Published in final edited form as:

Neuropharmacology. 2013 August ; 0: 154–163. doi:10.1016/j.neuropharm.2013.03.025.

Anti-inflammatory effects of angiotensin-(1-7) in ischemic stroke

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Abstract

Previously we demonstrated that central administration of angiotensin-(1-7) [Ang-(1-7)] into rats elicits significant cerebroprotection against ischemic stroke elicited by endothelin-1 induced middle cerebral artery occlusion. Ang-(1-7), acting via its receptor Mas, reduced cerebral infarct size, and rats exhibited improved performance on neurological exams. These beneficial actions of Ang-(1-7) were not due to inhibition of the effects of endothelin-1 on cerebral vasoconstriction or effects on cerebral blood flow, and so we considered other potential mechanisms. Here we investigated the possibility that the Ang-(1-7)-induced cerebroprotection involves an anti-inflammatory effect, since stroke-induced cerebral damage includes an excessive intracerebral inflammatory response. Our quantitative RT-PCR analyses revealed that central Ang-(1-7) treatment attenuates the increased expression of mRNAs for inducible nitric oxide synthase (iNOS), several pro-inflammatory cytokines and cluster of differentiation molecule 11b (microglial marker) within the cerebral cortex following endothelin-1 induced stroke. Western blotting confirmed similar changes in iNOS protein expression in the cerebral cortex. In support of these observations, immunostaining revealed the presence of immunoreactive Mas on activated microglia within the cerebral cortical infarct zone, and in vitro experiments demonstrated that lipopolysaccharide-induced increases in nitric oxide production in glial cultures are attenuated by Ang-(1-7) acting via Mas. Collectively these findings demonstrate an anti-inflammatory action of Ang-(1-7) in the brain, and suggest that the cerebroprotective action of this peptide in ischemic stroke may involve effects on nitric oxide generation by microglia.

1. Introduction

Stroke is the fourth leading cause of death in the United States and a major cause of serious, long-term disability (Roger et al., 2012). While there have been many efforts to develop therapeutic approaches for stroke, very little progress has been made to counteract stroke damage and limit long-term disability. Mounting evidence indicates that the renin-angiotensin system (RAS) is a potential therapeutic target for ischemic stroke, as over activation of the angiotensin converting enzyme/angiotensin II/angiotensin II type 1 receptor (ACE/Ang II/AT1R) arm of the RAS is highly involved in the processes that induce cerebral damage following ischemia. Specifically, numerous studies in animal models of

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experimental stroke have shown that ACE inhibitors and AT1R blockers (ARBs) decrease cortical/subcortical infarct size and the ensuing neurological deficits in animal models of stroke (Groth et al., 2003; Thone-Reineke et al., 2006). Importantly, a number of human clinical trials have also indicated that ACE inhibitors and ARBs can reduce cardiovascular risk and prevent stroke (Dahlof et al., 2002; Papademetriou et al., 2004; Reboldi et al., 2008).

While Ang II acting via AT1R activation is well known to exert deleterious actions in stroke and other cardiovascular diseases, there is accumulating evidence that the more recently discovered angiotensin converting enzyme 2/angiotensin-(1-7)/Mas (ACE2/Ang-(1-7)/Mas) axis of the RAS exerts beneficial actions in several cardiovascular diseases (Santos et al., 2008; Ferreira et al., 2010). Activating this protective arm of the RAS appears to have potential for treating hypertension, hypertension related pathology, pulmonary hypertension, myocardial infarction, and heart failure based on its ability to counteract the ACE/Ang II/AT1R axis (Castro-Chaves et al., 2010). In the brain, Ang-(1-7) is primarily generated by the action of ACE2 on Ang II, and its effects are mediated by its receptor, Mas (Santos et al., 2003). In recent studies, we demonstrated that the intracerebral damage and neurological deficits elicited by endothelin-1 (ET-1)-induced middle cerebral artery occlusion (MCAO), a model of ischemic stroke, are significantly reduced by intracerebroventricular (ICV) administration of either exogenous Ang-(1-7) or an activator of ACE2, prior to and during the stroke period (Mecca et al., 2011). These beneficial actions of Ang-(1-7) were not due to inhibition of the effects of ET-1 on cerebral vasoconstriction or effects on cerebral blood flow.

The aim of the present study was to investigate the mechanism of this Ang-(1-7) induced cerebroprotection, as understanding these processes would further support the rationale for activating the ACE2/Ang-(1-7)/Mas axis as a potential stroke therapy. Since stroke-induced cerebral damage includes an excessive intracerebral pro-inflammatory response leading to neuronal death (Jin et al., 2010; Iadecola and Anrather, 2011; Lamberts et al., 2012), in the current study we investigated whether the cerebroprotective actions of Ang-(1-7) in ischemic stroke are associated with anti-inflammatory actions of this peptide.

2. Materials and Methods

2.1 Animals and Ethical approval

For the experiments described here, we used adult male Sprague Dawley (SD) rats (250–275 g) or FVB mice (25–30 g) purchased from Charles River Farms (Wilmington, MA, USA). In addition, Sprague Dawley pups (derived from in-house breeding colony) were used to generate the cell cultures. Brains from FVB/N-*Mas*-deficient (*Mas*^{-/-}) mice were obtained from Dr. Michael Bader (Max Delbrück Center for Molecular Medicine, Berlin, Germany) and Dr. Robson Santos (Federal University of Minas Gerais, Belo Horizonte, Brazil). The University of Florida Institutional Animal Care and Use Committee approved all experimental procedures. In addition, these studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Academy of Sciences (eighth ed., 2011). Animals had *ad libitum* access to water and standard rat chow and were housed in a well-ventilated, specific pathogen-free, temperature-controlled environment (24 ± 1°C; 12 h-12 h light-dark cycle).

2.2 Anesthesia, Analgesia and Euthanasia

For surgical procedures, anesthesia was induced using 100% O₂/4% isoflurane, and was maintained throughout the surgeries by the administration of 100% O₂/2% isoflurane. During the surgeries/procedures, the level of anesthesia was monitored by checking the eye

blink reflex and a reaction to paw pinch, and was adjusted if necessary. Buprenorphine (0.05 mg/kg, s.c., Hospira Inc., Lake Forest, IL, USA) was administered to rats immediately following the survival surgeries. Animals were euthanized by placing them under deep anesthesia with 100% O₂/5% isoflurane, followed by decapitation.

2.3 Implantation of intracranial cannulas and osmotic pumps

Rats underwent two intracranial surgeries as detailed previously (Mecca et al., 2011). First, implantation of a stainless steel guide cannula (1.6 mm anterior and 5.2 mm lateral to bregma) for ET-1 or 0.9% saline injections adjacent to the MCA, and immediately following this placement of an ICV cannula (1.3 mm posterior and 1.5 mm lateral to bregma, 4.5 mm below the surface of the cranium) for infusion of Ang-(1-7) [1.1 nM; 0.5 μ L/h] or control artificial cerebrospinal fluid (aCSF; 0.5 μ L/h) via osmotic pumps. In one set of rats, the guide cannula was situated at 1.6 mm anterior and 3.2 mm lateral to bregma, in order to make the ET-1/0.9% saline injections at a site distant from the MCA (2 mm medial and 2 mm superior) and in both cases *not* produce an ischemic stroke.

2.4 ET-1 induced MCAO

Seven days after placement of intracranial cannulas, rats underwent MCAO via intracranial injection of ET-1 (3 μ L of 80 μ M solution; 1 μ L/min) through the guide cannula or sham MCAO (administration of 3 μ L 0.9% saline instead of ET-1) exactly as detailed previously (Mecca et al., 2011).

2.5 Experimental Protocols

Experiment 1—SD rats were infused ICV with Ang-(1-7) or aCSF for 7 days, then underwent ET-1 induced MCAO or sham MCAO as described above. ICV infusions of Ang-(1-7) or aCSF were continued post MCAO, and rats were euthanized either at 6 or 24 h post MCAO and their brains removed. Prior to being euthanized, each rat underwent neurological testing using forelimb flexion (Mecca et al., 2009) to ensure stroke had occurred in the ET-1 injected rats. For each brain, three coronal sections (2 mm each) were cut through the cerebrum ipsilateral to the ET-1 induced MCAO or sham MCAO, beginning caudally at approximately 6 mm rostral to the top of the ventral pons, and ending rostrally just before the end of the prefrontal cortex and olfactory bulbs. The most rostral section was used to assess intracerebral infarct size, as a further index of the effectiveness of ET-1 MCAO. The middle section was carefully dissected to separate the right cortex along the line of the corpus callosum; this right cortex sample was flash frozen in liquid nitrogen, stored at -80°C , and later used for mRNA analyses. The most caudal section was identically dissected, and the right cortex sample was flash frozen in liquid nitrogen, stored at -80°C , and processed as described below for protein analyses. The right side of the cerebral hemisphere is ipsilateral to the ET-1 MCAO. We focused on this cortical tissue for mRNA and protein expression analyses, as this sample of tissue was determined to reliably contain the ischemic core, penumbra, and some surrounding healthy tissue. Furthermore, including contralateral and/or more tissue were shown previously to dilute out changes in gene expression in the immediate region of stroke (Mecca et al., 2011).

Experiment 2—This protocol was identical to Experiment 1, with the following exceptions: ET-1/0.9% saline injections were made at a site distant from the MCA (2 mm medial and 2 mm superior) as to capture any direct effects of ET-1 on brain parenchyma without causing stroke. Rats were euthanized at 24 h only; analyses were restricted to infarct size and RNA, not protein.

Experiment 3—SD rat pups were euthanized, brains removed and the cerebral cortices were dissected and used for the preparation of mixed glial (microglia and astrocytes) cultures. These cultures were used to assess the direct effects of Ang-(1-7) on nitric oxide (NO) formation.

Experiment 4—SD rats (sham-stroked) were euthanized, brains removed and cut into 2mm sections as described above under Experiment 1. These sections were fresh frozen in Tissue-Tek® Optimal Cutting Temperature™ Compound (OCT; Sakura Finetech, Torrance, CA), stored at −20°C and used for immunostaining. FVB mice and FVB/*N-Mas*-deficient (*Mas*^{−/−}) mice were euthanized, brains removed, snap frozen (isopentane/dry ice), stored at −20°C and used for immunostaining.

Details of the post mortem experimental procedures used following the above experimental protocols are as follows.

2.6 Cerebral infarct measurement

Cerebral infarct volume was assessed as described previously (Mecca et al., 2011) by staining brain sections with 0.05% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 min at 37°C. All cerebral infarct analyses were performed in a blinded fashion.

2.7 mRNA analyses

We focused on analyzing the levels of mRNAs for cytokines, chemokines and markers of inflammatory cells that are known to be associated with the pro-inflammatory process during stroke. Further, we assessed the level of mRNA for *Mas*, the receptor for Ang-(1-7). Levels of iNOS, endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), pentraxin-3 (Ptx3), interleukin-6 (IL6), interleukin 1α (IL1α), interleukin 1β (IL1β), tumor necrosis factor α (TNFα), cluster of differentiation molecule 11b (CD11b), glial fibrillary acidic protein (GFAP), myeloperoxidase (MPO), monocyte chemotactic protein-1 (MCP-1), chemokine receptor type 4 (CXCR4), chemokine (C-X-C motif) ligand 12 (CXCL12) and *Mas* mRNAs within the right (ipsilateral) cerebral cortex samples were analyzed by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) as detailed previously (Li et al., 2008). RNA was extracted from the samples using an RNeasy Plus Kit (Qiagen, Valencia, CA, USA), reverse transcribed with a high-capacity cDNA reverse transcription kit (Bio-Rad Laboratories, Hercules, CA), and then analyzed *via* qRT-PCR using an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Oligonucleotide primers and TaqMan probes were obtained from Applied Biosystems (Carlsbad, California). Data were normalized to 18S rRNA.

2.8 Protein analyses

Protein from right (ipsilateral) cerebral cortex samples was extracted using a lysis buffer composed of the following: distilled water, 10× RIPA (Cell Signaling, Danvers, MA), protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO), and phenylmethanesulfonylfluoride (Sigma). Tissue was homogenized with a syringe, first using an 18-gauge needle then a 26-gauge needle until dissolved. Samples were allowed to incubate on ice for 30 min with occasional vortexing and were then centrifuged at 4°C for 30 min at 16.1g. Supernatants were frozen at −20°C, until analysis of iNOS protein by Western blotting, as follows. Extracted protein was added to an equal volume of 2× Laemmli's buffer with β-mercaptoethanol (Bio-Rad, Hercules, CA). After heating for 5 min at 90°C, samples were loaded into a precast TGX Criterion gel (Bio-Rad). The gel was run at 200V using Tris Glycine SDS running buffer and Dual Color MW markers (Bio-Rad). Transfer buffer was prepared using Tris Base 25mM, Glycine 192mM, and methanol 20%. The protein was then transferred from the gel to a nitrocellulose membrane (Bio-Rad) at 80V for 1 hour. After

transfer, the membrane was rinsed with Tris buffered saline (TBS), then TBS with Tween (TBST), and placed on a shaker in TBST + milk for 1 hour of blocking. The membrane was then incubated with primary antibodies [rabbit anti-iNOS (1:333) and rabbit anti-cofilin (1:10,000; used as a loading control)] overnight at 4°C with slow agitation on a shaker. The following day the membrane was washed six times with TBST (5 min each) on a shaker with fast agitation. Then, the secondary antibody (goat anti-rabbit IgG horseradish peroxidase [HRP]; 1:2,000) was added to the membrane, which was then placed on a shaker for 1 h. The membrane was then washed with TBST six times (5 min each) on a shaker with fast agitation, exposed to Enhanced Chemiluminescence Reagent (Promega, Madison, WI), and developed. Densitometry was used to quantify the blots using the integrated density (ImageJ, NIH). Samples were normalized to the housekeeping protein cofilin.

2.9 Mixed glial cultures

Mixed glial cultures were grown from the cortex and striatum newborn SD rat pups as detailed previously (Kopnisky and Sumners, 2000). Cells were grown at a density of 1×10^5 cells/dish in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and were used after 7 days in vitro for nitrite analyses as described in Experiment 3. At this time, cultures consisted of both astrocytes and microglia (~50:50% ratio), but not neurons, as determined by immunostaining with anti-GFAP, Iba-1, and NeuN antibodies (data not shown).

2.10 Nitrite analyses

Formation of NO in mixed glial cultures was determined by measuring the accumulation of nitrite, a stable breakdown product of NO, in the culture medium. This was achieved using the Greiss reaction, exactly as detailed previously (Kopnisky and Sumners, 2000).

2.11 Mas Immunohistochemistry

Mas immunoreactivity, and its co-localization with neurons, microglia, astroglia, endothelial cells and neutrophils in the cerebral cortex was assessed as follows.

Rats—Sections (at 5µm) were cut from the 2 mm coronal rat brain slices as described in Experiment 4 and air-dried at room temperature overnight. OCT was removed in a wash of $1 \times$ TBS for 5 minutes. Slides were drained and wiped, then blocked for 1 hour in 2% horse serum diluted in $1 \times$ TBS. Blocking buffer was replaced with antibody cocktails (rabbit anti-Mas [1:400] combined with either mouse anti-NeuN [Neuron specific protein; 1:100], anti-GFAP [glial fibrillary acidic protein, astrocyte marker; 1:100], anti-OX-42 [CD11b, microglial marker; 1:100], anti-RECA [rat endothelial cell antigen; 1:100] or anti-MPO [myeloperoxidase, neutrophil marker; 1:50] diluted in antibody diluent (Invitrogen, Carlsbad, CA), and incubated overnight at 4°C. Following two 5 min washes in TBS, Alexafluor donkey anti-rabbit 594 and donkey anti-mouse 488 were added to the slides, both at 1:500. After a 45 minute room temperature incubation, slides were again double washed in TBS. Sections were then post fixed for 5 minutes in 10% neutral-buffered formalin and washed twice before mounting in DAPI vectashield (Vector Labs, Burlingame, CA). Separate consecutive sections were treated as above except that the rabbit anti-Mas primary antibody was omitted in order to determine if this negated the observed Mas immunostaining.

Mice—Sections (20 µm) were cut from mouse brains (snap frozen using isopentane/dry ice), and used for immunostaining of Mas in the cerebral cortex as described above.

2.12 Peptides and Antibodies

Ang-(1-7) and A779 were purchased from Bachem Bioscience (Torrance, CA, USA). ET-1 was from American Peptide Company, Inc (Sunnyvale, CA, USA). Rabbit anti-Ang-(1-7) Mas receptor antibody was from Alomone Labs (Jerusalem, Israel). Mouse anti-rat RECA-1 was from AbD Serotec (Raleigh, NC). Mouse anti-MPO, mouse anti-GFAP, rabbit anti-iNOS (ab15323), rabbit anti-Cofilin (ab11062), and goat anti-rabbit IgG HRP (ab6721) antibodies were from Abcam (Cambridge, MA). Mouse anti-OX-42 antibody was from BD Biosciences (San Jose, CA). Mouse anti-NeuN was from Millipore (Bedford, MA). Rabbit anti-Iba-1 (01919741) was from Wako (Richmond, VA). Alexafluor donkey anti-rabbit 594 and anti-mouse 488 were from Molecular Probes [Invitrogen] (Carlsbad, CA, USA). Vectashield mounting medium with DAPI (4', 6-diamidino-2-phenylindole) was from Vector Labs (Burlingame, CA, USA). All other reagents/chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma (St. Louis, MO), or suppliers were noted in the previous sections.

2.13 Data analysis

Data are expressed as means $\pm$ SEM. Statistical significance was evaluated, as specified in the figure legends, with the use of a one-way ANOVA, Newman-Keuls test, Dunn's test, or t-test as appropriate. Differences were considered significant at $p < 0.05$.

3. Results

3.1 Ang-(1-7) reduces intracerebral infarct size 24 h after ET-1-induced MCAO

Previously we demonstrated that ICV treatment with Ang-(1-7) prior to and during induction of ischemic stroke via ET-1-induced MCAO (Mecca et al., 2011) decreased the size of the intracerebral infarct and improved performance on several neurological exams, when measured 72 h after MCAO. Furthermore, in the same study we demonstrated that Ang-(1-7) did not alter blood pressure, percent change in MCA branch vessel diameter or percent change in cerebral blood flow (Mecca et al., 2011). Using the protocol described in Experiment 1, we demonstrated that there was a visible intracerebral infarct at 6 h post ET-1 MCAO, an effect that was not discernibly altered by ICV Ang-(1-7) (1.1 nM; 0.5 μ L/h; data not shown). However, there was a significant beneficial effect of ICV administered Ang-(1-7) on intracerebral infarct size measured at 24 h (Figure 1) post MCAO, similar in magnitude to that obtained 72 h post MCAO (Mecca et al., 2011).

3.2 Effects of Ang-(1-7) on the expression of nitric oxide synthase (NOS) isozymes, pro-inflammatory cytokines, and inflammatory cell markers in the ipsilateral cerebral cortex following ET-1-induced MCAO

It is well known that the expression of many genes, including those for pro-inflammatory cytokines, chemokines and some NOS isozymes, is increased in the cerebral cortical infarct zone following ischemic stroke and contributes to neurotoxicity (Iadecola et al., 1997). We used the protocol described in Experiment 1 to test whether ICV administration of Ang-(1-7) would blunt the expression of genes for pro-inflammatory markers in the ipsilateral cerebral cortex, and thus represent a potential mechanism for the cerebroprotective actions of this peptide. Specifically, we were interested in determining the relative levels of mRNAs for the NOS isozymes, pro-inflammatory cytokines, chemokines and their receptors, and of inflammatory cell markers (MPO for neutrophils, CD11b for monocytes/microglia, and GFAP for astrocytes) under each treatment condition as a function of time following stroke. At 6 h post MCAO where the intracerebral infarct is only minor, levels of eNOS, IL1 α , IL1 β , CXCR4, and MPO mRNAs were all significantly increased, whereas levels of iNOS, nNOS, CXCL12, CD11b (Figure 2) and IL6 (not shown) were unaltered. While Ang-(1-7)

was largely ineffective at altering the expression of genes for the pro-inflammatory markers tested, it significantly decreased the levels of CXCL12 mRNA 6 h after MCAO, and also produced non-significant reductions in the post-MCAO expression of IL1 α and MPO (Figure 2).

In contrast to the results at 6 h post MCAO, there were much larger increases in the levels of genes for many pro-inflammatory markers at 24 h post MCAO. Specifically, iNOS, eNOS, Ptx3, IL1 α , IL1 β , IL6, TNF α , CXCR4, MCP-1, CD11b, and GFAP mRNAs were increased by factors of 300, 7, 100, 10, 10, 80, 10, 40, 1200, 12 and 5 respectively, when compared with rats that underwent sham MCAO (Figures 3 and 4), whereas nNOS, CXCL12, and MPO mRNA levels were unaffected. Of the genes that were increased in stroke, the expression of iNOS, IL1 α , IL6, CXCR4, and CD11b were all significantly blunted by ICV Ang-(1-7) administration. IL1 β , TNF α , MCP-1, and GFAP mRNAs all tended to decrease with Ang-(1-7) treatment, but the effects were not-statistically significant (Figures 3 and 4). Ang-(1-7) alone had no effects on gene expression in sham animals that did not undergo MCAO (Figures 2–4). To control for ET-1 effects on gene expression that were independent of stroke, we injected either ET-1 (3 μ L of 80 μ M; n = 3 rats) or 0.9% saline (n = 3 rats) at a site 2 mm medial and 2 mm superior to the normal coordinates used for induction of MCAO, as described in Experiment 2. Injections of ET-1 at this site failed to induce behavioral deficits or produce a cerebral infarct, according to TTC staining of brain sections from these rats. In addition, injection of ET-1 at this site did not alter the expression of several representative genes (iNOS, IL1 α , CD11b) at 24 h post MCAO, when compared with the saline-injected rats (data not shown). Since the inhibitory effects of Ang-(1-7) on iNOS mRNA levels were observed to be the most profound, we tested whether these changes were detectable at the protein level via Western blotting (Figure 5). Densitometry analysis showed that iNOS protein was increased by ~ 4-fold 24 h after MCAO. This increase was blunted by ICV Ang-(1-7) administration. As with the mRNA, Ang-(1-7) alone had no effect on the expression of iNOS protein in sham animals that did not undergo MCAO. These data were consistent with our earlier finding where levels of iNOS mRNA in the cerebral cortical infarct zone were reduced by Ang-(1-7) at 24 hours post ET-1 induced MCAO (Mecca et al., 2011), and also expanded our understanding of the potential mechanism of Ang-(1-7)'s cerebroprotective effects. It should also be noted that there was no effect of stroke or Ang-(1-7) treatment on Mas mRNA levels in the ipsilateral cerebral cortex (not shown).

3.3 Ang-(1-7) blunts lipopolysaccharide induction of NO in primary mixed glial cultures

Considering that macrophages/microglia within the cerebral cortex contain immunoreactive Mas, and that ICV infusion of Ang-(1-7) decreases the stroke-induced expression of certain pro-inflammatory cytokines, iNOS, and CD11b (suggesting reduced activation of microglia), we wanted to test the *direct* effects of Ang-(1-7) on NO production by glia. Since our hypothesis is that Ang-(1-7) exerts cerebroprotective actions by blunting inflammatory responses, we utilized a lipopolysaccharide (LPS)-induced in vitro model of inflammation. This model of inflammation was chosen to complement our in vivo stroke damage model because inflammatory processes are a major contributor to the damage due to stroke (Jin et al., 2010; Iadecola and Anrather, 2011; Lamberts et al., 2012), our in vivo results strongly suggest an anti-inflammatory action of Ang-(1-7) in stroke, and the LPS model of inflammation in mixed glia yields more reproducible, consistent results than other in vitro hypoxia models. This was achieved using primary glial cultures containing both microglia and astrocytes as described in the methods. Incubation of cultures with LPS (10ng/mL), which is well known to elicit pro-inflammatory cytokine production and secretion, produced a highly significant (3 fold) increase in NO production after 24 h, as demonstrated by accumulation of nitrite (a stable breakdown product of NO) in the growth media (Figure 6).

This increase in nitrite was significantly blunted by Ang-(1-7) treatment (0.1 nmol/mL), Figure 6. Note that three additions of Ang-(1-7) were used (12 h before LPS, immediately before LPS, and 12 h after LPS) as the peptide was shown to degrade in the growth media (data not shown). Furthermore, this effect of Ang-(1-7) was reversed by co-treatment with the Mas antagonist, A779 (1.0 nmol/mL), when administered at the same time points.

3.4 Cellular localization of Mas within rat cerebral cortex

Having demonstrated that Ang-(1-7) reduces the expression of various pro-inflammatory markers in the ipsilateral cortex during ischemic stroke, and reduces NO production in cultured glia following a pro-inflammatory challenge, we used immunostaining to investigate the cellular location of the Ang-(1-7) receptor, Mas in the brains of normal, healthy rats. The representative fluorescence micrograph shown in Figures 7A and D indicate that Mas immunoreactivity was widespread within normal rat cerebral cortex. Of note, Mas immunostaining was observed to exist in both non-nuclear and nuclear compartments. This presence of Mas in the nucleus is consistent with its original discovery as an oncogene (Young et al., 1986). Omission of the primary (Mas) antibody resulted in no specific Mas immunostaining (Figure 7G). While Mas immunoreactivity was primarily associated with neurons, as evidenced by co-localization with the neuron specific marker NeuN (Figures 7A–C), it was clear that Mas is also associated with non-NeuN positive cells. Further immunostaining experiments revealed the presence of Mas on macrophages/microglia, as illustrated by co-localization with immunoreactive CD11b (OX-42 immunostaining), a specific marker of these cells (Figures 7D–F). Weak Mas immunoreactivity was also observed within endothelial cells of small cerebral vessels and was abundant within the endothelium of large vessels such as the MCA (not shown), as demonstrated by co-localization with immunoreactive RECA. In contrast, Mas immunoreactivity was not associated with astroglia or neutrophils seen within blood vessels (not shown), based on a lack of co-localization with immunoreactive GFAP and MPO, respectively. Similar localization of Mas immunoreactivity was observed in the striatum of normal rats, i.e. presence of Mas on neurons, macrophages/microglia and endothelial cells, but not on astroglia nor on neutrophils (not shown). The specificity of the Mas antibody used here was further verified using brain sections from wild type and Mas-deficient (*Mas*^{−/−}) mice. Whereas wild type mice exhibited a similar pattern of Mas immunoreactivity in the cortex as observed in normal rats (Figure 7H), no specific Mas immunoreactivity was observed in the cortex of Mas deficient mice (Figure 7I). We also confirmed that each immunopositive area was a cell by co-localizing with DAPI nuclear staining (not shown).

4. Discussion

In this study we investigated potential mechanisms that underlie the cerebroprotective action of Ang-(1-7) in ischemic stroke. The major novel findings are: (i) Ang-(1-7) blunts the increases in the levels of iNOS, pro-inflammatory cytokines and CD11b (a marker of macrophage/microglial activation) that occur in the ipsilateral hemisphere following MCAO-induced ischemic stroke; (ii) Ang-(1-7) blunts LPS-induced increases in NO production in cultured glia (mixed culture of microglia and astrocytes); (iii) Qualitative immunostaining data which indicate that the Ang-(1-7) receptor Mas is present on microglia, one of the major resident immune cells in the brain, as well as on neurons. Considering these results, our findings suggest that the cerebroprotective action of Ang-(1-7) involves an anti-inflammatory effect, possibly via interruption of the excessive activation of microglia that occurs during stroke (Yenari et al., 2010; Thiel and Heiss, 2011). The current data are consistent with the results of a recent study, which indicates that the neuroprotective action of Ang-(1-7) in ischemic stroke involves Mas-mediated suppression of the inflammatory NFκB pathway (Jiang et al., 2012), and that Ang-(1-7) exerts anti-

inflammatory actions in macrophages (Souza et al., 2012). Despite our novel findings summarized above, the present study raises many important questions.

While it is clear that Mas is present in the cerebrum (Becker et al., 2007), one of the major issues concerns the precise cellular locus of action of Ang-(1-7) in reducing the damage due to stroke. Our studies point to involvement of microglia. Over-activation of microglia, increased production of pro-inflammatory cytokines and consequent increased iNOS expression are detrimental in stroke (Yenari et al., 2010; Thiel and Heiss, 2011). Furthermore, inhibiting iNOS and microglial activation has been shown to have a therapeutic effect in experimental models of stroke (Iadecola et al., 1997). Our data suggest that reduced activation of microglia and decreased production of excessive iNOS may contribute to the cerebroprotective action of Ang-(1-7) in ischemic stroke. Evidence for this is as follows. First, Mas is present on microglia so Ang-(1-7) may exert a direct action on this cell type to reduce activation and production of pro-inflammatory cytokines. This idea is supported by our data which indicate that Ang-(1-7) blunts the increases in CD11b and IL-1 α mRNAs during stroke, implying that fewer activated, pro-inflammatory cytokine secreting microglia are present in the ipsilateral cortex 24 h after MCAO. Further, the increases in iNOS mRNA and protein 24 h post MCAO are also blunted by Ang-(1-7). While inhibition of microglial activation, pro-inflammatory cytokines and iNOS production appears to be one locus of action of Ang-(1-7), the effects of this peptide do not appear to include regulation of Ptx3, which has been shown to modulate the phagocytic activity of microglia (Jeon et al., 2010). Our ongoing and future experiments include a detailed analysis of the effects of Ang-(1-7) on microglial activation and migration in situ.

While direct actions of Ang-(1-7) on microglia are a possibility, we cannot conclusively rule out other indirect mechanisms. For example, neurons within the cortex and striatum contain high levels of Mas immunoreactivity. Thus, it is possible that Ang-(1-7) has an effect on neurons, such as the observed reduction of MCP-1 production and secretion, which normally recruits monocytes/microglia, astroglia, or other inflammatory cells towards neurons (El Khoury et al., 2007; Schilling et al., 2009). Another possible site of the anti-inflammatory action of Ang-(1-7) is astroglia. These cells play an important role in cerebral innate immunity (Farina et al., 2007) and we demonstrated in the present study that GFAP mRNA is significantly increased following MCAO. In addition, a number of studies have demonstrated the presence of Mas on astroglia cultured from brain areas other than the cortex and striatum (Gallagher et al., 2006; Goo et al., 2010). However, we failed to observe Mas immunoreactivity associated with astroglia in the cerebral cortex and striatum (note that we have demonstrated Mas immunoreactivity on astroglia cultured from rat hypothalamus, using the same primary antibody; not shown). While the lack of Mas on astroglia in the cortex and striatum might suggest that these cells are not directly involved in the cerebroprotective action of Ang-(1-7), we cannot exclude the possibility that they have indirect involvement, especially since there was a strong trend to lower GFAP mRNA expression exerted by Ang-(1-7) in the current study.

A further possible site of action of Ang-(1-7) is endothelial cells. We observed the presence of Mas immunoreactivity within these cells, and it has been reported that Mas stimulation increases cerebral blood flow through increasing bradykinin secretion and eNOS activity 3 hours after stroke (Zhang et al., 2008). However, our previous measurements of cerebral blood flow with central Ang-(1-7) treatment during stroke do not support these alterations as a mechanism for cerebroprotection (Mecca et al., 2011). Further, our *in vitro* data with a cultured glial system support that Ang-(1-7) has a direct effect to decrease the NO production. Since the culture system lacked neurons or endothelial cells, it suggests that these cells are not required for the anti-inflammatory effect.

It is well known that induction of intracerebral pro-inflammatory mechanisms is an essential part of the processes that lead to neuron death following ischemic stroke. The changes in intracerebral gene expression elicited by Ang-(1-7) treatment are certainly consistent with the idea that Ang-(1-7) is exerting an anti-inflammatory effect. In particular, induction of iNOS mRNA and protein 24 h after MCAO was significantly blunted by Ang-(1-7), affirming our earlier study (Mecca et al., 2011). We focused much of our attention on iNOS, as there is substantial evidence in the literature to suggest it is highly involved in the pathology of stroke, by increasing NO levels (Iadecola et al., 1997). Interestingly, while we did not see an effect of Ang-(1-7) on eNOS mRNA expression at 6 or 24 h post MCAO, it is still possible that Ang-(1-7) may have increased eNOS activation, but not gene expression, at least in the early post stroke time period as described previously (Zhang et al., 2008). Further support for the anti-inflammatory effect of Ang-(1-7) is shown by the significant blunting of IL1 α and IL6 at 24 h post MCAO. There was also a strong trend for IL1 β and TNF α to be blunted by Ang-(1-7) at 24 h. Furthermore, MCAO-induced increases in these pro-inflammatory cytokines were only significantly blunted by Ang-(1-7) at 24 h post MCAO and not 6 h post MCAO. This suggests that there is not yet sufficient inflammation to reveal a clear and measurable effect of Ang-(1-7) 6 h after MCAO, even though there are small yet significant MCAO-induced increases in several pro-inflammatory cytokines at this time point. It is also worth mentioning that several of these trends may have reached statistical significance if we examined other time points, but we limited our examination to 6 and 24 h post MCAO.

In addition to the above pro-inflammatory cytokines, the chemokine CXCL12 and its receptor CXCR4 were examined. These molecules play important roles in many processes after ischemic stroke, including the inflammatory response, and the trafficking of bone marrow derived stem cells and neural progenitor cells to the site of injury (Magnon et al., 2011; Jaerve and Müller, 2012). While CXCR4 was significantly increased 6 h and 24 h post MCAO, CXCL12 showed only a trend to increase at these time points. Furthermore at 6 h, the increase in CXCR4 was significantly blunted by Ang-(1-7) treatment, as was the increase in CXCL12 at 24 h post stroke. CXCL12 has been shown to be upregulated in stroke in the ischemic penumbra (Lu et al., 2009). There are several differences between our study and that performed by Lu et al. It is possible that our samples contained less penumbral tissue relative to total tissue. While this might dilute the measurement, we preferred our method for reasons previously stated. Furthermore, it is possible that the ischemic penumbra in our study was simply had a different average volume due to the different model of ischemic stroke employed. In addition, it has been shown that the CXCL12/CXCR4 pathway stimulation can increase IL6 production in microglia, indicating a role for this pathway in activating microglia (Lu et al., 2009).

In conclusion, this study demonstrates that the beneficial effect of Ang-(1-7) during ischemic stroke is associated with an anti-inflammatory effect of this peptide. These results raise the theory that activation of the ACE2/Ang-(1-7)/Mas axis may prove to be a novel therapeutic target for other diseases where the underlying pathology involves inflammation, such as other cardiovascular diseases, type 2 diabetes, chronic kidney disease, and cancer (Manabe, 2011). There is accumulating evidence that the activation of this axis has anti-inflammatory effects in many tissues, including the heart (Giani et al., 2010), aortic valve (Peltonen et al., 2011) and kidneys (Giani et al., 2011). Since we demonstrate a central effect here, it also may prove to have therapeutic potential for other brain diseases that involve inflammation, such as Alzheimer's disease (Manabe, 2011).

Acknowledgments

This work was supported by grants from the American Heart Association Greater Southeast Affiliate (09GRNT2060421), the American Medical Association, and from the University of Florida Clinical and Translational Science Institute. Robert Regenhardt received predoctoral fellowship support from the University of Florida Multidisciplinary Training Program in Hypertension (T32 HL-083810). Adam Mecca is a NIH/NINDS, NRSA predoctoral fellow (F30 NS-060335). Support from University of Florida HHMI Science for Life (David Pioquinto) and University Scholars Program (Fiona Desland, David Pioquinto) undergraduate research fellowships. The authors thank Dr. Michael Bader (Max Delbrück Center for Molecular Medicine, Berlin, Germany) and Dr. Robson Santos (Federal University of Minas Gerais, Belo Horizonte, Brazil) for providing brains from Mas knockout mice.

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Highlights

- Ang-(1-7) reduces infarct & improves function 24 h after ischemic stroke in rat model
- Ang-(1-7) blunts stroke-induced increases in iNOS, cytokines, and CD11b/microglia
- Ang-(1-7) blunts LPS-induced increases in NO production in vitro, using cultured glia
- Ang-(1-7) receptor, Mas, is present on cortical microglia and neurons in vivo
- Suggests an anti-inflammatory role for Ang-(1-7)'s therapeutic effect in stroke

Figure 1. Intracerebral pretreatment with Ang-(1-7) reduces infarct size 24 h after ET-1-induced MCAO

Rats were pretreated via the ICV route with Ang-(1-7) (1.1 nM; 0.5 μ L/h; n=8) or aCSF (0.5 μ L/h; n=9) for 7 days prior to ET-1-induced MCAO as described in the methods. A control group of rats also underwent sham MCAO with 0.9% saline injection instead of ET-1 (n=5). Brains were removed for TTC staining 24 h after MCAO. Bar graphs are means + SEM showing the percentage infarcted gray matter in each treatment group. *P<0.05 compared to Sham/aCSF. †P<0.05 compared to ET-1/aCSF. Below are representative brain sections from each group showing infarcted (white) and non-infarcted (red) gray matter.

Figure 2. Gene expression in ipsilateral (right) cerebrocortical tissue 6 h after ET-1-induced MCAO

Rats underwent sham- or ET-1 induced MCAO in the absence or presence of seven days infusion of Ang-(1-7) (1.1 nM; 0.5 μ L/h) or aCSF (0.5 μ L/h) via the ICV route. At 6 h post MCAO rats were euthanized and expression of the indicated genes in the cerebral cortex ipsilateral to the stroke was assessed by qRT-PCR as detailed in the methods. Bar graphs are means + SEM. N=6-11/group. *P<0.05 compared to Sham/aCSF. †P<0.05 compared to ET-1/aCSF.

Figure 3. Gene expression of NOS isozymes and pro-inflammatory cytokines in ipsilateral (right) cerebrocortical tissue 24 h after ET-1-induced MCAO

Rats underwent sham- or ET-1 induced MCAO in the absence or presence of seven days infusion of Ang-(1-7) (1.1 nM; 0.5 μ L/h) or aCSF (0.5 μ L/h) via the ICV route. At 24 h post MCAO rats were euthanized and expression of the indicated genes in the cerebral cortex ipsilateral to the stroke was assessed by qRT-PCR as detailed in the Methods. Bar graphs are means + SEM. N=7–12/group. *P<0.05 compared to Sham/aCSF. †P<0.05 compared to ET-1/aCSF.

Figure 4. Gene expression of cell markers and migratory factors in ipsilateral (right) cerebrocortical tissue 24 h after ET-1-induced MCAO

Rats underwent sham- or ET-1 induced MCAO in the absence or presence of seven days infusion of Ang-(1-7) (1.1 nM; 0.5 μ L/h) or aCSF (0.5 μ L/h) via the ICV route. At 24 h post MCAO rats were euthanized and expression of the indicated genes in the cerebral cortex ipsilateral to the stroke was assessed by qRT-PCR as detailed in the Methods. Bar graphs are means + SEM. N=7–12/group. *P<0.05 compared to Sham/aCSF. †P<0.05 compared to ET-1/aCSF.

Figure 5. Levels of iNOS protein in ipsilateral (right) cerebrocortical tissue 24 h after ET-1-induced MCAO stroke or sham procedure

Rats underwent sham- or ET-1 induced MCAO in the absence or presence of seven days infusion of Ang-(1-7) (1.1 nM; 0.5 μ L/h) or aCSF (0.5 μ L/h) via the ICV route. At 24 h post MCAO rats were euthanized and expression of iNOS protein in the cerebral cortex ipsilateral to the stroke was assessed by Western blotting as detailed in the Methods. Bar graphs show levels of iNOS protein under each treatment condition, normalized against cofilin. Data are means + SEM. N=7–12/group. *P<0.05 compared to Sham/aCSF. †P<0.05 compared to ET-1/aCSF. Also shown are representative iNOS protein bands from each treatment, plus from a positive control (mixed cultured glial cells treated with LPS, 10 ng/mL, 24h).

Figure 6. Ang-(1–7) blunts LPS-induced increases in nitrite in the media of primary mixed glial cultures

Cells cultured as described in the Methods were treated as follows. Control solution (PBS); LPS (10 ng/mL); LPS + Ang-(1–7) (0.1 nmol/mL). LPS + Ang-(1–7) + A779 (1.0 nmol/mL). Note that Ang-(1–7) +/- A779 were administered 12 h before LPS, at the time of LPS treatment, and 12 h after LPS. Growth media were sampled 24 h after LPS or control treatment for analysis of nitrite (NO₂) via the Greiss reaction. Bar graphs are means + SEM showing the levels of NO₂ under each treatment condition. N=7 for control and N=10 for other treatment groups. *P<0.05 compared to control. †P<0.05 compared to LPS.

Figure 7. Cellular localization of immunoreactive Mas in cerebral cortex

Representative high power fluorescence micrographs taken at 40× magnification. Panels A, B and C show Mas immunoreactivity (red), NeuN immunoreactivity (green) and NeuN plus Mas co-localization, respectively, in normal rat cerebral cortex. Panels D, E and F show Mas immunoreactivity (red), OX-42 immunoreactivity (green) and OX-42 plus Mas co-localization, respectively, in normal rat cerebral cortex. Panel G shows no specific Mas immunoreactivity was observed when the Mas primary antibody was omitted. Panel H is Mas immunoreactivity in the cerebral cortex of a wild type (WT) mouse, and panel I shows no specific Mas immunostaining in the cortex of a Mas-deficient mouse. Images are representative of 3 animals per strain.