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 Guidelines¹ 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 previously²⁻⁵. 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 hyperemia⁶; 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 comparison³,⁷. 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 lipcalin-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. 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 ZnCl$_2$) or TACE buffer (100mmol/L NaCl, 50mmol/L Tris HCl, ph 7.5, 100μmol/L ZnCl$_2$, 10mmol/L CaCl$_2$) 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 previously 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 previously. 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 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, 2x10^5 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)