Diabetic microangiopathy: impact of impaired cerebral vasoreactivity and delayed angiogenesis after permanent middle cerebral artery occlusion on stroke damage and cerebral repair in mice

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Abstract

Diabetes increases the risk of stroke by 3, increases related mortality and delays recovery. We aimed to characterize functional and structural alterations in cerebral microvasculature before and after experimental cerebral ischemia in a mouse model of type 1 diabetes. We hypothesized that pre-existing brain microvascular disease in diabetics might partly explain increased stroke severity and impact on outcome.

Diabetes was induced in 4-week-old C57Bl/6J mice by intraperitoneal injections of streptozotocin (60 mg/kg). After 8 weeks of diabetes, the vasoreactivity of the neurovascular network to CO$_2$ was abolished, and was not be reversed by NO-donor administration; eNOS and nNOS mRNA, phospho-eNOS protein, nNOS and phospho-nNOS protein were significantly decreased; angiogenic and vessel maturation factors (VEGFa, Ang1, Ang2, TGFβ and PDGFβ) and blood-brain barrier occludin and ZO-1 expression were significantly decreased; microvessel density was increased without changes in ultrastructural imaging. After permanent focal cerebral ischemia induction, infarct volume and neurological deficit were significantly increased at D1 and D7, neuronal death (TUNEL+/NeuN+ cells) and blood-brain barrier permeability (extravasation of Evans blue) at D1. At D7, CD31+/Ki67+ double-immunolabeled cells, and VEGFa and Ang2 expression were significantly increased indicating delayed angiogenesis. We show that cerebral microangiopathy thus partly explains stroke severity in diabetes.

Keywords: Diabetes, stroke, cerebral ischemia, CO$_2$-vasoreactivity, angiogenesis

Nonstandard abbreviations used: BBB (Blood Brain Barrier), Nitric Oxide (NO), NO synthase (NOS), endothelial NOS (eNOS), neuronal NOS (nNOS), Cerebral Blood Flow (CBF), streptozotocin (STZ), mean blood flow velocity (mBFV), Permanent focal cerebral ischemia occlusion (pMCAo)
Diabetes is an independent risk factor for stroke, tripling its incidence, and affecting stroke severity (1) and related mortality (2). While stroke is classically considered as a macrovascular complication of diabetes due to accelerated atherosclerosis and carotid artery disease, there is increasing evidence that microvasculature of the brain is also severely affected in diabetic patients. Indeed, in addition to diffuse brain atrophy, leukoaraiosis, microbleeds and asymptomatic lacunar infarcts (<15 mm in diameter, often multiple) have been reported on brain MRI or in post-mortem studies (1). Moreover, the prevalence and severity of other microvascular diabetic complications such as retinopathy and proteinuria are correlated with increased stroke risk (1). Functional cerebral microcirculation such as decreased baseline regional cerebral blood flow (CBF) and impaired vasoreactivity to CO\textsubscript{2} have been shown in diabetic patients (3). Yet, vessel diameter is a key determinant of stroke extension. Under normal physiological conditions, vessel diameter is regulated by several pathways that ensure sufficient blood flow. After cerebral artery occlusion, the consecutive drop in local CBF is compensated for by collateral artery recruitment and vasodilation, which limit lesion extension, a phenomenon mediated by Nitric oxide (NO) (4,5). Later on, angiogenesis initiated during the first three days after brain injury participates in brain recovery by triggering neurogenesis and the migration of neural progenitors to the infarct (6).

The superimposition of a secondary injury on pre-existing cerebral microvascular disease could have deep effects on neurological function. However, the microvascular “response” during the acute and subacute phases of stroke in diabetics remains unstudied.

Our working hypothesis is that chronic hyperglycemia will result in dysfunctional cerebral microcirculation that in turn will contribute to enhance brain injury after focal cerebral ischemia, during the acute phase by compromising local collateral artery recruitment, an important step in tissue preservation, and during the subacute phase by compromising angiogenesis, an important step in brain repair. We first characterized functional and structural alterations in cerebral microvasculature in an experimental model of type-1 diabetes induced by streptozotocin (STZ) in adult male C57Bl/6J mice, and then evaluated the effects of experimental focal cerebral ischemia by permanent middle cerebral artery occlusion (pMCAo) one day and one week after stroke.
RESEARCH DESIGN AND METHODS

All experiments using animals were performed according to NIH guidelines for the care and use of laboratory animals (B 75-10-03). The study was specifically approved by our local institutional ethics committee (CEEALV/2012-05-01). All experiments were performed by investigators blind to the diabetic status of mice.

**Animals and diabetes induction.** Male C57Bl/6J mice (Janvier, France) aged 4 weeks with a mean body weight of 18-20 g were divided into two groups: one group received 5 consecutive daily intraperitoneal injections of STZ (60mg/kg in 100µl of citrate buffer) to induce diabetes, while the non-diabetic group received only buffer citrate. Glycemia was tested weekly for 8 weeks. Mice with sustained hyperglycemia (>300mg/dL) were considered to be diabetic.

**Distribution of groups.** All experiments were performed at the age of 12 weeks, 8 weeks following diabetes induction. We first studied functional and structural impairment of microcirculation in chronic diabetic mice before cerebral ischemia (A): we measured vasoreactivity to CO₂ and NO, characterized the expression of NO synthases and brain factors involved in vessel regulation using PCR, western blotting and immunohistochemistry, and performed Transmission Electron Microscopy (TEM) of cerebral capillaries and arterioles. Brain MRI was performed before cerebral ischemia in order to detect asymptomatic microvascular lesions. Next, we studied the impact of diabetic microangiopathy on stroke damage and cerebral repair (B): we evaluated infarct volume (cresyl violet staining and brain MRI), neurological deficit, and angiogenesis at D1 and D7. Blood-Brain-Barrier (BBB) proteins modification was measured at D1 (Fig.1).

**Physiological parameters.** Systolic blood pressure was measured in unanesthetized mice by the tail-cuff method (Kent Scientific Corporation, Torrington, USA) after daily acclimatization for 2 weeks.

**Doppler imaging of vasoreactivity to inhaled CO₂.** Thermoregulated mice were subjected to ultrasound measurements under 0.5% isoflurane anesthesia using an echocardiograph (Acuson S 3000, Erlangen, Germany) equipped with a 14-MHz linear transducer (14L5 SP) as previously reported (4). Heart rate, peak systolic, end-diastolic and time-averaged mean blood flow velocities (mBFVs) were measured in the basilar trunk (BT) before cerebral ischemia 1)
under air (all mice), 2) 5 min after starting to breathe a gas mixture of 16% O\textsubscript{2}, 5% CO\textsubscript{2}, 79% N\textsubscript{2} (n=7-10), or 3) 5 min after administration of an NO donor (NONOate, Sigma, France) (n=4-5) (1mg/kg, i.p.), in order to discriminate between endothelial and smooth muscle cell (SMC) dysfunction. Indeed, vasodilation is reestablished by the NO donor if endothelium only is dysfunctional. Vasoreactivity was estimated for each mouse as the percentage increase in mBFV recorded under gas mixture or NONOate compared to mBFV recorded under normoxic air; acidosis and hypercapnia were assessed by blood gas measurements.

**Real-time PCR analysis of mRNAs for Nitric Oxide Synthase (NOS) isoforms, factors regulating cerebral vessel and blood brain barrier (BBB) components.** The procedure was performed as previously described (n=5-8) (7) using custom-designed primers for all genes of interest (Table 1) and a ready-to-use primer for the housekeeping gene peptidylprolyl isomerase A (cyclophilin A) (Qiagen, France). We normalized the results for each gene to cyclophilin A levels. Results are expressed as arbitrary units (AU).

**Western blot analysis of NOS isoforms, factors regulating cerebral vessel and BBB components.** Animals (n=5-7) were sacrificed and brains rapidly dissected out on a cold plate. Cytosolic proteins were extracted (5), and equal amounts of proteins (40 µg) were resolved by SDS-PAGE electrophoresis and immunoblotted using the antibodies indicated in Table 1. Blots were scanned and analyzed using ImageJ (NIH, Bethesda, MD, USA).

**Permanent focal cerebral ischemia by middle cerebral artery occlusion (pMCAo) and infarct volume assessment.** Briefly, as previously described (7), thermoregulated (37±0.5°C) and anesthetized (2% isoflurane in air) mice were subjected to left middle cerebral artery electrocoagulation (pMCAo) using bipolar forceps. The overall mortality was less than 5% in 24 hours. Cortical infarct volume was evaluated on cresyl-violet-stained sections (every 8\textsuperscript{th} 30 µm-thick coronal section) using NIH ImageJ (n=5-9).

**Assessment of neurological deficit.** Neurological deficit was assessed 24 hours after pMCAo, based on 5 neurological tests: neurological score, grip and string tests, beam walking and the pole test, and used to calculate a global neurological score (/21) (n=7-9). The lower the neurological score, the more severe the deficit (8).
**Evaluation of BBB permeability.** A 2% solution of Evans Blue dye (Sigma-Aldrich Inc., St Louis, MO, USA) in PBS was administered through the tail vein of mice (4mL/kg) 24 hours after stroke (n=5-6). Two hours later, animals were transcardially perfused under isoflurane anesthesia, with 20 mL of 1% heparinized saline, until a colorless perfusion fluid was obtained. Each hemisphere was placed separately in 2 mL of formamide and allowed to soak for 72h at room temperature. The absorbance of the supernatant solution was measured against a pure formamide standard at 625nm using a spectrophotometer. The tissue was then dried in the oven at 95°C for 5 days (6), and relative absorbance measured (units/g dry weight). Results are expressed as the percentage of the value measured in non-diabetic mice (100%).

**Immunohistochemistry and morphological analysis.** Coronal 30µm-thick floating sections were incubated with primary antibody overnight at 4°C: anti-Ki67 (Thermo Fisher, USA, 1:200) and anti-CD31 (BD Biosciences, USA, 1:200) were used to detect proliferating cells and endothelial cells respectively, and double immunolabeling to detect angiogenesis (n=5-6). Appropriate Alexa Fluor 594 or 488-labeled secondary antibodies (Molecular Probes, Oregon, USA, 1:400) were applied for 1h at room temperature. Specificity was checked by omitting the primary antibody. Cell counts and microvessel density measurements were performed at 3 coronal brain levels, +0.80mm, -0.80mm and -1.20mm relative to bregma, that consistently contained the infarct area. Angiogenesis was assessed in 3 regions of interest (ROIs; 0.06mm² each) located in the peri-infarct area, and expressed as the average number of Ki67+/CD31+ cells per ROI. Microvascular density was evaluated after CD31 immunolabeling by calculating the integrated pixel density of the images using NIH ImageJ. The average integrated pixel density of one field of view (20×) located in the peri-infarct area was used for analysis.

**Transmission electron microscopy (TEM).** Mice (n=4) were transcardially perfused for 12 min with a fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde and 2mM CaCl2 in 0.1M cacodylate buffer (CB), pH 7.4 at room temperature (n=4). Brains were removed and 1mm³ brain fragments were post-fixed in the same fixative for 1 h at 4°C, extensively washed in CB and fixed in 1% OsO₄ in CB for 45min at 4°C. After washing in CB, samples were fixed in 1% aqueous uranyl acetate and finally rinsed in water. After dehydration in graded
ethanol, followed by propylene oxide, the fragments were embedded in Epon. Ultrathin (80nm) sections were prepared, stained with lead citrate and photographed with a Jeol S100 TEM equipped with a 2kx2k Orius 200-830 camera (Gatan-Roper Scientific, Evry, France).

**Magnetic Resonance Imaging (MRI).** Brain MRI was performed before and at D1 and D7 after cerebral ischemia, in order to detect pre- and post-stroke (secondary hemorrhagic transformation) hemorrhages in the mouse brain. All MR examinations were performed using a 7.0Tesla MR unit (PharmaScan, Bruker Biospin, Ettlingen, Germany) equipped with a surface coil with an internal diameter of 10mm, in thermoregulated and anesthetized (0.8-1% isoflurane in O₂ 30%/N₂O 70%) mice (n=5-6). Infarct volume was measured on T2-weighted images according to the RARE sequence type parameters; TR=6475.1 ms; TE=8894 ms; effective TE=53.36 ms; field of view=20x20 mm; matrix=128x128; slice thickness=0.5 mm (0.156x0.156=voxelx0.5 mm3); flip angle=180°; RARE factor=16; number of averages=2. Maximal gradient slope was 300mT/m. Gradient-echo T2-weighted images (T2-star) were obtained to evaluate the presence of hemorrhagic spots with the following parameters: TR=260 ms; TE=6 ms; field of view=20x20 mm; matrix =256x256; slice thickness=0.75 mm; flip angle=55°; number of averages=2. The brain of each mouse was imaged before and after an intraperitoneal injection of 0.1-mmol/kg gadopentetate dimeglumine (Gd-DTPA; Magnevist; Berlex Laboratories, Wayne, NJ) with a final set of post-contrast T1-weighted images with the following parameters: spin-echo sequence; TE=10 ms; TR=500 ms; FOV=20x20 mm; matrix=256x256; 12 slices; slice thickness=0.75 mm; total imaging time=2 min.

**Statistical analysis.** Statistical analyses were performed with Prism 5 software (Prism 5.03, GraphPad, San Diego, USA). Data are expressed as mean±standard deviation (SD). Comparisons between non-diabetic and diabetic mouse groups were carried out using the non-parametric Mann-Whitney U test. A P-value of <0.05 was considered statistically significant.
RESULTS

Mean blood glucose concentration (424±54 vs 161±10 mg/dl; \( P<0.001 \)) was significantly increased and body weight (23.7±1.1 vs 21.08±2.4 g, \( P=0.003 \)) significantly decreased in diabetic mice compared to non-diabetic mice. At 12 weeks, systolic, diastolic and mean arterial pressures were not significantly different between groups. Blood gas after CO\(_2\) inhalation showed significant acidosis and normoxic hypercapnia in both groups compared to measurements under air (Table 2).

A) Functional and structural impairment of microcirculation in chronic diabetic mice before experimental ischemia

**CO\(_2\) and NO-donor-mediated vasoreactivity are impaired in diabetic mice.** Inhalation of a CO\(_2\) gas mixture resulted in significant hypercapnia (\( P=0.017 \)) and acidosis (\( P=0.006 \)) in diabetic and non-diabetic mice (Table 2); mBFV in the BT was not significantly modified after CO\(_2\) inhalation in diabetic mice (air: 100±14.5% vs. CO\(_2\): 98.8±11.9%; \( P=1 \)), whereas mBFV in the BT was significantly increased in non-diabetic mice (air: 100±6.4% vs. CO\(_2\): 124.2±24.2%; \( P=0.0004 \)) (Fig.2A). After NO-donor injection, the BT mBFV of diabetic mice was not significantly modified (air: 100±13.8% vs. NO: 90.2±17.4%; \( P=0.2 \)) whereas it was significantly increased in non-diabetic mice (air: 100±6.2% vs. NO: 122.6±8.7%; \( P=0.0009 \)) (Fig.2B).

**Diabetes alters NOS isoforms.** There was a significant decrease in eNOS mRNA (0.63±0.4 vs. 1.44±0.7 AU; \( P=0.04 \)) and phospho-eNOS protein expression (0.59±0.32 vs. 1±0.10 AU; \( P=0.02 \)) in diabetic compared to non-diabetic mice, although eNOS protein levels were not significantly different (0.99±0.1 vs. 1±0.21 AU; \( P=1 \)) (Fig.3A). The mRNA for nNOS (0.59±0.37 vs. 1.32±0.61 AU; \( P=0.03 \)) and protein levels of nNOS (0.92±0.06 vs. 1±0.04 AU; \( P=0.05 \)) and phospho-nNOS (0.36±0.26 vs. 1±0.12 AU; \( P=0.002 \)) were significantly decreased in diabetic compared to non-diabetic mice (Fig.3B).

**Alteration of BBB protein expression in diabetic mice without ultrastructural or brain MRI modifications.** mRNA for the adherens junction molecule VE-cadherin was significantly decreased (0.71±0.49 vs. 1.39±0.61 AU; \( P=0.03 \)) in diabetic compared to non-diabetic mice while VE-cadherin protein levels (1.75±0.24 vs. 1.89±0.44 AU; \( P=0.5 \)) remained unchanged.
There was a significant decrease in mRNA (0.41±0.18 vs. 0.94±0.31 AU; \( p=0.001 \)) and protein levels (0.19±0.03 vs. 0.27±0.05 AU; \( p=0.01 \)) of the tight junction protein zona occludens 1 (ZO-1) in diabetic compared to non-diabetic mice (Fig.4A). However, TEM did not show any modification of tight junction structure in the microvessels of diabetic mice (Fig.4B). Brain MRI did not show any hemorrhages or gadolinium enhancement (data not shown).

**Alteration of factors regulating vessels expression in diabetic mice.** Cortical microvessel density was increased (mean gray surface value: 21.5±0.9 vs. 15.61±1.81 AU; \( P=0.02 \)) in diabetic versus non-diabetic mice. VEGFα mRNA (0.56±0.29 vs. 1.01±0.39 AU; \( P=0.02 \)) and protein expression (0.51±0.09 vs. 1.0±0.36 AU; \( P=0.05 \)) were significantly decreased in diabetic compared to non-diabetic mice; Ang2 mRNA (0.24±0.13 vs. 0.32±0.14 AU; \( P=0.3 \)) was not significantly modified although Ang2 protein levels were decreased (0.56±0.21 vs. 1±0.18 AU; \( P=0.001 \)) (Fig.4C). In addition, mRNAs for Ang1 (0.48±0.33 vs. 0.88±0.39 AU; \( P=0.05 \)), TGFβ (0.77±0.19 vs. 1.25±0.43 AU; \( P=0.02 \)) and PDGFβ (0.41±0.16 vs. 0.80±0.24 AU; \( P=0.003 \)) were significantly decreased in diabetic compared to non-diabetic mice.

**B) Impact of diabetic microangiopathy on stroke damage and cerebral repair**

**Diabetes worsens infarct volume and neurological deficit at D1 and D7 after cerebral ischemia.** Infarct volumes were significantly larger in diabetic compared to non-diabetic mice at D1 (17.72±2.82 mm\(^3\) vs. 14.02±0.99 mm\(^3\), \( P=0.03 \)) and D7 (14.24±4.50 mm\(^3\) vs. 2.84±0.50 mm\(^3\), \( P=0.04 \)) (Fig.5A, B). The neurological score (/21) was significantly worse at D1 (13±3 vs. 18±2; \( P<0.001 \)) and D7 (11±5 vs. 19±1, \( P=0.008 \)) in diabetic compared to non-diabetic mice, which did not show any neurological deficit (Fig.5C). No hemorrhagic transformation or suffusion of blood was visible on brain MRI at either time point (Fig.5D).

**Diabetes worsens BBB permeability at D1.** Evans Blue extravasation was increased in diabetic (114±9%) compared to non-diabetic mice (100±4%; \( P=0.02 \)) (Fig 6A). ZO-1 (0.12±0.02 vs. 0.16±0.04 AU; \( P=0.03 \)) and VE-cadherin protein levels (1.16±0.21 vs. 1.87±0.32 AU; \( P=0.03 \)) were significantly decreased in diabetic compared to non-diabetic mice (Fig 6B).
Diabetes delays angiogenesis. At D1, endothelial cell proliferation (36±11 vs. 46±11, \( P=0.2 \)) was not significantly different between diabetic and non-diabetic mice. Similar to pre-stroke conditions, VEGFa, TGF\( \beta \) and PDGF\( \beta \) mRNA levels at D1 were lower in diabetic compared to non-diabetic mice (data not shown). In contrast, at D7, there was a significant increase in endothelial cell proliferation (16±3 cells/ROI vs. 11±4 cells/ROI, \( P=0.05 \)) and a significant increase in VEGFa mRNA (0.67±0.20 vs. 0.42±0.12 AU, \( P=0.03 \)) and protein (0.89±0.49 vs. 0.37±0.13 AU, \( P=0.05 \)), and Ang2 mRNA (0.96±0.67 vs. 0.36±0.11 AU, \( P=0.05 \)) and protein levels (1.91±0.81 vs. 4.0±1.66 AU, \( P=0.05 \)) in diabetic compared to non-diabetic mice (Fig 6C). Ang1, PDGF\( \beta \) and TGF\( \beta \) mRNA levels were unchanged (data not shown).
DISCUSSION

The present study, performed in mice with STZ–induced type-1 diabetes, shows that cerebral microcirculation is affected after 8 weeks of hyperglycemia: vasoreactivity to inhaled CO\(_2\) is abolished and not reversed by NO-donor administration, while mRNA and protein levels of phospho-eNOS, nNOS and phospho-nNOS are decreased, suggesting smooth muscle and endothelial cell dysfunction. In addition, microvessel density is increased, while the downregulation of BBB proteins and the pattern of angiogenic factor expression suggest an immature vascular network. After the induction of experimental cerebral ischemia, infarct volume, neurological deficit and BBB permeability are significantly increased and angiogenesis, which is important for brain repair process, is delayed. Taken together, these findings could explain the greater damage during the acute phase of stroke and delayed brain repair in these mice.

To our knowledge, this is the first study to reproduce impaired cerebral vasoreactivity after CO\(_2\) inhalation in diabetic mice, facilitating the extrapolation of our methodology and results to humans. Our diabetic mice did not exhibit cerebral vasodilation after CO\(_2\) inhalation or NONOate injection, suggesting an SMC dysfunction. SMC alterations have already been reported in diabetes (9), and might be explained by primary reduced NO bioavailability or direct cell toxicity by increased production of reactive oxygen species and peroxynitrite. NO is the main vasodilator involved in the increased CBF induced by experimental hypercapnia in normal brains, since cerebral vasoreactivity is suppressed by NOS inhibitors and reversed by NO agonists (10). NO is produced in the brain by endothelial cells (eNOS) and neurons (nNOS) (11). However, the data concerning their involvement are conflicting (12-15). Although endothelial dysfunction in the cerebral arteries of diabetics is generally accepted (16), there is no modification of eNOS protein level in STZ-induced diabetic rats (17), down-regulation in high-fat-diet-induced diabetic mice (18) or up-regulation in obese diabetic Zucker rats (19). Up-regulation was unevenly interpreted as a compensatory mechanism to maintain acceptable NO levels. In our model, although there was no modification in eNOS protein levels, we did observe a decrease in the enzymatically active phosphorylated form of the protein (20). Since the endothelial production of NO requires the phosphorylation of specific eNOS residues by protein kinase B or Akt and since insulin-mediated Akt phosphorylation is impaired in diabetic mice, we can speculate that this might be responsible
for the downregulation of eNOS in the brain of diabetic mice. Consistent with these results, mice with the phosphomimetic "SD" mutation of eNOS display reduced infarct size and neurological deficit after MCAo compared to eNOS mice with the unphosphorylatable "SA" mutation (21). The expression of nNOS, whose enzymatic activity similarly depends on its phosphorylation (22), is decreased (17) or unchanged (19) in STZ-induced diabetic rats. We therefore also systematically investigated nNOS phosphorylation in the brain of diabetic mice, and demonstrate, to our knowledge for the first time, that both eNOS mRNA and phospho-eNOS protein as well as nNOS mRNA and nNOS and phospho-nNOS protein levels are decreased in diabetic mice, suggesting that a downregulation of both NOS isoforms may contribute to impaired CBF regulation in diabetic mice.

In acute stroke settings, collateral circulation can sustain tissue perfusion following a proximal arterial occlusion. The leptomeningeal collateral network represents the final route for the perfusion of ischemic territory distal to the proximal occlusion (23). After arterial occlusion, the reduction in CBF is more pronounced in the infarct core and less severe at the periphery or penumbra, due to NO-dependent collateral circulation recruitment (24). Furthermore, the inactivation of both nNOS and eNOS activity leads to lesion extension (5). Here, we have shown that a combination of endothelial and SMC dysfunction and eNOS and nNOS downregulation in 8-week diabetic mice prevents the dilatation of cerebral arterioles, compromising the adaptation of blood flow to metabolic rate, and potentially explaining the greater infarct volume and neurological deficit. One should thus encourage the development of therapeutic strategies that aim to improve the early recovery of vasoreactivity, for example by using statins or angiotensin type 1 (AT1) receptor blockers to modify the NO pathway (25).

Another mechanism that might take part in increased stroke severity is increased BBB permeability, as shown by Evans Blue extravasation, and as already demonstrated in a model of cerebral artery occlusion and reperfusion (tMCAo) in type-2 diabetic mice (26). We found no hemorrhagic transformation on brain MRI, in accordance with previous reports indicating that hemorrhage is mainly found in tMCAo (27). There was also no gadolinium enhancement, although BBB proteins levels were lower in diabetic mice, suggesting that gadolinium assessment might not be sufficient to detect these changes.
Blood vessel stabilization depends on the equilibrium between pro-angiogenic factors (VEGFα, Ang2) and maturation and stabilization factors (Ang1, PDGFβ and TGFβ). We have shown that prior to cerebral ischemia, the brain microvasculature in naive diabetic mice is immature, with a decrease in VEGFα, Ang2, Ang1, PDGFβ and TGFβ mRNA levels. This is consistent with another model of diabetes, the Goto-Kakisaki (GK) rat, in which there are more prominent unperfused new brain vessels and a decreased pericyte–to–endothelial cell ratio, suggesting a "destabilizing vessel profile" (28). However, this immature vascular phenotype is associated with increased cortical vascular density in GK rats, also reported in Lepr<sup>db/db</sup> mice by Prakash and colleagues (28, 29), and increased VEGFα protein levels in micro- but not macrovessels (28). This is in contrast with our own findings, where increased vessel density was instead associated with a downregulation of VEGFα mRNA and protein levels. In contrast to our experimental conditions, Prakash et al. (28,29) used a rat genetic model of type-2 diabetes with a shorter duration of diabetes, and measured VEGFα levels in microvessels and not the whole brain. As the duration of chronic hyperglycemia is longer in our protocol, it is possible that toxic metabolic end products due to the disease might interfere with pro-angiogenic pathways under chronic conditions (30).

At D1 post-stroke, mRNAs for angiogenic factors in diabetic mice were not different from values obtained before stroke, and still lower than those in non-diabetic mice. At D7, PDGFβ, TGFβ and Ang1 mRNA were not significantly different between diabetic and non-diabetic mice, whereas a significant upregulation of VEGFα and Ang2 mRNA and protein levels was observed, with increased angiogenesis. From these time points, we can deduce that the time course of vessel repair is delayed in our model, since these factors should have returned to basal levels otherwise (31). These results are in contrast to those of Cui et al. (26) who investigated the regulation of Ang1 and Ang2 one day after stroke in a model of type-2 diabetic mice, and found early modifications with decreased Ang1 and increased Ang2 levels. However, protein levels in their study were not compared to non-ischemic mice but to the contralateral hemisphere. Since there could be compensatory changes in the contralateral hemisphere, we chose to compare our ischemic diabetic mice to non-operated diabetic mice. Post-stroke angiogenesis results in increased vessel density from D1 that continues until D21 (31), and is maximal between D3 and D7. In a model of tMCAo in CD1 mice, VEGFα and its receptor flk1 (VEGFR2) were maximally upregulated from 3 hours until D3 for the ligand
and until D7 for the receptor, at which time they returned to baseline. Ang2 follows the same
time course, whereas Ang1 expression is delayed with maximal expression at D7. To our
knowledge, there is no report as to the time-course of pro-angiogenic factors after stroke. As
we have shown previously in C57Bl/6J mice following pMCAo that angiogenesis is also
maximal between D3 and D7 (6), we suggest that the time-course of expression of pro-
angiogenic factors is also comparable, despite the use of different models. To our knowledge,
this is the first time that these factors have been sequentially measured in diabetic mice after
stroke. NO is also a positive regulator of neovascularization by contributing to the pro-
angiogenic effect of growth factors (32), and its downregulation in diabetic mice might
prevent proper angiogenesis.

Although our experimental data were obtained from two different sets of animals and a
causal relationship thus cannot be strictly verified, we believe that cerebral microvessel
impairment, already present in non-operated diabetic mice, is at least partly responsible for
worsened stroke outcome. 1) After cerebral ischemia, the magnitude of vasoreactivity, i.e. the
recruitment of collateral circulation, is inversely proportional to brain damage (24), and the
vasoreactivity impairment in our model suggests that after stroke, compensatory mechanisms
such as collateral opening and patency are not sufficient to limit brain damage. 2) The
magnitude of BBB opening after stroke determines the extent of the infarct (33). This is also
observed in the diabetic mice in our study, whose cerebral vessels display an immature
phenotype with a relative decrease in pro-angiogenic factors and BBB proteins, suggesting
BBB “fragility”. 3) As shown in a previous work (6), cerebral repair is partly dependent on
angiogenesis, and we could speculate that if pro-angiogenic factors are already decreased
before stroke, they will be less effective in initiating an angiogenic response after stroke, as
also observed in the diabetic mice in our study. Nonetheless, we cannot exclude the
involvement of other factors such as brain-specific basal inflammation in diabetic mice in
worsened stroke outcome. Indeed, inflammation is a key contributor to brain damage after
stroke (34), and the level of inflammatory cytokines (interleukin-1β, tumor necrosis factor-α
and interleukin-6) has been shown to be increased in the hippocampus of obese and diabetic
db/db mice (35). The contribution of inflammation to brain damage STZ-induced diabetic
mice following stroke warrants further study.
Alterations of the microvasculature associated with peripheral diabetic neuropathy have also been reported. Demiot et al (36) have observed an impaired pressure-induced vasodilation response associated with neuropathy in male Swiss mice 8 weeks after they were given a high dose of STZ (200mg/Kg), and Jeong et al (37) have observed decreased vasa nervorum in the sciatic nerve in a similar model. In human diabetic neuropathy, a thickening of the basement membrane, endothelial fenestration, decrease of periendothelial coverage, reduction of nerve blood flow correlated with the severity of diabetic neuropathy (38-41), and a reduction (40-41) or increase of capillary density (42) have all been reported. The absence of microvascular structural abnormalities in our model at the time of analysis could indicate that such modifications are milder or occur later in the brain.

We chose to develop a chronic induced type-1 diabetic mouse model in order to exclude hypertension and dyslipidemia, which are also responsible of vascular impairment, and other confounding factors, from our analysis. Although type-1 diabetes only represents ~10% of diabetic patients, though this proportion is greater at younger ages (43), the relative stroke risk is 4 times greater in type-1 than in type-2 diabetes. Moreover, in most genetic type-2 diabetic models, the lack of the leptin receptor may introduce a bias since leptin is neuroprotective, and the lack of leptin signalling worsens damage after cerebral ischemia (44). The duration and degree of hyperglycemia in diabetes may also be critical for neurovascular outcomes. Most animal studies have been performed after acute or short-term (2-5weeks) hyperglycemia, which might not be sufficient to cause the microangiopathy that needs to be taken into account in the pathophysiology of diabetic stroke, in particular in repair mechanisms that occur later than purely metabolic effects. However, although STZ induction is a well-accepted model of type-1 diabetes (45), it does not recapitulate the complex pathophysiology observed in type-1 diabetic patients, and the extrapolation to our findings to these patients is thus premature.

Our results provide for the first time direct evidence that diabetes alters cerebral vasoreactivity, microvessel density, permeability and stability, all of which influence neuronal and vascular damage following ischemic brain injury not only in the short term but at longer times. These results offer another insight into the crucial role of the neurovascular network in the outcome of stroke. They highlight the fact that there is an urgent need to understand the basic mechanisms that limit or enhance brain repair after stroke and to develop appropriate
experimental models to lead to customized stroke therapies.

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**Conflict of interest:** the authors declare no conflict of interest.

**Author contributions:** MP designed experiments, performed experiments and participated in writing and reviewing the manuscript; PB performed the inhaled-CO2 vasoreactivity Doppler analysis and blood gas analyses, reviewed the manuscript and contributed to the discussion; CP and LR performed some of the PCR and western blots for BBB and microcirculation proteins; CS and AD did the brain MRI experiments; CCM helped with the NOS western blots, provided antibodies, reviewed the manuscript and contributed to the discussion; NK designed the experiments, wrote and edited the manuscript and is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and their accuracy.

**Acknowledgements:** We thank Bruno Palmier (EA4475 - Pharmacologie de la Circulation Cérébrale Université Paris Descartes, Faculté des Sciences Pharmaceutiques et Biologiques), for western blot guidance, the Centre d’explorations fonctionnelles—Imagerie (CEFI, Institut Claude Bernard) for MRI image acquisition and Bruno Saubaméa (INSERM U705, CNRS UMR 8206, Université Paris Descartes, Faculté des Sciences Pharmaceutiques et Biologiques), for performing ultrastructural imaging.
Table 1 - Sense and antisense sequences of custom-designed primers for RT-PCR (NOS: Nitric Oxide Synthase; eNOS: endothelial NOS; nNOS: neuronal NOS; VEGF: Vascular Endothelial Growth factor; Ang: Angiopoietin; PDGF: Platelet-Derived Growth Factor; TGF: Tumor Growth Factor; ZO-1: Zona Occludens 1; VE-cadherin: vascular endothelial cadherin) and antibodies for western blotting (WB).

<table>
<thead>
<tr>
<th>PCR</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
<tr>
<td>nNOS</td>
<td>CTGGCTCAACCGAATACAGG</td>
<td>GTAGGCAGTGTACAGCTCTCTGAAG</td>
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<tr>
<td>eNOS</td>
<td>AAGCTGCAGGTATTGTGATGC</td>
<td>TATAGCCCGCATAGCTC</td>
</tr>
<tr>
<td>VEGFa</td>
<td>CCTTAATCCAGAAAGCCTGACATG</td>
<td>AAAGTGCTCTCTGAAGAGTCTCC</td>
</tr>
<tr>
<td>Ang1</td>
<td>GGTCACAGAAATCGCCACTT</td>
<td>CCTGGTCCCCATTTGCTGT</td>
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<tr>
<td>Ang2</td>
<td>AATGTTCCGTGGAGTTTCAG</td>
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<tr>
<td>PDGFβ</td>
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<td>TGFβ</td>
<td>TTGCTTCAGCTCCACAGAGA</td>
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<td>ZO-1</td>
<td>ACCCAGCAAGGTTGTACAGG</td>
<td>CCGTAGGCGATGGTCATA</td>
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<td>VE-cadherin</td>
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<td>Abcam</td>
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<tr>
<td></td>
<td>p-eNOS</td>
<td>Ser1177, Santa Cruz</td>
<td>1/500</td>
</tr>
<tr>
<td></td>
<td>nNOS</td>
<td>Abcam</td>
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<tr>
<td></td>
<td>p-nNOS</td>
<td>Ser847, Abcam</td>
<td>1/1000</td>
</tr>
<tr>
<td></td>
<td>VEGFa</td>
<td>Millipore</td>
<td>1/1000</td>
</tr>
<tr>
<td></td>
<td>Ang2</td>
<td>Abcam</td>
<td>1/1000</td>
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<tr>
<td></td>
<td>VE-cadherin</td>
<td>Santa Cruz</td>
<td>1/100</td>
</tr>
<tr>
<td></td>
<td>ZO-1</td>
<td>Invitrogen</td>
<td>1/125</td>
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<tr>
<td></td>
<td>Beta-actin</td>
<td>Sigma</td>
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<tr>
<td>ERK2</td>
<td>Santa-Cruz</td>
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Table 2 - Comparisons between non-diabetic and diabetic mice (body weight (g), systolic pressure, diastolic pressure, mean arterial pressure (mmHg), under air and inhaled CO2 conditions (pH and pCO2). Data indicate mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic mice</th>
<th>Diabetic mice</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>23.7 ± 1.1</td>
<td>21.08 ± 2.4</td>
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<tr>
<td>Systolic pressure (mmHg)</td>
<td>129 ± 11</td>
<td>134 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>96 ± 7</td>
<td>103 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>Mean Pressure (mmHg)</td>
<td>101 ± 17</td>
<td>113 ± 11</td>
<td>NS</td>
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</tbody>
</table>

<table>
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<th>CO2</th>
<th>Air</th>
<th>CO2</th>
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<tr>
<td>pH</td>
<td>7.45 ± 0.02</td>
<td>7.29 ± 0.05</td>
<td>7.45 ± 0.02</td>
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<tr>
<td>pCO2</td>
<td>23.7 ± 2.8</td>
<td>34.0 ± 3.95</td>
<td>23.4 ± 3.9</td>
<td>36 ± 3.4</td>
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<tr>
<td>pO2</td>
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<td>98.5 ± 16.7</td>
<td>87.7 ± 4.0</td>
<td>89.2 ± 3.5</td>
<td>NS</td>
</tr>
</tbody>
</table>
REFERENCES


42. Britland ST, Young RJ, Sharma AK, Clarke BF. Relationship of endoneurial capillary abnormalities to type and severity of diabetic polyneuropathy. Diabetes 1990;39:909-13


Figure legends

Figure 1. Experimental protocol and time schedule

Figure 2. CO₂- and NO-vasoreactivity impairment in diabetic mice. A: In non-diabetic mice, mean blood flow velocity (mBFV) in the basilar trunk (BT) increases significantly by 25% under CO₂ inhalation, whereas in diabetic mice, there is no significant difference of BT mBFV between air and CO₂ inhalation conditions, indicating an absence of cerebral vasoreactivity in these mice (n=7-10). B: In non-diabetic mice, BT mBFV increases significantly by 22% after NO-donor (NONOate) injection, whereas it remains unchanged compared to basal conditions in diabetic mice, indicating an alteration in NO-vasoreactivity (n=4-5). Data are represented as a percentage of the values in non-diabetic mice (means ± SD). ***: P<0.001.

Figure 3. eNOS and nNOS expression levels are significantly decreased in diabetic mice. A: eNOS mRNA and phosphorylated protein expression levels are significantly decreased in diabetic mice compared to non-diabetic mice, while eNOS protein is unchanged (n=5-8). B: nNOS mRNA, protein and phosphorylated protein levels are significantly decreased in diabetic mice (n=5-8). Data represent mean ± SD. *: p<0.05.

Figure 4. A: Diabetes alters BBB mRNA (top) and protein expression (bottom) (n=5-8). Data are represented as AU (arbitrary units) ± SD. *: P<0.05, **: P<0.01. B: TEM of non-diabetic and diabetic microvessels (BL: basal lamina, SMC: smooth muscle cells, EC: endothelial cells, PC: pericytes, TJ: tight junctions) (n=4). C: Diabetes alters mRNA (top) and protein (bottom) levels of vessel regulating factors, VEGFa and Ang2 (n=5-8). Data are represented as AU ± SD. **: P<0.01, *: P<0.05.

Figure 5. A, B: Diabetes leads to a larger infarct volume and C: a more severe neurological deficit at D1 and D7 (n=5-9). D: Brain MRI at D1 and D7 post-stroke (axial T2 weighted slices) shows no hemorrhagic transformation in non-diabetic or in diabetic mice (n=5-6). Data represent mean ± SD. *P<0.05, **P<0.01, ***P<0.001
Figure 6. A: Diabetes induces an increase in BBB permeability as shown by Evans Blue extravasation (n=5-6), and a decrease in BBB proteins at D1 (n=5-8). B: Diabetes increases endothelial cell proliferation and C: angiogenic factors (VEGFa and Ang2) at D7 (n=5-8). Data represent mean ± SD. *P<0.05.
### A) Functional and structural characterization of microcirculation in chronic diabetic mice

<table>
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<td>COX and NO Doppler Reactivity</td>
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<td>NFκB inflammatory response</td>
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<td>Transmission Electronic Microscopy</td>
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### B) Impact of diabetic microangiopathy on stroke damage and cerebral repair

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<td>Expression of factors regulating vessels</td>
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<td>Endothelial cells proliferation</td>
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figure 2

661x881mm (96 x 96 DPI)
figure 3
661x881mm (96 x 96 DPI)
figure 4

Diabetes
Poittevin et al Figure n°5

**Figure 5**

661x881mm (96 x 96 DPI)
Diabetes

Poittevin et al Figure n°6

A

B

CD31+    KI67+    CD31+/KI67+

ND  40x  ND  40x  ND  40x

D      D      D

C

VEGFA mRNA (AU)

VEGFA (AU)

ND  D  ND  D

Ang2 mRNA (AU)  Ang2 (AU)

ND  D  ND  D

figure 6
661x881mm (96 x 96 DPI)