Sustained Neurological Recovery After Stroke in Aged Rats Treated With a Novel Prostacyclin Analog

Changjun Yang, PhD; Kelly M. DeMars, BS; Jon C. Alexander, PhD; Marcelo Febo, PhD; Eduardo Candelario-Jalil, PhD

Background and Purpose—Targeting the prostaglandin I₂ (PGI₂) prostanoid (IP) receptor to reduce stroke injury has been hindered by the lack of selective drugs. MRE-269 is the active metabolite of selexipag showing a high selectivity toward the IP receptor. Selexipag has been recently approved for clinical use in pulmonary hypertension. We hypothesized that postschismic treatment with MRE-269 provides long-lasting neuroprotection with improved neurological outcomes in a clinically relevant rat stroke model.

Methods—Aged male Sprague–Dawley rats underwent transient middle cerebral artery occlusion and were randomly selected to receive either vehicle or MRE-269 (0.25 mg/kg) intravenously starting at 4.5 hours post ischemia. Accelerating rotarot and adhesive removal tests were conducted before and at 3, 7, 14, and 21 days after stroke. Infarct volume was quantified by magnetic resonance imaging at 48 hours and 21 days post middle cerebral artery occlusion. In parallel experiments, cerebral cortex samples from stroke and nonstroke sides from vehicle- and MRE-269–treated groups were collected at 18 hours post middle cerebral artery occlusion for molecular biology analyses.

Results—Quantitative magnetic resonance imaging data showed that postschismic MRE-269 treatment significantly reduced infarct volume compared with vehicle-treated rats at both 48 hours and 3 weeks after stroke. MRE-269 treatment resulted in a significant long-term recovery in both locomotor and somatosensory functions after middle cerebral artery occlusion, which was associated with a reduced weight loss in animals receiving the IP receptor agonist. Postschismic MRE-269 treatment reduced proinflammatory cytokines/chemokines and oxidative stress. Damage to the blood–brain barrier, as assessed by extravasation of immunoglobulin G to the ischemic brain, was significantly reduced by MRE-269, which was associated with a reduction in matrix metalloproteinase-9 activity in the brain of stroked aged rats given the IP agonist at 4.5 hours after ischemia onset.

Conclusions—Our data suggest that targeting the IP receptor with MRE-269 is a novel strategy to reduce cerebral ischemia injury and promote long-term neurological recovery in ischemic stroke. (Stroke. 2017;48:1948-1956. DOI: 10.1161/STROKEAHA.117.016474.)

Key Words: blood–brain barrier ■ inflammation ■ matrix metalloproteinase-9 ■ oxidative stress ■ stroke

Prostacyclin, also known as prostaglandin I₂ (PGI₂), is an arachidonic-acid–derived eicosanoid, which is predominantly produced by the endothelium.¹ PGI₂ induces vasodilation, a potent inhibitor of platelet aggregation, and reduces microvascular permeability.¹ Mice deficient in PGI₂ prostanoid (IP) receptor displayed exacerbated neuronal death after brain ischemia.²³ In addition, PGI₂ analogs significantly reduced ischemic brain injury.²⁴⁻²⁶ However, the use of PGI₂ and known analogs have limited therapeutic value because of chemical instability, rapid metabolism (t½= 4 minutes),² and lack of selectivity for the IP receptor resulting in unsafe side effects.²⁷ Kuwano et al²⁷ discovered a novel, brain-permeable, and highly selective IP agonist coded as NS-304 (selexipag), a prodrug of the active form termed MRE-269 that is most active at a low nmol/L range.²⁸ Pharmacokinetics in rats, dogs, and humans showed a significantly improved t½ of 6 to 8 hours.²⁸ After successful clinical trials,²⁹³⁰ selexipag/MRE-269 has been approved for pulmonary hypertension by the Food and Drug Administration in the United States.

In an animal model of excitotoxic brain injury, MRE-269 was found to exert neuroprotective effects,¹¹ which is a significant finding because excitotoxicity contributes to neuronal death in stroke. There is limited information about the protective mechanisms and the potential to target the IP receptor pathway to reduce stroke injury and improve long-term neurological recovery. Here, we hypothesized that postschismic treatment with MRE-269 will provide long-lasting neuroprotection with improved neurological outcomes after ischemic stroke. Using an aged rat ischemic stroke model, we found that MRE-269 significantly reduced infarct volume and dramatically improved
long-term recovery of both locomotor and somatosensory functions after middle cerebral artery occlusion (MCAO). Neuroprotection of MRE-269 in ischemic stroke was through a reduction in proinflammatory cytokines/chemokines and oxidative stress. Moreover, postischemic treatment with MRE-269 significantly reduced brain matrix metalloproteinase-9 (MMP-9) activity and protected against stroke-induced blood-brain barrier (BBB) disruption. Collectively, these data suggest that targeting the IP receptor with MRE-269 is a novel strategy to reduce cerebral ischemia injury and promote long-term neurological recovery in ischemic stroke.

Methods
A detailed description of all the experiments is provided as an online-only Data Supplement. A brief description of the main methodology is provided below.

Animals and MCAO Model
All experimental procedures were in accordance with the National Institutes of Health guidelines and protocols approved by the University of Florida Institutional Animal Care and Use Committee. An a priori sample size calculation was performed using the G*Power v.3.1.3 software and detailed in Methods section in the online-only Data Supplement. A total of 57 aged male Sprague–Dawley rats (18–20 months, Hilltop Laboratories, Scottdale, PA) were used in this study and randomly assigned to treatment groups. Transient MCAO for 90 minutes was induced using an intraluminal silicone-coated filament as previously described by our group.13

Experimental Design and Drug Administration
For the intravenous administration of vehicle or MRE-269, a catheter was inserted into the right femoral vein of ischemic rats at the time of MCAO surgery. MRE-269 ([4-[(5,6-diphenylpyrazinyl)(1-methyl-ethyl)amino]butoxy]-acetic acid; Cat. No. 10010412; Cayman Chemical, Ann Arbor, MI) was dissolved in dimethyl sulfoxide and then diluted in sterile saline. For the infarct size and neurobehavioral tests experiments, 26 rats underwent transient MCAO and were randomly assigned to vehicle or treatment group with administration of 1% dimethyl sulfoxide in saline (n=13) or MRE-269 (0.25 mg/kg, n=13) starting at 4.5 hours post-MCAO. Additional doses were given every 12 hours for the first 48 hours, and then 1 injection daily for 7 days post-MCAO. The MRE-269 dose and treatment schedule were based on our preliminary findings in young rats (3–4 months) showing that MRE-269 (0.1–0.5 mg/kg, intravenous) given at 1.5 hours after stroke onset produced a dose-dependent reduction in infarct volume as measured by 2,3,5-triphenyltetrazolium chloride staining at 48 hours (data not shown). The optimal dose of 0.25 mg/kg was, therefore, used in this study in aged rats.

For the biochemical experiments, 31 rats were randomly assigned to the following groups: sham-operation (n=5), vehicle (1% dimethyl sulfoxide, n=13), or MRE-269 (0.25 mg/kg, n=13). Drug or vehicle was given intravenously starting at 4.5 hours after stroke, and an additional dose was given at 12 hours after stroke onset. Animals were euthanized at 18 hours post-MCAO and perfused with ice-cold saline. Samples from the ipsilateral and contralateral cerebral cortices were obtained for RNA isolation, immunoblotting, and lipid peroxidation analyses.

Magnetic Resonance Imaging, Image Analysis, and Behavioral Tests
At 48 hours and 21 days after MCAO, rats treated with vehicle or MRE-269 (0.25 mg/kg) were imaged in a Bruker 4.7-T magnetic resonance imaging scanner. Behavioral tests were performed pre-MCAO and at 3, 7, 14, and 21 days post-MCAO by an independent investigator blinded to the experimental groups. Somatosensory deficits were assessed using the adhesive removal test as described previously.14 Locomotor impairments were assessed with the accelerating rotarod as described previously.15 Five rats in the vehicle and 4 rats in the MRE-269 groups died during the 21 days post-MCAO period. Further details are described in the Methods section in the online-only Data Supplement.

RNA Extraction and Real-Time Polymerase Chain Reaction
Total RNA isolation from cortical tissue, cDNA synthesis, and real-time polymerase chain reaction were performed as described previously by our group.13 Results are presented as normalized expression relative to sham-operated group. Additional details are described in the Methods section in the online-only Data Supplement.

Immunoblotting
Details of the immunoblotting technique, antibody incubations, and signal detection are provided in the Methods section in the online-only Data Supplement.

Determination of 8-iso-Prostaglandin F<sub>2α</sub>
Levels of 8-iso-prostaglandin F<sub>2α</sub> (8-iso-PGF<sub>2α</sub>), a highly sensitive biomarker of oxidative stress, were measured using an ELISA kit (Cat. No. 516351, Cayman Chemical, Ann Arbor, MI) following the manufacturer’s protocol.

Statistical Analysis
All values were expressed as mean±SEM. Statistical analysis was performed by unpaired Student t test for comparisons between 2 groups, 1-way or 2-way ANOVA followed by Bonferroni post-tests for comparisons of multiple groups. GraphPad Prism 6 was used to conduct data analysis, and P<0.05 was considered statistically significant.

Results
Long-Lasting Neuroprotective Efficacy of Delayed MRE-269 Administration in Aged Rats After Transient MCAO
Because age is the single most important risk factor in stroke,17 it is important to know whether postischemic treatment with MRE-269 is able to confer long-lasting neuroprotection in aged rats subjected to ischemic stroke. Magnetic resonance imaging–based infarct size analysis was performed at 48 hours (acute phase) and at 21 days after stroke. As shown in Figure 1, delayed treatment with MRE-269 at 0.25 mg/kg resulted in a remarkable reduction in infarct volume, which was sustained for 3 weeks after stroke.

MRE-269 treatment resulted in a significant long-term recovery in both somatosensory (Figure 2A) and locomotor functions (Figure 2B) after MCAO compared with the vehicle group, which was associated with less body weight loss (Figure 2C) in animals receiving the IP receptor agonist. To determine whether there was any association between major physiological parameters and the neuroprotective effect of
MRE-269 in ischemic stroke, physiological variables including blood pH, blood oxygen (P$_{O_2}$), carbon dioxide saturation (P$_{CO_2}$), ion concentrations, blood glucose, hematocrit, hemoglobin, rectal temperature, and cerebral blood flow were measured 15 minutes before and after MCAO, respectively, as well as at 15 minutes after initial dose of vehicle (1% dimethyl sulfoxide) or MRE-269 (0.25 mg/kg) injection. As shown in the Table I and Figure I in the online-only Data Supplement, we did not observe a significant difference in these physiological parameters, including cerebral blood flow, at any time point between the vehicle- and MRE-269–treated groups. These data suggest that changes in major physiological variables are unlikely to explain the neuroprotective effects of MRE-269 in ischemic stroke.

**Effect of MRE-269 on Cortical Gene Expression of Cytokines and Chemokines in Aged Rats After Transient MCAO**

To examine whether the neuroprotective effect of MRE-269 in ischemic brain was through regulation of neuroinflammatory
mediators, cortical gene expression of proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and chemokine monocyte chemoattractant protein-1 (MCP-1) were measured by quantitative real-time polymerase chain reaction in sham-operated, vehicle-, and MRE-269–treated aged rats euthanized at 18 hours post-MCAO. Transient MCAO significantly increased mRNA expression of IL-1β (Figure 3A), TNF-α (Figure 3B), MCP-1 (Figure 3D), and trends toward a considerable increase in IL-6 (Figure 3C) in the ipsilateral cerebral cortex compared with the sham group. Postischemic treatment with MRE-269 significantly reduced the mRNA levels of IL-1β (Figure 3A), TNF-α (Figure 3B), and MCP-1 (Figure 3D) in the ipsilateral cerebral cortex compared with vehicle-treated rats. Taken together, these data suggest that delayed MRE-269 treatment can suppress transient MCAO-induced expression of proinflammatory cytokines IL-1β, TNF-α, and chemokine MCP-1 in the aged rat brain subjected to ischemic stroke.

Next we examined the effects of MRE-269 on measures of oxidative damage to the brain tissue. We first determined the protein level of gp91phox (NOX2), a glycosylated subunit of NADPH oxidase (NOX), which is a major source of superoxide generation. Immunoblot analyses showed a dramatic increase of the gp91phox subunit in the ipsilateral cortex of MCAO-treated aged rats at 18 hours post-MCAO compared with the sham group, and MRE-269 treatment significantly reduced the gp91phox levels induced by transient MCAO (Figure 4A and 4B). Also, we investigated the effects of delayed treatment with MRE-269 on lipid peroxidation, as assessed by quantification of 4-hydroxynonenal and 8-iso-PGF$_2$α, two sensitive biomarkers of oxidative stress in ischemic brain damage. As shown in Figure 5A through 5D, transient MCAO resulted in dramatic increases in both 4-hydroxynonenal and 8-iso-PGF$_2$α.
Reduced BBB Damage and MMP-9 Activity in Stoked Aged Rats Given MRE-269

Because increased oxidative stress and proinflammatory cytokines contribute to BBB breakdown after stroke, we next investigated the effects of MRE-269 on ischemia-induced BBB opening. At 18 hours after ischemia, we observed a dramatic increase in IgG extravasation in the ipsilateral cerebral cortex of aged rats given the vehicle. Rats receiving MRE-269 (0.25 mg/kg; intravenous) at 4.5 hours after stroke onset had significantly less BBB damage compared with the vehicle group (Figure 6A). Because increased MMP-9 activation after stroke is a key mediator of BBB disruption, we next measured MMP-9 activity in the ipsilateral and contralateral cortices in both treatment groups. Ipsilateral MMP-9 activity was significantly increased in vehicle and MRE-269 groups when compared with either sham or their respective contralateral values (Figure 6B). Postischemic treatment with MRE-269 significantly reduced MMP-9 activity in the ischemic brain as shown in Figure 6B.

Discussion

This study demonstrated for the first time that a 4.5-hour delayed administration of MRE-269, a highly specific and clinically used IP receptor agonist, provided sustained neuroprotection and improved long-term neurological recovery in aged rats subjected to ischemic stroke. Molecular studies indicated that MRE-269 reduced proinflammatory mediators, such as IL-1β, TNF-α, and MCP-1, and reduced oxidative stress and BBB damage, which likely contributed to the observed neuroprotective effect. MRE-269 is safe in the clinical setting. Repurposing its use as a potential treatment for stroke is highly significant. The marked and long-lasting neuroprotection seen with a 4.5-hour delayed administration of MRE-269 in aged rats add a significant translational value to our findings.

This is the first study to demonstrate the sustained neuroprotective effect of an IP receptor agonist given poststroke in a clinically relevant model in aged rats. Moreover, the effects of MRE-269, a highly selective IP agonist used in the clinic, have never been studied in any animal model of brain injury. Another novel aspect of this study is our finding that a delayed administration of MRE-269 significantly reduces inflammatory mediators, oxidative stress, BBB damage, and MMP-9 activity in the postischemic brain.

Prostacyclin/IP receptor activation has potent vasodilatory effects, which raises the possibility that increased brain perfusion could contribute to neuroprotection by IP agonists. However, low doses of PGI2 do not alter cerebral blood flow in humans. Consistent with these previous reports, we did not observe significant changes in cerebral blood flow between vehicle and MRE-269 groups, which suggests that the neuroprotection by MRE-269 at a dose of 0.25 mg/kg is not because of the improvement in microcirculatory reperfusion. At low doses, MRE-269 has no effect on mean arterial blood pressure or heart rate.

It has been reported that IP receptor gene deletion exacerbated neurological deficits and infarct size in both transient MCAO and permanent MCAO model in mice. Conversely, treatment with beraprost, an IP receptor agonist, significantly improved negative stroke outcomes in wild-type mice and reduced CA1 hippocampal neuronal death after global ischemia in aged mice. MRE-269 has a much higher selectivity at low nmol/L range and a longer half-life (6–8 hours) compared with the classical IP receptor agonists, such as beraprost and iloprost. More importantly, beraprost and iloprost also bind to other prostanoid receptors especially the EP3 receptor, potentially causing unsafe side effects such as marked hypotension, increased heart rate, impaired gastrointestinal function, and lung arterial contraction, which limits their clinical use. As a highly specific and clinically available IP receptor agonist used in the treatment of pulmonary hypertension, our data for the first time demonstrated that delayed treatment with MRE-269 reduced infarct volume in aged rats subjected to transient MCAO.
suggesting a potentially valuable application in the treatment of ischemic stroke. Because age is the single most important risk factor in stroke, it is of great clinical significance to know whether postischemic treatment with MRE-269 is able to confer long-lasting neuroprotection in aged rats subjected to ischemic stroke. Consistent with our findings in young rats, delayed treatment with MRE-269 (0.25 mg/kg) also exerted neuroprotective effects demonstrated by reduced infarct volume in aged rats subjected to transient MCAO. Importantly, sufficiently delayed drug administration is lacking in most experimental stroke studies which reduces their translational relevancy and may contribute to the discrepancy between the efficacy of pharmaceutical stroke treatment in animal studies and in the clinic. Also, considerable stroke research has been limited to the acute phase of focal ischemia injury in young animals rather than aged, which is less clinically relevant to most stroke populations in human. In this study, we tried to mimic a clinical setting by using aged rats tested for 3 weeks after MCAO to evaluate the efficacy of MRE-269 with an initial administration at 4.5 hours postocclusion. Delayed MRE-269 treatment resulted in a significant improvement of long-term recovery in both locomotor and somatosensory functions after MCAO. Collectively, our data provide a solid indication that postischemic intervention with MRE-269 is a promising neuroprotective therapy for ischemic stroke.

Ischemia/reperfusion triggers immune responses, excessive oxidative stress, adhesion molecule upregulation, and peripheral leukocyte recruitment, which ultimately cause inflammatory cell activation and infiltration. The activated inflammatory cells release many neuroinflammatory mediators, including cytokines, chemokines, and MMPs, thus, resulting in BBB disruption and neuronal cell death in ischemic stroke. In this study, we found that stroke-induced neurobehavioral deficits and infarction were associated with the upregulation of proinflammatory cytokines IL-1β, TNF-α, and IL-6 as well as MCP-1 in the ipsilateral cerebral cortex of aged rats. As reported previously in a rat distal transient MCAO model, both IL-1β and TNF-α mRNA are induced in the ischemic cortex as early as 1 hour after reperfusion, peaking between 3 and 6 hours post stroke, and remain elevated for up to 48 hours after MCAO. Administration of IL-1β to rats increases brain injury, and IL-1β–deficient mice showed smaller infarct volume compared with wild-type mice. MCP-1 is one of the most commonly expressed chemokines that regulate
migration and infiltration of monocytes/macrophages during neuroinflammation. Increased MCP-1 in the brain markedly exacerbated ischemic damage, which was correlated with inflammatory cell recruitment.35 Mice deficient in CCR2 (CCR2−/−), the receptor for MCP-1, had a dramatic reduction in infarct size, BBB permeability, and edema after focal transient MCAO compared with wild-type mice. The protection seen in CCR2−/− mice was associated with a marked reduction in immune cell infiltration and reduced expression/production of proinflammatory cytokines, such as IL-1β and TNF-α.36 In line with these reports, our data also found that transient MCAO significantly increased mRNA expression of IL-1β, TNF-α, and MCP-1, and trends toward a considerable increase of IL-6 in the ipsilateral cerebral cortex at 18 hours post stroke. Further findings indicated that delayed treatment with MRE-269 significantly reduced the mRNA levels of IL-1β, TNF-α, and MCP-1, but not IL-6 induced by MCAO, which suggests that suppression of IL-1β, TNF-α, and MCP-1 expression by MRE-269 may be one of the mechanisms underlying the neuroprotective effects of MRE-269 in ischemic stroke.

In addition to inflammatory cytokines/chemokines, oxidative stress also plays a critical role in the pathogenesis of ischemic stroke. In the central nervous system, there are several sources generating free radicals, including the mitochondria, xanthine oxidase, uncoupled nitric oxide synthase, and cyclooxygenases.37 However, the NOX family of enzymes seems to be a major contributor to oxidative stress after ischemia. NOX2 (namely gp91phox) is a critical contributor to worse stroke outcomes because it is a major source of superoxide generation and a key contributor to ischemic injury.38,39 In this study, we found for the first time that neurological impairment and brain infarction were associated with a significant increase of gp91phox protein after stroke in the aged rat brain, and delayed MRE-269 treatment dramatically attenuated its increase. Previous studies have shown that activation of the IP receptor reduces gp91phox levels in peripheral endothelial cells.40,41 This is the first study to show reduced gp91phox levels by a PGI 2 analog in stroke. In addition, a dramatic increase in 8-iso-PGF 2α and 4-hydroxynonenal-modified proteins were observed in the ischemic cerebral cortex after stroke. MRE-269 significantly reduced both 8-iso-PGF 2α and 4-hydroxynonenal levels in the aged ischemic brain. Collectively, these findings suggest that the neuroprotective effect of MRE-269 in stroke may be partly through the reduction of oxidative stress induced by transient MCAO.

Stroke-induced proinflammatory cytokines and free radicals have been shown to exacerbate brain injury, in part, by increasing BBB permeability.18,20 Treatment with MRE-269 resulted in less BBB opening, which was associated with a significant reduction in ischemia-induced MMP-9 activation. Although activation of the IP receptor has been shown to reduce ischemia-induced brain microvascular permeability,42,43 this is the first study showing that MRE-269 attenuates BBB damage after ischemic stroke likely by reducing MMP-9 activation.

In conclusion, we show that the delayed treatment with the highly selective IP receptor agonist, MRE-269, provided sustained protection, which correlated with a marked improvement in neurological function, reduction in infarct size, and reduced body weight loss in a stroke model in aged rats. Mechanistically, the downregulation of proinflammatory cytokines IL-1β, TNF-α, and chemokine MCP-1, as well as the reduction of oxidative damage and BBB disruption by MRE-269 likely contributed to the observed neuroprotective effect. These data strongly suggest that targeting the IP receptor with MRE-269 is a novel strategy to reduce cerebral ischemia injury and promote long-term neurological recovery in ischemic stroke. Repurposing the use of seleipag/MRE-269 for ischemic stroke is of great translational relevance because this clinically approved drug has therapeutic potential to ameliorate ischemic brain injury.

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Neuroprotection by MRE-269 in Ischemic Stroke

Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals, a priori sample size calculation, power analysis, blinding, and randomization

Fifty-seven aged male Sprague Dawley rats (18-20 months, Hilltop Laboratories, Scottdale, PA, USA) underwent 90 min of MCAO or sham operation. We performed an a priori sample size calculation using the G*Power v.3.1.3 software\(^1\). In order to calculate Cohen effect size \( (d) \), we compared two independent groups in a two-tailed unpaired \( t \)-test using \( \alpha=0.05 \), and \( \beta \) (type II error) of 0.1 with a power of 90%. We utilized means and standard deviations from our preliminary studies in this stroke model in aged rats. The present study was powered with the expectation that we will detect a difference in infarct size of at least 25% between vehicle- and MRE-269-treated groups, which is a biologically meaningful effect\(^2\). For the difference to become statistically significant, we calculated a sample size of \( n=11 \) with an effect size of \( d=1.34 \). A final sample size of \( n=13 \) per group (vehicle- or MRE-269-treated) was utilized for the experiments described in this report after adjusting for a 15% attrition rate. Corrected sample size=calculated sample size/(1-% attrition/100)). Five sham-operated control rats were also included for the molecular biology analyses performed with tissue collected after 18 h of ischemia. All rats were housed in individual cages in a controlled environment maintained under a 12-h light/dark cycle. Animals had free access to food and water, and they were acclimated to our animal facility for at least 7 days before any surgical procedure. All experimental animal procedures were performed in accordance with approved guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals, the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines), and the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida (protocol #201406503). Animals were randomly allocated to treatment groups using the GraphPad software randomization tool (http://www.graphpad.com/quickcalcs/randomize1.cfm). The investigators performing surgeries and euthanizing animals had no knowledge of the experimental group to which an animal belonged. An independent investigator administered either the vehicle or MRE-269 (coded vials) according to the randomization schedule to guarantee treatment allocation concealment. Similarly, investigators responsible for the assessment of outcomes (infarct size, behavior, and molecular biology analyses) were blinded to treatment groups (coded animals, samples, and MRI images).

Induction of transient MCAO in aged rats

Transient MCAO was induced using an intraluminal silicone-coated filament method as previously described by our group\(^3,4\). Briefly, animals were subjected to anesthesia by 3% isoflurane and maintained by inhalation of 1.5-2% isoflurane in medical grade oxygen during surgery. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed via a midline vertical incision in the anterior neck. The CCA was ligated with a 4-0 suture at the proximal portion from the carotid bifurcation. Another 4-0 suture was loosely tied around the ICA and ECA bifurcation and micro-aneurysm clips were temporarily placed on the ICA and ECA. A small arteriotomy was made in the CCA approximately 2 mm proximal to the carotid bifurcation, and a 3-0 silicone-coated nylon filament was inserted via the incision and advanced gently in the ICA approximately 20-22 mm from the carotid bifurcation until a mild resistance was felt. The end of the occluding filament was coated with silicone rubber (6-7 mm coating length and 0.48 mm in diameter). The clips on the ICA and ECA were removed during the induction of focal cerebral ischemia, and rats were allowed to recover from
anesthesia. After 90 min ischemia, rats were re-anesthetized and the filament was gently retracted to allow reperfusion. The skin was closed and anesthesia was discontinued, the animals were allowed to recover in a temperature-controlled chamber. Buprenorphine hydrochloride (Buprenex; 0.05 mg/kg; s.c.) was given as an analgesic immediately before surgery. Sham-operated rats underwent the same surgical procedures except for the advancement of the silicone-coated filament into the ICA. Ischemia and reperfusion conditions were confirmed by regional cerebral blood flow (CBF) detected by a laser Doppler flowmeter (moorVMS-LDF, Moor Instruments, Delaware, DE, USA) positioned 1.0 mm posterior to bregma and 5.0 mm lateral to the midline before, during, and after withdrawal of the occluding filament. Only rats showing sustained CBF reduction to less than 55% of pre-ischemic baseline values as well as successful reperfusion (>90% of baseline) were included in the experiments. Animals showing complete loss of spontaneous activity for a prolonged time (>2 h), subarachnoid hemorrhage upon tissue harvesting, or lack of neurological deficits after 90 min of stroke were excluded from the analysis. Blood was collected from the femoral vein (~0.2 mL) to measure physiological parameters with an iSTAT clinical analyzer using CG8+ cartridges (Cat. No. 600-9001; Abbott, Princeton, NJ) 15 min before and after MCAO, as well as 15 min after initial dose of vehicle or MRE-269 injection.

**Experimental design and drug administration**

For the intravenous administration of vehicle or MRE-269, a Micro-Renathane catheter was inserted into the right femoral vein of ischemic rats at the time of MCAO surgery. The catheter was placed in the distal portion of the vein to minimize disruption of blood flow in the leg. This technique has been utilized in long-term behavioral studies in rodents after stroke and results from our pilot studies indicate that distal catheterization of the femoral vein does not interfere with motor behavior in rats. MRE-269 (Cat No.10010412; Cayman Chemical, Ann Arbor, MI, USA) was dissolved in dimethyl sulfoxide (DMSO) and then diluted in sterile saline. The final concentration of DMSO was 1%. For the infarct size and neurobehavioral tests experiments, twenty-six rats underwent transient MCAO and were randomly assigned to vehicle or treatment group with administration of 1% DMSO in saline (n=13) or MRE-269 (0.25 mg/kg, n=13) starting at 4.5 h post-MCAO. Additional doses were given every 12 h for the first 48 h, and then one injection daily for 7 days post-MCAO. The MRE-269 dose and treatment schedule was based on our preliminary findings in young rats (3-4 months) showing that MRE-269 (0.1-0.5 mg/kg, i.v.) given at 1.5 h after stroke onset produced a dose-dependent reduction in infarct volume as measured by TTC staining at 48 h (data not shown). The optimal dose of 0.25 mg/kg was therefore used in the present study in aged rats.

Rats were scanned by MRI at 48 h and 21 days post-MCAO and neurobehavioral tests were performed before ischemia and at 3, 7, 14 and 21 days after stroke. Five rats in the vehicle and 4 rats in the MRE-269 groups died during the 21 days post-MCAO period. For the biochemical experiments, 31 rats were randomly assigned to the following groups: sham-operation (n=5), vehicle (1% DMSO, n=13) or MRE-269 (0.25 mg/kg, n=13). Drug or vehicle were given intravenously starting at 4.5 h after stroke and an additional dose was given at 12 h after stroke onset. One animal in the vehicle group died shortly after MCAO. Animals were sacrificed at 18 h post-MCAO and perfused with ice-cold saline. Samples from the ipsilateral and contralateral cerebral cortices were obtained for RNA isolation, immunoblotting, and lipid peroxidation analyses.
MRI and image analysis

At 48 h and 21 days after MCAO, rats treated with vehicle or MRE-269 (0.25 mg/kg) were brought to the University of Florida’s Advanced Magnetic Resonance Imaging and Spectroscopy Facility (AMRIS) for MRI measurements. During imaging sessions, rats were anesthetized under 1.5-2% isoflurane gas and placed supine on a custom-made bed and their heads accommodated inside a quadrature surface transmit/receive 1H radiofrequency coil tuned to 200 MHz (airMRI, LLC, Holden, MA). Diffusion and T2 weighted images were collected in a Magnex Scientific 4.7 Tesla MR scanner controlled by an Agilent VnmRJ 3.1 console. Respiratory rates and core body temperature were monitored and controlled continuously through the experiment (SA Instruments). For diffusion-weighted MRI, we collected eight interleaved coronal slices covering the entire rostral-caudal extent of the stroke lesion. The following parameters were optimized and used: conventional spin echo sequence with a repetition time (TR) = 2 sec, echo time (TE) = 37.5 ms, field of view (FOV) = 25.62 mm, slice thickness = 1.5mm, data matrix = 96 x 96 (for an in-plane resolution of 267 μm²). A trace weighted sequence was used with one B = 0 and three orthogonal B images = 1270 s/mm², ∆ = 17.5 ms, δ = 5.6 ms, and gradient (G) amplitude 18.61 Gauss/cm. Scan time was 13 minutes per rat. Region of interest (ROI) analysis for the stroke volume was used. Stroke volume was the primary measure for the present study. We quantified the apparent diffusion coefficient (ADC) for the side ipsilateral to the lesion and the contralateral side. The first image in the trace sequence was a T2 image that provided excellent contrast for delineating the outer borders of the lesion area across multiple slices. Manual ROI drawing for each subject at each time point was carried out using ITK-SNAP software. The T2 and diffusion weighted images were imported into NIH ImageJ software (http://rsbweb.nih.gov/ij) and ADC maps were constructed. ADC maps were set at a threshold between 0 and 200 s/mm² and ADC values extracted for the stroke lesion side and the contralateral side. Hyper-intense ischemic areas in the ADC and T2 weighted images were marked with a region of interest (ROI) tool in the ITK-SNAP software. The mean signal intensity of all pixels within the ROI was used to calculate infarct area. Stroke ROI volumes were converted to .vtk format and registered to an atlas of the rat brain in order to assess relative changes in lesion with and without MRE-269 treatment.

Behavioral tests

To examine the effects of MRE-269 on neurological outcomes in aged rats subjected to ischemic stroke, a battery of behavioral tests was performed pre-MCAO and at 3, 7, 14 and 21 days post-MCAO by an independent investigator blinded to the experimental groups. For the adhesive removal test, somatosensory deficits can be seen in the latency for the rat to notice the sticker and remove it from the paw as previously described with some modifications. Briefly, adhesive tape (113.1 mm² round) was placed on the ventral surface of the contralateral forepaw. The observer recorded the latency for each rat to remove the adhesive tape with its mouth up to a cut-off time of 2 min. Before surgery, the animals were pre-trained for 2 days. Once the rats could remove the tape within 10 seconds, they were subjected to MCAO. Three trials per animal were performed and the average of the two best (smallest) latency values was used for analysis.

For the rotarod test, locomotor impairments were assessed with the accelerating rotarod as described previously with minor modifications. Briefly, rats were placed on an accelerating rotarod (model ENV-575, MED Associates Inc., Fairfax, VT, USA) and the speed was slowly increased from 4 to 40 rpm during a 5-min period. Before surgery, the animals were pre-trained for 2 days. Latency to fall off the rotarod was recorded before ischemia and at 3, 7, 14 and 21 days after stroke. All animals were
required to stay on the accelerating rotarod for a minimum of 30 seconds. If they were unable to reach this criterion, the trial was repeated for a maximum of 5 times instead of 3 times. The average of the two best (largest) fall latency values was used for analysis and the motor test data were presented as percentage of mean latency compared with the internal baseline (before surgery).

RNA extraction and real-time PCR

As described previously by our group\(^3\), total RNA from cortical tissue was extracted using the PureZOL™ RNA isolation kit (Cat. No. 738-6830; Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. cDNA was synthesized using iScript Reverse Transcription kit (Cat. No. 170-8841; Bio-Rad) and quantitative real-time PCR was performed with 20 ng of cDNA in a total volume of 10 μl using Sso Advanced Universal SYBR Green Supermix (Cat. No. 172-5272; Bio-Rad) according to the manufacturer’s protocol. The following primers were used: IL-1β, GTGCTGTCTGACCCATGT (forward), TTGTGTTGCTTTGTCTCTCC (reverse); TNF-α, AGACCCCTACACTCAGATCA (forward), GTCTTTGAGATCCATGCCATTG (reverse); IL-6, CAGAGCAATACTGAAACCCTAGT (forward), CCTTCTGTGACCTAACTTCTCC (reverse); MCP-1, ATCTCTCTTCCCTCCACCTA (forward), GAATGAGTAGCAGCAGGTGAG (reverse); Ywhaz, GAAGAGTCGTACAAAGACAGCA (forward), GCTTTGCTTCGTCCTCCCTTG (reverse). PCR reactions were run in triplicate and cycle threshold (Ct) values were normalized to Ywhaz expression for each sample. Results are presented as normalized expression relative to sham-operated group.

Immunoblotting

Cortical tissue was homogenized in radioimmunoprecipitation (RIPA) lysis buffer containing protease and phosphatase inhibitors as detailed in our recent report\(^3\). Fifty micrograms of protein were incubated in non-reduced Laemmli’s sample buffer for 5 min at 100°C to measure gp91\(^{phox}\), while incubated at 60°C for 3 min to analyze 4-hydroxynonenal (4-HNE) as previously reported\(^10\). After the incubation step, samples were separated in 4-20% SDS-polyacrylamide gels, and then transferred onto nitrocellulose membranes. Membranes were then blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline (TBS) before overnight incubation at 4°C with antibodies against either gp91\(^{phox}\) (Cat. No. 611414, 1:1000; BD Biosciences, San Jose, CA), 4-HNE (Cat. No. ab48506, 1:200; Abcam, Cambridge, MA) or β-actin (Cat. No. A1978, 1:10,000; Sigma-Aldrich, Saint Louis, MO). Afterwards, membranes were washed and incubated for 1 hour with goat anti-mouse IRDye 800CW secondary antibody (1:30,000; Li-Cor, Lincoln, NE, USA) for detecting gp91\(^{phox}\) or 4-HNE, and donkey anti-mouse IRDye680LT (1:40,000; Li-Cor) for β-actin. Immunoreactive bands were visualized and densitometrically analyzed using Odyssey infrared scanner and Image Studio 2.0 software (Li-Cor).

Determination of 8-iso-prostaglandin F\(_2\)α

To determine the effects of delayed MRE-269 treatment on lipid peroxidation, 8-iso-prostaglandin F\(_2\)α (8-iso-PGF\(_2\)α), a highly sensitive biomarker of oxidative stress, was measured using a commercially available ELISA kit (Cat. No. 516351, Cayman Chemical, Ann Arbor, MI) following the manufacturer’s protocol. Briefly, the 8-iso-PGF\(_2\)α from brain cortical tissue was hydrolyzed with 15% (w/v) KOH before purification using an 8-isoprostane affinity sorbent protocol. Each sample was assayed in duplicate, and total 8-iso-PGF\(_2\)α in samples was determined based on a standard curve and expressed in nanogram per gram of tissue.
Immunoglobulin G (IgG) ELISA

To study the blood brain-barrier (BBB) permeability, the extravasation of IgG from the blood into the brain parenchyma was measured by a commercial IgG ELISA kit (Cat. No. E-25G; Portland, OR) according to the manufacturer's instructions. A total of 50 µg of protein extracted from ipsilateral and contralateral cerebral cortices of rat brain were used for the IgG measurement. All samples were assayed in duplicate and optical absorbance at 450 nm was measured with a Synergy™ HT Multi-Mode Plate Reader (Biotek Instruments, Winooski, VT). A standard curve was constructed (0-400 ng/ml) and the concentrations of IgG in brain tissue were determined from the standard curve.

Fluorometric immunocapture assay of matrix metallopeptidase (MMP)-9 enzymatic activity

Enzymatic activity of matrix metallopeptidase (MMP)-9 in cortical tissue was measured using a fluorescence resonance energy transfer (FRET) peptide immunoassay as described in our previous studies. Briefly, 96-well plates were coated with 5 µg/ml protein A/G for 2 hours at room temperature before the addition of the MMP-9 antibody (Cat. No. sc-6841R; Santa Cruz Biotechnology, Dallas, TX) for another 2 hours incubation. A total of 50 µg protein extracted from rat brain cerebral cortex was added to each well and incubated overnight at 4°C with gentle shaking. After incubation, wells were washed with TCNB buffer (50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, 0.05% Brij-35) and 1 µM of 520 MMP FRET substrate III (Cat. No. 60570-01; AnaSpec, San Jose, CA) was added. Plates were incubated for 48 h at 37°C, then relative fluorescence units (RFUs) were read and monitored at excitation/emission wavelengths of 485/528 nm in a Synergy™ HT Multi-Mode Plate Fluorescence Reader (Biotek Instruments, Winooski, VT). The average value from one paired substrate control wells was used to subtract baseline fluorescence from sample wells.

Supplemental References

1. Faul F, Erdfelder E, Lang AG, Buchner A. G*power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behavior research methods. 2007;39:175-191


Supplemental Table I. Physiological parameters in aged rats treated with vehicle- or MRE-269

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>MRE-269 (0.25 mg/kg; i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>15 min before MCAO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.33±0.01</td>
<td>7.30±0.04</td>
</tr>
<tr>
<td>PCO$_2$ (mmHg)</td>
<td>54.62±2.12</td>
<td>59.36±6.38</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>291.60±29.74</td>
<td>274.60±14.94</td>
</tr>
<tr>
<td>HCO$_3^-$ (mmol/L)</td>
<td>28.84±0.51</td>
<td>28.92±0.43</td>
</tr>
<tr>
<td>Na$^+$ (mmol/L)</td>
<td>135.00±0.45</td>
<td>135.00±1.45</td>
</tr>
<tr>
<td>K$^+$ (mmol/L)</td>
<td>3.90±0.09</td>
<td>3.68±0.17</td>
</tr>
<tr>
<td>Ca$^{2+}$ (mmol/L)</td>
<td>1.28±0.05</td>
<td>1.22±0.04</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>392.80±26.85</td>
<td>395.80±41.42</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>42.20±1.07</td>
<td>39.00±1.92</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.36±0.36</td>
<td>13.28±0.65</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.63±0.06</td>
<td>36.52±0.09</td>
</tr>
<tr>
<td><strong>15 min after MCAO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.39±0.06</td>
<td>7.33±0.02</td>
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<tr>
<td>PCO$_2$ (mmHg)</td>
<td>46.98±9.45</td>
<td>56.93±3.30</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>240.25±44.40</td>
<td>229.25±13.60</td>
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<tr>
<td>HCO$_3^-$ (mmol/L)</td>
<td>26.85±2.47</td>
<td>30.03±0.21</td>
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<tr>
<td>Na$^+$ (mmol/L)</td>
<td>135.50±0.87</td>
<td>135.25±1.31</td>
</tr>
<tr>
<td>K$^+$ (mmol/L)</td>
<td>4.18±0.14</td>
<td>4.18±0.21</td>
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<tr>
<td>Ca$^{2+}$ (mmol/L)</td>
<td>1.27±0.07</td>
<td>1.25±0.03</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>349.50±33.00</td>
<td>382.25±49.06</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>41.50±1.07</td>
<td>39.00±2.04</td>
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<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.13±0.55</td>
<td>13.25±0.70</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>37.09±0.52</td>
<td>36.77±0.17</td>
</tr>
<tr>
<td><strong>CBF (% of Pre-MCAO baseline)</strong></td>
<td><strong>41.74±3.31</strong></td>
<td><strong>35.78±3.91</strong></td>
</tr>
<tr>
<td><strong>15 min after treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.38±0.01</td>
<td>7.38±0.02</td>
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<tr>
<td>PCO$_2$ (mmHg)</td>
<td>51.68±1.81</td>
<td>55.43±3.79</td>
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<tr>
<td>PO$_2$ (mmHg)</td>
<td>368.20±36.84</td>
<td>396.67±48.64</td>
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<tr>
<td>HCO$_3^-$ (mmol/L)</td>
<td>30.74±1.05</td>
<td>30.74±1.05</td>
</tr>
<tr>
<td>Na$^+$ (mmol/L)</td>
<td>137.60±0.40</td>
<td>138.80±0.58</td>
</tr>
<tr>
<td>K$^+$ (mmol/L)</td>
<td>3.96±0.14</td>
<td>4.16±0.16</td>
</tr>
<tr>
<td>Ca$^{2+}$ (mmol/L)</td>
<td>1.21±0.05</td>
<td>1.23±0.05</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>325.40±25.06</td>
<td>275.00±31.47</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>39.40±0.75</td>
<td>38.00±2.49</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.40±0.26</td>
<td>12.92±0.82</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.91±0.25</td>
<td>36.42±0.40</td>
</tr>
<tr>
<td><strong>CBF (% of Pre-treatment baseline)</strong></td>
<td><strong>93.23±2.74</strong></td>
<td><strong>92.70±1.80</strong></td>
</tr>
</tbody>
</table>

Values are mean ± SEM. MCAO, middle cerebral artery occlusion; CBF, cerebral blood flow.
Supplemental Figure I. Temporal profile of regional cerebral blood flow (CBF) in rats subjected to 90 min of transient MCAO followed by 120 min of reperfusion. Regional CBF reduced to about 40% of baseline immediately after MCAO occlusion and restored to baseline after reperfusion in both vehicle- (n=5) and MRE-269-treated (n=5) groups. A single bolus injection of MRE-269 (0.25 mg/kg) at the start of reperfusion via the right femoral vein has no significant effect on regional CBF compared to vehicle-treated animals. Data are expressed as mean ± SD.
Table II. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

<table>
<thead>
<tr>
<th>Methodological and Reporting Aspects</th>
<th>Description of Procedures</th>
</tr>
</thead>
</table>
| Experimental groups and study timeline | ☐ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study.  
☐ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated.  
☐ An overall study timeline is provided. |
| Inclusion and exclusion criteria | ☐ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article. |
| Randomization | ☐ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided.  
☐ Type and methods of randomization have been described.  
☐ Methods used for allocation concealment have been reported. |
| Blinding | ☐ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible.  
☐ Blinding procedures have been described with regard to masking of group assignment during outcome assessment. |
| Sample size and power calculations | ☐ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided. |
| Data reporting and statistical methods | ☐ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups.  
☐ Baseline data on assessed outcome(s) for all experimental groups have been reported.  
☐ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms.  
☐ Statistical methods used have been reported.  
☐ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures. |
| Experimental details, ethics, and funding statements | ☐ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described.  
☐ Different sex animals have been used. If not, the reason/justification is provided.  
☐ Statements on approval by ethics boards and ethical conduct of studies have been provided.  
☐ Statements on funding and conflicts of interests have been provided. |

Only male rats were used in the current study due to potential neuroprotective effects of sex hormones in females. Future studies should be conducted in female rodents subjected to ischemic stroke.