Emerging evidence suggests that stroke comorbidities are associated with resistance to tissue plasminogen activator (tPA) reperfusion, higher risk of intracerebral hemorrhage, and worse neurological outcomes. In this study, we asked whether minocycline combined with intravenous tPA may ameliorate inflammation and brain injury after focal embolic stroke in type 1 diabetic rats.

Methods—Type 1 diabetic rats were subjected to a focal embolic stroke. Three treatment groups were used: (1) saline at 1.5 hours after stroke; (2) tPA alone at 1.5 hours after stroke; (3) combined minocycline (intravenously) at 1 hour plus tPA at 1.5 hours, and second treatment of minocycline (intraperitoneally) at 12 hours after stroke. Acute brain tissue damages were assessed at 24 hours after stroke. Inflammatory biomarkers interleukin-1β and matrix metalloproteinases 2 and 9 were examined in plasma. Neutrophil infiltration, microglia activation, matrix metalloproteinase activation, and degradation of the tight junction protein claudin-5 were examined in the brain.

Results—Compared with saline or tPA alone treatments, minocycline plus tPA combination therapy significantly reduced brain infarction, intracerebral hemorrhage, and hemispheric swelling at 24 hours after stroke. The combination also significantly suppressed the elevated plasma levels of matrix metalloproteinase-9 and interleukin-1β up to 24 hours after stroke. At 16 hours after stroke, neutrophil infiltration, microglia activation, matrix metalloproteinase-9, and tight junction protein claudin-5 degradation in the peri-infarct brain tissues were also significantly attenuated by the combination therapy.

Conclusions—Combination therapy with minocycline plus tPA may be beneficial in ameliorating inflammation and reducing infarction, brain swelling, and hemorrhage after ischemic stroke with diabetes mellitus/hyperglycemia.

Key Words: combination therapy ■ diabetes mellitus ■ embolic stroke ■ hyperglycemia ■ minocycline ■ tissue plasminogen activator

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the context of stroke patients with vascular comorbidities. A previous study has demonstrated that minocycline does not affect tPA fibrinolytic activity in vitro and does not compromise tPA clot lysis in the stroke animal model.14 We have previously shown that minocycline plus tPA may be effective in hypertensive rats.15 In this study, we now ask whether this combination therapy may also be effective in a rat model of hyperglycemia/type 1 diabetes mellitus.

Materials and Methods

Induction of Type 1 Diabetes Mellitus in Rats
All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight-week-old male Wistar rats (Charles River Laboratories, Wilmington, MA) with an initial body weight of 200 to 220 g were used to induce type 1 diabetes mellitus by a standard intraperitoneal injection of streptozotocin (60 mg/kg; Sigma, St. Louis, MO). Seven days after streptozotocin administration, rats with blood glucose concentrations >15 mmol/L were retained as diabetic rats as we have previously described.16

Focal Embolic Stroke Model and Treatment Groups
Fourteen-week-old (type 1 diabetes mellitus for 6 weeks) streptozoto-
cin-induced diabetic (male, Wistar) rats with blood glucose concentra-
tion of 18 to 31 mmol/L were subjected to focal embolic strokes as we have described previously.16 This focal embolic stroke rat model was originally established by Dr Chopp’s group, and its thrombolytic reperfusion time window and hemorrhagic transformation closely mimic clinical situation, which has been most commonly used for thrombolytic stroke studies.17 Totally, 63 rats were used in 2 sets of experiments. In the first set of experiments, 45 rats were divided randomly into 3 treatment groups: (1) intravenous saline at 1.5 hours after ischemia (n=15); (2) intravenous 10 mg/kg of tPA at 1.5 hours after ischemia (n=15); (3) combination of intravenous 10 mg/kg of minocycline at 1 hour plus tPA at 1.5 hours, and second minocycline treatment (45 mg/kg IP) at 12 hours after stroke (n=15). Minocycline dose was selected according to the established dose regimen;16,18 the relative higher dose used in rodents was based on the fact that the half-life of minocycline in rodents is >2 to 3 hours,16 whereas in humans the half-life is in the range of 2 to 24 hours.17,20 Regional cerebral blood flow was used to monitor reperfusion for 1.5 hours after induction of ischemia and then continuously monitored for 1 hour after treatments as we have previously described.16 Only animals that survived for 24 hours after stroke were included for outcome assessments; animals dead within 24 hours after stroke were counted for overall mortality rate to all groups. In the second set of experiments, totally 18 rats were used to investigate the therapeutic effects of minocycline plus tPA combination in metalloproteinase (MMP) activity, inflammatory responses, and vascular damage. Investigators were blinded to all drug treatments and experimental assessments.

Measurement of Ischemic Brain Infarction Volume, Hemispheric Swelling, and Intracerebral Hemorrhage Volume
To examine acute brain tissue outcomes, rats were euthanized 24 hours after ischemia. Seven coronal brain sections (2 mm thick) were stained with 2,3,5-triphenyltetrazolium chloride (Sigma) to quantify infarct volumes and hemispheric swelling using computer-assisted image analysis. 2,3,5-triphenyltetrazolium chloride–stained brain sections were used for quantification of intracerebral hemorrhage volume with spectrophotometric hemoglobin assay as we have previously described.16,21

Measurements of Plasma MMP-2/9 Activity and IL-1β Levels
To determine changes of systemic inflammatory biomarkers, blood plasma samples were collected before ischemia, at 1.5 hours after stroke (before tPA treatment), at 1 hour after tPA treatments (2.5 hours after stroke), and at 24 hours after stroke. The levels of MMP-2 and MMP-9 activity in plasma were measured by gelatin zymogram following our previously described techniques.21 Data were expressed as a percentage of human MMP-9 standards in each gel. A commercial ELISA kit (DY401; R&D Systems, Minneapolis, MN) was used to measure interleukin (IL)-1β concentrations in the plasma samples according to the manufacturer’s instructions. Ten microliters of plasma sample was applied in each well, and all the samples were run in duplicates.

In Situ MMP Zymography and Immunohistochemistry Analyses
We collected ischemic brain tissues at 16 hours after stroke and assessed in situ MMP zymography and immunohistochemistry analyses. In brief, we selected brain sections at +1.3, −0.7, −2.7, and −4.7 mm from bregma (axial sections 3, 4, 5, and 6 for quantification, because these represented locations of maximal cortical infarctions). MMP enzyme activity in tissue sections was conducted by following a standard method using a commercial kit (EnzCheck Gelatinase Assay Kit; Molecular Probes, Eugene, OR) as we have previously described.20 For semiquantification of the MMP activity, 2 cortical and 2 subcortical fields of ischemic boundary area and 4 fields of contralateral homologous area were acquired using a 20 objective through the fluorescent microscope (Nikon). All readings from 4 sections were averaged for each rat brain. Data were presented as a percentage of the integrated optical density of the saline. Immunohistochemistry was performed by following a standard method as we have previously described.21 Briefly, we used a rabbit anti–iba-1 antibody (Wako, Osaka, Japan) to examine microglia activation at a titer of 1:200, a polyclonal rabbit anti-human myelo-
peroxidase (MPO; DAKO, Carpinteria, CA) antibody to examine neutrophil accumulation at a titer of 1:500, and a mouse anti-rat claudin-5 antibody (Invitrogen, Grand Island, NY) to detect the tight junction degradation of blood-brain barrier at a titer of 1:200. For semiquantification of microglia activation or neutrophil infiltration, numbers of iba-1 or MPO positive cells were counted throughout 2 cortical and 2 subcortical fields of ischemic boundary area, respectively. For semiquantification of claudin-5 antigen, 2 cortical and 2 subcortical fields of ischemic boundary area and 4 fields of contralateral homologous area were acquired using a 20 objective through the fluorescent microscope (Nikon), and data were presented as a percentage of the positive immunoreactivity area of the contralateral area (pixel). Totally, 18 ischemic rat brains were examined. Fresh brain samples with PBS cardiac perfusion were used for MMP in situ zymography and claudin-5 immunohistochemistry analysis (n=3 per group), and paraformaldehyde-perfused brain samples were used for MPO and iba-1 immunohistochemistry analysis (n=3 per group).

Statistical Analysis
Data were expressed as mean±SD. All measurements were assessed with ANOVA, followed by Tukey–Kramer tests. Differences with P<0.05 were considered statistically significant.

Results

Streptozotocin-induced Type 1 Diabetes Mellitus in Rats
At 6 weeks after streptozotocin injection (14 weeks old), the range of blood glucose concentration was ~18 to 31 mmol/L, and glycosylated hemoglobin was significantly increased from normal baseline 3.9±0.2% to 9.7±0.7%. These findings confirmed that this standard model may mimic key aspects of hyperglycemia and type 1 diabetes mellitus in rats. Blood glucose concentrations were monitored at 0, 1.5, 2.5, and 24 hours after stroke, and there were no significant differences between groups at all time points (Figure I in the online-only Data Supplement).
Minocycline Combined With tPA Reduced Brain Tissue Damage at 24 Hours After Stroke
The standard rat dose tPA at 10 mg/kg alone and combined with pretreatment of intravenous 10 mg/kg minocycline only slightly increased reperfusion at 10.3% and 15.6% compared with saline control in the focal embolic stroke of diabetic rats, respectively (Figure II in the online-only Data Supplement). At 24 hours after stroke, tPA alone reduced brain infarction (16.6% reduction; Figure 1A and 1B) but had no effects on hemispheric swelling (Figure 1C). Instead, tPA alone significantly worsened intracerebral hemorrhage in these diabetic rats (Figure 1D). Combination treatment with minocycline plus tPA significantly reduced brain infarction (30.1% reduction; Figure 1A and 1B) and suppressed hemispheric swelling (63.9% reduction compared with saline; 60.6% reduction compared with tPA; Figure 1C). Furthermore, this minocycline–tPA combination also significantly decreased tPA-induced hemorrhagic transformation (20.8% reduction in ICH volume; Figure 1D). There was no significant difference in mortality within 24 hours after stroke among the 3 treatment groups (3/15 for saline, 4/15 for tPA alone, and 1/15 for the combination, respectively). Although we did not detect the significant difference in 24-hour mortality, the statistical significance of the reduced mortality by minocycline plus tPA might be reached with a larger sample size testing. In addition, physiological parameters measured before ischemia, 1.5 hours after ischemia, and 2.5 hours after ischemia remained within the normal range in all groups (data not shown).

Minocycline Combined With tPA Reduced MMP-9 Levels in Plasma and Ischemic Brains
After stroke onset, total plasma MMP-9 was significantly elevated, and combination of minocycline with tPA significantly suppressed the total plasma MMP-9 compared with the tPA alone treatment group (Figure 2A and 2B). Focal stroke dramatically increased MMP activity in the ischemic brain hemisphere, and the combination of minocycline plus tPA significantly lowered MMP activity compared with tPA alone (Figure 2C and 2D).

Minocycline Combined With tPA Decreased Inflammatory Biomarkers After Stroke
Total plasma IL-1β levels were significantly elevated after stroke. Minocycline combined with tPA significantly decreased total plasma IL-1β levels at 24 hours after stroke (Figure 3A). Activated and proliferating microglia/macrophages were observed in the peri-infarct area of ischemic brains at 16 hours after stroke. tPA significantly increased the number of activated microglia/macrophages, whereas it was significantly reduced by the combination of minocycline plus tPA. All the 3 treatments had no significant effect on the number of resting microglia in contralateral hemispheres (Figure 3B and 3C).

Figure 1. Measurements of acute brain tissue damages in a focal embolic stroke model of diabetic rats. A, Representative images for 2,3,5-triphenyltetrazolium chloride (TTC)–stained ischemic brain infarctions. B, Ischemic infarct volumes were quantified at 24 hours after stroke. C, Hemispheric swelling was quantified on TTC-stained brain slices. D, Intracerebral hemorrhage volumes were quantified at 24 hours after stroke by hemoglobin assay. Data are expressed as mean±SD; *P<0.05 (n=11–14 per group). tPA indicates tissue plasminogen activator.
Minocycline Combined With tPA Decreased Neutrophil Infiltration of Ischemic Brains

Numerous MPO-positive cells, presumably infiltrated neutrophils, were detected in the peri-infarct area of ischemic brains at 16 hours after stroke, which were significantly increased in the brains of tPA-treated stroke rats. However, minocycline combined with tPA treatment significantly reduced neutrophil infiltration in the peri-infarct areas (Figure 4A and 4B).
Minocycline Combined With tPA Ameliorated tPA-induced Tight Junction Protein Claudin-5 Degradation

At 16 hours after stroke, there was a significant reduction in claudin-5 antigen levels in the peri-infarct area of ischemic brains treated with tPA, indicating a degradation of tight junction integrity in cerebral microvessels. The degradation of claudin-5 was significantly diminished by minocycline combined with tPA treatment compared with tPA alone (Figure 5A and 5B).

Discussion

Experimental results from this study showed that minocycline plus tPA combination therapy significantly reduced brain infarction, intracerebral hemorrhage, and hemispheric swelling at 24 hours after stroke. Furthermore, our initial findings also suggest that these beneficial effects of minocycline plus tPA may be mediated by the amelioration of MMP-9, inflammatory responses, and microvascular tight junction degradation.

MMPs, especially MMP-9 activation triggers extracellular matrix proteolysis dysfunction that contributes to the ischemic brain tissue and cerebrovascular damage and tPA-mediated ICH.1,2,24 Animal studies have also shown that MMP plays an important role in cerebrovascular damage after permanent focal stroke of diabetic rats.25,26 Importantly, it has been reported that minocycline has potent inhibitory effects on both MMP-9 gene expression and proteolytic activity.14,15,18,27 Previous studies have suggested that the early increased plasma MMP-9 is released from circulating neutrophils.28,29 Results from present study showed that increased plasma MMP-9 and brain MMP activity after stroke and tPA thrombolysis was significantly inhibited by the combination of minocycline with tPA in diabetic rats. This effect may underlie the ability of the tPA–minocycline combination to reduce hemorrhagic transformation in our model.

Increased systemic and cerebrovascular inflammation is one of key pathophysiological features in diabetes mellitus and its vascular complications.30,31 Furthermore, ischemic stroke and tPA thrombolysis further stimulate acute inflammation.
cascades that contribute to ischemic brain damage and intracerebral hemorrhage transformation. It has been reported that minocycline has a strong anti-inflammatory effect in ischemic stroke animals. Hence, based on this rationale, we examined 3 well-established inflammatory biomarkers, that is, plasma IL-1β, microglia activation, and neutrophil infiltration into ischemic brain tissue. Our results showed that minocycline combined with tPA treatment significantly decreased total plasma IL-1β levels at 24 hours after stroke compared with tPA alone. In addition, at 16 hours after stroke, the combination significantly reduced the number of activated microglia/macrophages and MPO-positive neutrophils in the peri-infarct area compared with saline and tPA alone treatments. These results are consistent with the overall ability of minocycline to dampen ischemic inflammation.

Ischemic stroke and tPA thrombolysis cause vascular damage, which may cause ICH and edema formation. Recently, experimental studies have found exacerbated vascular damage.

Figure 4. Measurements of inflammatory neutrophil infiltration in ischemic brains of diabetic rats. A, At 16 hours after stroke, representative myeloperoxidase (MPO)-positive cells by immunostaining on rat brain sections treated with saline, tissue plasminogen activator (tPA), and minocycline combined with tPA, respectively. Scale bar, 100 μm. B, Quantitative data of neutrophil infiltration (MPO-positive cells). Data are expressed as mean±SD; *P<0.05 (n=3 per group).

Figure 5. Measurement of tight junction protein claudin-5 degradation in ischemic brains of diabetic rats. A, At 16 hours after stroke, representative claudin-5 expression by immunostaining on rat brain sections treated with saline, tissue plasminogen activator (tPA), and minocycline combined with tPA, respectively. Scale bar, 100 μm. B, Quantitative data of claudin-5 expression. Data are expressed as mean±SD; *P<0.05 (n=3 per group).
after focal ischemic stroke in both acute hyperglycemic and diabetic rats. In this study, we examined a commonly used biomarker for cerebrovascular damage, that is, degradation of claudin-5 protein, a key integral membrane protein and component of tight junction strands. We found that tPA alone significantly increased degradation of claudin-5 protein at 16 hours after stroke, but this was significantly reduced by the tPA–minocycline combination.

Taken together, our experimental results suggest that minocycline combined with tPA may lessen ischemic brain damage and reduce risk of intracerebral hemorrhagic transformation by suppressing MMP activity in circulation and brain tissues, decreasing brain inflammation, and protecting against cerebrovascular damage. We acknowledge that these complex pathways have many feedback loops. Nevertheless, we believe these mechanisms are critical for the beneficial properties of minocycline, that is, minocycline interrupts multifactorial pathogenic pathways that operate in parallel after stroke and tPA thrombolysis that result in overall synergistic protective effects.

There are several caveats and limitations in this work. First, our blood, tissue, and molecular data are collected only within an acute time frame. This may be consistent with the fact that poststroke hyperglycemia typically affects acute infarct development, and most tPA thrombolysis-associated ICH transformation occur within 12 to 36 hours after stroke. However, for translation and clinical use, further studies are required to confirm that these acutely beneficial results truly correlate with improved long-term neurological outcomes. Second, we only induced hyperglycemia to mimic type 1 diabetes mellitus in young male rats. These mechanisms may be affected by age and sex, so further studies to test female and aged models should be useful. Third, we were unable to detect early reperfusion after tPA or in combination with tPA for up to 1 hour. In part, this may be related to our relatively brief period of laser doppler flowmetry measurements in a restricted cortical region. The effects of our combination therapy on the late treatment window and prolonged profile of thrombolytic reperfusion warrant further studies. Finally, minocycline has many pleiotropic properties. Besides targeting MMPs, inflammation, and cerebrovascular tight junctions, other mechanisms involving antioxidative tissue damage and direct neuroprotection may also be taking place. Further experiments to dissect and potentially optimize these multifactorial pathways would be clinically important.

In summary, this study showed that minocycline combined with tPA significantly reduced ischemic brain infarction, hemispheric swelling, and tPA-associated intracerebral hemorrhagic transformation at 24 hours after focal embolic stroke of type 1 diabetic rats. These beneficial effects of tPA–minocycline combination therapy may be mediated, in part, by the suppression of MMP-9 activity, decrease in brain tissue inflammation, and protection against cerebrovascular damage. Further translation studies to test and develop this therapeutic approach for diabetic brains may be warranted.

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Disclosures
None.

References


Effects of Minocycline Plus Tissue Plasminogen Activator Combination Therapy After Focal Embolic Stroke in Type 1 Diabetic Rats
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Figure I. Blood glucose concentrations were measured at before, 1.5, 2.5, and 24 hours after stroke. Data were expressed as mean ± s.d., n=11-14 per group.

Figure II. Laser Doppler flowmetry monitored regional cerebral blood flow for up to 1 hour after treatment. Data were expressed as mean ± s.d., n=11-14 per group.