# Exacerbation of Thromboinflammation by Hyperglycemia Precipitates Cerebral Infarct Growth and Hemorrhagic Transformation

Jean-Philippe Desilles, MD, MSc; Varouna Syvannarath, MSc; Véronique Ollivier, PhD; Clément Journé, MSc; Sandrine Delbosc, PhD; Célina Ducroux, MD; William Boisseau, MD; Liliane Louedec, BSc; Lucas Di Meglio, MD; Stéphane Loyau, BSc; Martine Jandrot-Perrus, MD, PhD; Louis Potier, MD, PhD; Jean-Baptiste Michel, MD, PhD; Mikael Mazighi, MD, PhD; Benoit Ho-Tin-Noé, PhD

- **Background and Purpose**—Admission hyperglycemia is associated with a poor outcome in acute ischemic stroke. How hyperglycemia impacts the pathophysiology of acute ischemic stroke remains largely unknown. We investigated how preexisting hyperglycemia increases ischemia/reperfusion cerebral injury.
- *Methods*—Normoglycemic and streptozotocin-treated hyperglycemic rats were subjected to transient middle cerebral artery occlusion. Infarct growth and brain perfusion were assessed by magnetic resonance imaging. Markers of platelet, coagulation, and neutrophil activation were measured in brain homogenates and plasma. Downstream microvascular thromboinflammation (DMT) was investigated by intravital microscopy.
- *Results*—Hyperglycemic rats had an increased infarct volume with an increased blood–brain barrier disruption and hemorrhagic transformation rate compared with normoglycemic rats. Magnetic resonance imaging scans revealed that hyperglycemia enhanced and accelerated lesion growth and was associated with hemorrhagic transformation originating from territories that were still not completely reperfused at 1 hour after middle cerebral artery recanalization. Intravital microscopy and analysis of brain homogenates showed that DMT began immediately after middle cerebral artery occlusion and was exacerbated by hyperglycemia. Measurement of plasma serotonin and matrix metalloproteinase-9 indicated that platelets and neutrophils were preactivated in hyperglycemic rats. Neutrophils from hyperglycemic diabetic patients showed increased adhesion to endothelial cells as compared with neutrophils from normoglycemic donors in flow chamber experiments.

Conclusions—We show that hyperglycemia primes the thromboinflammatory cascade, thus, amplifying middle cerebral artery occlusion–induced DMT. DMT exacerbation in hyperglycemic rats impaired reperfusion and precipitated neurovascular damage, blood–brain barrier disruption, and hemorrhagic transformation. Our results designate DMT as a possible target for reduction of the deleterious impact of hyperglycemia in acute ischemic stroke. (Stroke. 2017;48:1932-1940. DOI: 10.1161/STROKEAHA.117.017080.)

Key Words: acute stroke ■ hyperglycemia ■ microvascular dysfunction ■ middle cerebral artery occlusion ■ polymorphonuclear neutrophils activation

A cute ischemic stroke (AIS) remains a leading cause of disability, cognitive impairment, and mortality worldwide, despite the development of revascularization therapies. Besides recanalization status, other prognostic factors in AIS patients are associated with clinical outcome. Among them, hyperglycemia has been found to be associated with hemorrhagic transformation (HT) and worsened neurological outcomes.<sup>1,2</sup> Given that 40% to 50% of AIS patients present with hyperglycemia,<sup>3</sup> understanding and reducing hyperglycemia-induced

neurovascular injury constitute an important clinical stake. Experimental studies have investigated extensively the relationship between hyperglycemia and poor outcome after AIS.<sup>4-6</sup> Notably, these studies have shown that preexisting hyperglycemia, whether acute or prolonged, was an important determinant of brain injury in AIS. In contrast, hyperglycemia induced after the period of ischemia had no effect on experimental stroke outcome.<sup>5</sup> Hyperglycemia was further shown to increase cerebral ischemia-reperfusion–induced blood–brain

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From the Laboratory of Vascular Translational Science, U1148 Institut National de la Santé et de la Recherche Médicale (INSERM), Univ Paris Diderot, Sorbonne Paris Cite, France (J.-P.D., V.S., V.O., C.J., S.D., C.D., W.B., L.L., L.D.M., S.L., M.J.-P., J.-B.M., M.M., B.H.-T.-N.); Department of Interventional Neuroradiology, Rothschild Foundation Hospital, Paris, France (J.-P.D., W.B., M.M.); DHU NeuroVasc, Paris, France (J.-P.D., M.M.); FRIM-Paris 7 University, Paris, France (C.J.); and Department of Diabetology AP-HP, Bichat Hospital, Paris, France (L.P.).

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Correspondence to Jean-Philippe Desilles, MD, MSc, Laboratory of Vascular Translational Science, U1148 INSERM, 46 rue Henri Huchard, 75018 Paris, France. E-mailsjpdesilles@gmail.com

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barrier (BBB) disruption, neurovascular damage, brain edema, and HT.<sup>6-8</sup> Oxidative stress and proteolysis, 2 important mediators of BBB disruption, were shown to be increased by hyperglycemia.<sup>9,10</sup> Other studies have shown that hyperglycemia was associated with decreased reperfusion after proximal recanalization with increased infarct volume.<sup>11,12</sup> Nevertheless, the mechanisms by which preexisting hyperglycemia contributes to exacerbated neurovascular injury and poor outcomes are still not fully understood.

Type I diabetes mellitus was recently shown to prime neutrophils, enhancing their response to any stimulus.<sup>13</sup> Neutrophils are among the first circulating cells to respond to AIS and contribute to BBB disruption, brain edema, and HT.<sup>14</sup> Experimental studies revealed that hyperglycemia enhanced endothelial activation and neutrophil infiltration in the ischemic brain.<sup>15,16</sup> In addition, we have shown that neutrophils were central actors of proximal occlusion–induced downstream microvascular thromboinflammation (DMT), which participates to neurovascular damage in AIS.<sup>17</sup> Neutrophils and DMT might, therefore, contribute to the deleterious impact of preexisting hyperglycemia on AIS. Here we investigated this possibility using a rat model of transient cerebral ischemia caused by proximal cerebral arterial occlusion.

#### **Materials and Methods**

#### **Induction of Hyperglycemia**

Hyperglycemia was induced as described previously.<sup>18</sup> Briefly, male Sprague–Dawley rats (Janvier, France) received a single intravenous injection of streptozotocin (35 mg/kg; Sigma-Aldrich, St. Quentin Fallavier, France). Hyperglycemic (HG) and weight-matched normo-glycemic (NG) rats were operated 4 weeks later. Body weight and capillary blood glucose were measured immediately before surgery.

#### Middle Cerebral Artery Occlusion and Reperfusion

Male Sprague–Dawley rats (330–400 g; Janvier, France) were subjected to 60 minutes transient monofilament middle cerebral artery occlusion (tMCAO), as described.<sup>17</sup>

#### Determination of Infarct Size, Brain Swelling, Neurological Deficit, HT, and BBB Disruption

Determinations of infarct size, brain swelling, and neurological deficit were performed as described.<sup>17</sup> ELISA-based methods for rat hemoglobin (Abcam; Hemoglobin rat ELISA kit) and rat IgG (Abcam; IgG rat ELISA kit) were used to quantitatively measure HT and BBB disruption in brain homogenates.

#### Magnetic Resonance Imaging

All magnetic resonance imaging (MRI) were performed on a 7-Tesla small animal scanner equipped with Paravision 5.1 software platform (Bruker, Ettlingen, Germany). Two scans were realized: the first  $\approx$ 15 minutes after the MCAO (ischemia MRI) and the second  $\approx$ 1 hour after monofilament withdrawal (recanalization MRI). The MRI protocol included 3-dimensional time-of-flight angiography; 2-dimensional diffusion-weighted imaging, including apparent diffusion coefficient map; and a perfusion imaging using arterial spine labeling sequence (see the online-only Data Supplement).

#### **Intravital Imaging of Cortical Microcirculation**

Intravital microscopy of downstream cortical branches of the middle cerebral artery was performed as described previously and in the online-only Data Supplement.<sup>17</sup>

# Measurement of Brain and Plasma Concentrations of Myeloperoxidase, Serotonin, Matrix

#### Metalloproteinase-9, and Thrombin–Antithrombin

Levels of myeloperoxidase (MPO), serotonin, matrix metalloproteinase-9 (MMP-9), and thrombin–antithrombin (TAT) complex were measured in plasma samples collected immediately before (baseline), during (immediately before recanalization), and at 1 hour and 24 hours after tMCAO. For measurements in brain homogenates, brains were recovered after intracardiac perfusion of saline (40 mL). The following kits were used: HycultBiotech MPO rat ELISA kit, Enzo Serotonin EIA kit, R&D, rat total MMP-9 quantikine ELISA kit, and Siemens Healthcare diagnostics, Enzygnost TAT micro.

#### Immunohistochemistry

Supplemental rats were subjected to 60-minute tMCAO. They were euthanized at 6 hours after recanalization (n=3 per group). Slices at +0.70 mm posterior to bregma were fixed in paraformaldehyde 3.7% and embedded in paraffin. After deparaffinization, tissue sections were permeabilized, washed, and incubated with primary antibodies to MPO (Dako) or rat IgG (ThermoFisher), followed by incubation with fluorescent secondary antibodies.

#### **Statistical Analysis**

The study was designed with 80% power to detect a relative 50% difference in infarct volume between HG and NG groups. Data were analyzed using a nonparametric analysis of variance (Kruskal–Wallis), followed by the Wilcoxon rank-sum test, for comparison of paired data, or by the Mann–Whitney U-test, for comparison of unpaired data. On the basis of preliminary data indicating that the average infarction volume in 60-minute tMCAO was 33% (SD, 15%), we used 16 rats for each group. Results are presented as mean±SD for continuous variables and percentage (count) for qualitative variables. For statistical analysis, PrismGraph 4.0 software (GraphPad Software, San Diego, CA) was used. Values of P<0.05 were considered statistically significant.

#### Results

#### Hyperglycemia Induces an Increased Infarct Volume With BBB Disruption and HT

The effect of preexisting hyperglycemia on ischemia/ reperfusion-induced cerebral injury was determined by comparing brain damage and stroke outcome after monofilament tMCAO in streptozotocin-treated HG and NG rats. Immediately before surgery, body weights were similar between HG and NG rats (358±31 versus 356±34 g, respectively), and capillary blood glucose were significantly elevated in HG compared with NG rats (27.6±3.2 versus 7.3±1 mmol/L; P<0.0001). HG significantly and markedly increased infarct volume and brain edema (Figure 1A). While IgG and hemoglobin content in nonischemic brain hemispheres were similar between NG and HG rats, they were increased in ischemic hemispheres of HG rats compared with NG rats (Figure 1B and 1C), thus, indicating that hyperglycemia was associated with a more severe BBB disruption and a greater risk of HT after tMCAO. Concordantly, the incidence of HT and the mortality rate, respectively, reached 77% (14/16) and 18% (3/16) in HG rats, whereas both rates remained null in NG rats. Accordingly, hyperglycemia was associated with worsened neurological outcome as indicated by the higher neurological severity score of HG rats compared with NG rats (Figure 1D).



**Figure 1.** Hyperglycemia increases infarct volume, blood–brain barrier disruption, and hemorrhagic transformation after transient middle cerebral artery occlusion. Infarct volume, brain edema, blood–brain barrier disruption, hemorrhagic transformation, and functional outcomes were assessed 24 hours after monofilament-induced transient focal cerebral ischemia in normoglycemic (NG) and hyperglycemic (HG) rats. **A**, Representative images of coronal brain sections of NG and HG rats stained with 2,3,5-triphenyltetrazolium chloride (TTC). The higher magnification view of the squared area show petechial bleeding spots (\*) in the infarct zone of a HG rat (**left**). Infarct volumes and brain edema in NG and HG rats (n=16 per group). Corrected infarct volumes are expressed as percentage relative to the contralateral hemisphere volume. Brain edema is expressed as percentage increase in ischemic hemisphere volume relative to contralateral hemisphere (**right**). **B**, Representative images of TTC (**top**) and anti-IgG (**bottom**, green fluorescence) staining of coronal brain sections of NG and HG rats. **C**, IgG (**left**) and hemoglobin (**right**) concentration in ischemic (Isch.) and nonischemic (Non-Isch.) brain hemispheres of NG (n=6) and HG (n=8) rats. **D**, Modified neurological severity scores in NG and HG rats (n=16 per group). \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001.

### Hyperglycemia Accelerates Neurovascular Damage During MCAO and Is Associated With Incomplete Reperfusion Despite Recanalization After tMCAO

To explore the parenchymal perfusion during MCAO and after recanalization, we realized MRI scans with diffusion and perfusion sequences during and 1 hour after tMCAO in HG and NG rats. Diffusion-weighted imaging revealed that during MCAO, HG rats had an increased apparent diffusion coefficient–based lesion volume indicative of an increased brain lesion volume (Figure 2A), as well as an increased hypoperfusion surface (Figure 2B). Remarkably, in addition to be associated with a higher apparent diffusion coefficient– based lesion volume and hypoperfusion surface, hyperglycemia tended to reduce the diffusion–perfusion mismatch. These results suggest that hyperglycemia not only extends downstream microvascular hypoperfusion but also accelerates hypoperfusion-related neurovascular damage (Figure 2D).

Regarding reperfusion, perfusion sequences acquired 1 hour after monofilament withdrawal revealed that despite complete MCA recanalization, there was a persistence of cerebral hypoperfusion in HG rats, whereas NG rats were fully reperfused (Figure 2C and 2D). Remarkably, HT (ie, petechiae) colocalized with these incomplete downstream reperfusion areas observed in HG rats (Figure 2D).

#### Hyperglycemia Exacerbates DMT

We have shown previously that occlusion of the MCA induces immediate adhesion of leukocytes and platelets in downstream pial vessels, a process that can lead to microvascular thrombosis, especially in the venous compartment.<sup>17</sup> Therefore, we investigated the impact of preexisting hyperglycemia on tMCAO-induced DMT.

Observation of pial surface vessels by intravital microscopy through a partial dura-sparing craniotomy revealed that HG rats presented an abnormal leukocyte rolling and firm adhesion in cortical veins at baseline. While no adherent leukocytes were observed in NG rats before tMCAO, rolling leukocytes and leukocytes firmly adhering to the venous vessel wall were systematically observed in HG rats (Figure 3A; Movie I in the online-only Data Supplement). There were no adhering leukocytes in the arterial compartment of HG and NG rats (Figure 3A; Movie I in the online-only Data Supplement). Immediately after occluding the MCA, a drop in venous and arterial blood flow occurred in both NG and HG rats (Movie II in the online-only Data Supplement). This abrupt decrease in blood flow was accompanied by the adhesion and accumulation of platelets and leukocytes mainly in veins (Figure 3A; Movie II in the online-only Data Supplement). Occlusion of the MCA also led to inversions of blood flow direction in small arteries of both NG and HG rats (Movie II in the onlineonly Data Supplement). After monofilament withdrawal, most leukocytes adherent to pial arteries were washed out, while leukocyte adhesion persisted and extravasation continued in the venous compartment of both NG and HG rats (Figure 3A; Movie III in the online-only Data Supplement). In all NG and HG rats analyzed, during the hour of MCAO, and before MCA recanalization, microthrombosis developed in postcapillary microvessels at sites of neutrophil and platelet accumulation (Figure 3B). At all stages of the observation after MCAO and recanalization, platelets and leukocytes accumulation, as



**Figure 2.** Comparison of cerebral perfusion and brain injury during occlusion and after recanalization of the middle cerebral artery in normoglycemic (NG) and hyperglycemic (HG) rats. Cerebral perfusion and brain injury were assessed by diffusion/perfusion magnetic resonance imaging (MRI) sequences. **A** and **B**, Apparent diffusion coefficient (ADC)–based lesions volume (**A**) and arterial spine labeling (ASL)–based hypoperfusion lesion surface (**B**) during occlusion of the middle cerebral artery. **C**, Reperfusion expressed as a percent-age relative to contralateral hemisphere perfusion at 1 hour after MCA recanalization in NG and HG rats. N=9 rats per group. \**P*<0.05, \*\**P*<0.01. **D**, Representative images of coronal slices of ASL (1st and 4th columns) and ADC (2nd column) sequences obtained during MCA occlusion and at 1 hour after recanalization in the 3rd columns show superimposition of the ASL-assessed hypoper-fused area at risk (outlined in blue) and the ADC-assessed infarct core (outlined in red). Note that the mismatch (gray area) between the 2 regions is markedly reduced in HG rats compared with NG rats. The asterisks in column 5 micrographs indicate petechial bleeding spots in the infarct area of a HG rat.

well as microvessel occlusion extension, appeared strikingly increased in HG rats compared with NG rats (Figure 3A; Movie III in the online-only Data Supplement).

To compare quantitatively the thromboinflammatory response to tMCAO between NG and HG rats, the levels of MMP-9, MPO, and TAT complex were measured in homogenates from brains obtained at 24 hours after tMCAO. MMP-9 and MPO levels, used as indicators of neutrophil recruitment, were considerably increased in ischemic hemispheres of HG rats compared with NG rats (Figure 3C). It should be noted that in line with intravital microscopy data indicating that abnormal baseline deposition of neutrophils occurs in brain vessels in the absence of tMCAO (Figure 3A), MMP-9 and MPO levels were also significantly elevated in nonischemic hemispheres of HG rats (Figure 3C). Measurement of TAT complex, used as a reflect of thrombin activation, showed that HG rats had an increased tMCAO-induced cerebral thrombotic response in their ischemic hemisphere compared with NG rats (Figure 3C).

To further investigate the location of tMCAO-induced thromboinflammation, immunostaining for neutrophils was performed on tissue sections from brains recovered at 6 hours after recanalization. Neutrophils were located within and around cortical surface vessels of ischemic hemispheres, but they were also found deeper in the cerebral cortex (not shown). No neutrophil was observed in nonischemic hemispheres. Remarkably, neutrophils were found in cortical hemorrhagic areas of HG rats (Figure 3D).

#### Hyperglycemia Primes Neutrophils and Accelerates Neutrophil, Platelet, and Coagulation Activation After tMCAO

The increased thromboinflammatory response of HG rats to tMCAO suggested us that hyperglycemia might affect actors of this process. We compared indicators of neutrophil, platelet, and coagulation cascade activity between NG and HG rats at baseline, during, and after tMCAO. There was no difference in basal total neutrophil and platelet counts between NG and HG rats (Figure 4A and 4B). However, basal neutrophil/lymphocyte ratios were significantly elevated in HG rats (Figure 4C). Furthermore, similarly to what was observed in cortical vessels of HG rats before tMCAO (Figure 3A), firm adhesion of leukocytes was also observed at baseline in unchallenged mesenteric venules of HG rats (not shown). These results are indicative of systemic inflammation and preactivation of circulating neutrophils in HG rats. Plasma levels



**Figure 3.** Hyperglycemia exacerbates transient middle cerebral artery occlusion (tMCAO)–induced downstream microvascular thromboinflammation. **A**, Rhodamine 6G-labeled platelets and leukocytes were observed by intravital microscopy in cortical microvessels downstream the middle cerebral artery (MCA). At baseline (before MCAO, **top**), although rare in normoglycemic rats (NG), marginating leukocytes were systematically found in abundance in cortical venules of hyperglycemic rats (HG). At 35 minutes after MCAO (**middle**), the decrease in venous and arterial blood flow was accompanied by the adhesion and accumulation of platelets and leukocytes mainly in veins. At 1 hour after MCA recanalization (**bottom**), extravasation of leukocytes from the venous compartment was observed in both NG and HG rats, but was more pronounced in HG rats. The asterisks indicate areas of strong leukocyte margination or extravasation. The arrows indicate an occluded venule. The images are representative of 6 rats per group. **B**, Representative images of MCAO-induced downstream microthrombosis of NG and HG rats (n=5 per group). Thrombosis during MCAO was evidenced by the accumulation of fibrinogen (green), neutrophils (red), and platelets (magenta) detected using intravenous injection of antibodies to neutrophils, platelets, and fibrinogen. The images shown here were taken 50 minutes after occluding the MCA, before recanalization in a HG rat. Bar=100  $\mu$ m. **C**, Quantification of matrix metalloproteinase-9 (MMP-9), myeloperoxidase (MPO), and thrombin–antithrombin (TAT) complexes in homogenates of ischemic and nonischemic hemispheres obtained at 24 hours after tMCAO in NG (n=7) and HG (n=9) rats. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001. **D**, Immunostaining of MPO in the infarct zone of HG rats showing the colocalization of neutrophils with microhemorrhage areas (\*) detected by the presence of extravasated red blood cells in differential interference contrast (DIC) microscopy. Bar=100  $\mu$ m.

of MPO and MMP-9 were significantly increased at baseline in HG rats compared with NG rats (Figure 5A and 5B). At 1 hour after occluding the MCA, and before recanalization, although MMP-9 plasma level was still comparable to baseline level in NG rats, it was already clearly increased in HG rats (Figure 5A). MMP-9 plasma level did elevate significantly in plasma samples of NG rats collected 1 hour after recanalization, but it remained much lower than in corresponding samples of HG rats (Figure 5A). MMP-9 plasma level had returned to baseline levels at 24 hours after recanalization in both NG and HG rats (Figure 5A). Whether in NG or HG rats, an increase in MPO plasma level compared with baseline level was detected in samples collected 1 hour after recanalization and at 24 hours after recanalization (Figure 5B). Again, the MPO plasma level in these samples was higher in HG rats (Figure 5B). Regarding the activation of platelets and that of the coagulation cascade, baseline plasma levels of serotonin were slightly but significantly increased in HG compared with



Figure 4. Effect of hyperglycemia on blood cell counts. A–C, Baseline neutrophil (A) and platelet (B) counts and neutrophil to lymphocyte ratio (C) in normoglycemic (NG; n=10) and hyperglycemic (HG; n=9) rats. \*P<0.05.



**Figure 5.** Priming of the thromboinflammatory cascade by hyperglycemia leads to increased and accelerated transient middle cerebral artery occlusion (tMCAO)–induced thromboinflammation. Plasma ELISA-based methods assessment of matrix metalloproteinase-9 (MMP-9; **A**), myeloperoxidase (MPO; **B**), serotonin (**C**), and thrombin–antithrombin (TAT) complexes (**D**) in normoglycemic (NG; n=15) and hyperglycemic (HG; n=16) rats. T0, baseline; T1, during ischemia (immediately before monofilament withdrawal); T2, 1 hour after recanalization, and T24, 24 hours after tMCAO. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

NG rats, whereas baseline plasma levels of TAT complex were similar in NG and HG rats (Figure 5C and 5D). Serotonin plasma level increased rapidly at 1 hour after occluding the MCA in both NG and HG rats, but it raised significantly more in HG rats (Figure 5C). It remained elevated at 1 hour after recanalization and diminished at 24 hours postrecanalization in the 2 groups, but remained significantly elevated compared with baseline in both groups (Figure 5C). Like for MMP-9 and serotonin, the kinetic and amplitude of tMCAO-induced TAT plasma level elevation was enhanced in HG rats compared with NG rats (Figure 5D). In fact, while in NG rats TAT plasma level increased briefly 1 hour after occluding the MCA and solely increased at 24 hours post-MCA recanalization, it was already significantly elevated 1 hour after recanalization in HG rats, compared with both their baseline value and NG rats (Figure 5D).

Finally, to explore whether preexisting hyperglycemia also primes human neutrophils and favors their interactions with the endothelium, we collected neutrophils from diabetic type 1 patients and age- and sex-matched healthy volunteers and compared their ability to firmly adhere to endothelial cells under venous flow conditions. While control donors were all NG with fasting plasma glucose levels inferior to 5 mmol/L, diabetic patients had a mean fasting plasma glucose level of  $10.2\pm6.4$  mmol/L (n=8). The adhesion of human neutrophils to endothelial cells was significantly increased in the diabetic group compared with controls (Figure 6).



**Figure 6.** Neutrophils from diabetic patients are primed for interactions with endothelial cells. Purified neutrophils at 3×10<sup>6</sup>/mL were perfused for 5 minutes on human umbilical vein endothelial cells under venous flow conditions (1 dyn cm<sup>-2</sup>) for 5 minutes, and neutrophils adherent to endothelial cells were quantified after a 3-minutes rinse. **A**, Representative images of endothelial layers after perfusion with neutrophils from control donors (**top**) or diabetic patients (**lower**). **B**, Neutrophil adhesion was quantified as the number of adherent neutrophils in 10 fields of view (×20 objective). Results are expressed as percent of mean control values. n=18 to 21 runs out of 8 donors per group (2–3 runs per donor). \*\*\*P<0.0001.

#### Discussion

Here, using a tMCAO stroke model combined with streptozotocin-induced hyperglycemia in rats, we explored the impact of preexisting hyperglycemia on DMT secondary to proximal arterial occlusion. Diffusion and perfusion MRI, as well as real-time intravital imaging of cortical vessels, performed during and after the MCAO period showed that DMT was amplified in HG rats compared with NG rats. All downstream microvascular events and their deleterious consequences were increased in HG rats. Those events included early platelet and leukocyte adhesion and accumulation in cortical microvessels, leukocyte extravasation, BBB disruption, and postcapillary microthrombosis. In agreement with previous studies,<sup>11,12</sup> the exacerbation of tMCAO-induced DMT by hyperglycemia was associated with incomplete downstream reperfusion, an increased infarct volume and neurological deficit, and a higher rate of HT. Regarding the latter HT phenomenon, petechial bleeding colocalized with areas that were still not fully reperfused at 1 hour after MCA recanalization according to MRI. This observation somewhat challenges the current paradigm that HT would be a direct consequence of reperfusion.<sup>19</sup> Our results suggest that, at least in the case of petechial HT, it might also be a consequence of the mechanisms impairing reperfusion, thus, pointing to a possible contribution of DMT in this process. This change of paradigm may lead to consider new therapeutical approaches aimed at limiting or reversing DMT for the prevention of HT. One of the characteristic features of petechial bleeding is that it develops at sites of neutrophil extravasation in postcapillary venules.<sup>20</sup> It is worth noting that, confirming our previous results,17 tMCAO-induced leukocyte recruitment took place almost exclusively in the venous compartment. One could then hypothesize that the exacerbated downstream neutrophil influx from cortical postcapillary venules of HG rats contributes to their higher propensity to develop HT. Because neutrophils are recruited at sites of cerebral ischemia and are important sources of proteases and reactive oxygen species capable of damaging the BBB, they have been previously incriminated as possible culprits contributing to BBB disruption and HT.14,21 Furthermore, in recent years, neutrophil to lymphocyte ratio has emerged as a predictor of short- and long-term outcomes in ischemic stroke patients and of risk of HT in stroke patients treated with intravenous thrombolysis.<sup>22</sup> In line with this clinical data, we found that HG rats had an increase in neutrophil to lymphocyte ratio compared with NG rats. Although neutrophils likely contribute to HT, other factors could participate to this complication in HG rats as well. Other sources of injurious products (eg, microglial and astrocyte cells), abnormalities of the basement membrane, or impaired vascular repair mechanisms might also favor bleeding complications of AIS in HG patients. Hyperglycemia has indeed been shown to be associated with microglial activation, impaired hemostasis,23 modifications of the basement membrane structure in microvessels, and platelet hyperreactivity, which have been shown to continuously prevent inflammatory bleeding, including in the tMCAO model of AIS.<sup>20,24,25</sup>

The expression time is brain underscores the fact that stroke outcome is extremely time-dependent, with a risk of permanent disability or death increasing with every minute from

stroke onset.<sup>26</sup> Our results indicate that time issues in stroke management could be even more critical in HG patients. In fact, we show here that in addition to be amplified, the thromboinflammatory response to tMCAO is also accelerated in HG rats. An elevation of plasma levels of MMP-9, serotonin, and TAT, which are respective indicators of neutrophil, platelet, and coagulation activation, was already detected in HG rats at 1 hour after occluding the MCA, while at this stage, no change was detected concerning MMP-9, and only a transient increase of TAT was detected in NG rats. More importantly, MRI data acquired during tMCAO revealed that although NG rats displayed a classical diffusion-perfusion mismatch with a clearly identifiable ischemic penumbra, HG rats had less diffusion-perfusion mismatch. Therefore, it seems that the accelerated downstream microvascular thromboinflammatory response and the associated increased collateral failure lead to precipitated neurovascular damage in HG rats.

It is noteworthy that early platelet activation was detectable at the plasma level as a raise in plasma serotonin before recanalization in both NG and HG rats. These data strengthen our previous<sup>17</sup> and current intravital observations showing that the thromboinflammatory response to tMCAO is an early event that is initiated before recanalization. From a clinical perspective, it suggests that therapeutical strategies aiming at dampening AIS-associated DMT might require to be administered as early as possible after stroke onset and before recanalization to be efficient whether in NG or HG patients.

The acceleration and exacerbation of tMCAO-induced DMT in HG rats raised the question of a possible priming of neutrophils in HG rats. In fact, neutrophils are not only the first leukocytes to be recruited at sites of cerebral ischemia, they have also been increasingly recognized as being capable of directly promoting intravascular thrombus formation,27 especially in the venous compartment subjected to blood flow reduction,<sup>28</sup> which, as we showed previously<sup>17</sup> and confirm here, is the main site of DMT. Our intravital observation of an abnormal firm adhesion of leukocytes to venules, including cortical venules, at baseline in HG rats provided evidence that neutrophils were primed in these animals. Basal neutrophil margination in HG rats was corroborated by the increased MPO and MMP-9 content in the nonischemic hemispheres of HG rats compared with NG rats. Moreover, human neutrophils isolated from diabetic patients also showed increased interactions with endothelial cells when perfused in vitro in venous flow conditions. It was shown previously in a rat model of type 2 diabetes mellitus that both sides of the neutrophil-endothelial interface were primed prior to tMCAO and remained significantly more activated during ischemia and reperfusion compared with nondiabetic rats.<sup>29</sup> An in vitro study using human cells also described that hyperglycemia increased neutrophil adhesion and expression of endothelial adhesion molecules.30 Our results are in agreement with these studies and further suggest that hyperglycemia-induced neutrophil priming is sufficient to induce neutrophil-endothelial cell adhesion and contributes to fuel increased DMT in case of large vessel occlusion. A remarkable feature of neutrophils is their short lifespan that does not exceed 24 hours in peripheral blood. Thus, circulating neutrophils are exposed to plasma glucose only for a limited time. This implies that priming of neutrophils by hyperglycemia is a relatively rapid process, which could explain why induction of hyperglycemia shortly before focal ischemia was sufficient to worsen stroke outcome in various experimental studies using models of cerebral ischemia/reperfusion.<sup>4,5,31,32</sup> Supporting this hypothesis, it was shown that exposure of human neutrophils to glucose concentrations similar to HG levels activate these cells within minutes.<sup>33</sup>

In conclusion, we show that preexisting hyperglycemia accelerates and amplifies tMCAO-induced DMT, thus, impairing reperfusion and precipitating neurovascular damage and HT. Our study, thus, provides a link between hyperglycemia and increased cerebral ischemia/reperfusion injury. We further show that hyperglycemia causes neutrophil priming and margination in the venous compartment, including cortical venules, where most of the microvascular deleterious events are initiated and occur after occlusion of the MCA. Considering our results and the previously reported procoagulant state and platelet hyperactivity of HG patients,<sup>34</sup> it seems that hyperglycemia generates a dangerous environment ideal for rapid initiation and development of thromboinflammation. Finally, because hyperglycemia-induced priming of the thromboinflammatory cascade seems to precede ischemia, insulin therapy to control hyperglycemia after AIS onset might turn futile. In a similar manner, and more generally, the thromboinflammatory cascade being initiated immediately after MCA occlusion, and not after recanalization, potential therapeutic strategies aiming at preventing thromboinflammation in AIS stroke patients, might also face a serious challenge because the time to treatment from stroke onset in these patients often exceed several hours.35

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**Disclosures** 

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### Exacerbation of Thromboinflammation by Hyperglycemia Precipitates Cerebral Infarct Growth and Hemorrhagic Transformation

Jean-Philippe Desilles, Varouna Syvannarath, Véronique Ollivier, Clément Journé, Sandrine Delbosc, Célina Ducroux, William Boisseau, Liliane Louedec, Lucas Di Meglio, Stéphane Loyau, Martine Jandrot-Perrus, Louis Potier, Jean-Baptiste Michel, Mikael Mazighi and Benoit Ho-Tin-Noé

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# SUPPLEMENTAL MATERIAL

# **Supplementary Materials and Methods:**

# **Magnetic Resonance Imaging**

All MRI were performed on a 7-Tesla small animal scanner equipped with Paravision 5.1 software platform (Bruker, Ettlingen, Germany). Two scans were realized: the first approximately 15 minutes after the MCAO (ischemia MRI) and the second approximately 1 hour after monofilament withdrawal (recanalization MRI). Rats were placed in the prone position in the scanner and the head was secured with a stereotaxic frame. Animals were imaged with the following MR sequences: 3-dimensional time-of-flight (TOF) angiography; 2-dimensional diffusion weighted imaging (DWI) including apparent diffusion coefficient (ADC) map; perfusion imaging using arterial spine labeling (ASL) sequence. The field of view scanned for 3-dimensional MRI sequences was 40x40x25 mm. The repetition time for the TOF sequence was 15 ms, echo time was 2.5 ms, flip angle was 75°, and number of excitations were 1 with a matrix size of 256x256x128 giving 14.5 mm/pixel resolution in a scan time of 6 minutes. For DWI, 15 0.8-mm axial slices were imaged with repetition time/echo time=6250/25, number of excitations=4, field of view=40x40 mm. matrix=128x128, resolution=31,2 mm/pixel using 7 b values=0, 100, 200, 400, 600, 800, 1000 s/mm2, giving a scan time of 7 minutes 55 seconds. For PWI, Single-shot/multi-phase ASL measurements (inversion recovery time (TIR) = 30 ms, increment of TIR = 100 ms, number of TIR = 22) were performed in one trans axial slice intersecting the central region of the brain (Bregma  $-3.8\pm0.5$  mm).

# Intravital imaging of cortical microcirculation

Platelets, leukocytes, and fibrinogen were observed in real-time before and during MCAO, and until 1 hour post-recanalization, by monitoring the accumulation of Alexa 555-conjugated hamster anti-rat CD42d (0.1 mg/kg, BD Pharmingen), Alexa 647-conjugated mouse anti-rat granulocytes (0.1 mg/kg, BD Pharmingen), and FITC-conjugated polyclonal rabbit anti-fibrinogen (1 mg/kg, Dako). Alterntatively, platelets and leukocytes were stained using rhodamine-6G (3 mg/kg). All fluorescent markers were administered intravenously into the tail vein.

# Human blood collection and neutrophil isolation

Whole blood was obtained from either diabetic patients or healthy volunteers exempt of medication, after full informed consent was obtained, according to the Declaration of Helsinki. Blood was collected via venipuncture into siliconized Vacutainer<sup>TM</sup> tubes (Becton Dickinson, Le Pont de Claix, France) containing K<sub>2</sub>EDTA. Neutrophil isolation was performed using the human MACSxpress<sup>®</sup> Neutrophil Isolation Kit (Miltenyi Biotec Inc, Paris, France) according to the manufacturer instructions. Isolated neutrophils (PMNs,  $\geq$  95% purity) were suspended at 3 x 10<sup>6</sup> PMNs/ml in PMN flow buffer (132 mmol/L NaCl, 20 mmol/L Hepes, 6 mmol/L KCl, 1 mmol/L MgSO<sub>4</sub>, 1.2 mmol/L K<sub>2</sub>HPO<sub>4</sub>, 1 mmol/L CaCl<sub>2</sub>, 0.5% BSA, 0.1% glucose, pH 7.4).

# **Endothelial cells**

Human umbilical vein endothelial cells (HUVECs, Promocell, Heidelberg, Germany) were seeded in perfusion chamber (Vena8 Endothelial+<sup>TM</sup> Biochips, Cellix, Dublin, Ireland) and grown under flow condition (KIMA<sup>TM</sup> pump, Cellix, Dublin, Ireland) following the manufacturer instructions. Briefly, each microchannel of the biochip was coated overnight

with 100  $\mu$ g/ml of human fibronectin (Promocell, Heidelberg, Germany). After the coating period, 2 x 10<sup>5</sup> cells were added in each microchannel and the biochip was placed at 37°C at 5% CO<sub>2</sub> for 2 hours before connection to the pump. HUVECs were then cultured under flow for 3 days in endothelial cell growth medium 2 (Promocell, Heidelberg, Germany).

# Neutrophil adhesion under flow conditions

PMNs at 3 x  $10^6$  /ml were perfused with an Exigo pump (Cellix, Dublin, Ireland) for 5 min at a venous shear stress of 1 dyne/cm<sup>2</sup> over the microchannels containing confluent layers of unstimulated HUVECs. Channels were then rinsed with PMN flow buffer for 3 min. Phase contrast images were obtained from at least ten different microscopic fields (Mosaic). Area coverage of PMNs was analyzed off-line using Histolab software (Microvision, Evry, France).

**Supplemental Movie I.** Intravital imaging of rhodamine-labeled leukocytes and platelets in pial microvessels of normoglycemic (NG) and hyperglycemic rats (HG) before MCAO. Venules and arterioles are indicated by the letter V and A, respectively. Note the important margination of leukocytes in venules of HG rats compared to NG rats.

**Supplemental Movie II.** Intravital imaging of rhodamine-labeled leukocytes and platelets in pial microvessels of normoglycemic (NG) and hyperglycemic rats (HG), before and 35 min after occluding the MCA. Venules and arterioles are indicated by the letter V and A, respectively. \* indicates an occluded venule.

**Supplemental Movie III.** Intravital imaging of rhodamine-labeled leukocytes and platelets in pial microvessels of normoglycemic (NG) and hyperglycemic rats (HG), 35 min after occluding the MCA, and 1 hour after MCA recanalization. Venules and arterioles are indicated by the letter V and A, respectively. \* indicates an occluded venule.