NIH Public Access
Author Manuscript
Neurobiol Dis. Author manuscript; available in PMC 2012 July 1.

Published in final edited form as:
Neurobiol Dis. 2011 July ; 43(1): 285–292. doi:10.1016/j.nbd.2011.04.005.

Angiopoietin-Tie2 pathway mediates type 2 diabetes induced vascular damage after cerebral stroke

Xu Cui[a], **Michael Chopp**[a,b], **Alex Zacharek**[a], **Xinchun Ye**[a], **Cynthia Roberts**[a], and **Jieli Chen**[a,*]

[a] Department of Neurology, Henry Ford Hospital, Detroit, MI 48202, USA

[b] Department of Physics, Oakland University, Rochester, MI 48309, USA

Abstract

We investigated the changes and the molecular mechanisms of cerebral vascular damage after stroke in type-2 diabetic (T2DM) mice. Adult male db/db T2DM and wild-type (WT) mice were subjected to transient middle cerebral artery occlusion (MCAo) and sacrificed 24 hours after MCAo. T2DM-mice exhibited significantly increased blood glucose, brain hemorrhagic rate, mortality and cerebrovascular density, but decreased cerebrovascular diameter, arteriolar density and arterial mural cell numbers in the ischemic brain compared with WT-mice. The hemorrhagic rate was significantly correlated with the mortality (r=0.85). T2DM-mice also exhibited increased blood-brain barrier leakage and concomitantly, increased Angiopoietin2, but decreased Angiopoietin1, Tie2 and tight junction protein expression in the ischemic brain. Angiopoietin1 gene expression also significantly decreased in the common carotid artery (CCA) in T2DM-mice compared with WT-mice after stroke. To further test the effects of T2DM on cerebrovascular damage, we performed in vitro studies. The capillary-like tube formation of primary cultured mouse brain endothelial cells (MBECs) significantly increased, but artery cell migration in the primary CCA cultures significantly decreased both in Sham and MCAo T2DM-mice compared with the WT-mice. Angiopoietin1 treatment significantly increased artery cell migration in T2DMCCA after MCAo. Tie2-FC, a neutralized Tie2 antibody, significantly decreased artery cell migration in WT-CCA after MCAo. Therefore, decreased Angiopoietin1/Tie2 and increased Angiopoietin2 expression may contribute to diabetes-induced vascular damage after stroke.

Keywords

diabetes; vascular damage; angiopoietin; stroke; mice

Introduction

Diabetes mellitus (DM) is a major health problem associated with both microvascular and macrovascular disease and is a high risk factor for ischemic stroke (Basu et al., 2005).

© 2011 Elsevier Inc. All rights reserved.

* Correspondence to Jieli Chen, MD, Neurology Research, E&R Building, Room #3091, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202. jieli@neuro.hfh.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures
None.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Clinical studies show that hyperglycemia increases mortality and leads to poor functional recovery in both diabetic and nondiabetic patients after stroke (Capes et al., 2001). Hyperglycemia and DM increase the blood-brain barrier (BBB) permeability and infarct volume (Ennis and Keep, 2007; Mooradian et al., 2005) after stroke in rats. However, the molecular mechanisms underlying DM-induced vascular damage after stroke require clarification.

Angiopoietin-1 (Ang1) belongs to a family of endothelial growth factors, and promotes migration, sprouting, and survival of endothelial cells and mediates vascular remodeling through activation of signaling pathways triggered by the Tie2 tyrosine kinase receptor (Suri et al., 1996). Transgenic over-expression of Ang1 increases vascularization (Suri et al., 1998), prevents plasma leakage in the ischemic brain, and consequently decreases ischemic lesion volume (Zhang et al., 2002). An Ang1 peptide mimetic treatment accelerates wound healing in diabetes animals (Liu et al., 2008; Van Slyke et al., 2009). Angiopoietin-2 (Ang2), as an antagonist for Ang1, inhibits Ang1-promoted Tie2 signaling and decreases blood vessel maturation and stabilization. In a model of oxygen-induced retinopathy, Ang2 over-expression results in enhanced preretinal and intraretinal neovascularization (Feng et al., 2007). Increased Ang2 in the vitreous fluid is associated with angiogenic activity in patients with diabetic retinopathy (Watanabe et al., 2005). However, whether angiopoietins/Tie2 is involved in DM-induced vascular damage after ischemic brain stroke has not been investigated.

Previous studies show that type-2 DM (T2DM) rats [Goto-Kakizaki (GK)] have more bleeding than their normoglycemic controls (Wistar) after stroke (Elewa et al., 2009). There is significantly more frequent hematoma formation in the ischemic hemisphere and changes in vessel architecture in GK rats as opposed to controls, and these changes in blood vessels in the diabetic rats increase the risk for hemorrhagic transformation, possibly exacerbating neurovascular damage due to cerebral ischemia/reperfusion (Ergul et al., 2007). We reported that db/db T2DM mice exhibit significantly increased blood glucose, lesion volume, white matter damage, and have worse neurological outcome after stroke compared with non-DM mice (Chen et al., 2010). However, whether T2DM induces vascular damage in the ischemic brain and the molecular mechanisms underlying the vascular damage after stroke have not been investigated. In this study, we investigate the vascular damage in T2DM and non-DM wild type (WT) mice subjected to stroke. In addition, we test the hypothesis that the Ang1/Ang2/Tie2 signaling pathway contributes to vascular damage after stroke in T2DM mice.

Materials and Methods

All experiments were conducted in accordance with the standards and procedures of the American Council on Animal Care and Institutional Animal Care and Use Committee of Henry Ford Health System.

Middle cerebral artery occlusion model and experimental groups

A total of 45 adult male T2DM (BKS.Cg-*m* +/+ *Lepr^{db}/J,* db/db) mice and 35 adult male non-diabetic WT (m+/+ db) mice (2-3 months), purchased from Jackson Laboratory (Wilmington, MA) were employed in the present study. Four T2DM and 4 WT mice were randomly selected as Sham group. All other animals were subjected to transient (1 hour) right middle cerebral artery occlusion (MCAo) using the filament model, as previously described (Liu et al., 2007). Briefly, MCAo was induced by advancing a 6-0 surgical nylon suture (8.0-9.0 mm determined by body weight) with an expanded (heated) tip from the external carotid artery into the lumen of the internal carotid artery to block the origin of the MCA. Sham-operated animals underwent the same surgical procedure without suture insertion. All survival animals (23 T2DM and 23 WT mice) were sacrificed 24 hours after

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

MCAo. The animals were divided into four sets: the first set of MCAo mice (n=11/group) were used for histochemical and immunohistochemical staining, a second set of MCAo mice (n=4/group) were used for BBB leakage measurement, a third set of MCAo mice (n=4/group) were used for Western blot, angiogenic protein array and real time-PCR (RT-PCR) assays, and a fourth set of MCAo mice and all Sham-operated mice (n=4/group) were used for isolation of primary mouse brain microvascular endothelial cells (MBEC) and the common carotid artery (CCA).

Blood glucose measurement

Blood glucose was measured before and 24h after MCAo by using test strips for glucose (Polymer technology System, Inc. Indianapolis, IN 46268 USA).

Mortality rate

The number of dead animals in each group was counted 24h after MCAo (n=18, in T2DM group; n=8, in WT group) in the four sets of stroke animals, and the mortality rate is presented as a percentage of the total number of stroke animals (n=41, in T2DM group; n=31, in WT group).

Quantitative evaluation of Evans blue dye extravasation

2% Evans blue dye in saline was injected intravenously as a BBB permeability tracer at 1 hour before sacrifice. The entire ischemic hemisphere was collected for BBB leakage measurement. Evans blue dye fluorescence intensity was determined by a microplate fluorescence reader (excitation 620nm and emission 680nm). Calculations were based on the external standards dissolved in the same solvent. The amount of extravasated Evans blue dye was quantified as micrograms per ischemic hemisphere.

Histological and hemorrhagic assessment

The first set of mice (n=11/group) were sacrificed 24 hours after MCAo. The brains were fixed by transcardial-perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde before being embedded in paraffin for immunostaining. For calculation of brain hemorrhagic rate, all brains from dead animals (8 WT, 18 T2DM) were also immersed in 4% paraformaldehyde and embedded in paraffin. Using a mouse brain matrix (Activational Systems Inc., Warren, MI), the cerebral tissues were cut into seven equally spaced (1 mm) coronal blocks. For cerebral hemorrhage analysis, a series of adjacent 6 μm thick sections were cut from each block and stained with hematoxylin and eosin (HE). The HE staining section was analyzed under a 10X microscope. The hemorrhagic rate was calculated by the number of animals with hemorrhage divided by the total number of animals including those that died and survived. All analyses were performed by investigators blinded to the experimental groups.

Immunohistochemical staining

For immunostaining, a standard paraffin block was obtained from the center of the lesion (bregma −1mm to +1mm). A series of 6 μm thick sections were cut from the block. Every 10th coronal section for a total of 5 sections was used. Antibody against von Willebrand Factor [vWF, an endothelial cell (EC) marker, rabbit polyclonal IgG, 1:300, Dako, Carpenteria, CA, USA], alpha smooth muscle actin [αSMA, a marker of smooth muscle cell (SMC) and pericyte, mouse monoclonal IgG, 1:800, Dako], Ang1 (rabbit polyclonal IgG, 1:2,000, Abcam, Cambridge, MA, USA), and Ang2 (rabbit polyclonal IgG, 1:2,000, Abcam) imminostaining were employed. For Tie2 and Occludin (tight junction protein) immunofluorescent staining, the sections were directly incubated with the antibody against Tie2 (rabbit polyclonal IgG antibody, 1:80, Santa Cruz Biotechnology, Santa Cruz, CA,

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

USA) or Occludin (mouse monoclonal IgG antibody, 1:200, Zymed, San Francisco, CA, USA) conjugated with cyanine-3 (Cy3, 1:200, Jackson Immunoresearch Laboratories, West Groove, PA, USA). Control experiments consisted of staining brain coronal tissue sections as outlined above, but non-immune serum was substituted for the primary antibody.

For immunostaining measurement, five sections with each section containing 8 fields of view within the cortex and striatum from the ischemic boundary zone (IBZ) (Cui et al., 2009) were digitized using a 40X objective (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970MD) interfaced with an MCID computer imaging analysis system (Imaging Research, St. Catharines, Canada). The IBZ is defined as the area surrounding the lesion, which morphologically differs from the surrounding normal tissue. The immunostaining analysis was performed by an investigator blinded to the experimental groups.

Vascular density and diameter measurement

For measurement of vascular density and diameter, the total number of vWF-immunostained positive vessels was counted and divided by the total tissue-area to determine vascular density. The diameter of a total of 20 enlarged thin walled vessels and vascular density in the IBZ were measured in each referenced coronal section using the MCID computer imaging analysis system (Length Trace function).

Arteriolar density and αSMA-positive cell density measurement

αSMA immunoreactivity was employed to identify arterioles (Ho et al., 2006). αSMA was also used as a marker of vascular mural cells including vascular smooth muscle cells (VSMCs) and pericytes. The numbers of αSMA-positive arterioles in the IBZ and αSMA-positive cell numbers in the arterioles were counted. The density of αSMA-positive arterioles was analyzed with regard to small and large vessels (mean diameter ≥ 10 μm) in the IBZ, and the total number of αSMA-positive coated vessels per mm^2 area is presented. The number of αSMA-positive cells in artery wall was counted. Data are presented as the average number of a total 10 largest arteries in the IBZ.

Quantification of Ang1, Ang2, Occludin and Tie2 immunostaining

For quantitative measurement of Ang1, the number of Ang1-positive cells in each 40X field was counted. Data are presented as the number of Ang1-positive cells per 40X field. For measurement of Ang2, Tie2 and Occludin, the percentage of immunoreactive-positive area of Ang2, Tie2 or Occludin in the vessel wall was measured, respectively. Data are presented as the percentage of positive area to the vessel wall. Data were analyzed in a blinded manner.

Angiogenic protein array

The entire ipsilateral hemisphere of WT and T2DM mouse brains were collected 24 hours after MCAo for angiogenic protein array analysis. Briefly, brain tissue samples were suspended in Phosphate Buffered Saline (PBS) with 1 μl/ml protease inhibitor cocktail set III (Calbiochem, San Diego, CA, USA). Samples were sonicated then centrifuged to pellet for 5 min at 12,000 rpm. Supernatant was transferred to new tube and protein concentration was measured by BCA protein assay kit (Thermo Scientific). 200 μg of protein was used to run the assay following standard protocol for the R&D Proteome Profiler Antibody Array Mouse Angiogenesis Array Kit. Data were analyzed with the MCID image analysis system.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Primary artery cell culture and migration measurement

To investigate DM-induced arterial damage, and to further elucidate whether the mechanisms underlying DM-decreased artery cell migration after stroke is related to the Ang1/Tie2 pathway, a primary artery culture model was employed. The CCAs were surgically removed from Sham-WT, Sham-T2DM, and MCAo-WT and MCAo-T2DM mice 24 hours after surgery, respectively. The CCAs were divided into 6 groups as following: 1) Sham-WT; 2) Sham-DM; 3) MCAo-WT; 4) MCAo-DM; 5) MCAo-WT + Tie2-FC (recombinant mouse Tie2/FC, 2μg/ml, Chimera, R&D System, Cambridge, MA); 6) MCAo-DM + Ang1 (100 ng/ml, Chemicon, Temecula, CA). The CCA was placed in Matrigel. Arterial cultures were allowed to grow for 5 days before being photographed and the ten longest distances of outgrowth were measured under a microscope at 4X magnification, processed with the MCID and averaged. n = 6/group.

Primary MBEC culture and capillary-like tube formation assay

The entire brains from Sham-WT and Sham-DM mice and the entire ipsilateral hemisphere brains from MCAo-WT and MCAo-DM mice were collected 24 hours after MCAo. The brain tissue was isolated and digested in collagenase/dispase, and the microvessels separated by centrifugation in a Percoll (Sigma) gradient. Microvessels were seeded in flasks coated with rat-tail collagen and the medium was changed every 2-3 days. Capillary tube formation assay was performed. Briefly, 0.1 ml growth factor reduced Matrigel (Becton Dickinson) was added per well of a 96 well plate, and MBECs (2×10^4 cells) were incubated for 5 hours (n=6/group). For quantitative measurements of capillary tube formation, Matrigel wells were digitized under a 4X objective (Olympus BX40) for measurement of total tube length of capillary tube formation using a video camera (Sony DXC-970MD) interfaced with the MCID image analysis system at 5 hours. Tracks of MBECs organized into networks of cellular cords (tubes) were counted and averaged in randomly selected 3 microscopic fields. The total length of tube formation was quantitated.

RT-PCR

Tissues from the ischemic area of the ipsilateral hemisphere, the CCA, and primary cultured MBECs from both WT and T2DM mice were isolated 24 hours after MCAo. Total RNA was isolated using a standard protocol. Quantitative PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA) using 3-stage program parameters provided by the manufacturer. Each sample was tested in triplicate, and analysis of relative gene expression data using the $2^{-\Delta\Delta CT}$ method. The following primers for RT-PCR were designed using Primer Express software (ABI). **Ang1:** Fwd: TAT TTT GTG ATT CTG GTG ATT; Rev: GTT TCG CTT TAT TTT TGT AATG. **Ang2:** Fwd: GTC TCC CAG CTG ACC AGT GGG, Rev: TAC CAC TTG ATA CCG TTG AAC; **Tie2:** Fwd: CGG CCA GGT ACA TAG GAG GAA; Rev: TCA CAT CTC CGA ACA ATC AGC. **GAPDH:** Fwd: AGA ACA TCA TCC CTG CAT CC; Rev: CAC ATT GGG GGT AGG AAC AC.

Western blot assay

Equal amounts of brain-tissue from the ischemic area the ipsilateral hemisphere and the homologous tissue from the contralateral hemisphere lysate from WT and T2DM mice were subjected to Western blot analysis. Specific proteins were visualized using a SuperSignal West Pico chemiluminescence kit (Pierce). The primary antibodies were used: anti-Ang1 (1:1000 dilution, Abcam), anti-Ang2 (1:1000 dilution, Abcam), and anti-Tie2 (1:500 dilution, Santa Cruz), and anti-β-actin (1:2000; Santa Cruz) for 16 hours at 4°C. The membranes were washed with blocking buffer without milk, and then incubated with horseradish peroxidase-conjugated secondary antibody in blocking buffer.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Statistical Analysis

Two-way ANOVA was performed on data of the vascular density, vascular diameter, arteriolar density and αSMA-positive cell in arteries, percentage of positive area for Occludin, Ang1, Ang2 and Tie2 in the vessels, Ang1, Ang2 and Tie2 protein expression tested by Western blot in the ischemic brain, and primary artery cell migration and MBEC tube formation in vitro. If an overall treatment group effect was detected at $P < 0.05$, Tukey test after Post Hoc Test was used for multiple comparisons. Independent Samples T-Test was used for testing BBB leakage, Ang1 protein expression measured by angiogenic protein array, primary artery cell migration and Ang1/Ang2/Tie2 mRNA expression measured by RTPCR in the brain tissues and arterial cultures between two groups. Pearson partial correlations after Bivariate correlation were used to analyze the correlation of the brain hemorrhagic rate with the mortality. All data are presented as mean ± Standard Error (SE).

Results

T2DM mice increase blood glucose

T2DM mice significantly increased blood glucose (before MCAo: 396.9 ± 37.1; after MCAo: 402.4 ± 40.8mg/dl) levels compared to WT mice (before MCAo: 170.5 ± 11.5; after MCAo: 185.9 ± 13.8mg/dl, p<0.05), respectively.

T2DM mice exhibit increased cerebral hemorrhagic and mortality rate after stroke

The cerebral hemorrhagic rate in T2DM mice (14/29=48.3%) was significantly higher than that in WT mice (2/19=10.5%). The mortality in the T2DM mice (18/41=43.9%) was also significantly higher than that in the WT mice (8/31=25.8%). Moreover, the cerebral hemorrhagic rate is significantly correlated with mortality (P<0.05, r=0.85).

T2DM mice exhibit increased vascular damage in the ischemic brain after stroke

To test whether diabetes affects vascular change and BBB function, the Evans blue dye extravasation assay was employed to identify the change of BBB function, and vWF- (ECs), αSMA- (SMCs and pericytes) and tight junction protein- (Occludin) immunostaining were performed. The vascular density/diameter, arteriolar density and αSMA-positive cell numbers in the arteries were counted both in the IBZ and contralateral hemispheres. **Fig.1A-C** show that the BBB leakage significantly increased (p<0.05, n=4/group) in T2DM mice compared with WT mice 24 hour after MCAo. **Fig.1D-I** show that the vWF-vascular density was significantly increased, but the vWF-vascular diameter were significantly decreased in the ipsilateral hemisphere in T2DM mice compared with the WT mice after stroke (p<0.05, n=11/group). **Fig.1J-O** show that the αSMA-arteriolar density and αSMA-cell numbers in the arterial vessel wall both in the ipsilateral and contralateral hemisphere in T2DM mice were significantly decreased compared with the WT mice after stroke (p<0.05, n=11/group). Additional studies are warranted to investigate vascular changes in T2DM without stroke. **Fig.1P-T** show that T2DM mice has significantly decreased Occludin expression in vessels in the ipsilateral hemisphere compared with WT mice after stroke (p<0.05, n=11/group). These data indicate that both the BBB function and vascular structure were more damaged in T2DM mice than in WT mice after stroke.

T2DM mice show decreased Ang1/Tie2 and increased Ang2 expression in the ischemic brain after stroke

The Ang1/Tie2 system controls recruitment of pericytes and their precursors, and EC survival, and is implicated in blood vessel formation and vascular stabilization (Iurlaro et al., 2003; Metheny-Barlow et al., 2004). Ang2, as an antagonist of Ang1, is associated with endothelial apoptosis and BBB breakdown (Nag et al., 2005). To test the mechanism by

NIH-PA Author Manuscript

which diabetes induces vascular damage after stroke in the ischemic brain, Ang1, Tie2 and Ang2 gene and protein expression were measured. **Fig.2** shows that T2DM significantly decreased Ang1 and Tie2 expression, but increased Ang2 expression in the ischemic ipsilateral hemisphere measured by immunostaining compared to WT mice ($p<0.05$, n=11/group).

To confirm the immunostaining data, Western blot and angiogenic protein array were also employed. **Fig.3A** and **3B** show that T2DM mice significantly decreased Ang1 and Tie2 protein level, but increased Ang2 protein level in the ipsilateral hemisphere compared to WT mice ($p<0.05$, n=4/group). There is no significant difference in Ang1/Ang2 and Tie2 protein levels in the contralateral hemisphere. Using angiogenic protein array, **Fig.3C** shows that Ang1 protein level significantly decreased in both the ipsilateral and contralateral hemisphere in T2DM mice compared with WT mice ($p<0.05$, n=4/group). Using RT-PCR measurement, T2DM also significantly decreased Ang1 and Tie2 gene expression in the ipsilateral hemisphere compared with WT mice (**Fig.2D**, $p<0.05$, n=4/group).

T2DM mice show decreased primary artery cell migration in the CCA with or without stroke; Ang1/Tie2 pathway mediates DM-induced artery cell migration after stroke

To confirm the in vivo findings, an in vitro arteriogenesis study was performed using primary artery cell migration models. The CCAs derived from both DM and WT mice were cultured in vitro. **Fig.4A-C** show that the arterial cell migration significantly decreased in the CCA derived from Sham-DM mice compared with the CCA derived from Sham-WT mice. Similarly, **Fig.4D** and **4E** show that the artery cell migration in the CCA derived from MCAo-DM mice significantly decreased compared with the CCA derived from MCAo-WT mice. However, Tie2-FC treatment of arterial cells (**Fig.4F**) from the CCA obtained from the MCAo-WT group significantly decreased arterial cell migration compared to arterial cells from the CCA obtained from the MCAo-WT control group ($p<0.05$, n=6/group). **Fig. 4G** shows that Ang1 treatment significantly increased artery cell migration in DM-CCA after MCAo compared with non-treatment DM-CCA control group. In addition, Ang1 gene expression measured by RT-PCR significantly decreased in T2DM ipsilateral CCA compared with WT ipsilateral CCA after stroke (**Fig.4I**, $p<0.05$, n=4/group). These data suggest that the decreased arteriogenesis in T2DM mice after stroke are related to the Ang1/Tie2 pathway.

T2DM mice show increased angiogenesis with or without stroke

To confirm the in vivo angiogenesis findings, in vitro angiogenesis assays were performed using primary capillary tube formation models. The MBECs derived from both T2DM and WT mice with or without MCAo were cultured in vitro. **Figure 5** shows that the capillary tube formation was significantly increased in T2DM-MBECs compared with WT-MBECs both in the Sham and in the MCAo groups ($p<0.05$, n=6/group).

Discussion

In this study, we found that T2DM mice show significantly increased mortality, brain hemorrhagic rate, vascular damage and decreased BBB function at 24 hour after stroke. The increased brain hemorrhage is correlated with mortality after stroke. T2DM mice show significantly increased Ang2, but exhibit decreased Ang1/Tie2 expression in the ischemic brain compared to WT mice after stroke. The Ang1/Ang2/Tie2 signaling pathway may contribute to the vascular destabilization and BBB dysfunction after stroke in T2DM mice.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

T2DM mice show increased vascular damage and decreased BBB function after stroke

Targets for enhancement of angiogenesis are being considered to improve functional outcome after stroke (Zhang and Chopp, 2009). However, pathological angiogenesis may worsen functional outcome after stroke. In addition, vascular stabilization and maturation is important in functional angiogenesis. Angiogenesis is an essential biological process in the progression of diabetes. Disequilibrium of angiogenesis promoters and inhibitors in DM can lead to exuberant but dysfunctional neovascularization, as seen in the diabetic retina, as well as vascular destabilization, as observed in skeletal and cardiac muscle, thus supporting a high degree of heterogeneity of diabetic vascular pathology (Ebrahimian et al., 2005; Weihrauch et al., 2004). In this study, we are the first to find that T2DM (db/db) mice exhibit a pathological increase in angiogenesis and BBB dysfunction as measured by the increased cerebral vascular/arteriolar density and MBEC capillary-like tube formation, but decreased vascular diameter and tight junction protein in the ischemic brain vessels compared with WT mice after stroke. Our findings that T2DM (db/db) mice exhibit increased vascular density are consistent with data, in which, T2DM (Goto-Kakizaki) rats also show an increased vessel density in the ischemic brain after MCAo (Li et al., 2010).

Vascular remodeling is a complex phenomenon associated with restructuring of the vessel wall as a consequence of disruption of vascular homeostasis. Development of coronary collateral vessels (arteriogenesis) is impaired in diabetic patients (Weihrauch et al., 2004). Altered remodeling of arterial collaterals as well as de novo vascularization plays a role in impaired recovery from ischemia in DM (Waltenberger, 2001). The vascular wall is mainly composed of ECs and mural cells (pericytes and VSMCs). Progressive dysfunction and death of VSMCs and pericytes is a pathophysiological hallmark of diabetic retinopathy (vom Hagen et al., 2005). VSMC recruitment and SMC coverage in the neovessels of the border zone of infarcted myocardium are severely reduced and are accompanied by decreased arteriole formation in db/db mice (Chen and Stinnett, 2008a; Chen and Stinnett, 2008b). Consistent with these findings, in the present study, we found that T2DM mice show significantly decreased αSMA-positive (a marker of VSMC and pericyte) cell numbers in the artery walls both in the ischemic ipsilateral and contralateral and decreased artery cell migration in the CCA compared with WT mice with or without stroke. Therefore, T2DM mice decreased mural cell recruitment may contribute to pathological cerebral vasculogenesis and also disrupt the integrity of the BBB (Badillo et al., 2007).

Angiopoietin/Tie2 activity may contribute to the increased vascular damage after stroke in T2DM mice

Angiopoietins (Ang1 and Ang2) and their receptor, Tie2, play a role in vascular integrity and neovascularization in DM. Hyperglycemia may suppress Ang1-induced vascular protection and provoke endothelial dysfunction and vascular disease (Singh et al., 2010). Hyperglycemia-enhanced ischemic brain damage in mutant manganese superoxide dismutase mice is associated with suppression of hypoxia-inducible factor-1alpha (HIF-1α) (Bullock et al., 2009). Tie2 expression was significantly attenuated, whereas Ang2 was increased in db/db mice subjected to myocardial ischemia (Chen and Stinnett, 2008b). Overexpression of Ang2 in the retina enhances vascular pathology, indicating that Ang2 plays an essential role in diabetic vasoregression via destabilization of pericytes (Pfister et al., 2010). Intravitreal injection of Ang2 in rats produced a significant increase in retinal vascular permeability (Rangasamy et al., 2011). Dysregulation of the angiopoietins/Tie2 system result in an impairment of VSMC recruitment and vascular maturation, which contributes to impaired angiogenesis in db/db diabetic mice after myocardia ischemia (Chen and Stinnett, 2008b).

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

The Ang1, Ang2/Tie2 system modulates EC, VSMC and pericyte recruitment (Chen and Stinnett, 2008b; Pfister et al., 2008). The impaired VSMC recruitment and vessel outgrowth were rescued with Ang1 gene therapy (Chen and Stinnett, 2008b). Ang1 gene therapy inhibits HIF-1α-prolyl-4-hydroxylase-2, stabilizes HIF-1α expression, and normalizes immature vasculature in db/db mice (Chen and Stinnett, 2008a). In the present study, we find that T2DM mice show a significantly decreased Ang1/Tie2, but increased Ang2 expression in the cerebral vascular walls in the ischemic brain and arteries after stroke. In vitro study shows that Tie2-FC significantly decreased WT-CCA arterial cell migration, but Ang1 treatment significantly increased T2DM-CCA arterial cell migration after stroke. These data support the hypothesis that the increased Ang2 and the decreased Ang1/Tie2 in T2DM mice contribute to the vascular damage and decreased BBB function in the ischemic brain. Therefore, targeting the Ang1/Ang2/Tie2 signaling pathways may have a beneficial effect in decreasing retinal vascular permeability in patients with diabetic retinopathy and decreasing angiogenic activity and vascular permeability in the DM patients. In addition, decreasing angiogenesis will decrease pathological angiogenesis-associated BBB dysfunction and may improve functional outcome in the diabetes population with stroke.

In summary, T2DM mice have increased vascular damage in the ischemic brain compared with WT mice. Ang1/Ang2/Tie2 signaling pathways may contribute to diabetes-induced vascular damage in the ischemic brain after stroke. Our results have important implications for the clinical treatment of diabetic retinopathy, diabetic micro/macroangiopathy and diabetes patients with ischemic stroke.

Acknowledgments

The authors thank Qinge Lu and Sutapa Santra for technical assistance.

Sources of Funding

This work was supported by National Institute on Aging RO1 AG031811 (J.C.), National Institute of Neurological Disorders and Stroke PO1 NS23393 (M.C.), and 1R41NS064708 (J.C.), and American Heart Association grant 09GRNT2300151 (J.C.).

References

Badillo AT, et al. Treatment of diabetic wounds with fetal murine mesenchymal stromal cells enhances wound closure. Cell Tissue Res. 2007; 329:301–11. [PubMed: 17453245]

Basu AK, et al. Risk factor analysis in ischaemic stroke: a hospital-based study. J Indian Med Assoc. 2005; 103:586, 588. [PubMed: 16570759]

Bullock JJ, et al. Hyperglycemia-enhanced ischemic brain damage in mutant manganese SOD mice is associated with suppression of HIF-1alpha. Neurosci Lett. 2009; 456:89–92. [PubMed: 19429140]

Capes SE, et al. Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: a systematic overview. Stroke. 2001; 32:2426–32. [PubMed: 11588337]

Chen J, et al. White matter damage and the effect of matrix metalloproteinases in type 2 diabetic mice after stroke. Stroke. 2011; 42:445–52. [PubMed: 21193743]

Chen JX, Stinnett A. Ang-1 gene therapy inhibits hypoxia-inducible factor-1alpha (HIF-1alpha)-prolyl-4-hydroxylase-2, stabilizes HIF-1alpha expression, and normalizes immature vasculature in db/db mice. Diabetes. 2008a; 57:3335–43. [PubMed: 18835934]

Chen JX, Stinnett A. Disruption of Ang-1/Tie-2 signaling contributes to the impaired myocardial vascular maturation and angiogenesis in type II diabetic mice. Arterioscler Thromb Vasc Biol. 2008b; 28:1606–13. [PubMed: 18556567]

Cui X, et al. Nitric oxide donor up-regulation of SDF1/CXCR4 and Ang1/Tie2 promotes neuroblast cell migration after stroke. J Neurosci Res. 2009; 87:86–95. [PubMed: 18711749]

Ebrahimian TG, et al. Dual effect of angiotensin-converting enzyme inhibition on angiogenesis in type 1 diabetic mice. Arterioscler Thromb Vasc Biol. 2005; 25:65–70. [PubMed: 15528473]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Elewa HF, et al. Early atorvastatin reduces hemorrhage after acute cerebral ischemia in diabetic rats. J Pharmacol Exp Ther. 2009; 330:532–40. [PubMed: 19478137]

Ennis SR, Keep RF. Effect of sustained-mild and transient-severe hyperglycemia on ischemia-induced blood-brain barrier opening. J Cereb Blood Flow Metab. 2007; 27:1573–82. [PubMed: 17293843]

Ergul A, et al. Increased hemorrhagic transformation and altered infarct size and localization after experimental stroke in a rat model type 2 diabetes. BMC Neurol. 2007; 7:33. [PubMed: 17937795]

Feng Y, et al. Impaired pericyte recruitment and abnormal retinal angiogenesis as a result of angiopoietin-2 overexpression. Thromb Haemost. 2007; 97:99–108. [PubMed: 17200776]

Ho TK, et al. Increased angiogenic response but deficient arteriolization and abnormal microvessel ultrastructure in critical leg ischaemia. Br J Surg. 2006; 93:1368–76. [PubMed: 16952207]

Iurlaro M, et al. Rat aorta-derived mural precursor cells express the Tie2 receptor and respond directly to stimulation by angiopoietins. J Cell Sci. 2003; 116:3635–43. [PubMed: 12876214]

Li W, et al. Adaptive cerebral neovascularization in a model of type 2 diabetes: relevance to focal cerebral ischemia. Diabetes. 2010; 59:228–35. [PubMed: 19808897]

Liu L, et al. Age-dependent impairment of HIF-1alpha expression in diabetic mice: Correction with electroporation-facilitated gene therapy increases wound healing, angiogenesis, and circulating angiogenic cells. J Cell Physiol. 2008; 217:319–27. [PubMed: 18506785]

Liu XS, et al. Comparison of in vivo and in vitro gene expression profiles in subventricular zone neural progenitor cells from the adult mouse after middle cerebral artery occlusion. Neuroscience. 2007; 146:1053–61. [PubMed: 17428613]

Metheny-Barlow LJ, et al. Direct chemotactic action of angiopoietin-1 on mesenchymal cells in the presence of VEGF. Microvasc Res. 2004; 68:221–30. [PubMed: 15501241]

Mooradian AD, et al. Statins ameliorate endothelial barrier permeability changes in the cerebral tissue of streptozotocin-induced diabetic rats. Diabetes. 2005; 54:2977–82. [PubMed: 16186401]

Nag S, et al. Increased angiopoietin2 expression is associated with endothelial apoptosis and blood-brain barrier breakdown. Lab Invest. 2005; 85:1189–98. [PubMed: 16056241]

Pfister F, et al. Pericyte migration: a novel mechanism of pericyte loss in experimental diabetic retinopathy. Diabetes. 2008; 57:2495–502. [PubMed: 18559662]

Pfister F, et al. Retinal overexpression of angiopoietin-2 mimics diabetic retinopathy and enhances vascular damages in hyperglycemia. Acta Diabetol. 2010; 47:59–64. [PubMed: 19238311]

Rangasamy S, et al. A Potential Role for Angiopoietin 2 in the Regulation of the Blood-Retinal Barrier in Diabetic Retinopathy. Invest Ophthalmol Vis Sci. 2011 in press.

Singh H, et al. High glucose and elevated fatty acids suppress signaling by the endothelium protective ligand angiopoietin-1. Microvasc Res. 2010; 79:121–7. [PubMed: 20079751]

Suri C, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell. 1996; 87:1171–80. [PubMed: 8980224]

Suri C, et al. Increased vascularization in mice overexpressing angiopoietin-1. Science. 1998; 282:468–71. [PubMed: 9774272]

Van Slyke P, et al. Acceleration of diabetic wound healing by an angiopoietin peptide mimetic. Tissue Eng Part A. 2009; 15:1269–80. [PubMed: 18939935]

vom Hagen F, et al. Early loss of arteriolar smooth muscle cells: more than just a pericyte loss in diabetic retinopathy. Exp Clin Endocrinol Diabetes. 2005; 113:573–6. [PubMed: 16320154]

Waltenberger J. Impaired collateral vessel development in diabetes: potential cellular mechanisms and therapeutic implications. Cardiovasc Res. 2001; 49:554–60. [PubMed: 11166268]

Watanabe D, et al. Vitreous levels of angiopoietin 2 and vascular endothelial growth factor in patients with proliferative diabetic retinopathy. Am J Ophthalmol. 2005; 139:476–81. [PubMed: 15767056]

Weihrauch D, et al. Chronic hyperglycemia attenuates coronary collateral development and impairs proliferative properties of myocardial interstitial fluid by production of angiostatin. Circulation. 2004; 109:2343–8. [PubMed: 15136506]

Zhang ZG, Chopp M. Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. Lancet Neurol. 2009; 8:491–500. [PubMed: 19375666]

Zhang ZG, et al. Angiopoietin-1 reduces cerebral blood vessel leakage and ischemic lesion volume after focal cerebral embolic ischemia in mice. Neuroscience. 2002; 113:683–7. [PubMed: 12150788]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

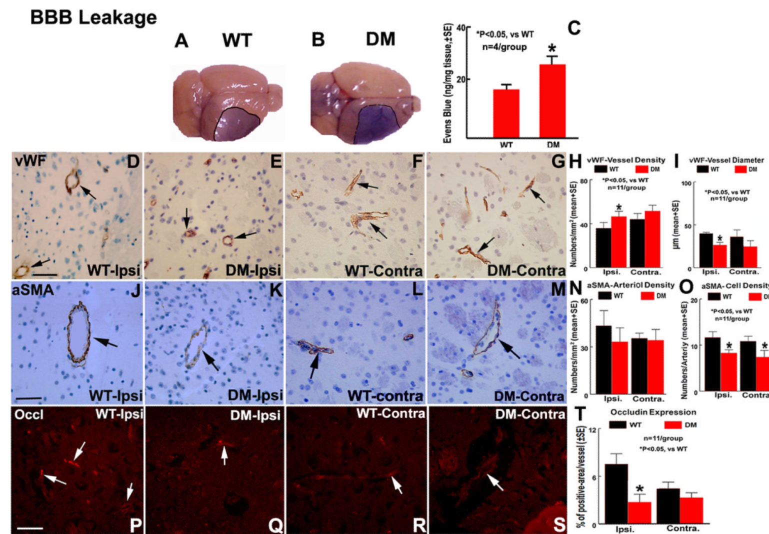

Fig.1.

T2DM increase BBB leakage and vascular density, decrease vascular/arteriolar diameter, arteriolar wall mural cell number, and decrease tight junction protein expression in the ischemic brain after stroke. **A-C:** BBB leakage and quantitative data measured by Evens Blue dye perfusion and extravasation, n=4/group; **D-I:** vWF immunostaining and quantitative data; **J-O:** αSMA immunostaining and quantitative data; **P-T:** Occludin immunofluorescent staining and quantitative data. n = 11/group, Scale bar in D, J and P = 40 μm.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

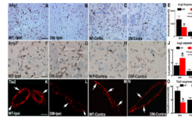

Fig.2.
T2DM decrease Ang1 and Tie2, increase Ang2 expression in the ischemic brain after stroke measured by immunostaining. **A-E:** Ang1 immunostaining and quantitative data; **F-J:** Ang2 immunostaining and quantitative data; **K-O:** Tie2 immunofluorescent staining and quantitative data. Scale bar in A = 40 μm, in F = 20 μm, in K = 50 μm. n = 11/group.

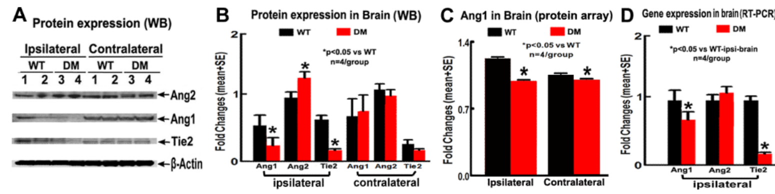

Fig.3.

T2DM decrease Ang1 and Tie2, but increase Ang2 expression in the ischemic brain after stroke measured by Western blot, protein array and RT-PCR. **A** and **B:** Ang1, Ang2 and Tie2 protein expression in the ischemic ipsilateral and contralateral brain (A) and quantitative data (B) measured by Western blot assay; **C:** Ang1 expression in the ischemic ipsilateral and contralateral brain measured by protein array; **D:** Ang1, Ang2 and Tie2 gene expression in the ischemic ipsilateral brain measured by RT-PCR. n = 4/group.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

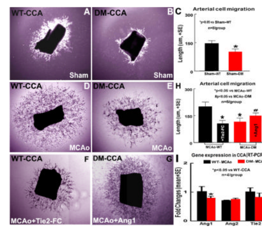

Fig.4.

T2DM decrease artery cell migration in the primary cultured artery both in Sham and MCAo group; Tie2-FC attenuate artery cell migration in WT-CCA; Ang1 treatment increase T2DM-artery cell migration after stroke. **A-C:** primary artery cell migration in CCA derived from Sham WT (A) and DM mice (B), and quantitative data (C); **D-H:** primary artery cell migration in CCA derived from WTMCAo (D), DM-MCAo (E), WT-MCAo + Tie2-FC 2 μg/ml (F), DM-MCAo + Ang1 100 ng/ml (G), and quantitative data (H); **I:** Ang1, Ang2 and Tie2 gene expression in the CCA derived from WT-MCAo and DM-MCAo measured by RT-PCR. n = 6/group.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

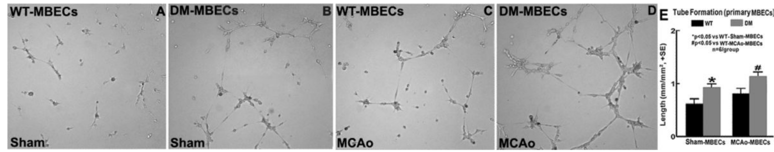

Fig.5.

T2DM increase capillary-like tube formation in the primary cultured MBEC both in Sham and MCAo group. **A:** WT-Sham, **B:** DM-Sham, **C:** WT-MCAo, **D:** DM-MCAo, **E:** quantitative data of tube formation. n = 6/group.