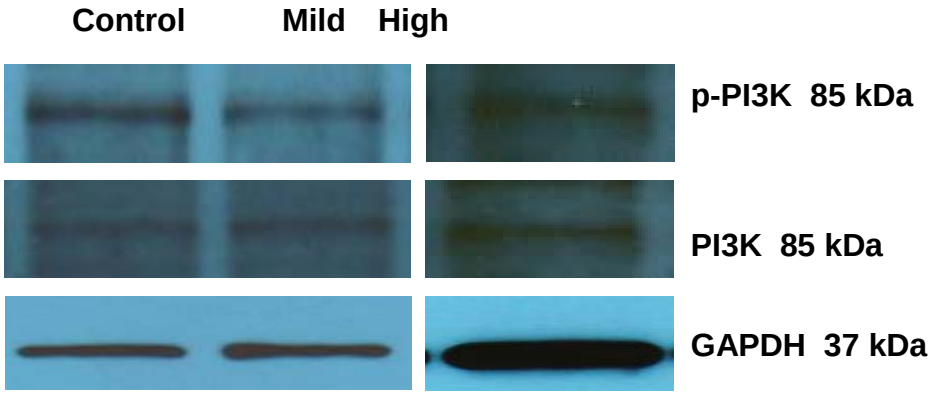


Figure 3.11. No change in Src protein expresssion in STZ-induced diabetic animals. Western analysis showed that global phosphorylation did not change in Src, the downstream target of Flk-1 at tyrosine 951, in diabetic animals compared to control. (n=8 for control; n=4 for mild; n=4 for high)



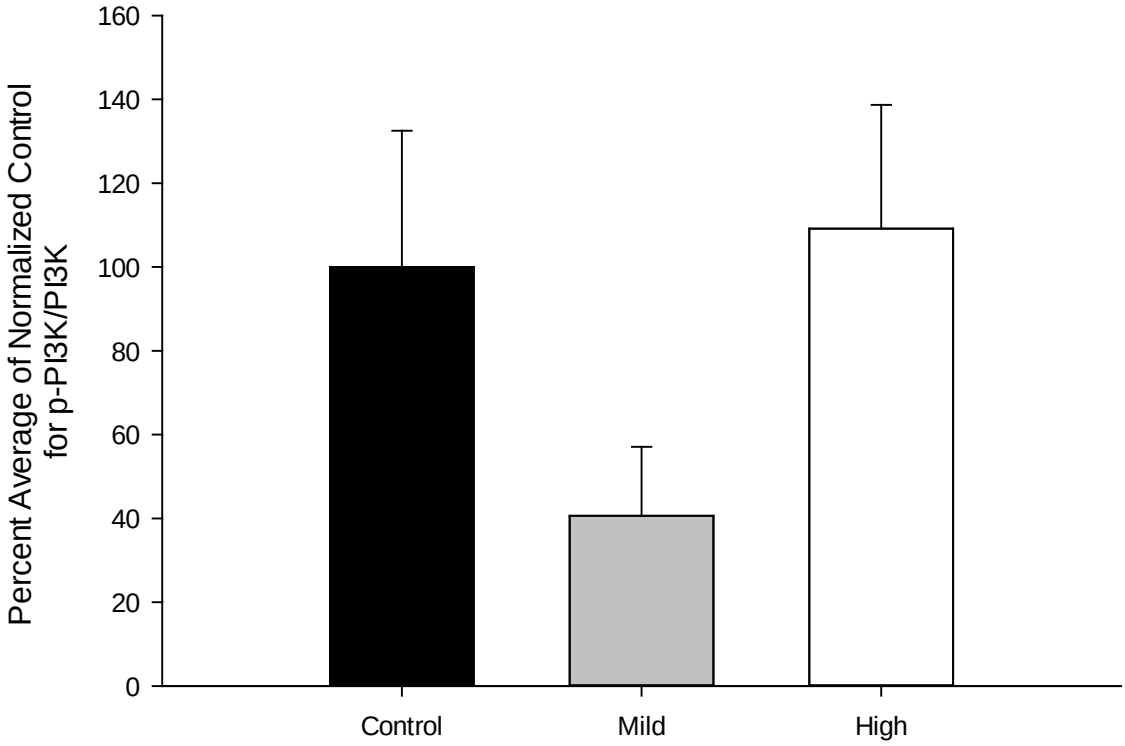
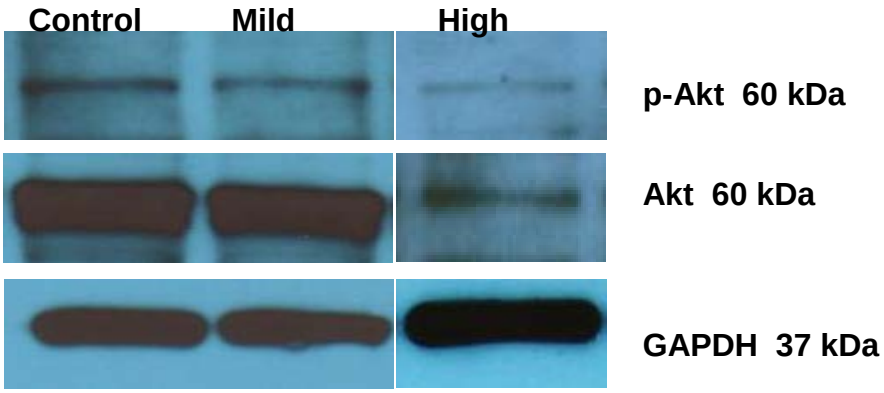


Figure 3.12. No change in protein expression of PI3K in STZ-induced diabetic animals. Phosphorylation of PI3K, the downstream target of Flk-1 at tyrosine 1175, did not change in diabetic animals compared to control. (n=13 for control; n=7 for mild; n=6 for high)



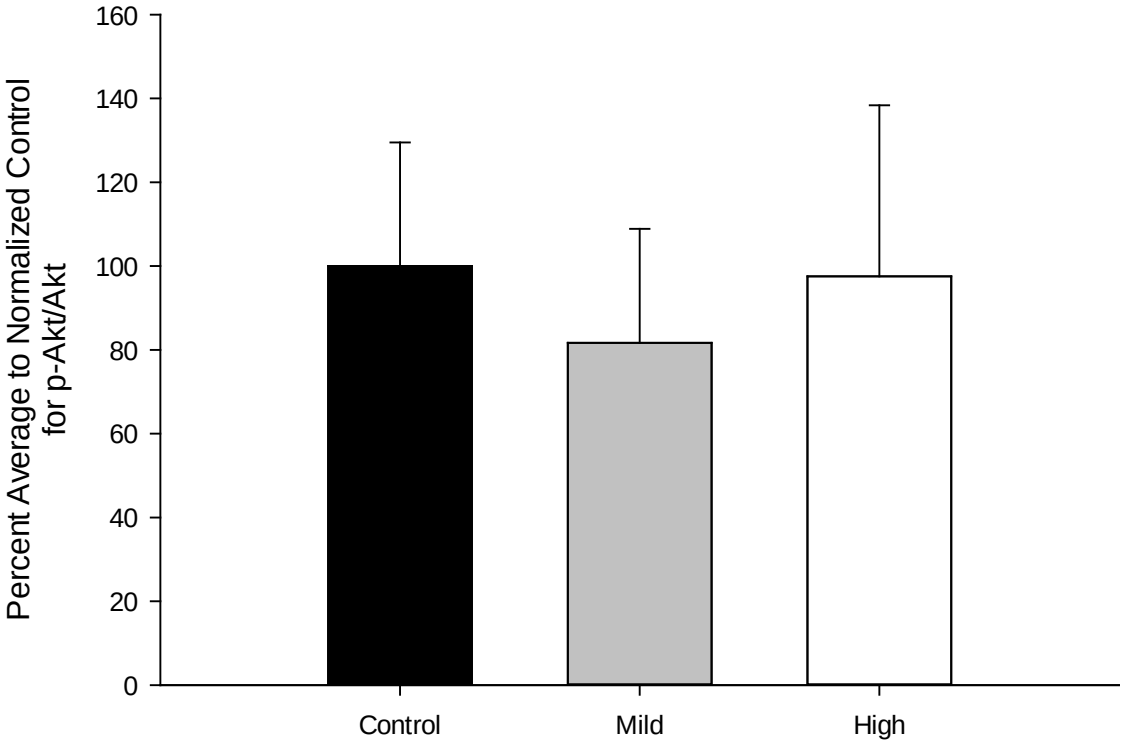


Figure 3.13. No change in protein expression of Akt in STZ-induced diabetic animals. Phosphorylation of Akt, the downstream target of Flk-1 at tyrosine 1175, did not change in diabetic animals compared to control. (n=15 for control; n=9 for mild; n=6 for high)

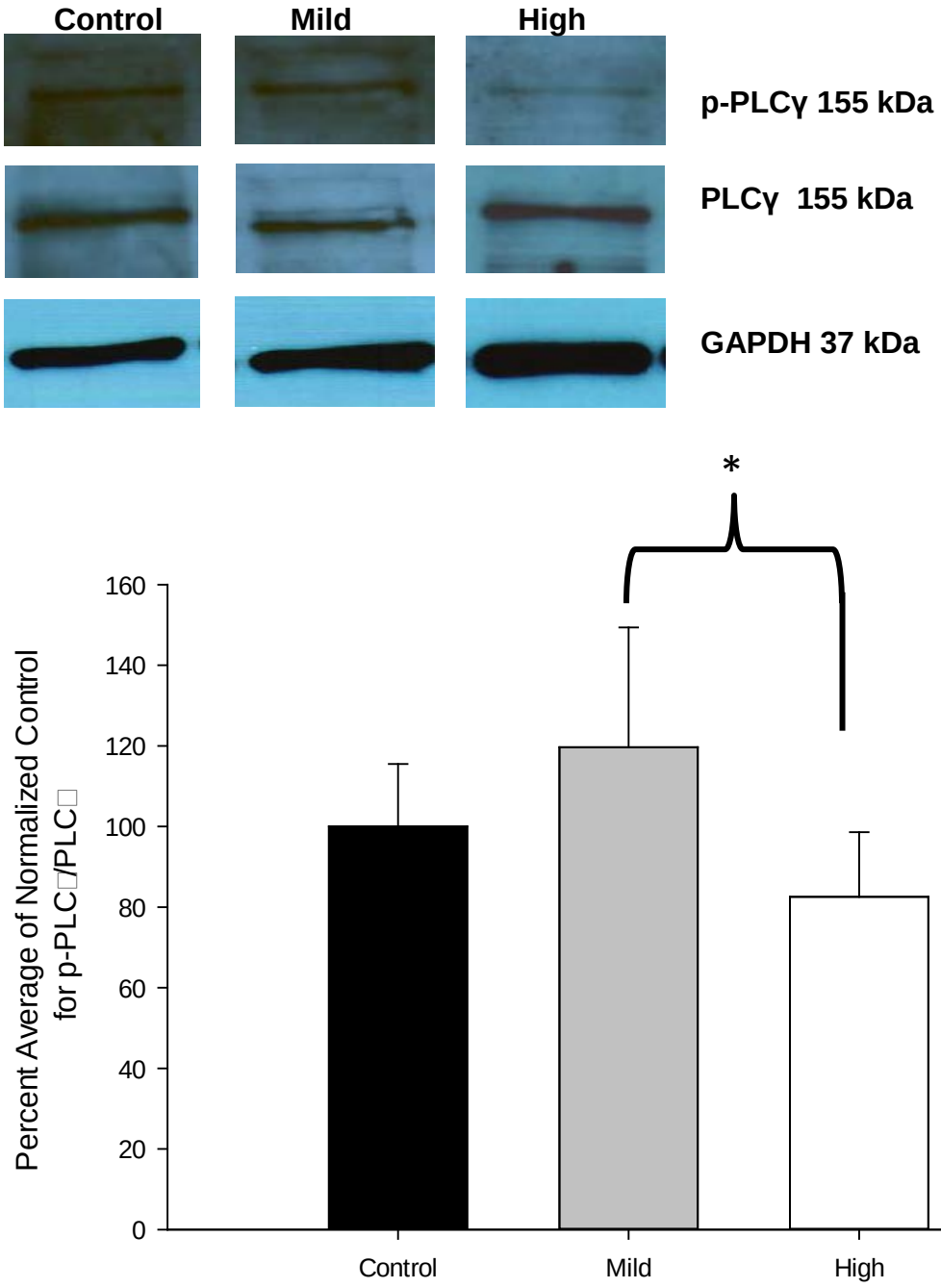


Figure 3.14. Protein expression of PLCγ in STZ-induced diabetic animals. Western analysis showed that there was a significantly greater phosphorylation of PLCγ in diabetic animals with mild glucose levels compared to diabetic animals high blood glucose levels. (n=12 for control; n=6 for mild; n= 6 for high)

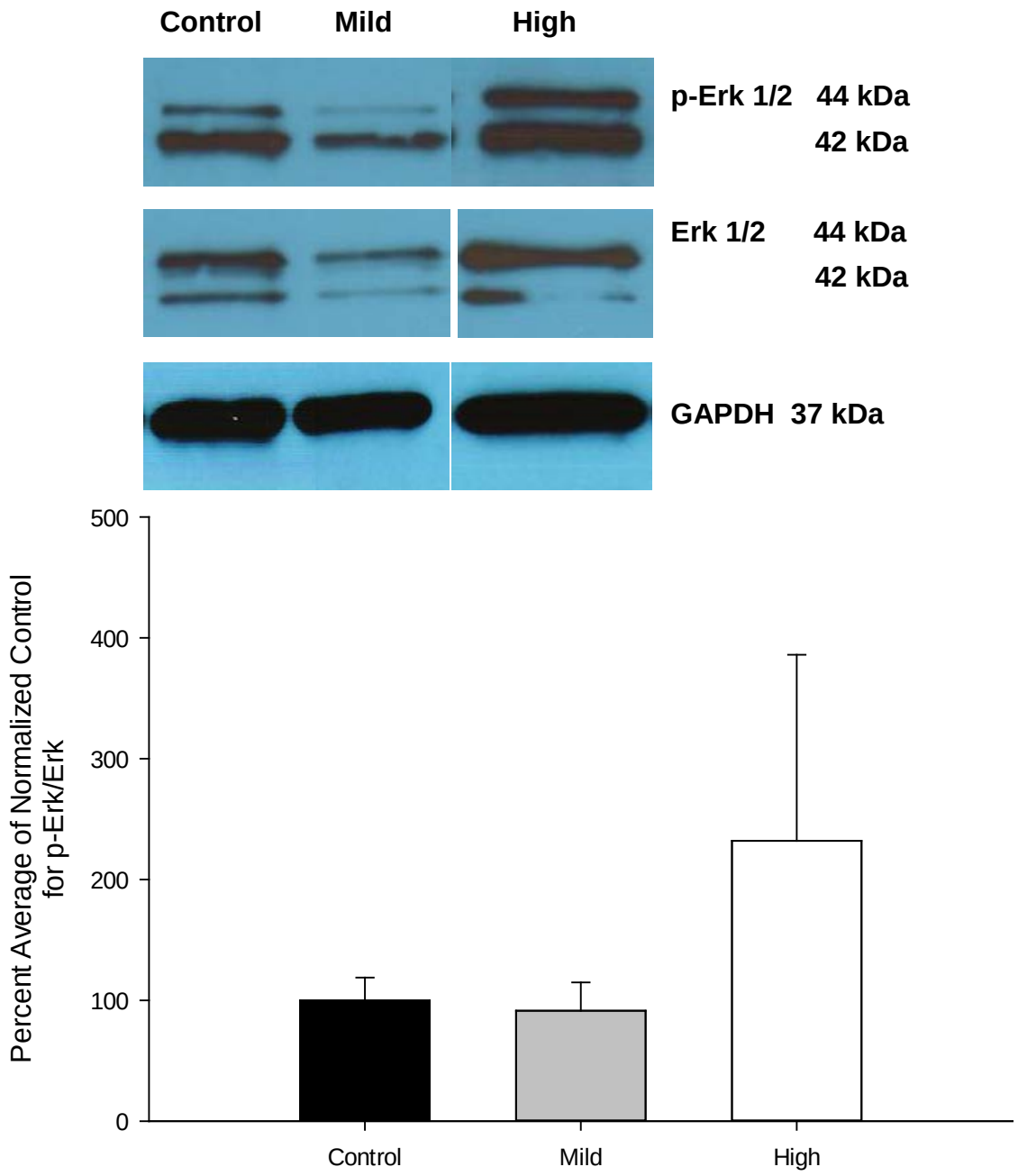


Figure 3.15. No change in protein expression of Erk 1/2 in STZ-induced diabetic animals. Western analysis showed that there was no change in phosphorylation of Erk 1/2 in diabetic animals compared to control. (n=14 for control; n=9 for mild; n=5 for high)

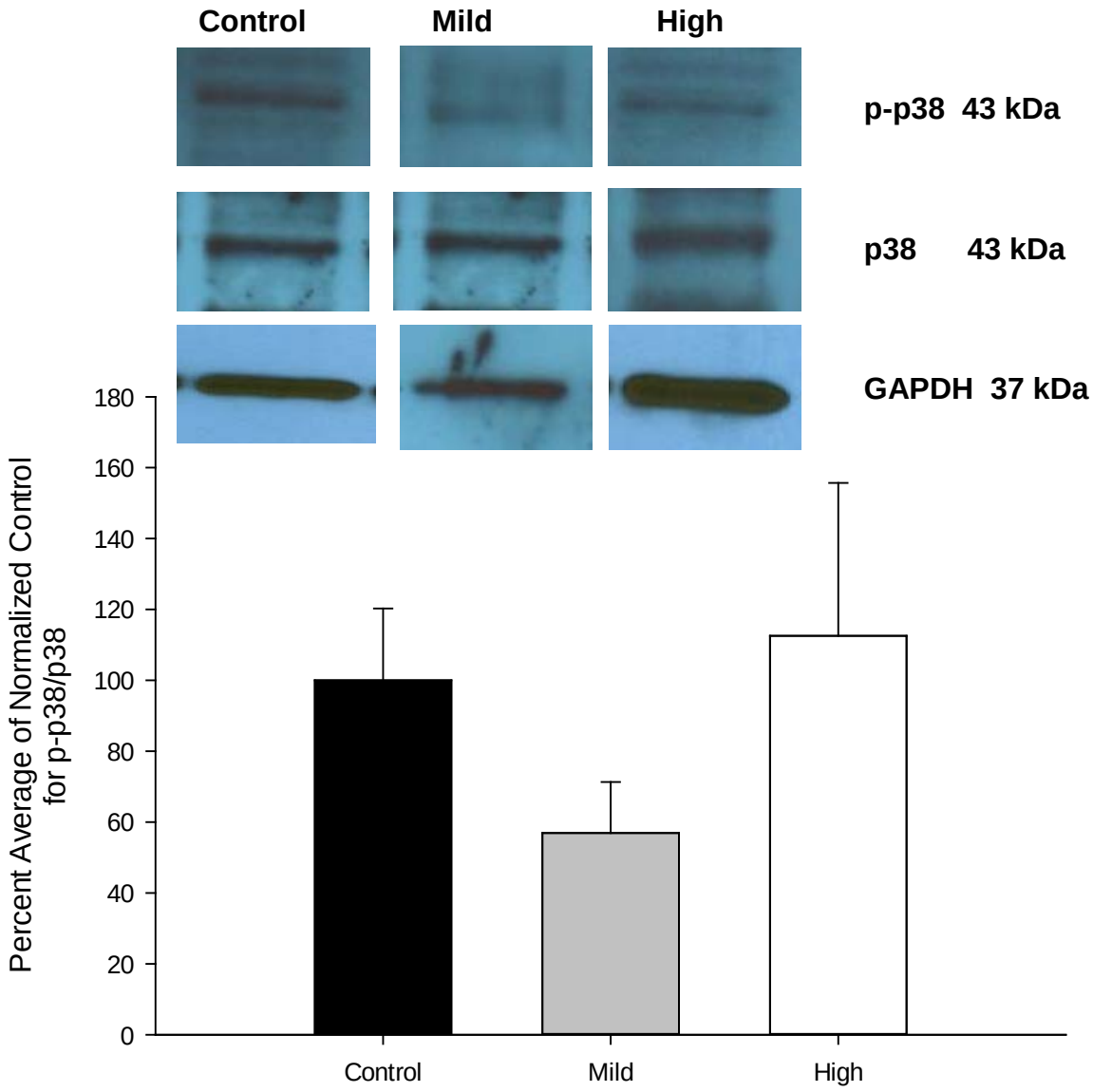


Figure 3.16. No change in protein expression of p38 in STZ-induced diabetic animals. There was no change in phosphorylation of p38 in diabetic animals compared to control. (n=10 for control; n=6 for mild; n=4 high)

to decrease Flk-1 levels at the plasma membrane through modification and degradation (Ewan et al., 2006).

Another explanation for the lack of effect of STZ treatment on Flk-1 expression is that receptor levels are decreased in the post-natal brain (Kremer et al., 1997). Therefore, it is possible that Flk-1 receptor expression is low in this diabetic model. Maturation of Flk-1 involves glycosylation, resulting in 200 and 230 kDa forms of the receptor. However, we were unable to detect Flk-1 at these molecular weights. A fraction of the NP-1 receptor can be modified with either heparin or chondroitin sulfate, and this modification is able to post-transcriptionally regulate Flk-1 expression (Shintani et al., 2006). Although NP-1 protein levels did not change, it is possible that there is enough modification of NP-1 receptors that could influence Flk-1 expression. VEGF has been shown to mediate Flk-1/NP-1 complex (Soker et al., 2002). Since VEGF protein levels did not change, then the Flk-1/NP-1 complex is less likely to form.

No change was observed in SEMA 3A protein levels in the diabetic animals. Furthermore, the VEGF/SEMA 3A ratio was unchanged between control and diabetic groups. An imbalance in this ratio in favor of VEGF would likely lead to activation of several pathways involved in angiogenesis and permeability, such as migration and proliferation. SEMA 3A is also a permeability factor acting through the neuropilin receptor (Acevedo et al., 1998). Therefore, SEMA 3A competes with VEGF in binding with the neuropilins. Additionally, VEGF has been shown to internalize the NP-1 in endothelial cells when competing with SEMA 3A (Narazaki and Tosato, 2006). Although the VEGF/SEMA 3A ratio did not change, this result could offer explanation for no change in NP-1 protein levels.

Although mRNA levels of VEGF, its receptors, and SEMA 3A increased in diabetic animals compared to control, we did not see any changes at the protein level. Recent studies have shown that circulating microRNA can play a significant role in diabetes (Guay et al., 2011; Rottiers et al., 2012). MicroRNA are short, non-coding, single-stranded RNAs, which regulate expression mRNA either by 1) complete homology resulting in mRNA degradation or 2) imperfect homology leading to translation repression. Circulating miRNAs that were significantly altered in STZ-induced diabetic rats include miRNAs which regulate insulin receptors and insulin binding proteins. In addition, there were miRNAs which could be involved in regulating VEGF signaling. There was a significant up-regulation of miR-361 and a down-regulation of miR-127, both of which target VEGF. Although there was no change in VEGF protein levels, these miRNAs may be regulating VEGF expression. Additionally, there was an up-regulation in miR-330, -26b, -let-7a/c, and -139-5p. SEMA 3A is a potential target for miR-330, and up-regulation of miR-330 may contribute to the mismatch between our SEMA 3A mRNA and protein measurements. There was also a down-regulation in miR-497, which targets Flk-1. An increase in Flk-1 protein expression would be expected. These discrepancies between mRNA and protein could be due to miRNA interaction with AU-rich element binding proteins (ARE-BPs), which together can regulate gene expression (Chang et al., 2011). Additionally, there could be Dicer silencing, which would lead to total reduction of miRNAs and impairment of VEGF-induced cellular processes (Suarez et al., 2008; Chang et al., 2011).

STZ treatment did not change Flk-1 phosphorylation at any of the tyrosine residues. The neuropilins can regulate Flk-1, which could explain the lack of Flk-1

phosphorylation and/or lack of VEGF-mediated formation of Flk-1/NP-1 complex. Although Flk-1 phosphorylation did not change, we continued to look at downstream targets of Flk-1. Phosphorylation of PI3K and Akt via activation of p-Flk-1 (tyrosine 1175) did not change in the diabetic groups compared to the control. Even though this pathway has been shown to increase permeability in endothelial cells, it is not surprising that phosphorylation of these downstream targets did not change since pFlk-1 levels did not change. However, phosphorylation of PLCγ, also via activation of Flk-1 (tyrosine 1175) was significantly increased in diabetic animals with mild glucose levels compared to animals with high glucose ($P<0.012$), but neither group showed significant difference with the control animals. This pathway stimulates proliferation of endothelial cells, which is important in angiogenesis, but it can also lead to eNOS activation and subsequent permeability. Additionally, permeability can occur via PLCγ- dependent influx of calcium (Holmes et al., 2007). However, Erk 1/2, the downstream target of PLCγ, did not change in the diabetic group versus control. This is not surprising since Erk 1/2 activity can be regulated by other proteins such as Ras (Hood et al., 2003) and reactive oxygen species (Wang et al., 2004). Ras has also been shown to mediate VEGF expression in Ras-induced Erk and HIF-1α activation (Wang et al., 2004). To complicate matters, Ras activation is also dependent on integrins, and these integrins can coordinate signaling pathways with VEGF (Hood et al., 2003). Activation of Flk-1 (tyrosine 951 and 1214) also did not yield changes in Src and p38 MAPK, respectively, in the diabetic groups compared to control. A study by Kawamura et al. (2008) showed that activation of p38 MAPK required both Flk-1 and NP-1 receptors. In our model,

neither receptor showed any change in diabetic versus control groups. Therefore, it is unlikely that p38 MAPK is involved in BBB permeability in diabetes.

Although there was an increase in gene expression of VEGF, its receptors, and SEMA 3A, our study showed that protein levels did not change. Protein expression of the components of the VEGF signaling system could be regulated by the abundance of specific miRNAs. In addition, there was no change in the phosphorylation status of the Flk-1 receptor. However, we were able to show a significant increase in PLCγ phosphorylation between the two subgroups of the STZ-treated animals. Therefore, the regulation of VEGF and its signaling pathways may, in part, be a mechanism for the observed permeability increase in this diabetic animal model.

Chapter 4: Discussion and Conclusions

Discussion

Diabetes is a significant problem in the United States, and complications include retinopathy, cardiovascular disease, peripheral artery disease, nephropathy, and neuropathy (Bonora et al., 2004; Pambianco et al., 2006). Many of these complications are linked to microvascular pathologies. Diabetic patients are at risk for cerebrovascular disease and neurodegeneration, and research has been aimed at the role of the blood brain barrier (BBB) in this process. Several studies indicate that these complications can arise from the loss of integrity of the BBB (Abbott, 2002; Zlokovic, 2008). Thus, the BBB is unable to effectively maintain its barrier characteristics and becomes "leaky." This leakiness is associated with decreased expression of tight junction proteins, which serve to keep the endothelial cells of the BBB in close contact and restrict paracellular transport (Hawkins et al., 2007). The first part of the study was to determine if the molecular changes at the BBB could be attenuated by minocycline, a tetracycline derivative. Furtherermore, I hypothesized that VEGF plays a role in minocycline treatment. VEGF is a potent angiogenic and permeability factor that has been implicated in vascular complications at the blood-retinal barrier in diabetes (Antonetti et al., 1998). Minocycline has been shown to have an inhibitory effect on VEGF. The aim of the second part of the study was to evaluate the potential role of VEGF in diabetic BBB dysfunction.

Minocycline Studies

The STZ model of diabetes is a well-studied model, and we were able to show that diabetic animals were hyperglycemic and exhibited increased ketone levels, which are characteristics of TID. Additionally, BBB integrity is compromised in diabetes.

Therefore, we hypothesized that minocycline would attenuate the observed BBB changes in diabetes. We were able to show that minocycline does not affect blood glucose levels or brain weights of these animals, suggesting that the drug does not affect diabetes itself. Studies have shown that minocycline reduced infarct volumes in MCAO models of ischemia (Yrjanheikki et al., 1999; Nagal et al., 2008; Matsukawa et al., 2009). On the other hand, treatment with minocycline increased infarct volumes in hypoxia-ischemia studies (Tsuji et al., 2004). These conflicting observations might be due to start of treatment times, as the above studies reported treatment prior to ischemic events, and duration of minocycline treatment. In our study, we treated the animals with minocycline seven days after STZ injection in order to mimic a long term treatment. Minocycline significantly decreased BBB permeability to sucrose in both control and STZ-treated animals. Because previous studies in the laboratory have reported a decrease in tight junction protein expression in this animal model in conjunction with increased permeability (Hawkins et al., 2007), we predicted that minocycline might exert its effects on these tight junction proteins. Treatment with minocycline had no effect on occludin and ZO-1 protein expression; however, minocycline significantly increased claudin-5 protein expression in both control and STZ-treated animals. This observation was interesting because claudin-5 is considered to be the primary seal for tight junctions (Hawkins and Davis, 2005). Minocycline may, in part, function to improve claudin-5 expression. However, the mechanism of this increase in expression is not known. Claudin-5 can be regulated by different mechanisms, such as storage, recycling, post-translational modification, and inhibition of specific signaling pathways such as the PKC pathway. Furthermore, MMP activity

also affects claudin-5 expression. It would be interesting to investigate these various avenues to determine how claudin-5 is regulated with minocycline treatment.

The next step was to determine if VEGF is involved in this minocycline-induced increase in claudin-5. Previous studies have shown that VEGF regulates claudin-5 expression (Argaw et al., 2009). Furthermore, minocycline is able to promote VEGF expression (Hollborn et al., 2010). We did not see any change in VEGF protein expression in control and diabetic animals. However, we were able to show that VEGF significantly increased in STZ-treated animals with minocycline, and a similar trend was observed in control animals treated with minocycline. This increase in VEGF may be due to a decrease in MMP expression and activity since minocycline has been shown to decrease MMPs (Wasserman and Schlichter, 2007). MMPs degrade the extracellular matrix, and previous work in the laboratory demonstrated an increase in MMP activity in STZ-treated animals (Hawkins et al., 2007). The observed increase in VEGF levels with minocycline treatment suggests that the extracellular matrix is relatively intact and the majority of VEGF is bound to it. Future experiments could investigate whether minocycline treatment decreases MMP activity in these animals.

We also found a significant increase in Flt-1 in control animals treated with minocycline, and observed a similar trend in the minocycline-treated diabetic animals. Interestingly, Flk-1 protein expression did not change with minocycline treatment, and neither did the neuropilin receptors. Flt-1 is a decoy receptor, and may be scavenging any VEGF that is secreted and thus inhibiting VEGF from binding the Flk-1 receptor. Inhibiting VEGF binding would prevent the activation of downstream signaling pathways that would lead to a permeability increase. It would be interesting to see if these

signaling pathways affect claudin-5 expression in both control and minocycline-treated diabetic animals.

STZ Studies

VEGF increased upon minocycline treatment in diabetic animals (Chapter 2). Therefore, we studied the effects VEGF signaling might have on diabetes. Furthermore, studies have demonstrated that VEGF can induce a permeability increase and a decrease in tight junction expression in the blood vessels of the diabetic eye (Antonetti et al., 1998). We were able to show that gene expression of VEGF, its receptors, and its endogenous antagonist, SEMA 3A, increased in STZ-treated rats compared to control in both cerebral microvessels and whole brain samples. VEGF and VEGFR (Flt-1 and Flk-1) mRNA expression was higher in whole brain samples than in cerebral microvessels. This result is expected since VEGF and its receptors are present in other cell types in the brain (McCloskey et al., 2008). An increase in VEGF and Flk-1 mRNA expression has been demonstrated in other studies (Hoehn et al., 2002). However, we found that protein levels of VEGF did not change in the microvessels or plasma of either group. This result was unexpected, as we would have expected an increase in VEGF protein levels in the diabetic brain microvasculature. Plate et al. (1992) demonstrated a low steady state level of expression of VEGF in blood vessels in the normal brain. VEGF expression is up-regulated in various pathological events. Since we observed an increase in mRNA expression of components of the VEGF signaling system, but no change at the protein level, we speculated that VEGF may undergo translational modification or that VEGF mRNA is regulated. In collaboration with Dr. Santanam's laboratory, we studied the potential

regulation of VEGF via microRNAs. MicroRNAs are single stranded RNA that regulate expression of mRNA. If the miRNA has 100% homology to the mRNA, then degradation of the mRNA occurs. If there is partial homology, the mRNA undergoes translation repression. Results indicate that up-regulation of miR-361 and down-regulation of miR-127 in diabetic animals could offer an explanation as to why VEGF did not change in this model of diabetes. miRNA-361 and -127 are known miRNAs that target VEGF mRNA. Furthermore, VEGF may be bound to the extracellular matrix in our model, which may also explain why we did not see any change in VEGF protein expression in either the microvessels or plasma. Additionally, other cell types, such as astrocytes, are able to secrete VEGF. Thus, additional experiments would be needed to evaluate VEGF protein levels in the extracellular matrix and astrocytes.

Interestingly, we also did not see any change in protein expression of the VEGF receptors, Flt-1, Flk-1 and NP-1. In the normal brain microvasculature, Flk-1 is down-regulated due to a decrease in angiogenesis post-natally (Kremer et al., 1997). We speculated that receptor expression would increase in the diabetic brain. An increase in Flk-1 protein expression was observed in STZ-treated retinas (Hammes et al., 1998). Additionally, it has been shown that VEGF is capable of up-regulating both mRNA and protein expression of Flk-1 (Shen et al., 1998). However, our results are not fully consistent in what has been reported in the literature. Flk-1 undergoes glycosylation, and we were able to show Flk-1 expression at 150 kDa, which is the immature receptor. Intermediate glycosylated and mature Flk-1 receptor are present at 200 and 230 kDa, respectively. As the receptor undergoes glycosylation, it will move to the plasma membrane of the cell. We were not able to observe the fully mature and functional

receptor, which may offer an explanation for the lack of change in receptor expression at the two-week time point. We were, however, able to measure the activated receptor via its phosphorylation. Therefore, it would be beneficial to extend the time point to see if there are receptor changes. The Flt-1 receptor has higher affinity for VEGF than Flk-1, and acts as adecoy receptor to sequester VEGF (Ferrara et al., 2003; Cebe-Suarez et al., 2006). Studies have shown that Flt-1 protein expression increased in intraretinal vessels in proliferative diabetic retinopathy (Smith et al., 1999). Additionally, it has been reported that VEGF is a major stimulus for Flt-1 up-regulation, especially in tumor angiogenesis (Barleon et al., 1997). However, VEGF levels did not change in STZ-induced diabetes, and may explain the lack of increase in Flt-1 expression.

The neuropilins function as co-receptors for VEGF signaling. We showed that there was no change in NP-1 protein expression. We were unable to show NP-2 expression. NP-2 also functions to promote VEGF-stimulated pro-angiogenic activity. Like NP-1, NP-2 can increase Flk-1 phosphorylation. It would be important to look at NP-2 expression in this model. An important question to ask is if NP-1 and NP-2 levels differ in this model, and if so, how does that affect VEGF signaling? NP-1 and NP-2 are primarily expressed at the arterial and venous ends, respectively (Sulpice et al., 2008). It is possible that the level of enhanced VEGF signaling is dependent on the location of these co-receptors. Furthermore, it would be interesting to examine if there are different affinities for Flk-1 binding between NP-1 and NP-2.

SEMA 3A is a ligand for the neuropilin receptors, and a competitive antagonist of VEGF. When it binds to the neuropilin receptors, it inhibits VEGF-mediated angiogenesis but can also induce permeability (Acevedo et al., 2008). We did not see

any change in SEMA 3A expression in our diabetic animals. Furthermore, we did not see changes in the VEGF:SEMA 3A ratio. If the ratio had changed in favor of VEGF, it is possible that VEGF-induced signaling pathways might be more evident in this model. It would be of interest to study SEMA 3A-induced permeability if the ratio had changed in favor of SEMA 3A. Once SEMA 3A binds to the neuropilins, it must form a complex with plexin A1, the signaling receptor for semaphorins. However, we were unable to detect the plexin A1 protein expression in our model; however, it is not known if it is absent altogether or if it was due to limitation of the antibody.

The lack of any observed changes in VEGF and its receptors prompted us to look at how the VEGF signaling system is regulated. In collaboration with Dr. Santanam, we investigated whether expression of VEGF is modulated by microRNAs. Although not entirely complete, these results indicate that microRNAs might play an important part in regulating the VEGF signaling system. In particular, the up-regulation of miR-361 and the down-regulation of miR-124 are likely indicators of how VEGF expression is modulated. One miRNA may be more effective in regulating VEGF expression due to a closer match in homology. The studies of the miRNAs are incomplete, and further investigation is needed to determine the role other miRNAs play in VEGF signaling.

Finally, we looked at phosphorylation of Flk-1 and the downstream targets of its signaling pathways. We looked at three phosphorylation sites (tyrosine 951, 1175, and 1214), but were unable to see any significant changes in the total phosphorylation. However, it appears that there is a trend for an increase in phosphorylation for all sites in diabetic animals with mild blood glucose, and a decrease in phosphorylation for all

sites in diabetic animals with high blood glucose. This result could mean that in mildly diabetic animals signaling pathways are activated, but by the time diabetes is at uncontrolled and high glucose levels, the Flk-1signaling pathways are overwhelmed. We looked at downstream targets of the Flk-1 phosphorylation sites. We did not see any changes in PI3K and Akt, the downstream target of tyrosine 1175. This result was somewhat unexpected because Akt will lead to the activation of eNOS to induce vascular permeability. However, we did see a significant change in phosphorylation of PLCγ, another downstream target of tyrosine 1175. To our knowledge, this result is a novel finding. However, a downstream target of PLCγ, Erk 1/2, did not exhibit any changes in phosphorylation. This observation is not discouraging since Erk 1/2 activation can be modulated by other signaling molecules, such as Ras. This change in PLCγ is of particular interest because this is a known pathway of VEGF-induced tight junction regulation. Inhibition of PLCγ activity leads to a hyperphosphorylation of tight junction proteins (Ward et al., 2002). This hyperphosphorylation is believed to be due to PKCα/β regulation of tight junction proteins. It would be beneficial to explore this avenue as a permeability increase could be a multi-pathway event. Last, we did not see any changes in Src and p38, the downstream targets of tyrosine 951 and 1214, respectively.

It must also be remembered that we are looking at a single time point in a dynamic process. Changes in the expression and activation levels of the VEGF system are likely to be modulated throughout the time of the disease. Thus, future studies would probably benefit from looking at multiple time points and perhaps concentrating on either mild or high blood glucose levels.

Conclusions

Vascular permeability and a decrease in tight junction protein expression are evident in diabetes. My results show that the increased permeability can be attenuated by the treatment of minocycline. The decreased permeability was in conjunction with an increase in claudin-5 protein expression, known as the primary seal of the tight junction. These results show promise for pharmacological treatment of cerebrovascular complications of diabetes. It has been shown in these studies that VEGF protein expression increased in diabetic animals upon minocycline treatment, perhaps making VEGF more available. Thus, we wanted to further investigate VEGF in the observed BBB changes in diabetes. The mechanism of increased permeability had not been elucidated in the literature, but studies have been shown that VEGF is implicated in vascular permeability in the diabetic eye. Therefore, we looked to see if VEGF is involved in the vascular permeability. Although VEGF and its receptors did not exhibit any changes at the protein level, we did observe a significant change in one of the downstream targets, PLCγ, which is involved in gene expression and proliferation of endothelial cells. Although the mechanism is not complete, this result is a starting point to further investigate the role of the PLCγ signaling system in vascular permeability.

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Appendix A

MARSHALL UNIVERSITY
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Office of Research Integrity

August 30, 2012

Aileen Marcelo
1 Springwood Drive
Huntington, WV 25705

Dear Ms. Marcelo:

This letter is in response to the submitted dissertation abstract titled "The Role of Vascular Endothelial Growth Factor at the Blood-Brain Barrier in Diabetes." After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study involves publicly available data and does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(f) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director
Office of Research Integrity

AILEEN J. MARCELO

Contact Information

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Huntington, WV 25705
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Education

2007-present Ph.D. Graduate Student in Biomedical Sciences under the direction of Richard Egleton, Ph.D., Marshall University School of Medicine (MUSOM), Huntington, WV

2001-2003 MS in Biomedical Sciences, Marshall University School of Medicine (MUSOM), Huntington, WV

1997-2001 BS in Biology, West Virginia University, Morgantown, WV

Professional Experience

January 2011 – May 2011

Adjunct Professor in the Division of Allied Health and Life Sciences, Mountwest Community and Technical College, Huntington, WV. Taught BIOL 257: Introduction to Anatomy and Physiology.

August 2003 – August 2007

Research Assistant in the Department of Pharmacology, Physiology, and Toxicology, MUSOM, Huntington, WV under the direction of Kelley Kiningham, Ph.D.

Laboratory Techniques

- Animal handling
- Rat cerebral microvessel isolation
- Rat cerebral spinal fluid isolation
- Rat brain sectioning
- Blood-gas analysis
- Western analysis
- Real-time PCR
- Immunohistochemistry
- Cell culture techniques

Publications and Abstracts
Peer-Reviewed Journal Publications:

Brown, K.C., Lau, J.K., Dom, A.M., Witte, T.R., Luo, H., Crabtree, C.M., Shah, Yashoni H., Shiftett, B.S., **Marcelo, A.J.,** Hardman, W.E., Egleton, R.D., Proper, N.A., Chen, Y.C., Mangiarua, E.I. and Dasgupta, P. (2011). MG624, an alpha7-nAChR antagonist, inhibits angiogenesis via the Egr-1/ FGF2 pathway. *Angiogenesis* (in press).

Dom, A.M., Buckley, A.W., Brown, K.C., Egleton, R.D., **Marcelo, A.J.**, Proper, N.A., Weller, D.E., Shah, Y.H., Lau, J.K., and Dasgupta, P. (2010). The α7-nicotinic acetylcholine receptor and MMP-2/9 pathway mediate the proangiogenic effect of nicotine in human retinal endothelial cells. *Invest. Ophthalmol. Vis. Sci,* June 16, 2010 ; doi:10.1167/iovs.10-5461.

Herdman, M., **Marcelo, A.,** Huang, Y., Niles, R.M., Dhar, S., and Kiningham, K.K. (2006). Thimerosal Induces Apoptosis in a Neuroblastoma Model via the c-Jun N-Terminal Kinase Pathway. *Toxicological Sciences, 92*(1): 246-53.

Abstracts:

June 2012: **Marcelo, A.** and Egleton, R. *The Role and Regulation of Vascular Endothelial Growth Factor (VEGF) at the Blood-Brain Barrier in a Rat Model of Diabetes.* **Selected for Oral Presentation** at the Gordon Research Seminar **and Poster Presentation** at the Gordon Research Conference, Colby Sawyer College, New London, NH.

November 2011: Furby, R., Shah, P., **Marcelo, A.**, Cook, C., Egleton, R., and Santanam, N. *Circulating MicroRNAs Regulating Insulin Signaling in Type I Diabetic Rat Model*. Selected for Poster Presentation at the Annual Biomedical Research Conference for Minority Students, St. Louis, MO.

November 2011: Shah, P., Furby, R., Marcelo, A., Egleton, R., Cook, C., and Santanam, N. *Circulating miRNAs Regulating Angiogenesis Signaling in Type 1 Diabetic Rat Model.* Selected for Poster Presentation at the Annual Biomedical Research Conference for Minority Students, St. Louis, MO.

July 2011: Levin-Nielson, E., **Marcelo, A.**, and Egleton, R. *Regulation of Vascular Endothelial Growth Factor Signaling (VEGF) in the Choroid Plexus (CP) in Diabetes*. Selected for Poster Presentation at the 10th Annual WV-INBRE Summer Research Symposium, Marshall University, Huntington, WV.

July 2011: McNeel, B.S., Proper, N.A., **Marcelo, A.J.**, and Egleton, R.D. *Effect of*

Minocycline (MIN) on Tight Junction Proteins of the Choroid Plexus (CP). Selected for Poster Presentation at the 10th Annual WV-INBRE Summer Research Symposium, Marshall University, Huntington, WV.

July 2011: Furby, R., Shah, P., Kocher, C., Cook, C., **Marcelo, A.**, Egleton, R., and Santanam, N. *MicroRNA Involvement in Type I Diabetic Rat Models*. Selected for Poster Presentation at the 10th Annual WV-INBRE Summer Research Symposium, Marshall University, Huntington, WV.

May 2011: **Marcelo, A.J**., and Egleton, R.D. *Vascular Endothelial Growth Factor (VEGF) Signaling and its Potential Role at the Blood Brain Barrier in Diabetes.* Selected for Poster Presentation at the XXVth International Symposium on Cerebral Blood Flow, Metabolism, and Function & Xth International Conference on Quantification of Brain Function with PET, Barcelona, Spain.

March 2011: **Marcelo, A.**, and Egleton, R. *Modulation of the Blood Brain Barrier (BBB) by Vascular Endothelial Growth Factor (VEGF) in Diabetes.* Selected for Poster Presentation at the 23rd Annual Research Day, MUSOM, Huntington, WV. **Winner for best basic science poster presentation**

June 2010: **Marcelo, A.**, and Egleton, R. *The Potential Role of Vascular Endothelial Growth Factor (VEGF) at the Blood Brain Barrier in Diabetes*. **Gordon-Kenan Research Seminar** on Barriers of the CNS, Colby-Sawyer College, New London, NH.

June 2010: **Marcelo, A.**, and Egleton, R. *The Potential Role of Vascular Endothelial Growth Factor (VEGF) at the Blood Brain Barrier in Diabetes*. **Gordon Research Conference** on Barriers of the CNS, Colby-Sawyer College, New London, NH.

March 2010: **Marcelo, A.**, and Egleton, R. *The Potential Role of Vascular Endothelial Growth Factor (VEGF) in the Diabetic Brain Microvasculature.* **Selected for Poster Presentation** at the 22nd Annual Research Day, MUSOM, Huntington, WV; **Winner for best basic science poster presentation**

.

October 2009: **Marcelo, A.**, and Egleton, R. *The Role of VEGF Signaling in the Modulation of Diabetic Brain Vasculature.* Society of Neuroscience Meeting, Chicago, IL.

October 2008: **Marcelo, A.**, and Egleton, R. *Blood Brain Barrier Dysfunction in a Rat Streptozotocin Model of Diabetes*. University of Kentucky Cardiovascular Research Day, Lexington, KY.

June 2008: **Marcelo, A.**, and Egleton, R. *Expression of Angiogenic Regulatory*

Receptors at the Blood Brain Barrier. Gordon Research Conference: Barriers of the CNS, Tilton, NH

August 2007: Hunter, V., Silvis, A., **Marcelo, A**., and Kiningham, K. *N-Acetyl-$_L$-Cysteine (NAC) Enhances All-Trans Retinoic Acid (ATRA)-Mediated Differentiation through Modulation of Retinoid Receptor Transcriptional Activity.* 2007 WV-INBRE Summer Research Symposium, Huntington, WV.

November 2006: Kiningham, K., Spitz, D., Cook, C., and **Marcelo, A.** *Upregulation of Manganese Superoxide Dismutase mRNA, Protein and Activity by All-Trans Retinoic Acid (ATRA): Potential Roles of NFκB and the Retinoic Acid Receptor-α* – 13th Annual Society for Free Radical Biology and Medicine Meeting, Denver, CO.

November 2006: Asbury, M., Tassone, M., **Marcelo, A.**, Kiningham, K. *Acute Dopamine Stimulation in SK-N-MC Neuroepithelioma Cells Causes Activation of the Redox-Sensitive AP-1 Transcription Factor, Oxidative Stress, and Apoptosis*– 13th Annual Society for Free Radical Biology and Medicine Meeting, Denver, CO.

March 2006: **Marcelo, A.**, Herdman, M., Cook, C., Kiningham, K. *N-acetylcysteine (NAC) and Trolox Attenuate Thimerosal-Induced Oxidative Stress and Apoptosis* – **Selected for Poster Presentation** at the 19th Annual Research Day, MUSOM, Huntington, WV; **Winner for best basic science poster presentation**

August 2005: Davis, T., Cook, C., Humphrey, M., **Marcelo, A.**, Tassone, M., Kiningham, K., Spitz, D. *Upregulation of Manganese Superoxide Dismutase Protein and Activity by All-trans Retinoic Acid (ATRA): Potential Roles for NFκB and Retinoic Acid Receptors (RAR)* – 4th Annual WV-INBRE Summer Research Symposium, Marshall University, Huntington, WV

April 2005: **Marcelo, A.**, Cook, C., Humphrey, M., Stewart, J., Kiningham, K. *Antioxidants Block Thimerosal-Induced Stress Response and Attenuate Cellular Damage in a Human Neuroblastoma Cell Line (SK-N-SH)* – Poster presentation at the 15th Annual Sigma Xi Research Day, Marshall University, Huntington, WV

March 2005: **Marcelo, A.**, Cook, C., Humphrey, M., Stewart, J., Kiningham, K. *Antioxidants Block Thimerosal-Induced Stress Response and Attenuate Cellular Damage in a Human Neuroblastoma Cell Line (SK-N-SH)* – **Selected for Oral Presentation** at the 18th Annual Research Day, MUSOM, Huntington, WV; **Winner for best basic science oral presentation**

April 2004: **Marcelo, A.**, Cook, C., Humphrey, M., Kiningham, K. *All-trans Retinoic Acid (ATRA) Protects Against Cisplatin-Mediated Apoptosis* – Poster presentation at the 14th Annual Sigma Xi Research Day, Marshall University, Huntington, WV

Extracurricular Activities

March 2012 — Volunteer at fourth annual Brain Expo at Marshall University. Presented interactive station to teach elementary school children about the retina at Marshall University's Brain Expo as part of a global Brain Awareness campaign organized by the Dana Alliance for Brain Initiatives

March 2011 — Volunteer at third annual Brain Expo at Marshall University. Presented interactive learning station about reflexes to area school children as part of International Brain Awareness Week.

May 2010-April 2011: President of the Graduate Student Organization for the Marshall University Biomedical Sciences Program.

March 2010: Volunteer at second annual Brain Expo at Marshall University. Presented information and hands-on activity to area school children about vision as part of International Brain Awareness Week.

May 2009-April2010: Vice-President of the Graduate Student Organization for the Marshall University Biomedical Sciences Program. Duties include attending Graduate Studies Committee meeting to act as a liaison between faculty and fellow graduate students and assisted the President in the decision making and planning of various fundraising events.

March 2009: Volunteer at first annual Brain Expo at Marshall University. Presented information and hands-on activity to area school children about the different parts of the brain as part of International Brain Awareness Week.

Awards

April 2010: Recipient of the NASA West Virginia Space Grant Consortium for the proposed project entitled **"The Potential Role of Vascular Endothelial Growth Factor (VEGF) in the Diabetic Brain Microvasculature." Monetary Amount: $12,000.**

June 2010: Recipient of Registration Fee Waiver for the Gordon-Kenan Research Seminar on Barriers of the CNS, Colby-Sawyer College, New London, NH.

June 2010: Recipient of Travel Award for the Gordon Research Conference on Barriers of the CNS, Colby-Sawyer College, New London, NH.

References

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