Activation of the Neuroprotective Angiotensin-Converting Enzyme 2 in Rat Ischemic Stroke

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Abstract—The angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas axis represents a promising target for inducing stroke neuroprotection. Here, we explored stroke-induced changes in expression and activity of endogenous angiotensin-converting enzyme 2 and other system components in Sprague–Dawley rats. To evaluate the clinical feasibility of treatments that target this axis and that may act in synergy with stroke-induced changes, we also tested the neuroprotective effects of diminazene aceturate, an angiotensin-converting enzyme 2 activator, administered systemically post stroke. Among rats that underwent experimental endothelin-1–induced ischemic stroke, angiotensin-converting enzyme 2 activity increased in the cerebral cortex and striatum in the 24 hours after stroke. Serum angiotensin-converting enzyme 2 activity was decreased within 4 hours post stroke, but rebounded to reach higher than baseline levels 3 days post stroke. Treatment after stroke with systemically applied diminazene resulted in decreased infarct volume and improved neurological function without apparent increases in cerebral blood flow. Central infusion of A-779, a Mas receptor antagonist, resulted in larger infarct volumes in diminazene-treated rats, and central infusion of the angiotensin-converting enzyme 2 inhibitor MLN-4760 alone worsened neurological function. The dynamic alterations of the protective angiotensin-converting enzyme 2 pathway after stroke suggest that it may be a favorable therapeutic target. Indeed, significant neuroprotection resulted from poststroke angiotensin-converting enzyme 2 activation, likely via Mas signaling in a blood flow–independent manner. Our findings suggest that stroke therapeutics that target the angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas axis may interact cooperatively with endogenous stroke-induced changes, lending promise to their further study as neuroprotective agents.

Key Words: angiotensin converting enzyme 2 ■ stroke

Stroke, a disease for which hypertension remains the leading modifiable risk factor, is a devastating reality each year for many millions of people worldwide and is a leading cause of death and disability.1 The renin–angiotensin system (RAS) holds promise as a potential target for novel stroke therapies, especially so with the recent discovery of a counter-regulatory arm of the RAS that exerts opposite effects of angiotensin II (Ang II) type 1 receptor (AT1R) signaling via activation of other receptors, including the angiotensin-(1–7) (Ang-(1–7)) receptor Mas. Ang-(1–7) is formed from angiotensin II by the action of angiotensin-converting enzyme 2 (ACE2).2 Further characterization of the actions of ACE2 in various pathologies has expanded our view of the therapeutic potential of small-molecule activators of this pathway.2

Significant research effort in the field of neuroprotective stroke therapies has been aimed at the discovery of novel treatments that may be administered to salvage penumbral tissue that is uniquely vulnerable to collapse.3 The ACE2/Ang-(1–7)/Mas pathway has been highlighted as a promising target for induction of stroke neuroprotection,4 and has proven efficacy in reducing infarct size and improving neurological function in preclinical models of ischemic5–11 and hemorrhagic stroke.12 Stroke neuroprotection has been demonstrated in both young5,6 and aged10 animals, and methods for activating the axis have included direct intracerebroventricular administration of Ang-(1–7),5,7,11 delivery of ACE2-primed endothelial progenitor cells,8 and neuronal ACE2 overexpression.9 Pharmacological activation of this axis has recently become more feasible with the identification of diminazene aceturate, trade name Berenil, as an activator of ACE2.13 We have shown that when given by intracerebroventricular infusion before, during, and after stroke, diminazene reduced infarct size in rats, an effect that was reversed by coadministration with Mas antagonist A-779.5

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An important consideration for the continued study of the therapeutic effects of the ACE2/Ang-(1–7)/Mas axis in stroke is the characterization of changes that occur in the endogenous components of this system during stroke in the absence of targeted interventions. It has been recently reported that expression of this protective axis is altered after stroke in the rat cerebral cortex\(^\text{14}\) and rostral ventrolateral medulla\(^\text{15}\) although it is unknown whether ACE2 activity levels are affected or whether this is accompanied by changes in the deleterious ACE/Ang II/AT1R axis. Our objective was to test whether components of the RAS, including the ACE2/Ang-(1–7)/Mas pathway, are altered in rats after stroke. Furthermore, we assessed the hypothesis that poststroke systemic administration of an ACE2 activator, diminazene, in rats would result in neuroprotection.

**Methods**

Description of experimental procedures is available in Materials and Methods in the online-only Data Supplement.

**Results**

**Effect of Stroke on ACE2 in Rat Brain and Serum**

Ischemic stroke induction, as described in experiment 1 of the Materials and Methods in the online-only Data Supplement, resulted in significantly increased ACE2 activity in the cerebral cortex ipsilateral to the stroke when compared with control activity levels from sham-operated rats at 4 hours, 12 hours, and 1 day after ischemia (Figure 1A), along with an increase at 12 hours in the ipsilateral striatum compared with both shams and contralateral striatum (Figure 1B). At 12 hours, ACE2 activity in the contralateral cortical samples was also significantly increased compared with respective sham levels (Figure 1A). ACE2 activity levels in the cerebral cortex and striatum had returned to sham levels by 3 days (Figure 1A and 1B). There was not a significant change in ACE2 mRNA levels in the ipsilateral cortex either 1 or 3 days after ischemia (Figure 1C). Stroke resulted in an initial minor decrease of ACE2 activity in rat serum measured at 4 hours post stroke when compared with normalized prestroke levels, followed by a significant rebound increase 3 days post stroke (Figure 1D).

**Effect of Stroke on Other RAS and Related Components in Ischemic Cerebral Cortex**

ACE2 is thought to exert neuroprotective effects, in part, via conversion of Ang II to Ang-(1–7), which subsequently binds and signals through the Ang-(1–7) receptor Mas. Therefore, we assessed the impact of stroke on mRNA levels of Mas and also of the neuroprotective AT2R, and did not find significant differences at 1 day after middle cerebral artery occlusion (MCAO; Figure 2A). In addition to these protective arms of the RAS, components of the classical ACE/Ang II/AT1R pathway were evaluated. Compared with sham, there was an increase in ACE mRNA levels in the ipsilateral cortex, but no change in AT1R mRNA was
observed (Figure 2A). As expected, we also observed increased mRNA levels of LCN2 (lipocalin 2), a marker of astrocyte activation, and CD11b, a marker of activated microglia (Figure 2B). We further evaluated the levels of tumor necrosis factor-α-converting enzyme (TACE, also named ADAM17), an enzyme that forms soluble ACE2 by cleaving ACE2 from its membrane-bound form via its sheddase activity, in the ipsilateral cortex, from the ipsilateral cortex and found a significant increase of TACE mRNA but not activity at 1 day post stroke relative to shams (Figure 2C and 2D). Interestingly, although TACE mRNA expression levels were not significantly different between sham and stroke groups at 3 days, TACE activity was significantly decreased in samples from the ischemic cortex at this time point. Serum TACE activity was not different between sham and stroke groups at 1 day post stroke (data not shown).

To further characterize the stroke-induced changes in Mas expression in neurons and other brain cells, we used immunohistochemical staining to label NeuN and Mas immunoreactive cells. Representative fluorescence micrographs (Figure 3A) show NeuN and Mas staining within the stroke penumbra from sham and stroke rats 1 day post stroke. Semiquantitative analysis of immunofluorescent staining showed that the percentage of fractional area of both NeuN and Mas immunopositive staining in the ipsilateral cerebral cortex was significantly decreased compared with sham rat cerebral cortex (Figure 3B and 3C).

Neuroprotective Effects of Poststroke Administration of Peripheral Diminazene, an ACE2 Activator

The observed increase in ACE2 activity in the ipsilateral cerebral cortex after stroke represents a promising target for inducing stroke neuroprotection. We have previously reported that prestroke activation of central ACE2 using diminazene by chronically coadministering the Mas antagonist A-779 or 0.9% sterile saline vehicle via the intracerebroventricular route. Blockade of central Mas receptors by infusion of A-779 in rats cotreated with poststroke peripheral diminazene resulted in significantly larger infarct sizes compared with saline infusion (Figure 4E).

We assessed the Mas-dependency of the neuroprotection induced by poststroke intraperitoneal diminazene injections by chronically coadministering the Mas antagonist A-779 or 0.9% sterile saline vehicle via the intracerebroventricular route. Blockade of central Mas receptors by infusion of A-779 in rats cotreated with poststroke peripheral diminazene resulted in significantly larger infarct sizes compared with saline infusion (Figure 4E).

To examine the role of endogenous central ACE2 in stroke, we infused MLN-4760, an ACE2 inhibitor, via the intracerebroventricular route for 5 days before and 3 days after stroke in the absence of any treatment. This resulted in significantly worse neurological function at 4 hours and 3 days post stroke without significantly increasing infarct volume (Figure 4F and 4G).

Effect of Diminazene on CBF During Baseline and Stroke

Ang-(1–7) has vasodilatory actions, which may potentially contribute to the neuroprotection induced by ACE2 activation.
To test its effect on CBF, we administered diminazene to rats at neuroprotective doses during endothelin-1–induced MCAO, not poststroke, as described in experiments 3 and 4 of the Materials and Methods in the online-only Data Supplement. Relative CBF was not increased by diminazine during stroke when compared with control as measured by laser Doppler flowmetry and ≤4 hours post stroke (Figure 5A). Also, there was not an observed increase in the baseline levels of absolute CBF, as assessed by the microsphere injection method, among rats chronically treated by intracerebroventricular infusion with diminazene (Figure 5B) when compared with saline-infused rats.

**Discussion**

This study aimed to assess endogenous poststroke changes within the RAS, with a focus on ACE2 activity, and to test the neuroprotective efficacy of poststroke activation of ACE2 by systemic administration of diminazene that might work synergistically with changes in the endogenous RAS. We found significant increases of endogenous ACE2 activity, but not expression, within the ischemic cerebral cortex and striatum of rats during the first 24 hours after stroke, whereas serum ACE2 activity levels were initially decreased, followed by rebound increases. At 1 day after stroke, levels of immunoreactive Mas were significantly decreased in the ischemic penumbra, and mRNA levels of the deleterious RAS component ACE were increased. Administration of poststroke diminazene resulted in significant neuroprotection, as well as increased serum ACE2 activity. Finally, studies of the effect of diminazene on CBF suggest that this protection may not be associated with increased cerebral perfusion.

An increasing number of studies indicate that activation of the ACE2/Ang-(1–7)/Mas axis is neuroprotective in stroke. Although these studies provide an essential foundation, there are several limitations that our current design helps to address. First, changes in expression and activity of the endogenous components of this pathway in the brain after stroke warranted further investigation. The recent demonstration of increases in levels of ACE2, Ang-(1–7), and Mas in the 48 hours after stroke in the ischemic cerebral cortex of rats was an important first look. A second study of transient MCAO in rats that examined axis components in the rostral ventrolateral medulla showed slightly different findings in that region, with decreases in Ang-(1–7) and Mas at 1 day after stroke. In light of these findings, our results, which show an increase in ACE2 activity in the ischemic cortex (Figure 1A) but a decrease in Mas immunoreactivity (Figure 3C), are intriguing. The reductions in immunoreactive Mas observed in penumbral regions of the ischemic cortex at 1 day after stroke agreed with findings from the rostral ventrolateral medulla and may be because of an overall decrease in penumbral Mas+$^+$ cortical neurons, as immunostaining of neuronal nuclear marker NeuN was decreased at this time point (Figure 3B and 3C). Additional work is needed to clarify the various changes that we and others have observed. It is likely that the difference in the stroke models, transient versus permanent MCAO, plays a distinctive role in the induction of RAS components. Our
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data showing increased ACE2 activity in the cerebral cortex support the overall consensus that stroke activates the protective axis.

Second, in all but one of the previous studies, the treatment conditions were started before stroke onset, either by genetic modification or prestroke administration of activating compounds. Using a more clinically relevant protocol, Chen et al\(^8\) were the first to use a poststroke treatment protocol to study ACE2-mediated neuroprotection by delivering endothelial progenitor cells, with or without ACE2 priming, by tail vein injection starting 2 hours after stroke in mice. Because these cells were protective even in the absence of ACE2 priming, and because ACE2 enhanced their beneficial effects, the isolated effects of activating the ACE2/Ang-(1–7)/Mas pathway after stroke onset were unclear. Here, we administered an ACE2 activator by peripheral injection starting at 4 hours after stroke onset. This allowed for assessment of therapeutic efficacy in a setting that overlaps favorably with the 4.5-hour treatment window for the delivery of tissue-type plasminogen activator to human stroke victims, as well as the endogenous stroke-induced changes to the RAS (Figures 1–3). It is also relevant that systemic administration is a preferred route in humans in emergent settings. There is evidence to indicate that peripheral diminazene can cross the intact blood–brain barrier,\(^17\) but in the

![Figure 4](image-url)

**Figure 4.** Poststroke administration of diminazene (DIZE), an angiotensin-converting enzyme 2 (ACE2) activator, results in decreased infarct volume and improved neurological function. A, Average infarct volumes at 3 days post MCAO for groups of rats that received intraperitoneal (IP) injections after stroke of vehicle (H\(_2\)O, n=20) or different doses of DIZE (0.75 mg/kg, n=11; 2.5 mg/kg, n=11; 7.5 mg/kg, n=15; and 15 mg/kg, n=6) or of vehicle after sham surgery (n=10). Representative 2,3,5-triphenyltetrazolium chloride (TTC)–stained sections are shown for each treatment condition. Neurological function was assessed at 1 day after stroke using the Bederson (B) and Garcia (C) scales. D, Levels of serum ACE2 activity in serum samples at 1, 2, and 3 days after stroke from control-treated (n=10) or DIZE-treated (7.5 mg/kg, n=4) stroke rats. E, Average infarct volumes from DIZE-treated rats cotreated with vehicle (NaCl, n=10) or Mas antagonist A-779 (n=11) by continuous intracerebroventricular (ICV) infusion. F, Average infarct volumes from rats given ACE2 inhibitor MLN-4760 by ICV infusion (1 mmol/L infused at a rate of 0.5 μL/h; n=6) for 5 days before and 3 days after endothelin-1 MCAO when compared with NaCl infusion (n=7). G, Neurological function was assessed at 4 hours, 1 day, and 3 days post stroke from MLN-4760 or NaCl infused rats. Data are means±SEM. *P<0.05 compared with respective controls (1-way ANOVA with post hoc Student t test for A–C; Mann–Whitney test for D–G).

![Figure 5](image-url)

**Figure 5.** Neuroprotective doses of diminazene (DIZE) administered systemically or centrally do not increase cerebral blood flow (CBF). A, Averaged laser Doppler flow recordings from rats undergoing endothelin-1 middle cerebral artery occlusion (MCAO) that received a single intraperitoneal (IP) injection of vehicle (H\(_2\)O, n=5) or DIZE (7.5 mg/kg, n=5) 30 minutes before stroke induction; black arrow indicates MCAO. B, Levels of relative CBF during baseline conditions as measured by injection of fluorescent microspheres after 5 days of intracerebroventricular (ICV) infusion of vehicle (0.9% NaCl, n=6) or DIZE (19.4 mmol/L infused at a rate of 0.5 μL/h, n=5).
setting of stroke, well-characterized barrier leakiness allows many compounds to cross.16

A third limitation of previously published studies that we can now begin to address involves the scarcity of data describing the ACE2/Ang-(1–7)/Mas axis in human stroke. Higher levels of ACE2 were found among patients who experienced cardioembolic stroke versus other stroke subtypes18 although baseline or control ACE2 levels were not assessed for comparison. It has also been shown that ACE2 gene polymorphisms may be associated with increased risk for stroke.19 Serum measures of protective RAS components are limited, but direct assessment of human serum ACE2 activity holds promise as a clinical marker in stroke. Our study is the first to assess ACE2 enzymatic activity in animal serum after stroke (Figure 1D). It is encouraging for the translational potential of the animal data that the changes after stroke in ACE2 activity in rat serum (Figure 1D) show a similar pattern to changes we have observed in preliminary studies using human serum obtained from patients with stroke (unpublished data). Although human studies of treatments that target the Ang-(1–7)/Mas axis have not been performed in stroke, it has been shown that application of recombinant human ACE2 in healthy volunteers is well tolerated,20 making plausible the idea of future studies in patients with stroke.

Since the recent indication that diminazene activates ACE2,13 it has been shown to have efficacy in treating a variety of inflammatory diseases, including stroke.2 We are the first to show that poststroke administration of this small-molecule activator results in significant neuroprotection (Figure 4A–C). Importantly, a recent report questioned whether diminazene activates ACE2, showing that it had no effect when incubated in vitro with recombinant ACE2 and ex vivo with kidney lysates.21 We found similar results in unpublished experiments using recombinant human ACE2 and lysates of cerebral cortex. However, our assays of serum ACE2 activity in animals that received systemic diminazene over the course of several days after stroke revealed significant increases in serum ACE2 activity (Figure 4E), a finding similar to that from a study of diminazene in myocardial infarction.22 Diminazene may only increase ACE2 activity in vivo over a period of time or under conditions of disease or stress through as yet undiscovered effects on transcription, translation, or protein modification of ACE2 or related molecules. Regardless, the observed increase in ACE2 activity implies an increase in Ang-(1–7) generation and subsequent Mas signaling as a result of poststroke injections of diminazene, which is further validated by our data showing significantly worse infarct volume with coadministration of A-779 (Figure 4D). The vasodilatory action of Ang-(1–7) was suggested to contribute to its neuroprotective effects in stroke,23 but our data do not indicate that diminazene increases regional CBF during endothelin-1 MCAO or absolute CBF during baseline conditions (Figure 5A and 5B). Nonetheless, Ang-(1–7) may still act to increase perfusion at the level of the microcirculation in such a way that it is not detected by our measures of CBF. Other potential non–ACE2-mediated effects of diminazene, specifically those that may independently reduce inflammation,17 cannot be ruled out as contributing to the neuroprotective effects we have observed. Because the current study is limited to exploring the neuroprotective benefits of targeting this axis in the acute and subacute phase of ischemic stroke, there remains the important question of the impact of such treatments on longer term outcomes and late mortality. We anticipate that future studies in this area will address these and other translationally relevant questions.

Few studies have examined the effect of stroke on the induction of components of the classical RAS. It has been reported that Ang II levels are increased in the stroke cortex 1 day after stroke,24 and others have shown Ang II to upregulate ACE, AT1R, and TACE and decrease ACE2 expression,25,26 which might account for some of the changes that we have observed 1 day post stroke (Figures 1C and 2A and 2D). The role of TACE as an ACE2 sheddase has received recent attention as a possible contributing factor to the development of neurogenic hypertension, where it has been hypothesized that TACE-mediated shedding impairs brain ACE2 compensatory activity.28 Along these lines, evidence from a study in cell culture showed that Ang II–induced proteolytic cleavage of membrane-bound ACE2 was mediated by increased activity of TACE, which was accompanied by an increase in plasma ACE2 activity,29 presumably as a result of an increase in soluble ACE2. Our findings that show significantly increased expression of TACE mRNA in the cortex (Figure 2C) coinciding with early rebound increases in serum ACE2 activity starting at 1 day after stroke (Figure 1D) suggest that brain ACE2, once cleaved from the membrane, may move into the circulation after stroke-induced blood–brain barrier breakdown. This seems unlikely to completely account for the increased serum ACE2 activity at 3 days after stroke because the levels of brain TACE activity are significantly attenuated by that time (Figure 2D). Other explanations for the observed poststroke changes in serum ACE2 activity levels (Figure 1D) include alterations in the release of soluble ACE2 from the membrane of endothelial cells.28 Evidence is not sufficient to positively identify what signaling processes could initiate such alterations in ACE2 shedding after stroke, but it seems plausible that Ang II, which has been shown to increase TACE,27 is involved. Another explanation incorporates bone marrow–derived hematopoietic cells as a potential source of soluble ACE2. In atherosclerotic plaques, ACE2 is expressed in CD34+ cells, a marker for endothelial or hematopoietic progenitor cells. Evidence suggests that activation of mononuclear cells leads to an increase of ACE228 and of TACE,30 which could result in increased shedding of ACE2 from activated leukocytes. Furthermore, levels of CD34+ cells in peripheral blood after stroke in humans may follow a similar pattern to that observed in serum ACE2 activity (Figure 2D) with an initial decrease followed by a rebound increase.31 Taken together, these findings suggest that ACE2 shedding from brain, endothelial, or activated hematopoietic cells may contribute to changes in serum ACE2 activity after stroke. The physiological relevance of changes in serum ACE2 activity is not clear,3 but the evidence seems to indicate that it is closely linked with the activity of TACE as regulated by Ang II signaling.
Perspectives
The characterization of ACE2-mediated protection in a variety of cardiovascular diseases, including hypertension and stroke, has opened a promising avenue for development of novel treatments, which will rely on a thorough characterization of pathophysiologic changes in the RAS. In this study, we characterized endogenous changes in this pathway in the hours and days after stroke in rats. Future work in this area may deepen our understanding of the cell subtype-specific regulation of stroke-induced changes in protective and deleterious RAS components, as well as the physiological significance of changes in systemic versus central ACE2 activity. Our results also provide the first evidence that activation of ACE2 by peripheral administration of a small-molecule drug after stroke is neuroprotective, which adds to an already compelling body of evidence that indicates great promise for the clinical potential of pharmaceutical formulations that target ACE2/Ang-(1–7)/Mas in stroke.

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Disclosures
None.

References


**Novelty and Significance**

**What Is New?**
- This is the first study to characterize angiotensin-converting enzyme 2 (ACE2) activity after stroke.
- We tested noninvasive administration of an ACE2 activator beginning after stroke as a clinically relevant approach.

**What Is Relevant?**
- The finding of stroke-induced changes within the ACE2 pathway is an important step for understanding ways it can best be targeted using potential stroke treatments.

- The discovery of neuroprotection by giving an ACE2 activator after stroke is compelling evidence for the continued study of this pathway toward clinical application.

**Summary**

Stroke resulted in dynamic changes of ACE2 activity in brain and serum from rats. Poststroke systemic administration of an ACE2 activator resulted in improved infarct size and neurological function, likely by activation of Mas, the Ang-(1–7) receptor.
ONLINE SUPPLEMENT

ACTIVATION OF THE NEUROPROTECTIVE ANGIOTENSIN CONVERTING ENZYME 2 IN RAT ISCHEMIC STROKE

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EXPANDED MATERIALS, METHODS

Animals and Housing

For these experiments, we used a total of 208 nine week old male Sprague Dawley rats (275-300g) purchased from Charles Rivers Farms (Wilmington, MA, USA) and were drug and test naïve prior to inclusion in this study. Rats were housed two per cage in well-ventilated, specific pathogen-free, temperature-controlled facilities (24 ± 1 °C; 12–12 h light–dark cycle). Rats had ad libitum access to water and standard rat chow. The Institutional Animal Care and Use Committee approved all procedures adhering to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The ARRIVE Guidelines to improve the design, analysis, and reporting of research using animals were applied in all experiments.

Anesthesia, analgesia, and euthanasia

Anesthesia for animal surgeries was induced using 100% O₂/4% isoflurane and maintained using 100% O₂/2% isoflurane. Post-operative analgesia was provided using buprenorphine (0.05 mg/kg, s.c., Hospira Inc., Lake Forest, IL, USA). Animals were euthanized by decapitation under deep anesthesia (5% isoflurane), with immediate brain removal and tissue processing and storage.

Endothelin-1 induced middle cerebral artery occlusion

Adult Sprague Dawley rats underwent transient ischemia by occlusion of the right middle cerebral artery (MCAO) induced by stereotaxic microinjection of endothelin-1 (3µl of 80 µmol/L solution) under isoflurane anesthesia as described previously. This model of focal ischemic stroke was selected for several reasons: 1) the gradual reperfusion of the tissue more closely resembles cerebral blood flow in human stroke than other temporary occlusion models associated with surge reperfusion and hyperemia; 2) the relatively non-invasive surgical procedure rarely results in surgical complications thus reducing experimentally-related mortality; and 3) we have effectively utilized the endothelin-1 model in this species and strain to establish the neuroprotective effects of manipulating of the ACE2/Ang-(1-7)/Mas axis pre-stroke, and we sought to build upon and extend these findings in the same model for comparison. To induce temporary occlusion of the middle cerebral artery (MCAO), a Hamilton syringe was lowered 7.5mm below the skull surface through a burr hole drilled stereotaxically at 5.2mm lateral and 1.6mm anterior to Bregma, and endothelin-1 (ET-1, 3µl of 80µmol/L solution) or sterile saline (3µl of 0.9% solution) was injected (1µl/min). For rats in Experiment 2 described below, we additionally implanted a stainless steel guide cannula 5 days before ET-1 MCAO at the same coordinates used for the burr hole through which the Hamilton syringe was lowered to induce stroke. During surgery, body temperature and depth of anesthesia were controlled, and the latter was monitored using the eye blink reflex, reaction to paw pinch, and visual monitoring of depth and frequency of respirations.

Experimental protocols
**Experiment 1**

This experiment was designed to assess post-stroke changes in the RAS and related systems in the absence of targeted interventions, with ACE2 activity levels assessed as the primary outcome measure and other molecular markers as secondary outcomes. Rats were randomly assigned to undergo sham stroke (n = 31) or ischemic stroke (n = 35) by endothelin-1 MCAO. Tail clip blood samples (~150μl) were collected at one pre-stroke time point and again at 4h, 12h, 1d, 2d, and 3d after stroke in the same animal, where possible, followed by centrifugation (13,200rpm for 15m) and decanting of serum, which was stored at -80 °C until analysis. Groups of rats were euthanized at 4h, 12h, 1d, and 3d after stroke for brain tissue collection. Brains were immediately placed in cold 0.9% saline and cut into 2mm coronal sections. Two sections from 1 to 5mm rostral to bregma were separated into left (contralateral to the stroke) and right (ipsilateral to the stroke) cortex and striatum before tissue homogenization and storage at -80 °C for use in mRNA and enzymatic activity assays. For the 1d group, a 2mm section from 1mm caudal to 1mm rostral to bregma was immediately saved in optimal cutting temperature compound (Sakura, #4583) and frozen at -20 °C in preparation for immunohistochemical analyses.

**Experiment 2**

To determine whether post-stroke administration of diminazene exerted protective effects, rats (n = 80) were randomly assigned to undergo either sham surgery or endothelin-1 MCAO followed by post-stroke intraperitoneal injections of 0.3mL drug vehicle (H₂O) or diminazene (0.75 – 15mg/kg) at 4h, 1d, and 2d, with percent infarct volume assessed as the primary outcome measure and neurological function as a secondary outcome. The effect of intraperitoneal diminazene on baseline blood pressure was assessed via indirect tail cuff method as detailed previously². Several rats also underwent serial collection of serum once daily at 1, 2, and 3d following stroke. To assess the role of central Mas during diminazene treatment, rats (n = 24) to receive post-stroke diminazene intraperitoneal injections were randomized to treatment centrally for five days before and three days after stroke with the Mas antagonist A-779 (1mmol/L infused at a rate of 0.5μL/h) or sterile saline (0.9%) via implantation of Alzet® osmotic mini-pumps and stainless steel intracerebroventricular cannulas at 1.5mm lateral and 1.3mm posterior to bregma. Neurological function was assessed at 1d and 3d using the Bederson (perfect score = 0) and Garcia scales (perfect score = 18) as we have described previously²⁻⁵. Upon euthanasia, the brains were immediately sectioned and two 2mm sections from 1 to 5mm rostral to bregma were used for infarct volume analysis.

In a related experiment to evaluate the role of central ACE2 in stroke, randomly assigned rats (n = 16) were treated centrally for five days prior to and three days after stroke with the ACE2 inhibitor MLN-4760 (100μmol/L infused at a rate of 0.5μL/h) or sterile saline (0.9%) via intracerebroventricular infusion as above. Following endothelin-1 MCAO, neurological function
was assessed at 4h, 1d, and 3d, and brains were harvested at 3d post-stroke for infarct volume analysis as above.

**Experiment 3**

To assess the effect of peripherally administered diminazene on relative CBF (primary outcome measure) during endothelin-1 MCAO, rats (n = 10) were randomized to injection with vehicle or diminazene (7.5 mg/kg) 30 minutes prior to stroke induction. CBF was recorded by laser Doppler flowmetry starting 10 minutes before MCAO and concluding 4h afterward.

**Experiment 4**

This experiment was used to measure the effect of central intracerebroventricular infusion of diminazene (19.4mmol/L infused at a rate of 0.5µl/h) or vehicle on levels of absolute CBF (primary outcome measure) during baseline conditions. Randomly assigned rats (n = 12) were implanted with Alzet® osmotic mini-pumps and intracerebroventricular cannulae as described in *Experiment 2* above. One week later, absolute CBF was assessed by the microsphere method described above. The absolute measurements reported are from the left hemispheres only, which were not different from levels in the right hemispheres (data not shown).

**mRNA analyses**

Angiotensin converting enzyme (ACE), angiotensin II type 1 receptor (AT1R), angiotensin converting enzyme 2 (ACE2), Mas, angiotensin type 2 receptor (AT2R), ADAM metallopeptidase domain 17 (ADAM17 or TACE), cluster of differentiation 11b (CD11b), and lipocalin-2 (LCN2) mRNA levels were assessed using real-time reverse transcription-PCR (qRT-PCR) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as detailed previously[^4]. Oligonucleotide primers and Taqman probes were from Applied Biosystems. Data were normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA.

**ACE2 and TACE activity assays**

Rat brain and serum samples were collected and stored at -80 °C. Samples of cerebral cortex and striatum were homogenized and centrifuged in radioimmuno-precipitation assay buffer with phosphatase and protease inhibitors, and the supernatant was assayed to determine protein concentration using the Bradford protein assay. Brain samples were then diluted (12µg protein per well) in ACE2 buffer (1mol/L NaCl, 75mmol/L Tris HCl, ph 7.5, and 50µmol/L ZnCl₂) or TACE buffer (100mmol/L NaCl, 50mmol/L Tris HCl, ph 7.5, 100µmol/L ZnCl₂, 10mmol/L CaCl₂) and were incubated in black flat-bottomed 96-well plates in 100µl of reaction mixture containing ACE2 buffer, 10µmol/L captopril, and 50µmol/L fluorogenic Mca-YVADAPK(Dnp)-OH ACE2 substrate (R&D Systems, Inc., #ES007), or TACE buffer and 10µmol/L fluorogenic TACE substrate (Enzo Life Sciences, #BML-P132-0500), respectively. For the quantification of TACE activity, brain samples were co-incubated with 50µmol/L TAPI-
2, a TACE inhibitor, to identify and subtract out non-specific peptidase activity. Relative fluorescence (RFU) for all samples was measured for 60 minutes using a Synergy Mx Microplate Reader (BioTek Instruments, Inc.) with excitation at 320nm and emission at 405nm. Samples of rat serum (6µl per well) were assayed undiluted in reaction mixture containing 25µmol/L Mca-YVADAPK(Dnp)-OH ACE2 substrate. We screened control samples to verify that no correction for inner filter effect was required. The slope of the fluorescence curve from 30-60 minutes was used to calculate RFU per minute. Substrate concentrations were selected following determination of reaction Km and Vmax using control samples and recombinant human ACE2 (R&D Systems, Inc., #933-ZN-010) as a positive control, and all samples were run in duplicate.

**Immunohistochemistry and Semi-quantitative analysis**

Mas immunoreactivity and its co-localization with neurons was assessed as we have described previously³ using 20µm brain sections obtained from fresh frozen brains in optimal cutting temperature compound as described in Experiment 1 in the methods. Sections were primary labeled with mouse anti-NeuN (Neuron specific protein; 1:100) and rabbit anti-Mas (1:100), and secondary labeled with Alexafluor donkey anti-mouse 488 (1:500) and goat anti-rabbit 594 (1:750) before mounting in DAPI vectashield followed by fluorescence imaging. In order to perform a semi-quantitative analysis of levels of NeuN and Mas immunofluorescence, we averaged the fractional area of immunopositive staining from three standardized 40x imaging fields from each of six 20 µm sections through the ischemic region, for a total of 18 fields from each brain. For each section, three standardized fields were imaged from the infarcted cerebral cortex and included images 1) from near midline, 2) a region of cortex at the dorsal edge (penumbra) or 3) fully within (core) the infarct area as typically defined by infarct volume analysis. Fractional area was calculated using ImageJ software (NIH) to identify immunopositive versus total area.

**Measurement of cerebral blood flow**

To measure relative cerebral blood flow by laser Doppler flowmetry, a Standard Pencil Probe was placed just posterior to the MCAO burr hole, and using a Blood Flow Meter coupled to a Powerlab 4/30, we measured several minutes of baseline flow before stroke induction followed by continuous flow monitoring during vessel occlusion, as described previously⁵, ⁷. We routinely observed 40-60% reductions in the level of relative CBF following endothelin-1 induced MCAO using this method (see Figure 5B). For the measurement of absolute cerebral blood flow, we followed the protocol described by Engelhorn et al.⁸ which utilizes a reference arterial blood sample to which levels of tissue perfusion are normalized. Rats were anesthetized and the abdominal aorta was catheterized for the collection of arterial reference blood, after which a thoracotomy was performed to expose the heart. Instead of radiolabeled spheres, 2x10⁵ fluorescent microspheres (15µm, Triton Technology, Inc., San Diego, CA, USA) in 0.2mL of the manufacturer’s suspension solution were injected over several seconds directly into the left ventricle. One minute of reference blood sampling using a constant withdrawal pump was started
5 seconds prior to microsphere injection and continued 55 seconds afterward for a total sample volume of 1mL (1 mL/min). Animals were immediately euthanized for harvesting of the brain, which was separated into left and right hemispheres. Isolation of microspheres was accomplished by a sedimentation method described in detail in the Manual for Using Fluorescent Microspheres to Measure Regional Organ Perfusion from the Fluorescence Microsphere Resource Center at the University of Washington, available for free online at http://fmrc.pulmcc.washington.edu/documents/fmrcman99.pdf. Quantification of isolated spheres was performed by fluorescence emission detection using black 96-well flat bottomed plates in a Synergy Mx Microplate Reader. We applied an internal standard to each sample using fluorescent microspheres of a different color to normalize for differences in microsphere recovery. The calculation of absolute CBF was performed as previously described previously⁹.

**Intracerebral infarct volume determination**
We measured cerebral infarct size by staining with 2,3,5-triphenyltetrazolium chloride (TTC; 0.05%) for 30 minutes at 37 °C as our group has detailed previously⁴,⁷. Brain sections were scanned on a flatbed scanner (Canon) and the volume of healthy (stained) tissue from the contralateral hemisphere was compared to the volume of infarcted (unstained) tissue from the hemisphere ipsilateral to the ET-1 MCAO using ImageJ software (NIH). We assessed the infarct volume using two 2mm coronal sections from 1 to 5mm rostral to bregma which contain the stroke core and penumbra. Infarct volume analyses were performed by a blinded investigator.

**Chemicals**
Endothelin-1 (ET-1) was purchased from American Peptide Company, Inc. (Sunnyvale, CA, USA). A-779 was from Bachem Bioscience (Torrance, CA, USA), and diminazene aceturate was from Sigma-Aldrich (St Louis, MO, USA) and Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mouse anti-NeuN was from Millipore (Bedford, MA, USA), and rabbit anti-Ang-(1-7) Mas receptor antibody was from Alomone Labs (Jerusalem, Israel). Alexafluor donkey anti-rabbit 594 and anti-mouse 488 were from Molecular Probes [Invitrogen] (Carlsbad, CA, USA). Vectashield mounting medium with DAPI was from Vector Laboratories (Burlingame, CA, USA). Unless otherwise noted, all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA).

**Inclusion and exclusion criteria**
Of the rats that underwent MCAO, five died shortly after stroke induction. Additionally, a small number were excluded: three exhibited atypical contralateral or bilateral strokes, confirmed by post-mortem staining; two had malfunctions of the guide cannula during MCAO; five had no signs of stroke on infarct volume analysis; and two exhibited abnormal CBF recordings.

**Randomization and allocation concealment**
Animals were identified by an assigned number and randomized using the randomize function in Microsoft Excel. Neurological assessments and analyses of all samples were performed by investigators blinded to group allocation.
Data analyses
Data are expressed as means ± SEM. Sample size determination was based on the primary outcome as specified in each experimental protocol and assumed a standard deviation of 15%, an effect size of 15%, power of 0.8, and alpha < 0.05. Statistical significance was evaluated with the use of the Kruskal-Wallis test, Mann Whitney test, one-way ANOVA for groups of equal variances, or student’s T-test. Differences were considered significant at p<0.05.

References

Figure S1: Effects of ACE2 activation on blood pressure and baseline cerebral blood flow (CBF). (A) Systolic blood pressure was not altered 30 minutes following an intraperitoneal injection of vehicle (n = 6) or diminazene at a dose of 7.5 mg/kg (n = 6). (B) Averaged laser Doppler flow recordings from anesthetized rats baseline conditions that received a single intraperitoneal injection of vehicle (H2O, n = 4) or diminazene (7.5 mg/kg, n = 4) at beginning of recording period (black arrow). (DIZE = diminazene; IP = intraperitoneal)