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

Douglas M. Bennion[1], **Emily Haltigan**[1], **Alexander J. Irwin**[1], **Lauren L. Donnangelo**[1], **Robert W. Regenhardt**[1], **David Pioquinto**[1], **Daniel L. Purich**[2], and **Colin Sumners**[1]

[1]Department of Physiology and Functional Genomics & McKnight Brain Institute, University of Florida, Gainesville, Florida, USA

[2]Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida, USA

Abstract

The angiotensin converting enzyme 2/angiotensin-(1-7)/Mas axis represents a promising target for inducing stroke neuroprotection. Here, 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. Amongst rats that underwent experimental endothelin-1-induced ischemic stroke, angiotensin converting enzyme 2 activity in the cerebral cortex and striatum increased in the 24 hours after stroke. Serum angiotensin converting enzyme 2 activity was decreased within 4h post stroke, but rebounded to reach higher than baseline levels 3d post-stroke. Treatment following 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 following stroke suggest that it may be a favorable therapeutic target. Indeed, significant neuroprotection resulted from post-stroke 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.

Corresponding Author: Colin Sumners, Ph.D., Department of Physiology & Functional Genomics, University of Florida, 1600 SW Archer Road, PO Box 100274, Gainesville, FL 32610-0274; Tel:352-392-4485; Fax:352-294-0191; csumners@ufl.edu.

Conflict of Interest

None.

Keywords

Angiotensin Converting Enzyme 2; Angiotensin-(1-7); Mas; Stroke; Renin-angiotensin system; Neuroprotection; tumor necrosis factor-α-converting enzyme (TACE or ADAM17)

Introduction

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 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 (Ang 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 ischemic[5-11] and hemorrhagic stroke[12]. Stroke neuroprotection has been demonstrated in both young[5, 6] and aged[10] 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 co-administration with Mas antagonist A-779[5].

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 recently been reported that expression of this protective axis is altered following stroke in the rat cerebral cortex[14] and rostral ventrolateral medulla[15], although it is unknown whether ACE2 activity levels are affected or whether this is accompanied by changes in the deleterious angiotensin converting enzyme/angiotensin II/angiotensin II type 1 receptor (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 following stroke. Further, we assessed the hypothesis that *post-stroke systemic* administration of an ACE2 activator, diminazene, in rats would result in neuroprotection.

Methods

For the description of experimental procedures, refer to the Materials and Methods please see http://hyper.ahajournals.org to access 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* in the Methods, resulted in significantly increased ACE2 activity in the cerebral cortex ipsilateral to the stroke when compared to control activity levels from sham-operated rats at 4h, 12h, and 1d after ischemia (Fig. 1A), along with an increase at 12h in the ipsilateral striatum compared to both shams and contralateral striatum (Fig. 1B). At 12h, ACE2 activity in the contralateral cortical samples was also significantly increased compared to respective sham levels (Fig. 1A). ACE2 activity levels in the cerebral cortex and striatum had returned to sham levels by 3d (Fig. 1A&B). There was not a significant change in ACE2 mRNA levels in the ipsilateral cortex either 1d or 3d following ischemia (Fig. 1C). Stroke resulted in an initial minor decrease of ACE2 activity in rat serum measured at 4h post-stroke as compared to normalized pre-stroke levels, followed by a significant rebound increase three days post-stroke (Fig. 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. We therefore assessed the impact of stroke on mRNA levels of Mas and also of the neuroprotective AT2R, and did not find significant differences at 1d following MCAO (Fig. 2A). In addition to these protective arms of the RAS, components of the classical ACE/Ang II/AT1R pathway were evaluated. Compared to sham, there was an increase in ACE mRNA levels in the ipsilateral cortex, but no change in AT1R mRNA (Fig. 2A). As expected, we also observed increased mRNA levels of LCN2, a marker of astrocyte activation, and CD11b, a marker of activated microglia (Fig. 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 samples from the ipsilateral cortex, and found a significant increase of TACE mRNA but not activity at 1d post-stroke relative to shams (Fig. 2C&D). Interestingly, although TACE mRNA expression levels were not significantly different between sham and stroke groups at 3d, 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 1d post-stroke (data not shown).

To further characterize the stroke-induced changes in Mas expression in neurons and other brain cells, we employed immunohistochemical staining to label NeuN and Mas immunoreactive cells. Representative fluorescence micrographs (Fig. 3A) show NeuN and Mas staining within the stroke penumbra from sham and stroke rats 1d post-stroke. Semi-quantitative 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 to sham rat cerebral cortex (Fig. 3B&C).

Neuroprotective Effects of Post-stroke Administration of Peripheral Diminazene, an ACE2 Activator

The observed increase in ACE2 activity in the ipsilateral cerebral cortex following stroke represents a promising target for inducing stroke neuroprotection. We have previously reported that *pre-stroke* activation of central ACE2 using diminazene results in significant neuroprotection when administered by intracerebroventricular infusion prior to, during, and after endothelin-1 MCAO[5]. Here, we tested a more clinically relevant treatment protocol, as described in *Experiment 2* of the Methods, which employed post-stroke intraperitoneal injections of drug or vehicle at 4h, 1d, and 2d following MCAO, times that overlap with endogenous stroke-induced changes to ACE2/Ang-(1-7)/Mas axis (Figs. 1-3).

To identify the maximally efficacious dose of diminazene for post-stroke peripheral administration, we performed a set of dose-response experiments in rats. Stroke resulted in large infarct volumes among vehicle-treated versus sham-operated rats. Compared to vehicle-treated controls, infarct volume was significantly decreased by post-stroke intraperitoneal injections of diminazene at doses of 0.75, 2.5, and 7.5 mg/kg, but not 15 mg/kg (Fig. 4A). The accompanying stroke-induced neurological deficits were significantly attenuated by 7.5 mg/kg diminazene at 1d (Fig. 4B-C) but not at 3d (data not shown). The data presented in Fig. 4D demonstrate that post-stroke administration of diminazene (7.5 mg/kg, intraperitoneal) at 4h, 1d, and 2d resulted in significantly higher levels of serum ACE2 activity at 2d post-stroke with a similar though not significant increase at 3 days. Based on these collective results and the finding that intraperitoneal administration did not affect levels of baseline blood pressure or cerebral blood flow (CBF, Fig. S1), the 7.5 mg/kg dose was used for all subsequent experiments.

We assessed the Mas-dependency of the neuroprotection induced by post-stroke intraperitoneal diminazene injections by chronically co-administering 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 co-treated with post-stroke peripheral diminazene resulted in significantly larger infarct sizes compared to saline infusion (Fig. 4E).

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

Effect of Diminazene on CBF during Baseline and Stroke

Ang-(1-7) has vasodilatory actions[2] 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 ET-1-induced MCAO, not post-stroke, as described in

Experiments 3&4 in the Methods. Relative CBF was not increased by diminazine during stroke as compared to control as measured by laser Doppler flowmetry and up to 4h post-stroke (Fig. 5A). Also, there was not an observed increase in the baseline levels of absolute CBF, as assessed by the microsphere injection method, amongst rats chronically treated by intracerebroventricular infusion with diminazene (Fig. 5B) as compared to saline-infused rats.

Discussion

This study aimed to assess *endogenous* post-stroke changes within the RAS, with a focus on ACE2 activity, and to test the neuroprotective efficacy of post-stroke 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, while serum ACE2 activity levels were initially decreased, followed by rebound increases. At 1d after stroke, levels of immunoreactive Mas were significantly decreased in the ischemic penumbra, and mRNA levels of the of deleterious RAS component ACE were increased. Administration of post-stroke diminazene resulted in significant neuroprotection, as well as increased serum ACE2 activity. Lastly, 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[16]. While 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 48h after stroke in the ischemic cerebral cortex of rats was an important first look[14]. A second study of transient MCAO in rats that examined axis components in the rostral ventrolateral medulla (RVLM) showed slightly different findings in that region, with decreases in Ang-(1-7) and Mas at 1d following stroke[15]. In light of these findings, our results, which show an increase in ACE2 activity in the ischemic cortex (Fig. 1A) but a decrease in Mas immunoreactivity (Fig. 3C), are intriguing. The reductions in immunoreactive Mas observed in penumbral regions of the ischemic cortex at 1d after stroke agreed with findings from the RVLM[15] and may be due to an overall decrease in penumbral Mas^+ cortical neurons, as immunostaining of neuronal nuclear marker NeuN was decreased at this time point (Fig. 3B&C). Further work is needed to clarify the various changes 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 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 pre-stroke administration of activating compounds. Using a more clinically-relevant protocol, Chen and colleagues were the first to utilize a post-stroke 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[8]. As these cells were protective even in the absence of ACE2 priming, and since 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 4h 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 plasminogen activator to human stroke victims, as well as the endogenous stroke-induced changes to the RAS (Figs. 1-3). It is also relevant that *systemic* (Fig. 4A-C) as well as central[5] administration of diminazene demonstrated neuroprotective efficacy, as intravenous drug 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 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 subtypes[18], 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 following stroke (Fig. 1D). It is encouraging for the translational potential of the animal data that the changes following stroke in ACE2 activity in rat serum (Fig. 1D) show a similar pattern to changes we have observed in preliminary studies using human serum obtained from stroke patients (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 stroke patients.

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[5]. We are the first to show that post-stroke administration of this small molecule activator results in significant neuroprotection (Fig 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 following stroke revealed significant increases in serum ACE2 activity (Fig. 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 and/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 post-stroke injections of diminazene, which is further validated by

our data showing significantly worse infarct volume with co-administration of A-779 (Fig. 4D). The vasodilatory action of Ang-(1-7) was suggested to contribute to its neuroprotective effects in stroke[16], but our data do not indicate that diminazene increases regional CBF during endothelin-1 MCAO or absolute CBF during baseline conditions (Fig. 5A&B). 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. As 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 one day after stroke[23], and others have shown Ang II to upregulate ACE, AT1R, and TACE, and decrease ACE2 expression[24, 25], which might account for some of the changes that we have observed one day post-stroke (Fig. 1C, Fig. 2A&D). 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[26]. 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[27], presumably as a result of an increase in soluble-ACE2. Our findings that show significantly increased expression of TACE mRNA in the cortex (Fig. 2C) coinciding with early rebound increases in serum ACE2 activity starting at 1d after stroke (Fig. 1D) suggest that brain ACE2, once cleaved from the membrane, may move into to 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 as the levels of brain TACE activity are significantly attenuated by that time (Fig 2D). Other explanations for the observed post-stroke changes in serum ACE2 activity levels (Fig 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 following 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 ACE2[29] and of TACE[30], which could result in increased shedding of ACE2 from activated leukocytes. Further, levels of CD34+ cells in peripheral blood following stroke in humans may follow a similar pattern to that observed in serum ACE2 activity (Fig 2D) with an initial decrease followed by a rebound increase[31]. Taken together, these findings suggest that ACE2 shedding from brain, endothelial, and/or activated hematopoietic cells may contribute to changes in serum ACE2 activity following stroke. The physiologic relevance of changes in

serum ACE2 activity is not clear[2], but the evidence seems to indicate that it is closely linked with the activity of TACE as regulated by Ang II signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Perspectives

The characterization of ACE2-mediated protection in a variety of cardiovascular diseases[2], including hypertension[2] and stroke[7], 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 following 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 physiologic 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 the ACE2/Ang-(1-7)/Mas in stroke.

Novelty and Significance

1) What is new?

- This is the first study to characterize ACE2 activity following stroke.
- We tested non-invasive administration of an ACE2 activator beginning after stroke as a clinically-relevant approach.

2) 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.

3) Summary

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

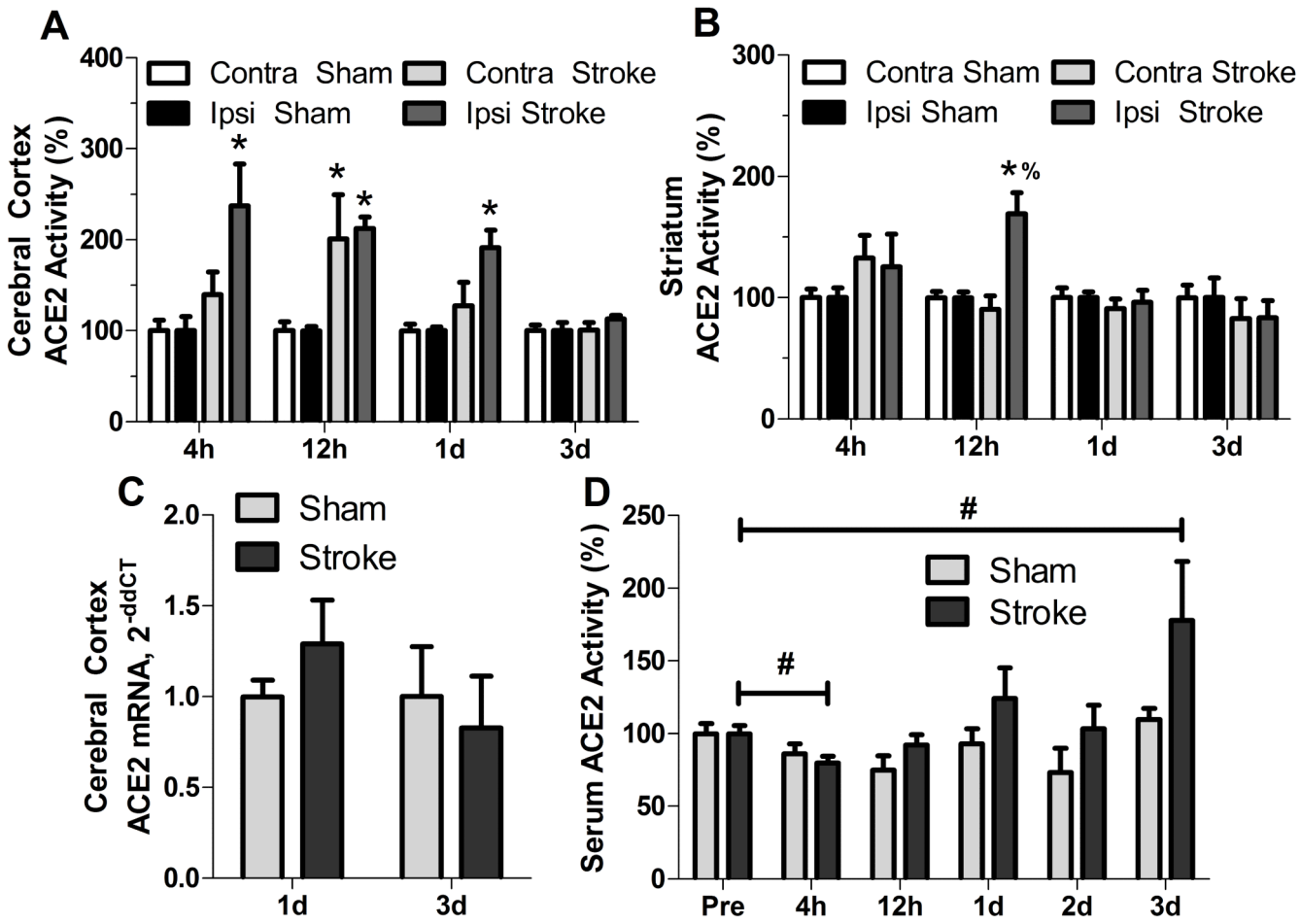

Figure 1. Activity of ACE2 in brain and serum is altered following stroke in rats

Rats underwent sham surgery or MCAO, followed by serial serum collections, euthanasia, and tissue harvesting at the time points indicated. Bar graphs are the average percent activity levels of ACE2 post MCAO, in tissue from (**A**) cerebral cortex or (**B**) striatum contralateral (Contra) and ipsilateral (Ipsi) to ischemia. Data are normalized to the corresponding sham values for each time point (A: n=4-6 rats at 4h, 12h, and 3d and n=12 rats at 1d time points; B: n=6-8 per time point). (**C**) mRNA levels of ACE2 at 1d and 3d post-stroke. Data are normalized to GAPDH and corresponding sham values (n=17 at 1d and n=4-6 at 3d). (**D**) Bar graphs are the average percent activity levels of ACE2 in serum at the indicated time points post MCAO. Serum was collected from the same sham- or stroked rat where possible. Data are normalized to pre-stroke values for either sham or stroke groups, respectively (n=19 sham and 31 stroke for pre-stroke, n=31 sham and 27 at 4h, n=6 sham and 8 stroke at 12h, n=19 sham and 21 stroke at 1d, n=7 sham and 10 stroke at 2d and 3d time points). Data are means ± SEM. ***** $p < 0.05$ vs. respective sham controls; % $p<0.05$ vs. contralateral stroke values; # $p<0.05$ vs. respective pre-stroke values (Kruskal-Wallis test with post-hoc Mann-Whitney test for panels A&B; Mann-Whitney test for other panels).

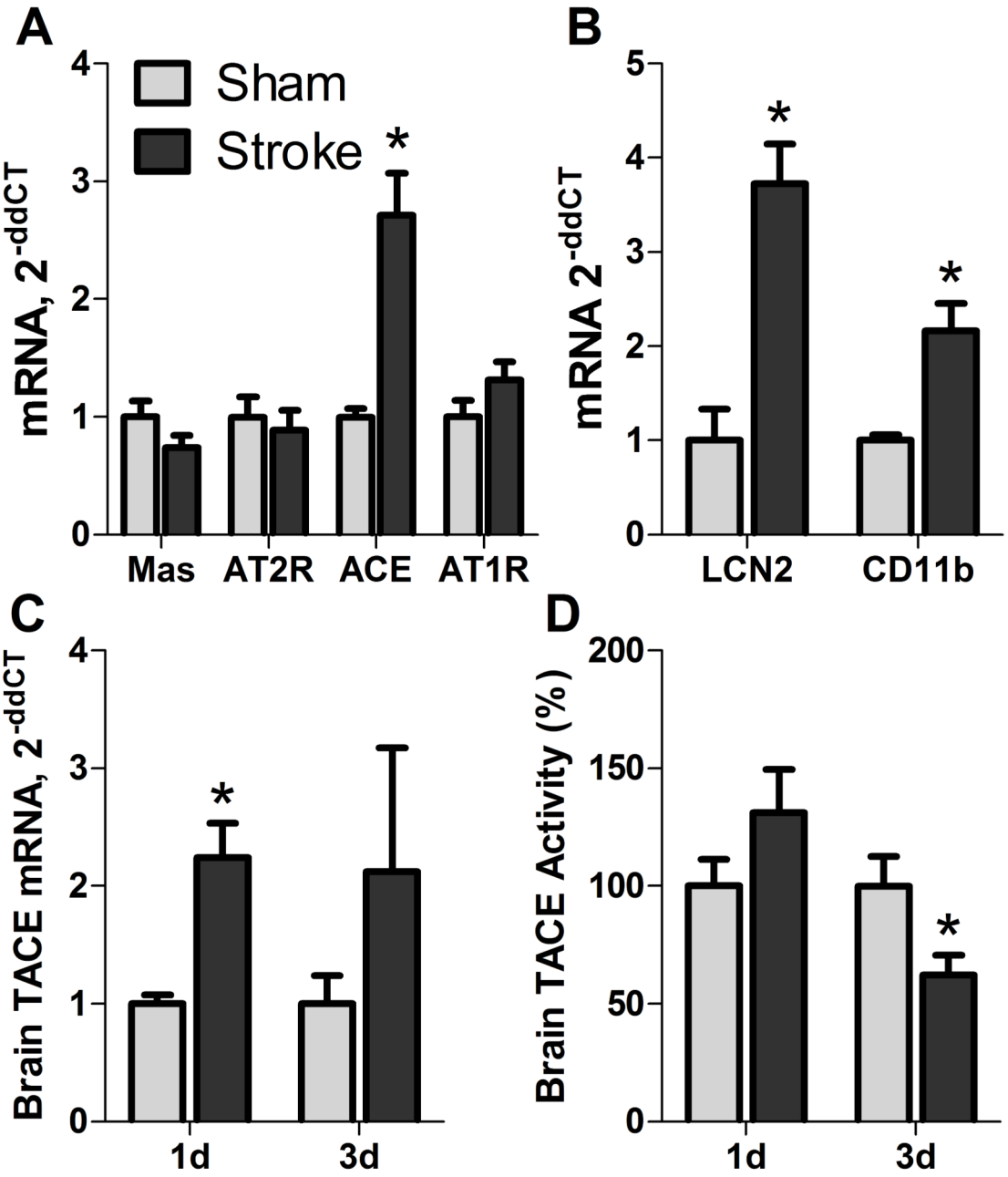

Figure 2. Alterations in RAS component mRNAs and TACE mRNA/activity following stroke in rats

Ipsilateral cerebral cortical samples were collected from sham and stroke rats at 1d post-MCAO. (**A**) mRNA levels of Mas, AT2R, ACE, and AT1R (normalized to GAPDH and corresponding sham values). (**B**) mRNA levels of LCN2 and CD11b. (**C**) Levels of TACE mRNA expression following stroke (normalized to GAPDH and corresponding sham values for each time point). (**D**) Levels of ipsilateral cerebral cortical TACE activity (normalized as percent of sham values). Data are means ± SEM from 10-12 rats per time point. *p<0.05 compared to respective sham control (Mann-Whitney test).

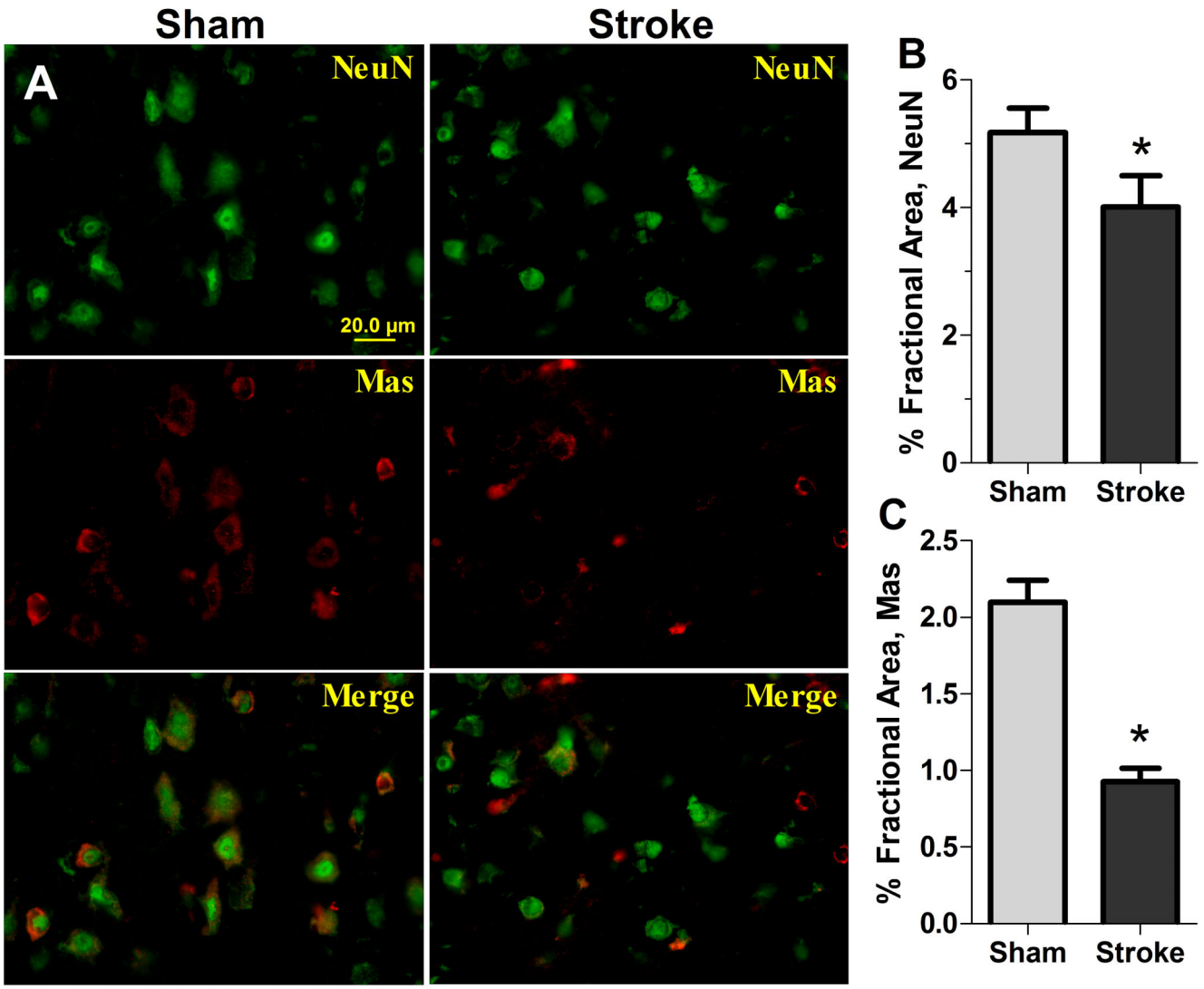

Figure 3. Immunoreactive Mas is decreased in the cerebral cortex following stroke in rats

Representative high power fluorescence micrographs from the ipsilateral cerebral cortex of sham (**A**, first column, n=6) and stroke (**A**, second column, n=4) brains harvested at 1d post-MCAO show immunoreactive NeuN (green, neuronal marker) and Mas (red), and NeuN plus Mas co-localization (merge). Semi-quantitative comparisons of average % fractional area of (**B**) NeuN and (**C**) Mas immunopositive staining are shown. Data are means ± SEM. *p<0.05 compared to sham control (Mann-Whitney test).

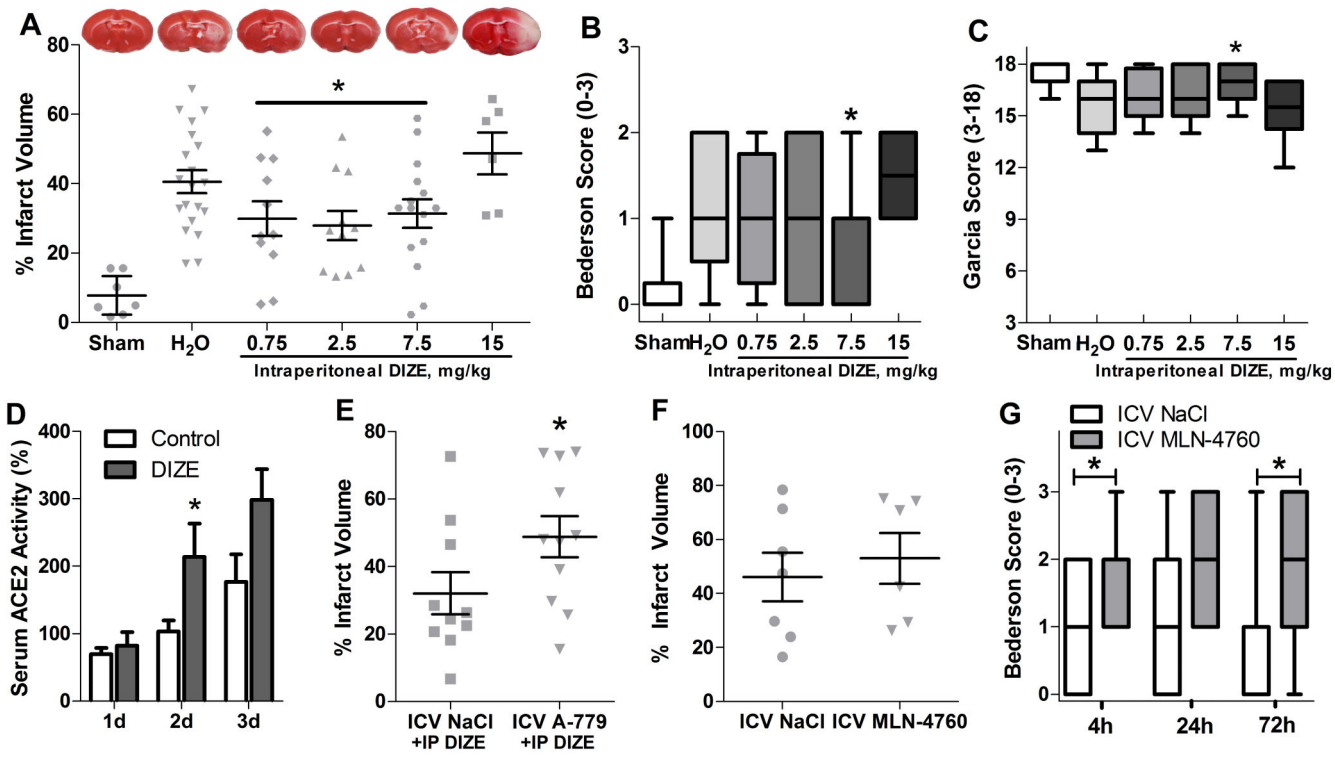

Figure 4. Post-stroke administration of Diminazene, an ACE2 activator, results in decreased infarct volume and improved neurological function

(**A**) Average infarct volumes at 3d post-MCAO for groups of rats that received intraperitoneal injections after stroke of vehicle (H_2O, n=20) or different doses of diminazene (0.75 mg/kg: n=11; 2.5 mg/kg: n=11; 7.5 mg/kg: n=5; 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 1d after stroke using the Bederson (**B**) and Garcia (**C**) scales. (**D**) Levels of serum ACE2 activity in serum samples at 1d, 2d, and 3d after stroke from control-treated (n=10) or diminazene-treated (7.5 mg/kg, n=4) stroked rats. (**E**) Average infarct volumes from diminazene-treated rats co-treated with vehicle (NaCl, n=10) or Mas antagonist A-779 (n=11) by continuous intracerebroventricular infusion. (**F**) Average infarct volumes from rats given ACE2 inhibitor MLN-4760 by intracerebroventricular infusion (100μmol/L infused at a rate of 0.5μL/h, n=6) for five days before and three days after ET-1 MCAO as compared to NaCl infusion (n=7). (**G**) Neurological function was assessed at 4h and 3d post-stroke from MLN-4760 or NaCl infused rats. Data are means ± SEM. *p<0.05 compared to respective controls. (one-way ANOVA with post-hoc student's T test for panel A-C, Mann-Whitney test for panels D-G; DIZE=diminazene; ICV=intracerebroventricular; IP=intraperitoneal).

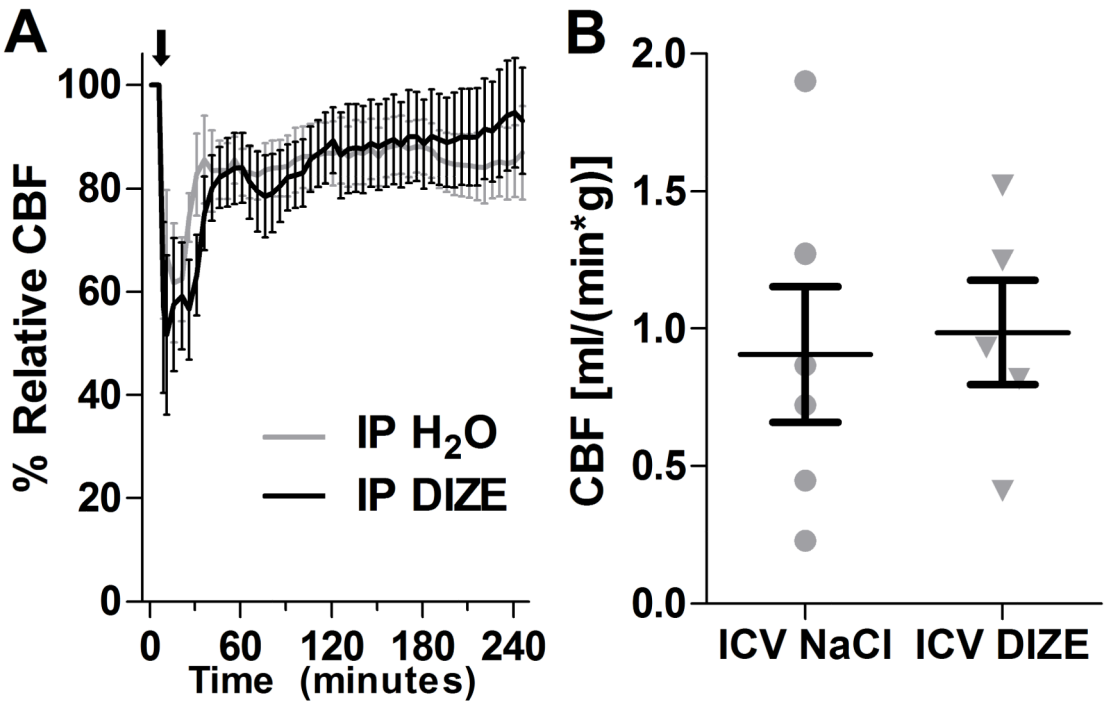

Figure 5. Neuroprotective doses of Diminazene administered systemically or centrally do not increase CBF

(**A**) Averaged laser Doppler flow recordings from rats undergoing endothelin-1 MCAO that received a single intraperitoneal injection of vehicle (H_2O, n=5) or diminazene (7.5 mg/kg, n=5) thirty minutes prior to stroke induction; black arrow indicates MCAO. (**B**) Levels of relative CBF during baseline conditions as measured by injection of fluorescent microspheres following 5 days of intracerebroventricular infusion of vehicle (0.9% NaCl, n=6) or diminazene (19.4mmol/L infused at a rate of 0.5μl/h, n=5; DIZE=diminazene; ICV=intracerebroventricular; IP=intraperitoneal).