Targets of vascular protection in acute ischemic stroke differ in type 2 diabetes

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1Department of Physiology, Georgia Regents University Augusta, Augusta, Georgia; 2Program in Clinical and Experimental Therapeutics, Georgia Regents University Augusta, Augusta, Georgia; 3Department of Biostatistics, Georgia Regents University Augusta, Augusta, Georgia; and 4Charlie Norwood Veterans Administration Medical Center, Augusta, Georgia

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Kelly-Cobbs AI, Prakash R, Li W, Pillai B, Hafez S, Coucha M, Johnson MH, Ogbi SN, Fagan SC, Ergul A. Targets of vascular protection in acute ischemic stroke differ in type 2 diabetes. Am J Physiol Heart Circ Physiol 304: H806–H815, 2013. First published January 18, 2013; doi:10.1152/ajpheart.00720.2012.—Hemorrhagic transformation is an important complication of acute ischemic stroke, particularly in diabetic patients receiving thrombolytic treatment with tissue plasminogen activator, the only approved drug for the treatment of acute ischemic stroke. The objective of the present study was to determine the effects of acute manipulation of potential targets for vascular protection [i.e., NF-κB, peroxynitrite, and matrix metalloproteinases (MMPs)] on vascular injury and functional outcome in a diabetic model of cerebral ischemia. Ischemia was induced by middle cerebral artery occlusion in control and type 2 diabetic Goto-Kakizaki rats. Treatment groups received a single dose of the peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), the nonspecific NF-κB inhibitor curcumin, or the broad-spectrum MMP inhibitor minocycline at reperfusion. Poststroke infarct volume, edema, hemorrhage, neurological deficits, and MMP-9 activity were evaluated. All acute treatments reduced MMP-9 and hemorrhagic transformation in diabetic groups. In addition, acute curcumin and minocycline therapy reduced edema in these animals. Improved neurological function was observed in varying degrees with treatment, as indicated by beam-walk performance, modified Bederson scores, and grip strength; however, infarct size was similar to untreated diabetic animals. In control animals, all treatments reduced MMP-9 activity, yet bleeding was not improved. Neuroprotection was only conferred by curcumin and minocycline. Uncovering the underlying mechanisms contributing to the success of acute therapy in diabetes will advance tailored stroke therapies.

minocycline; curcumin; 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III); nuclear factor-κB; vascular protection

WITHIN THE NEXT DECADE, the number of individuals living with diabetes is expected to rise dramatically (8). By 2030, the estimated global prevalence of the disease will exceed 437 million (53), and the vascular damage sustained during the course of the disease will increase the likelihood that these affected individuals will develop micro- and macrovascular complications, including acute ischemic stroke (AIS) (41). Historically, individuals 65 yr and older are disproportionately affected, but for the first time ever, there is an alarming increase in the number of Americans who are diagnosed at a younger age and face a prolonged course of diabetes (46). This trend poses a serious problem because recent findings suggest that the risk of AIS increases 3% with each year of diabetes and triples after 10 yr (5). In addition, diabetes complicates ischemic injury, leading to increased morbidity and poor functional recovery (7), but how diabetes worsens ischemic stroke is not fully delineated. Understanding these subtleties is essential in identifying targets for neurovascular protection and for developing therapeutic strategies tailored to this burgeoning at-risk population.

In a series of studies, we showed that there is extensive cerebrovascular remodeling and dysfunctional neovascularization that is characterized by increased matrix metalloproteinase (MMP)-2 and -9 activity in a lean and mild model of type 2 diabetes (T2D) (18, 28). Preexisting diabetes in conjunction with ischemia-reperfusion injury causes a rapid loss of myogenic tone via increased oxidative stress (i.e., peroxynitrite) (35), augments hemorrhagic transformation (HT), and worsens functional outcome despite the relatively smaller infarct size (19). Based on these findings, we asked the following question: “Does acute peroxynitrite scavenging improve stroke outcomes?” Proinflammatory responses initiated during ischemia-reperfusion involve the activation of the nuclear transcription factor NF-κB, which is responsible for upregulating genes coding for cytokines and growth factors and can directly modulate MMP activity (25, 47). Accordingly, the present study investigated the effect of NF-κB inhibition on MMP activity and neurovascular injury and outcomes in diabetic stroke. Moreover, prevention of cerebrovascular remodeling by glycemic control with metformin or MMP inhibition with minocycline started at the onset of diabetes reduces HT and improves outcome (18). Given that MMPs, and especially MMP-9, are associated with the breakdown of the blood-brain barrier (BBB) (3, 24) and ensuing HT in experimental models of stroke that used normoglycemic animals (12, 55), in the present study, we hypothesized that pharmacological inhibition of MMP-9 at reperfusion either directly or indirectly by inhibition of NF-κB or peroxynitrite will prevent HT and improve functional outcomes in diabetes (Fig. 1).

METHODS

Animals. In previous studies, our group has shown that 3 h of middle cerebral artery (MCA) occlusion (MCAO) and 21 h of reperfusion coincided with the development of smaller infarcts and greater vascular injury (i.e., edema and HT) in the T2D Goto-Kakizaki (GK) rat model compared with normoglycemic control rats (18, 19, 38). Therefore, we selected this nonobese model of diabetes to study the effects of acute NF-κB and MMP inhibition on edema, HT, and neurological outcomes after transient focal cerebral ischemia. This spontaneously diabetic rat strain was derived from the repeated in-breeding of glucose-intolerant Wistar rats (27). For this reason, we...
Ischemia/reperfusion

FeTPPs

ONOO-

NFkB

MMP

Minocycline

Curcumin

↑ Edema, HT

Neurological deficit

Pre-existing Diabetes

Fig. 1. Schematic of experimental hypothesis. Excess peroxynitrite formation and inflammation during ischemia-reperfusion injury in preexisting diabetes amplifies the proteolytic activity of matrix metalloproteinases (MMPs), thereby contributing to greater vascular injury [i.e., edema and hemorrhagic transformation (HT)] and neurological deficit. FeTPPs, 5,10,15,20-tetrakis(4-sulfonatophenyl)prophyrinato iron (III).
that a treatment had a different effect on an outcome dependent on disease status. A Tukey’s test was used to adjust for the multiple comparisons to assess significant interaction effects from all analyses. For analysis of mortality, a Fisher’s exact test was performed, due to small frequencies, within each treatment to examine whether mortality rates were different in control and diabetic mice. Zele’s exact test for homogeneity was used to examine whether the mortality rates were homogeneous across treatments. If mortality was homogeneous across treatments, an exact Cochran-Mantel-Haenszel test was used to examine whether the mortality rates were different in control and diabetic mice.

**RESULTS**

**Metabolic parameters and mortality in experimental animals.** Average body weights and blood glucose values before MCAO surgery are shown in Table 1. Weight-matched (270–320 g) diabetic animals displayed moderate levels of hyperglycemia compared with normoglycemic control animals. Furthermore, there were no differences in poststroke mortality rates between groups.

**Evaluation of HT.** Arterial blood gases in control and diabetic groups after the induction of anesthesia before MCAO were pH 7.5 ± 0.003 versus 7.5 ± 0.003, Pco₂ 42.9 ± 1.4 versus 51.5 ± 1.5 mmHg, and Po₂ 522.8 ± 22.1 versus 545.7 ± 22.4 mmHg. Whereas there were no differences in the percent drop in cerebral perfusion right after occlusion, the percent increase in flow in the first 5–10 min of reperfusion was significantly lower in all experimental diabetic groups compared with normoglycemic groups (Table 2).

Unstained brain slices from each animal were examined for evidence of stroke-induced macroscopic bleeding (Fig. 2, A and B). Twenty-one hours after ischemia, there was minimal bleeding in normoglycemic groups, primarily in the form of diffuse individual petechiae. Since a large number of animals had no bleeding (a score of 0), vehicle versus treatment groups were not statistically compared. Evaluation of macroscopic bleeding in T2D rats indicated that macroscopic bleeding was more severe compared with untreated normoglycemic rats (P < 0.05; Fig. 2B). There was a trend for decreased macroscopic bleeding by all treatments (P = 0.71). Microscopic bleeding in the ischemic hemisphere was also measured as indicator of HT in all experimental groups (Fig. 2C). Poststroke microscopic bleeding was present in untreated normoglycemic animals. Furthermore, stroke-induced microscopic bleeding was similar in all treated normoglycemic animals except those receiving curcumin therapy. Surprisingly, single-dose administration of curcumin at reperfusion exacerbated microscopic bleeding in normoglycemic animals (P < 0.05 vs. untreated animals). Similarly, significantly higher levels of hemoglobin were detected in untreated diabetic animals compared with their normoglycemic counterparts (P < 0.05; Fig. 2C). Administration of FeTPPs at reperfusion reduced microscopic bleeding to undetectable levels. Curcumin and minocycline therapies were also effective in reducing microscopic bleeding in diabetic animals (P < 0.05 vs. untreated diabetic animals). Analysis of the bleeding data using 2 × 2 ANOVA indicated that a disease and drug interaction such that both acute treatment with FeTPPs and minocycline reduced microscopic bleeding in diabetic animals but not in the corresponding normoglycemic animals (P = 0.0003 and P = 0.0011, respectively; Fig. 2C). Curcumin, on the other hand, augmented microscopic bleeding in the ischemic brain hemispheres of control animals but reduced bleeding in diabetic animals (P = 0.0016).

**Evaluation of infarct and edema.** Infarcted regions were identified on TTC-stained brain slices to determine the extent of neuronal damage (Fig. 3A). Infarct size, expressed as a percentage of the total ischemic hemisphere, was determined for all experimental groups (Fig. 3B). FeTPPs therapy did not alter the extent of neuronal damage in normoglycemic animals; however, both curcumin and minocycline treatments reduced infarct size by ~20% (P < 0.05 vs. untreated normoglycemic animals). Edema formation, expressed as edema/infarct size, was quantified as the percent difference between ischemic and nonischemic hemispheres, correcting for ischemic lesion volume. Stroke-induced edema formation was similar in all normoglycemic treatment groups, with the exception of animals receiving minocycline at reperfusion (Fig. 3C); brain edema in

### Table 1. Metabolic parameters and mortality in experimental stroke groups

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>FeTPPs (10 mg/kg ip)</th>
<th>Curcumin (250 mg/kg ip)</th>
<th>Minocycline (20 mg/kg ip)</th>
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<tbody>
<tr>
<td></td>
<td>NG D</td>
<td>NG D</td>
<td>NG D</td>
<td>NG D</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>293 ± 4</td>
<td>307.6 ± 3</td>
<td>309.4 ± 7</td>
<td>307.2 ± 5</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>102 ± 4</td>
<td>241 ± 22*</td>
<td>105 ± 5</td>
<td>255 ± 16*</td>
</tr>
<tr>
<td>Mortality, no. of</td>
<td>3/15 (20)</td>
<td>1/16 (6.3)</td>
<td>3/8 (37.5)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>animals/total (%)</td>
<td></td>
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Results are given as means ± SE. NG, normoglycemia; D, diabetes; FeTPPs, 5,10,15,20-tetrakis(4-sulfonatophenyl)prophyrinato iron (III). *P < 0.05 vs. untreated NG.

### Table 2. Percent changes in cerebral perfusion after occlusion and reperfusion in experimental stroke groups

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>FeTPPs (10 mg/kg ip)</th>
<th>Curcumin (250 mg/kg ip)</th>
<th>Minocycline (20 mg/kg ip)</th>
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<tbody>
<tr>
<td></td>
<td>NG D</td>
<td>NG D</td>
<td>NG D</td>
<td>NG D</td>
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<tr>
<td>Percent decrease</td>
<td>45 ± 2</td>
<td>47 ± 2</td>
<td>43 ± 2</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>Postocclusion</td>
<td></td>
<td></td>
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<tr>
<td>Percent increase</td>
<td>29 ± 2</td>
<td>13 ± 5*</td>
<td>27 ± 3</td>
<td>9 ± 13*</td>
</tr>
<tr>
<td>Postreperfusion</td>
<td></td>
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</table>

Results are given as means ± SE. *P < 0.01 vs. NG.
animals but not normoglycemic animals (\(P < 0.0001\); Fig. 3B). Furthermore, the infarcted zones were typically confined to the striatum in diabetic animals (Fig. 3A), whereas ischemic lesions in normoglycemic animals were found in both striatal and cortical brain regions (Fig. 3A). Infarct size in diabetic animals did not change with acute FeTPPs treatment; however, curcumin and minocycline therapies mitigated brain edema in diabetic animals. A disease-treatment interaction was detected with curcumin such that it reduced edema in diabetic animals but not in normoglycemic animals (\(P = 0.0013\)). Similarly, acute minocycline treatment reduced edema in diabetic animals but not in normoglycemic animals (\(P = 0.0001\)).

**Neurological assessment.** A beam walk test was used to observe vestibulomotor function after stroke in control and diabetic animals (Fig. 4A). Postoperative beam walk performance was poor in untreated normoglycemic animals; these animals could not traverse the beam, but they could stay balanced. Acute treatment with FeTPPs or minocycline did not improve beam walk performance in untreated normoglycemic animals. Curcumin therapy significantly improved poststroke beam walk scores (\(P < 0.01\) vs. untreated animals). A modified Bederson test was used to determine the impact of stroke on global neurological function (Fig. 4B). Abnormal postural responses were observed in all untreated normoglycemic animals after stroke. Administration of FeTPPs, curcumin, or minocycline at reperfusion did not improve modified Bederson scores in normoglycemic animals. Poststroke forelimb strength
Global neurological function was significantly reduced poststroke grip strength deficits in normoglycemic animals; FeTPPs treatment had no effect. Diabetic animals consistently developed smaller infarcts than normoglycemic animals. Single-dose administration of FeTPPs, curcumin, or minocycline at reperfusion did not alter the magnitude of infarcted brain tissue in diabetic animals. C: ischemic stroke induced brain edema in normoglycemic animals. Acute treatment with FeTPPs or curcumin had no effect on edema formation in normoglycemic animals; however, there was a slight increase in brain edema with minocycline therapy. Edema was greater in untreated diabetic animals compared with untreated normoglycemic animals. Edema formation in animals with preexisting diabetes was not reduced by FeTPPs or curcumin when administered in a single dose at reperfusion. Acute minocycline treatment effectively reduced brain edema in diabetic animals. Results are given as means ± SE; n = 5–13. *P < 0.05 vs. untreated normoglycemic or diabetic animals; ***P < 0.001 vs. untreated normoglycemic animals; aP = 0.0033, bP = 0.016, *P = 0.0013, and cP = 0.0001, disease by treatment interaction compared with untreated control and diabetic animals.

was evaluated in all experimental groups (Fig. 4C). Untreated normoglycemic animals displayed grip strength deficits after stroke. Acute treatment with FeTPPs or curcumin did not improve postoperative performance. However, minocycline therapy effectively reduced poststroke grip strength deficits in normoglycemic animals. Poststroke beam walk performance in untreated diabetic animals was similar to their untreated normoglycemic counterparts (Fig. 4A). Single-dose administration of FeTPPs and curcumin but not minocycline improved poststroke beam walk performance in diabetic animals (P < 0.05 and P < 0.01 vs. untreated animals). Global neurological function was significantly worse in untreated diabetic animals compared with untreated normoglycemic animals (P < 0.05; Fig. 4B). Acute FeTPPs or minocycline therapies did not improve modified Bederson scores in diabetic animals. Only curcumin treatment effectively improved poststroke Bederson test performance in diabetic animals (P < 0.05). Furthermore, a disease-treatment interaction was detected in which curcumin therapy improved poststroke Bederson test performance in diabetic but not normoglycemic animals (P = 0.0016). Stroke-induced grip strength deficit in untreated diabetic animals was greater than in normoglycemic animals (Fig. 4C). FeTPPs treatment did not correct lost grip strength in diabetic animals; however, curcumin and minocycline improved grip strength deficit in diabetic animals (P < 0.05 vs. untreated

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**Image Descriptions**

**Fig. 3.** Evaluation of infarct volume and edema in normoglycemic and diabetic experimental stroke groups. A: representative digital images of necrotic tissue (white) in 2,3,5-triphenyltetrazolium chloride-stained coronal brain slices (slice D was selected for each group). B: quantification of infarct size. Untreated normoglycemic animals developed infarcts that occupied 60% of the total ischemic hemisphere. Single-dose administration of either curcumin or minocycline reduced infarct volume in normoglycemic animals; FeTPPs treatment had no effect. Diabetic animals consistently developed smaller infarcts than normoglycemic animals. Poststroke beam walk performance in untreated diabetic animals was similar to their untreated normoglycemic counterparts. By treatment interaction compared with untreated normoglycemic animals (P < 0.05 and P < 0.01 vs. untreated animals). Global neurological function was significantly worse in untreated diabetic animals compared with untreated normoglycemic animals (P < 0.05; Fig. 4B). Acute FeTPPs or minocycline therapies did not improve modified Bederson scores in diabetic animals. Only curcumin treatment effectively improved poststroke Bederson test performance in diabetic animals (P < 0.05). Furthermore, a disease-treatment interaction was detected in which curcumin therapy improved poststroke Bederson test performance in diabetic but not normoglycemic animals (P = 0.0016). Stroke-induced grip strength deficit in untreated diabetic animals was greater than in normoglycemic animals (Fig. 4C). FeTPPs treatment did not correct lost grip strength in diabetic animals; however, curcumin and minocycline improved grip strength deficit in diabetic animals (P < 0.05 vs. untreated...
normoglycemic animals). These analyses also pointed to differences in response to treatment in control versus diabetic animals. There was a disease-treatment interaction for FeTPPS (P = 0.034) such that the deficit was greater in control animals compared with diabetic animals after treatment (Fig. 4C). Curcumin treatment had no effect in normoglycemic animals but improved the deficit in diabetic animals (P = 0.049).

Measurement of Cerebrovascular MMP Activity

MMP-9 gelatinolytic activity measured in the macrovessels from ischemic brain hemispheres was similar between untreated normoglycemic and diabetic animals. All treatments reduced MMP-9 activity (Fig. 5). There were no changes in MMP-2 activity (not shown).

Fig. 4. Poststroke functional deficits in normoglycemic and diabetic animals. A: beam walk performance was impaired in untreated normoglycemic animals after stroke. Neither FeTPPS nor minocycline treatment altered beam walk performance in normoglycemic animals, but curcumin alone was effective in improving poststroke beam walk scores. Modest improvements in poststroke functional deficits in diabetes could be achieved by acute therapies given at reperfusion. Vestibulomotor function impairments in untreated diabetic animals were similar to untreated normoglycemic animals. Neutralizing peroxynitrite using FeTPPS poststroke beam walk scores. Modest improvements in poststroke functional deficits in diabetes could be achieved by acute therapies given at reperfusion. The frequency of pathological postural responses was greater in untreated diabetic animals compared with control animals. Acute treatment with FeTPPS and minocycline failed to prevent neurological impairments diabetic animals. A single dose of curcumin at reperfusion reduced poststroke disability in diabetic animals. C: stroke-induced inflammatory responses using curcumin also improved beam walk scores.
DISCUSSION

Diabetic patients and experimental animal models sustain greater neurovascular injury and functional deficits in AIS. Disruption of cerebrovascular integrity is associated with increased MMP-9 activity and expression. Therefore, the objective of the present study was to determine whether the acute inhibition of MMP-9 at reperfusion by minocycline or through inhibition of its upstream activators peroxynitrite and NF-κB would improve HT and functional outcomes in diabetic stroke. Major findings from this study indicate that 24 h poststroke, bleeding was improved by a single-dose administration of FeTPPs, curcumin, or minocycline at reperfusion in diabetic animals and that this was associated with reduced MMP-9 activity. Varying degrees of improvement in neurological deficits were observed in diabetic animals receiving these acute therapies. Infarct size, however, was not altered by direct peroxynitrite scavenging, suppression of inflammation, or inhibition of MMPs. The multiple disease and drug interactions noted in this study strongly suggest that these treatments have differential effects on control and diabetic animals, and, as such, therapeutic targets for neurovascular protection may differ in disease states.

Of the three therapies, single-dose administration of curcumin at reperfusion offered the most benefit by reducing edema, bleeding, and neurological deficit after focal cerebral ischemia in diabetes. A nonspecific NF-κB inhibitor, curcumin has emerged as a promising candidate in the treatment of neurodegenerative diseases because of its antioxidant, anti-inflammatory, and antiapoptotic properties (29, 61). A recent report by King et al. (37) investigated the potential role for curcumin in mitigating neurovascular injury in a model of intracerebral hemorrhage. The researchers demonstrated that curcumin administration (150 mg/kg ip) decreased hematoma size, BBB permeability, and edema postinjury. Evidence in experimental stroke models suggests that a single dose within 30 min to 1 h of focal cerebral ischemia protects against peroxynitrite-mediated BBB disruption (32, 57). Delayed treatment within 4 h of ischemia has also been observed to reduce infarct size and improve neurological outcomes after stroke (14). In the present study, we did not anticipate further reductions in infarct volume in diabetic animals because the lesion size was already very small in untreated animals. However, consistent with earlier studies in normoglycemic animals, curcumin was effective in reducing infarct size in the control group. Given its ability to resolve hematoma within 72 h postinjury in the intracerebral hemorrhage model, we expected only modest improvements in bleeding with a single dose of curcumin at reperfusion. Visual inspection of macroscopic bleeding, however, indicated that curcumin treatment at reperfusion reduced bleeding in both control and diabetic animals. Interestingly, microscopic bleeding was reduced by half in the diabetic group that received curcumin treatment. Even more surprising was the curcumin-mediated increase in microscopic bleeding in the control group despite a reduction in MMP-9 activity. While we do not have an explanation for this finding, it is possible that curcumin is also inhibiting potential vasoprotective mechanisms in otherwise health animals.

NF-κB activation prompts the release of proinflammatory cytokines that promote increases in BBB permeability, thereby allowing neurotoxic blood elements to leak into the infarcted area and exacerbate ischemic injury (43). Improvement of neurological deficits in diabetic animals may be an indirect effect of reducing edema and hemorrhage in these animals. Conversely, failure to attenuate bleeding in control animals may have contributed to worse functional outcomes in this treatment group.

We chose to use minocycline for MMP inhibition in the present study because a phase 2 clinical trial (21) recently showed that minocycline reduces plasma MMP-9 activity in acute ischemic stroke patients, which will be further tested in a phase 3 trial. In the present study, acute minocycline treatment also limited vascular damage after acute stroke but only modestly improved poststroke deficits in diabetic animals. Minocycline is a tetracycline derivative that possesses neuroprotective properties that are distinct from its antimicrobial effects (9, 23, 54). Another study (59) reported that neuropro-
tection was conferred by block of activation of microglia and proinflammatory cytokines. Minocycline has also been documented to prevent extracellular matrix degradation by inhibiting MMP activity. Using a rat model of focal ischemia, Machado et al. (40) determined that minocycline treatment (a single dose at reperfusion with a followup dose 12 h postocclusion) significantly reduced the expression of MMP-2 and -9 after stroke. Data from our group examining the effects of chronic minocycline treatment diabetic animals on indexes of remodeling and HT showed that MMP-9 activity was reduced and MMP-mediated pathological remodeling was prevented (18). Furthermore, chronic treatment with minocycline reduced HT in diabetic animals. Finally, a recent report by Schildknecht et al. (50) has shown that neuroprotection by minocycline can be attributed to the drug’s ability to act as a direct and specific scavenger of peroxynitrite. Thus, in the present study, we anticipated that minocycline would confer neuroprotection by reducing infarct volume in treated control not diabetic rats. We also assumed that acute minocycline treatment would attenuate vascular injury in diabetic animals. Consistent with our hypothesis, single-dose administration of minocycline at reperfusion (20 mg/kg ip) was effective in reducing both macro- and microscopic bleeding and edema in diabetic animals after stroke. Furthermore, macroscopic bleeding in control animals was undetectable, and minocycline had no effect on microscopic bleeding in these animals. Data from the present study also indicate that edema is worsened by acute minocycline treatment in control animals, through an unknown mechanism. Xing et al. (58) demonstrated that at high concentrations, minocycline is cytotoxic to macrovascular endothelial cells, despite its ability to reduce MMP-9 levels. The investigators posited that the dose-dependent cell death was mediated by calpain and caspase activation. In the present study, modest improvements in functional deficits were observed in both treatment groups and may indicate that despite reduced vascular injury in these animals, a single dose of minocycline is not sufficient to reverse stroke-induced behavioral deficits.

Direct peroxynitrite scavenging by FeTPPs reduced stroke-induced macro- and microscopic bleeding and vestibulomotor function deficits in diabetes. Thiyagarajan et al. (57) demonstrated that delayed administration of FeTPPs at 2 and 6 h reduced infarct size, edema, and neurological deficits. The investigators concluded that these effects were mediated by reductions of peroxynitrite and inhibition of apoptosis (52, 57). In the present study, we anticipated decreased infarct volume in FeTPPs-treated control animals. We also predicted that direct peroxynitrite scavenging with FeTPPs would reduce bleeding and edema in diabetic animals by preventing peroxynitrite-mediated loss of myogenic tone, thus confirming data reported in a previous ex vivo study from our group (35). Since hypoxia-induced loss of myogenic tone was not restored by FeTPPs in MCAs isolated from control animals, we hypothesized that this vascular dysfunction would contribute to hyperperfusion and more bleeding. On the contrary, FeTPPs did not have an effect on either macro- or microscopic bleeding in treated control animals. Improvements in neurological deficits were limited to vestibulomotor function in diabetic animals, suggesting that single-dose administration of FeTPPs at reperfusion is not sufficient to reduce neurological impairments.

The present study focused on MMP-9 for a number of reasons. MMPs, particularly MMP-2 and -9, can be activated by oxidative stress, inflammation, or by other MMPs and have biphasic actions in AIS (44). A prolonged opening of the BBB occurs within 24–48 h after stroke and can last for several days (48). During this phase, MMP-9 is associated with increased BBB permeability, vasogenic edema, and hemorrhagic transformation (13). MMP-9 knockout mice are protected from HT (4). In AIS patients, elevated plasma MMP-9 levels are associated with infarct expansion, increased hemorrhage after thrombolytic therapies, and worsened stroke prognosis (36). Data from our group reported that elevated MMP-9 levels were associated with greater HT in a moderately hyperglycemic model of diabetes and that chronic glycemic control or minocycline intervention improved bleeding and stroke outcomes (18). Based on these findings, we anticipated that direct inhibition of MMPs or their upstream regulators (i.e., NF-κB and peroxynitrite) would lower MMP-9 activity in the cerebrovascular and prevent HT. Evidence from the present study indicates that in diabetes, bleeding and MMP-9 activity were reduced with all therapies. On the other hand, despite inhibition of MMP-9 activity by all treatments, HT was not reduced in any groups in the normoglycemia arm, raising the possibility that mediators of vascular injury may be different in control and disease states. It is also possible that other proteolytic enzymes may be contributing to the improvements observed in this study. One potential candidate could be MMP-3, also known as stromelysin-1, which has been shown to be activated by neurons and microglia in the ischemic brain (31). In a thrombolytic model of MCAO using MMP-3- and MMP-9-deficient mice, Suzuki et al. (56) demonstrated that tissue plasminogen activator-induced intracerebral hemorrhage was attenuated in MMP-3- -/- but not MMP-9 -/- mice, suggesting that MMP-3 and not MMP-9 is more important in tissue plasminogen activator-induced hemorrhage (56).

The data from the present study would certainly be strengthened by measurements of NF-κB expression, tight junction protein expression in cerebral microvessels, MMP-3 activity, and markers of peroxynitrite generation (e.g., nitrotyrosine or lipid peroxidation). We could not perform those measurements in the present study because of the limited amount of cerebrovascular tissue isolated from our experimental groups. Interpretation of the present findings is also limited by the fact that only a single dose was used and that all end points were measured at 24 h poststroke. We also detected that the percent increase in cerebral blood flow within 5–10 min after suture was removed was significantly less in diabetic animals. It is possible that due to existing vascular dysfunction diabetic animals take longer to fully reestablish blood flow. These limitations, however, do not outweigh the important findings of the present study. In line with current Stroke Therapy Academic Industry Roundtable preclinical recommendations, our study evaluated potential stroke therapies in a moderately hyperglycemic model of T2D with preexisting vascular disease. Experimental stroke studies investigating the therapeutic potential of agents that preserve BBB health and vascular integrity in long-term diabetes are underrepresented in the literature (58, 64, 65). By studying stroke in the context of diabetes, we were able to demonstrate that although MMP-9 was reduced by FeTPPs, curcumin, and minocycline at reperfusion, the targets may differ in control animals and in animals.
with preexisting diabetes, thus culminating in different stroke outcomes. In conclusion, we have shown that targets of vascular protection in AIS are different in T2D and that future studies are needed to understand why acute treatments at reperfusion conferred greater benefit in our diabetic model of stroke compared with treated control animals. In this way, we will reduce the gap in knowledge of how preexisting diabetes contributes to stroke pathophysiology and will potentially aid in the development of novel therapeutic strategies tailored to the diabetic population.

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DISCLAIMER
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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