CEREBROVASCULAR REMODELING AND PLASTICITY IN DIABETES: MECHANISMS AND RELEVANCE TO STROKE RECOVERY

ROSHINI PRAKASH
(Under the mentorship of ADVIYE ERGUL)

ABSTRACT

Diabetes-mediated complications spanning from kidney disease to stroke increase the mortality and morbidity and almost all diabetic complications have a vascular component. While diabetes is known to increase the risk and recurrence rate of stroke and worsen outcomes, the impact of diabetes on the regulation cerebrovascular structure which is in the pathophysiology of stroke as well as in the recovery after stroke remains ambiguous. The objectives of this study were to investigate the cerebral vascular remodeling, neovascularization patterns, delineate the underlying mechanisms that contribute to the regulation of cerebrovascular restructuring and explore the brain's ability to revascularize and functionally recover after ischemic reperfusion injury in type-2 diabetes. Pial cerebral vasculature was visualized by vascular corrosion casting revealed increased vascular tortuosity, collateralization, intra-arterial anastomoses and collateral diameters. A fluorescent space filling model demonstrated the existence of dysfunctional cerebral vasculature in diabetes. Cerebral vessels also displayed increased perfused volume and surface areas with a major contribution from macrovessel enlargement and profuse microvasculature. These vessels had greater susceptibility to vascular injury and bleeding after stroke. Primary microvascular endothelial cells derived from the type-2 diabetic rats expressed increased activation of vascular endothelial growth factor (VEGF) receptors, c-src, MMP-2 and MT1-MMPs in diabetes. Early events in angiogenesis like the cell proliferation, migration and

tube formation were enhanced in these cells. This study also elucidated the molecular interplay of VEGF and peroxynitrite, a critical mediator of oxidative stress. Cerebral neovascularization is seemingly necessary post stroke and is being considered as a protective therapeutic strategy to rewire and recover brain functions. Diabetic rats subjected to ischemic reperfusion injury display impaired reparative cerebral neovascularization and increased astrogliosis associated with poor functional outcome. Lastly, this study provides beneficiary roles of metformin in diabetes by preventing vascular complications when used as a glycemic control strategy and improving reparative cerebral neovascularization when used as an interventional therapeutic target. In conclusion, our study is the first report demonstrating dysfunctional cerebral neovascularization mediated by diabetes via vascular endothelial growth factor and peroxynitrite that renders the cerebrovasculature more susceptible to ischemic injury and debilitates the brain's ability to revive after stroke.

INDEX WORDS: Type-2-diabetes, stroke, cerebral neovascularization, vascular remodeling, glycemic control, oxidative stress, matrix metalloproteases, vascular endothelial growth factor, hemorrhagic transformation.

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By

ROSHINI PRAKASH

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ROSHINI PRAKASH

Major Professor: Adviye Ergul

Committee: Susan C Fagan
Azza B El-remessy
Somanath PR Shenoy
Shuang Huang

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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PROBLEM STATEMENT AND SPECIFIC AIMS

The objective of this study is to understand how type 2 diabetes affects brain neovascularization before and after stroke and ultimately stroke outcomes and recovery. We have previously shown that ischemia/reperfusion (I/R) injury in a mild and lean model of type-2 diabetes (T2D), Goto-Kakizaki (GK) rats results in greater hemorrhagic transformation associated with infarction. In addition diabetic GK rats have severe neurological outcomes 24 hours after stroke. Diabetes alone can exacerbate vascular disease; however the dynamics of cerebrovascular remodeling and its long-term consequences on stroke are unclear.

The **central hypothesis** is that diabetes-mediated dysfunctional cerebral neovascularization compromises the vasculature making it susceptible to secondary bleeding or hemorrhagic transformation as a result of reperfusion. Furthermore, dysfunctional vasculature impairs adaptive angiogenesis and functional recovery long-term after stroke. It is further postulated that increased vascular endothelial growth factor (VEGF) together with peroxynitrite contributes to dysfunctional cerebral neovascularization in diabetes. These hypotheses will be tested in 3 aims using GK model of T2D and respective controls rats.

Aim 1: Test the hypothesis that diabetes exacerbates the cerebral neovascularization and structural alterations differentially in the regions susceptible to ischemic reperfusion injury.

a) The extent of cerebral arteriogenesis that will be assessed after infusing the pial circulation with a polyurethane resin in control and diabetic GK rats treated with vehicle, metformin (glycemic control) and minocycline (MMP inhibitor). We will assess the

arteriogenic parameters like tortuosity, collateralization, vessel diameter as well as MMP expression and activation. It is expected that therapeutic inhibition of MMPs or glycemic control can reduce cerebral arteriogenesis.

b) Indices of cerebral neovascularization such as vascular density, volume and surface areas will be evaluated to study if the changes occurring in the pial vasculature extend into the deep cortical and subcortical vasculature. This study will also focus on spatial and temporal distribution of vasculature in different brain regions and compare them to other vascular beds. We will also determine the maturity of the vasculature by pericyte coverage and ratio of perfused and non-perfused vasculature.

c) We will investigate the existence of dysfunctional cerebral neovascularization in other models of T2D. Branch density, tortuosity and parenchymal vessel diameters will be assessed in micro and macrovascular beds to understand structural alterations.

Aim 2: Test the hypothesis that increased VEGF production in diabetes mediates the angiogenic response by a peroxynitrite-dependent mechanism in brain microvascular endothelial cells (BMVEC).

Early angiogenic events such as proliferation, migration and tube-formation properties of primary BMVECs isolated from control and GK rats will be measured in the absence and presence of pharmacological and molecular inhibitors of VEGF, peroxynitrite, membrane type-1 (MT1) MMP and MMP-2. The Working hypothesis is that increased VEGF signaling via VEGFR2 and c-src activates MT1-MMP, MMP-2 and these stimulate EC migration and tube formation.

Aim 3: Test the hypothesis that diabetes mediated dysfunctional cerebral angiogenesis prior to stroke impairs vascular and functional plasticity after I/R injury.

Indices of neovascularization will be assessed in sham operated animals and compared to cerebral neovascularization long-term after stroke in control and diabetes. Neurological outcomes will be tested temporally for about 14 days after ischemic reperfusion injury. We will employ glycemic control using metformin to determine the impact of hyperglycemia on neurovascular recovery after stroke. It is expected that glycemic control improves angiogenic response and functional outcomes after I/R injury in diabetes.

At the end of proposed studies, this project will provide novel information on potential mechanisms underlying cerebral complications of diabetes. These are important to develop preventive and therapeutic strategies for stroke in diabetic patients and discover pivotal mechanisms of cerebral angiogenesis that increases the symptomatic hemorrhage rate in diabetic stroke while providing an excellent training opportunity for the applicant.

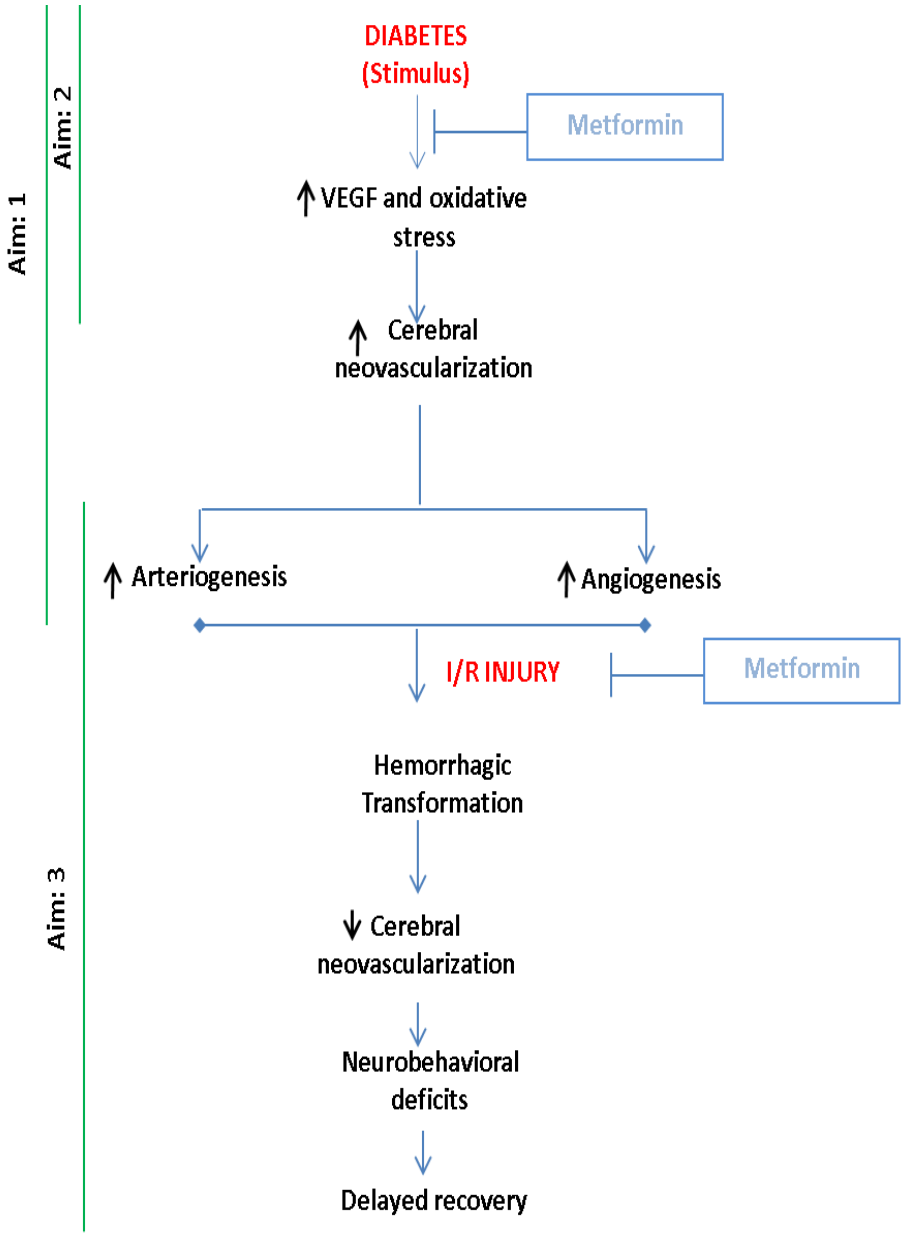

Figure 1-1. Schematic diagram of overall hypothesis.

CHAPTER 1

REVIEW OF THE RELEVANT LITERATURE AND RATIONALE

OVERVIEW OF DIABETES MELLITUS AND COMPLICATIONS

Diabetes is a global threat which affects more than 347 million patients worldwide and this number is expected to double by the year 2030 with greater increase in the developing countries [1, 2]. The global estimate of the prevalence of type-2-diabetes (T2D) is about 3.8% of the current population. In recent years it has been observed that the risk of diabetic and prediabetic conditions in the younger population has increased significantly thereby causing an even higher economic burden. Insulin is a key regulatory hormone that is involved in maintaining blood glucose level. T2D is symptomatically characterized by blood glucose levels >120mg/dL, elevated HbA1Cs and impaired glucose tolerance. In T2D resistance to the actions of insulin occurs by decreased sensitivity to insulin and downregulation of insulin receptor, as a result there is an increased demand to produce insulin from the pancreatic β-cells to maintain normal blood glucose which progressively results in β-cell exhaustion, insulin resistance and chronic hyperglycemia. Metabolic dysregulation through chronic state of hyperglycemia develops long term complications in the vasculature [1, 3, 4].

Diabetes mediated vascular complications

Vascular complications are fundamental causes of increasing morbidity and mortality in diabetic patients. Chronic hyperglycemic status in T2D increases the odds of organ dysfunction [5]. Diabetic complications are generally characterized to be a) macrovascular, clinically resulting in coronary artery disease (CAD), peripheral arterial disease (PAD) and stroke, b)

microvascular in nature causing nephropathy and retinopathy [6]. Large prospective prevention studies, The U.K. prospective diabetes study (UKPDS) conducted on T2D patients emphasize a significant correlation between metabolic indices such as hyperglycemia, HbA_{1C} and increased risk of stroke, cardiovascular morbidity and mortality [7]. Multi risk intervention trials and epidemiological studies, EPIC and ARIC studies provide convincing evidence of 2-4 fold increased risk of cardiovascular events in diabetic patients compared to non-diabetic individuals. Not only chronic or acute elevations in glucose but also glucose fluctuations pose vascular risks.

Approximately 3.5 million people in United States are affected with peripheral arterial disease with a majority of the population being African American [8]. Macrovascular complications in the lower extremities have been reported in diabetic patients possibly due to increased arterial resistance, increased arterial stiffness limiting flow volume resulting in hypercoagulable state ultimately resulting in foot lesions and occlusive arterial disease [9, 10].

Hyperglycemic stature reduces retinal blood flow leading to hypoxic conditions, capillary damage and blockage [11]. Microvascular complications as seen in diabetic retinopathy can lead to total or partial loss of vision through vitreous hemorrhages, macular edema, retinal vascular leakage and retinal detachment. Duration of diabetes is also known to increase the prevalence and severity of diabetic retinopathy [12]. In a Reykjavik study, examined the relationship between retinopathic lesions and their predisposition to cerebral microvascular signs and bleeding. This study provided a positive correlation between retinal and cerebral microbleeds. Decreased total cerebral blood flow and increased cerebral microbleeds also lowered cognitive performance in the diabetic population [13-17]. The Rotterdam study suggested that retinal vessel caliber can be a good determinant of cerebral infarction and hemorrhage; however identification of high risk groups needs to be determined [18].

Cerebrovascular diseases comprise 20% of death among the diabetic population and leading to severe neurological deficits and disability [1]. The damage caused by diabetes in the brain can lead to Alzheimer's disease, dementia, vascular cognitive impairment, stroke, and depression. A detailed focus on stroke is provided in the next section. Our knowledge of how diabetes affects vascularization and global morphological alterations in the cerebrovasculature in early diabetes is undefined. The key focus of this dissertation was to understand the development of dysfunctional vasculature and structural alterations mediated by moderate diabetes in the brain that is prone to bleeding. This study also provides a comprehensive outlook on diabetic vasculature by comparing different vascular beds.

Diabetes mediated neurological complications

Diabetes not only affects the vascular system and contributes to microangiopathies but also causes mild to severe damage to the nervous system. 60-70% of diabetic patients are predisposed to impaired sensation, pain in the extremities during the initial stages, and may be subjected to lower-extremity amputations in the severe form [1]. Nerve demyelination and axonal thickening progress into axonal loss in diabetic condition. Basement membrane hypertrophy, loss of pericytes, derangements in cytoskeletal microfilaments can also contribute to neuropathy by reducing the blood supply to these neurons [11].

Impaired neuropsychological functioning has been shown in type-2 diabetic patients. Diabetes is also considered as a major contributor to amyloid angiopathies seen in Alzheimer's disease and dementia. In the CASCADE trial (The study of cardiovascular determinants of dementia) studied the association of brain lesions by magnetic resonance imaging in a diabetic population. The pooled data demonstrated a direct relationship between cortical brain atrophy

and diabetes, with greater interactive effects exerted with hypertension [19]. Diabetic patients are twice as likely to have depression that can complicate diabetes management [1].

A proportional association in cognitive dysfunction with the severity and duration of diabetes has also been indicated in the diabetic population. The Framingham study demonstrated strong independent interactions of diabetes and hypertension with poor cognitive function in the elderly [20]. This study also provided direct correlations of 10-year stroke risk with cognitive performance in larger populations with greater subclinical cerebrovascular disease burden [21-23].

Harten et.al, reviewed brain imaging studies conducted on diabetic patients using CT, MRI, MRS and various tomography methodologies. The review reports not only apparent association between diabetes and white matter lesion, but also significant contribution of diabetes to silent infarcts. Lacunar nature of the infarcts was more frequently observed in the diabetic patients. Cerebral cortical atrophy with ventricular enlargement which is presumed to be related to neurodegeneration was also reported to be associated with vascular risk factors [24, 25].

With emerging concepts of diabetes affecting the brain and cognition [26], we sought to investigate behavioral and cognitive alterations in early diabetes and also after an event of vascular injury. This will be addressed in short in the Aim: 3 of this dissertation.

Mechanistic overview

Experiments conducted in cell cultures and animal models provide a unifying hypothesis that demonstrates the role of hyperglycemia in micro and macrovascular complications. Hyperglycemia or pronounced elevation in plasma glucose is the hallmark of diabetes. Chronic hyperglycemia triggers inflammatory response, activation and secretion of several cytokines that

mediate pro-inflammatory gene expression in various organs, including pancreas causing β-cell dysfunction and insulin resistance [27]. Mitochondrial superoxide generation tips the redox status of cells and leads to oxidative stress. Excess reactive oxygen species reduces eNOS activity resulting into vascular dysfunction. Hyperglycemia also leads to glycosylation of proteins generating higher levels of advanced glycation end products. Activation of protein kinase C through the hexosamine pathway leads to overproduction of vascular growth factors, NADPH oxidase system, collagen deposition and intimal thickening and capillary occlusion. Decreased activity of eNOS and increased endothelin-1 affects blood-flow properties and produces a cascade of signaling events leading to endothelial dysfunction and altered vascular response. Increased VEGF (Vascular Endothelial Growth Factor) affects vascular permeability leading to increased pathological angiogenesis [28].

Given the knowledge of several biochemical links leading to vascular alterations and angiopathies, further studies are warranted to integrate molecular mechanisms that lead to vascular alterations in the micro and macrovasculature.

DIABETES MELLITUS, STROKE AND HEMORRHAGIC TRANSFORMATION

Stroke etiology

Stroke or cerebral infarction affects 15 million people globally, with one third of the affected population having permanent disability, impacting ones quality of life [29, 30]. Stroke is caused by an acute interference or blockage of vascular supply in the brain resulting in severe impairment of neurological functions. It is estimated that stroke compromises 43.7 million disability-adjusted life-years in the adult stroke population [29]. Two categories of stroke subtypes have been observed a) *Ischemic type* arising due to blockage of arteries that can be of

cardiac origin or due to the detachment of an atherosclerotic plaque and this comprises of 88% of all strokes, while b) *hemorrhagic type* of stroke caused due to rupture of vessels in the brain leading to either sub-arachnoid or intracranial hemorrhage. The most critical region that suffers a total blood loss and undergoes necrosis represents the *core* of the infarct. The vulnerable area surrounding the core, which has partially compromised blood flow and structural integrity, is the *penumbra*.

Clinically, stroke is a heterogeneous disease owing to a myriad of risk factors that can significantly contribute to the occurrence of stroke and these include, *social factors* such as smoking, alcohol consumption, poor dietary habits; *pathological disease states* such as hypertension, diabetes, obesity; psychological factors such as stress and depression [29]. The duration of the insult, localization, severity of ischemia and genetics also adds to complexity.

The main aim of therapies targeted acutely after stroke are recanalization, thrombolysis, and neuroprotection to salvage the ischemic penumbra and prevent the expansion of the core. Currently rtPA (recombinant tissue plasminogen activator) is the single approved therapy used as a clot buster. Not all patients can undergo tPA treatment due to the limitations in the window of treatment, number of contraindications of tPA, reperfusion injury and co-morbid conditions associated with stroke. Even the patients that are treated with tPA have a greater risk of thrombolysis induced bleeding and worsening neurological function. Therefore, there is an urgent need to investigate novel therapeutics based on the existing knowledge of stroke pathophysiology that can replace tPA or work in synergy [31].

Diabetes is an independent risk factor of ischemic stroke:

It is estimated that 30-40 % of adult strokes present with hyperglycemia with or without diabetes. Epidemiological studies suggest that about 20 % of the stroke patients have diabetes as

a co-morbid condition. The relative risk of cerebrovascular disease or stroke is 2 to 6-fold higher in diabetes [32]. Diabetes leads to vascular dysfunction and atherosclerosis that accelerates the risk of stroke apart from aging, and this has been reflected in the age of the stroke patients associated with diabetes, who are relatively younger compared to the patients without co-morbid conditions [33]. Diabetes also increases stroke related mortality, severity and rate of recurrent stroke [34, 35] and predicts early neurologic deterioration following ictus [36, 37]. The Framingham heart study, Honolulu Heart Program and the Copenhagen Heart Study greatly relate the increased risk of stroke associated with diabetes with a greatest odds ratio in the African American, followed by Caucasians and Hispanics. The majority of the clinical trials so far conducted use a measure of vascular related risk factor to assess the risk of stroke. The UKPDS-60 trial derived diabetes-specific risk factors such as duration of diabetes and age at diagnosis in stroke [38]. Although patients with diabetes have an increased number of additional atherosclerotic risk factors including hypertension, obesity, and hyperlipidemia; diabetes has been identified as a stroke risk factor independent of these comorbidities. There exists a bidirectional link between dysglycemia and ischemic stroke. First, diabetes alone doubles the risk of stroke. Secondly, ischemic stroke can cause disturbances in glucose homeostasis and metabolism leading to harmful stroke outcomes [39]. Identification of insulin resistance and pre-diabetes as a risk factor of stroke are now being evaluated [40].

Deleterious effects of T2D on stroke outcomes:

Clinical presentation of stroke in diabetic patients differs from the nondiabetic patients, in that diabetic patients have a greater risk of ischemic stroke than the hemorrhagic type [41]. Hyperglycemia poses a greater risk and deficit in stroke patients than diabetes itself. Generally stroke has been viewed as a disease of the large vessel [42]. However with more studies

reporting microbleeds and silent infarction; the microvasculature has added to this complexity. Symptomatic cerebral bleeding and lacunar infarcts are much more common in diabetic stroke [43, 44]. There is also strong evidence of increased incidence of silent infarction associated with cognitive decline in diabetes.

Admission hyperglycemia is a strong predictor of neurological deterioration in stroke patients [45]. A J- shaped association between serum glucose and neurological outcomes have been drawn [46]. Our knowledge of negative effects of diabetes on stroke outcome mainly came from observational studies. Two NIH-funded feasibility trials including Treatment of Hyperglycemia in Ischemic Stroke (THIS) and Glucose Regulation in Acute Stroke Patients (GRASP) started addressing the important clinical problem of hyperglycemia management in AIS [47, 48]. These recent clinical trials provided important relevant information that rationalized the importance of the studies in this dissertation. 1) More than 80% of the patients in these trials had diabetes. 2) These studies also showed that the blood glucose levels in hyperglycemic stroke patients range 200-220 mg/dl on day 1 and 180-200 mg/dl on day 2. These levels are very similar to that observed in the diabetic model of rats used for the experimental approach in this study. Past studies on experimental hyperglycemic and diabetic stroke included animal models that present with extreme plasma glucose levels >300 mg/dl, unlike these studies, our lab used spontaneously diabetic Goto-Kakizaki (GK) rat model that display moderate hyperglycemia and insulin resistance similar to these clinical trials. This animal model when subjected to ischemic reperfusion injury had infarcts associated with bleeding in the subcortical cerebral regions. This formed the basis of studying the detrimental effects extended by the vasculature into the neuropil in diabetic conditions.

Most studies in experimental models report increased infarct, edema and hemorrhage in hyperglycemic animals after reperfusion [33, 49-51]. While these studies emphasize the importance of glycemia in acute ischemic stroke setting, blood glucose was very high in these experimental models (≥300 mg/dl). Most, if not all, of these studies were conducted mainly under acute hyperglycemic conditions induced by glucose injection or STZ-injection just 2-3 days prior to ischemic injury and thus do not really provide information on diabetic ischemic injury. It is seen that the effects of acute hyperglycemia on stroke outcomes are more detrimental compared to diabetes. This possibly can be due to lactic acid release and tissue acidosis. Mild increase in blood glucose is some studies show a reduction in the infarct, a protective effect of hyperglycemia that provides energy and reduces depolarization. Experimental studies on diabetic brain injury are limited. In db/db mice, a well characterized type 2 model which lacks the leptin receptors, edema and infarct size are increased. In this model blood glucose levels were >400 mg/dI [52]. Furthermore, leptin has neuroprotective properties and lack of its receptors may have additional compounding effects. Studies involving chronic and acute hyperglycemia in rodents have shown vascular leaks and microbleeds associated with ischemic reperfusion injury modeling stroke [33, 53-57]. There have been contrasting observations with hyperglycemia leading to either smaller or larger infarcts. However given the number of studies focusing on the effect of diabetes, hyperglycemic on stroke, little is known about the micro and macrovascular complications in the brain, mechanisms that lead to secondary bleeding in diabetic stroke and its effect on long-term outcomes after stroke. Our studies bridge this gap in knowledge about cerebral micro and macrovascular structural complications in diabetes and their role in stroke pathogenesis. We have discussed these results in chapter 1 and 2. We evaluated the long term effects of diabetes that is known to be associated

with secondary bleeding on a rodent model of T2D that is spontaneously diabetic. This is discussed in Aim: 3.

Hemorrhagic transformation, a secondary complication of stroke in diabetes

Depending on the duration of the occlusion, breakdown of the blood brain barrier (BBB) progresses and secondary hemorrhage known as hemorrhagic transformation (HT) may develop further complicating the stroke outcome. Early studies by Garcia et al. suggested that microvascular changes upon reperfusion promote HT [58]. Blood brain barrier is comprised of endothelial cell, tight junctions, the basal lamina and the astrocytic foot processes [59]. Cellular components of blood, particularly the red cells, enter the parenchyma when this entire unit fails [60, 61]. The degree and duration of occlusion is also a major determinant of HT [62-64]. HTs are commonly triggered by MMPs, due to oxidative stress developed due to a pre-existing metabolic condition or due to the release of excess free radicals that are formed during reperfusion post ischemia [65-68].

Most clinical trials performed earlier classified HT as symptomatic hemorrhage that was defined as any clinical deterioration due to HT seen within 36 hours of ischemic injury. However, the European co-operative acute ischemic stroke study (ECASS) classified HT into two which includes a) *Hemorrhagic infarction HI* that was defined as small petechial hemorrhages seen in and around the infarcts. They were further classified into type I and II based on their confluence. Type I HI referred to small petechial hemorrhages that were seen around the area of infarcts while type II referred to the presence of who had small confluent petechial hemorrhages within the infarcted zone. b) *Parenchymal hematomas (PH*) had space occupying effect. Upon X-ray examination bleeding was seen as hypo dense areas which are the regions of hemorrhage. They were further classified into type I which consisted of slight hypodense areas ≤

30% the cerebral tissue and type II consisted of dense hematomas ≥ 30% of the cerebral tissue [69-71].

The results of the ECASS trial II suggests that PHs were seen associated with hyperglycemia and they were linearly associated with plasma blood glucoses. Older patients had a higher risk of PH upon thrombolysis. Type II PH had worsened outcomes and caused disability within 3 months of the event. PHs are probably caused due to delayed reperfusion with thrombolytic therapy or pre-existing disease states like hyperglycemia unlike HIs which are considered to occur as a result of reperfusion due to thrombolytic therapy. The risk of PH increases linearly above 110mg/dL of blood glucose.

The Rotterdam study shows that cardiovascular risk determinants, lacunar infarction and white matter lesions have a greater prevalence of cerebral microbleeds. Cerebral microbleeds are considered to be asymptomatic and precede symptomatic intracerebral hemorrhage [72, 73]. Intracerebral hemorrhages have been blamed on the pre-existing hypertension, whereas the secondary hemorrhages are mainly due to vascular anomalies, thrombolysis and hyperglycemia. Hyperglycemia has become a growing concern in acute ischemic stroke. Demchuk et.al, reported serum glucose and diabetes to be significant independent predictors of symptomatic intracerebral hemorrhages following thrombolysis with rtPA [74]. These results were reproduced by PROACT II trial. 36% of stroke patients with blood glucose >200mg/dL had hemorrhages. Furthermore, the safety and efficacy of rtPA in hyperglycemic stroke patients is compromised. The NINDS rtPA trial also reported diabetes and hyperglycemia as a strong predictor of HT; diabetes worsened bleeding after tPA administration. Experimental data also supports the plausibility of increased HT with hyperglycemia [75]. However, a clear idea of how diabetes related hyperglycemia affects reperfusion injury is missing [76].

Diabetes precipitates a pro-thrombotic condition in the vasculature. A series of biochemical events disrupt hemostatic and inflammatory pathways causing thrombosis during occlusive vascular disease. Previous findings from a vast literature including our lab are suggestive of diabetes mediated endothelial dysfunction. Endothelial dysfunction activates plasminogen activator inhibitor-1, MMPs, and ROS that are strongly correlated with HT. Del Zoppo and colleagues have recently reported the involvement of microglia mediated matrix metalloprotease during HT [77]. Our lab also demonstrated that diabetic rats subjected to ischemic reperfusion injury presented infarcts associated with HT but not after permanent ischemia.

Bleeding into the brain parenchyma is undoubtedly a vascular associated risk. To investigate what leads to HT, a good understanding of vascular pathophysiology in terms of structural and functional changes mediated by diabetes is essential. The following section will elaborate on diabetes mediated vascular remodeling in the brain.

VASCULAR REMODELING IN HEALTH AND DISEASES

Vascular growth and remodeling begins during organogenesis and continues for the entire life of an organism. The first known assessment of capillary network and complex branching was first carried out in the tail of eel by Leeuvenhoek in the seventeenth century following the advent of the light microscope. Blood vessels form complex patterns, owing to their irregularity and are in general is organized into arcade and tree like arrangements with sequential branching decreasing in diameter but more frequent in number. A careful review of the literature reiterates that angioarchitecture has been well characterized in many organs and tissue. Vascular characteristics are highly specialized based on the requirements of the tissue demands to oxygen

and nutrients. Brain vascular supply is profuse and tightly regulated and receives approxiamately 15-20% of the cardiac output despite the fact that it comprises of 2% its significant lower constitution in body weight compared to other organs. Differences in capillary length and diameter also regulate capillary pressure changes, myogenic tone and flow throughout a vascular tree. Capillaries also provide a large surface for the exchange of blood components such as oxygen and nutrients to the supplying tissue. The topographic, anatomic features and the rheological assessments have been extensively studied and reviewed in the skeletal muscles by Popel and Johnson. Vascular structural and functional adaption continually occurs in response to changes in arterial pressures, hypoxic conditions, inflammatory response, glucose levels, pressure, hemodynamic factors and carcinogens. Dynamic changes in the metabolic parameters can activate molecular signals to remodel vasculature. Vascular remodeling can occur at level of individual small arteries and collectively affecting branching vascularity and organization by neovascularization processes [78].

Remodeling in individual segment of the arteries is studied by measuring media to lumen ratio, stress and strain produced on the vascular walls, myogenic tone and autoregulatory capacity. The degree of vascular autoregulation change with respect to size, length of the vascular segment, localization, and functional nature of the organ. Two types of vascular remodeling are observed; eutrophic and hypertrophic remodeling with inward or outward fashion based on the dynamic changes mediated by the hemodynamic and metabolic stimuli.

Neovascularization is a very complex phenomenon that can be both adaptive and pathological [79]. Neovascularization process occurs by vasculogenesis (new vessel formation from progenitor cells), angiogenesis (capillary sprouting), collateral growth and/or arteriogenesis defined as remodeling of native collaterals to functional arterioles [79, 80]. *Arteriogenesis* occurs

in response to changes in hemodynamic conditions such as wall stress, shear stress and blood flow properties. Arteriogenesis results in a positive outward remodeling of preexisting collateral arteries into larger caliber vessels, which are fully perfused, functional, and have the ability to bypass sites of occlusion [79-82]. *Angiogenesis* is a complex and tightly regulated process that involves the participation of endothelial cells degradation of basement membrane, migration, and proliferation thereby forming capillary sprouts. This process involves numerous stimulators (e.g. growth factors), inhibitors and extracellular matrix components. Angiogenesis is a tissue response to hypoxia and occurs in situations where a reduction in blood supply has sufficiently compromised oxygen delivery to cells [80]. Although these processes share similarities they are distinct and are not independent of each other.

Vascularization is continuously being studied during embryonic development, adaptation to metabolic changes as seen in stroke, diabetic retinopathy, hypertension related cardiac abnormalities, peripheral vascular diseases and in tumors. The central nervous system is highly vulnerable to any disruption in oxygen and nutrient supply. Vascular occlusive diseases mainly stroke has been an impetus to investigate cerebrovascular system. Before investigating diabetes associated vascular disorganization, it is important to gain insights into the normal vascular architecture to compare with.

Cerebral angioarchitecture in normal physiology

The earliest known comprehensive report on rat cerebral vasculature was published by EH Craigie in 1921. Cerebral cortical vascularity was characterized in five distinct areas and was found to be richly perfused in the parietal areas compared to the insular region. This research was the earliest observation showing differences in vascular density [83]. Regional differences between brain vascular supply in the cortical structures were also characterized by Cavaglia et al.

in Sprague-Dawley rats. They show the perpendicular orientation of parenchymal vessels into the cerebral cortex, transition of vascular supply between gray and white matter and that the former had greater vascular density than the latter, vascularization pattern differed in relation to the neuronal orientation [84]. Shih et al. investigated vascular and neuronal interactions and employed two-photon microscopy to study blood flow and neurovascular coupling in the mice brain. Measuring vascular density has been the most conventional method adopted, however lacks the 3-dimensional feature and spatial resolution which the current imaging methods can offer. Risser et al. in 2009 described the complex patterning and integration of adult angiogenesis in primate cortex. They devised 3-dimensional imaging and quantification methods to evaluate the patterns of intra-cortical vessels in newborn and adult monkeys. Their findings provided the following conclusions- a) vascular volume in the gray matter ranges from 2-4.3 % of the total tissue volumes, b) macrovascular volumes composed of 44% of the total vasculature and the rest being capillaries c) with the increase in cortical depth the proportions of macrovascular volumes linearly declined [85]. Exchange between blood and the tissue occur mainly at the capillary level; therefore measuring three-dimensional cylindrical surface areas of the vasculature provide knowledge of the available areas for exchange of vascular components, and permeability. Currently noninvasive imaging techniques like micro-CT and MRI blood volume measurements, x-ray tomographic microscopy are being validated and compared to ex-vivo data obtained from the vasculature to ascertain their feasibility and use in rodent models. Human cerebral cortical vasculature has been characterized by several researchers. Corrosion cast created on the pial cerebral arteries were shown to have interconnections and anastomoses. The increase or decrease in vascular density has been attributed to differences in neuronal contacts and glucose demands [86]. A large topographical estimation by 3-D imaging of the cortical microvasculature was

studied by Cassot et al. The studies show that the major arteries have tree like arrangements while the capillaries have a netted appearance. Vascular branching patterns influence vascular resistance and over blood flow in an organ. A clear identification of branching ratios, connectivity to the parent vessel has been detailed in the human cerebral microcirculation [87-89]. Although these studies only show structural and taxonomic differences, they have potential implications to identify vascular aberrations. Any change not in line with normal laws of patterning can provide cues to detect pathophysiological conditions.

Cerebral vasculature in diabetes

A good association between retinal microvascular structural abnormality and a greater risk of mortality from ischemic heart disease and stroke has been reported by Witt and colleagues [90]. Wiernsperger has reviewed the interrelations between microcirculation and metabolic syndrome [91]. Studies conducted in animal models of diabetes to assess vascular organization are sparse. However there is a large body of literature that discusses the structural alterations in a vessel segment. Studies on STZ treated rats for about 8 weeks shows basement membrane thickening and decreased capillary density. Tomassoni and colleagues studied the simultaneous changes occurring on the astrocytes, neurons and vasculature in STZ induced diabetes. They observed decreased capillary density, thickening of the basement membrane, loss of cerebrocortical neurons, and increased expression of GFAP in the astrocytes. However little research to this effect has been studied in T2D in animal models or in humans [92]. Velchava et al. report increased blood viscosity and whole blood volume in type-2 diabetic patients. Reduced cerebral blood flow velocity measured using transcranial Doppler is indicated in T2D patients [93]. Measuring carotid intima-media thickness (cIMT) is relatively easier unlike imaging whole

brain to assess cerebral microangiopathy. cIMT have been used as a biomarker to predict cerebrovascular dysfunction in diabetic patients [94-96].

Effect of diabetes on remodeling and neovascularization is most extensively studied in the retinal circulation [97-101]. It is well established that hyperglycemia-mediated oxidative damage to microvascular endothelial cells triggers a cascade of events that cause excessive angiogenesis and result in vascular proliferative retinopathy [102-105]. These immature vessels then break and leak worsening vascular and neuronal damage [106]. Retinal microaneurysms and hemorrhages are consequences of increased growth factors and cytokines in diabetes. Retinal hemorrhages have also been shown to be associated with cerebral microbleeds. On the other hand, neovascularization in the coronary and peripheral circulation is impaired in diabetes resulting in increased coronary artery disease and peripheral vascular disease risk, respectively [107, 108]. These well demonstrated observations emphasize the importance assessing different vascular beds and regional differences to angiogenic response mediated by diabetes.

Thus, we see that the knowledge of vascular structural patterning of the brain in normal physiology is considerably established and mainly in the cerebral cortical angioarchitecture; however the knowledge of striatal vasculature in terms of vascular patterning in comparison with the cortex, interconnections between cortex and striatum is poorly known. In pathological states, the primary research focus has been on small vessel remodeling and impaired autoregulatory mechanisms in individual vessel segments. We clearly lack in the cognizance of global information about vascular networking in pathological states especially diabetes. The studies provided in this dissertation bridge the gap in knowledge and emphasize on a) the consequences of neovascularization in early diabetes, b) contrasts vascular networking and organization in normal and pathological states, and c) remodeling due to diabetes and relative role of micro and

macrovasculature in rodent cerebral circulation. We also provide a conceptual molecular mechanism of dysfunctional angiogenesis in the brain microvascular endothelial cells mediated by diabetes.

MEDIATORS OF NEOVASCULARIZATION

Angiogenesis in the brain is highly regulated and strictly controlled process. Growth factors, cytokines, metabolic and neuronal factors drive this process. Endothelial cells are the first effectors of angiogenesis. Structural and functional alterations in the vasculature require intercommunications between various cell types and during diabetes involve the glucose dependent signaling pathways. Adaptions occurring at the cellular level cause heterogeneity and structural alterations of the tissue. Micro and macrovascular endothelial cells differ in function and protein expression at the organ level. William C Aird suggests the existence of substantial heterogeneity in the endothelial cell characteristics of various vascular beds. Endothelial cells in the kidney adapt to the low levels of oxygen, while liver sinusoidal endothelial cells can aid clearance. Insights into various endothelial properties can be therapeutically used in other vascular beds to modulate their characteristics. While the brain microvascular endothelial cells lack fenestrations and prevent the passage of molecules through the blood brain barrier. They have low pinocytic activity and limit the paracellular diffusion by tight junction proteins. In association with the pericytes they regulate the BBB permeability [109, 110]. Our primary focus was to investigate a potential mechanism mediated by diabetes in the cerebral microvascular endothelial cells.

Endothelial cells in the brain communicate with cells in the surrounding milieu to regulate barrier functions, hemostasis, cerebral autoregulation and neurotransmission. This niche

of endothelial cells, together with the neurons, astrocytes and other supporting cells is termed as the *'Neurovascular unit'*. Pathological conditions affecting any component on the neurovascular unit results in altered vascularization patterns and functions.

Vascular endothelial growth factor (VEGF): initiator of vascular complications

VEGF acts as a prime mediator of angiogenesis to design the vascular events and is a master regulator of the angiogenic cascade. There are six known VEGF ligands that interact with different receptors. These receptors are transmembranc tyrosine kinase receptors that dimerize to elicit the activity of VEGF. Upon VEGF binding to the dimerized receptors, tyrosine residues are phosphorylated. Furthermore, these phosphorylated sites act as docking sites and relays signal to the downstream molecules. VEGF plays central role in organ development, wound healing and tumor angiogenesis. VEGF-A exists in five different isoforms, $VEGF_{121}$ is a freely diffusible and $VEGF_{165}$ is both diffusible and bound to the extracellular matrix while $VEGF_{189}$ is completely sequestered to the ECM. $VEGF_{165}$ is the most available form and has a higher bioactivity compared to the other isoforms in the endothelial cells. VEGF dynamically regulates capillary number and density. Inflammatory cytokines, reactive oxygen species, NO and mechanical stimuli can induce the expression and activity of VEGF. Hypoxia is a strong stimulus for angiogenesis during which the cells release HIF-1α. HIF-1α activates VEGF and in a feedback loop to reestablish the oxygen and nutrient supply through vessel formation. VEGF regulates multiple biological actions in the endothelium. It is a potent vascular permeability factor and is essential in endothelial cell differentiation, proliferation, migration and tubulogenesis. Most, if not all of these processes are signaled through VEGF binding to VEGFR-2. PI3K, PLCγ interacts with these activated receptors and signal proliferation and cell survival. VEGF binding to

VEGFR-2 induces endothelial cell migration through activation of src family of kinases [111-115].

VEGF induced angiogenesis differs in physiological conditions and during a disease state. Under physiological conditions VEGF activity and angiogenesis are highly controlled process. Diabetes is majorly viewed as a vascular disease. Diabetes mediated endothelial dysfunction causes a reduction of VEGF expression in the peripheral arteries thus increasing neuropathy. VEGF is highly expressed in diabetic retinopathy as evidenced in rodents and humans and cause vascular lesions, blood-retinal barrier break down and increased retinal neovascularization. Not only the vascular cells but also neurons and astrocytic reactivity are altered. Thus, potential angiogenic therapies can be used to increase or decrease vascular perfusion to prevent vascular abnormalities.

Recently VEGF-A has been implicated in the central nervous system. VEGF increases BBB permeability after ischemia and mediate inflammatory responses. Inflamatory cytokines like IL-2 and interferons can upregulate TGF-β and VEGF. A key objective of stroke treatment is to repair both damaged vasculature and neurons. It is now established that VEGF is involved in neurogenesis, synaptic plasticity, neuroprotection and neuroregeneration. VEGF links both the signaling pathways between angiogenesis and neurogenesis, however intervention with this therapy should be approached with caution as at low doses it can confer neuroprotection and be ineffective for angiogenesis, while at high doses it can be an effective angiogeneic therapy but detrimental to the neurons [116, 117].

Oxidative stress in diabetes

Oxidative stress has been defined as any imbalance between the anti-oxidant and the oxidizing agents that can cause potential damage. Traditionally, the metabolic disturbances

arising in diabetes lead to the generation of various derivatives of nitrogen and oxygen species such as the superoxide, hydroxyl anion, hydrogen peroxide, nitric oxide and peroxynitrite that are unstable and highly reactive. Mitochondrial electron transport chain, NADPH oxidase system, xanthine oxidase and cycloxoygeneses are the major sources of reactive oxygen species (ROS). Nitric oxide is one of the most important molecules that can signal normal physiological functions. In the endothelium it is generated by eNOS. Due to its highly diffusive nature, it is ubiquitously present in all cells and it is involved in flow-mediated dilation of blood vessels through the activation of cGMP and regulates vascular tone. Insulin resistance and diabetes lead to disruption and uncoupling of oxidative reactives and generate large amounts of free radicals [118]. Research on animal models of retinopathy clearly show that both hyperglycemia and increased fatty acid levels acts as excess energy sources producing electron donors such as NADH and FADH. Hyperglycemia leads to mitochondrial dysfunction and activation of stress pathways. As this system continues to saturate, excess superoxides are produced through the electron transport chain. Superoxides combine with NO and form a potent free radical called the peroxynitrite. Peroxynitrite has been demonstrated to promote atherosclerosis by thickening of the intimal layers and endangering blood flow [118, 119]. Peroxynitrite activates various kinases and phosphatases signaling molecules both up- and downstream of the cascade [120]. This occurs in a concentration dependent fashion and the actions are specific to cell types. *In-vitro* peroxynitrite has been known to activate src kinases in neuronal, endothelial and blood cells [121-123]. Human diabetic conditions parallel these findings. The earliest event during diabetes is the endothelial dysfunction which is mainly mediated by ROS through alterations in glucose chemistry. While these reactive compounds in controlled levels are essential to mediate normal physiological process, oxidative damage can be caused by them above the physiological

threshold. Free radicals modify the proteins by nitration of tyrosine residues, nitrosylation and oxidation. Extensive literature supports the notion that reactive oxygen and nitrogen species lead to oxidative stress induced by hyperglycemia in the early stages of diabetes that progressively contributes to vascular complications in diabetes [124, 125].

The sensitivity of the brain is highest to changing demands in oxygen. Due to highly lipophilic nature of the brain, it is susceptible to lipid peroxidation and BBB damage. Strikingly higher levels of NO were detected in the plasma samples of diabetic stroke patients than nondiabetic stroke patients that can cause a cumulative oxidative damage imparted by both the diseases in combination. Decline in cellular levels of antioxidants noted in diabetes increases the susceptibility to oxidative stress. Plasma VEGF was significantly higher in type 1 diabetes with no clinical signs of vascular complications [126]. From animal studies it is identified that the release and activation of VEGF can be mediated by peroxynitrite in diabetic retinopathy. Oxidative stress mediated increased production of growth factors lead to increased vascular remodeling [127]. Scavenging these reactive molecules has been the goal of therapy in diabetes.

Matrix metalloproteinases (MMPs)

MMPs are cardinal to tissue remodeling and their role has been well studied in physiological processes as well as during pathological states. Extracellular matrix holds the vascular cells together and degradation of the matrix components is essential to initiate angiogenesis to enhance movement of the cells. MMPs engage in development of the tissue, wound healing processes, implantation and angiogenesis [128]. These are also implicated in inflammation, vascular abnormalities, atherosclerosis, oncogenesis and also in neurodegenerative disorders. MMPs are zinc dependant proteases that exist mainly as an inactive pro-enzyme and removal of the propeptide is essential for their activation. Tissue inhibitors of matrix

metalloproteases (TIMPs) regulate MMP activity. Major classes of MMPs include the collagenases, gelatinases, stromelysins and Membrane type MMPs. Gelatinase A or MMP-2 has vital role in angiogenesis. It appears constitutively in the brain and the cerebrospinal fluid. MMP-2 activation requires the formation of a trimolecular complex on the cell surface between the pro-MMP, TIMP-2 and membrane type-1 MMP (MT1-MMP). MT-1 MMP partially activates the pro-MMP2 and this further undergoes autocatalysis to form the fully active MMP-2. MMP-2 can also be activated via thrombin [129]. Contribution of MT1-MMP to endothelial migration has been evaluated in human microvascular endothelial cells. Hyperglycemia induced activation of MMP-2 and MT-1 MMP has also been reported during diabetic retinopathy [130]. Chronic hyperglycemic exposure to endothelial cells also shows upregulation of MMP-9 activity on the endothelial cells, setting a stage for the inflammatory mediators via oxidative stress [131]. Previous studies conducted in our lab on the diabetic GK rats also demonstrate similar finding. Both MMP-2 and MMP-9 expression and activity were elevated in the cerebral microvessels [78]. Glycemic control prevented this upregulation in the micro and macrovasculature [132].

ROS can activate MMPs in the event of cerebral ischemia and cause edema, BBB breakdown, inflammation and cell death [133]. During cerebral ischemia, the MMP levels rise acutely within a few hours of stroke and peaks at 12 hours and remains steadily until 24 hours. This elevation can be consistent upto 5 days after stroke. Acutely they cause vasogenic edema, blood brain barrier disruption by digesting the extracellular matrix proteins, increasing vascular permeability and increased movements of vascular components in and out of the tissue. In the delayed phase, some of these MMPs are essential for the onset of vascular repair and movement of proliferating endothelial cells from the vascular milieu. Upregulation of gelatinases has been

observed even after several months in human stroke with a possibility of playing a major role in aiding reparative neovascularization [134-136].

Our goal was to investigate the sequence of molecular events that occur in the diabetic endothelial cells. The mechanisms of diabetes-induced angiogenesis have been extensively studied in the retinal circulation. Revisiting the roles of oxidative stress mediators, VEGF and MMPs in cerebral vasculature will explain their role in cerebral neovascularization in diabetes and their contribution to bleeding during stroke.

PERICYTES

The abluminal surface of the capillary endothelium is encircled by highly specialized supporting cells designated as pericytes. Pericytes interact with endothelial cells bidirectionally and are mainly found surrounding the capillaries and post-capillary venules. Functionally, pericytes are involved in vascular development, maturation, stabilization, and remodeling. Endothelial cells secrete platelet derived growth factors (PDGF) that attract and recruit pericytes though its cognate receptors, PDGF-Receptor [137]. They have contractile properties and hence are also known to regulate blood flow by changing the capillary diameter [138, 139]. They can differentiate into vascular smooth muscle cells, fibroblasts and other types of mesenchymal cells. Armulik et.al, demonstrated the intercommunication between pericytes, endothelial cells and astrocytes in the microcapillaries in regulating blood brain barrier permeability and hemostasis [140]. Pericytes express a wide variety of proteins, including α-smooth muscle actin, NG-2, PDGFR-2, RGS5 and several others [141]. Due to their high degree of adaption and differential protein expression, there is no definite marker to disseminate their identity. However they are identified with their characteristic differences in the shape of nuclei and encircling the

endothelial layer [141]. High glucose levels initiate apoptosis in the pericytes and capillary dysfunction through the activation of PKC, reactive oxygen species and inflammatory mediator, NF-κB. Loss of pericytes in the microvessels has been observed in diabetic retinopathy and systemic hypertension [142]. Understanding the changes in pericyte function and maintenance of blood brain barrier is critical to develop new treatments for microvascular abnormalities.

PLASTICITY AFTER STROKE

'Plasticity' can be defined as any adaption in terms of anatomical and functional origin resulting in better outcomes to maintain homeostasis. Angioadaption or matching capillary density to the demands of oxygen or glucose availability occurs in an adult brain. Vascular plasticity has also been implicated in response to chronic ischemia. After an ischemic injury visible endogenous repair mechanisms, initiate 3 days after stroke. Shin et.al, reported a spatial overlap between neurogenic and angiogenic mechanisms after focal cerebral ischemia. Proliferating endothelial cells were in close connection with increased microcapillarization [143]. Restoring blood flow to the region of ictus depends upon two key parameters, presence of collaterals that can redirect blood flow and formation of new capillaries. In addition, distal to the site of injury the endothelial morphology has non-sprouting angiogenesis [144]. Stimulation of vascular plasticity may possibly increase vascular perfusion, neuronal survival and functional recovery.

Eng Lo reviewed that most molecular mediators of injury during stroke have biphasic roles. The same mediators that kick in during the acute phase are necessary to signal molecules and transition the brain to a repair phase. Several molecules can be reiterated to have this effect [145]. VEGF that has the capability to increase permeability, disrupt blood brain barrier, and is

also involved in recapillarization during the repair phase of stroke. Working in concert with angiopoietins, VEGF levels are also upregulated during hypoxic, ischemic conditions [146]. Acute administration of VEGF leads to increased activity and opening of the BBB and late upregulation of VEGF after stroke mediate neovascularization around the ischemic zone [147]. Similar conclusions are supported by the use of transplanted stem cells that secrete VEGF, effectively improve functional outcomes with early and increased neovascularization [148]. VEGF also regulates formation of vascular collaterals of ischemic brain. Studies conducted in stroke patients have shown greater capillary density in the ischemic hemisphere compared to the contralateral side. There is also a significant good correlation between vascular density and survival rates of these patients [149]. VEGF-A is also reported to form native collaterals and regulate collateral growth after ischemia [150]. MMPs also signal BBB disruption, edema, hemorrhage, and cell death. MMP-9 shoots acutely after stroke within 24 hours and degrade the neurovascular matrix. The only approved clot buster tPA also generates MMPs. Inhibiting MMPs, has been shown as an effective strategy to prevent HT. Despite these negative roles, MMPs are also involved in neurovascular remodeling. Degradation and proteolysis of the extracellular matrix by MMP-9 prepares a stage for VEGF mediated initialization of repair and angiogenic events in endothelial cells [151]. Proliferation of endothelial cells ensues as early as 12-24 hours after stroke in the peri-infarcted region and the activity steadily increases during day3-4 and peaks a day-7 [152]. There is also a fine line delineating the good sides of free radicals being generated during and after stroke. Undoubtedly, a large amount of free radicals can damage the cellular proteins, cause lipid peroxidation and DNA damage. Controlled physiological amounts of NO are capable of maintaining blood flow in the ischemic penumbra, and excessive NO may be essential to rescue further reductions in blood flow. A little over this

threshold limits causes a redox imbalance and potentiates damaging effects on the entire brain. Free radicals also participate in remodeling the brain after stroke. A better understanding of these molecules and their nature of change from damaging to remodeling can advance the field of stroke therapy.

While there are extensive studies being conducted to assess brain remodeling and plasticity in stroke, the vascular and metabolic risk factors associated with this complex event is relatively less explored. Treating patients that display a multifaceted metabolic dysfunction such as diabetes, hypertension, hypercholesterolemia in addition to aging warrants more studies. It is estimated that 25 % of the stroke patients have previously suffered diabetes, while the leading co-morbidity being hypertension that is presented by three fourth of the stroke patients [153, 154]. Stroke outcomes are poor in diabetic patients [155]. Studies conducted in animal models of diabetes show impaired vascular remodeling [52, 156]. Our studies add to this existing knowledge as to how different brain regions recoup after the injury in diabetic stroke and as well as investigate the response from the non-lesional hemisphere.

Functional deficits almost always results due to a stroke. Angiogenesis and neurogenesis rewire the brain and improve functional outcomes after cerebral injury [157-160]. Restoration of blood flow after stroke is closely related to improved neurological functions. Increasing motor functions, physical activity can improve brain functions by synaptogenesis. Electrical stimulation, deep brain stimulation evaluated in animal models show faster functional recovery in experimental animal models. Clinical recovery more often depends upon the frequency and ability to use the affected limb. As functional recovery occurs, cortical maps reorganize and brain functions become malleable based on the need. Cortical infarction induced in adult primates show newer anatomical connections in the ipsilesional area and in the distant cortical

areas [161]. Motor functions in the upper and lower extremities recover within one month of stroke [162, 163]. Recovery in cognitive and language deficits take up to one year after stroke.

Understanding the changes associated with the vasculature to undergo spontaneous recovery will provide a better insight in the processes responsible for modulation of angiogenesis and improvement in functional outcomes. Microvascular plasticity and synaptogenesis has been investigated in the adult brain to assess the influence of neural activity and learning associated with increased physical activity.

This comparison of improvement in functional recovery between diabetics and non-diabetics is still unknown. Our studies provide long term vascular structural re-organization in control and diabetic rats in relation to reparative neovascularization after stroke.

GLYCEMIC CONTROL TO PREVENT VASCULAR COMPLICATIONS IN DIABETES AND STROKE

Stimulation of new vessel formation from existing vessels has long been considered as an option to improve diseases associated with tissue hypoxia such as coronary artery disease, peripheral arterial disease and stroke [164-169]. Therapeutic angiogenesis can be achieved by enhancement of natural recovery mechanisms[170]. Reduction but not elimination of oxidative stress by acute angiotensin II receptor antagonism at reperfusion creates a proangiogenic state which improves functional outcome [171]. This study provided further evidence that angiogenesis involves complex interactions between pro and anti-angiogenic mediators and the microenvironment is very important. Therapeutic angiogenesis can be further stimulated with various growth factors like VEGF and FGF as well as cell based approaches as recently reviewed [172, 173]. However, use of this approach in ischemic cardiovascular disease has been

discouraging. Despite promising results in animal models, phase 1-3 clinical trials showed very limited clinical benefit. This conflict was recently addressed elegantly in a review article[172]. It has been suggested that one potential reason may be that patients with coronary artery disease are vastly different from the young and healthy animals used for preclinical studies. Despite these unfortunate failures, the timing of growth factor stimulation is being reviewed as an option to improve post-stroke angiogenesis. Whether this therapy would be effective in diabetic patients who already have elevated VEGF levels prior to stroke is difficult to determine. With the glycemic control strategies being much widely investigated, we evaluated the use of metformin as an interventional strategy in diabetic stroke and reassessed its beneficiary roles in angiogenesis and improving functional outcomes.

The CONTROL (collaborators on trials of lowering glucose) group performed a meta-analysis from the findings of four major clinical trial comprising of the ACCORD (Action to control cardiovascular risk in diabetes), ADVANCE (Action of Diabetes and vascular disease: Preterax and Diamicron Modified Release Controlled Examination), UKPDS (U.K. prospective diabetes study) and the VADT (veterans affairs diabetes trail) trials. The major conclusion drawn by CONTROL suggested that an intensive glycemic control decreased the number of primary outcomes, death due to cardiovascular disease, nonfatal stroke and myocardial infarction by about 9% compared to the less intensive glycemic arm. Despite these positive results, there were two major setbacks: a) there were severe episodes of hypoglycemia associated with intensive glycemic control, and b) there was a trend towards 10% increase in the all-cause mortality and cardiovascular events. Patient stratification based on the duration of diabetes, HbA1c values showed that ACCORD and VADT had patients with advanced diabetes with extreme HbA1c values and hence showed increased risk of cardiovascular events with intensive glycemic

control. In fact, the glycemic control in the ACCORD study was stopped after one year due to a 35% increase in cardiovascular mortality. Whereas ADVANCE had recruited patients with median duration of diabetes with high normal HbA1c of about 7.5% and UKPDS composed of newly diagnosed diabetes with borderline HbA1c of 7.1 %. The meta-analysis conclude that intensive glycemic control was beneficial in early diabetes but not during the advanced stages [174-179].

Metformin is a first line choice of drug commonly used to treat T2D patients. Metformin exerts its anti-hyperglycemic actions by reducing hepatic gluconeogenesis, increasing peripheral glucose uptake, sensitivity to insulin and fatty acid oxidation, while conferring vascular protection independent of its glycemic control properties. Metformin exerts its protective role independent of its antihyperglycemic actions. Metformin improved anti-oxidant capacity in T2D patients who display excessive increased oxidation and glycation of albumin in the serum [180]. Effects of metformin in GK rats have been reported by Rosen and Wiernsperger who show improved anti-oxidant defense with the use of metformin [181]. Studies conducted in our lab show vasculoprotective effects of metformin when it is used as a glycemic control strategy in GK rats [132]. Metformin prevented remodeling of the arteries by it actions on ET-1 [182]. Not only does metformin limit the production of free radicals and ROS but also activates AMP-kinase that is a modulator of glucose and lipid homeostasis. These studies founded the rationale behind using glycemic control on diabetic animal model used in the studies addressed in the dissertation. The study evaluates the effectiveness of glycemic control in preventing early vascular complications in diabetes.

Both acute hyperglycemia prior to stroke and chronic hyperglycemia due to diabetes amplify the severity of stroke outcomes. Indeed, acute elevations in blood glucoses without frank

diabetes prior to stroke increases infarct sizes and neurological deficits compared to diabetes. The results are true both with clinical and experimental studies. Nevertheless to say evaluating the strategies to achieve glycemic control has become a priority and a must especially in stroke patients. Clinical trials conducted so far debates over glycemic target goal after stroke with little evidence on diabetic stroke. Previous studies performed in our lab show that metformin not only improved pial cerebrovascular remodeling in diabetes but also corrected HT in the GK model of diabetic stroke [132]. Studies proposed in Aim: 3 provided results pertaining to the use of glycemic control as an interventional strategy post stoke in diabetes.

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CHAPTER 2

ENHANCED CEREBRAL BUT NOT PERIPHERAL ANGIOGENESIS IN THE GOTO-KAKIZAKI MODEL OF TYPE 2 DIABETES INVOLVES VEGF AND PEROXYNITRITE SIGNALING

Roshini Prakash, Payaningal R. Somanath, Azza B El-Remessy, Aisha Kelly-Cobbs, Javier E Stern, Paula Dore-Duffy, Maribeth Johnson, Susan C Fagan, and Adviye Ergul

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ABSTRACT

We previously reported enhanced cerebrovascular remodeling and arteriogenesis in experimental type 2 diabetes. This study tested the hypotheses: 1) cerebral but not peripheral angiogenesis is increased in a spatial manner, and 2) peroxynitrite orchestrates VEGF-mediated brain angiogenesis in diabetes. Stereology of brain, eye and skeletal muscle microvasculature was evaluated in control and diabetic rats using 3-D images. Migration and tube formation properties of brain microvascular endothelial cells (BMEC) were analyzed as markers of angiogenesis. Vascular density, volume and surface area were progressively increased from rostral to caudal sections in both cerebral cortex and striatum in diabetic rats. Unperfused new vessels were more prominent and pericyte/endothelial cell ratio was decreased in diabetes. Vascularization was greater in the retina but lower in the peripheral circulation. VEGF and nitrotyrosine levels were higher in cerebral microvessels of diabetic animals. Migratory and tube formation properties were enhanced in BMECs from diabetic rats which also expressed high levels of basal VEGF, nitrotyrosine and membrane type matrix metalloprotease (MT1-MMP). VEGF neutralizing antibody and inhibitors of peroxynitrite, src kinase or MMP blocked the migration. Diabetes increases and spatially regulates cerebral neovascularization. Increased VEGF-dependent angiogenic function in BMEC is mediated by peroxynitrite and involves c-src and MT1-MMP activation.

INTRODUCTION

Major complications spanning from coronary artery disease (CAD), peripheral arterial disease (PAD), and retinopathy to stroke contribute to the increased morbidity and mortality in diabetes. Increasing occurrence of diabetes in younger individuals is especially alarming when one considers the development of these complications over the course of the disease. While impaired collateralization and angiogenesis play an important role in CAD and PAD, in diabetic retinopathy, excess angiogenesis leads to increased edema, bleeding and ultimately resulting in blindness [1-3]. Our understanding of how diabetes affects the cerebral vascularization is not equally clear.

Neovascularization can involve vasculogenesis, angiogenesis, and arteriogenesis [4-6]. We reported extensive vascular remodeling and arteriogenesis in the pial vessels in Goto-Kakizaki (GK) rats, a lean and mild model of Type 2 diabetes. We also showed that when an ischemic stroke is superimposed on this existing vascular condition, diabetic animals develop hemorrhagic transformation (HT), i.e. bleeding into the infarct, and perform poorly on neurobehavioral tests [7]. Prevention of pial remodeling by glycemic control or inhibition of matrix metalloproteinases (MMPs) was associated with reduced HT and improved neurologic outcome [8]. The spatial and molecular regulation of angiogenesis and its impact on stroke outcome remain to be determined.

Reactive oxygen species (ROS) are involved in the regulation of neovascularization [9, 10]. While low levels of ROS, peroxynitrite in particular, can propagate the angiogenic signal of VEGF, excess ROS can be detrimental and inhibit VEGF-mediated cell survival [11-13]. Inhibition of peroxynitrite prevents neovascularization in ischemic retinopathy [14]. Building on these findings, we tested the hypotheses that 1) cerebral but not peripheral angiogenesis is

increased in a spatial manner in the GK model of diabetes, and 2) peroxynitrite orchestrates VEGF-mediated angiogenic signal in the brain microvasculature in diabetes.

RESEARCH DESIGN AND METHODS

Assessment of cerebral neovascularization.

Weight-matched control and diabetic rats GK rats (male, 270-310 g) were used for the study in accordance with National Institute of Health guidelines for the care and use of animals in research and under protocols approved by the Georgia Health Sciences University. Diabetic rats had higher blood glucoses (95.3 ± 3.0 vs. 176.2 ± 7.5 mg/dl, n=19). Animals were injected with 500µl of 50mg/ml FITC-Fluorescein Isothiocyanate-Dextran (molecular weight 2,000,000, Sigma-Aldrich, St. Louis, MO) through the jugular vein under pentobarbital anesthesia. Brains were cut into 2 mm slices (labeled A-G rostral to caudal, Fig 2-1A upper panel), and processed in 4% paraformaldehyde (24 h) and 30% sucrose in phosphate buffered saline (PBS). Z-stacked confocal images of 100 µm sections from Region B (anterior to the MCA comprising the frontal cortex-sensory, bregma 3 to 1), C (medial where the MCA branches out to supply the frontal motor cortex, bregma 1 to -1) and D (posterior to the MCA comprising of the parietal cortex, bregma -1 to -3) were acquired using Zeiss LSM 510 confocal microscope in the regions of interest (ROIs) within the cortex and striatum (Fig 2-1A, lower panel indicated by yellow and orange squares). ROIs were based on our previous findings demonstrating the location of infarcts and hemorrhage in the diabetic and control rats (Fig 2-1A). An overall representation of these regions was obtained by imaging ROIs from three different sections obtained from one slice. Values were obtained from each ROI, and a mean value of 3 images from each section was calculated. Each measurement from one animal was comprised of an average of 9 images from

either the cortical or striatal region. Retinal flat mount, gastrocnemius and soleus muscles were prepared similarly in the same animals to assess neovascularization in different vascular beds. Z-step was defined as 1.984 μm, image size 512 x 512 pixels, 25 X lens). Image stacks were imported into Volocity (Improvision, Lexington, MA), and reconstructed in 3D. The FITC channel was classified to establish an intensity threshold, set to intensities 5X above background immunofluorescence (calculated from random adjacent areas where no vasculature was observed).

Vascular density refers to the density of FITC stained vasculature from the merged planes over the total area of the section. This parameter determines the change in vascularization in a given reference area and is independent of Z-function. Vascular volume refers to the ratio of the volume of the vasculature to the total volume (reference volume) of the section on a Z-stack [15]. Surface area represents absolute surface area of the vasculature and a proportional increase in surface area with vascular volume represents increased vasculature.

To differentiate perfused and nonperfused immature vasculature, FITC stained brain sections were co-stained with biotinylated isolectin B4 (Vector Laboratories Inc. Burlingame, CA) overnight at 4^0 C. The sections were then incubated with Texas Red Avidin D (Vector Laboratories Inc. Burlingame, CA) for 2 h at room temperature. Images were acquired using Zeiss confocal microscope and colocalization measurements were carried out using Image J.

***In vitro* angiogenesis assays.**

Cell proliferation, migration and tube formation assays were used as the indices of angiogenic potential of endothelial cells. Primary BMECs were isolated from control and GK rat brains by an immunomagnetic method of separation using dynabeads. Whole brains were extracted under aseptic conditions and the pial macrovasculature and the white matter was discarded. The

cerebrum was minced and incubated overnight with collagenase/dispase (Roche, Indianapolis, IN). Following incubation, the digestate was filtered through a 100 µm sieve, centrifuged, and the fraction containing microvascular segments were washed twice with PBS. Next, this fraction was incubated with CD31 antibody (BD Biosciences, Bedford, MA) for 4 h at 4^0 C and re-incubated with secondary coated dynabeads (Invitrogen, Carlsbad, CA) for 1 h. Cells attached to the dynabeads were pulled down using a magnet, suspended in growth medium containing 10% fetal bovine serum, 5% bovine calf supplement, 5 mM glucose, endothelial cell growth supplement and cultured on fibronectin coated flasks under standard 5/95% CO_2/air conditions.

Cell proliferation assay was carried out by plating 70,000 cells and the number of cells, cell volume and diameters were measured using Scepter automated cell counter (Millipore, Billerica, MA) 24 and 48 h after plating.

For the cell migration assay, cells were grown until confluency and serum starved for 8 h before performing the assay [16]. A wound/scratch was created with a sterile pipette tip and the distance uncovered was measured 24 h post-scratch. During this time, no exogenous growth factors were added. An average of three measurements were taken and % recovery of scratch distance was calculated as [(total scratch distance - average distance uncovered)/total scratch distance]*100. To determine the role of endogenous VEGF and downstream signaling in cerebral angiogenesis, the assay was repeated using cells pretreated with VEGF neutralizing antibody (0.5µg/mL R&D systems, Minneapolis, MN), peroxynitrite decomposition catalyst 5,10,15,20-Tetrakis(4-sulfonatophenyl) porphyrinato Iron (III) chloride (FeTPPs 2.5µM, EMD Biosciences, San Diego, CA), src kinase inhibitor PP2 (1 µM, Calbiochem Cambridge, MA) and MMP inhibitor minocycline (100 µM, Sigma Aldrich St. Louis, MO) for 2 or 24 h before the migration assay. In addition, control cells were cultured with conditioned medium collected from

BMECs of diabetic rats with or without a VEGF neutralizing antibody after the scratch was made.

For tube formation assay, 70,000 BMECs were suspended in reduced matrigel (BD Biosciences, Bedford, MA) mixed with serum free media and allowed to polymerize at 37^0 C. Tube-like structures were counted in a unit area at 24 and 48 h.

Assessment of pericytes.

For preparation of brain capillaries, tissue was homogenized as reported previously [17, 18]. The pellet was re-suspended in 15% dextran in DMEM and passed through an 80mm nylon mesh. Microvessels (MV) were collected and re-suspended in DMEM. MV were >95% viable by trypan blue exclusion. In addition, exclusion of large vessels and the capillary nature of the preparation were confirmed by analysis on the Meridian ACAS 470 laser cytometer with computer-generated size determinations. Staining of MV preparations indicated that there were no neurons or glial cell contaminants [17-19]. Samples were allowed to adhere to cover slips and stained with DAPI. To determine the number of pericytes, round nuclei were counted versus elongated nuclei (endothelial cells) [17, 18].

Isolation of cerebral vessels.

Macro- and microvessels were isolated and homogenized in radioimmunoprecipitation assay (RIPA) buffer as previously described [20]. Homogenates were immunoblotted with VEGF and nitrotyrosine antibodies as described below.

Immunoblotting.

To measure the endogenous production of VEGF, BMEC supernatants were collected after serum starvation and incubated with 30µL of heparin agarose beads overnight at 4^0C. After centrifugation, beads were boiled with loading buffer for 10 min, separated on 10% SDS gels,

transferred to a PVDF membrane and incubated with anti-VEGF antibody (R&D Systems) overnight. Following incubation with secondary antibody, bands were visualized using ChemiGlow from Alpha Innotech Corporation (San Leandro, CA). All blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading.

To study the effect of peroxynitrite formation on VEGF signaling, BMECs were pretreated with 2.5 μM FeTPPS for 30 min and then challenged with 30 ng VEGF for 10 min. Published studies and our pilot findings (data not shown) showed that this protocol causes a rapid increase in peroxynitrite formation and VEGFR phosphorylation [14]. Cell lysates prepared in RIPA buffer (35 μg) were immunoblotted using antibodies against native and phosphorylated VEGF-R2 (Cell Signaling Technology, Danvers, MA) anti-nitrotyrosine (Millipore, Billerica, MA), native and phosphorylated c-src, MMP-2, and MT1-MMP (Calbiochem, Cambridge, MA). The proteolytic activity of MMP-2 in cell culture supernatants prepared from each group was determined by gelatin zymography [21].

Statistical Analysis.

Data are expressed as mean ± SE. Data were evaluated for normality and appropriate transformations were used when necessary. Exact two-group Wilcoxon tests were used to study the effect of disease (control vs. diabetes). Exact tests are appropriate when a data set is small, sparse or skewed. A 2X2 mixed model repeated measures analysis of variance (RMANOVA) was used to study the effect of disease (control vs. diabetes) and area of the brain (cortex vs. striatum) and their interaction on log percent vascular density, vascular volume and surface area, and the ratio of percent non-perfused vasculature to total vasculature. A 2X2 RMANOVA was also used to study the effect of disease and time (24 h vs. 48 h) and their interaction on log number of tubes per loops and percent increase in proliferation. A one-way ANOVA was used to

assess the effect of treatment for controls (none, diabetic ECM, diabetic ECM+anti-VEGF antibody) on percent recovery of scratch distance in 24 h. A 2X3 ANOVA was used to study the effect of disease and treatment and their interaction using VEGF neutralizing antibody, FeTPPs, PP2, and minocycline as the treatments and measuring percent recovery at 24 h as the outcome. A Tukey's test was used to adjust for the multiple comparisons for significant effects in the previous analyses. A one-way ANOVA within disease was used to study the effect of treatment (Untreated, FeTPPs, VEGF, and FeTPPs+VEGF) on the ranked data for pVEGF-R2 to VEGF-R2 ratio, nitrotyrosine, and p-src to src ratio. A Dunnett's test was used to compare treated to untreated groups for significant effects. Statistical significance was determined at alpha=0.05 and due to the small sample sizes for some of the variables a statistical trend was determined at alpha=0.10. SAS® version 9.2 was used for all analyses (SAS Institute, Inc., Cary, NC).

RESULTS

Diabetes-mediated cerebral angiogenesis is spatially regulated.

In all sections (frontal sensory cortex (B), frontal motor cortex (C) and parietal cortex (D), Fig. 2-1A and B), cortical vascular density, volume and surface area were greater than in that observed in the striatum (Fig. 2-1D-F). Furthermore, diabetes significantly increased all these parameters especially in the C-section. There was a progressive increase (rostral to caudal) in both cortical and striatal vascular density, volume and surface area in the diabetic group (Fig. 2-1D-F).

Diabetes uniquely mediates cerebral neovascularization while regresses the peripheral vasculature.

Isolectin co-staining of FITC-filled sections allowed differentiation of perfusing (FITC and isolectin colocalization) and nonperfusing (isolectin alone) vessels. In accordance with the Volocity data, the total vasculature was relatively greater in the diabetic group and more so in the cortex compared to control. Greater colocalization of FITC and isolectin in the cortex and striatum of the control group (Fig. 2-2A, Supplementary Fig. A) indicated that vessels were more mature and more likely to be perfused. There was relatively more isolectin staining in diabetic sections indicating that there are more newly formed and nonperfusing vessels in both cortex and striatum in the diabetic group (Fig. 2-2B, Supplementary Fig. 2-1 A). The ratio of pericyte to endothelial cell was significantly reduced in the diabetic group compared to control (Fig. 2-2B) providing further evidence for immature nature of the vessels in diabetes.

Neovascularization was compared in three different vascular beds. While the vessels appeared remodeled and larger in diabetic animals, vascular density was significantly decreased in gastrocnemius muscles in the diabetic rats compared to control (Fig. 2-2C, Supplementary Fig. 2-1B). There was a nonsignificant increase in vascular density in the diabetic retinal vasculature exhibiting collateralization (Fig. 2-2C and Supplementary Fig. 2-1B). Retinal capillaries also appeared kinked along the abluminal surface.

Angiogenic factors are increased in cerebral microvessels of diabetic animals.

Since VEGF is the major angiogenic factor involved in diabetic retinopathy, VEGF protein levels were measured in micro- and macrovessel preparations. The VEGF dimer detected around ~45kDa was greater in the microvessels but not macrovessels of diabetic animals (Fig. 2-3A). Slot-blot analysis showed that these vessels also exhibit greater nitrotyrosine levels (Fig. 2-3B).

Cerebral microvascular endothelial cells exhibit increased angiogenesis.

BMECs from diabetic animals showed tube-like structures within 1 day of incubation while the control endothelial cells underwent tubulogenesis 2 days after incubation (Fig. 2-4A and B). Cell proliferation was also significantly increased in diabetes (Fig. 2-4C). BMECs from diabetic rats were morphologically different as indicated by smaller diameter and volume (Fig. 2-4D and E).

Spontaneous cell migratory response was greater in diabetes (Fig 2-5A and B). To study if a growth factor released by the diabetic endothelial cells had the ability to increase cell migratory response in control endothelial cells, the diabetic BMECs were serum starved and the endothelial conditioned media was collected after 24 h. Control BMECs grown in this conditioned media showed greater cell migration compared to control and the presence of VEGF neutralizing antibody restored the cell migratory properties to control levels (Fig 2-5A-C). VEGF-A levels, especially the dimer form, were significantly increased in the culture media obtained from BMECs of diabetic rats (Fig 2-5D and E).

Roles of peroxynitrite, c-src and MMPs in endothelial cell migration.

To demonstrate the involvement of VEGF and peroxynitrite signaling in the increased angiogenic response in diabetes, we first determined the basal levels of native and phosphorylated VEGF receptor 2 (VEGF-R2), nitrotyrosine, native and phosphorylated c-src as well as MT1-MMP and MMP-2. Phospho-VEGFR2, phospho-c-src and nitrotyrosine levels were greater in BMECs from diabetic animals as compared to control cells (Fig 2-6A, B and C). While there was no difference in secreted MMP-2 (pro- or active form), cellular MMP-2 and MT1-MMP were higher in the diabetic BMECs compared to control (Fig 2-6D-F).

To determine the roles of endogenous VEGF and downstream signaling molecules including peroxynitrite, src kinase and MMPs in mediating the angiogenic response in BMECs,

cells were pretreated for 2 or 24 h with either a VEGF neutralizing antibody, FeTPPs, PP2 or minocycline, respectively, and cell migration at 24 h after treatment was assessed. With all treatments, there was a disease and treatment interaction such that treatments had no effects on the migratory response of control cells in the absence of an exogenous growth factor stimulation but reduced the migratory response of diabetic BMECs to levels seen in control cells (Fig 2-7A-D).

Effect of peroxynitrite inhibition on VEGF signaling.

In order to determine how peroxynitrite modulates VEGF signaling, cells were first pretreated with FeTPPs and then stimulated with VEGF. The greater basal VEGFR and c-src activation as well as tyrosine nitration observed in diabetic cells were all reduced with FeTPPs (Fig 2-8A-C) suggesting that peroxynitrite modulates VEGFR activation. While VEGF stimulation increased VEGFR and c-src phosphorylation in control cells as expected, in diabetic cells there was no further increase. Pretreatment with FeTPPs prevented VEGF-stimulated VEGFR and c-src activation as well as protein tyrosine nitration in control cells. In diabetic cells, VEGF stimulation in the presence of FeTPPs yielded similar results to FeTPPs alone.

DISCUSSION

We have previously shown that cerebral neovascularization is stimulated in a mild model of Type 2 diabetes as evidenced by increased collateral number and diameter in the pial circulation [7, 8, 22]. The goals of the current study were 1) determine whether neovascularization also involves angiogenesis in this model, 2) if so, understand the spatial regulation and mechanism of this enhanced neovascularization, and 3) investigate whether these vascular changes are unique to the cerebral circulation. The results provide intriguing new evidence that mild diabetes

stimulates cerebral angiogenesis in a spatial manner while it has an opposing effect on the peripheral vasculature. Furthermore, VEGF and peroxynitrite together are the key regulators of angiogenesis in cerebral microvascular endothelial cells via sequential activation of c-src followed by MMP-2 and MT1-MMP.

Diabetes is an exponentially expanding epidemic disease that leads to severe complications. Most diabetic complications have a significant vascular component and have been classified as either microvascular (nephropathy, retinopathy and neuropathy) or macrovascular (heart disease, stroke and peripheral arterial disease) [23, 24]. Macrovascular classification of stroke was mainly based on accelerated atherosclerosis in diabetes leading to narrowing of the carotid arteries. However, accumulating evidence suggests that small vessel disease is also important for neurological disorders such as dementia and stroke in patients with diabetes [25-27]. These patients develop both large artery and terminal arteriole (lacunar) infarcts associated with increased incidences of bleeding [28-31]. This line of clinical evidence emphasizes the need for preclinical studies focusing on the microvasculature of the brain in diabetes. Tissue-specific and spatial regulation of angiogenesis in experimental diabetes as we demonstrate in this study may be particularly significant for several reasons. First, we have reported that diabetic GK rats develop greater HT, especially around the infarcted area upon temporary middle cerebral artery occlusion and have poor functional outcome [7, 8]. Differences in cerebrovascular architecture were previously observed within human cerebral cortex and between the striatum in rats and mice [32-34]. Similarly in this study we observed not only greater cerebral density of perfusing microvasculature in the cortex than in the white matter but also demonstrated that this difference is more pronounced in diabetic rats which may partially explain smaller cortical infarcts in this model. Second, we now provide evidence that in addition

to overall greater vascular density, there is more nonperfused new vessel formation in brain sections where we reported overt macroscopic bleeding in our past studies. Furthermore, there is less pericyte support indicative of immature nature of these vessels, which may render the diabetic vessels more prone to reperfusion injury leading to greater HT. Third, this pronounced neovascularization appears to be unique to the cerebral and retinal vasculature. As past studies reported, we also found impaired neovascularization of the skeletal muscle [35] while there was increased retinal vascular density and remodeling as seen in proliferative retinopathy [36, 37]. Collectively, our results suggest that angiogenesis in the brain may explain the increased bleeding and vascular permeability that we reported in the diabetic animal model.

Endothelial cells are the first target of oxidative stress, matrix degradation and metabolic changes occurring in diabetes. Micro- and macrovascular endothelial cells differ in function and protein expression at the organ level [38, 39]. Although past studies provided information on the molecular mechanisms of angiogenesis in retinal and coronary endothelial cells, little is known about cerebral microvascular endothelial cells [40-43]. Furthermore, most if not all studies used either endothelial cell lines or primary cells isolated from normal animals to investigate the VEGF-mediated angiogenic response. The current study provide novel data by first studying brain microvascular endothelial cells and second by comparing the angiogenic potential of cells isolated from control vs. diabetic rats. VEGF A is a 22-KDa glycoprotein with 5 different isoforms and it is one of the best studied angiogenic factors [44]. $VEGF_{165}$ is activated in diabetic retinopathy [14, 45, 46] and associated with retinal hematomas. Autophosphorylation of the VEGFR2 upon VEGF binding and subsequent activation of c-src mediate the angiogenic effects. In our model, we found increased expression of soluble VEGF A isoforms and its cognate receptor. Basal levels of the

phosphorylated VEGFR2 as well as c-src were higher providing evidence that the endogenous VEGF system contributes to enhanced angiogenic potential of brain microvascular endothelial cells in diabetes. Since these cells are already exposed to higher endogenous VEGF levels, further stimulation with exogenous VEGF does not have an additional effect on VEGFR2 activation that is seen in control cells suggesting differential regulation of angiogenic signals in control and disease models.

Growing evidence suggest that reactive oxygen and nitrogen species such as peroxynitrite can act as signaling molecules and mediate VEGF's angiogenic properties [47]. As depicted in Fig 8C, we hypothesized that VEGF causes sequential stimulation of VEGFR2, peroxynitrite, c-src and MMPs, and that peroxynitrite sustains VEGFR2 phosphorylation augmenting the angiogenic signal. Consistent with our hypothesis and our data with microvessel preparations and BMECs at baseline conditions, inhibition of VEGF, peroxynitrite, c-src or MMPs completely prevented the greater migratory response in diabetic cells. When cells were stimulated with VEGF, control cells responded with increased VEGF and c-src phosphorylation as reported in the literature with retinal microvascular endothelial cells but diabetic cells did not show a further increase in VEGF or c-src activation indicating that the system may be saturated. When cells were treated with FeTPPs, however, under baseline or VEGF stimulation conditions, VEGFR2 phosphorylation was significantly decreased confirming previous reports that peroxynitrite sustains VEGFR2 activation and modulates the angiogenic signal in brain microvascular endothelial cells.

MMPs are involved in angiogenesis by degrading the matrix and allowing cells to migrate [48, 49]. MMP-2 and MT1-MMP, a secreted and membrane-bound form, respectively, are essential to initiate angiogenic responses. We have previously reported that increased MMP-

2 and -9 activities in brain micro- and macrovessels in our diabetic model [9, 21]. In the current study, we found increased cellular MMP-2 activity and MT1-MMP protein. Moreover, treatment of cells with a broad spectrum MMP inhibitor minocycline prevented the cell migration in diabetes. We previously reported that chronic treatment of diabetic rats with minocycline prevents vascular remodeling and pial neovascularization in this model. Taken together, MMP activation contributes to angiogenic response of brain vascular endothelial cells. Interestingly, we found that cellular but not secreted MMP-2 was increased in BMECs from diabetic animals suggesting that either cells retain MMP-2 for another purpose or there is a defect in its secretion which needs further investigation.

There are several limitations of the current study. First, we measured neovascularization and angiogenesis in a mild and lean model of diabetes unlike the clinical conditions where diabetes and obesity are generally co-morbid conditions. Blood glucose levels and resulting oxidative stress may be an important factor in the regulation of angiogenesis. However, this model helps us to tease out how moderate increases in blood glucose can contribute to pathological angiogenesis. As such, further studies are needed to compare and contrast our findings with other models of diabetes. Second, we used an *in vitro* culture model to study mechanisms of increased angiogenesis. However, cells isolated from diabetic rats retained their angiogenic potential and served as a good model. Third, we focused on VEGF but involvement of other angiogenic factors cannot be ruled out. Nevertheless, there are novel and innovative aspects of this study which include: 1) Spatial characterization of angiogenesis in different brain regions that are relevant to neurovascular injury, 2) Comparison of molecular signaling mechanisms that regulate emergence of capillaries under control and diabetic conditions, and

3) Comparison of the angiogenic response in different vascular beds affected by diabetes. Collectively, our data advance our knowledge of cerebral angiogenesis in health and disease.

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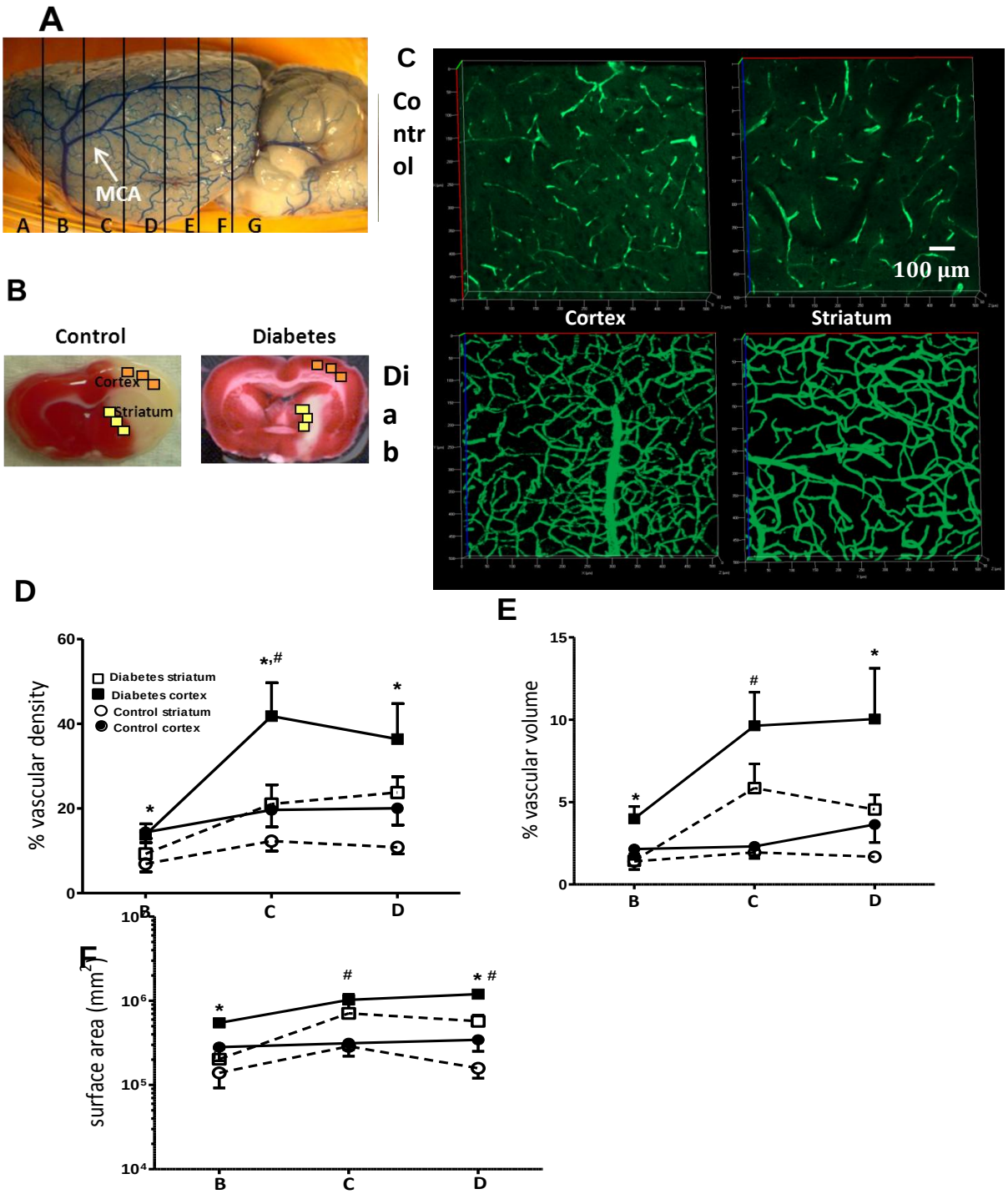
Figure 2-1. Comparison of cerebral vascularization and its spatial distribution in control and diabetic GK rats. (A) Represents different brain regions and (B) shows regions of interest (ROIs) where angiogenic parameters were assessed. (C) Representative FITC perfused cerebrovascular images from control and diabetic rats showing differences in angiogenesis in the cerebral cortex and striatum. (D-F) Significant differences in vascular density, volume and surface area were observed between the cortex and striatum in both control and diabetic groups however diabetic rats exhibited more than 2 fold increase in vascular density in the regions of interest in section C and D. (E-F) Vascular volume and surface area were markedly increased in both cortex and striatum of the diabetic group compared to control. *$p<0.01$ cortex vs striatum, #$p<0.05$ diabetes vs control. Mean ± SEM, n= 4-11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Striatum
D
% vascular density
Diabetes striatum
Diabetes cortex
Control striatum
Control cortex
E
% vascular volume
F
surface area (mm²)
B
C
D

Figure 2-2. Immature cerebral microvessels are more abundant in diabetes. . (A) Diabetic group had significantly increased cortical and striatal non-perfused vessels compared to control. (B) Pericyte to endothelial ratio was decreased in the diabetic group. (C) There was a visual but not statistically significant increase in retinal vasculature whereas peripheral vasculature was decreased in diabetes. P=0.0016 diabetes vs control, $^{\Psi}$p=0.01, **p=0.029 diabetes vs control. Mean ± SEM, n=3-4 (Exact Wilcoxon test).

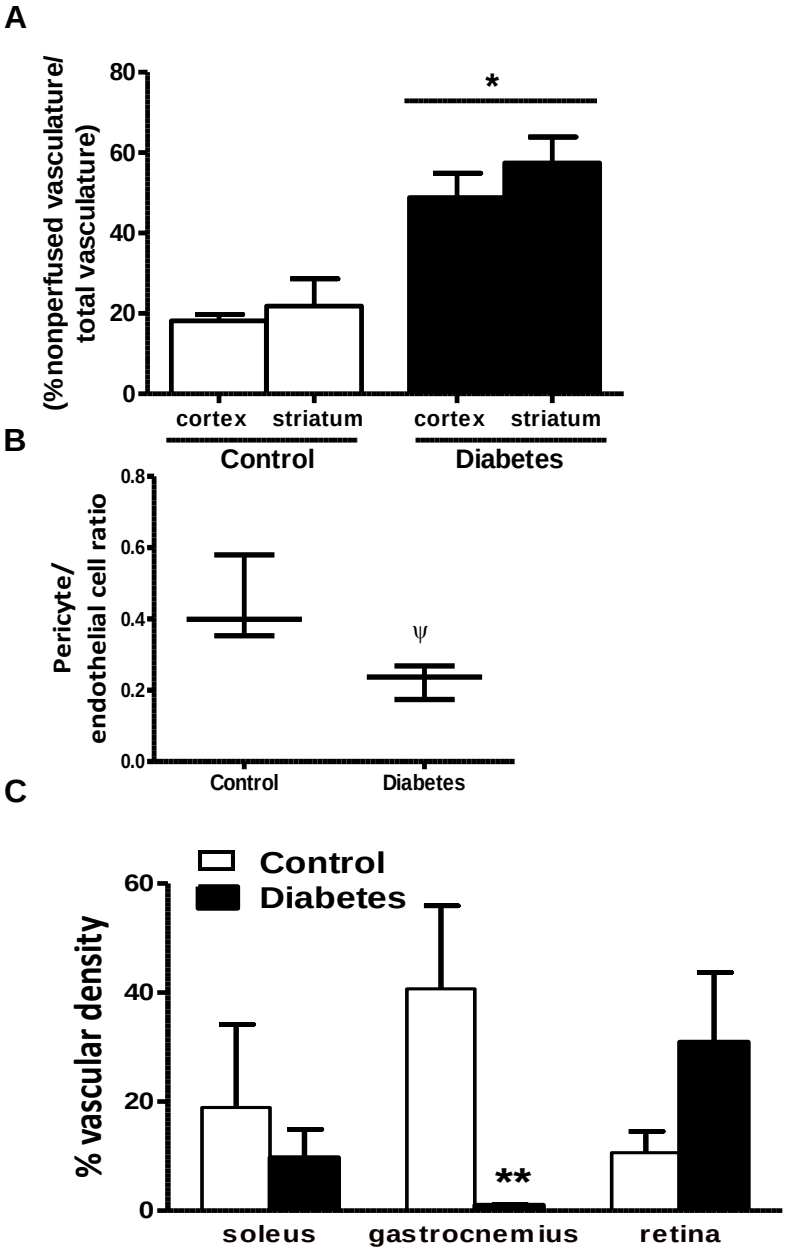

A
(%nonperfused vasculature/ total vasculature)
80
60
40
20
0
*
cortex
striatum
cortex
striatum
Control
Diabetes
B
Pericyte/ endothelial cell ratio
0.8
0.6
0.4
0.2
0.0
ψ
Control
Diabetes
C
Control
Diabetes
% vascular density
60
40
20
0
**
soleus
gastrocnemius
retina

Figure 2-3. Increased VEGF expression and tyrosine nitration status of micro and microvasculature in diabetes. (A) VEGF levels were significantly increased in the cerebral microvasculature but not the macrovasculature in diabetes. (B) Both diabetic micro and macrovasculature had significantly increased tyrosine nitration compared to control. *p= 0.001, **p=0.05 diabetes vs control, Mean ± SEM, n=4 (Exact Wilcoxon test).

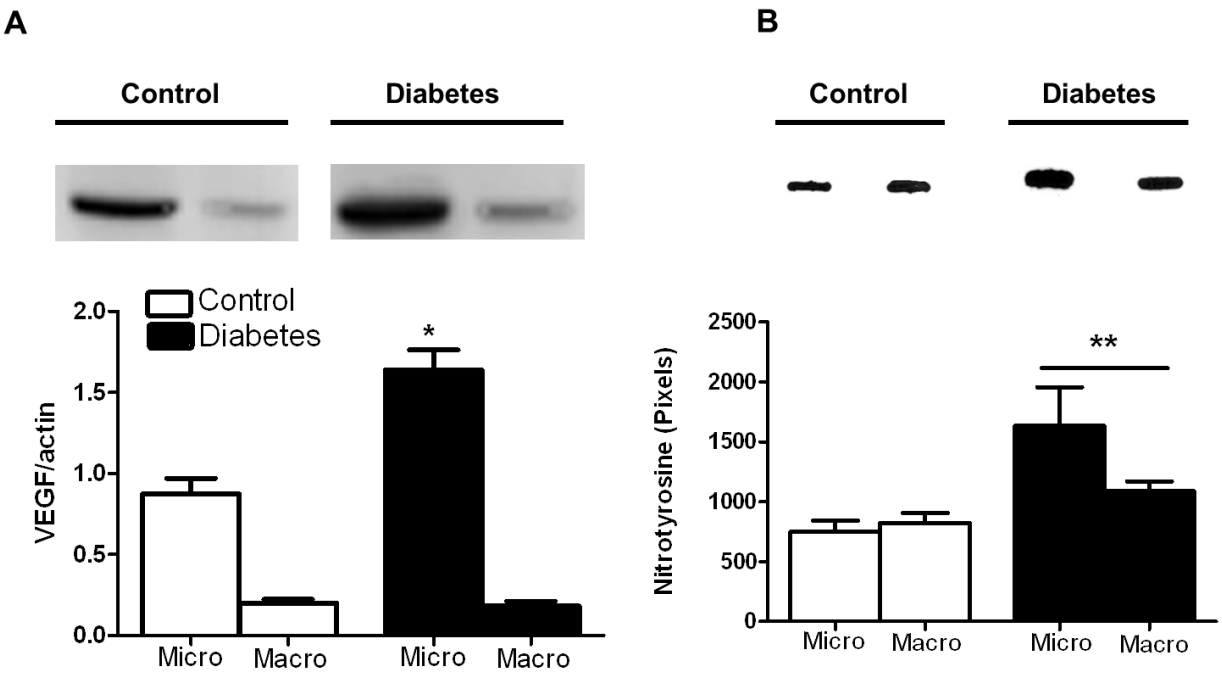

A
Control
Diabetes
Control
Diabetes
VEGF/actin
2.0
1.5
1.0
0.5
0.0
*
Micro
Macro
Micro
Macro
B
Control
Diabetes
Nitrotyrosine (Pixels)
2500
2000
1500
1000
500
0
**
Micro
Macro
Micro
Macro

Figure 2-4. Diabetic BMECs show significant increases in tubologenesis over time. (A) Representative images of BMECs showing more tube formation in diabetes group after 24 and 48h of plating on reduced matrigel. (B) Diabetic BMECs have significantly increased tube formation properties after 24 and 48 h of plating on reduced matrigel than in control cells. (C) Diabetic BMECs have significantly higher % increase in proliferation after 24 and 48 h. (D, E) Diabetic BMECs have significantly smaller cell mean diameter and volume compared to control cells respectively. *$p<0.01$ diabetes vs control, #$p<0.01$ 24 h vs 48 h, **$p=0.0022$ diabetes vs control. Mean ± SEM, n=3-6.

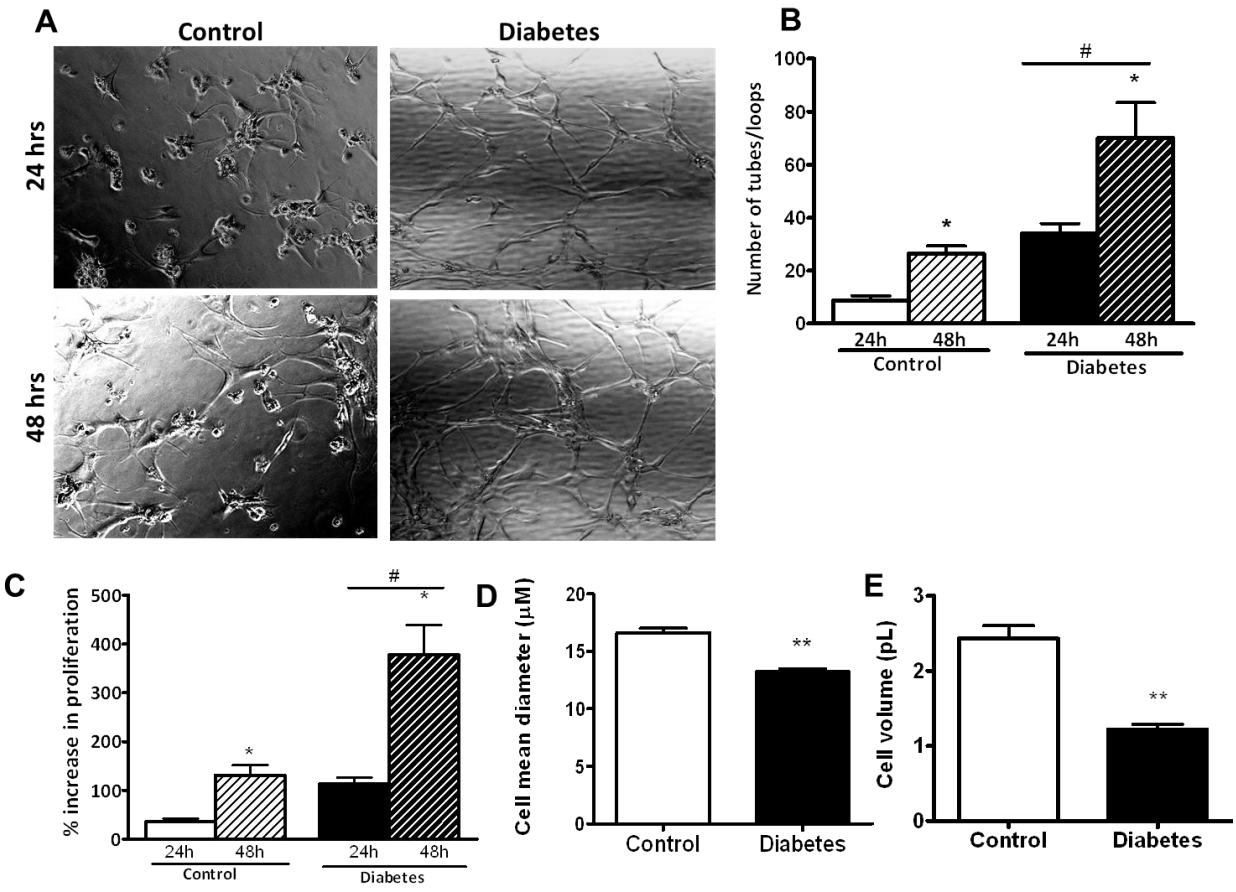

A
Control
Diabetes
24 hrs
48 hrs
B
Number of tubes/loops
100
80
60
40
20
0
#
*
*
24h
48h
24h
48h
Control
Diabetes
C
% increase in proliferation
500
400
300
200
100
0
#
*
*
24h
48h
24h
48h
Control
Diabetes
D
Cell mean diameter (μM)
20
15
10
5
0
**
Control
Diabetes
E
Cell volume (pL)
3
2
1
0
**
Control
Diabetes

Figure 2-5. Diabetic BMECs have increased cell migration that is mediated by VEGF in an autocrine manner. (A) Representative images of BMECs showing increased spontaneous cell migration. (B) Representative images of control cells treated with conditioned medium from diabetic cells in the presence and absence of VEGF neutralizing antibody. Control BMECs treated with diabetic BMEC conditioned media show increased cell migration and anti-VEGF antibody inhibits this effect significantly. (C) Quantitative analysis of data shown in panels A and B. Diabetic BMECs plated on fibronectin show significantly increased spontaneous cell migration after 24h. Control cells showed enhanced migratory properties when treated with diabetic endothelial cell conditioned media and VEGF neutralizing antibody significantly abrogated this response. *$p=0.0026$ across control groups. **$p<0.05$ vs other control groups by Tukey's post-hoc analysis. Mean ± SEM, n=5-7. (D, E) Diabetic endothelial cells secrete relatively higher levels of native and dimerized VEGF-A. $^{\#}p=0.016$ diabetes vs control. Mean ± SEM, n=3-8 (Exact Wilcoxon test).

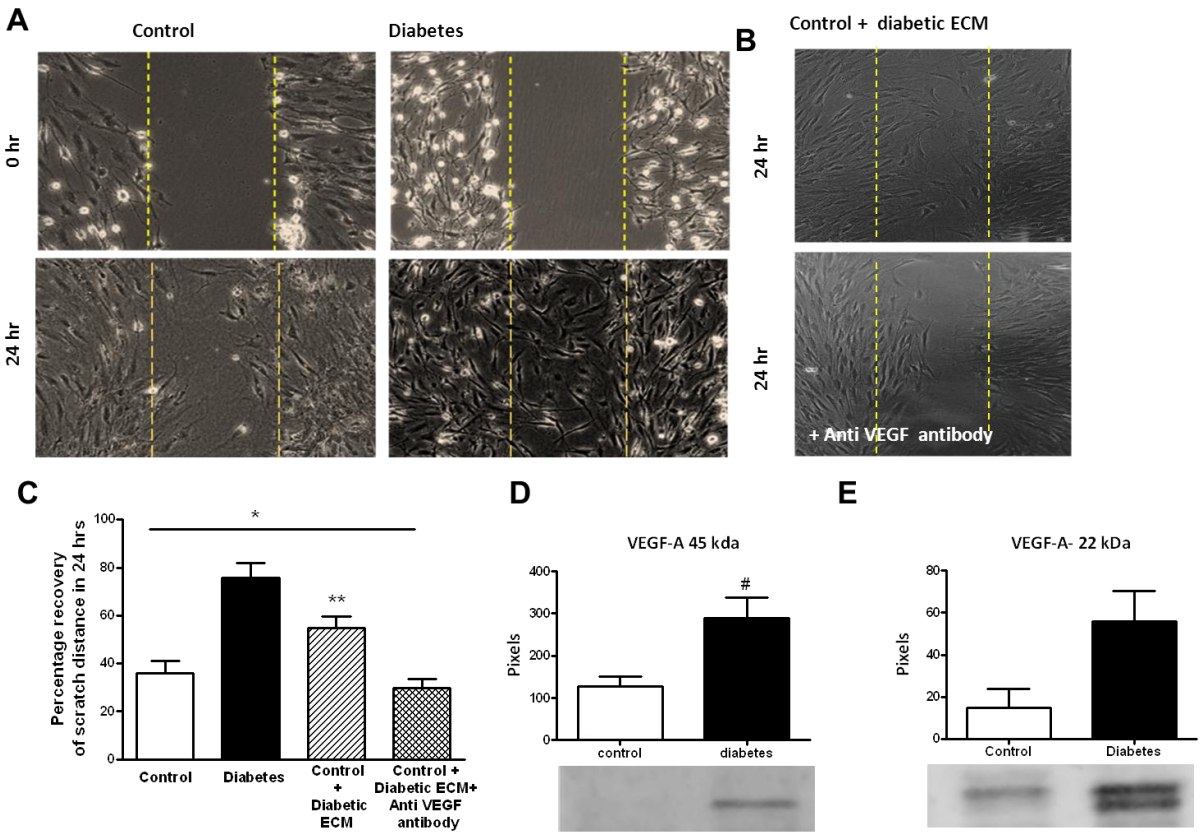

A
Control
Diabetes
0 hr
24 hr
B
Control + diabetic ECM
24 hr
24 hr
+ Anti VEGF antibody
C
Percentage recovery of scratch distance in 24 hrs
Control
Diabetes
Control + Diabetic ECM
Control + Diabetic ECM+ Anti VEGF antibody
D
VEGF-A 45 kda
Pixels
control
diabetes
E
VEGF-A- 22 kDa
Pixels
Control
Diabetes

Figure 2-6. Effect of diabetes on basal expression and phosphorylation status of angiogenesis mediators. (A) Native and phosphorylated VEGF-R2 levels were determined by immunoblotting. Phospho VEGF was increased in diabetic BMECs. Mean ± SEM, n=3. (B) In parallel with increased VEGF-R2 activation, c-src phosphorylation was also increased in diabetes. Mean ± SEM, n=4. (C) Diabetic endothelial cells have elevated protein tyrosine nitration compared to control. Mean ± SEM, n=6-11. (D-E) MMP-2 activity was assessed by gelatin zymography. While there was no difference in secreted latent and active MMP-2, cell associated MMP-2 was significantly increased. F) Membrane type (MT1)-MMP levels determined by immunoblotting were greater in diabetes. Mean ± SEM, n=3-4. *p=0.0079 diabetes vs control, #p=0.029 diabetes vs control, $^{\Psi}$p=0.015 diabetes vs control, **p= 0.057 diabetes vs control (Exact Wilcoxon test).

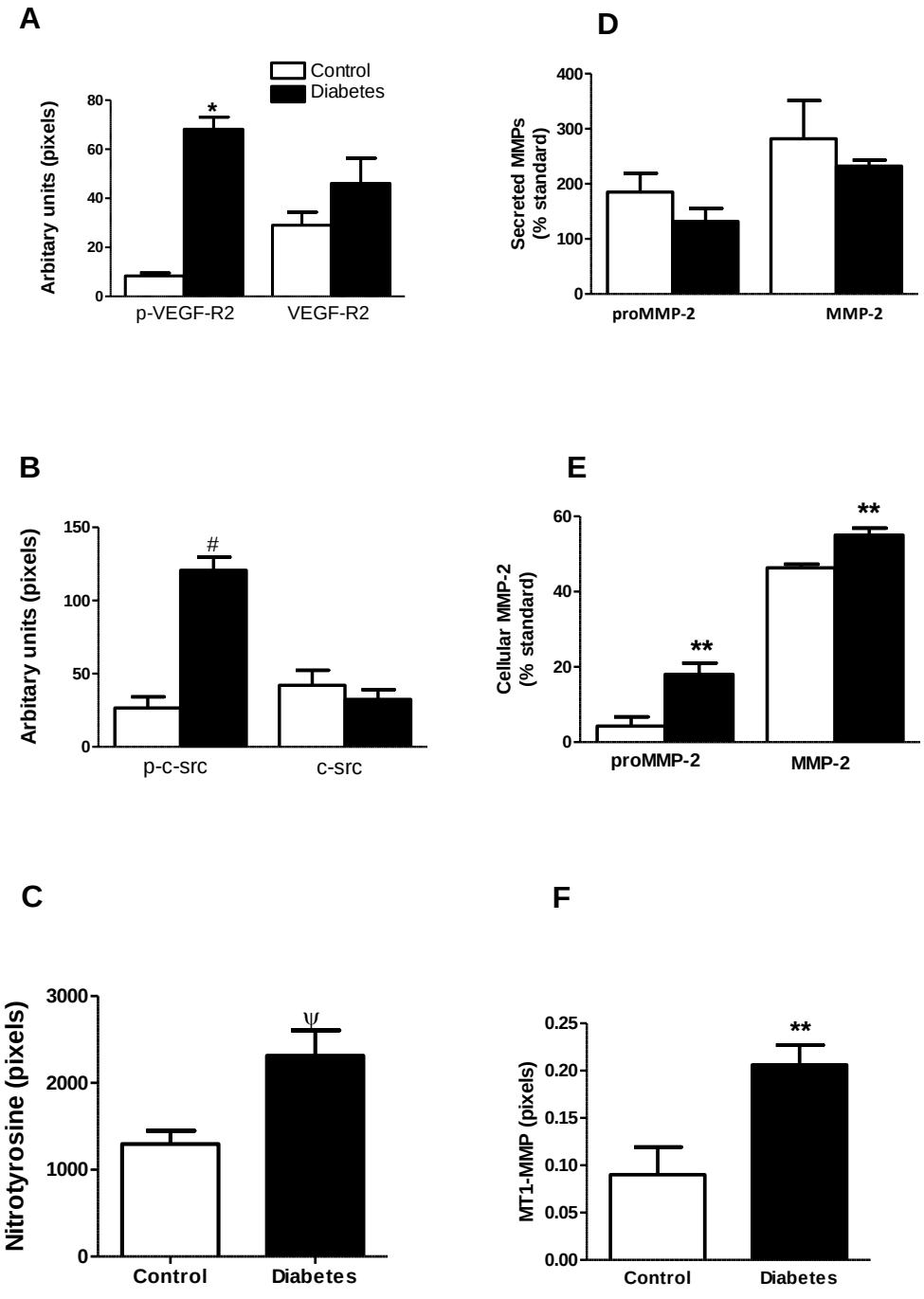

A
Control
Diabetes
Arbitary units (pixels)
80
60
40
20
0
*
p-VEGF-R2
VEGF-R2
D
Secreted MMPs (% standard)
400
300
200
100
0
proMMP-2
MMP-2
B
Arbitary units (pixels)
150
100
50
0
#
p-c-src
c-src
E
Cellular MMP-2 (% standard)
60
40
20
0
**
**
proMMP-2
MMP-2
C
Nitrotyrosine (pixels)
3000
2000
1000
0
ψ
Control
Diabetes
F
MT1-MMP (pixels)
0.25
0.20
0.15
0.10
0.05
0.00
**
Control
Diabetes

Figure 2-7. Evidence for involvement of endogenous VEGF signaling in increased migration in diabetes. The role of various angiogenic proteins were assessed by using respective inhibitors on cell migration assays. 2 or 24 h pretreatment with anti-VEGF antibody (A), peroxynitrite decomposition catalyst FeTPPs (B), src inhibitor PP2 (C) or MMP inhibitor minocyline (D) significantly reduced migration of BMEC in diabetic but not control endothelial cells. #$p<0.05$ vs untreated diabetes, $^{*}p<0.01$ untreated diabetes vs control. Mean ± SEM, n=4-7.

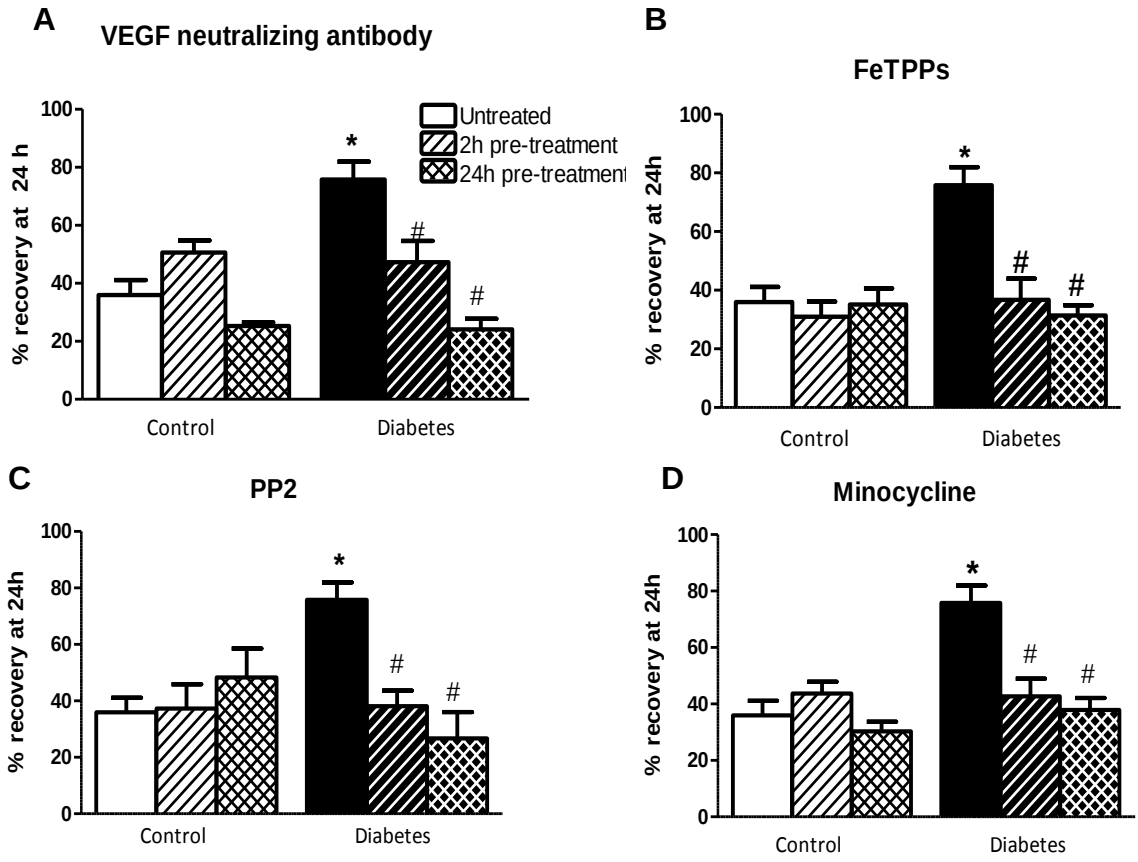
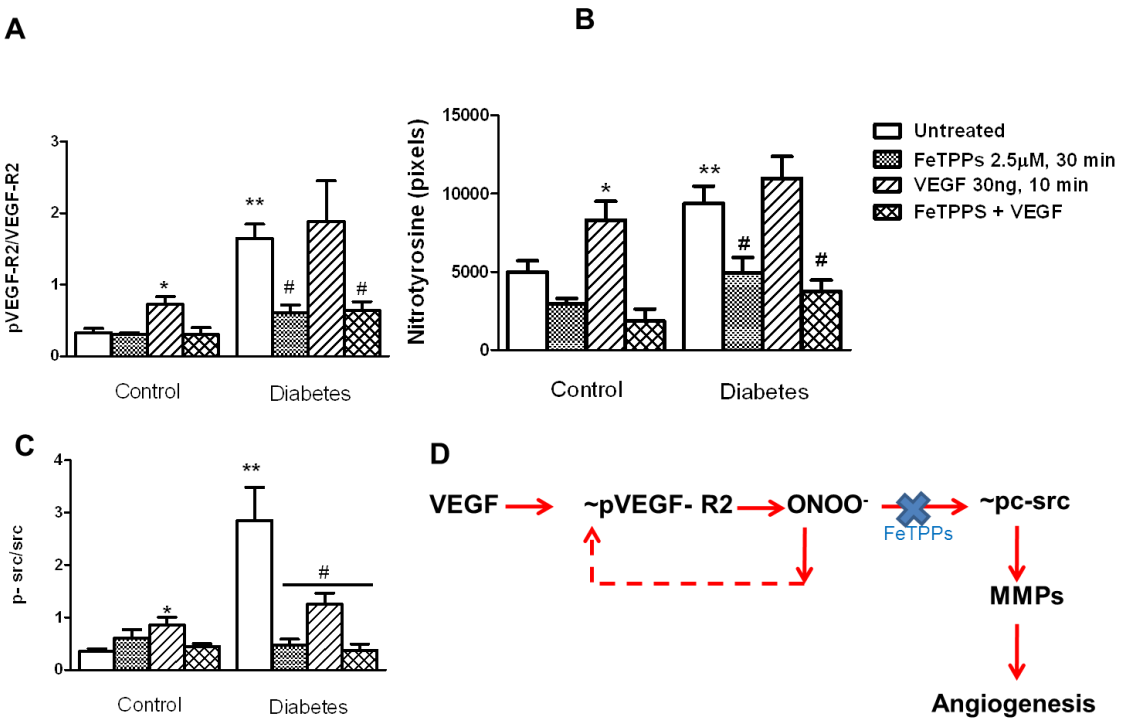
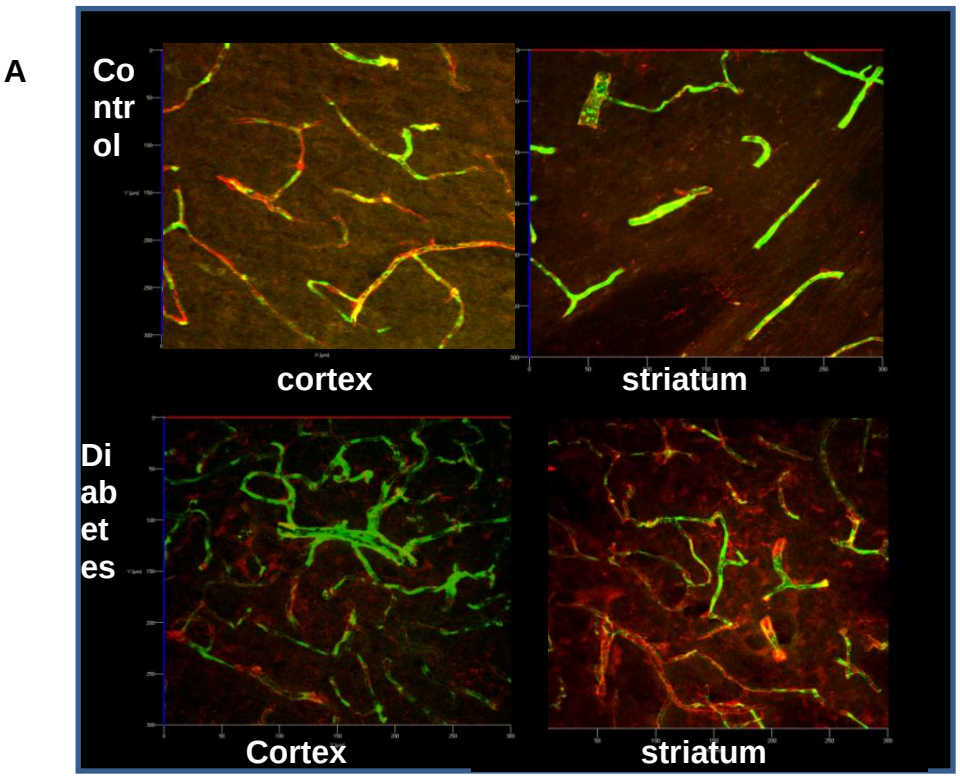
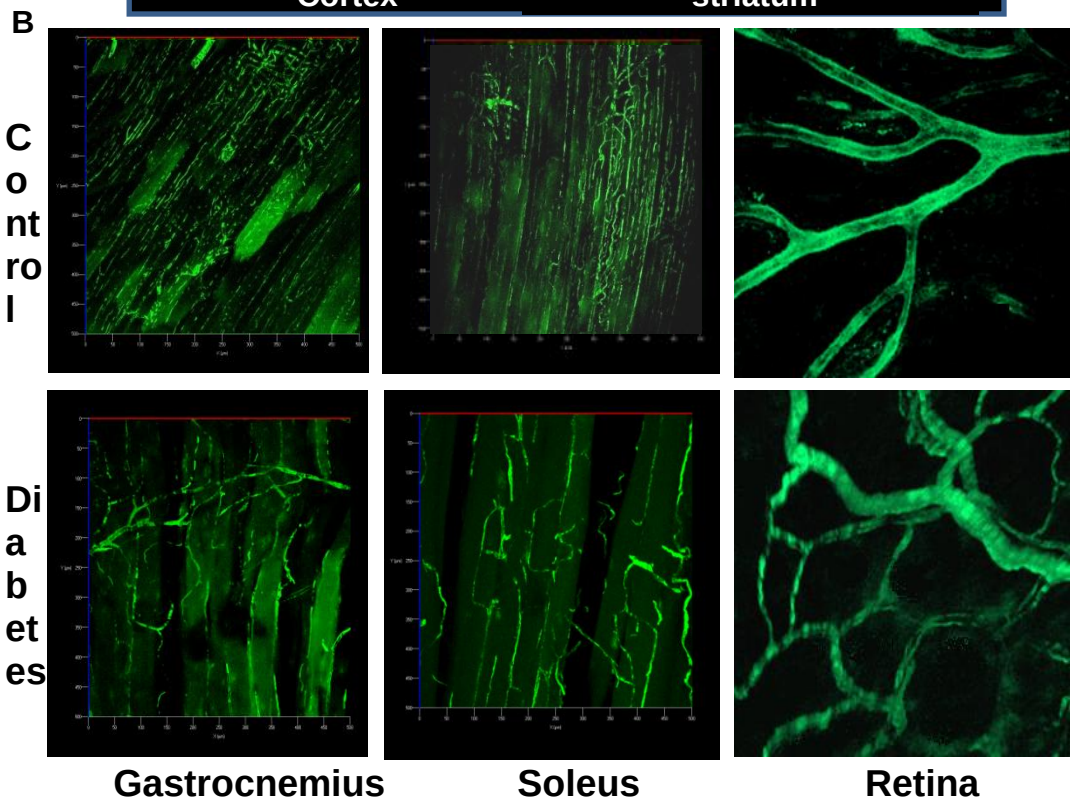

A
VEGF neutralizing antibody
Untreated
2h pre-treatment
24h pre-treatment
% recovery at 24 h
Control
Diabetes
B
FeTPPs
% recovery at 24h
C
PP2
D
Minocycline

Figure 2-8. The differential effect of exogenous VEGF on VEGF signaling in control and diabetic cells. Cells were treated with vehicle, peroxynitrite decomposition catalyst FETPPs alone, VEGF alone or VEGF plus FeTPPs. VEGF receptor activation (p~VEGF-R2/VEGF-R2 ratio), peroxynitrite formation and c-src activation (p~c-src/c-src ratio) were determined by immunoblotting as shown in Panels A-C, respectively. Exogenous VEGF treatment stimulated VEGF-R2 activation, c-src activation and tyrosine nitration in control cells and co-treatment with FeTPPS prevented this activation. Diabetic cells which show increased basal VEGF-R2, c-src and nitrotyrosine activation do not show further elevation in response to exogenous VEGF but respond to FeTPPS treatment indicating that peroxynitrite sustains VEGF-R2 phosphorylation and also mediates downstream signaling in these cells. D) Schematic representation of the role peroxynitrite in modulation of the angiogenic signal in brain microvascular endothelial cells. *$p<0.05$ vs other control groups, **$p<0.01$ vs untreated control, #$p<0.05$ vs untreated diabetes.


A
pVEGF-R2/VEGF-R2
3
2
1
0
*
**
#
#
Control
Diabetes
B
Nitrotyrosine (pixels)
15000
10000
5000
0
*
**
#
#
Control
Diabetes
Untreated
FeTPPs 2.5μM, 30 min
VEGF 30ng, 10 min
FeTPPS + VEGF
C
p- src/src
4
3
2
1
0
*
**
#
Control
Diabetes
D
VEGF
~pVEGF- R2
ONOO-
FeTPPs
~pc-src
MMPs
Angiogenesis

Supplementary Figure 2-1: (A) Representative images of cerebral cortex and striatum showing perfused (green-FITC dextran) and non-perfused vessels (Red- Isolectin). Quantitative assessment shown in Figure 2-2A. (B) Representative images of retinal and peripheral vasculature in diabetes and control groups. Arrows indicate collateralization and decreased microvasculature. Quantitative assessment shown in Figure 2-2C


A
Control
cortex
striatum
Diabetes
Cortex
striatum


B
Control
Diabetes
Gastrocnemius
Soleus
Retina

CHAPTER 3

CEREBRAL NEOVASCULARIZATION AND REMODELING PATTERNS IN TWO MODELS OF TYPE 2 DIABETES.

Roshini Prakash, Maribeth Johnson, Susan C Fagan and Adviye Ergul

Submitted to [PLOSone], 10/26/2012

ABSTRACT

We previously reported intense pial cerebral collateralization and arteriogenesis in a mild and lean model of type 2 diabetes (T2D), Goto-Kakizaki (GK) rats. Increased cerebral neovascularization differed regionally and was associated with poor vessel wall maturity. Building upon these findings, the goals of this study were a) to compare and contrast this pathological neovascularization pattern in db/db mice and GK models of diabetes, and b) determine the effect of glycemic control on erratic cerebral neovascularization. Vascular volume, surface area and structural parameters including microvessel/macrovessel ratio, non-FITC (fluorescein) perfusing vessel abundance were measured by 3D reconstruction of FITC stained vasculature. Lean GK rats exhibited an increase in all of these parameters. Glycemic control with metformin prevented these changes. In obese db/db mice, microvascular density was increased but there was no change in non-FITC perfusing vessels. Increased PA branch density was associated with reduced branch diameter. These results suggest that T2D leads to cerebral neovascularization and remodeling but structural characteristics of newly formed vessels differ between lean and obese models that have mild or severe hyperglycemia, respectively. The prevention of dysfunctional cerebral neovascularization by early glucose control suggests that hyperglycemia is a mediator of this response.

INTRODUCTION

Diabetes-mediated microvascular disease of the brain is increasingly recognized as a risk factor for neurodegenerative diseases like vascular cognitive impairment and stroke [1]. Changes in cerebrovascular structure and function can lead to altered blood brain barrier (BBB) permeability and cerebral blood flow not only contributing to the development of the disease but also impairing the recovery after an ischemic event like stroke [2]. Earlier we showed greater pial cerebral arteriogenesis characterized by increased collateralization and tortuosity in the Goto-Kakizaki (GK) model of diabetes [3, 4]. This model also develops new cerebral blood vessels that have greater permeability and reduced wall maturity thus making them liable to bleeding during an ischemic insult [4, 5]. It is known that vascular morphogenesis in a healthy animal is dependent upon physiologic, metabolic and local factors [6]. The presence of a disease state and its severity affects these factors in relation to the altered functional needs of a particular tissue [7]. Therefore, understanding of the cerebrovascular architecture that is important for the delivery of oxygen and nutrients in different experimental models of diabetes is critical to identify and develop novel therapeutic targets for prevention and treatment of cerebral microvascular complications in diabetes. Given that the GK rat is a lean model of diabetes that present with mild to moderate hyperglycemia but no dyslipidemia, several important questions remained to be answered: 1) Are these pathological changes in diabetes present in other models of diabetes?, 2) Does diabetes severity and presence of confounding factors make a difference?, and 3) Is glycemic control effective in the prevention of dysfunctional cerebral angiogenesis? The current study sought to address these questions using three-dimensional images of the cerebral angioarchitecture from the lean and moderately hyperglycemic GK rat, metformin-treated euglycemic GK rat and the obese db/db mice that have severe hyperglycemia.

METHODS

Animal preparation and glycemic control

All procedures on animals for the study were carried out in accordance with National Institute of Health guidelines for the care and use of animals in research and under protocols approved by the Georgia Health Sciences University. Glycemic control in the GK rats was achieved using metformin (150-300mg/kg body weight, dose escalated with age to a maximum of 300 mg/kg) in artificially sweetened drinking water to mask the metallic taste of the drug. Euglycemia was targeted in GK rats starting at 6 weeks of age at the onset of diabetes until 11 weeks when established vascular disease is seen in the untreated parallel GK group. The control group was not treated with metformin due to ethical reasons as they begin to lose weight and deteriorate upon treatment. Metabolic parameters are given in Table 3-1.

Imaging of cerebral vasculature

Vascularization patterns and density were measured using the space-filling FITC-Fluorescein IsoThioCyanate-dextran method as we recently described. Brains were processed in 4% paraformaldehyde (24 h) and 30% sucrose in phosphate buffered saline (PBS), sectioned into 100μm slices and mounted on slides. Z-stacked confocal images of the regions proximate to the middle cerebral artery (MCA) and its branches that supply the frontal motor cortex, bregma 1 to -1 were acquired using Zeiss LSM 510 upright confocal microscope. Cortical parenchymal vessels that dive in from the surface vessels and its immediate first order branches were imaged at 10X in this region. A mean of 3 values from this region was recorded as an observation. Each measurement from one animal was comprised of an average of 9 images from either the cortical or striatal region. Retinal flat mount, gastrocnemius and soleus muscles were prepared similarly

in the same animals to assess neovascularization in different vascular beds. Axial distance of 1 µm spaced images was obtained using 25X objective to assess 3 dimensional parameters.

Indices of neovascularization

Vascular volume refers to the ratio of the volume of the FITC stained vasculature to the total volume (reference volume) of the section on a Z-stack as this represents the unit volume of blood being supplied to the brain tissue in the region of interest [5, 8]. Absolute *surface area* represents the area of diffusion of vascular nutrients to the surrounding tissue [8]. Penetrating arterioles, defined as the macrovessels, were selected on each image using Volocity Improvision and their respective volumes and surface area measurements were derived. Microvascular measurements were obtained by subtracting the macrovascular measures from the total vascular parameters.

To differentiate vessels not perfused with FITC, brain sections were co-stained with biotinylated isolectin B4 (Vector Laboratories Inc. Burlingame, CA) that binds to basement membrane and marks all endothelial cells [5].

Vessel structure and morphometry was assessed using Fiji software and axially projected into an 8-bit image[9]. Centerlines reduced to 1-pixel size were extracted to obtain binary skeletonized images which were then assessed to determine the tortuosity, diameter of the penetrating arterioles (PA) and its first order branches, and the number of branch points associated with the penetrating arterioles. The centerline line extracted images were run through to analyze vessel tortuosity by longest-shortest distance method without pruning the ends to measure the actual length of the vessels [10, 11]. A ratio of this value over the euclidean distance provided the tortuosity or skewness of the vessel. The values obtained from this analysis were sorted in a descending fashion. Diameter measurements were drafted manually as the parallel distance between internal walls of the vessels after outlining the lumen using Fiji software. An average of

3-4 values was recorded as mean diameter of a given vessel. Branch density refers to the number of branch points found over unit length of a vessel and indicated on Fig 2 [12, 13]. (Methodology shown on Supplementary Fig 1)

Astrocytic Structure

The FITC stained sections were co-stained with GFAP (Millipore). Surface area of the GFAP stained astrocytes were determined. A smaller region of interest was selected on each image and evaluated for astrocytic surface density. A ratio of the surface area over the total number of astrocytes in the image was evaluated as the astrocytic surface density.

Statistical Analysis

Data are expressed as mean ± SE. Data were evaluated for normality and appropriate transformations were used when necessary. One-way ANOVAs on the ranks of the data were used to compare control (Wistar), Diabetes (GK), and GK + Metformin groups of rats for all variables. A Tukey's test was used to adjust for multiple comparisons for significant group effects. T-tests on the ranks of the data were used to compare control (C57BL) and diabetic ($Lepr^{db/db}$) mice for all variables. A test for the homogeneity of slopes among the groups of rats or mice for the relationship between percent vascular surface area and percent vascular volume was performed using ANCOVA on the ranked data. Statistical significance was determined at alpha=0.05 and due to the small sample sizes for some of the variables a statistical trend was determined at alpha=0.10. SAS® version 9.3 was used for all analyses (SAS Institute, Inc., Cary, NC).

RESULTS

Glycemic status in the two models of diabetes

Blood glucoses were measured in the two models of diabetes and compared to the respective controls. GK exhibit mild hyperglycemia and are lean (Table 3-1). However the db/db mice are extremely diabetic and in addition present thrice the body weight compared to their controls.

Neovascularization characteristics are different in 2 models of Type 2 diabetes

Vascular architecture was assessed in different models of Type 2 diabetes, a lean and mild rat model (GK rats) and obese and severe mice model (db/db mice). Measurements were made in the cortical and striatal regions that are susceptible to vascular injury if these animals are subjected to ischemic brain injury as we reported before. The GK model displayed increased total vascular density, volume and surface area in both cortex and striatum (Fig 3-1A-C). On the other hand, there was no difference in vascular density in db/db mice as compared to control mice. There was, however, an increase in 3-D indices like vascular volume and surface area (Fig 3-1 G-I). When the relationship between vascular volume and surface area was analyzed, there was a linear correlation between the surface area and volume in control groups- Wistar and c57 black mice. However there was disproportional increase in these parameters in the GK group, suggesting a contribution from both macro and microvasculature. These results also suggested that in GK rats there were some vessels that are getting larger (more volume) without a parallel increase in area indicative of larger vessel remodeling. We then analyzed the relative density of micro and microvasculature in these sections. As shown in Fig 3-1D and E, GK rats displayed an increase in both micro and macrovessel volume and

surface area. db/db mice, however, showed an increase only in microvascular volume and area (Fig 3-1G and H).

Since FITC-dextran is a space-filling model, it is assumed that vessels visualized are perfused with FITC. In order to differentiate vessels that are not perfused with FITC, the same sections were labeled with isolectin. The nonperfused/perfused vessel ratio which we reported to be greater in GK rats was normalized with glycemic control. In contrast to the GK model, the nonperfused/perfused vessel ratio was not different between control and diabetic animals in the db/db model (Supplementary Fig 3-2)

Glycemic control prevents dysfunctional neovascularization

Glucose control with metformin initiated right after onset of diabetes lowered blood glucose levels to control levels in GK rats (Table 3-1). Glycemic control completely prevented the increase in all indices of neovascularization (Fig 3-1A-E) and also corrected the relationship between vascular volume and surface area (Fig 3-1F).

Diabetes augments cerebral vascular tortuosity and branching in both GK rat and db/db mice

Both animal models exhibit significantly enhanced branch density of the PAs (Fig 3-2A and B). The diameter of these vessels was increased in the GK model but it was reduced in the db/db mice (Fig 3-3A and B). Diabetes also significantly increased the tortuosity of cortical penetrating arterioles in both the animal models (Fig 3-4A and B). Euglycemia achieved by treating with metformin prevented these pathological features in the cerebrovasculature (Figs 3-2, 3-4).

Peripheral neovascularization is impaired in both models of diabetes

The decreased vascular density in the peripheral skeletal muscle in GK rats was prevented by metformin treatment (Fig 3-5 A and B). There was also a profound decrease in the peripheral vascular density in the db/db mice. Interestingly, just like in the brain, vascular density was greater in the retina in both GK and db/db models and metformin treatment abrogated these effects in GK rats (Fig 3-5 C, D).

Diabetes increases reactive astrocytes

Since the PAs are surrounded by astrocytes which are critical cells in communicating the signals from neurons to the vessels for proper regulation of blood flow to provide the metabolic needs of neurons, we next assessed the presence of reactive astrocytes. There is increased surface area per astrocyte; referred to as astrocytic surface density, in both of the diabetic models (Fig 3-6 A, B) compared to the respective control groups. We also observe differences in the density of astrocytes between the cortex and striatum. The diabetic group exhibits increased perivascular projections wrapping the vessels with relatively smaller soma compared to the control groups. Glycemic control with metformin prevented astrocytic reactivity caused by diabetes.

DISCUSSION

The brain is an important target organ for complications associated with diabetes. Since changes in vascular function and structure play a major role in diabetic complications, understanding of cerebrovascular networking and neovascularization patterns in multiple models of diabetes is critical to identify commonalities and differences so that therapeutic targets and strategies can be developed for the prevention and treatment of these

complications. Accordingly, this study investigated cerebrovascular architecture in lean (GK rats) and obese (db/db mice) models of diabetes with different hyperglycemia profiles. The major findings are: 1) While both microvessel and macrovessel densities in the brain are increased in the lean and mildly diabetic GK rats, db/db mice show a significant increase in the microvasculature; 2) Branch density and tortuosity of penetrating arterioles are increased in both models of diabetes, 3) Lumen diameter of penetrating arterioles are increased in GK rats but decreased in db/db mice; 4) Peripheral neovascularization is impaired in both models and this decrease is profound in the db/db mice; 5) There is increased retinal neovascularization in both models, and 6) Glycemic control started at the onset of diabetes prevented dysfunctional neovascularization of the brain, retina and peripheral skeletal muscles.

As recently reviewed, numerous studies reported ultrastructural changes such as basal membrane thickening, collagen deposition and endothelial degeneration in the cerebral microvasculature in multiple models of diabetes [14].What is less clear is how diabetes impacts cerebrovascular networking and neovascularization patterns in the brain. We have recently reported that there is enhanced cerebral neovascularization that is spatially and regionally regulated in the GK model of diabetes [5]. The current study further expanded on these findings to determine the relative contribution of microvessels and macrovessels to this increased neovascularization response. In a given volume of tissue, an increase in vascular volume which measures mainly the vascular lumen space can be due to either remodeling of the vessel and getting larger lumen and/or due to new vessel formation. In the latter, an increase in surface area, a measure of the area vessel wall occupies, accompanies increased volume. As such when one looks at the relationship between volume and surface area, a linear association suggests that the increase in these two

parameters go hand-in-hand and there is significant new microvessel formation. The vascularization pattern in the db/db model exactly fits this model. In the GK model, however, there is extreme and disproportional increase in these parameters as a result of profuse microvasculature as well as macrovascular remodeling. Increased surface area is indicative of the large vascular surface area available for exchange of components between the tissue and the vasculature. These vessels have been already reported to have high vascular permeability [15]. Thus, these pathological alterations in both models of diabetes may contribute to greater vascular damage and bleeding associated with stroke or neurodegenerative processes in diabetes.

Astrocytes bridge the neurovascular interaction and they have been reported to regulate cerebral microcirculatory responses and neurovascular remodeling [16, 17]. They also regulate brain metabolism and are involved in synaptic plasticity [18, 19]. Astrocytes reactivity in the hypothalamic regions and retina is increased in type 1 diabetes induced by streptozotocin [20, 21]. Astrocytes exhibit hypertrophy in the areas surrounding brain lesions and are associated with oxidative stress [22]. We report increased astrocytic reactivity in both the models of type 2 diabetes. The diabetic groups show numerous finer astrocytic processes with smaller somatic volumes compared to the control groups. The increased astrocytic reactivity together with dysfunctional cerebral vasculature may be the major contributors to increased risk of stroke, and poor vascular and functional recovery post-stroke.

Our study is the first to report an increase in the cerebral microvasculature in the db/db model. A previous study showed impaired angiogenesis after stroke in this model but vascularization between control and diabetic animals in the absence of an ischemic injury was not compared in that study [23]. Previous studies have reported that the microvasculature undergoes rarefaction in the peripheral vascular beds in this model later in the disease. This study was

conducted in relatively younger animals to determine the early changes in the cerebrovasculature. Whether rarefaction occurs in the brain later in the disease remains to be determined but our finding that the vascular density is already quite dramatically reduced in the skeletal muscle argues that brain vascularization is differentially regulated.

The similarities between the cerebrovascular networking patterns in these models included enhanced surface area, volume, and branch density as well as increased tortuosity. A major difference that stood out was the lumen diameter of penetrating arterioles and their first order branches, which were increased in GK rats but decreased in the db/db mice. This is consistent with our findings discussed above that in the GK model; there is an increase in the % macrovasculature indicative of vascular remodeling. Diabetes and obesity leads to hypertrophic remodeling of blood vessels as observed in both older animal models and in diabetic patients [24]. The difference may be due to the presence of hyperlipidemia and obesity in this model and or due to the differences between the glucose levels. Mild hyperglycemia in the early phases of diabetes in the GK rats may increase the lumen diameter of the penetrating arterioles and its immediate branches whereas obese and severe diabetic model shows decreased lumen diameter suggestive of progression of medial thickening and increasing the risk of ischemia and peripheral arterial disease.

Penetrating arterioles are considered the bottleneck of cerebral blood flow regulation as they reach deep into the brain parenchyma, they are in close association with astrocytes that provide the bidirectional communication, known as neurovascular coupling and functional hyperemia, between neurons and vessels to meet the metabolic demands of the brain [25]. In this study, the branch density and tortuosity of parenchymal arterioles are increased in both models of diabetes. Higher degree of branching and tortuous patterns has been demonstrated

to be an effective determinant of blood flow changes [13, 26]. Reduced cerebral blood flow has been described in diabetic patients as well as in animal models of type 1 diabetes (T1D) [27, 28]. While we did not investigate the penetrating arteriole function in this study, we recently reported that functional hyperemia is blunted and cerebral blood flow is lower in the GK rat model [29]. Increased tortuosity and branching of penetrating arterioles may be a factor contributing to cerebral blood flow changes and need to be further confirmed in the db/db model.

Glycemic control is an effective preventive strategy, as evidenced from randomized control trials such as DCCT, UKPDS, there exists a significant direct correlation between glycemia contributing to both vascular and neurological complications [30, 31]. While the impact of glycemic control on prevention of macrovascular complications is still being debated, intensive glycemic control with other agents or treatment with metformin reduced the diabetes related endpoints and microvascular complications [32]. Metformin in experimental animal models has shown to prevent the progression of diabetes [33, 34]. We chose to achieve euglycemia with metformin, a first line choice of drug for Type-2-diabetes that has been safely used with minimal side effects. We limited this treatment only to the GK rats as it is the most appropriate model to study the role of glycemic control. GK rats exhibit spontaneous diabetes whose etiology is closely related to the human diabetic models however lacks the obesity factor. While db/db mice provided both the confounding factors of hyperglycemia and increased body weight compared to the GK model of diabetes, this model is leptin receptor deficient. Early glycemic control with metformin abrogated abnormal responses like cerebral neovascularizations, tortuosity and branching mediated by diabetes. Metformin was also effective in preventing peripheral vascular regression and retinal hypervascularizations.

CONCLUSION

Our studies provide new information comparing the effect of moderate and severe glycemic status on microvascular changes that may contribute to diabetic complications. From this study we conclude that the severity of hyperglycemic status with/without obesity in diabetic conditions affects the cerebral vascularization differentially as they have different pathological attributes to the cerebral microvascularization and macrovascular remodeling. Moderate diabetes begins with an incessant increase in both micro and macrovasculature while severe diabetes progresses with augmented increase in the microvasculature. Understanding mechanisms involved in micro and macrovascular alterations can provide a better understanding of a) dynamic changes in vascular structural alteration with the progression of diabetes, b) early prognosis of microvascular events, and c) better approaches to treat diabetes with and without obesity and reduce the risk of macrovascular events.

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Figure 3-1. Comparative evidence of cerebral vascularization between lean and obese models of type-2 diabetes. (A, G) Vascular density is significantly increased in both diabetic cortex and striatum in the GK rats, while the db/db mice show no significant change. (B-C, H-I) Vascular volume and surface were markedly increased in both cortex and striatum of the diabetic groups compared to their respective controls. (D-E) Moderate diabetes in GK rats significantly alters both micro and microvasculature compared to control, (J-K) while severe diabetes as seen in the $Lepr^{db/db}$ mice affects only the microvasculature. (F, L) linear regression graphs depicting the correlation between the vascular volume and surface are of the vasculature. (F) Diabetic vascular correlations are extremely disproportionate and the slopes of the two lines are significantly different, (L) shows a trend towards a proportional increase in surface are with an increase in vascular volume. Glycemic control normalized the effect of diabetes in the GK rats. *$p<0.05$ cortex vs striatum, **$p<0.01$ diabetes vs control and treatment. Mean ± SEM, n= 4-9

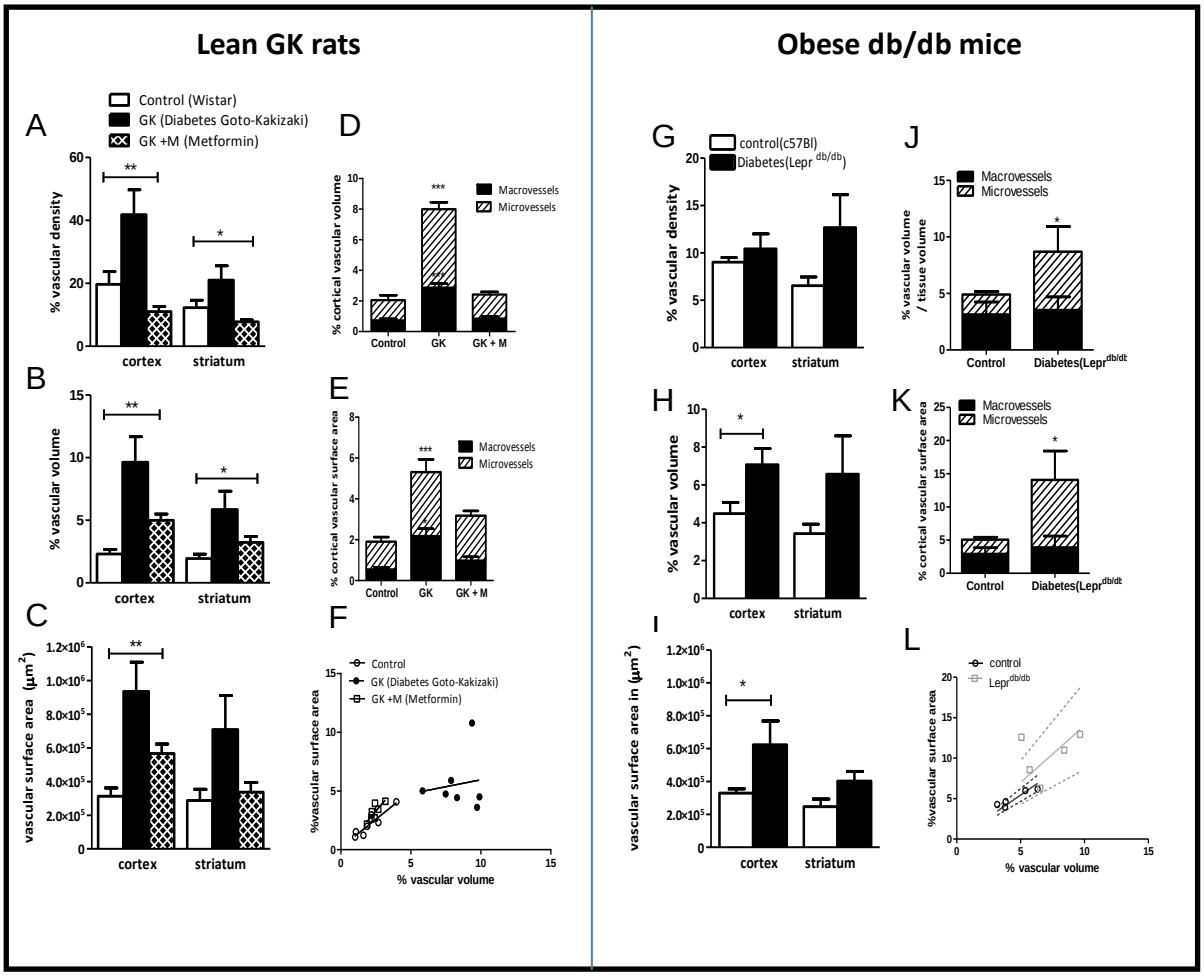
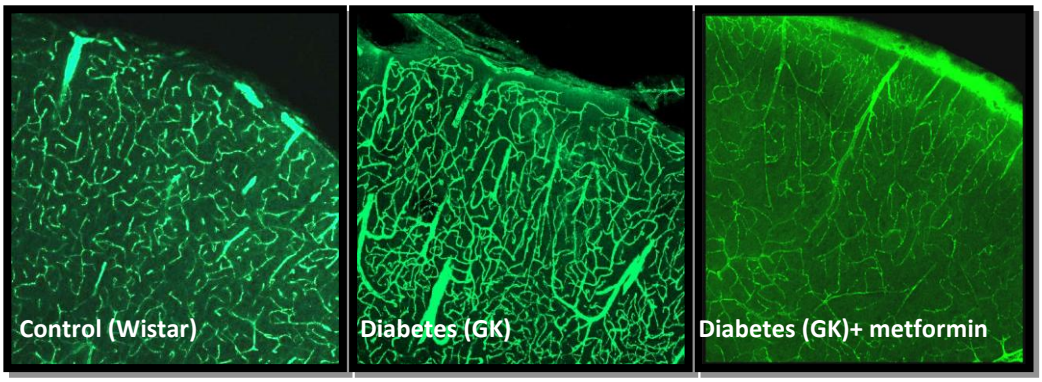
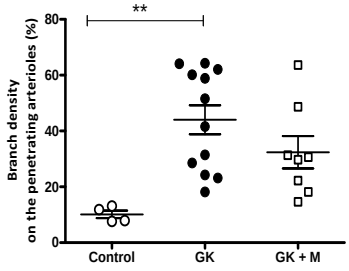
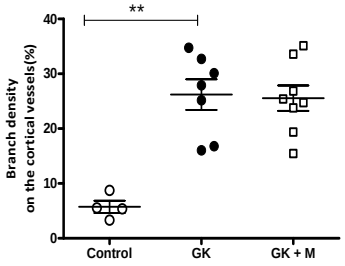

Lean GK rats
Obese db/db mice
Control (Wistar)
GK (Diabetes Goto-Kakizaki)
GK +M (Metformin)
A
B
C
D
E
F
G
H
I
J
K
L
% vascular density
% vascular volume
vascular surface area (µm²)
% cortical vascular volume
% cortical vascular surface area
%vascular surface area
% vascular volume
cortex
striatum
Control
GK
GK + M
Macrovessels
Microvessels
control(c57Bl)
Diabetes(Lepr db/db)
% vascular volume / tissue volume
vascular surface area in (µm²)
Diabetes(Leprdb/db)
control
Leprdb/db

Figure 3-2. Diabetes increases branch density of the penetrating arterioles and its subsequent branches. (A, B) Representative images showing vascular branching on the penetrating arterioles and surface cortical vessels taken under 10X. (A) GK rats exhibit profound increase in branch density on the penetrating arterioles. Similarly (B) $Lepr^{db/db}$ mice exhibit significantly increased branching compared to controls. There was a trend towards decrease in branching of the penetrating arterioles with glycemic control. *$p<0.05$ cortex vs striatum, **$p<0.01$ diabetes vs control. Mean ± SEM, n= 4-8

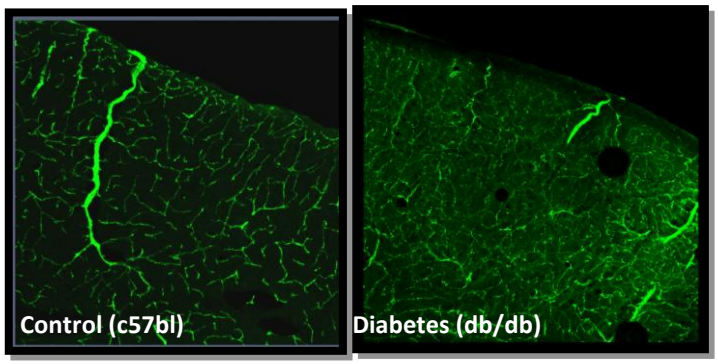
A

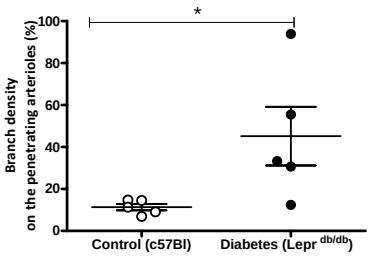

Control (Wistar)
Diabetes (GK)
Diabetes (GK)+ metformin


**
Branch density on the penetrating arterioles (%)
0
20
40
60
80
Control
GK
GK + M


**
Branch density on the cortical vessels(%)
0
10
20
30
40
Control
GK
GK + M

B


Control (c57bl)
Diabetes (db/db)


*
Branch density on the penetrating arterioles (%)
0
20
40
60
80
100
Control (c57Bl)
Diabetes (Lepr db/db)

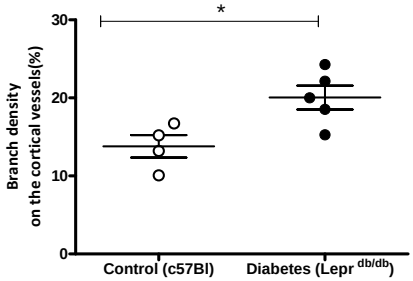
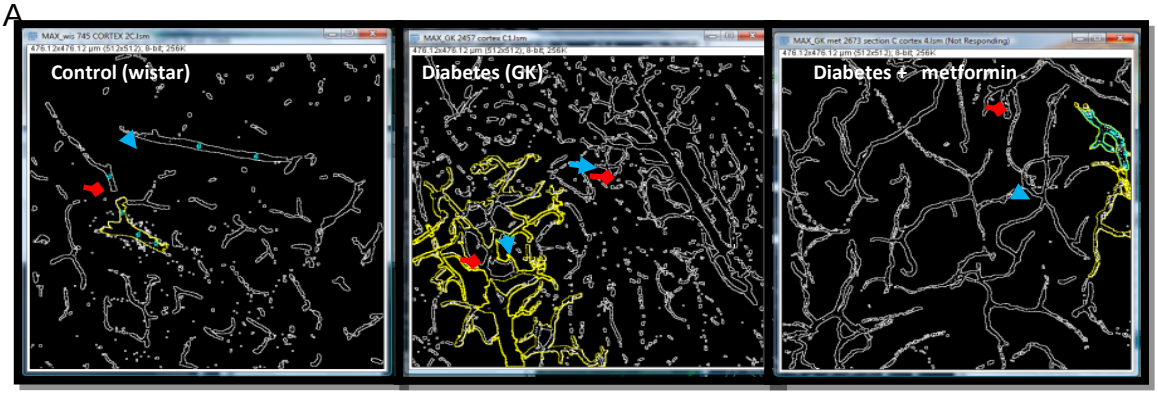
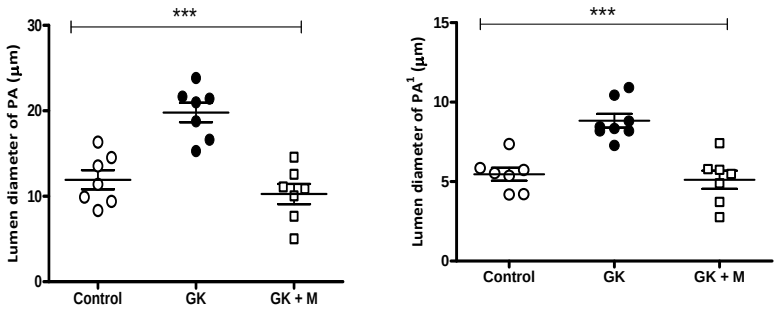

*
Branch density on the cortical vessels(%)
0
10
20
30
Control (c57Bl)
Diabetes (Lepr db/db)

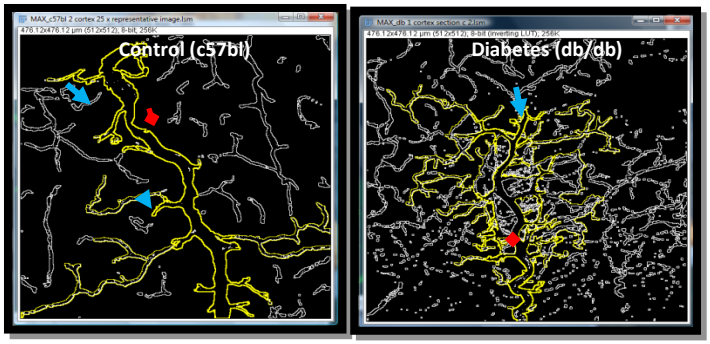
Figure 3-3. Diabetes dysregulates vascular morphology. (A, B) Representative pictures showing inner vessel walls outlined using the Fiji software. Red arrows represent the Penetrating arterioles (PA) and the blue arrows depict the immediate branched from the PA (PA^1), outlined yellow. (A) Lumen diameter in both PA and PA^1 are increased in moderate diabetes, in GK rats, while in severe diabetes as seen in $Lepr^{db/db}$ mice (B), there is a trend towards decrease in lumen diameters. Metformin treatment prevented this alteration in vascular morphology. ***p=0.005 diabetes vs control and treatment, Mean ± SEM, n=4-8.

A


Control (wistar)
Diabetes (GK)
Diabetes + metformin


***
Lumen diameter of PA (µm)
30
20
10
0
Control
GK
GK + M
***
Lumen diameter of PA-1 (µm)
15
10
5
0
Control
GK
GK + M

B


Control (c57bl)
Diabetes (db/db)

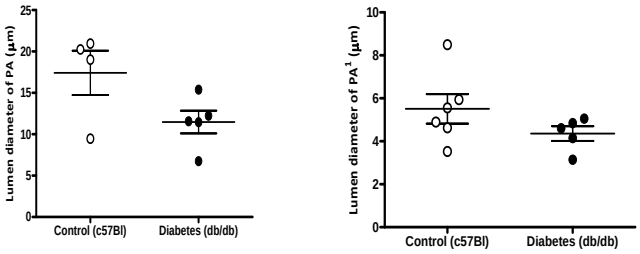

Lumen diameter of PA (µm)
25
20
15
10
5
0
Control (c57Bl)
Diabetes (db/db)
Lumen diameter of PA-1 (µm)
10
8
6
4
2
0
Control (c57Bl)
Diabetes (db/db)

Figure 3-4. Diabetes augments cortical vessel tortuosity. (A, B) Representative pictures showing tortuous cortical vessels imaged under 25X objective. Tortuosity index is significantly increased in both the diabetic models compared to their respective controls. Glycemic control with metformin prevented the tortuosity ***$p<0.0001$ diabetes vs control and treatment. Mean ± SEM, n=5-8.

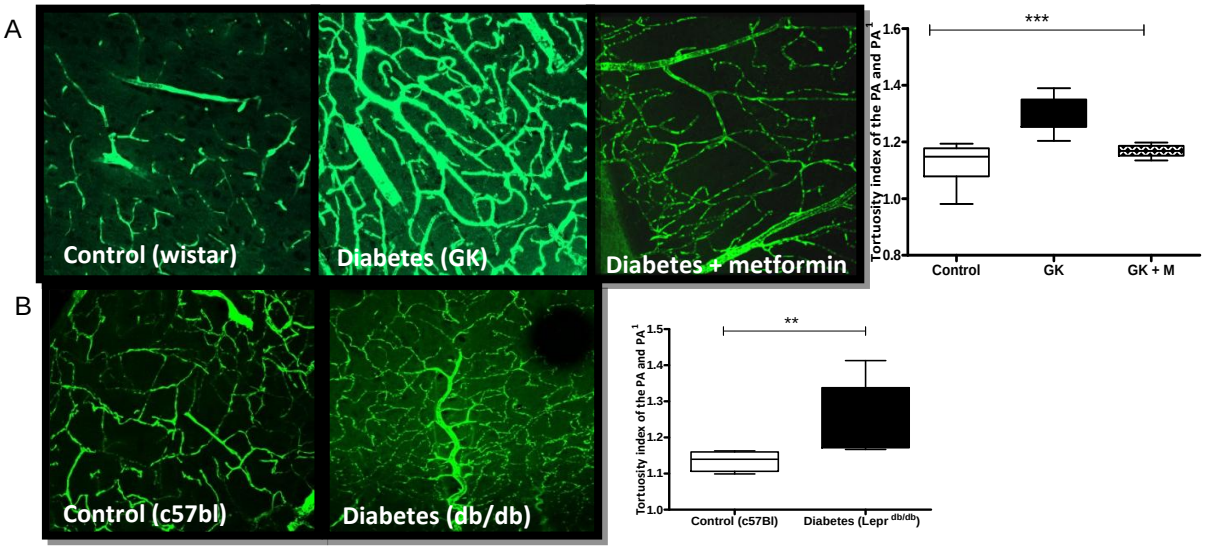

A
Control (wistar)
Diabetes (GK)
Diabetes + metformin
***
Tortuosity index of the PA and PA-1
1.6
1.4
1.2
1.0
0.8
Control
GK
GK + M
B
Control (c57bl)
Diabetes (db/db)
**
Tortuosity index of the PA and PA-1
1.5
1.4
1.3
1.2
1.1
1.0
Control (c57Bl)
Diabetes (Lepr db/db)

Figure 3-5. Peripheral vasculature is impaired in both the models of diabetes. (A, B) Representative images of blood vessels in the Gastrocnemius and Soleus muscles. Both the diabetic models exhibit significantly reduced peripheral vasculature as seen in the gastrocnemius muscles. (C, D) Representative images of retinal vasculature. Both the models of diabetes mediate retinal hypervascularization. Glycemic control with metformin restores the peripheral blood vessels and prevents increased retinal vascularization. *p=0.05 across control groups. Mean ± SEM, n=5-7.

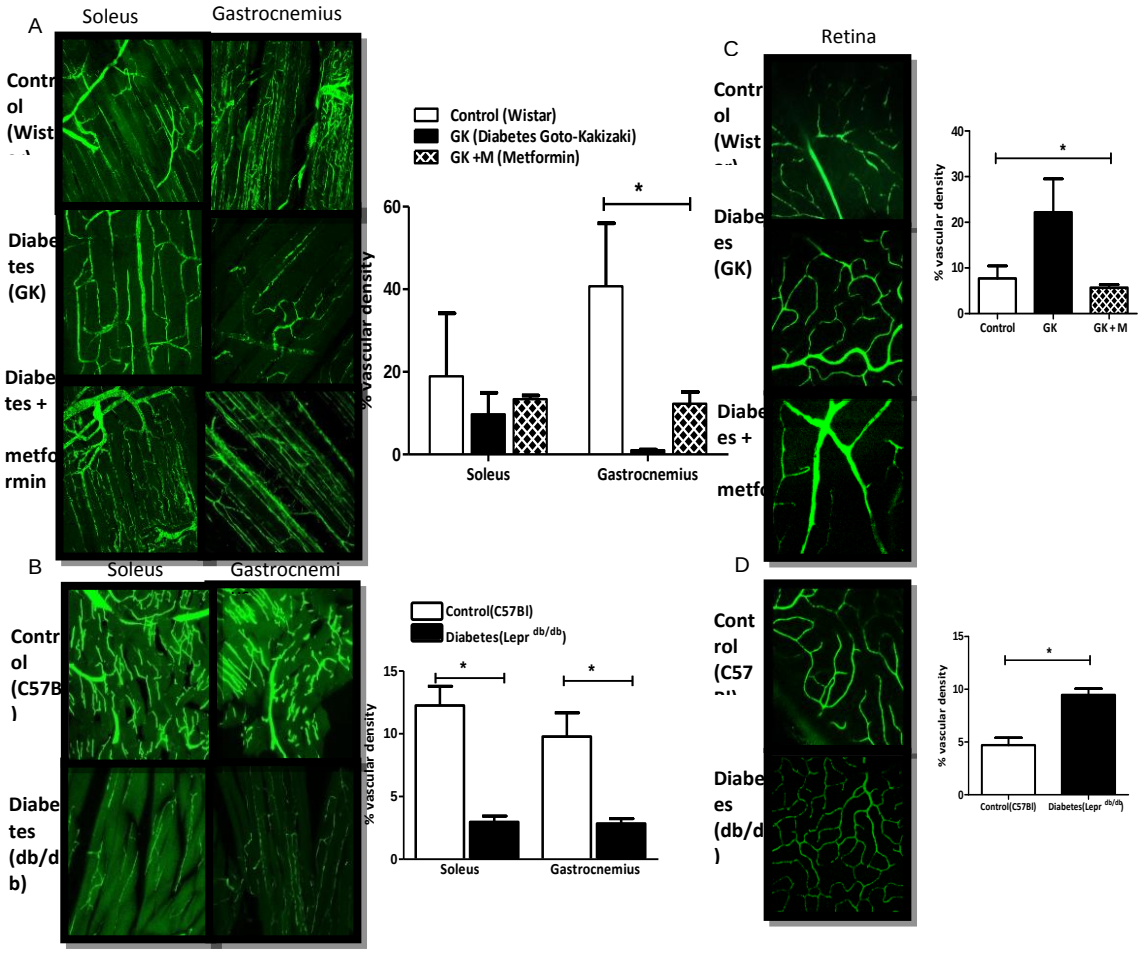
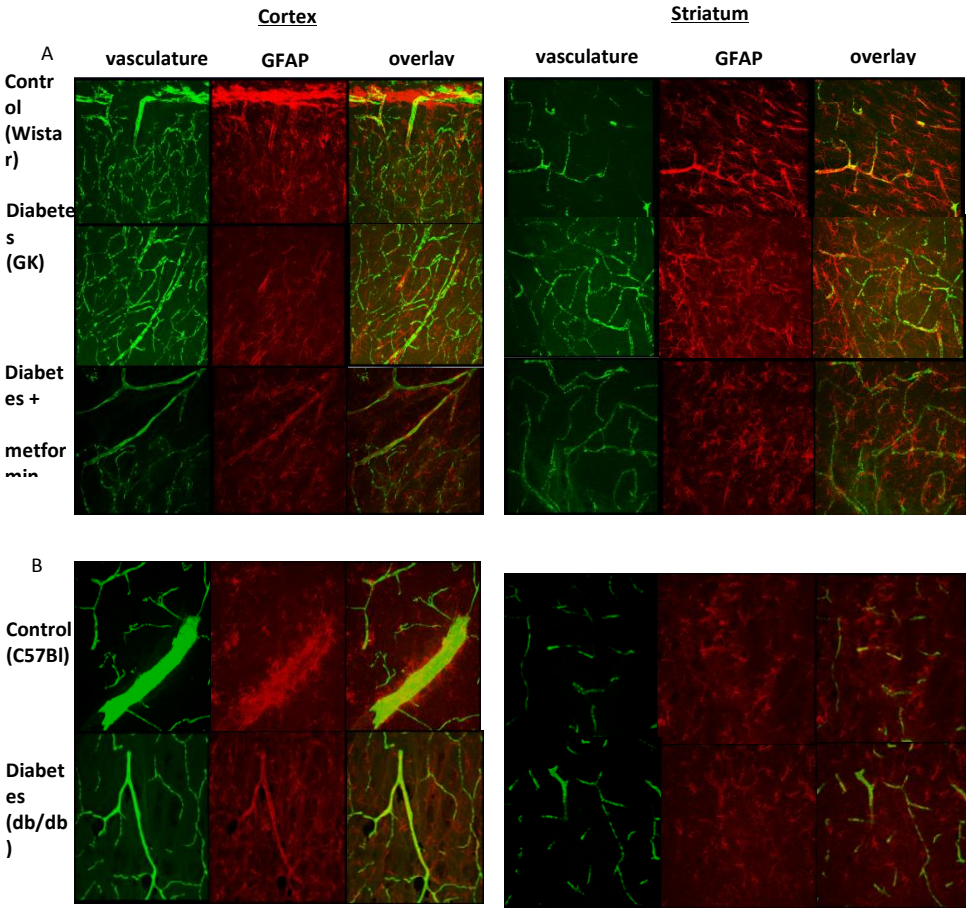
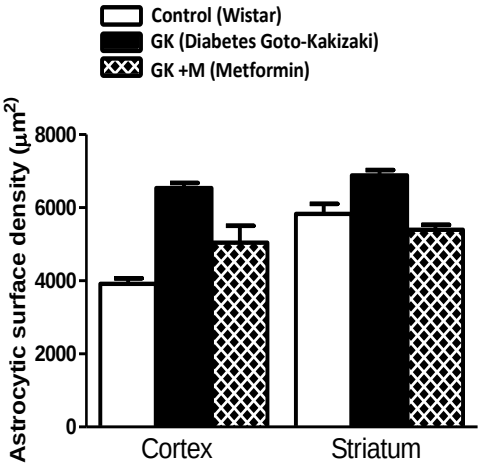
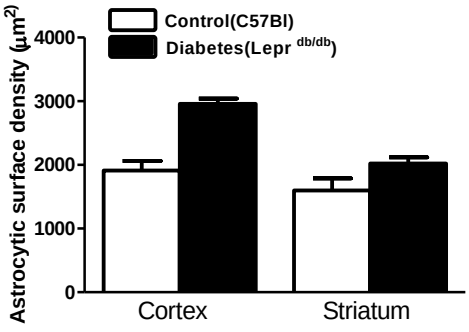

A
Soleus
Gastrocnemius
Control (Wistar)
GK (Diabetes Goto-Kakizaki)
GK +M (Metformin)
% vascular density
Soleus
Gastrocnemius
B
Soleus
Gastrocnemi
Control(C57Bl)
Diabetes(Lepr db/db)
% vascular density
Soleus
Gastrocnemius
C
Retina
% vascular density
Control
GK
GK + M
D
% vascular density
Control(C57Bl)
Diabetes(Lepr db/db)

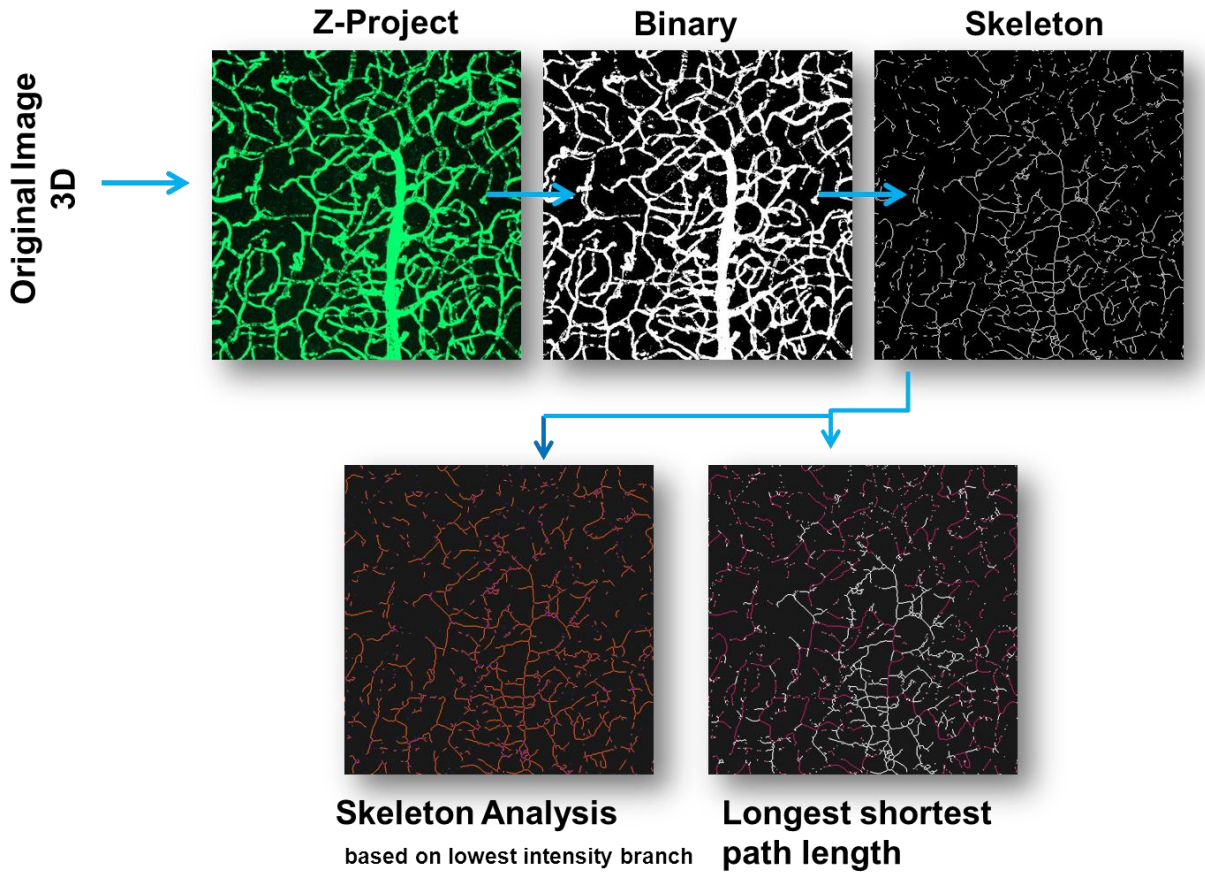
Figure 3-6. Astrocytic structural alterations are prominent in diabetes. (A, B) Representative images showing GFAP stained astrocytes (in red) wrapping around the vessels perfused with FITC (in green). Diabetic groups have decreased number of astrocytes with more perivascular projection and smaller soma. Diabetes show increased astrocytic surface density in both cortex and stratum compared to the control groups. * $p<0.05$,** $p<0.005$ diabetes vs control and treatment.Mean ± SEM, n=3-4. n.s –not signigicant.


Cortex
Striatum
A
vasculature
GFAP
overlay
vasculature
GFAP
overlay
Control (Wistar)
Diabetes (GK)
Diabetes + metformin
B
Control (C57Bl)
Diabetes (db/db)


Control (Wistar)
GK (Diabetes Goto-Kakizaki)
GK +M (Metformin)
Astrocytic surface density (µm2)
8000
6000
4000
2000
0
Cortex
Striatum


Control(C57Bl)
Diabetes(Lepr db/db)
Astrocytic surface density (µm2)
4000
3000
2000
1000
0
Cortex
Striatum

Supplementary Figure 3-1. Schematics showing tissue procession performed using Fiji software explained in the methodology.


Z-Project
Binary
Skeleton
Original Image
3D
Skeleton Analysis
based on lowest intensity branch
Longest shortest
path length

Supplementary Figure 3-2. Immature cerebral microvessels are more abundant in diabetes. (A, B) Representative images of cerebral striatal vasculature showing perfused (green-FITC dextran) and non-perfused vessels (Red- Isolectin). GK rats exhibit increased immature vasculature, however this was not observed in the $Lepr^{db/db}$ mice striatum. Glycemic control with metformin reduced the immature vasculature. ** $p<0.005$ vs control. Mean ± SEM, n=4-7.

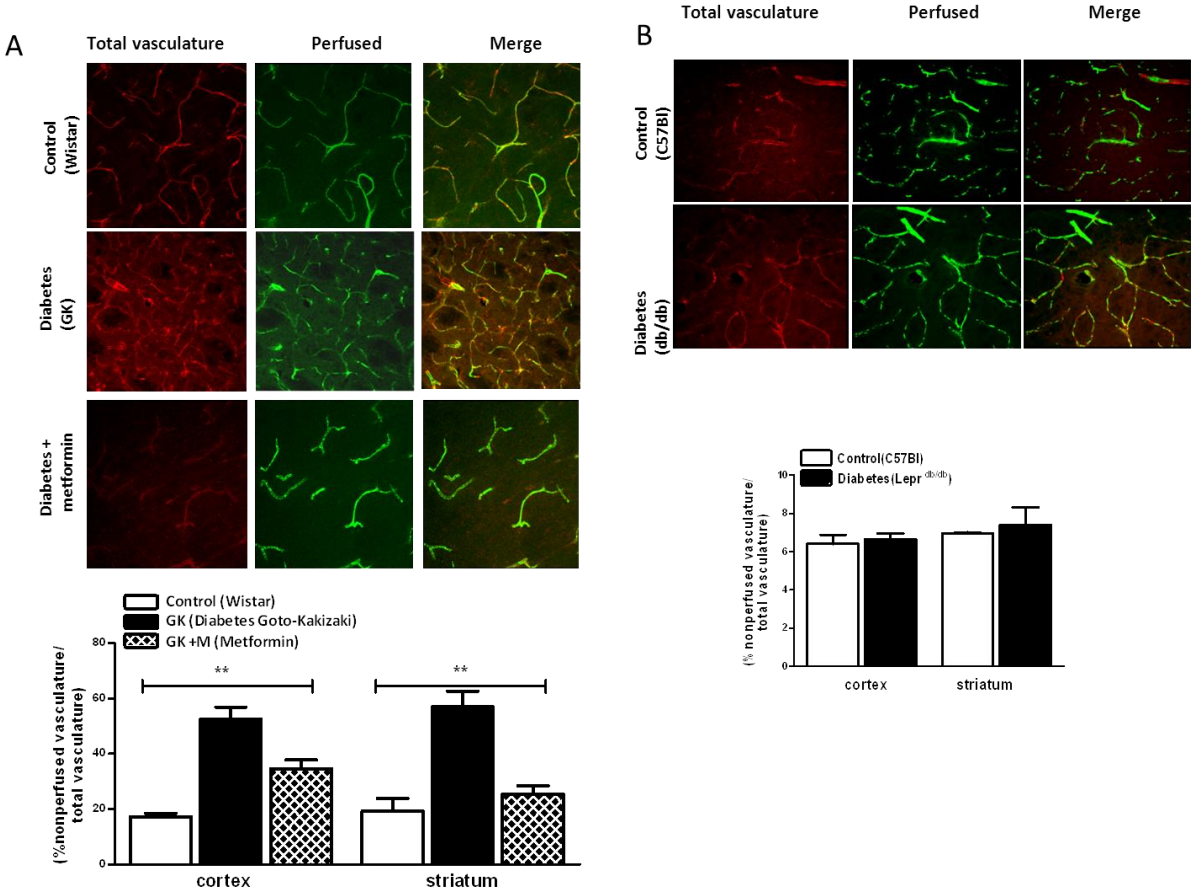

A
Total vasculature
Perfused
Merge
Control (Wistar)
Diabetes (GK)
Diabetes + metformin
Control (Wistar)
GK (Diabetes Goto-Kakizaki)
GK +M (Metformin)
(%nonperfused vasculature/ total vasculature)
**
**
cortex
striatum
B
Total vasculature
Perfused
Merge
Control (C57Bl)
Diabetes (db/db)
Control(C57Bl)
Diabetes (Lepr db/db)
(% nonperfused vasculature/ total vasculature)
cortex
striatum

Table: 1 Metabolic parameters of both the diabetic models.

Groups/Parameters	Body Weight (g)	Blood Glucose (mg/dL)
Control (Wistar)	301.3 ± 2.4	98 ± 3
Diabetes (GK)	285.3 ± 5.3	183 ± 8***
Diabetes +Metformin	291.2 ± 3.5	96 ± 3
Control (c57Bl)	29.10 ± 0.6	97 ± 3
Diabetes (db/db)	60.98 ± 1.2***	306 ± 31***

*** p<0.0001 vs. control

CHAPTER 4

VASCULARIZATION PATTERN AFTER ISCHEMIC STROKE IS DIFFERENT IN CONTROL VERSUS DIABETIC RATS: RELEVANCE TO STROKE RECOVERY

Roshini Prakash, Weiguo Li, Zhi Qu, Maribeth Johnson, Susan C Fagan and Adviye Ergul

To be submitted to PLOS biology

ABSTRACT

Background: Pre-existing diabetes worsens brain functionality in ischemic stroke. We have previously shown that type-2-diabetic rats subjected to cerebral ischemic reperfusion injury develop hemorrhagic transformation (HT) and greater neurological deficits. These diabetic rats also exhibit enhanced dysfunctional cerebral neovascularization that increases the risk of bleeding post-stroke. However, our knowledge of vascular and functional plasticity during the recovery phase of diabetic stroke is limited. This study tested the hypothesis that there is enhanced vascular regression in the post-stroke period in diabetes and this is associated with poor sensorimotor and cognitive function. We further hypothesized that glycemic control prevents impaired vascularization and improves functional outcome in diabetes. Methods: Vascularization was assessed in the lesional and non-lesional areas in control, diabetes and diabetes plus metformin groups 14 days after ischemic reperfusion injury as well as in respective sham controls. 3-dimensional reconstruction of the FITC stained vasculature were obtained by confocal microscopy and stereological parameters including vascular volume and surface area were measured. Astrogliosis was determined by GFAP staining. The relative rates of sensorimotor recovery, cognitive decline and spontaneous activity were assessed. Results: Vascular density in the peri-infarct area is significantly reduced in diabetes whereas there is reparative neovascularization in control rats. Astroglial swelling and reactivity was more pronounced in diabetic stroke compared to control stroke. Diabetes blunted the rate of sensorimotor recovery and also exacerbated anxiety-like symptoms and cognitive decline post-stroke relative to control. Glycemic control started after stroke partially prevented these changes. Conclusion: Diabetes impairs post-stroke reparative neovascularization and impedes the

recovery. Glycemic control after stroke can improve neurovascular repair and improve functional outcome.

INTRODUCTION

Stroke or cerebral infarction affects 15 million people globally, with one third of the affected population having permanent disability, impacting quality of life [1, 2]. Diabetes, hypertension, hypercholesterolemia and/or aging adds to the complexity of stroke outcomes. It is estimated that more than 25% of the stroke patients have diagnosed with diabetes and these patients suffer from a greater risk of hemorrhagic transformation, increased mortality and slower recovery [3-7]. Recent clinical studies suggest that diabetes also contributes to cognitive decline and dementia that can have a negative impact in the event of stroke and compromise functional outcomes [8-12] . Therefore, there is a great need for better understanding of stroke recovery in disease models.

Harmonized regulation of angiogenesis and neurogenesis is very important for brain repair and improvement of functional outcome after cerebral injury [13-17]. Indeed, stimulation of angiogenesis is being evaluated as a therapeutic modality in stroke [13, 14, 16]. We have shown that diabetes causes dysfunctional cerebral neovascularization that is characterized by increased microvascular density, augmented remodeling and permeability. These vessels also lack sufficient pericyte coverage rendering them more susceptible to reperfusion injury [18-21]. However, the impact of ischemia/reperfusion (I/R) on cerebrovascular repair in diabetes is unknown. A recent study reported that neuronal plasticity and functional recovery after stroke is blunted in type 1 diabetic rats [22]. Understanding the changes associated with the vasculature will provide a better insight into the processes responsible for modulation of reparative angiogenesis and neurogenesis and ultimately improvement of functional outcomes. Building on these studies, the current study tested the hypotheses that 1) there is enhanced vascular regression in the post-stroke period in diabetes, 2) motor and cognitive recovery is blunted after

diabetic stroke, and 3) glycemic control in the post-stroke period improves cerebrovascularization and this is associated with better functional outcome.

METHODS

Animal procedures and experimental design

All surgical and behavioral procedures on animals for this research were carried out in accordance with National Institute of Health guidelines for the care and use of animals in research and under protocols approved by the Georgia Health Sciences University. Wistar rats were purchased from Harlan, (Indianapolis, ID, USA) and the Goto-Kakizaki rats (GK) were purchased from Taconic (Hudson, NY, USA). We used five cohorts of rats: control and diabetic rats subjected to sham surgery, control and diabetic rats subjected to I/R, and diabetic rats subjected to I/R injury and treated with metformin (300 g/kg/day) for 14 days after stroke. Only male rats were used for all the experiments that were weight matched (250-300g) and were about 10-12 weeks old at the time of ischemic reperfusion injury. Blood glucose levels ranged from 86-103 mg/dL in control rats, 155-217 in the diabetic group and metformin treatment lowered the glucose levels to 81-115 mg/dL.

Method of ischemic stroke

I/R injury was achieved by occluding the middle cerebral artery (MCA) with a nylon suture under anesthesia maintained at 3% isoflurane mixed with nitrogen. The skin on the cervical region was incised and reached for the common carotid artery. The external carotid artery was separated, ligated, and severed. The nylon suture with rounded-tip was inserted into the internal carotid artery to reach until the origin of MCA. The nylon suture occluding the MCA was secured along with the external carotid artery at its base and the incision was closed. Ischemia was induced for a period of 90 minutes following which the animals were re-anesthetized, and

the occlusion suture was removed to allow reperfusion. Laser Doppler (PIM-3, Perimed, Stockholm, Sweden) was used to confirm reduction if in cerebral perfusion among groups induced by surgical ischemia. Animals were maintained warm throughout the procedure using a heating pad. Animals were returned to their home cages after MCAO with easy access to food and water. 2 ml of 0.9% saline was injected intraperitoneally for the first 2 days after stroke. One cohort of the diabetic rats was treated with metformin (300mg/kg body weight, provided in the drinking water) to achieve euglycemia. Treatment was started 2 days after stroke when blood glucose reached over >140 mg/dl. All the animals were tested for functional outcomes for a period of 14 days at regular intervals. Blood glucoses were monitored to maintain euglycemic status.

Measurement of post-stroke vascularization

Vascularization patterns were assessed in cortex and striatum around the infarct territories as well as in corresponding regions in the contralateral hemisphere using the space-filling model as reported earlier [19]. Regions of interests are depicted in Fig 4-1. Each observation from one animal was comprised of an average of 9 images from either the cortical or striatal region. Vascular images were obtained using 25X objective to assess 3 dimensional parameters. In addition, vascularization right at the edge of infarction was measured. For these studies, slides were co-stained with NeuN and GFAP (Chemicon, Temecula, CA) to identify the neurons and surrounding astrocytes, respectively, and regions where the measurements were made are indicated on Fig 4-2.

Vascular volume representing volume of the vasculature perfusing the brain tissue in the region of interest [19, 23] and s*urface area* representing the area available on the vasculature for the

exchange of vascular components in the surrounding tissue were measured using the Volocity Improvision software after thresholding the images and eliminating the background [23].

Assessing astrogliosis and swelling

The FITC stained section were co-stained with anti-GFAP antibody and imaged at 63X immediately around the infarcts and in the corresponding contralateral hemispheres of the same sections. Astrocytic swelling was evaluated by measuring the somatic volumes using Volocity software. Number of processes projecting from individual astrocytes was counted as the astrocytic reactivity using the Fiji software after skeletonizing the images.

Evaluation of neurological outcomes

All the rats were housed in 12h light dark cycles and all the behavior tests were performed during the day with ambient light of about 30 lux. Animals were handled for 5-7 days prior to behavior testing in rooms where behavior testing was to be carried out. Neurobehavioral analysis was done in a blinded fashion. Bederson's score, beam walk and grip strength tests which assess sensorimotor functions were performed before and after stroke for a period of 14 days.

Bederson's score for each rat was obtained by using 3 parameters which include (a) observation of spontaneous ipsilateral circling, graded as 2 (no circling), 1 (partial circling), 0 (continuous circling), (b) contralateral hindlimb retraction, (c) forelimb retraction which measures the ability of the animal to replace the limb after it is displaced laterally by 2 to 3 cm, graded 1 (immediate replacement) and 0 (replacement after minutes or no replacement). Maximum score of 5 was allotted to a normal rat. A lower score represents a poor neurological outcome.

Beam walking ability graded based on 7-point scale method. Score 7 for a rat that readily traverses a 2.4-cm-wide, 80-cm-long beam with no more than 2 foot slips, 6 for rat that crosses the beam with the help of the affected paw but slips more than twice, 5 for a rat that crosses the

beam with limited use of the affected limb, 4 for a rat that crosses the beam and puts the affected paw on the beam but not use it for movement, 3 for a rat that crosses the beam dragging the feet, 2 for a rat that puts the affected the limb on the horizontal surface and maintains balance for 5 sec, and 1 for a rat unable to place the affected hindpaw on the horizontal surface of the beam.

Grip strength was measured with a standard grip strength meter (Columbus Instrument). The rat is gently held with their forepaws grasping the mesh under tension, attached the grip strength meter and then pulled back consistently with its tail. The digital recording obtained from 3 trials is averaged and recorded as one observation. Composite neurological scores composed of sum of the Bederson's score and the scores obtained from the beam walking test.

For evaluation of cognition and memory-related tasks, spontaneous novel object recognition (NOR) test was performed using a grey plastic box of (63L x 38W x 42H cm) that was layered with animal bedding. Animals were habituated to the box one day prior to the day of testing with no objects in it. Objects with greater intricacy and details and similar in appearances with equal and unbiased preferences for one over the other were chosen to perform the test. On the day of testing, the rats were allowed to explore two identical objects during the A/A session for a period of 5 minutes. The rats were returned to their home cages for a delay/retention interval of 15 minutes following which the rats were confronted to A/B sessions in the consisting of 5 minutes, during which a novel object was paired with a familiar object used in the A/A session. All objects were cleaned after each session with 30% ethanol and the bedding was ruffled and cleaned to discard cues. The objects were placed equidistant from the walls of the box, in the center and spaced 20 cm apart and the rats were placed in between both the objects at the start of the experiment. The time spent in exploring each object during the A/B session was recorded and

recognition indices were calculated by the ratio of time spent in exploring the novel object over the total time spent in exploring both the objects.

Anxiety like symptoms were assessed using the elevated plus maze experiments. The apparatus consisted of 2 open arms and 2 closed arms elevated 20" from the ground. Each rat was placed in the central region at the junction of the open and closed arms and its behavior was recorded for about 5 minutes. The time spent in the open and closed arms of the maze arm was recorded. Time spent in the center was also measured as the freezing time. High anxiety states are directly related to the degree to which the rodent avoids the open arms of the maze.

Spontaneous arm alterations were also tested using a T-maze made of plexi-glass that consisted of 3 arms. This memory test is based on the fact that animals will alternate the arms if they remember which arm was entered last. The end of each arms and the surrounding were marked with different symbols that served as cues for the rats to identify the arena. The rats were placed in the start arm and were allowed to make a choice. The first turn was considered as a choice trial and the sliding door was let down after the first choice that allowed the rat to remain in the choice arm for about 30 seconds. After which the rats were taken out and the three sample trials/chances were given to alternate from the choice trial. Graded scores were allotted based on the number of trials taken by the rat to make a correct choice or the alternate turn from that of the choice trial. Correct choice in the first sample trial was scored as 3 and third trial was recorded as 1. No correct alternation as scored as 0.

Statistical Analysis

All data points are expressed in mean ± SEM. 2-way measure of ANOVA were performed comparing the vascularization between the sham, control and diabetes group after stroke. The

effect of disease on the ischemia and the total interactions were performed among various groups to test the level of significance. One-way ANOVA was used to test the level of significance of astrocytic swelling and processes. A one-way repeated measure of ANOVA was used to compare the behavioral deficits between groups and the effect of metformin treatment over a period of time. Tukey's and Bonferroni's test were used to adjust for multiple comparisons for significant group effects. Statistical significance was determined at alpha=0.05 using GraphPad prism.

RESULTS

Vascularization in the ischemic and contralateral hemispheres

Post-stroke vascular volume and surface area was dramatically enhanced in the ischemic and contralateral hemispheres both in the cortex and striatum in the control group compared to the respective shams (Fig 4-1). The greater increase in surface area indicates that significant portion of this increase comes from the enhancement of microvasculature. In diabetic animals, on the other hand, vascular volume was significantly decreased in the ischemic cortex and striatum. There was a decrease in the surface area only in the ischemic cortex. Metformin intervention initiated after stroke to achieve euglycemia prevented the decrease in vascular volume and surface area restoring it to control levels. Surprisingly, when vascularization was assessed directly at the border of the infarct zone (Fig 4-2C), there were FITC-perfused vessels feeding into the infarcted area and diabetic rats show decreased vascular density. There was evident astrocyte activation as shown by enhanced GFAP staining around the infarct. Metformin treatment improved vascularization in the diabetic group (Fig 4-2B).

Diabetes aggravates astrogliosis post-stroke

Astrocytic swelling was evaluated by measuring the somatic volumes. Diabetes shows increased swelling compared to the control groups. Diabetic group also show increased number of astrocytic processes both around the zone of infarction and in the contralateral hemisphere. Metformin treatment reduced both the astrocytic swelling and number of astrocytic processes around the region of infarction (Fig 4-3 A-C).

Glycemic intervention post stroke improves sensorimotor functions in diabetes

Neurological scores were similar at baseline and dampened much more in the diabetic group compared to control rats 24 hours post stroke. Control group gradually improved sensorimotor functions with maximum improvement observed after 10 days of ischemic reperfusion injury. The recovery in the diabetic group was significantly attenuated (Fig 4-4A). Metformin treatment rescued the sensorimotor deficits immediately after day 5 in the diabetic group and the composite neurological scores were similar to that of the control group. Parallel results were obtained with the forelimb grip strength post stroke (Fig 4-4B).

Diabetes induces anxiety-like behaviors and is further exacerbated after stroke

Longer time spent in the closed arm of elevated plus maze is indicative of anxiety like behavior. At baseline diabetic rats were seen associated with anxiety like behaviors as they spent longer time in the closed arm. After ischemic reperfusion injury, both groups showed a similar pattern (Fig 5B). This response improved in subsequent days in the control group and At Day 14, control animals spent significantly less time than diabetics. Percent time spent in the open arm was pretty stable in diabetes and in the control group by day 14, there was a significant improvement which was not seen in diabetes (Fig. 4-5A). Metformin treatment reduced anxiety like behavior only by day 14 (Figs 4-5A and B). Freezing time or the time in

the center of the maze was greater in diabetes but metformin treatment did not have an effect (Fig. 4-5C).

Spontaneous T-maze alterations tested on these groups show that both groups had a dramatic decrease at 24 hours post- stroke. Animals including metformin-treated group progressively improved but the recovery was less in the diabetic rats. Clearly metformin treatment proved beneficial in improving spontaneous t-maze alteration scores to better than baseline scores (Fig. 4-5D).

Comparison of the effects of glycemic intervention on cognition after stroke

Short-term memory/cognitive function was assessed by novel object recognition task. At baseline diabetes causes a reduction in the novel object recognition index indicating the negative impact of even short-term diabetes on cognition. All groups experienced a similar degree of deficits in the first few days following stroke but rats recovered moderately from cognitive dysfunction that is seen after stroke. Recovery in the diabetic stroked rats was impaired and metformin partially restored the cognitive function (Fig 4-6B).

DISCUSSION

Diabetes increases the risk and severity of stroke ultimately resulting in poor outcomes. Clinical evidence suggests that no history of diabetes is a predictor of recovery implicating that diabetes hampers recovery after stroke [8, 24, 25]. However, preclinical evidence as to how diabetes influences stroke recovery is scarce. This study addressed this important gap in our knowledge and investigated the impact of diabetes a) on cerebrovascular remodeling and vascularization patterns, and b) motor and cognitive recovery following stroke. Studies conducted on human and animal models reveal a critical place for therapeutic angiogenesis

and recovery after stroke [26, 27]. Angiogenesis around the infarct boundary in the ipsilateral ischemic hemisphere had been well characterized in animal models [16, 26]. Increased angiogenesis has been observed around the infarction in stroke patients. Morbidity, survival rates and neurological recovery are directly co-related to the degree of angiogenesis, microvascular density and restoration of blood flow after stroke [14, 28, 29]. Only a handful of studies have been so far conducted to assess the brain repair in diabetic stroke and these reports suggest impaired vascular restoration after diabetic stroke. Type-2 diabetic mice subjected to ischemia showed decreased number of microvessels after stroke in the ischemic hemisphere [30, 31]. Another study conducted on diabetic GK rats, similar to these studies also show reduced rate of angiogenesis 7-days after stroke [32]. Most, if not all, of these studies employed conventional 2-dimensional strategies to assess microvascular density in the ischemic hemisphere and nonischemic hemisphere was used as control tissue. We now provide a comprehensive report of 3-dimensional changes occurring in the brain vasculature in the infarcted zone as well as in peri-lesional cortical and subcortical regions in both ischemic and nonischemic hemispheres 14-days after stroke as compared to sham-operated animals. This approach not only allowed us to compare the vascularization in control and diabetic animals but also assess the impact of stroke on cerebrovasculature architecture in the nonischemic hemisphere. As recently reviewed [17, 33], most believe that angiogenesis only occurs in the ipsilateral hemisphere. Our novel findings show that there is an increase in vascular density and remodeling in both ipsilateral and contralateral hemispheres in control animals. On the other hand, diabetic rats which have increased yet dysfunctional angiogenesis at baseline exhibit a dramatic decrease in vascular volume and surface area after stroke in both hemispheres. These results strongly suggest that nonischemic hemisphere is also affected

from ischemic injury and responds to the damage and repairs under normal conditions but presence of a confounding disease such as diabetes prevents this reparative response.

It is known that angiogenesis peaks one week after stroke followed by pruning and maturation of growing blood vessels [26, 27]. Similar to these previous studies, stroke induced in normal rats stimulated an angiogenic response in the current study. While we did not directly study angiogenic markers, greater increase in surface areas is indicative of increased microvasculature. In a given volume of tissue, an increase in vascular volume which measures mainly the vascular lumen space can be due to either remodeling of the vessel and getting larger lumen and/or due to new vessel formation. In the latter, an increase in surface area, a measure of the area vessel wall occupies, accompanies increased volume. As such when one looks at the relationship between volume and surface area, a linear association suggests that the increase in these two parameters go hand-in-hand and there is significant new microvessel formation. In this study, we detected increased vascular volume and even a greater increase in surface area. However, 14-days after stroke the diabetic GK rats had severely impaired vasculature around the infarct borders. Decreased vascularization was also observed in the nonischemic hemisphere. Greater reduction in vascular volume suggests that there is significant vascular regression of existing vessels. Potential mechanisms underlying this response merit further investigation.

We have earlier provided evidence of glycemic control being an effective preventive strategy that confers vascular protection and reduces the risk of HT after ischemic reperfusion injury [34]. Although clinical trials focusing on the impact of glycemic control on cardiovascular outcomes resulted in debatable information, tight glycemic control has been an effective treatment for prevention of microvascular complications such as diabetic nephropathy and

retinopathy [35-41]. We used metformin as an interventional strategy to target hyperglycemic status in diabetic rats. Clearly this data suggest metformin effectively prevents vascular impairment and promotes remodeling.

Glial cells, especially astrocytes, respond to vascular damage by cellular swelling and increased reactivity. Astrogliosis has been reported around the ischemic core and is increased progressively closer to the infarct border associated with glial scarring [42]. This study provides evidence that stroke in association with diabetes heightens astrogliosis by increasing the swelling and number of astrocytic process densities in the peri-lesional zones. Marked increase in astrogliosis in the brain may establish neurovascular coupling to restore blood flow to the damage regions, causes inflammatory and trophic response [43, 44].

Clinical and preclinical studies report memory and cognition deficits associated with metabolic alterations and after ischemic reperfusion. Acutely after stroke, motor coordination is weakened that can propagate to permanent paralysis of the extremities. Studies report increased synaptic plasticity and neuronal reorganization with increased motor learning and activities [45]. Animal behavior assessed after stroke also provides evidence of synaptic plasticity and progressive improvement in functional behavior associated with learning [46]. A recent study showed that cortical plasticity in diabetic stroke is challenged compared to the stroke injury on animals without diabetes. Functional restoration was also compromised long-term after diabetic stroke [22]. The current study evaluated temporal changes in sensorimotor functions, anxiety like symptoms, spontaneous activity and cognitive function after stroke with or without diabetes. Sensorimotor deficits occurred due to ischemic reperfusion injury improved and saturated after 10 days of stroke in the control rats. The extent of recovery was

diminished in diabetic rats. Glycemic intervention with metformin improved motor functions at day 5 (Fig 4-4 A, B) that were comparable to the controls.

Emerging evidences suggest that both type-1 and type-2 diabetic patients have poor neuropsychological functions affecting cognition [10, 12, 47-49]. In our current study we elaborated on the temporal profile of cognitive functions after stroke in control and diabetic rats. Our results with novel object recognition and spontaneous alteration at T-maze experiments show that even at baseline memory and cognition-related tasks are impaired in diabetes. When stroke is overlayed on this pathology, recovery of cognitive function is severely affected. Glycemic control in the post-stroke period improved this deficit. Our past studies showed that chronic glycemic control initiated after the onset of diabetes prevent pathological remodeling and dysfunctional vascularization. Collectively, these results provide compelling evidence that glycemic control is an effective strategy in prevention and or improvement of cerebrovascular complications in diabetes.

In conclusion, these studies show that brain responds to repair ischemic injury by increasing vascularization even in remote sites but in the presence of diabetes this response is completely reversed and there is vascular regression. These changes are associated with delayed functional recovery after stroke. Our results lend a strong support for glycemic control in the pre and post-stroke period to improve vascular and neurological recovery.

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Figure 4-1. Diabetes impairs post-stroke neovascularization in the peri- and non-lesional hemispheres. (A) Representative images contrasting peri-lesional and non-lesional zones across the groups. (B) Representative brain section depicting the region of interest in the peri- and non-lesional hemisphere shown in red and yellow squares. (C, D) Vascular volume and surface areas are significantly increased in the control group while diabetes impairs reparative vascularization after stroke. a= $p<0.05$, b=$p<0.01$, c=$p<0.005$, d= $p<0.001$, e= $p<0.0005$. Mean ± SEM, n=6-9

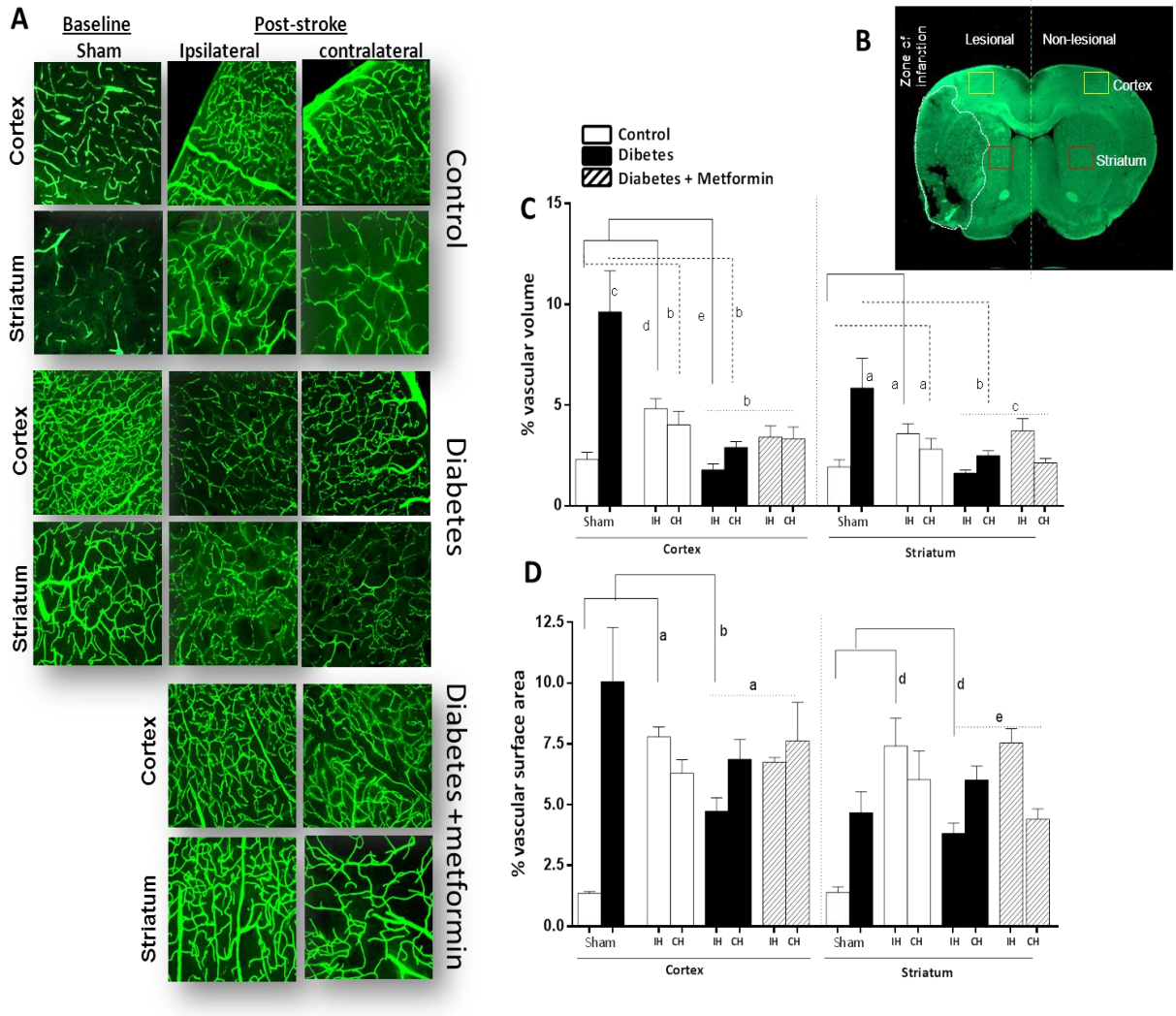

A
Baseline
Sham
Post-stroke
Ipsilateral
contralateral
Cortex
Striatum
Control
Diabetes
Diabetes +metformin
B
Zone of Infarction
Lesional
Non-lesional
Cortex
Striatum
Control
Dibetes
Diabetes + Metformin
C
% vascular volume
Sham
IH
CH
Cortex
Striatum
D
% vascular surface area

Figure 4-2. Diabetes impairs post stroke cerebral neovascularization at the infarct border zone. (A) Representative image depicting the localization of the infarct border zone which is used for vascular density measurements. (B) Representative images comparing vascular density at infarct border zone taken under 10 x objective. (C) Graphical representation of the % vascular density around the area of infarction in all the groups. c= $p<0.005$ Mean ± SEM, n=4-6

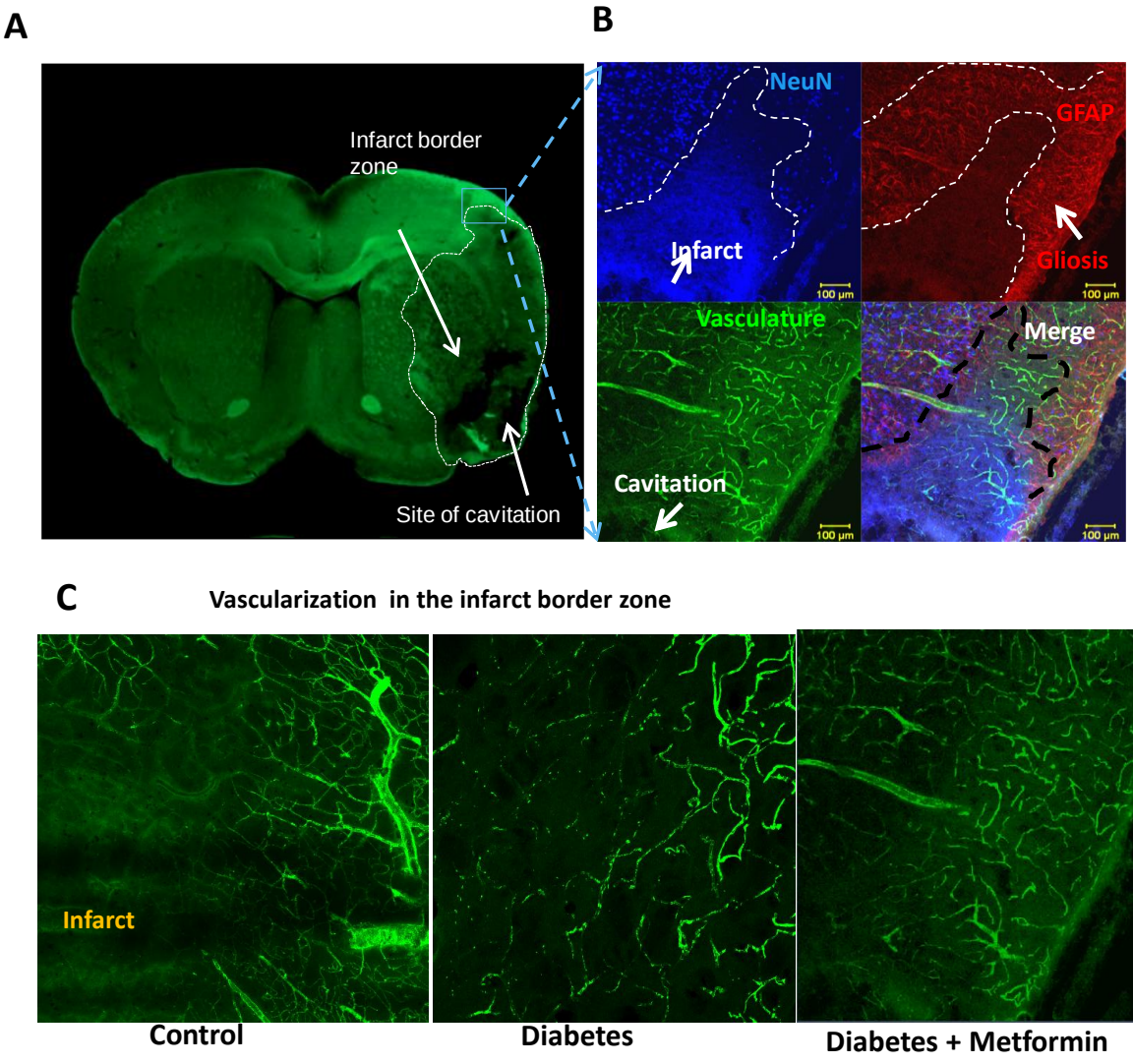

A
B
Infarct border zone
Site of cavitation
NeuN
Infarct
GFAP
Gliosis
Vasculature
Cavitation
Merge
100 µm
C
Vascularization in the infarct border zone
Infarct
Control
Diabetes
Diabetes + Metformin
% vascular density (infarct border zone)
40
30
20
10
0
c
Control
Diabetes
Diabetes + Metformin

Figure 4-3. Diabetes exacerbates astrogliosis post-stroke. (A) Representative images showing astrocytic morphology in the infarct border zone and non-lesional hemisphere as well as in the corresponding regions in sham animals (B) Diabetic stroke dramatically increases astrocytic swelling compared to control strokes and metformin treatment decreased swelling. (C) Number of astrocytic projections is significantly increased even after 14 days in diabetic stroke compared to control and glycemic intervention conserved astrocytic processes. f=$p<0.0001$, d=$p<0.001$vs. control Mean ± SEM, n=3-4

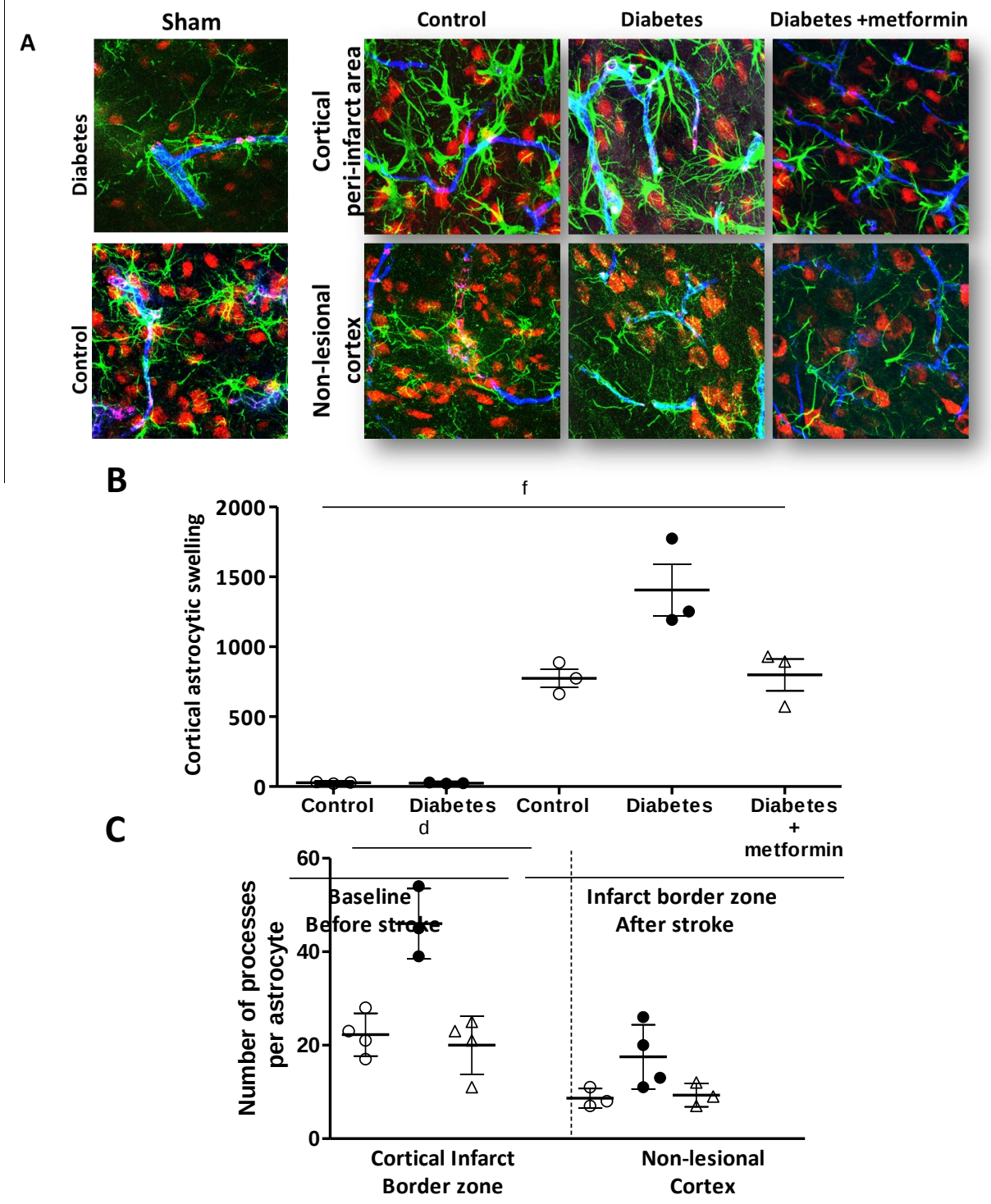

A
Sham
Control
Diabetes
Diabetes +metformin
Diabetes
Control
Cortical peri-infarct area
Non-lesional cortex
B
f
Cortical astrocytic swelling
2000
1500
1000
500
0
Control
Diabetes
Control
Diabetes
Diabetes + metformin
C
d
Number of processes per astrocyte
60
40
20
0
Baseline
Before stroke
Infarct border zone
After stroke
Cortical Infarct Border zone
Non-lesional Cortex

Figure 4-4. Glycemic intervention post stroke improves sensorimotor functions in diabetes. (A, B) Temporal profile of sensorimotor functions represented as composite neurological score. Diabetes significantly reduced the neurological scores after stroke. Improvement in the neurological scores was slower in the diabetic stroke group compared to control stroke. Glycemic intervention with metformin improved and restored the composite neurological scores comparable to the control group. (B) Temporal profile of forelimb grip strength shows a similar trend. The drop in the forelimb grip strength is not restored 14-days after stroke in the diabetic group compared to control. Glycemic intervention with metformin improvised the forelimb grip strength. f=p<0.0001vs. control Mean ± SEM, n=6-8

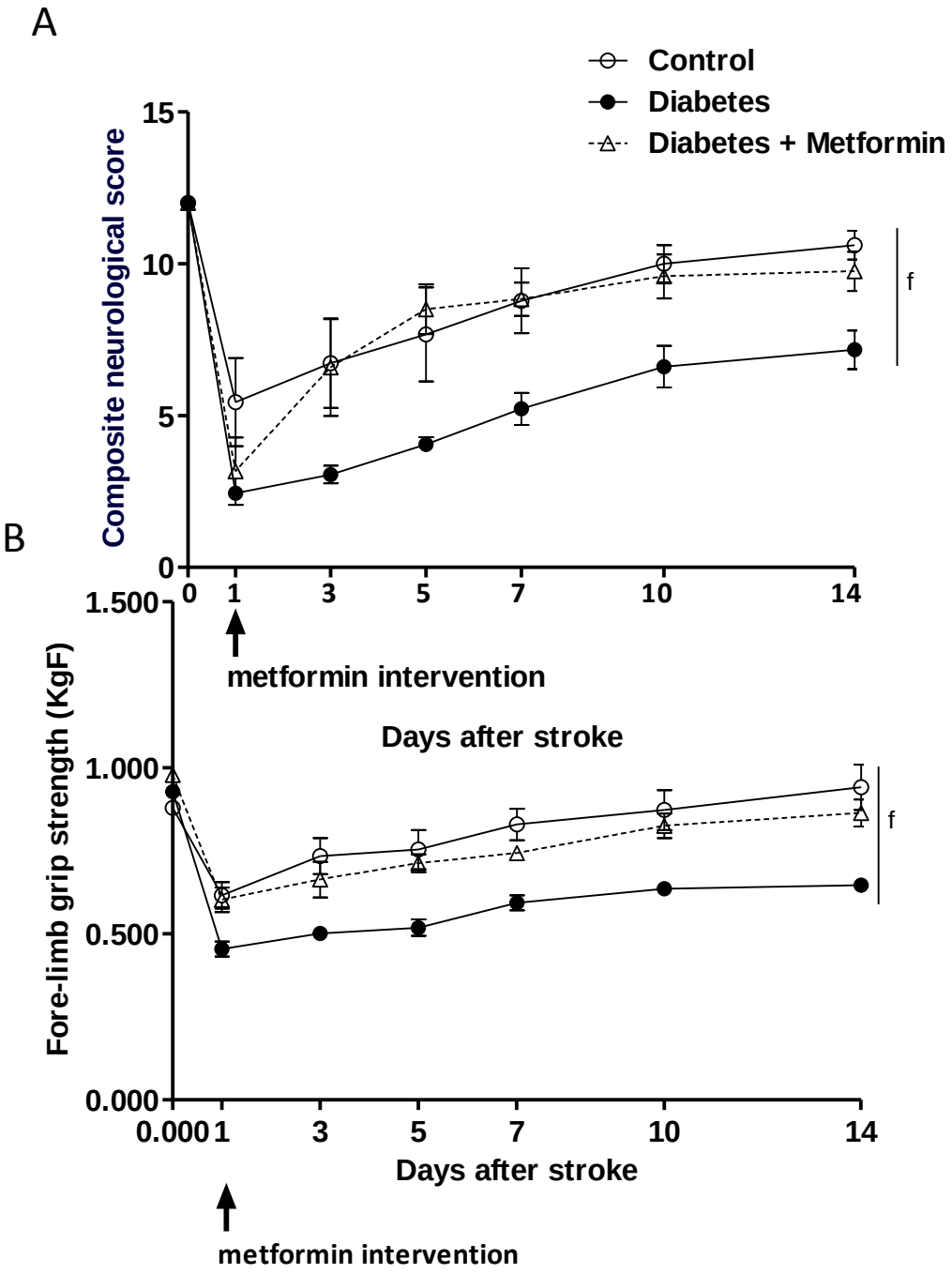

A
Control
Diabetes
Diabetes + Metformin
Composite neurological score
15
10
5
0
0
1
3
5
7
10
14
f
metformin intervention
Days after stroke
B
Fore-limb grip strength (KgF)
1.500
1.000
0.500
0.000
0.000
1
3
5
7
10
14
f
Days after stroke
metformin intervention

Figure 4-5. Spontaneous T-maze activity and anxiety like symptoms are aggravated in diabetic stroke. (A, B) Graphical representation of the temporal changes in the percentage of time spent in the open and closed arm of the elevated plus maze. Diabetic groups tend to spend more time in the closed arm compared to the control groups and ischemic reperfusion injury worsened this outcome. (C) Freezing time was dramatically increased after stroke in the diabetic group, that already showed augmented time spent in the center of the maze before stroke. (D) Graphical plots of graded scores given to the rats based on the delay in correct choice made on the spontaneous T-maze task. Diabetic group had decreased scored depicting poor outcome in the T-maze test. Stroke further worsened this activity in the diabetic group more so compared to the control. Glycemic intervention with metformin restored this response comparable to the control group. a= $p<0.05$, b=$p<0.01$, c=$p<0.005$, e= $p<0.0005$*=$p=0.00503$ vs. control Mean ± SEM, n=6-8

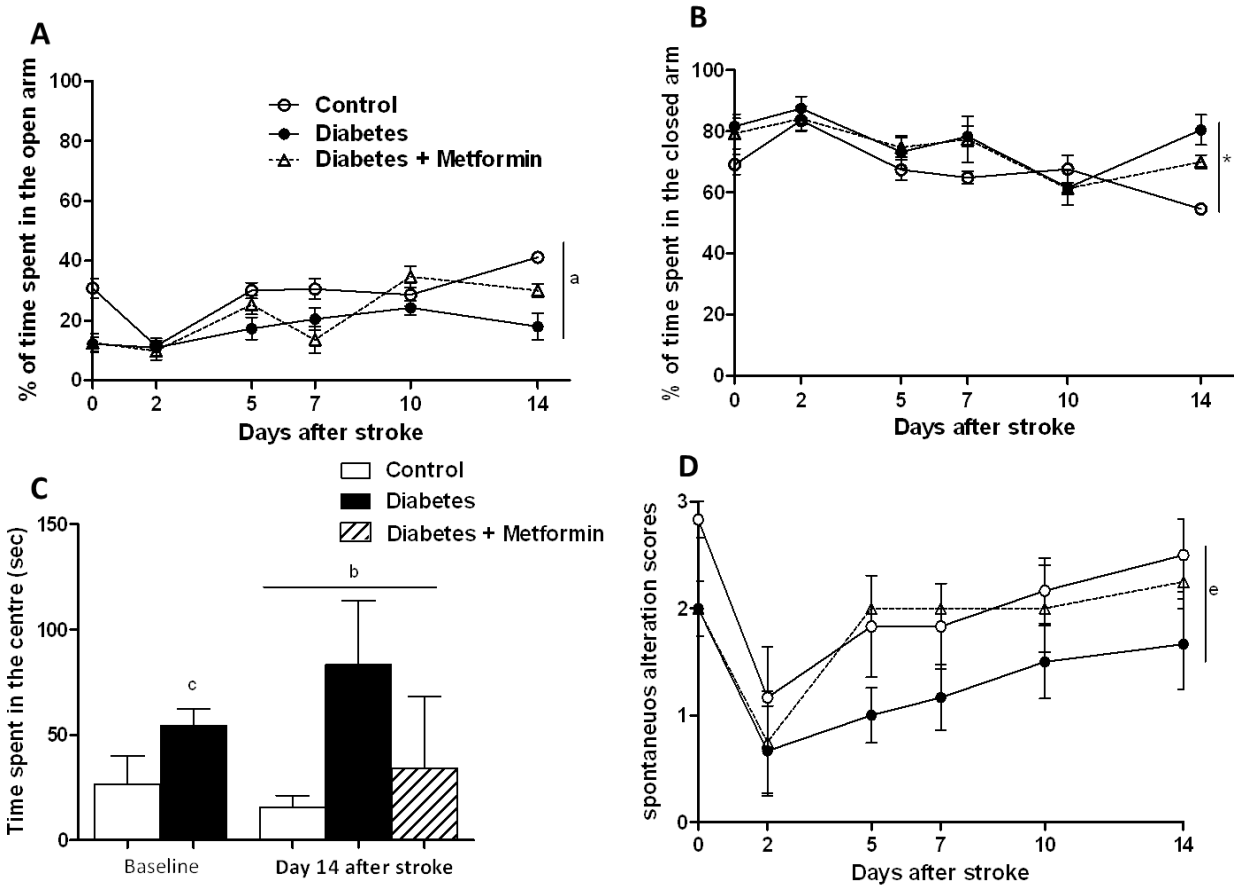
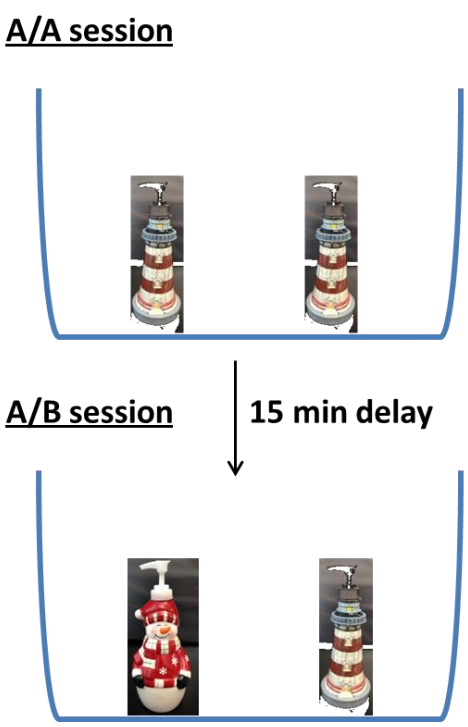
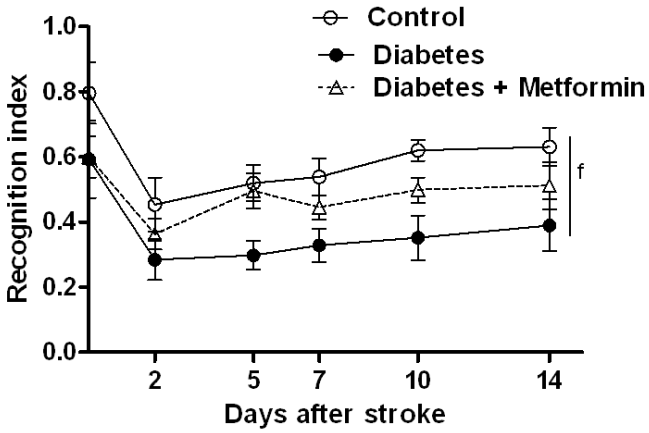

A
% of time spent in the open arm
Control
Diabetes
Diabetes + Metformin
a
Days after stroke
B
% of time spent in the closed arm
*
Days after stroke
C
Control
Diabetes
Diabetes + Metformin
Time spent in the centre (sec)
b
c
Baseline
Day 14 after stroke
D
spontaneuos alteration scores
e
Days after stroke

Figure 4-6. Diabetes dampens cognitive index. (A) Schematic description of the Novel Object Recognition task performed on the rats. (B) Plot of the recognition index of all the groups at baseline and after stroke at various time points show that diabetes dampens the recognition index and stroke injury further worsens this index. 14 days after stroke control rats show improvement in the recognition index while there is no significant change in the diabetic stroke. Metformin intervention restored the recognition index in the diabetic stroke group. f=$p<0.0001$ vs. control Mean ± SEM, n=6-8


A/A session
A/B session
15 min delay


Control
Diabetes
Diabetes + Metformin
Recognition index
1.0
0.8
0.6
0.4
0.2
0.0
2
5
7
10
14
Days after stroke
f

CHAPTER 5

INTEGRATED DISCUSSION

The aim of the current dissertation was to determine the effect of diabetes on cerebrovascular architecture and its subsequent implications in the event of stroke in GK rats. Previous studies conducted in our laboratory provided evidence that even short duration of diabetes causes cerebrovascular remodeling and dysfunction. With more number of stroke patients being diagnosed with diabetes, our group began investigating the consequences of diabetes on stroke. The backbone of this entire project surmounts on the data published in 2007 from our group which showed that diabetic GK rats subjected to 3 hours of ischemia followed by reperfusion developed smaller subcortical infarcts that were almost always associated with HT [1]. Despite smaller infarcts the neurological deficits manifested by diabetic stroke were severe. From these studies, it was apparent that HT due to reperfusion injury in diabetes was a major contributor of functional deficits seen within a day of stroke. We hypothesized that diabetes mediates cerebrovascular complications that predisposes these vessels to greater injury during stroke. Several studies from our group as well as others suggest vascular alterations in small vessel segments from the brain in terms of hypertrophic remodeling and compromised myogenic tone during diabetes [2, 3]. There were several articles reporting the global vascular organization in different animal models and human, brain. However there were no reports of vascular patterning, organization and derangements occurring in the diabetic brain.

To address this, we infused a polyurethane resin into aorta in a retrograde manner and created cerebrovascular casts of the pial vessels that showed increased tortuosity, collateralization and

anastomoses in diabetic GK rats (Appendix –Article 1). Glycemic control used as a preventive approach with metformin counteracted this adaptive arteriogenesis. We also reported the involvement of MMPs concordant with pial cerebral remodeling in diabetes. Since MMPs are involved in blood brain barrier disruption and increased vascular permeability, we reported elevated MMP-2 and 9 being major contributors of increased arteriogenesis in diabetes and bleeding during stroke (Appendix- Article 2).

Given the knowledge of diabetes remodeling the pial vasculature and complicating stroke outcomes, we hypothesized that neovascularization seen in the pial vessels promulgates deeper into the cerebral vasculature. Most studies so far employed conventional 2-dimensional methodology to assess vascular patterning and density, however, to get more meaningful data that will increase the sensitivity of the findings, provide vascular lumen volume and exchange surface area between the vessels and brain tissue; we employed 3-dimensional approach to assess vascular stereology. Fluorescein space-filling model demonstrated that the organization of the cerebrovasculature is deranged and is spatially regulated as one progresses from the rostral to the caudal regions of the brain (Fig 2-1). Cortical neovascularization differed from the striatum and also between the control and diabetic groups. This data provided information only about the perfused functional vessels, while we lacked in our knowledge of growing or immature vasculature. Further investigation reported that diabetic rats also displayed a large number of non-perfused vasculature and lesser degree of pericyte coverage around the microvessels (Fig 2-2). Thus, diabetes resulted in increased neovascularization that was highly immature in nature. This can be probably due to a greater angiogenic potential of the cerebral vessels that increases endothelial migration to form newer connections or due to inability of the vasculature to mature. We investigated a conceptual mechanism that involved the sequential activation of

neovascularization mediators such as VEGF, c-src, peroxynitrite and MMPs in the brain microvascular endothelial cells. These expression and activity of these mediators were significantly enhanced in the microvascular endothelial cells (Fig 2-6). BMECs also had greater angiogenic potential in the diabetic rats (Fig 2-4, 2-5). Extensive studies conducted on the retinal vasculature already provided clues of these molecules involved in retinal hypervascularization and retinal hemorrhages and aneurysms. We revisited these molecules in the diabetic brain and demonstrated a clear interplay between VEGF and peroxynitrite (Fig 2-8). One salient mechanism stood out that suggested that only with the availability of peroxynitrite, VEGF binding to it receptor lead to the activation of the downstream effector molecule c-src. Thus, diabetes mediated oxidative stress participates with enhanced VEGF through chronic hyperglycemia resulting in enhanced cerebral neovascularization.

Effect of diabetes on neovascularization is most extensively studied in the retinal circulation [4-8]. It is well established that hyperglycemia-mediated oxidative damage to microvascular endothelial cells triggers a cascade of events that cause excessive angiogenesis and result in vascular proliferative retinopathy [9-12]. On the other hand, neovascularization in the coronary and peripheral circulation is impaired in diabetes resulting in increased coronary artery disease and peripheral vascular disease risk, respectively [13, 14]. We also provided a broader perspective of various vascular pools such as the peripheral circulation with that of the retina and the brain. Unlike the retina and the brain peripheral vasculature was regressed in both the models of diabetes (Fig 2-2, 3-5). This suggests that the vessels closer to the central nervous system have a differential response compared to the periphery. More studies need to be conducted to understand the differences in the endothelial nature and the mechanisms.

We see the prevalence of enhanced cerebral neovascularization in the GK model of diabetes. It is highly questionable if this effects were truly mediated by diabetes or if it is a strain dependent phenomenon. We addressed this critical question several ways:

a) We looked into the pial vasculature at 5 weeks of age that is at the onset of diabetes in this model and we do not observe pial collateralization.

b) Glycemic control with metformin prevented cerebral neovascularization (Appendix- Article2, Fig 3-1) which suggested that chronic hyperglycemia was a mediator of this response.

c) We investigated cerebral neovascularization in a mice model of type-2-diabetes. The db/db mice are leptin receptor deficient and develops obesity associated with diabetes. Db/db mice displayed similar results of increased cerebral neovascularization indices as seen in the GK model of diabetes (Fig 3-1). Both models displayed increased cortical tortuosity and parenchymal arteriole branching (Fig 3-2, 3-3). The results obtained from comparing these two models of diabetes suggested that this phenomenon may be common to diabetic models during the early phase of diabetes. When we compared the relative contribution of micro and macrovasculature to this effect, db/db mice showed increased microvasculature alone compared to GK model that displayed both increased micro and macrovessels. This can be due to an added confounding factor of obesity as seen in the db/db mice unlike the GK rats that are lean and can also be due to the extreme plasma glycemic status of the db/db mice. This is highly correlated with the parenchymal arterioles beginning to reduce the lumen diameter in the db/db mice while the GKs show increased parenchymal arteriole diameters. We also did not observe an increased level of non-perfused vasculature as in the GK model of rats (supplementary figure 3-2). Another question arises as to why there should be highly dense network of capillaries in early diabetes? Blood flow is an important parameter that can be deregulated by any changes in the flow

properties, glucose chemistry and pressure. It can be reasoned that a high sheer stress and increased viscosity of blood caused by increased glucose levels in diabetic conditions may kick in growth factors that might remodel the vessels [15-18]. Increased cerebral neovascularization can also be due to this incessant increase in VEGF or to compensate for the decreased cerebral blood flow seen in diabetes.

The results obtained from our last set of studies show that diabetes adversely impairs reparative cerebral neovascularization compared to the control group (Fig 4-2). Both the lesional and the non-lesional hemisphere have decreased reparative neovascularization compared to the respective diabetic shams. In contrast to the controls, in diabetes we see slightly increased neovascularization in the non-lesional hemisphere compared to the lesional hemisphere (Fig 4-1). Can the non-lesional hemisphere in diabetes compensate this impairment? However, this needs to be further confirmed. Passive rates of recovery from functional deficits inflicted by the reperfusion injury on diabetes are observed (Fig 4-4, 4-5, 4-6). A wide range of functional deficits in terms of sensorimotor functions, spontaneous alteration activity, anxiety like symptoms and cognitive deficits were assessed. Cognitive regain was severely affected long-term after diabetic stroke. There were increased anxiety like symptoms and induction of higher levels of fear as determined by the time spent in the closed arm of the maze.

We noticed beneficial roles of metformin in preventing the initiation or the progression of diabetes mediated cerebrovascular complications when administered at the onset of diabetes. Metformin was also instrumental in repairing post-stroke vasculature and improving functional outcomes when administered long-term after stroke. Thus metformin not only conferred vascular protection but also neurological improvement which may be through vascular correction or direct protective effects on neurons.

Taken together, it can be said that dense vascular networks and inter connections in the vasculature may confer protection that can allow the blood to shunt locally and act as bypass arteries. Notably these vessels also contribute to hemorrhages following ischemia. Despite their perfused and functional nature, these collaterals are immature and nonperfused. This clearly elucidates that the extent of vasculopathy can dictate the degree of neurodegeneration.

The novel findings in this study that can advance the field of both diabetes and stoke are that: a) Diabetes increases cerebral but not peripheral neovascularization; b) neovascularization patterns differ between micro and macrovasculature and in different brain regions where infarct and HT are observed; 3) molecular signaling mechanisms that regulate emergence of capillaries in diabetic brain involve VEGF and peroxynitrite signaling; and 4) I/R impairs the ability of the brain cerebrovasculature to promote repair and this is associated with poor recovery. Therefore, strategies to prevent dysfunctional neovascularization in diabetes are likely to reduce neurovascular injury, improve outcome and promote recovery after stroke.

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CHAPTER 6

SUMMARY

In summary, type-2-diabetes leads to cerebral neovascularization by adaptive arteriogenesis and angiogenesis. Diabetes also spatially regulates this cerebral neovascularization in different brain regions. Diabetes not only mediates structural alterations, vascular branching and tortuosity but also perturbs vascular maturity in the brain through chronic hyperglycemia. Neovascularization closer to the central nervous system are regulated differentially compared to the peripheral vasculature which is regressed in diabetic conditions. VEGF and peroxynitrite interplay is critical to mediate early angiogenic events in diabetic brain microvascular endothelial cells. As a consequence of this mechanistic co-ordination, sequential augmented activation of MMPs is seen in diabetes.

Long-term after ischemic reperfusion injury diabetes impairs reparative neovascularization. Impairment in neovascularization co-existed with greater neurological deficits caused due to stroke in diabetes. Diabetes associated with stroke undermined the rates of functional recovery such as sensorimotor deficits, cognition, anxiety like symptoms and alteration activity. Glial cells that were affected by diabetes had severely magnified swelling and astrocytic processes densities after stroke compared to stroke without diabetes.

Antidiabetic drug metformin, used to normalize plasma glycemic stature had prominent beneficial effects to protect diabetes induced vascular remodeling. As an interventional therapy, metformin, not only ameliorated vascular impairment in diabetic stroke but also improvised the rates of neurobehavioral recovery.

There are several limitation to the current study that are currently being addressed in the lab-a) mediators that are dysregulated after diabetic stroke, b) the signaling pathways that lead to vascular degeneration or apoptosis and c) adjunct factors released from the vascular cells to confer neuroprotection and improve functional outcomes.

APPENDIX

Includes 2 published manuscripts

1. **Adaptive cerebral neovascularization in a model of type 2 diabetes: relevance to focal cerebral ischemia.**

   Weiguo Li, Roshini Prakash, Aisha I. Kelly-Cobbs, Safia Ogbi, Anna Kozak, Azza B El-Remessy, Derek A. Schreihofer, Susan C. Fagan, Adviye Ergul.

   Diabetes. 2010 Jan;59(1):228-35.

2. **Vascular protection in diabetic stroke: role of matrix metalloprotease-dependent vascular remodeling**

   Mostafa M Elgebaly, Roshini Prakash, Weiguo Li, Safia Ogbi, Maribeth H Johnson, Erin M Mezzetti, Susan C Fagan, and Adviye Ergul

   J Cereb Blood Flow Metab. 2010 December; 30(12): 1928–1938.

ORIGINAL ARTICLE

Adaptive Cerebral Neovascularization in a Model of Type 2 Diabetes

Relevance to Focal Cerebral Ischemia

Weiguo Li,[1] Roshini Prakash,[2] Aisha I. Kelly-Cobbs,[1] Safia Ogbi,[1] Anna Kozak,[2,3] Azza B. El-Remessy,[2,3] Derek A. Schreihofer,[1] Susan C. Fagan,[2,3,4] and Adviye Ergul[1,2,3]

OBJECTIVE—The effect of diabetes on neovascularization varies between different organ systems. While excessive angiogenesis complicates diabetic retinopathy, impaired neovascularization contributes to coronary and peripheral complications of diabetes. However, how diabetes influences cerebral neovascularization is not clear. Our aim was to determine diabetes-mediated changes in the cerebrovasculature and its impact on the short-term outcome of cerebral ischemia.

RESEARCH DESIGN AND METHODS—Angiogenesis (capillary density) and arteriogenesis (number of collaterals and intratree anostomoses) were determined as indexes of neovascularization in the brain of control and type 2 diabetic Goto-Kakizaki (GK) rats. The infarct volume, edema, hemorrhagic transformation, and short-term neurological outcome were assessed after permanent middle–cerebral artery occlusion (MCAO).

RESULTS—The number of collaterals between middle and anterior cerebral arteries, the anastomoses within middle–cerebral artery trees, the vessel density, and the level of brain-derived neurotrophic factor were increased in diabetes. Cerebrovascular permeability, matrix metalloproteinase (MMP)-9 protein level, and total MMP activity were augmented while occludin was decreased in isolated cerebrovessels of the GK group. Following permanent MCAO, infarct size was smaller, edema was greater, and there was no macroscopic hemorrhagic transformation in GK rats.

CONCLUSIONS—The augmented neovascularization in the GK model includes both angiogenesis and arteriogenesis. While adaptive arteriogenesis of the pial vessels and angiogenesis at the capillary level may contribute to smaller infarction, changes in the tight junction proteins may lead to the greater edema following cerebral ischemia in diabetes. ***Diabetes* 59:228–235, 2010**

From the [1]Department of Physiology, Medical College of Georgia, Augusta, Georgia; the [2]Program in Clinical and Experimental Therapeutics, University of Georgia College of Pharmacy, Augusta, Georgia; the [3]Charlie Norwood Veterans Affairs Medical Center, Augusta, Georgia; and the [4]Department of Neurology, Medical College of Georgia, Augusta, Georgia.
Corresponding author: Adviye Ergul, aergul@mail.mcg.edu.
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Type 2 diabetic patients hold two- to fourfold higher risk for cerebrovascular disease and stroke (1,2), and 70% of patients with a recent stroke have overt diabetes or pre-diabetes distinguished by impaired fasting glucose or impaired glucose tolerance (3). However, the underlying basis of this increased risk remains unclear. Diabetic vascular complications are well studied in diabetic retinopathy, nephropathy, peripheral arterial disease, and coronary artery disease. However, diabetes-induced structural and functional changes in the cerebral vasculature are unknown.

It is becoming clear that the integrity of cerebral blood vessels is critical in the pathophysiology of stroke. While type 2 diabetes accounts for ~90–95% of all diagnosed cases of diabetes in adult patients, most of the experimental studies are focused on type 1 diabetes induced by streptozotocin (STZ) injection, which is associated with high-level and short-term elevations of blood glucose. We have recently shown that diabetic Goto-Kakizaki (GK) rats develop smaller infarct and greater hemorrhagic transformation after stroke. These animals also showed cerebrovascular remodeling characterized by increased vessel tortuosity, vascular endothelial growth factor (VEGF) expression, and matrix metalloproteinase (MMP)-2 and -9 activity (4). Generated from glucose-intolerant Wistar rats, GK rat is a nonobese model of spontaneous type 2 diabetes with moderately elevated glucose levels (5,6). In light of previous reports that showed greater ischemic damage in acute hyperglycemic models of stroke (7–9), we hypothesized that chronic moderate hyperglycemia as seen in the GK model promotes neovascularization that affects the extent of ischemic injury. Considering that neovascularization may originate from new vessel formation as well as functional remodeling of existing vessels, the goals of the current study were *1*) to determine the effect of diabetes on capillary density and arteriogenesis, *2*) to determine the influence of diabetes on blood-brain barrier (BBB) baseline permeability and expression of proteins important for vascular integrity, and finally *3*) to evaluate the effect of ischemic injury on infarct and hemorrhagic transformation development in diabetic rats with preexisting vascular disease, all of which may impact the outcome of ischemic injury.

RESEARCH DESIGN AND METHODS

Male Wistar (Harlan Laboratories, Indianapolis, IN) and GK (in-house bred, derived from the Tampa colony) rats (10–12 weeks old, 250–270 g) were maintained at a constant temperature (21–23°C) with a 12/12-h light/dark cycle and allowed access to food and water ad libitum. Body weight and blood glucose measurements were performed twice weekly. Glucose measurements

were taken from the tail vein and measured using a Free Style glucose meter (Abbott Diabetes Care, Alameda, CA). A1C was measured to evaluate long-term glucose levels with an A1C Now$^+$ kit (Bayer Healthcare, Sunnyvale, CA).

Collateral number and size of pial vessels. To determine the effect of diabetes on the pial vessel arteriogenesis, the collateral number and size of cerebrovasculature were measured in 5- and 10-week-old animals. After being anesthetized with sodium pentobarbital (100 mg/kg), the animals were injected with 3 ml freshly prepared polyurethane elastomer PU4ii (vasQtec, Zurich, Switzerland) through the aorta in 1 min. PU4ii mixture was prepared by mixing the blue-stained ethylmethylketone (30% of the final mixture) and 0.8 g of PU4ii hardener shortly before casting as previously described (10). The rats were decapitated immediately, and the brain was removed and immersed in 4% paraformaldehyde for 48 h.

Stereomicroscopic images of the perfused brains were captured, and actual vessel length of cortical branches of middle cerebral arteries (MCAs) starting from its origin was measured using National Institutes of Health Image-J software. Each hemisphere was divided into six grids of equal area, and the total number of collaterals between the anterior cerebral arteries (ACAs), posterior arteries (PCAs), and MCAs were counted manually (11). A collateral was defined as an anastomosis between MCAs and ACAs or PCAs. Arteriole-to-arteriole anastomoses between the MCA branches were also counted and defined as intratree anastomosis. The diameter was measured at the midpoint of the collaterals. At least eight measurements of the diameters were taken on each hemisphere, and the average of all these measurements was considered as the average diameter of the collaterals.

Vascular density. Cerebral vessel density was measured with modified fluorescein isothiocyanate–dextran assay (12). To stain the vasculature, 1 ml of 5% FITC-dextran (150 kDa; Sigma, St. Louis, MO) in saline was injected through the femoral vein and circulated for 30 min under isoflurane anesthesia. Brains were enucleated and fixed in 4% paraformaldehyde for 48 h. Brain sections (50 μm) with 600-μm intervals were mounted onto slides. By means of a computer-controlled platform, the hemisphere of the sections was stereologically captured with the Lucivid system (MicroBrightField, Williston, VT) and analyzed by the Spaceball protocol of Stereo Investigator software (13) (MicroBrightField). Each section was randomly counted at 12–18 points, and the average number was analyzed for statistics. Vessel density was defined as the length of fluorescence stained capillaries within the observation area (expressed as $\mu m/\mu m^3$).

Vascular permeability. The BBB permeability was assessed by measuring FITC-BSA (Molecular Probes, Carlsbad, CA) extravasations as previously described (14). In brief, 10 mg/kg FITC-BSA was injected through femoral vein 30 min before the rats were killed. Plasma fluorescence intensity in each animal was measured with a fluorescence spectrophotometer (BioTek, Winooski, VT) using standard curves of FITC-BSA in normal rat plasma. Fluorescence intensity in the cortex of brain sections were analyzed with a fluorescence microscope (Carl Zeiss, Thronwood, NY). The average cerebral fluorescence intensity was normalized with the plasma fluorescence intensity of each animal for statistics.

Isolation of cerebral vessels and evaluation of structural proteins. After the major vessels (basilar, MCA, ACA, PCA, and connecting arteries of the Circle of Willis) were removed and saved as macrovessel preparation, the brain was homogenized with ice-cold PBS (0.01mmol/l, pH 7.4). The homogenate was centrifuged at 2,000*g*, 4°C for 10 min. The supernatant was saved as brain homogenate (15). The pellet was washed in PBS and gently layered on top of a dextran solution (15%, MW 38,400) and centrifuged at 4,000*g*, 4°C for 20 min. The final pellet was saved as cerebral microvessel preparation. Both macro- and microvessels were homogenized in radioimmunoprecipitation buffer as previously described (16). Homogenates were immunoblotted with occludin and claudin-5 (Zymed Laboratories, Carlsbad, CA), collagen IV (Abcam, Cambridge, MA), MMP-2, and MMP-9 (Calbiochem, Gibbstown, NJ) antibodies, respectively. All blots were probed with actin (Calbiochem) for loading control. Densities of protein bands were analyzed with Gel Pro Analyzer software (Media Cybernetics, Bethesda, MD). Total MMP activities were measured in macrovessels with gelatin zymography as previously described (16). Brain-derived neurotrophic factor (BDNF) level in the homogenates was measured using an enzyme-linked immunosorbent assay (ELISA) kit from Promega (Madison, WI).

Focal cerebral ischemia. The unilateral permanent MCAO was achieved by intraluminal filament technique (4,17). The animals were anesthetized with isofluorane inhalation in a glass chamber prior to stroke procedure. The right femoral artery was catheterized for blood sampling for arterial blood gas analysis. The anesthesia was kept at 2% isofluorane in 70% nitrous oxide and 30% oxygen during surgery. The cerebral perfusion was measured with a PIM 3 scanning laser Doppler imaging system (Perimed, Stockholm, Sweden) to evaluate basal perfusion as well as changes in perfusion following MCAO to confirm successful occlusion. Body temperature was maintained constant at 37.5°C with a heating pad and monitored by a rectal probe.

TABLE 1
Physiological parameters of control and diabetic rats in the study

	Control	Diabetes
n	12	9
Body weight (g)	262 ± 6	255 ± 8
Blood glucose (mg/dl)	106 ± 3	145 ± 6*
A1C (%)	4.6 ± 0.1	7.3 ± 0.7*
pH	7.44 ± 0.01	7.39 ± 0.06
pCO_2 (mmHg)	44.7 ± 0.8	49.1 ± 6.6
pO_2 (mmHg)	168 ± 5	159 ± 4

Data are means ± SE. *$P < 0.0001$ vs. control.

At 24 h after occlusion, cerebral blood perfusion was evaluated with PIM 3 again and the animal was immediately killed. Brains were enucleated and sliced in the coronal plane with 2-mm intervals. Section images were scanned before and after 2,3,5-triphenyltetrazolium chloride (TTC) staining. The infarct size was evaluated as previously described (4). Hemorrhagic transformation was assessed macroscopically in a binary fashion since our previous study showed overt hematoma formation but not diffuse bleeding in GK rats. Edema was determined as the difference in volume of ischemic and nonischemic hemispheres and normalized to infarct volume.

Neurobehavioral tests. Neurological outcome of ischemic injury was assessed by a Bederson test (16) and elevated-body swing test (EBST). The Bederson test was combined with contralateral hind-limb retraction, beam walking ability, and bilateral forepaw grasp tests (18). Scores were given to each item from 0 to 3 for a total of 12 for maximal deficit. The animals with a score lower than six after MCAO were excluded for analysis. These tests were repeated at 24 h before the animals were killed. EBST was assessed to evaluate motor asymmetry (19).

Statistical analysis. A two-tailed unpaired *t* test, 95% CI, was used to compare the average data for all the studies between two groups. Data are expressed as means ± SE. Differences were considered significant at $P < 0.05$.

RESULTS

Physiological parameters. Metabolic parameters are summarized in Table 1. A1C and blood glucose was significantly higher in diabetic animals. There was no significant difference in arterial blood gases and body weight.

Effect of diabetes on cerebral neovascularization. Neovascularization may result from angiogenesis (capillary sprouting) and/or arteriogenesis (collateral formation and growth as a result of remodeling of native collaterals to functional arterioles). Number and diameter of collaterals formed between pial MCAs and ACAs were evaluated as measures of arteriogenesis. In addition, number of anostomoses within the MCA tree was determined. Visualization of the surface vasculature demonstrated that cerebral vessels display increased tortuosity and typical corkscrew pattern in 10-week-old GK rats as we previously reported (Fig. 1). Diabetes significantly improved the number (Fig. 1*E*) and diameter (Fig. 1*F*) of the collaterals and intratree arteriole-to-arteriole anastomoses (Fig. 1*G*) between MCA branches. To determine whether these changes are inherent to the GK model or develop as a result of diabetes, we evaluated collateral numbers in younger (5-week-old) control and GK rats before the onset of diabetes and no difference was found between the groups (Fig. 1*E*). Capillary density was measured as an index of angiogenesis. There was prominent microvessel stained with FITC-dextran and the quantification using unbiased stereological analysis with the Spaceball protocol in Stereo Investigator software demonstrated increased capillary density in diabetic animals (Fig. 1*H*).

Effect of diabetes on cerebrovascular integrity. Since there was an increase in capillary density, in order to determine whether the vascular structure alterations induced by diabetes contribute to increased leakiness, the BBB permeability was

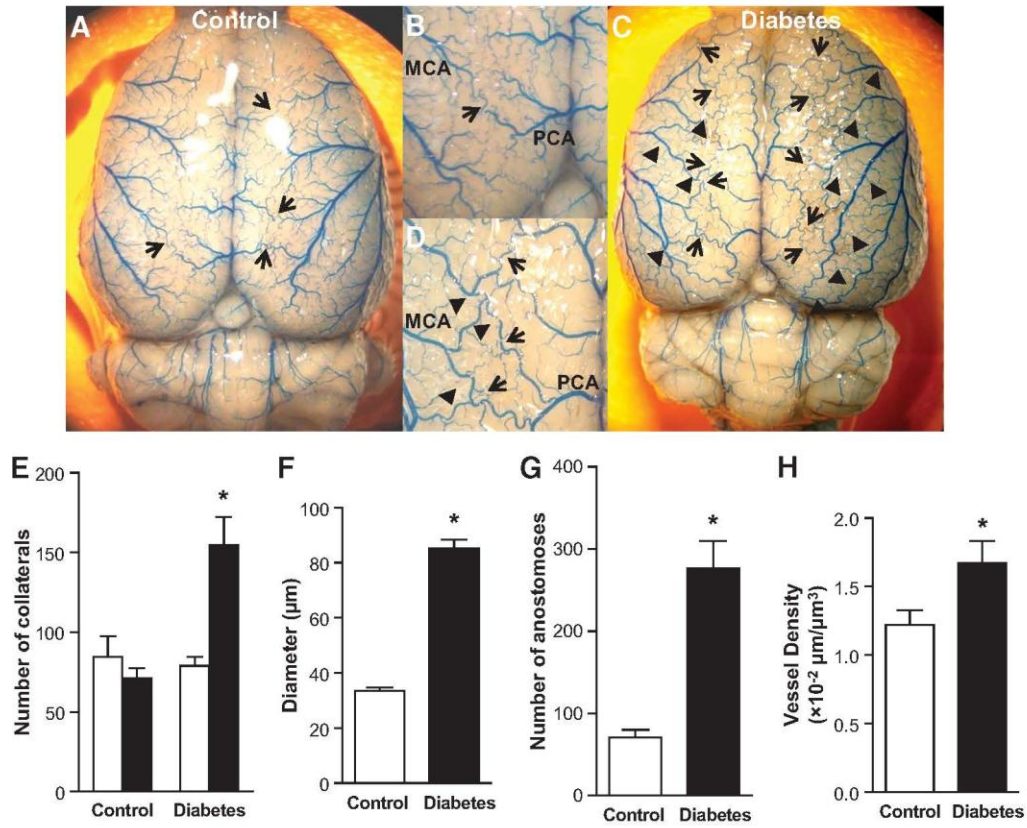

FIG. 1. Increased angiogenesis and arteriogenesis in diabetic GK rats. *A–D*: Representative brain images of 10-week-old control (*A* and *B*, $n = 9$) and diabetic GK (*C* and *D*, $n = 7$) rats infused with Pu4ii indicate increased vascular tortuosity (corkscrew pattern) indicative of vascular remodeling and collateralization in diabetes. Arrow: collaterals between MCA and PCA or ACA; arrow head: anastomoses between MCA branches. *E*: Number of collaterals was significantly increased in 10-week-old GK rats compared with control but not in younger 5-week-old animals ($n = 5$ per group) before the onset of diabetes. In 10-week-old animals, the diameter of the collaterals (*F*), the anastomoses within the MCA tree (*G*), and the total microvessel density (*H*) were all increased in diabetes ($n = 6$ per group). *$P < 0.05$ vs. control. □, 5 weeks; ■, 10 weeks. (A high-quality color digital representation of this figure is available in the online issue.)

examined with the FITC-BSA extravasation. There was a 1.5-fold increase in fluorescence intensity in diabetes compared with control indicating increased baseline permeability in diabetes (Fig. 2*A*). Next, tight junction proteins occludin and claudin-5 as well as collagen IV, a key component of basal lamina, were measured in cerebral micro and macrovessels isolated from control and diabetic animals. A 65-kDa band corresponding to occludin was detected in all samples and it was decreased in the microvasculature of diabetic animals as compared with controls (Fig. 2*B*). There was no difference in occludin levels in the macrovessels. A lower band around 63 kDa was also detected in both vascular beds with no difference between groups (data not shown). Claudin-5 and collagen IV levels were similar in micro- and macrovessels from both experimental groups (Figs. 2*C* and *D*).

MMPs are involved in the regulation of vascular remodeling and BBB breakdown following ischemic injury. Thus, MMP-2 and MMP-9 proteins were measured in the same macro- and microvessel preparations used for evaluation of tight junction proteins. MMP-9 was more abundant in both micro- and macrovessels from diabetic GK rats than in controls (Fig. 3*A*). MMP-2, on the other hand, was significantly higher in the macrovessels, but not microvessels, of diabetic rats (Fig. 3*B*). MMP-2 and MMP-9 activity of macrovessels was assessed by gelatin zymography. Despite increases in MMP-2 protein levels in diabetic rats, there was no difference in MMP-2 activity between control and GK rats. MMP-9 activity, on the other hand, was increased in diabetic rats. Microvessel MMP-2 activity was also similar between groups (data not shown). Lytic bands corresponding to microvessel MMP-9 were very faint, so they were not quantified. To determine whether increased MMP activities were developed as a result of diabetes, we also measured macrovessel MMP activities in 5-week-old rats before the onset of diabetes. Total MMP activities were 108 ± 15 vs. 99 ± 11 pixels in control and diabetic rats ($n = 4$), respectively.

A recent study (20) provided evidence that BDNF released from endothelial cells protects the neurons from a wide array of insults. Accordingly, BDNF levels in the vessel preparations were measured by ELISA. The macrovessels of both control and diabetic animals showed

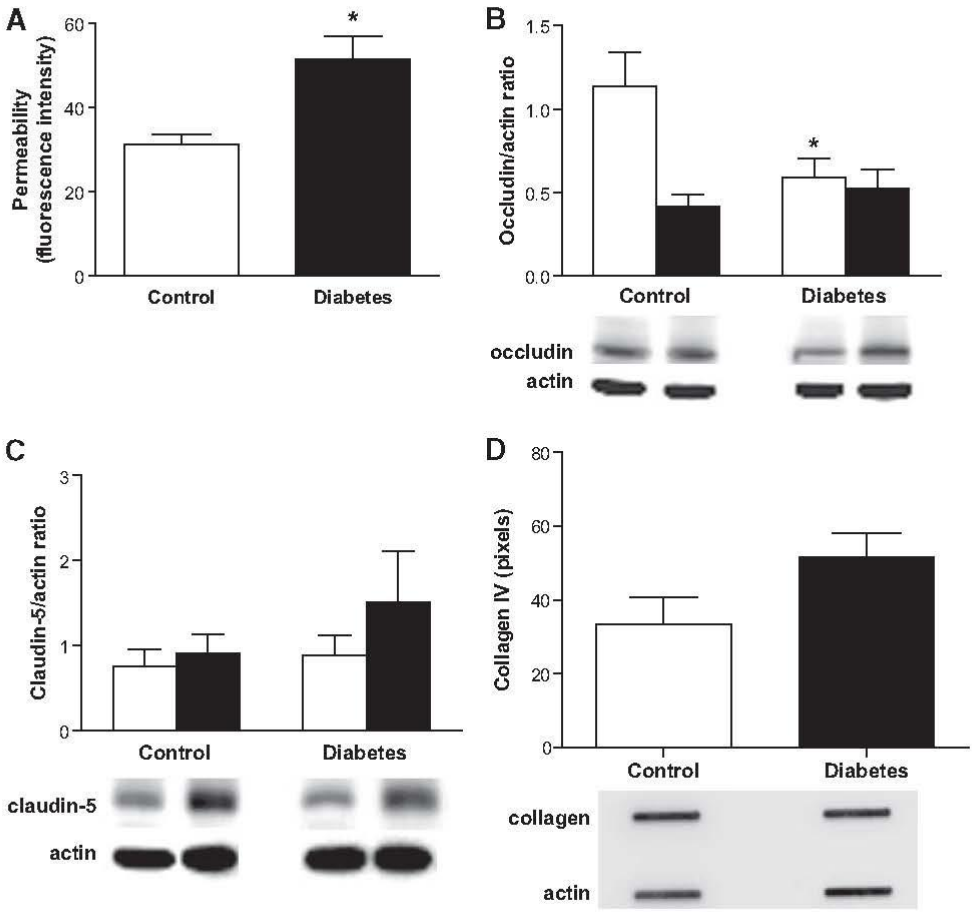

FIG. 2. Cerebrovascular permeability and matrix proteins are altered in diabetic GK rats. *A*: **BBB permeability was significantly increased in diabetes ($n = 6$ per group). *$P < 0.01$.** *B*: **Occludin protein levels, evaluated by immunoblotting of brain microvessel and macrovessel homogenates and normalized to actin levels, were decreased in the microvasculature of diabetic rats ($n = 8$ per group). *$P < 0.05$ vs. control. There was no difference in microvessels or microvessel claudin-5 levels (*C*) or microvascular collagen IV levels (*D*) in control and diabetic animals ($n = 8$ per group). □, microvessel; ■, macrovessel.**

high levels of BDNF, while the microvessels in diabetic group had higher level than controls (Fig. 3*D*).

Infarct size, hemorrhagic transformation, and edema. Infarct size was smaller (29%) in diabetic animals than in controls (49%) (Fig. 4*A*). Consistent with our previous findings, the infarcts were mainly in the striatum in GK rats, while that of Wistar rats had both cortical and subcortical localization. Edema on the other hand was higher in diabetes (Fig. 4*B*). In contrast to our previous finding of overt hematoma formation following 3 h MCAO/21 h reperfusion (4), there was no macroscopic hemorrhagic transformation after 24 h permanent MCAO in either strain.

Neurobehavioral outcome. The modified Bederson test score was 0 in both groups before surgery and increased to 10.8 ± 0.3 in control and to 9.7 ± 0.4 in diabetic rats at 24 h after MCAO (Fig. 4*C*). As shown in Fig. 4*D*, MCAO induced a significant increase in left-swing responses in both groups with no obvious difference between the groups.

Cerebral perfusion. Single-point laser Doppler probe has been widely used at monitoring cerebral blood perfusion in MCAO experiments. However, the single-point blood flow alteration is less representative for the overall perfusion. In this study, we used the scanning laser Doppler imaging system to monitor the real time subcranial cerebral blood perfusion. At 5 min after MCAO, the extent of perfusion decrease was similar in both control and diabetic rats (Fig. 5*A*), indicating that the extent of occlusion was comparable between the groups. However, at 24 h and immediately before they were killed, GK rats showed a slight recovery of perfusion, whereas there was no change in control rats (Fig. 5*B*).

DISCUSSION

Both clinical and experimental studies have shown that elevations in blood glucose due to preexisting diabetes or the acute stress response at the time of stroke is associ-

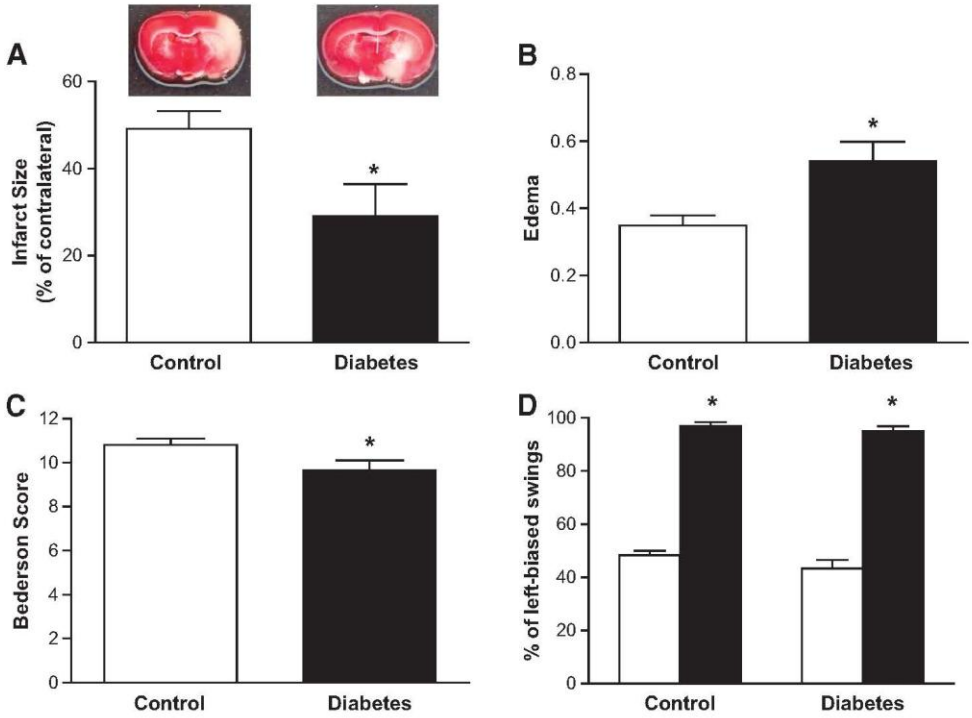

FIG. 4. Infarct size is smaller in diabetic rats after permanent MCAO. *A*: Summary of cerebral infarct size from TTC-stained brain sections of control and diabetic rats. Infarct size was calculated as percentage of contralateral hemisphere (n = 13 for control and 9 for diabetes). *P = 0.001. *B*: Edema formation was assessed as the volume difference between ischemic and nonischemic hemispheres and normalized to infarct volume. *$P < 0.05$. There was a small but significant difference in Bederson score (*C*) but not in EBST (*D*) between the groups. *$P < 0.05$. *D*: □, before; ■, after. (A high-quality color digital representation of this figure is available in the online issue.)

view. It is well established that diabetes modulates neovascularization depending on the vascular bed. While excessive pathological angiogenesis leads to diabetic retinopathy (28,30), neovascularization is impaired in the myocardium and skeletal muscle contributing to coronary artery disease and peripheral arterial disease, respectively. Accordingly, we first addressed the question whether there was neovascularization in diabetic rats. Neovascularization may result from vasculogenesis (new vessel formation from progenitor cells), angiogenesis (capillary sprouting), collateral growth, and/or arteriogenesis defined as remodeling of native collaterals to functional arterioles (31–33). As the vessels remodel and collaterals form, vessels present with increased diameter and a typical corkscrew pattern as we detect in the pial vessels of the GK model (34). Accordingly, we evaluated capillary density as a measure of angiogenesis and pial collateral number and diameter as a measure of arteriogenesis in our model, all of which were increased in diabetes. These findings suggest that cerebrovasculature undergoes adaptive arteriogenesis and angiogenesis in moderate diabetes.

We next evaluated permeability as a measurement of cerebrovascular integrity in diabetes prior to an ischemic event. Increased permeability may be due to diabetes-induced damage to the BBB function and/or due to the immature nature of the newly formed vessels. Cerebrovascular permeability was increased in diabetes as reported in early diabetic retinopathy (14,35). Since tight junction proteins such as occludin and claudin-5 have essential roles in regulating BBB stability (36), we evaluated abundance of occludin, claudin-5, and collagen IV in the isolated micro- and macrovascular vessels from the brain. Occludin was lower in the microvasculature but not macrovasculature of the diabetic group. Chehade et al. (37) reported that occludin but not zona occludens-1 levels are decreased at 2 weeks after the induction of diabetes by STZ injection. Another study reported increased MMP-2 and MMP-9 activity and rapid degradation of occludin after temporary focal ischemia (38). In the current study, MMP-9 was increased in microvessel preparation along with decreased occludin. Taken together, these changes in the structural components of microvessels may be contributing to increased permeability in the diabetic GK rats. While we do not have direct evidence on the regulation of these proteins following an ischemic insult in our model, it is highly possible that these changes also play a role in the development of increased edema formation following permanent focal MCAO as we report in the current study as well as the development of overt hematomas following temporary occlusion in the GK rats as we reported previously (4).

Cerebral perfusion is a key determinant of the extent of ischemic injury. Thus, we confirmed that the extent of MCA occlusion was similar in control and diabetic rats using a scanning-laser Doppler imaging system. While it is recognized that cerebral blood flow needs to be assessed by more quantitative approaches, we found that 24 h after permanent MCAO, there was restoration of cerebral per-

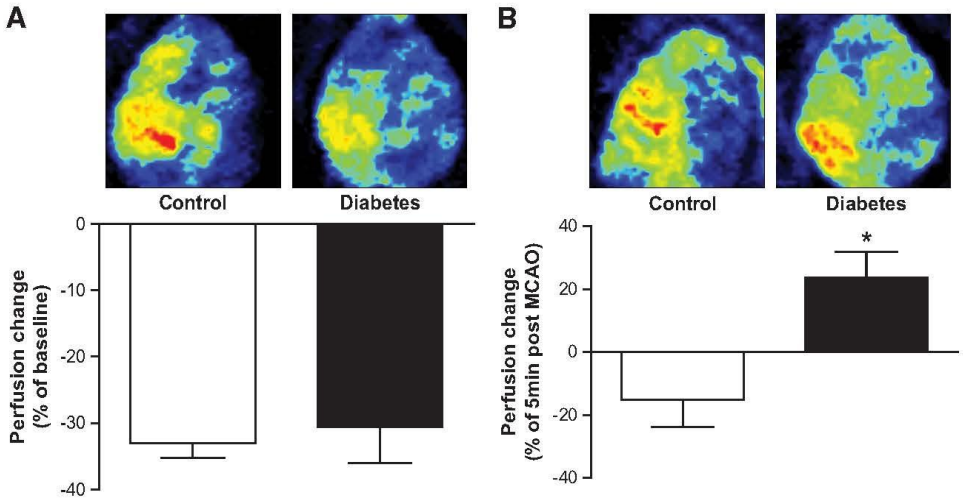

FIG. 5. Cerebral perfusion before and after MCAO. *A*: Cerebral perfusion was decreased to the same extent in both groups following MCAO. Percent decrease (5 min post-MCAO versus baseline) in perfusion was summarized in the bar graph. *B*: Cerebral perfusion was reevaluated before sacrifice at 24 h after occlusion and percent change (24 h post-MCAO versus 5 min post-MCAO) indicated recovery of flow in diabetic rats (n = 13 for control and 9 for diabetes). *$P < 0.001$. (A high-quality color digital representation of this figure is available in the online issue.)

fusion in the diabetic group consistent with increased collateralization in GK rats.

Numerous past experimental studies have reported exacerbation of the ischemic damage by hyperglycemia (7,21,24,25,39). These studies also reported increased hemorrhagic transformation only if blood flow was reestablished following occlusion. Consistent with these results, in the current study we did not observe any hematoma formation in either group. The interesting finding of the current study is consistent with our previous finding that infarct size is smaller in diabetic GK rats even after permanent occlusion. Careful review of the literature on diabetes and focal brain ischemia demonstrated that most studies used animal models in which blood glucose was elevated acutely by glucose injection just prior to occlusion or diabetes was induced by STZ injection a few days prior to surgery. In leptin receptor–deficient *db/db* mice, edema and infarct size after hypoxic-ischemic injury was increased as compared with nondiabetic animals (40), but it has to be recognized that this model is associated with obesity, and leptin is neuroprotective. An earlier study by Warner et al. reported that acutely hyperglycemic, but not diabetic, rats are more vulnerable to global ischemia despite similar levels of glycemia, suggesting some degree of protection in diabetes (41). Observational studies support this concept. Hyperglycemic nondiabetic acute ischemic stroke patients appear to suffer the most from stroke (26,42). Interestingly, myocardium is reported to be resistant to ischemic injury in diabetes as a result of metabolic preconditioning (43). We detected higher BDNF levels in the vessels of diabetic rats. A recent in vitro study (20) provided very intriguing evidence for neuroprotection by endothelium-derived BDNF secretion mediated by integrin signaling. It is possible that in our model, all the neovascularization events taking place may stimulate BDNF synthesis and release. Based on our intriguing results, we speculate that longer duration of hyperglycemia as seen in our GK model preconditions the brain, although mechanisms by which this occurs remain to be determined.

There are limitations to our study. First of all, the GK rats are a model of moderate type 2 diabetes without confounding factors like obesity and dyslipidemia that are frequently found in stroke patients. However, diabetes is a risk factor for stroke independent of these comorbidities, and thus GK rats allow us to study effects of hyperglycemia alone. GK rats are an in-bred strain, and vascular changes may be inherent to the model. However, the fact that there was no difference in collateral numbers and MMP activity in younger GK rats before the onset of diabetes as compared with control rats at that age argues against this possibility. We also used relatively young animals. As with any animal model of disease, the diversity of acute ischemic stroke patients in clinical setting cannot be replicated with this model. Second, this study included only a short-term functional evaluation after stroke. Edema and microvascular responses may still be evolving at this point and functional recovery at later time points need to be assessed. Finally, we only studied the effect of short and moderate elevations in blood glucose, but duration and degree of hyperglycemia in diabetes may be critical for neurovascular outcomes following ischemic injury. Nevertheless, our results provide direct evidence that diabetes alters cerebrovascular density, neovascularization patterns, and microvascular permeability, all of which affect the neuronal and vascular damage following ischemic brain injury. When compared with the literature, these findings also suggest that the pattern of ischemic injury under diabetic and hyperglycemic conditions differs. Given that therapeutic angiogenesis after stroke is an active area of research, an enhanced understanding of cerebrovascular networking in the setting of diabetes is fundamentally important to develop preventive and therapeutic strategies for stroke in high-risk patients as well as improving therapeutic angiogenesis after stroke. The effect of longer duration of diabetes on cerebrovascular function, structure, and ultimately on ischemia/reperfusion injury requires further study.

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No potential conflicts of interest relevant to this article were reported.

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Vascular protection in diabetic stroke: role of matrix metalloprotease-dependent vascular remodeling

Mostafa M Elgebaly[1], Roshini Prakash[1], Weiguo Li[2], Safia Ogbi[2], Maribeth H Johnson[3], Erin M Mezzetti[2], Susan C Fagan[1,4] and Adviye Ergul[1,2,4]

[1]*Department of Clinical Pharmacy, Program in Clinical and Experimental Therapeutics, University of Georgia, College of Pharmacy, Augusta, Georgia, USA;* [2]*Department of Physiology, Medical College of Georgia, Augusta, Georgia, USA;* [3]*Department of Biostatistics, Medical College of Georgia, Augusta, Georgia, USA;* [4]*Charlie Norwood VA Medical Center, Augusta, Georgia, USA*

Temporary focal ischemia causes greater hemorrhagic transformation (HT) in diabetic Goto-Kakizaki (GK) rats, a model with increased cerebrovascular matrix metalloprotease (MMP) activity and tortuosity. The objective of the current study was to test the hypotheses that (1) diabetes-induced cerebrovascular remodeling is MMP dependent and (2) prevention of vascular remodeling by glucose control or MMP inhibition reduces HT in diabetic stroke. Control and GK rats were treated with vehicle, metformin, or minocycline for 4 weeks, and indices of remodeling including vascular tortuosity index, lumen diameter, number of collaterals, and middle cerebral artery (MCA) MMP activity were measured. Additional animals were subjected to 3 hours MCA occlusion/21 hours reperfusion, and infarct size and HT were evaluated as indices of neurovascular injury. All remodeling markers including MMP-9 activity were increased in diabetes. Infarct size was smaller in minocycline-treated animals. Both metformin and minocycline reduced vascular remodeling and severity of HT in diabetes. These results provide evidence that diabetes-mediated stimulation of MMP-9 activity promotes cerebrovascular remodeling, which contributes to greater HT in diabetes. Metformin and minocycline offer vascular protection, which has important clinical implications for diabetes patients who are at a fourfold to sixfold higher risk for stroke.
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Keywords: diabetes; hemorrhagic transformation; MMP; stroke; vascular remodeling

Introduction

Diabetes is an increasingly growing epidemic affecting 21 million Americans over 65% of whom will eventually die of a macrovascular event such as stroke (Lloyd-Jones *et al*, 2009). As diabetic patients are at a higher risk of stroke and have poorer prognosis compared with the nondiabetic population, a better understanding of diabetes-induced vascular pathology and the underlying mechanisms is pivotal for developing better vascular protection strategies before and after an ischemic insult (Poppe *et al*, 2009).

Traditional vascular complications of diabetes are categorized as (1) microvascular (nephropathy, neuropathy, and retinopathy) and (2) macrovascular (stroke, coronary artery disease, and peripheral arterial disease) (Brownlee, 2005). In both cases, the vascular wall structure and function are affected by remodeling changes. These may include vascular smooth muscle cells proliferation, degeneration of endothelial cells, basement membrane thickening, and a state of coagulopathy (Gabbay, 1975). In established disease, there is vascular wall growth as a result of increased collagen deposition or vascular smooth muscle cell hypertrophy/hyperplasia (Endemann *et al*, 2004). The matrix metalloprotease (MMP) system is involved in restructuring of the vessels and the surrounding matrix by degrading as well as stimulating matrix deposition (Olszynski

Correspondence: Dr A Ergul, Department of Physiology, Medical College of Georgia, 1120 15th Street, CA-2094, Augusta, GA 30912, USA.
E-mail: aergul@mcg.edu
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and Zimowska, 2009). The MMP-2 and MMP-9 expression and activity are elevated under hyperglycemic conditions (Signorelli *et al*, 2005). We previously showed increased tortuosity and MMP activity of the cerebral vessels as evidence of early vascular structure changes in diabetes (Ergul *et al*, 2007). We recently reported that there is increased cerebral angiogenesis and arteriogenesis associated with augmented microvessel and macrovessel MMP activity in diabetic animals (Li *et al*, 2009). We also showed that when diabetic animals are exposed to ischemic brain injury, they suffer greater hemorrhage and edema, suggesting that preexisting neovascularization exacerbates stroke injury as also seen in diabetic retinopathy (Ergul *et al*, 2007; Li *et al*, 2009). Given that MMP-2 and MMP-9 rise acutely after stroke leading to edema and hemorrhagic transformation (HT) that develop secondarily to prolonged ischemia (Alvarez-Sabin *et al*, 2004), the relative contribution of diabetes-induced MMP activation and vascular remodeling to ischemic brain injury remained to be determined.

Cerebral vasculature has autoregulatory properties to adjust myogenic tone, a critical functional aspect of the cerebral circulation for adequate blood flow under normal conditions and more so in ischemia/reperfusion injury. Cipolla *et al* (1997) reported that intrinsic myogenic tone of posterior cerebral arteries is diminished in response to increased glucose concentration *in vitro*. However, Zimmermann *et al* (1997) showed a constriction of middle cerebral arteries (MCAs) in diabetic rats. A third study reported that posterior cerebral artery tone is enhanced in diabetes (Jarajapu *et al*, 2008). Collectively, these studies point out to the importance of understanding more about cerebral vessel structure and function under physiologic circumstances and pathologic alterations that may cause deleterious outcomes. As discussed above, we reported increased cerebrovascular remodeling and neovascularization in diabetes. Whether and to what extent these changes influence vascular tone, integrity, and ultimately the magnitude of ischemia/reperfusion injury are yet to be determined. Taken together, our working hypothesis for the current study was that MMP-mediated cerebrovascular remodeling in diabetes augments vascular damage after ischemia/reperfusion injury. We also hypothesized that tight glycemic control or MMP inhibition serve as vascular protection strategies.

Materials and methods

Animals

The institutional animal care and use committee of the Medical College of Georgia approved all protocols used in animal work. Male Wistar and Goto-Kakizaki (GK) rats were purchased from Harlan (Indianapolis, ID, USA) and Taconic (Hudson, NY, USA) Laboratories, respectively. For all studies, weight-matched rats (270 to 310 g, 9 to 11 weeks) were used.

Metformin was titrated to maintain euglycemia in GK rats (~300 mg/kg per day based on blood glucose levels) and was given in drinking water artificially sweetened by noncaloric sweetener. Minocycline was also given in drinking water (5 mg/kg per day). Both treatments were chronic, starting with the onset of diabetes in GK rats (5 to 6 weeks of age) till the animals reached the weight range used for MCA occlusion (MCAO), which averaged about 5 weeks. Minocycline treatment was stopped 3 days before MCAO to allow for a wash-out period. Blood glucose levels were measured from the tail vein blood using a glucometer (Freestyle, Alameda, CA, USA).

Measurement of Remodeling Indices

Tortuosity index, collateral number, and diameter were measured as indices of remodeling in a masked manner. After being anesthetized with sodium pentobarbital (100 mg/kg), the animals were injected with 3 mL freshly prepared polyurethane elastomer PU4ii (vasQtec, Zuerich, Switzerland) through the aorta within a minute. PU4ii mixture was prepared by mixing the blue-stained ethylmethylketone (30% of the final mixture) and 0.8 g of PU4ii hardener shortly before casting as previously described (Krucker *et al*, 2006). The rats were decapitated immediately, and the brain was removed and immersed in 4% paraformaldehyde for 48 hours.

Stereomicroscopic images of the perfused brains were captured and actual vessel length of cortical branches of MCA starting from its origin was traced on a Wacom tablet 493-3 using Image J I-36 software. Each hemisphere was divided into six grids of equal area and the total number of collaterals between the anterior, posterior, and middle cerebral arteries were counted manually (Chalothorn *et al*, 2007). A collateral was defined as an anastomosis between MCA and anterior or posterior cerebral arteries. Arteriole-to-arteriole anastomoses between the MCA branches were also counted and defined as an intra-tree anastomosis. The diameter was measured at the midpoint of the collaterals. At least eight measurements of the diameters were taken on each hemisphere, and the average of all these measurements was reported as the average diameter of the collaterals. Tortuosity index was defined as the ratio of the vessel length over straight-line distance between two vessel ends. In each grid, two middle size vessels were traced, and the average of 12 measurements was used as the Tortuosity index per animal.

Evaluation of Middle Cerebral Artery Vascular Structure and Myogenic Tone

One of the MCAs collected immediately after decapitation was mounted on the pressurized arteriography (Living Systems Instrumentation, Burlington, VT, USA), to measure media thickness, lumen and outer diameters with a video dimension analyzer at different pressures ranging from 5 to 180 mm Hg at 20 mm Hg pressure increments. The system was equilibrated in Krebs-HEPES (4-(2-hydroxyethyl)-1-piperazinethananesulfonic acid) buffer free of calcium to obtain measurements under passive conditions and media thickness, lumen and outer diameters and

vessel cross-sectional areas were determined as follows: wall thickness (WT, μm) = outer diameter − lumen diameter (i.e., OD−LD); (M/L) ratio = WT/LD; cross-sectional area (μm^2) = outer vessel area − lumen area. Myogenic tone and stiffness of MCAs (β-coefficient) were calculated as follows: myogenic tone (% tone) = $(1-(OD_{active}/OD_{passive}))\times 100$; stiffness ($\beta$-coefficient) was obtained from the slope of the stress versus strain curve using the equation: $y = ae^{\beta x}$ (y = stress, x = strain, a = intercept, and β = slope) (Rigsby *et al*, 2007).

Isolation of Cerebral Vessels

The animals were subjected to ischemic brain injury as described below. At 24 hours, animals were killed and macrovessels were isolated immediately from ischemic and nonischemic side separately, snap frozen in liquid nitrogen and kept at −80°C for later protein work. Macrovessels are defined as basilar artery, MCA, circle of Willis, and ACA. Vessels were homogenized using RIPA (RadioImmunoPrecipitation Assay) buffer to extract MMPs and a standard Bradford protein assay was performed before running immunoblots or zymograms to determine the amount of loaded protein. In an additional group of animals treated with vehicle, metformin, or minocycline, macrovessels were isolated at the end of the treatment period without any ischemic injury.

Matrix Metalloprotease-9 Expression and Activity

The MMP-2 and MMP-9 expression was determined by immunoblotting. A measure of 50 μg proteins were directly loaded on SDS-PAGE gel and separated under reducing conditions. After electrophoresis, proteins were transferred to a nitrocellulose membrane. In all, 5% milk-TTBS (Tween Tris Buffered Saline) was used for blocking and band detection was performed using primary antibody against MMP-2 or MMP-9 (Calbiochem, San Diego, CA, USA) and a peroxidase-conjugated goat antimouse secondary antibody. The chemiluminescent signal was detected using (Alpha Imager, Santa Clara, CA, USA) and bands intensity quantified by image analysis using GelPro analyzer (Media Cybernetics, Bethesda, MD, USA).

The MMP-2 or MMP-9 activity was assessed by gelatin zymography. A measure of 30 μg protein was loaded on an SDS-PAGE gel containing 0.1% gelatin and separated under nonreducing conditions. After electrophoresis, the gel was washed in 2.5% 'Triton X-100' for 20 minutes twice and incubated for 20 hours in a substrate buffer- 50 mmol Tris-HCl, 5 mmol $CaCl_2$ + 0.02% NaN_3− pH = 7.5 at 37°C. A recombinant MMP-2 and MMP-9 standard was run as positive control. After incubation, the gel was stained overnight using 'Coomassie blue' then destained. The zymogram was digitized and bands intensity was quantified by image analysis using GelPro.

Middle Cerebral Artery Occlusion and Cerebral Perfusion Measurement

Three hours MCAO/21 hours reperfusion model was used. Animals were anesthetized using isoflurane in an induction chamber then maintained for about 15 minutes on 3% isoflurane for the procedure. A midline cervical incision was made to expose the common carotid artery. The external carotid artery separated, ligated, and severed. A rounded-tip, by heating, 4-0 nylon monofilament suture was inserted into the internal carotid artery to occlude the origin of MCA (Longa *et al*, 1989). The occlusion suture was secured with one-silk suture at the stump of external carotid artery, and the incision was closed. After 3 hours, the animals were reanesthetized, and the occlusion suture was removed to allow reperfusion. Laser Doppler (PIM-3, Perimed, Stockholm, Sweden) was used to confirm a consistent drop in perfusion among groups. Core body temperature was maintained using a heating pad and monitored through a rectal probe during the surgery and on a heating pad under the cage till the end of 24 hours. Animals were singly housed before and after MCAO with free access to food and water.

Evaluation of Infarct Size, Edema, and Hemorrhagic Transformation

At 24 hours after occlusion, cerebral blood perfusion was evaluated with PIM-3 again and the animal was immediately killed. Brains were removed and sliced in the coronal plane with 2 mm intervals, labeled A–F, front to back and were used to calculate infarct size, edema, and HT. Visual inspection of hemorrhage if present was reported as a qualitative score for the frequency of macroscopic bleeding. 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co., St Louis, MO, USA) was used to outline the infarct area. Images analysis was performed in a masked manner using the Image-J (NIH, Bethesda, MD, USA) software. After staining, hemispheres were separated; snap frozen at −80°C for later hemoglobin direct enzyme linked immunosorbent assay (ELISA) quantification (Hilali *et al*, 2004). Edema was expressed as a percentage of infarct hemisphere size to control hemisphere.

Neurobehavioral Assessment

Short-term neurobehavioral functional outcomes of ischemic injury was assessed by a battery of tests including Bederson, forepaw grasp, beam walk, hind limb retraction, and elevated body swing tests at 24 hours before the kill. Bederson test was scored for the presence of forelimb flexion, decreased resistance to push, and ipsilateral circling with each item given 1 point. A score of 3 is consistent with an MCAO. Bederson score was combined with beam walking ability, and bilateral forepaw grasp tests to determine a composite score (Li *et al*, 2009). Scores were given to each item from 0 to 3 for a total of 9 for maximal deficit. Bederson's score was also recorded at the end of 3 hours occlusion period to verify proper occlusion (Kozak *et al*, 2008).

Statistics

The distributions for the measures of stroke severity (infarct size, percent edema, and bleeding) as well as the measures of behavior and vascular remodeling were found

to be skewed. The difference between stroke versus nonstroke side of the brain for MMP-2 and MMP-9 levels was calculated, and the difference was adjusted for the nonstroke value in the analysis. A rank transformation was used before the analysis of all measures. The analysis for the effect of minocycline on Wistar and GK rats was performed using a 2-disease (Wistar versus GK) × 2-treatment (vehicle versus minocycline) analysis of variance. An interaction between disease and treatment would indicate a differential effect of minocycline treatment that is dependent on disease status. The analysis for the effects of minocycline and metformin on GK rats was performed using a 3-treatment (vehicle, metformin, and minocycline) one-way analysis of variance. A Tukey's test was used to adjust for multiple comparisons when determining mean differences for significant analysis of variance effects.

Results

Diabetes Promotes Vascular Remodeling

Blood glucose levels (mg/dL) were 110.0 ± 11 and 106.8 ± 7 in control and control + minocycline groups, respectively (Table 1). In diabetic animals, metformin treatment provided glycemic control and blood glucose levels were 212.0 ± 19, 102.2 ± 8, and 177.4 ± 21 in vehicle, metformin, and minocycline groups, respectively. Cerebrovascular structure was evaluated by two different methods: (1) visualization and evaluation of cerebral pial vessels by PU4ii injection and (2) pressurized arteriographic assessment of isolated MCAs. Tortuosity index, the number of collateral and anastomoses were significantly higher in diabetic rats than in control group. Both metformin and minocycline treatments in diabetes reduced them significantly to control values (Figures 1A to 1D). The collateral inner diameter (mm) was greater in diabetic GK rats and minocycline reduced it significantly in both control and diabetic animals (Figure 1E). Glycemic control with metformin also reduced inner diameter in GK rats.

Wall remodeling indices obtained from pressure arteriography included MCA inner and outer diameters (μm), cross-sectional area (μm^2), and M/L ratio. In addition, myogenic tone and stiffness were assessed as a measure of vascular function and mechanics at an intraluminal pressure of 80 mm Hg, which represents the estimated pressure experienced by MCA *in vivo*. There was a disease and treatment effect on inner ($P = 0.0023$) and outer diameters ($P = 0.01$) such that minocycline increased these parameters in controls but reduced them in diabetes. There was no difference in cross-sectional area or M/L ratio between groups (Figure 2). Myogenic tone was significantly higher in diabetic rats than in controls at 80 mm Hg pressure and both metformin and minocycline significantly reduced it to less than control levels (Figure 3A). There was a disease by treatment effect on vascular stiffness. Minocycline reduced stiffness in controls but increased it in diabetes (Figure 3B). Metformin had no effect on any of these parameters.

Diabetes Augments Stroke-Induced Increase in Matrix Metalloprotease Expression and Activity

The MMP-9 is involved in the regulation of vascular remodeling and blood–brain barrier breakdown after ischemic injury. Thus, MMP-9 activity was measured in macrovessel homogenates prepared from ischemic and nonischemic hemispheres after MCAO. When nonischemic hemispheres were compared as an indicator of baseline macrovascular MMP-9 activity, diabetic rats displayed greater enzyme activity than controls and glycemic control with metformin prevented this increase (Figure 4A). Ischemic injury increased MMP-9 activity in both control and diabetic animals and metformin treatment prevented ischemia-induced MMP-9 activity in diabetic animals. Minocycline treatment abolished gelatinolytic activity in both control and diabetic animals. There was no difference in MMP-2 activity among the study groups or between ischemic and nonischemic macrovessels (Figure 4B). To ensure that MCAO procedure itself is not affecting MMP levels on the nonischemic side, macrovascular MMP expression/activity was determined at baseline without any exposure to ischemia in an additional group of animals treated with vehicle, metformin, or minocycline. Both MMP-2 and MMP-9 activity were greater in the diabetic animals than in controls (Figure 4C). Metformin treatment reduced MMP-2 but not MMP-9 activity in diabetic animals. Minocycline treatment lowered MMP-9 but not MMP-2 activity in both control and diabetic rats. The MMP-2 protein levels followed the same pattern seen in activity results (Figure 4D). The MMP-9 protein levels were higher

Table 1 Baseline metabolic parameters

	Control	*Diabetes*	*Diabetes+ metformin*	*Control+ minocycline*	*Diabetes+ minocycline*
BW (g)	293.6 ± 9	279.2 ± 10	256.0 ± 4	316.3 ± 4	270.9 ± 3
BG (mg/dL)	110.0 ± 11*	212.0 ± 19	106.8 ± 7*	102.2 ± 8*	177.4 ± 21

BG, blood glucose; BW, body weight.
*$P < 0.001$ versus diabetes.

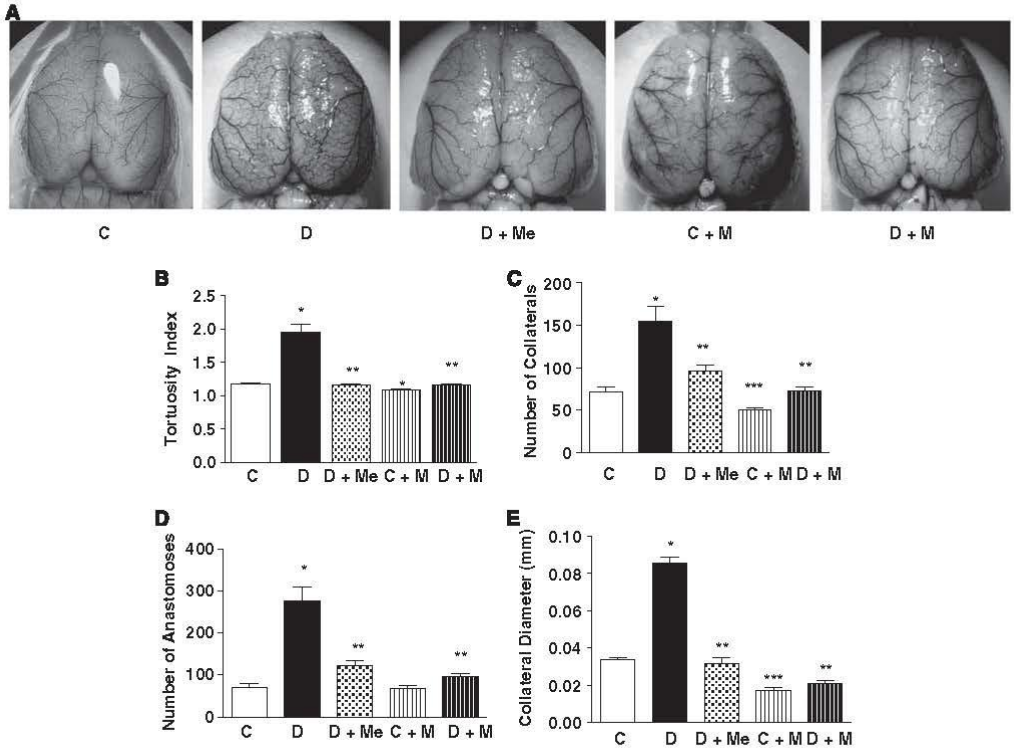

Figure 1 Diabetes promotes remodeling of cerebral vessels. (**A**) Representative images of the pial vessels after PU4ii injection to visualize middle, anterior, and posterior cerebral arteries trees in control (C), diabetes (D), diabetes + metformin (D + Me), control + minocycline (C + M), or diabetes + minocycline (D + M) groups. Tortuosity index (**B**), number of collaterals (**C**), number of anastomoses (**D**), and collateral diameter (**E**) were significantly higher in diabetes all of which were prevented by metformin or minocycline treatments. Mean ± s.e.m., $n = 7$ to 14, $^{*}P < 0.0001$ versus C, $^{**}P < 0.001$ versus D, $^{***}P < 0.0002$ versus C.

in diabetic vessels but treatment with either metformin or minocycline did not have an effect.

Neurovascular Injury Is Exacerbated in Diabetes

The drop in perfusion after MCAO was consistent among groups (45% to 55%, data not shown). The control group did not differ from diabetic animals with regard to mean arterial blood pressure, pH, pCO_2, and pO_2. Infarct size was significantly smaller in diabetic rats as reported by our group before (Figures 5A and 5B). Treatment with metformin did not have an effect on infarct size but minocycline reduced infarct in both control and diabetic animals.

Edema and HT were evaluated as indices of ischemia-induced vascular damage. The HT was assessed qualitatively by measuring the incidence of intracerebral bleeding and quantitatively by a previously validated hemoglobin ELISA. Diabetes caused greater vascular damage in diabetic rats than controls as shown by higher incidence of bleeding (Figure 6A) and greater HT severity (Figure 6B). There was a disease and treatment interaction such that minocycline significantly reduced HT incidence and severity in diabetes but not in control rats. Metformin treatment also reduced HT severity. Edema was bigger in diabetes (Figure 6C). Metformin reduced it and there was a disease and treatment interaction where minocycline significantly reduced it in diabetes.

Neurologic Outcome Is Worsened in Diabetes

Multiple neurobehavioral tests were used to assess the short-term functional outcome of ischemia/reperfusion injury at 24 hours. The composite neurodeficit score was significantly higher in diabetic animals indicating worse functional outcome (Figure 7A). Treatment with metformin improved functional outcome. Minocycline treatment showed a disease–drug interaction with no effect on control animals but reduced deficit in diabetic rats. There

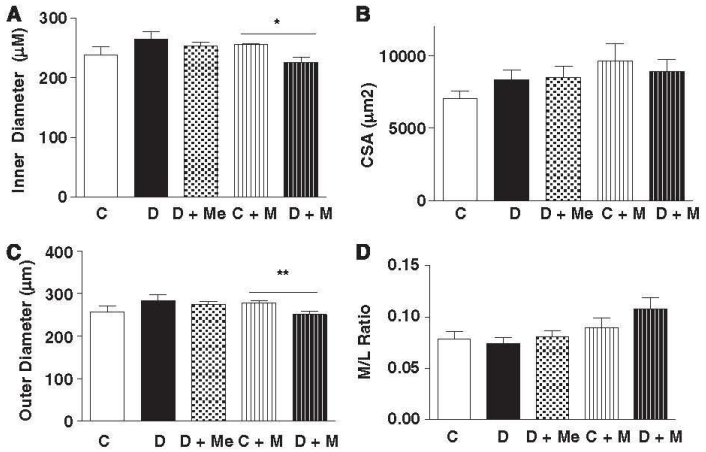

Figure 2 Diabetes does not increase wall thickness or M/L ratio in diabetes. Middle cerebral artery (MCA) wall remodeling parameters including (**A**) inner diameter, (**B**) cross-sectional area (CSA), (**C**) outer diameter, and (**D**) M/L ratio were measured by pressure arteriography at 80 mm Hg. Mean ± s.e.m., $n = 6$ to 9, $^{*}P = 0.0023$ and $^{**}P = 0.01$, disease by treatment interaction. M/L, media/lumen.

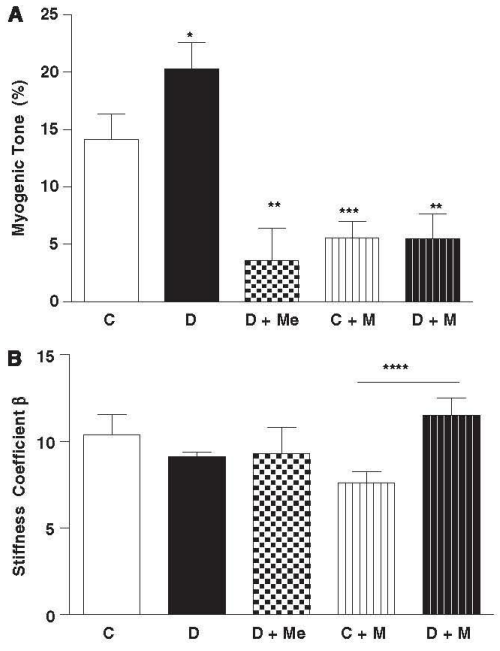

Figure 3 Diabetes increases myogenic tone of isolated middle cerebral arteries (MCAs). (**A**) The MCA myogenic tone was increased in diabetic animals and both metformin and minocycline reduced the tone. (**B**) There was a disease and treatment interaction such that minocycline decreased stiffness in controls but increased it in diabetic rats. Mean ± s.e.m., $n = 7$ to 14, $^{*}P < 0.05$ versus C, $^{**}P = 0.011$ versus D, $^{***}P = 0.0046$ versus C; $^{****}P < 0.0001$. C, control; D, diabetes.

was no significant difference in elevated body swing test between groups (Figure 7B). Minocycline improved forepaw grasp in both control and diabetic animals (Figure 7C).

Discussion

The current study was designed to address the following important questions: (1) Is diabetic remodeling of the cerebrovasculature MMP dependent? (2) Does MMP-mediated cerebrovascular remodeling contribute to the augmented stroke injury seen in diabetes? and (3) Does glycemic control prevent MMP activation/remodeling and reduce neurovascular damage after ischemic brain injury? Our findings provide evidence that even after a short duration of relatively mild hyperglycemia, there are structural changes in the cerebral vessels as indicated by increased tortuosity, number of collaterals, and collateral diameter all of which can be prevented by MMP inhibition. When these structural alterations are inhibited by minocycline treatment, vascular damage ensuing ischemic brain injury is significantly reduced. Glycemic control prevents remodeling, reduces bleeding, and improves short-term functional outcome in diabetes.

Clinical data have shown remarkably worse outcomes, slower short- and long-term functional recovery, and higher mortality in diabetic patients after stroke compared with the nondiabetic population (Idris *et al*, 2006). Experimental studies in streptozotocin-induced model of diabetes with very high blood glucose levels also showed greater infarct development (de Courten-Myers *et al*, 1992). Our understanding of the mechanisms involved in

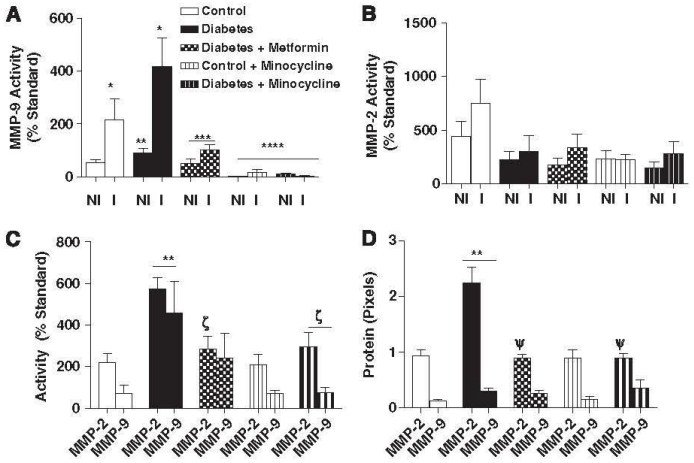

Figure 4 Diabetes augments ischemia-induced stimulation of MMP-9 activity in isolated cerebral vessels. (**A**) The MMP-9 activity in macrovessels isolated from ischemic (I) and nonischemic (NI) hemispheres of animals subjected to middle cerebral artery occlusion (MCAO) was measured using gelatin zymography. The MMP-9 activity was greater in both the NI and I hemispheres in diabetes indicating increased baseline and ischemia-induced augmentation of MMP-9 activity. Both metformin and minocycline caused a dramatic reduction in enzyme activity. (**B**) The MMP-2 activity of the same groups did not show any difference. (**C**) When lytic activity was assessed in the same cerebral macrovessels isolated from animals not subjected to MCAO, both MMP-2 and MMP-9 baseline activities were greater in diabetes. (**D**) Protein levels of MMP-2 and MMP-9 were increased in diabetes. Metformin and minocycline treatments significantly reduced MMP-2 level but not MMP-9. Mean ± s.e.m., $n = 5$ to 8, $^{*}P < 0.01$ versus NI, $^{**}P = 0.05$ versus C, $^{***}P = 0.012$ versus D, $^{****}P < 0.0001$ versus vehicle C or D, $^{\varsigma}P = 0.031$ versus D, $^{\Psi}P = 0.003$ versus D. C, control; D, diabetes; MMP, matrix metalloprotease.

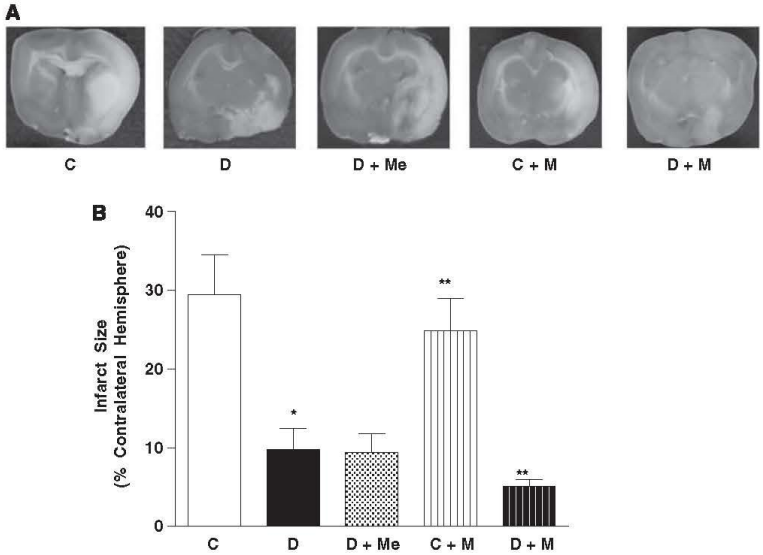

Figure 5 Infarct size is smaller in diabetes. (**A**) Representative images of 2,3,5-triphenyltetrazolium chloride-stained coronal sections of the brain after middle cerebral artery occlusion (MCAO). (**B**) Quantitative analysis of infarct size indicated smaller infarcts in diabetes. Although metformin had no effect on infarct size minocycline reduced infarct in both groups. Mean ± s.e.m., $n = 6$ to 9, $^{*}P < 0.0001$ versus C, $^{**}P = 0.05$ versus vehicle C or D. C, control; D, diabetes.

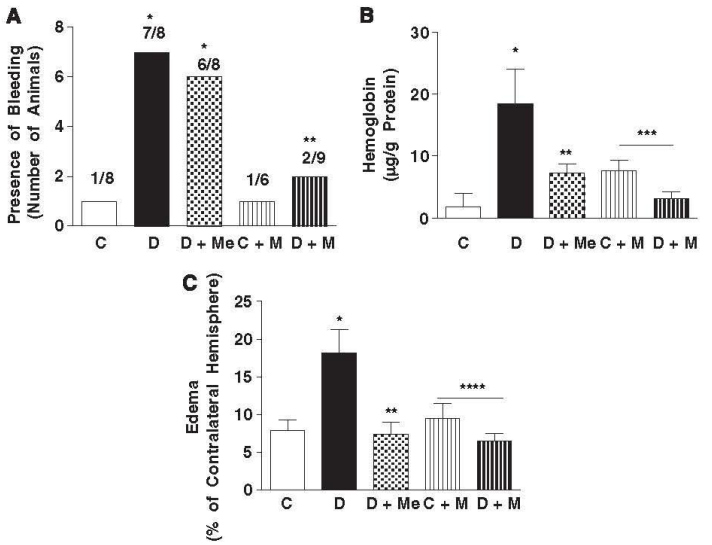

Figure 6 Vascular injury is greater in diabetes. (**A**) Occurrence of macroscopic intracerebral bleeding was significantly higher in diabetes compared with control and was reduced by chronic minocycline treatment. (**B**) ELISA measurements of hemoglobin show significant increase in hemorrhagic transformation (HT) in diabetes and a vasoprotective effect of minocycline and metformin. There was a disease and treatment interaction showing minocycline preventing HT in diabetes but no effect on control animals. (**C**) Edema was significantly higher in diabetes compared with control and both metformin and minocycline reduced it. Minocycline had no effect on edema in control animals, indicating a disease/treatment interaction. Mean ± s.e.m., $n = 6$ to 11, $^{*}P < 0.05$ versus C, $^{**}P = 0.001$ versus D. $^{***}P = 0.0009$ and $^{****}P = 0.0024$ are disease/treatment interaction for minocycline. C, control; D, diabetes; ELISA, enzyme linked immunosorbent assay.

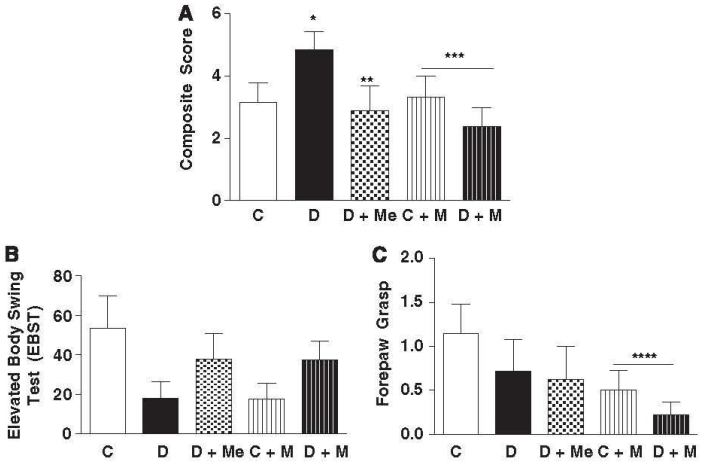

Figure 7 Short-term (24 hours) neurologic outcomes after middle cerebral artery occlusion (MCAO) in all treatment groups. (**A**) A composite score for multiple neurobehavioral tests shows worse functional recovery in diabetes compared with control. Both metformin and minocycline treatment improved the score significantly. Minocycline showed a differential effect indicated by no change in control animals but improvement of score in diabetic animals. Although there was no difference in individual scores of elevated body swing test (**B**) and forepaw grasp (**C**), forepaw grasp was improved in minocycline-treated animals. Mean ± s.e.m., $n = 6$ to 13, $^{*}P < 0.05$ versus C, $^{**}P = 0.05$ versus D, $^{***}P = 0.035$ disease/treatment interaction for minocycline, $^{****}P = 0.0031$ versus vehicle C or D. C, control; D, diabetes.

augmented ischemic injury in diabetes is limited. Although much emphasis is focused on neuronal damage after stroke, it is becoming clear that vasculature has an important role not only in the pathophysiology but also the recovery of ischemic brain injury (Li *et al*, 2009). We recently extended studies on diabetic stroke to a lean model of diabetes that presents with glucose levels (~200 mg/dL) that are comparable to levels seen in most acute ischemic stroke patients enrolled in various clinical trials (Bruno *et al*, 2008). Our studies have shown that cerebrovasculature undergoes extensive remodeling leading to increased tortuosity and neovascularization that is associated with increased MMP activity in early diabetes (Li *et al*, 2009). We also reported that when these animals are subjected to temporary focal ischemia, the occurrence rate of overt HT increases significantly but infarcts are smaller (Elewa *et al*, 2009; Ergul *et al*, 2007). Numerous reports also documented the importance of MMPs especially during the acute phase of ischemia in damaging the neurovascular unit (Lee *et al*, 2007; Lo, 2008). The resulting loss of its crucial barrier function leads to edema and extravasations of red blood cells into brain parenchyma particularly with prolonged periods of ischemia. If the MMP system is dysregulated as it occurs in diabetes, vascular wall integrity may be weakened setting the stage for an aggravated damage in case of stroke. The results of the current study show that chronic inhibition of MMPs by minocycline starting at the onset of diabetes prevents cerebrovascular remodeling and reduces HT incidence and severity. As acute activation of MMPs during ischemia is important for brain injury, in the current study we stopped minocycline treatment 3 days before surgery to allow for a wash-out period to separate the effect of acute MMP inhibition on ischemic injury from that on vascular remodeling and neovascularization. Although there was no change in MMP-9 protein levels in diabetes with chronic minocycline treatment, enzymatic activity on the ischemic and nonischemic hemispheres were abolished compared with untreated diabetic rats. A possible explanation is that 3-day withdrawal is not sufficient to eliminate the inhibitory effect of minocycline on MMPs. Further studies are needed to clarify this issue. Another interesting finding was that MMP-2 and MMP-9 activity patterns measured in the nonischemic side of stroked animals were different than those detected in nonstroked animals. We first compared MMP activity in the nonischemic hemispheres of control and diabetic animals to determine baseline differences, which showed higher MMP-9 but not MMP-2 activity in diabetes. However, when the same experiments were repeated in control and diabetic rats not subjected to MCAO, both MMP-2 and MMP-9 activities were increased in diabetic rats. These results suggest that MCAO procedure itself may affect the expression/activity patterns. These findings also highlight the possibility that the use of nonischemic side as a control may be limiting in stroke studies and sham animals will be a better control.

Minocycline, although a nonspecific MMPs inhibitor, was previously shown to inhibit cerebral MMP activity efficiently in experimental stroke (Lee *et al*, 2007; Machado *et al*, 2006). We also used it as it is a generic drug with a well-known safety profile and because there is an ongoing clinical trial Minocycline to Improve Neurologic Outcome to evaluate its use as a neurovascular protective agent. In addition to its MMP inhibitory effects, minocycline has antiinflammatory, antiapoptotic, and neuroprotective properties (Li and McCullough, 2009; Yrjanheikki *et al*, 1999). In the current study, minocycline-treated animals showed a small but significant decrease in infarct sizes as compared with vehicle-treated control and diabetic animals. Thus, in the ischemia model used in this study, minocycline was both neuroprotective and vasoprotective. It is possible that either reduction of bleeding because of MMP inhibition decreases infarct size or direct neuroprotective effects contribute to this finding.

An important feature of the cerebral circulation is the ability to regulate blood flow within a wide pressure range to maintain the nutrient and oxygen supply to the brain. The mechanism behind this autoregulatory capacity is the myogenic reactivity of vascular smooth muscle cell (Schubert and Mulvany, 1999). We wanted to study the effects of diabetes on this important functional feature of cerebral vessels. This is perceivable taking into account that structural wall alterations may affect the vascular wall function as well. This is especially true as previous studies showed different myogenic reactivity in different diabetes models (Cipolla *et al*, 1997; Jarajapu *et al*, 2008; Zimmermann *et al*, 1997). In the current study, the myogenic reactivity across the pressure range (40 to 120 mm Hg) was preserved in diabetic animals. Although there was an increase in myogenic tone at 80 mm Hg pressure, given that the lumen diameter is not different between control and diabetic rats at this pressure, it is unlikely to affect cerebral blood flow under normoxic conditions. However, it has to be recognized that we only measured myogenic reactivity and not neuronal or endocrine mechanisms that are involved in the regulation of vessel diameter. In addition, whether vascular reactivity is altered under hypoxic conditions remains to be determined. Unexpectedly, both metformin and minocycline treatment reduced the tone significantly compared with vehicle-treated animals, which deserves further investigation.

The chronic hyperglycemia present in diabetes ultimately leads to both microvascular and macrovascular remodeling changes. One of the goals behind glycemic control in diabetes is to prevent both these changes and hence prevent and reduce diabetes-dependant vascular events. It is well established that early and good glycemic control reduces the microvascular complications in both types of diabetes. However, the relation between glycemic control and

macrovascular events prevention has been only proven in type 1 diabetes (Akalin *et al*, 2009). The most recent major prospective randomized controlled clinical trial Action to Control Cardiovascular Risk in Diabetes was prematurely stopped because of the increased macrovascular events (Akalin *et al*, 2009; Dhar, 2009; Karalliedde and Gnudi, 2008; Kravetz and Federman, 2009; Lebovitz, 2008; Schatz, 2009; Skyler *et al*, 2009). However, ADVANCE (Action in Diabetes and Vascular Disease: Pretrax and Diamicron MR Controlled Evaluation) and VADT (Veterans Affairs Diabetes Trial) did not replicate the same findings (Buse *et al*, 2007; Chalmers *et al*, 2006; Duckworth *et al*, 2009). The latest consensus statement by the American Diabetes Association highlights that the long-term follow-up of DCCT and UKPDS showed that early glycemic control is associated with long-term reduction in macrovascular events and supports the overall benefits of glycemic control in reducing vascular events in addition to the importance of controlling other comorbidities (Skyler *et al*, 2009). Previous work from our group showed that glycemic control prevents microvascular remodeling in mesenteric bed (Sachidanandam *et al*, 2009). Yet, impact of glycemic control on cerebrovascular complications of diabetes remained unclear and the current study addressed this gap. It has to be recognized that metformin possesses some neuroprotective properties independent of its blood glucose lowering effect through its antioxidant and AMP-activated protein kinase stimulatory properties, which may have contributed to our findings (Correia *et al*, 2008; Poels *et al*, 2009).

In summary, our results provide evidence that diabetes-induced MMP-9 activity upregulation promotes cerebrovascular remodeling and affects vascular myogenic reactivity as well. This remodeling is associated with higher vascular damage after ischemia/reperfusion injury, which may explain at least in part why diabetic patients have a worsened injury compared with the nondiabetic population. Glycemic control is important to reduce vascular damage associated with ischemic brain injury. The clinical application of minocycline to reduce incidence and severity of HT as well as edema and infarct makes it a neurovasculoprotective agent. This is a safe and feasible strategy that can be used to benefit patients with diabetes who are at higher odds of ischemia/reperfusion injury.

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