Exacerbated VEGF up-regulation accompanies diabetes-aggravated hemorrhage in mice after experimental cerebral ischemia and delayed reperfusion

Abstract
Reperfusion therapy is the preferred treatment for ischemic stroke, but is hindered by its short treatment window, especially in patients with diabetes whose reperfusion after prolonged ischemia is often accompanied by exacerbated hemorrhage. The mechanisms underlying exacerbated hemorrhage are not fully understood. This study aimed to identify this mechanism by inducing prolonged 2-hour transient intraluminal middle cerebral artery occlusion in diabetic Ins2^{Akita/+} mice to mimic patients with diabetes undergoing delayed mechanical thrombectomy. The results showed that at as early as 2 hours after reperfusion, Ins2^{Akita/+} mice exhibited rapid development of neurological deficits, increased infarct and hemorrhagic transformation, together with exacerbated down-regulation of tight-junction protein ZO-1 and up-regulation of blood-brain barrier-disrupting matrix metalloproteinase 2 and matrix metalloproteinase 9 when compared with normoglycemic Ins2^{+/+} mice. This indicated that diabetes led to the rapid compromise of vessel integrity immediately after reperfusion, and consequently earlier death and further aggravation of hemorrhagic transformation 22 hours after reperfusion. This observation was associated with earlier and stronger up-regulation of pro-angiogenic vascular endothelial growth factor (VEGF) and its downstream phospho-Erk1/2 at 2 hours after reperfusion, which was suggestive of premature angiogenesis induced by early VEGF up-regulation, resulting in rapid vessel disintegration in diabetic stroke. Endoplasmic reticulum stress-related pro-apoptotic C/EBP homologous protein was overexpressed in challenged Ins2^{Akita/+} mice, which suggests that the exacerbated VEGF up-regulation may be caused by overwhelming endoplasmic reticulum stress under diabetic conditions. In conclusion, the results mimicked complications in patients with diabetes undergoing delayed mechanical thrombectomy, and diabetes-induced accelerated VEGF up-regulation is likely to underlie exacerbated hemorrhagic transformation. Thus, suppression of the VEGF pathway could be a potential approach to allow reperfusion therapy in patients with diabetic stroke beyond the current treatment window. Experiments were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong ([CULATR 3834-15 (approval date January 5, 2016); 3977-16 (approval date April 13, 2016); and 4666-18 (approval date March 29, 2018)].

Keywords: blood-brain barrier; brain injury; diabetes mellitus; hemorrhagic transformation; infarct; ischemia/reperfusion injury; middle cerebral artery occlusion; mouse model; stroke; vascular endothelial growth factor

Chinese Library Classification No. R453; R364; Q2
Introduction
Diabetes mellitus is a prominent disease worldwide (Saeedi et al., 2019). Type 1 and type 2 diabetes increases the risk of ischemic stroke by 6.3-fold and 2.3-fold, respectively (Janghorbani et al., 2007), and one study reported that 31% of patients with ischemic stroke had diabetes (Reeves et al., 2010). Diabetic stroke is associated with a poor prognosis and high mortality (Eriksson et al., 2012; Lau et al., 2019).

The current standard intervention of large vessel occlusion is thrombolysis combined with endovascular thrombectomy (Shi et al., 2018). However, such reperfusion therapies are hindered by their narrow time window (for thrombolysis (alteplase): 4.5 hours after symptom onset; for endovascular thrombectomy: within 24 hours of last known normal to patients meeting DAWN trial eligibility criteria. DAWN excludes patients with baseline blood glucose > 400 mg/dL (22.2 mM), and for these patients, endovascular thrombectomy must be initiated within 6 hours of symptom onset; Jovin et al., 2017; Powers et al., 2019). In combination with a concern of increased intracranial hemorrhage risk in patients with diabetes/hyperglycemia (Celik et al., 2004) following reperfusion therapies (Reeves et al., 2010; Jiang et al., 2015), the rate of patients with diabetes receiving these beneficial treatments is lower than patients without diabetes (Reeves et al., 2010; Nathaniel et al., 2019; Saber et al., 2020).

Diabetic/hyperglycemic-exacerbated hemorrhage is likely to be the result of an aggravated inflammatory response primed by diabetic/hyperglycemic conditions (Jiang et al., 2021). Elevated serum matrix metallopeptidase 9 (MMP-9) has been associated with aggravated outcomes in clinical studies (Abdelnasseer et al., 2015; Zhong et al., 2017), as well as with cerebral hemorrhagic transformation in ischemia-reperfused animals (Elgebaly et al., 2010; McBride et al., 2020) and patients (Montaner et al., 2003). Furthermore, MMP-9 levels upon ischemic stroke have been found to be aggravated under hyperglycemic conditions in human serum (Setyopranoto et al., 2018) and in the cerebrocerebri of diabetic mice (Kumari et al., 2011). Another matrix metallopeptidase, MMP-2, has also been considered to participate in the early stage of ischemic pathology (Yang and Rosenberg, 2015) and has found to be up-regulated very soon after experimental ischemia in baboons (Heo et al., 1999; Chang et al., 2003). MMP-2 has also been shown to destroy vascular integrity (Liu et al., 2012) and induce hemorrhagic transformation upon ischemia in mice (Lu et al., 2013). However, the mechanism/s underlying MMP-2/9 aggravation in diabetes upon ischemic stroke have not been well studied.

Although ischemic stroke has been investigated using hyperglycemic/diabetic animal models, most existing mice model studies induced either a short ischemia length (0.5–1.5 hours) or permanent occlusion in favor of reduced animal mortality (Tsuchiya et al., 2003; Villalba et al., 2018). These experimental paradigms have left the mechanism of diabetes-exacerbated outcomes under prolonged ischemia and delayed reperfusion largely unstudied. Thus, there is still limited progress in extending the window of reperfusion therapies for patients with diabetic stroke. However, a fast and complete reperfusion is important for outcome improvement in patients with stroke (Shi et al., 2018).

To fill this gap between basic research and the clinical situation, this study used Ins2+/+ mice and 2-hour transient intraluminal MCAO to mimic delayed mechanical thrombectomy in patients with diabetic stroke, and investigated the causal mechanism of exacerbated intracerebral hemorrhage after prolonged ischemia in diabetes. Ins2+/+ mice are a widely accepted model of type 1 diabetes (Lai and Lo, 2013) and have been used to study diabetic complications in various organ systems. The present results could aid the development of interventions to extend the treatment window of mechanical thrombectomy in patients with diabetic stroke by mitigating the exacerbated hemorrhage through the identification of suppression targets.

Materials and Methods

Animals
Twelve-week-old male Ins2+/+Icr (n = 74; 23.7 ± 0.2 g) and Ins2+/− (n = 69; 25.3 ± 0.2 g) mice were generated from C57BL/6-Ins2+/+Icr and C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME, USA). They were kept under a 12-hour light/dark cycle and given ad libitum access to food and water. Two to five mice were housed per cage. Blood glucose level was measured using a glucometer (Ascensia Elite XL, Bayer Healthcare AG, Leverkusen, Germany).

The mice of both genotypes were randomly assigned to the sham or MCAO groups for each of the two time-points of assessment (2 hours ischemia/2 hours reperfusion or 2 hours ischemia/22 hours reperfusion), resulting in a total of eight groups (Table 1).

Experiments were conducted according to local and institute regulations and were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong [CULATR 3834-15 (approval date January 5, 2018); 3977-16 (approval date April 13, 2016); and 4666-18 (approval date March 29, 2018)], as well as in compliance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. This study was reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Percie du Sert et al., 2020).

Induction of ischemia and reperfusion
Transient focal cerebral ischemia was induced using the intraluminal method (Lo et al., 2005; Li et al., 2012; Yang et al., 2012). In brief, mice were randomly assigned and were subjected to 2% isoflurane (IsoFlo; 50019100, Zoetis Inc., Kalamazoo, MI, USA) inhalation anesthesia (2% isoflurane in 70% N2/30% O2 for maintenance). A filament coated with vinylpolysiloxane material (ESPE 7302, 3M Dental Products, St. Paul, MN, USA) was inserted into the right internal carotid artery and further advanced until reaching the middle cerebral artery. After 2 hours of ischemia, the filament was removed to commence reperfusion. Animals in the sham groups received the same procedures except for filament insertion. Relative cerebral blood flow in the middle cerebral artery territory was monitored by a laser Doppler flowmeter (Periflux 5000, Perimed AB, Järfälla, Sweden), with the reading at 5 minutes before ischemia set as 100%. Body temperature was maintained at 37 ± 0.5°C throughout the surgery. Mice were placed in an intensive care unit set at 30 ± 0.5°C during ischemia and for 4 hours after reperfusion commenced. Twelve-week-old male Ins2+/+ mice and Ins2+/− mice were subjected to 2 hours of middle cerebral artery occlusion (MCAO) or sham surgery. Two hours later, the filament was removed to commence reperfusion in mice of the MCAO group, while mice in sham group received another sham surgery. At either 2 hours (2h ischemia) or 2 hours reperfusion; abbreviated as 2h I/2h R) or 22 hours (2 hours ischemia/22 hours reperfusion) or 22 hours (2 hours ischemia/22 hours reperfusion) of reperfusion (Figure 1).

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Survival rate and neurological assessments
Experimental mice were closely monitored and survival rate was recorded. At the end of the reperfusion period (either 2 hours or 22 hours after reperfusion), neurological deficits were evaluated by an observer blinded to the genotype of the mouse (Lo et al., 2005; Li et al., 2012; Yang et al., 2012). The scoring system was as follows: 0 – no observable neurological deficits (normal); 1 – left wrist drop and walks straight (mild); 2 – left wrist drop and walks in a circular motion (moderate); 3 – loss of righting reflex (severe); and 4 – dead.

Tissue collection
Following decapitation under anesthesia with intraperitoneal injection of a mixture of ketamine (100 mg/kg, Alfasan International BV, Woerden, Netherlands) and xylazine (6 mg/kg, Alfasan International) at either 2 hours or 22 hours after reperfusion, mouse brains were cut into five 2 mm-thick coronal slices. For histological analyses, brain slices were stained with 2% 2,3,5-triphenyltetrazolium chloride and fixed in 10% formalin (see below). For molecular analyses, infarct and penumbra areas of brain slice number two to four were collected at 1 mm away from the midline, snap-frozen in liquid nitrogen and homogenized together in 10 mM phosphate buffered saline (PBS), pH 7.4, containing protease inhibitors (4693159001, Roche Applied Science) and phosphatase inhibitors (524628, Merck KGaA, Darmstadt, Germany), followed by protein or mRNA extraction (see below).

Measurement of infarct, swelling, and hemorrhagic transformation
Freshly cut brain slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; T8877, Sigma-Aldrich, St. Louis, MO, USA) in PBS at 37°C for 7.5 minutes and then fixed overnight in 10% buffered formalin (Merck KGaA). The red live and white infarct areas on the posterior side of brain slices were measured using SigmaScan Pro (SPSS Inc., Chicago, IL, USA) by a researcher who was blinded to the genotypes of each mouse. Infarct areas and volume were estimated indirectly to minimize inaccuracy due to swelling (Swanson et al., 1990), and hemispheric swelling was estimated using the following equation: (ipsilateral volume – contralateral volume)/contralateral volume × 100%. Hemorrhagic transformations, identified as dark-brown areas on the posterior side of brain slice number three (at approximately bregma –0.34 mm), were presented as percentages of infarct and contralateral areas.

Histochemistry and immunohistochemistry
To further verify the presence of hemorrhage, the fixed brain slices were embedded in paraffin (T565, Fisher Chemical, Thermo Fisher Scientific, Waltham, MA, USA) and cut into sections of 7 μm thickness using a microtome (HM 315R, Microm International GmbH, Walldorf, Germany). Sections were dehydrated in ascending ethanol gradients followed by toluene for 3 times for 5 minutes each, followed by toluene and xylene to be dried and deparaffinized at 57°C overnight in an oven set to 37°C. Sections were differentiated in acid alcohol for 5 minutes, the sections were dehydrated in ascending ethanol gradients followed by xylene for 3 times for 5 minutes each, followed by xylene and toluene to be dried, mounted with Permount (SP15-500, Fisher Chemical) and covered with cover slips. Microscopic photos of infarct cores were taken using a light microscope (Eclipse 80i; Nikon, Tokyo, Japan) equipped with a digital camera (SPOT RT3 25.4 M2 Mp slider; Diagnostic Instruments, Inc., Sterling Heights, MI, USA), and patches of orange red indicated the presence of hemorrhage.

Western blot analysis
Lysates of infarct and penumbra areas of brain slices two to four were mixed with 2× ice-cold radioimmunoprecipitation assay lysis buffer in a 1:1 ratio. Supernatants were collected following centrifugation at 16,100 × g and 4°C for 30 minutes, mixed with Laemmli reducing loading dye at 95°C for 5 minutes, separated by SDS-PAGE at 30 mA (4% stacking gel and 10% separating gel, 10 μg protein per lane), and transferred onto polyvinylidene fluoride membranes (IPVH00010, Merck KGaA) at 300 mA for 2 hours on ice. The membranes were blocked with 5% skimmed milk (Nestlé S.A., Switzerland) in Tris-buffered saline with 0.1% Tween 20 detergent (0.1% TBST) and incubated with primary antibodies (Additional Table 1) diluted with 5% bovine serum albumin (USB Corporation, Affymetrix, Santa Clara, CA, USA) in 0.1% TBST at 4°C overnight followed by peroxide-conjugated anti-rabbit IgG (PI-1000, 1:2000, Vector Laboratories, Burlingame, CA, USA) or anti-mouse IgG (PI-2000, 1:5000, Vector Laboratories) secondary antibodies at room temperature for 1 hour. Immunoreactivities were detected using enhanced chemiluminescence reagents (RPN2106, GE Healthcare, Buckinghamshire, UK or K-12042-D10, Advanta, CA, USA) and light-sensitive films (47410 19291, Fujifilm, Tokyo, Japan), quantified using ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA), and normalized using an endogenous protein (actin or α-tubulin), except for phospho-(p)-Akt, p-Erk1/2, and p-p38 MAPK, which were normalized with their respective total expressions.

Real-time PCR analysis
After mixing lysates of infarct and penumbra areas of brain slices two to four with ice-cold RNAiso plus (9109, Takara Bio Inc., Japan) at a ratio of 1:4, total RNA was extracted by the phenol:chloroform extraction method, and cDNA was prepared from 2 μg of the extracted RNA (SuperScript VILO; 11754050, Life Technologies, USA), both according to the manufacturers’ instructions. Real-time PCR reactions were performed using the StepOnePlus system and SYBR Green technology (4385610, Life Technologies) with the primers listed in Additional Table 2. The relative mRNA expression levels were shown as fold changes to the Ins2+/+ sham group after normalizing with the endogenous gene β-actin, following the manufacturer’s protocol.

### Table 1 Number of experiments performed and body weight of mice used

|                      | Ins2+/+ Sham | Ins2Δα/Δα+ MCAO | Ins2+/+ MCAO | Ins2Δα/Δα+ MCAO | Ins2+/+ Sham | Ins2Δα/Δα+ MCAO | Ins2+/+ MCAO | Ins2Δα/Δα+ MCAO |
|----------------------|--------------|-----------------|--------------|-----------------|--------------|-----------------|--------------|----------------|
| Number of experiments performed | 10 | 9             | 24           | 21              | 10           | 10              | 25           | 34             |
| Excluded due to incomplete occlusion/reperfusion | –  | –             | 4            | 1               | –            | –               | 1            | 0              |
| Included             | 10           | 9              | 20           | 20              | 10           | 10              | 24           | 34             |
| Body weight before experiment (g) | 25.3±0.5     | 24.1±0.4       | 26.0±0.4**   | 24.0±0.4**      | 24.6±0.5     | 23.8±0.4        | 25.1±0.3     | 23.4±0.2**     |

This table shows the number of mice used for each group. Mice were randomly drawn for the experiments. In mice with MCAO performed, relative cerebral blood flows were measured using a laser Doppler at 5 minutes before and during ischemia as well as 5 minutes after reperfusion, and animals were excluded from analysis if the measurements indicated any incomplete occlusion or reperfusion. In mice subjected to MCAO, the body weight of Ins2Δα/Δα+ mice were significantly lower than that of Ins2+/+ mice. *p < 0.05, vs. Ins2Δα/Δα+ Sham; **p < 0.01, vs. Ins2+/+ MCAO; one-way analysis of variance followed by Tukey’s HSD post hoc test; mean ± SEM. I: Ischemia; MCAO: middle cerebral artery occlusion; R: reperfusion.
Statistical analysis
No statistical methods were used to predetermine sample sizes; however, the sample sizes of this study (~20 animals in each group) were similar to those reported in our previous publication (Li et al., 2012). The total number of mice used in each group is shown in Table 1. Data are expressed as the mean ± standard error of mean (SEM) and statistical tests were performed using GraphPad Prism (v5.02; GraphPad Software, Inc., USA). Survival rate and the neurological score were analyzed using the log-rank test and Mann-Whitney U test, respectively. All other measurements were analyzed using a one-way analysis of variance followed by Tukey’s HSD post hoc tests or unpaired Student’s t-tests, except for the comparison of hemorrhagic transformation areas, in which case the Mann-Whitney U test was used due to non-normality of the data. A P-value of < 0.05 was considered statistically significant.

Results
Physiological parameters of Ins2+/− and Ins2Akita/+ mice
Ins2Akita/+ mice displayed hyperglycemia from 4 weeks of age and a decreased body weight from 13 weeks of age (Figure 2). Ins2Akita/+ mice had higher relative cerebral blood flow during ischemia, which was similar in both genotypes during reperfusion (Table 2).

Figure 2 | Ins2Akita/+ mice displayed hyperglycemia and decreased body weight.
Ins2Akita/+ mice displayed hyperglycemia (A) and decreased body weight (B) from 4 and 13 weeks of age, respectively, when compared with Ins2+/− mice (n = 10–19; * P < 0.05, ** P < 0.01, and *** P < 0.001, unpaired Student’s t-test; mean ± SEM).

Table 2 | Physiological conditions of Ins2Akita/+ mice and their wildtype littermates before and after ischemia

Relative cerebral blood flows were measured using a laser Doppler at 5 minutes before and during ischemia as well as 5 minutes after reperfusion. The reading of relative blood flow at 5 minutes before ischemia was set as 100%, and the sequential changes of blood flow were calculated as a reference to this. Relative cerebral blood flows were similar in both Ins2Akita/+ and Ins2+/− mice after reperfusion, but they were faster in Ins2Akita/+ mice during ischemia. * P < 0.05, vs. Ins2+/− MCAO at 22 h R (22 hours after reperfusion), unpaired Student’s t-test. Data are expressed as the mean ± SEM. I: Ischemia; MCAO: middle cerebral artery occlusion; R: reperfusion.

Ins2Akita/+ mice subjected to prolonged ischemia showed decreased survival rate and worse neurological outcomes
Ins2Akita/+ mice had a significantly lower survival rate than Ins2+/− mice after reperfusion (Figure 3A and B). The majority of lethality was observed in the first 4 hours after reperfusion in Ins2Akita/+ mice, while death of Ins2+/− mice mostly occurred later. Moreover, Ins2Akita/+ mice suffered from more severe neurological deficits at 2 hours after reperfusion (Figure 3C), whereas there was no between-group difference at 22 hours after reperfusion (Figure 3D).

Figure 3 | More severe neurological deficits and lower survival rate in Ins2Akita/+ mice after 2 hours of MCAO.
(A & B) Survival rate after 2 hours of ischemia recorded at various time points after reperfusion. The majority of deaths of Ins2+/− mice occurred before 4 hours of reperfusion. (C & D) Neurological deficits were graded from 0 to 4 at the end of the assigned period of reperfusion. A significantly higher neurological score was observed in Ins2Akita/+ mice compared with Ins2+/− mice at 2 hours after reperfusion, but the difference diminished at 22 hours after reperfusion (2h I/22h R: Ins2−/− (n = 20), Ins2+/− (n = 20); 2h I/22h R: Ins2−/− (n = 24), Ins2+/− (n = 34); * P < 0.05, Log-rank (Mantel-Cox) test; **P < 0.05, Mann-Whitney U test; mean ± SEM).

Ins2Akita/+ mice displayed accelerated infarct development and increased hemorrhage after prolonged ischemia
In the second group of experiments, Ins2+/− and Ins2Akita/+ mice displayed accelerated infarct development and increased hemorrhage after prolonged ischemia (Figure 4A and B). Ins2+/− mice had significantly larger infarct area and infarct volume compared with Ins2+/− brains (Figure 4C), with significantly larger infarct volume compared with Ins2+/− brains and a trend towards greater hemispheric swelling (Figure 4E and F). However, these differences between Ins2+/− and Ins2Akita/+ mice diminished at 22 hours after reperfusion (Figure 4D, G, and H).

Figure 4 | Increased development rate of infarct area and infarct volume upon 2h I/22h R ischemic challenge in Ins2Akita/+ mice.
Representative TTC-stained brain slices of mice after 2h I/2h R (A) and 2h I/22h R (B). Calculated infarct area, infarct volume, and hemispheric swelling after 2h I/22h R are shown in C, E, and F; those after 2h I/2h R are shown in D, G, and H, respectively. 2h I/22h R: Ins2+/− middle cerebral artery occlusion (MCAO); (n = 12), Ins2−/− MCAO (n = 10), Ins2+/− MCAO (n = 10), Ins2−/− MCAO (n = 10); * P < 0.05, ** P < 0.01, unpaired Student’s t-test; mean ± SEM. I: Ischemia; R: reperfusion; TTC: 2,3,5-triphenyltetrazolium chloride.
Hemorrhage (red-pink spreading) was observed in ipsilateral \textit{Ins2}^{AKita} brains as early as 2 hours after reperfusion (Figure 5A and B), while eosin staining revealed its presence around blood vessels in both the infarct core (Figure 5C–F) and penumbra area (data not shown). Quantitative analysis revealed a significantly larger hemorrhagic area in brain slice number three of \textit{Ins2}^{AKita} mice at 2 hours after reperfusion (Figure 5G and H), which was robustly exacerbated at 22 hours after reperfusion (Figure 5I and J).

Figure 5 | Increased hemorrhage in \textit{Ins2}^{AKita} mice as early as 2 hours after reperfusion following 2 hours of ischemia, which was further advanced at 22 hours after reperfusion. General view of the ipsilateral side of the brain of \textit{Ins2}^{WT} (A) and \textit{Ins2}^{AKita} (B) mice after 2h/2h R challenge. The black line outlining the reddish area indicates the presence of hemorrhage. Representative regions of the infarct core located in the cerebral cortex after 2 hours (C & D) and 22 hours (E & F) of reperfusion are shown. Sections were stained with hematoxylin and eosin, with which red blood cells were stained in an orange-red color. Scale bars: 50 μm. Quantification of the hemorrhagic area was presented as a ratio of the infarct core as well as a ratio of the contralateral side in brain slice number three of mice after 2 hours (G & H) and 22 hours (I & J) of reperfusion. *P < 0.05, **P < 0.01, Mann-Whitney U test; mean ± SEM. I: Ischemia; R: reperfusion.

Decreased tight junction protein ZO-1 level and increased blood-brain barrier-disrupting MMP expressions in \textit{Ins2}^{AKita} brains after prolonged ischemia

Infarct and penumbra areas of operated mice were subjected to Western blot analysis. At 2 hours after reperfusion, there was a reduced level of tight junction protein ZO-1 in operated \textit{Ins2}^{WT} mice upon MCAO, which was further exaggerated in \textit{Ins2}^{AKita} mice (Figure 6A). Likewise, at 22 hours after reperfusion, only \textit{Ins2}^{AKita} mice showed a significant reduction in ZO-1 level (Figure 6B).

Western blot analysis of MMP-2, which is known to disrupt the blood-brain barrier following stroke, was significantly up-regulated only in \textit{Ins2}^{AKita} ipsilateral brains at 2 hours after reperfusion (Figure 6C). Interestingly, at 22 hours after reperfusion, the previously up-regulated MMP-2 expression in \textit{Ins2}^{AKita} mice returned to a normal level that was similar to that of sham-operated controls; however, MMP-2 expression in \textit{Ins2}^{WT} mice was significantly up-regulated (Figure 6D).

The expression of MMP-9 was also significantly increased in \textit{Ins2}^{AKita} mouse brains at 2 hours after reperfusion when compared with that of the sham-operated \textit{Ins2}^{WT} mice (Figure 6E), but not at 22 hours after reperfusion (Figure 6F).

Up-regulation of vascular endothelial growth factor (VEGF) and p-Erk1/2 were remarkably over-expressed in \textit{Ins2}^{AKita} ipsilateral brains when compared with \textit{Ins2}^{WT} mice (Figure 7A and C). The expression of p-p38 mitogen-activated protein kinase (p-p38 MAPK) was also significantly higher in \textit{Ins2}^{AKita} ipsilateral brains than that of sham-operated \textit{Ins2}^{WT} controls (Figure 7E). In contrast, there were no significant changes in p-Akt level in operated mice of both genotypes when compared with levels of sham-operated mice (Figure 7G).

Similarly, at 22 hours after reperfusion, VEGF expression only remarkably increased in \textit{Ins2}^{AKita} mice (Figure 7B). While there was a significant increase of p-Erk1/2 expression in the ipsilateral brains of both genotypes, its level in \textit{Ins2}^{AKita} mouse brains was significantly higher than that of \textit{Ins2}^{WT} mice (Figure 7D). The expression of p-p38 in both genotypes was also significantly higher than that of the sham-operated controls (Figure 7F). No significant differences in p-Akt level were found between any of the groups (Figure 7H).

Increased endoplasmic reticulum stress markers expression in \textit{Ins2}^{AKita} brains during the early stage of reperfusion

The expression of endoplasmic reticulum (ER) stress markers in ipsilateral brains was revealed by RT-PCR. CHOP was significantly up-regulated in both \textit{Ins2}^{WT} and \textit{Ins2}^{AKita} brains at 2 hours after reperfusion, with a more pronounced increase in \textit{Ins2}^{AKita} mice (Figure 8A). In contrast, a significant increase in BiP expression was only found in \textit{Ins2}^{AKita} mice when compared with sham-operated \textit{Ins2}^{WT} controls (Figure 8A). These increases vanished at 22 hours after reperfusion (Figure 8B).

Figure 6 | Vulnerable blood vessel integrity in \textit{Ins2}^{AKita} mice at 2 hours and 22 hours after reperfusion following 2 hours of ischemia. Protein expressions of ZO-1, MMP-2, and MMP-9 for the two experimental groups, 2h I/2h R (A, C, & E) and 2h I/22h R (B, D & F), were semi-quantified using Western blot analysis. The corresponding fold changes of these proteins are shown in the bottom panel. WT: \textit{Ins2}^{WT}; AK: \textit{Ins2}^{AKita}; A, C, & E: \textit{Ins2}^{WT} Sham; n = 4, \textit{Ins2}^{WT}/Sham: n = 4, \textit{Ins2}^{AKita} 2h R: n = 5, \textit{Ins2}^{AKita}/Sham: n = 4, \textit{Ins2}^{AKita} 22h R: n = 5, \textit{Ins2}^{AKita}/Sham: n = 5; *P < 0.05, **P < 0.01, ***P < 0.001, vs. \textit{Ins2}^{WT}/Sham; #P < 0.05, ##P < 0.01, and ###P < 0.001, vs. \textit{Ins2}^{WT}/Sham; +P < 0.01, vs. \textit{Ins2}^{WT} 2h R or \textit{Ins2}^{WT}/Sham; one-way analysis of variance followed by Tukey’s HSD post hoc test; mean ± SEM. I: Ischemia; MCAO: middle cerebral artery occlusion; MMP: matrix metalloproteinase; R: reperfusion.
mice could mimic the exacerbated outcomes mice at 2 hours after reperfusion mice, Up-regulation of VEGF and its endothelium BiP R: reperfusion. Ins2 R: ischemic challenge followed by 2 hours (A) or 22 hours (B) of reperfusion. mRNA expressions of ER stress-targets were analyzed after 2 hours of increased in the Figure 8, which is involved in the ER stress pathway, was further demonstrated by ZO-1 down-regulation in Ins2 mice after 2 hours of reperfusion following 2 hours of ischemia. Further, this rapid compromise of blood vessel integrity was associated with a quicker and heavier compromise of blood vessel integrity, which was shown to be more severe neurological deficits, earlier mortality, increased hemorrhagic transformation (Zhang et al., 2000; Kaya et al., 2005). Meanwhile, antagonism of VEGF signaling has been found to significantly reduce brain swelling in both normal adult (van Bruggen et al., 1999; Shimamura et al., 2006; Yoo et al., 2010; Wu et al., 2014), but its role in exacerbating diabetic ischemic stroke has not been well studied. We observed a remarkable and persistent VEGF up-regulation during reperfusion in Ins2/mice, which suggests that diabetic conditions play a role in magnifying VEGF overexpression after prolonged ischemia. The deleterious role of VEGF reported in rodents after stroke includes increasing infarct volume, blood-brain barrier breakdown, and hemorrhagic transformation (Zhang et al., 2000; Kaya et al., 2005). Meanwhile, antagonism of VEGF signaling has been found to significantly reduce brain swelling in both normal adult (van Bruggen et al., 1999) and diabetic mice (Kim et al., 2018) at 1 day after reperfusion. In parallel with worse neurological deficits, earlier mortality, increased hemorrhagic transformation, and blood-brain barrier breakdown, as demonstrated by ZO-1 down-regulation in Ins2/mice, the current study further substantiated the deleterious role of VEGF in exacerbating diabetic ischemic stroke during reperfusion.

Erk1/2 and p38 MAPK are two downstream factors of the VEGF pathway that are responsible for the proliferation and migration of endothelial cells during angiogenesis, respectively, while Akt is another downstream factor and induce hemorrhagic transformation upon ischemia in mice (Lu et al., 2013), has been found to be up-regulated very soon after MCAO in baboons (Heo et al., 1999; Chang et al., 2003). Furthermore, MMP-9 has been associated with hemorrhagic transformation following ischemia in multiple animal and clinical studies (Montaner et al., 2003; del Zoppo, 2010; Elgebaly et al., 2010; Khati et al., 2012; McBride et al., 2020). In the current study, we observed a premature increase of MMP-2 in Ins2/mice at 2 hours after reperfusion compared with Ins2/mice, in which the increase was only evident at 22 hours after reperfusion. We also observed a potentially higher MMP-9 expression in Ins2/mice at 2 hours after reperfusion, yet this difference was not significant, which may be due to the limited sample size. Together with a greater decline in ZO-1 levels starting at 2 hours after reperfusion, these results indicate that early increased expressions of MMPs in diabetic animals after experimental stroke result in a quicker and more severe compromise of blood vessel integrity and therefore hemorrhagic transformation, which may account for the worse outcomes and earlier mortality in Ins2/mice.

Discussion

In the current study, we demonstrated that induction of prolonged ischemia followed by reperfusion in diabetic Ins2/mice could mimic the exacerbated outcomes observed in patients with diabetes upon ischemic stroke but with delayed reperfusion. Multiple studies have reported more severe neurological deficits, larger infarcts, and hemorrhagic transformation in diabetic animals after experimental stroke (Young et al., 2000; Kusaka et al., 2004; Elgebaly et al., 2010; Chen et al., 2011; Ye et al., 2011; Cui et al., 2012; Ning et al., 2012; Yan et al., 2012; Liao et al., 2014; Mishiro et al., 2014). After inducing 2-hour long ischemia, we found early hormone and hemorrhagic transformation in diabetic Ins2/mice at as early as 2 hours after reperfusion, with a robust increase in hemorrhagic transformation at 22 hours after reperfusion. This study revealed that the diabetes-exacerbated hemorrhagic transformation was due to a quicker and heavier compromise of blood vessel integrity, which was shown by the greater loss of tight junction protein ZO-1 from 2 hours of reperfusion in Ins2/mice. Furthermore, this rapid compromise of blood vessel integrity was associated with accelerated VEGF and p-Erk1/2 up-regulation in Ins2/mice, which suggests that VEGF is a potential target for attenuating hemorrhage following delayed reperfusion in diabetic stroke. MMP-2, which can destroy vascular integrity (Liu et al., 2012)
mice, which resulted in the mice also increased, but to a lesser extent. These results suggest that the rapidly exaggerated VEGF expression found in diabetic Ins2+/− mice is largely driven by a pro-survival UPR to CHOP-driven pro-apoptotic UPR. Alternatively, the chronic inflammation and oxidative stress environment that is exacerbated under diabetic conditions might play a role in the rapid exacerbation of hemorrhage transformation during delayed reperfusion therapy in patients with diabetes beyond the therapeutic window. By considering the relationship between diabetes-exacerbated hemorrhage transformation and the HIF-1α-VEGF pathway as a possible therapeutic target, the potential of suppressing the VEGF pathway in attenuating diabetes-exacerbated stroke outcomes was not explored in the present study due to the high mortality of mice following prolonged ischemia. In addition, neurological score was used as a measurement of neurological deficit in this study. Although carried out in a blinded manner, the subjective nature of the assessment could intrinsically bring inaccuracy. More objective methods for assessing neurological deficit, such as the adhesive removal test, should be included in future research.

Conclusion

We demonstrated that diabetic conditions might play a key role in the rapid exacerbation of hemorrhage transformation at the reperfusion phase following prolonged ischemia. We postulate that this rapid exacerbation seen under diabetic, but not normoglycemic conditions, is at least partially underpinned by diabetes-aggravated premature VEGF up-regulation during delayed reperfusion. This has deleterious effects via rapid triggering of vascular disintegration and, hence, hemorrhagic transformation, and therefore exacerbated other outcomes upon prolonged ischemia and reperfusion.

Limitations

Although this study postulates the relationship between diabetes-exacerbated hemorrhage transformation and aggravated VEGF up-regulation during delayed reperfusion, the potential of suppressing the VEGF pathway in attenuating diabetes-exacerbated stroke outcomes was not explored in the present study due to the high mortality of mice following prolonged ischemia. In addition, neurological score was used as a measurement of neurological deficit in this study. Although carried out in a blinded manner, the subjective nature of the assessment could intrinsically bring inaccuracy. More objective methods for assessing neurological deficit, such as the adhesive removal test, should be included in future research.

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for a better clinical outcome. In future research, the effect of VEGF antagonism during delayed reperfusion in diabetic animals should be explored by the use of VEGF antagonists like bevacizumab and sunitinib.

Author contributions: Study design: AKWL; literature search, manuscript preparation: AKWL, TCN; experimental studies and data acquisition: AKWL, VKLH, KCT; data analysis: AKWL, TCN, VKLH; manuscript editing and review: TCN, CWC, SJC, ACYL; study concept and design, literature search, experimental studies, data analysis, guarantor: ACYL. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Editor note: ACYL is an Editorial Board member of Neural Regeneration Research. She was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal’s standard procedures, with peer review handled independently of this Editorial Board member and their research groups.

Financial support: This study was supported by Health and Medical Research Fund, the Food and Health Bureau, The Government of the Hong Kong Special Administrative Region (03142256); General Research Fund, Hong Kong Research Grants Council (GRF #HKU773613M); Seed Funding Programme for Basic Research (201811159123, 201910159191), The University of Hong Kong (all to ACYL).

Institutional review board statement: The animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong [CULATR 3834-15 (approval date January 5, 2016); 2977-16 (approval date April 13, 2016); and 4666-18 (approval date March 29, 2018)].

Author statement: The abstract has been presented at Neuroscience 2016 (San Diego, CA, USA; Nov 12-16, 2016), 14th International Symposium on Healthy Aging (Hong Kong SAR, China; March 16–17, 2019), and Neuroscience 2019 (Chicago, IL, USA; October 19–23, 2019), respectively.

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Additional files:

Additional Table 1: Antibodies used in Western blot analysis.
Additional Table 2: Primer sequences used in real-time PCR analysis.
Additional Figure 1: Original blot images for Figure 6.
Additional Figure 2: Original blot images for Figure 7.

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### Additional Table 1 Antibodies used in Western blot analysis

| Target         | Dilution | Cat#  | Supplier                                      |
|----------------|----------|-------|-----------------------------------------------|
| α-Tubulin      | 1:20000  | sc-5286 | Santa Cruz Biotechnology, Santa Cruz, CA     |
| Actin          | 1:5000   | MAB1501 | Merck KGaA, Darmstadt, Germany                |
| MMP-2          | 1:1000   | 4022   | Cell Signaling Technologies, MA              |
| MMP-9          | 1:1000   | 2270   | Cell Signaling Technologies, MA              |
| ZO-1           | 1:1000   | 40-2300 | Zymed Laboratories, South San Francisco, CA  |
| VEGF           | 1:1000   | sc-7269 | Santa Cruz Biotechnology, Santa Cruz, CA     |
| Total Akt      | 1:1000   | 9272   | Cell Signaling Technologies, MA              |
| p-Akt          | 1:1500   | 9277   | Cell Signaling Technologies, MA              |
| Total Erk1/2   | 1:2000   | 9107   | Cell Signaling Technologies, MA              |
| p-Erk1/2       | 1:2000   | 9106   | Cell Signaling Technologies, MA              |
| Total p38 MAPK | 1:1000   | 9212   | Cell Signaling Technologies, MA              |
| p-p38 MAPK     | 1:1000   | 9211   | Cell Signaling Technologies, MA              |

MAPK: Mitogen-activated protein kinase; MMP: matrix metallopeptidase; VEGF: vascular endothelial growth factor.
### Additional Table 2 Primer sequences used in real-time PCR analysis

| Target  | Primer sequence (5' to 3') | Reference                  |
|---------|----------------------------|----------------------------|
| β-actin | Forward: GACGGCCAGGTCTACATATTG  | Binet et al., 2013        |
|         | Reverse: CCACAGGATTCCATACCCAAGA |                            |
| BiP     | Forward: AAGGTGAAACGACCCCTAACAAA | Binet et al., 2013        |
|         | Reverse: GTCAACTCGGAGAATACCATAACATCT |            |
| CHOP    | Forward: GTTGAAGATGACGCGTGGGCAGC | Wang et al., 2012        |
|         | Reverse: GCACGTGGACCAGGTTCTGCTT |                            |

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