Interplay between brain stem angiotensins and monocyte chemoattractant protein-1 as a novel mechanism for pressor response after ischemic stroke

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A B S T R A C T

Pressor response after stroke commonly leads to early death or susceptibility to stroke recurrence, and detailed mechanisms are still lacking. We assessed the hypothesis that the renin–angiotensin system contributes to pressor response after stroke by differential modulation of the pro-inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) in the rostral ventrolateral medulla (RVLM), a key brain stem site that maintains blood pressure. We also investigated the beneficial effects of a novel renin inhibitor, aliskiren, against stroke-elicited pressor response. Experiments were performed in male adult Sprague–Dawley rats. Stroke induced by middle cerebral artery occlusion elicited significant pressor response, accompanied by activation of angiotensin II (Ang II)/type I receptor (AT1R) and AT2R signaling, depression of Ang-(1–7)/MasR and Ang IV/AT4R cascade, alongside augmentation of MCP-1/C–C chemokine receptor 2 (CCR2) signaling and neuroinflammation in the RVLM. Stroke-elicited pressor response was significantly blunted by antagonism of AT1R, AT2R or MCP-1/CCR2 signaling, and eliminated by applying Ang-(1–7) or Ang IV into the RVLM. Furthermore, stroke-activated MCP-1/CCR2 signaling was enhanced by AT1R and AT2R activation, and depressed by Ang-(1–7)/MasR and Ang IV/AT4R cascade. Aliskiren inhibited stroke-elicited pressor response via downregulating MCP-1/CCR2 activity and reduced neuroinflammation in the RVLM; these effects were potentiated by Ang-(1–7) or Ang IV. We conclude that whereas Ang II/AT1R or Ang II/AT2R signaling in the brain stem enhances, Ang-(1–7)/MasR or Ang IV/AT4R antagonizes pressor response after stroke by differential modulations of MCP-1 in the RVLM. Furthermore, combined administration of aliskiren and Ang-(1–7) or Ang IV into the brain stem provides more effective amelioration of stroke-induced pressor response.

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Abbreviations: ACE, angiotensin converting enzyme; aCSF, artificial cerebrospinal fluid; Ang II, angiotensin II; Ang IV, angiotensin IV; Ang-(1–7), angiotensin-(1–7); APN, aminopeptidase N; ARBs, angiotensin receptor blockers; AT1R, angiotensin II type I receptor; AT2R, angiotensin II type II receptor; AT4R, angiotensin II type IV receptor; ATRs, angiotensin receptors; BBB, blood–brain barrier; BP, blood pressure; CCL2, chemokine (C–C motif) ligand 2; CCR2, C–C chemokine receptor 2; CSF, cerebrospinal fluid; DBP, diastolic blood pressure; GFAP, glial fibrillary acidic protein; HR, heart rate; i.v., intravenous; ICF, intracellular free water; ICV, intracerebroventricular; ICU, intensive care unit; MasR, Mas receptor; MBBP, mean blood pressure; MCAO, middle cerebral artery occlusion; MCP-1, monocyte chemoattractant protein-1; mNSS, modified neurological severity score; MRI, magnetic resonance imaging; NeuN, neuron specific nuclear protein; RAS, renin–angiotensin system; RVLM, rostral ventrolateral medulla; SBP, systolic blood pressure; TTC, 2,3,5-triphenyltetrazolium chloride.

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Introduction

Stroke is a major global health problem because it is the second leading cause of death worldwide (Donnan et al., 2008). Pressor response after stroke is observed in 60–75% patients (Potter et al., 2005) and is a common complication that leads to poor outcome, including early death in hospital, neurological deficiency at discharge (Zhang et al., 2011) and susceptibility to recurrent stroke (Geegang et al., 2011). At present, the causes, effects and optimal management of pressor response immediately after stroke remain a hotly debated and sometimes controversial issue (Lattanzi et al., 2013; Sykora et al., 2010). Further delineation of the pathophysiological mechanisms of pressor response after stroke that leads to effective therapeutic management is therefore warranted.

The rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons in the brain stem are located, is responsible for maintaining sympathetic vasomotor tone and stable blood pressure; pharmacological or electrical activation of the RVLM elicits pressor response (Spyer, 1994). Clinically, vascular compression of the RVLM is identified in 26.8% of acute ischemic stroke patients who manifested greater blood pressure variability that is related to poorer prognosis (Aoki et al., 2011). Furthermore, rats in a middle cerebral artery occlusion (MCAO) stroke model (Marks et al., 2001; Mogi et al., 2006) exhibit disturbed cardiovascular responses because of altered neurotransmission in the ventrolateral medulla (Ally et al., 2002). MCAO also augmented Fos-like immunoreactivity in medullary neurons (Wu and Ling, 1998). It follows that the RVLM may participate in pressor response after stroke and is a suitable target for mechanistic delineation.

The condition of up to one-third of stroke patients worsens after hospital admission, and the management of pressor response after stroke is still controversial (Lattanzi et al., 2013; Sykora et al., 2010). Early systematic application of angiotensin receptor blockers (ARBs) reportedly prevents pressor response after stroke (Linders, 2007) and reduces severity (Lee et al., 2012). Other studies, however, indicate that careful blood pressure lowering treatment with ARBs does not improve the neurological outcomes of patients with stroke (Rhoney and Moser, 2011; Sandset et al., 2011). Whereas angiotensin II (Ang II), the principal effector molecule of the central renin–angiotensin system (RAS), induces tonic sympathoexcitatory and pressor response by acting on Ang II type 1 receptors (AT1R) in the RVLM (Chan et al., 2007, 2010), a decrease of AT1R in the RVLM mediates hypotension induced by lipopolysaccharide administration (Chan et al., 2003). Recent work (Jiang et al., 2012; Faure et al., 2006) further showed that angiotensin–(1–7) (Ang-(1–7)) and angiotensin IV (Ang IV), two metabolites when Ang II is degraded respectively by angiotensin converting enzyme (ACE) 2 or aminopeptidase N (APN) (McKinley et al., 2003), play a protective role during stroke by reducing the infarct volume and subsequent neurological deficits (Faure et al., 2006; Mecca et al., 2011). Considering the importance of the central RAS in blood pressure homeostasis, a systematic evaluation of the roles of Ang–(1–7) and Ang IV and their respective receptor subtypes, Mas receptor (MasR) and AT4R, in addition to Ang II and the classical AT1R and AT2R, in the RVLM in stroke-induced pressor response is therefore of interest.

Monocyte chemoattractant protein-1 (MCP-1), also named chemokine (C–C motif) ligand 2 (CCL2), is a pro-inflammatory chemokine that exhibits potent chemoattractant activity for monocyte/macrophage infiltration to the injured area, of which triggers an intense inflammatory reaction and contributes to worsen stroke brain injury indicated by increased infarct size and impaired neurological outcome (Iadecola and Alexander, 2001). Moreover, MCP-1 contributes to inflammatory reactions during stroke via an action on C–C chemokine receptor 2 (CCR2) (Losy and Zaremba, 2001; Jiang et al., 2008); clinical studies indicated that polymorphism of MCP-1 gene is associated with the susceptibility to stroke (Buraczynska et al., 2010). Elevation of MCP-1 is observed in cerebrospinal fluid (CSF) (Losy and Zaremba, 2001) or in circulation (Arakelyan et al., 2005) of stroke patients and in brain of rats after MCAO (Jiang et al., 2008). In addition, the level of MCP-1 mRNA and MCP-1 concentration in plasma is significantly higher in hypertensive patients (Sardo et al., 2008). Of note is that Ang II induces AT1R-mediated upregulation of MCP-1 or CCR-2, and secretion of MCP-1 in ventricular cardiomyocytes (Omura et al., 2004) or human U937 mononuclear cells (Ko et al., 2007). It follows that brain stem MCP-1 may underlie RAS-mediated pressor response after stroke.

Aliskiren, an orally active, non-peptide renin inhibitor, is an antihypertensive agent that blocks renin, the first and rate-limiting enzymatic step of angiotensin synthesis (Sawhney, 2010). Intracerebroventricular (i.c.v.) infusion of aliskiren markedly inhibits the increase in Ang II levels in rat CSF and blood pressure (BP) elicited by i.c.v. infusion of renin (Huang et al., 2012). Furthermore, pretreatment with systemic application of aliskiren reduces the expression of inflammatory marker genes in the cerebral ischemic core after MCAO (Schmerbach et al., 2010). Because of the controversy surrounding the efficacy of ARBs against pressor response after stroke, whether aliskiren-mediated reduction of inflammatory response presents itself as an alternative therapeutic strategy warrants further delineation.

Employing a MCAO stroke model, the present study was undertaken to assess the hypothesis that Ang II, Ang–(1–7) or Ang IV, and AT1R, AT2R, MasR or AT4R, contribute to pressor response after stroke by differential modulations of pro-inflammatory MCP-1 in the RVLM. We also investigated the relationship between these cellular mechanisms and the beneficial effects of aliskiren.

Materials and methods

All experimental procedures carried out in this study have been performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, approved by the Institutional Animal Care and Use Committee of the Kaohsiung Chang Gung Memorial Hospital, and were in compliance with the guidelines for care and handling of animals set forth by that committee.

Animals

Adult male Sprague–Dawley rats (285–337 g, n = 219) purchased from the Experimental Animal Center of the National Science Council, Taiwan, Republic of China were used. Animals were housed in groups of 2–3 in individually ventilated cages in an AAALAC International-accredited Center for Laboratory Animals, with free access to rat chow and water. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Blood pressure and heart rate measurement

Systolic and diastolic blood pressure (SBP and DBP), mean BP (MBP) and heart rate (HR) of conscious rats were measured between 0900 and 1200 h, using a non-invasive tail cuff plethysmography (Visitech Systems, Apex, NC, USA). Averages of 10 inflation/deflation cycles were conducted to obtain MBP.

MCAO stroke model

Transient MCAO was induced by intraluminal vascular occlusion as described previously (Marks et al., 2001; Mogi et al., 2006; Ally et al., 2002; Wu and Ling, 1998). Briefly, the left MCA in rats anesthetized with 2–3% isoflurane was effectively occluded by a silicon-coated 4-0- monofilament (Doccol, Sharon, MA, USA) for 120 min, followed by reperfusion. Successful establishment of experimental stroke was confirmed on day 1 after MCAO by magnetic resonance imaging (MRI) and staining with 2,3,5-triphenyltetrazolium chloride (TTC; Sigma). Specifically, the presence of edema was determined by high resolution T2 images obtained from a 9.4 T Animal MR scanner (Biospec 94/20, Bruker, Ettlingen, Germany). For TTC staining, rats deeply anesthetized with pentobarbital sodium were perfused intracardially with warm saline that contains...
Values are mean ± SEM. * P < 0.001 versus Sham control group in the post hoc Scheffé multiple-range test. bpm, beat per minute.

**Table 1**

<table>
<thead>
<tr>
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<th>Basal</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
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<tbody>
<tr>
<td><strong>MBP (mmHg)</strong></td>
<td>Sham: 127.22 ± 4.04</td>
<td>133.35 ± 4.24*</td>
<td>134.26 ± 3.96*</td>
<td>134.86 ± 5.97*</td>
</tr>
<tr>
<td></td>
<td>MCAO: 125.45 ± 1.67</td>
<td>161.51 ± 2.35*</td>
<td>144.74 ± 5.33*</td>
<td>134.06 ± 5.03*</td>
</tr>
<tr>
<td><strong>SBP (mmHg)</strong></td>
<td>Sham: 146.04 ± 3.79</td>
<td>147.57 ± 3.43*</td>
<td>147.12 ± 4.02*</td>
<td>147.34 ± 7.06*</td>
</tr>
<tr>
<td></td>
<td>MCAO: 145.15 ± 1.62</td>
<td>180.40 ± 2.13*</td>
<td>163.07 ± 3.29*</td>
<td>143.56 ± 5.01*</td>
</tr>
<tr>
<td><strong>DBP (mmHg)</strong></td>
<td>Sham: 114.41 ± 4.30</td>
<td>119.06 ± 5.25*</td>
<td>108.38 ± 6.07*</td>
<td>121.97 ± 6.62*</td>
</tr>
<tr>
<td></td>
<td>MCAO: 114.02 ± 1.86</td>
<td>151.96 ± 2.70*</td>
<td>135.71 ± 4.40*</td>
<td>124.57 ± 5.99*</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>Sham: 362.76 ± 2.52</td>
<td>365.55 ± 3.30*</td>
<td>373.48 ± 2.50*</td>
<td>380.30 ± 2.99*</td>
</tr>
<tr>
<td></td>
<td>MCAO: 368.24 ± 1.08</td>
<td>391.56 ± 2.18*</td>
<td>407.49 ± 3.54*</td>
<td>419.30 ± 4.96*</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>Sham: 244.75 ± 12.06</td>
<td>247.50 ± 9.58*</td>
<td>247.30 ± 4.21*</td>
<td>247.30 ± 4.21*</td>
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<tr>
<td><strong>mNSS</strong></td>
<td>Sham: 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MCAO: 10.80 ± 1.80*</td>
<td>9.25 ± 2.5*</td>
<td>6.0 ± 1.1*</td>
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</table>

**Evaluation of neurological functions after stroke**

Two technicians blinded to the experimental groups determined stroke-induced neurological deficits of the rats based on the modified neurological severity score (mNSS). The averaged scores graded independently on a scale of 0–14 (normal score, 0; maximal deficit score, 14) were used as our final results.

**Microinjection or systemic administration of test agents**

Microinjection bilaterally of test agents into the RVLM (stereotaxic coordinates: 4.5–5.5 mm posterior to lambda, 1.8–2.1 mm lateral to midline, and 8.1–8.4 mm below the dorsal surface of cerebellum), each at a volume of 50 nl, was carried out stereotaxically and consecutively via a glass micropipette connected to a 0.5-μl Hamilton (Reno, NV, USA) micropipette. Tests agents used (see Supplementary Table S1 for their pharmacological effects) included aliskiren (MedChemexpress, Shanghai, China), candesartan cilexetil (Carbosynth, Compton, Berkshire UK), PD123319 (Tocris, Plymouth, TX, USA), AT799 (American Peptide, Sunnyvale, CA, USA), antiserum against AT1R, AT2R, MasR or AT4R in RVLM (Santa Cruz Biotechnology) or c-Fos (Santa Cruz Biotechnology) and a mouse monoclonal antiserum against glial fibrillary acidic protein (GFAP). AT1R, AT2R, MasR, AT4R, c-Fos, and GFAP, were carried out on proteins extracted from the RVLM and detected by immunofluorescence staining coupled with laser scanning confocal microscopy (n = 3 per group).

**Collection of tissue samples**

We routinely collected tissue samples for biochemical evaluations one day after MCAO or in sham-control animals. Animals were first deeply anesthetized to receive transcardiac perfusion with warm saline, and RVLM tissues were immediately obtained by micropunch. In some experiments, RVLM tissue samples were collected on day 3 or 7 after MCAO following the same procedures.

**Determination of mRNA levels**

We used a RT2 profiler PCR array (SABiosciences™, Frederick, MD, USA) to determine global changes of 84 hypertension-related genes in the RVLM (see Supplementary Table S2 for detailed information). The mRNA levels of genes of interest and gadph control were further quantified by real-time PCR analysis (see Supplementary Table S3 for list of primers).

**Measurement of protein concentration**

The protein concentration in cell lysate from the RVLM was determined by commercial ELISA kits, coupled with spectrophotometry. Kits for angiotensinogen (Shanghai BlueGene Biotech, Shanghai, China), Ang II (Biovendor, Brno, Czech Republic), Ang-I(1–7) (Shanghai BlueGene Biotech), APN (Cusabio, Wuhan, Hubei, China), MCP-1 (Biosciences, San Diego, CA, USA), and CCR2 (Cusabio) were used.

**Western blot analysis**

Western blot analysis for AT1R, AT2R, MasR, AT4R, c-Fos, and GFAP, was carried out on proteins extracted from the RVLM and detected by immunofluorescence staining coupled with laser scanning confocal microscopy (n = 3 per group).
against a specific neuron marker, neuron-specific nuclear protein (NeuN; EMD Millipore), GFAP (Life Technologies) or OX-42 (AbD Serotec). The secondary antisera (Molecular Probes, Eugene, OR, USA) used included a goat anti-rabbit IgG conjugated with Alexa Fluor 488 for AT1R, AT2R, MasR, AT4R, CCR2 or c-Fos, and a goat anti-mouse IgG conjugated with Alexa Fluor 568 for NeuN, GFAP or OX-42. Tissues similarly processed but omitting the primary antisera served as our negative controls. Immunoreactivity was viewed under a Fluorview FV10i laser scanning confocal microscope (Olympus, Tokyo, Japan).

**Histology**

In some animals in which biochemical analyses were not performed, the brain stem was removed at the end of the physiological experiment and processed for histological verification of the microinjection sites.

**Statistical analysis**

All values are expressed as mean ± SEM. The averaged value of MBP, SBP, DBP and HR, the concentration, mRNA or protein expression of...
interest in the RVLM, body weight or mNSS prior to or after stroke was used for statistical analysis. One-way or two-way ANOVA with repeated measures was used, as appropriate, to assess group means. This was followed by the Dunnett or Scheffé multiple-range test for post hoc assessment of individual means. $P < 0.05$ was considered to be statistically significant.

**Fig. 2.** Stroke-induced brain damage and distribution of angiotensin receptors (ATRs) and CCR2 in RVLM neurons and correlation between angiotensin isoforms and pressor response after stroke. A and B: Representative MRI images (A) and TTC staining (B) confirming brain infarct on day one after MCAO. C: Representative laser scanning confocal microscopic images showing cells in the RVLM that were immunoreactive to a neuronal marker, neuron-specific nuclear protein (NeuN; red fluorescence) and additionally stained positively for AT1R, AT2R, MasR, AT4R or CCR2 (green fluorescence) in sham-control (SC) or MCAO group one day after stroke. Note that the position of individual nucleus is denoted by #, + or *. These results are typical of 4 animals from each experimental group. Scale bar, 6 μm. D: Linear regression analysis of the correlation between concentration of Ang II, MCP-1, Ang-(1–7) or Ang IV in the RVLM and changes of mean blood pressure relative to baseline ($\Delta$MBP) in SC and MCAO groups. Dotted lines denote 95% confidence intervals, and $r^2$ denotes coefficient of correlation.
**Results**

**Pressor response after MCAO**

One hundred and twenty minutes of MCAO followed by reperfusion elicited a significant elevation of MBP, SBP, or DBP measured by the tail cuff plethysmography, alongside increases of neurological deficits and decreases in body weight. All these events peaked on day 1 after stroke and progressively returned to baseline over 7 days (Table 1); the augmented HR sustained 7 days.

**Differential activation of the RAS in RVLM**

Results from a hypertensive RT-PCR array, which profiles 84 key genes that regulate constriction and dilation of blood vessels (Supplementary Fig. S1), revealed that the transcript level of 57 genes in the RVLM was upregulated one day after stroke, and 27 genes were downregulated. Of note is that the mRNA level of a majority of the RAS family, including Ang II receptor type 1a (agtr1a), agtr1b, agtr2, angiotensin converting enzyme (ace), ace2, and renin (ren), was significantly upregulated (Supplementary Fig. S1). Therefore, angiotensin isomers and receptor subtypes in the RVLM after stroke were targeted in our subsequent experiments.

Time-course analysis by RT-PCR, ELISA or Western blot showed that the augmented mRNA level of agtr1a, agtr1b or agtr2 (Fig. 1A), protein concentration of Ang II (Fig. 1B), and protein expression of AT1R or AT2R (Fig. 1C) in the RVLM peaked on day 1 after stroke. Likewise, whereas the mRNA level of mas1, agtr4 variant 1 (agtr4-v1) or agtr4-v2 exhibited a significant increase on days 3 and 7 (Fig. 1A), the decrease in concentration of Ang-(1–7) and Ang IV (Fig. 1B) or protein expression of MasR and AT4R (Fig. 1C) also peaked on post-stroke day 1. Since the most significant pressor response after stroke and changes in RAS mRNA and protein levels were always observed on day 1, these events were targeted in our subsequent experiments.
protein expressions in the RVLM also occurred one day after stroke, we have focused our mechanistic investigations on this time-point.

**Differential activation of angiotensin receptors (ATRs) in RVLM neurons after stroke**

We confirmed that brain damage occurred on day 1 after stroke by the presence of brain edema in T2-weighted MRI (Fig. 2A) or by the increase in infarct volume in TTC staining (Fig. 2B). Double immunofluorescence staining coupled with laser scanning confocal microscopy revealed that the differential changes in ATRs observed in our biochemical experiments indeed occurred in RVLM neurons. Cells in the RVLM that stained positively with the neuronal marker NeuN showed enhanced immunoreactivity of AT1R and AT2R, and reduced immunoreactivity of MasR and AT4R one day after stroke (Fig. 2C).

**Activation of AT1R or AT2R signaling in RVLM promotes pressor response after stroke**

Linear regression analysis showed that the increase in MBP after stroke was significantly ($r^2 = 0.88$) and positively correlated to the concentration of Ang II (Fig. 2D) in the RVLM. In addition, pretreatment with microinjection bilaterally of candesartan (AT1R antagonist; 5 nmol) or PD123319 (AT2R antagonist; 5 nmol) into the RVLM 120 min after MCAO significantly blunted the stroke-induced increase in MBP, SBP and DBP (Fig. 3A); stroke-elicited elevation in HR was antagonized by candesartan and potentiated by PD123319 (Fig. 3A). Furthermore, those hemodynamic parameters were potentiated by Ang II (10 nmol) (Fig. 3A). Of note is that intraperitoneal administration of valsartan (5 or 25 mg/kg), an AT1R antagonist that acts only on peripheral AT1R because of its inability to cross the blood–brain barrier (BBB), did not significantly affect the stroke-induced increase in MBP, SBP, DBP or HR (Fig. 3B).

**Activation of Ang-(1–7)/MasR or Ang IV/AT4R signaling in RVLM antagonizes pressor response after stroke**

Linear regression analysis also showed that the increase in MBP after stroke was significantly and negatively correlated to the concentration of Ang-(1–7) ($r^2 = 0.82$) and Ang IV ($r^2 = 0.95$) (Fig. 2D) in the RVLM. Furthermore, pretreatment with Ang-(1–7) (200 pmol) or Ang IV (1 nmol) significantly antagonized or reversed the stroke-induced increase in MBP, SBP and DBP (Fig. 3A); whereas A779 (MasR antagonist; 500 pmol) and AT4R antiserum (1:20) were ineffective. The stroke-induced elevation in HR was blunted by Ang-(1–7), unaffected by Ang IV and potentiated by A779 and AT4R antiserum (Fig. 3A).

**MCP-1 in RVLM contributes to pressor response after stroke**

The mRNA level of ccl2 (Fig. 1A) and concentration of the pro-inflammatory MCP-1 (a common name for CCL2) (Fig. 1B) in the RVLM also underwent an increase that is significantly ($r^2 = 0.81$) and positively correlated with the augmented MBP after stroke (Fig. 2D). Both the mRNA (Fig. 1A) and protein levels (Fig. 1B) of CCR2 in the RVLM, and CCR2-immunoreactivity in RVLM neurons (Fig. 2C) remained unaltered. In addition, the stroke-induced increase in MBP, SBP, DBP or HR was significantly antagonized by microinjection into the bilateral RVLM of bindarit (MCP-1 synthesis inhibitor; 10 nmol), BMS CCR2 22 (CCR2 antagonist; 10 pmol) or propagermanium (CCR2 antagonist; 10 pmol) (Fig. 3C).
Stroke-activated MCP-1 in RVLM is differentially modulated by AT1R or AT2R and Ang-(1–7)/MasR or Ang IV/AT4R signaling

The stroked-induced elevation of MCP-1 in the RVLM was blunted by pretreatment with candesartan, PD123319, combination of candesartan and PD123319, Ang-(1–7), Ang IV or bindarit, but not by A779, AT4R antiserum, combination of A779 and AT4R antiserum, BMS CCR2 22 or propagermanium (Fig. 4A). Furthermore, whereas Ang II potentiated the stroke-induced augmentation of MCP-1 and elevation of CCR2 concentration in the RVLM, PD123319 or combination of PD123319 and candesartan significantly reduced CCR2 concentration (Fig. 4B). Other pretreatments were ineffective against CCR2 concentration in RVLM.

Dual effects of aliskiren on changes of angiotensin isoforms and MCP-1 in RVLM and pressor response after stroke

Microinjection bilaterally of the renin inhibitor aliskiren (1 nmol) into the RVLM significantly blunted the elevation of Ang II and exacerbated the reduction of Ang-(1–7) or Ang IV induced by stroke in the RVLM (Fig. 5A). Aliskiren also blunted the stroke-induced augmentation of MCP-1 and reduced CCR2 concentration (Fig. 5B), accompanied by an amelioration of the increase in MBP, SBP, DBP or HR. As a control, the concentration of angiotensinogen in the RVLM was not affected by stroke or pretreatments with aliskiren (Fig. 5A), candesartan, PD123319, A779 or AT4R antiserum (Fig. 6). Likewise, those pharmacological treatments did not change Ang II, Ang-(1–7) or Ang IV concentration in the RVLM after stroke (Fig. 6).

Aliskiren exerts beneficial effects against neuronal activation and inflammation in RVLM after stroke

At the cellular level, pretreatment with aliskiren (1 nmol) produced significant antagonism of stroke-induced neuronal activation in the RVLM, which was indicated by reducing the increase in c-Fos immuno-reactivity (c-Fos-IR) in RVLM neurons. Aliskiren similarly reduced the activation of astrocytes and microglia, two cellular markers of

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**Fig. 5.** Effects of aliskiren (AK) on concentration of angiotensins in RVLM after stroke and combination of AK and Ang-(1–7) or Ang IV produces efficacious antagonism of pressor response and tachycardia after stroke. Changes (A) in concentration of Ang II, ATG, Ang-(1–7) or Ang IV in the RVLM of stroked rats that received pretreatments with AK (1 nmol; renin inhibitor) or Veh, 120 min after MCAO. Changes (B) in MBP, SBP, DBP or HR relative to baseline and changes (C) of MCP-1 and CCR2 concentrations in the RVLM of rats that received pretreatments of AK (1 nmol), combination of AK (0.5 nmol) and Ang-(1–7) (100 pmol) or Ang IV (0.5 nmol), or Veh, 120 min after MCAO. Values are mean ± SEM, n = 5–7 animals per experimental group. *P < 0.05 versus SC group, and + P < 0.05 versus Veh + MCAO group in the post hoc Scheffé multiple-range test.
inflammation, as demonstrated by a decrease of the elevated GFAP-IR orOX–42-IR in the RVLM after stroke (Fig. 7). Comparable findings wereobtained from Western blot analysis (Fig. 7). Aliskiren pretreatment sig-nificantly blunted stroke-induced augmentation of protein expression ofc-Fos, GFAP andOX–42 in the RVLM (Fig. 7).

Combination of aliskiren and Ang-(1–7) or Ang IV exerts beneficial effects against pressor response after stroke

Combined pretreatment with only half of the doses of aliskiren (0.5 nmol) andAng-(1–7) (100 pmol) or Ang IV (0.5 nmol) produced significantantagonism of the stroke-induced increase in MBP, SBP, DBP or HR, to a degree that was comparable to or more than that elicited bythose test agents when delivered alone at full dose (cf. Fig. 3). Similarfindings were obtained for the changes in MCP-1 concentration in theRVLM (Figs. 4 and 5).

Discussion

Pressor response after stroke predisposes early death, more severe neurological deficit and frequent recurrence of stroke. An effective ther-apeutic strategy is still controversial, possibly because the key mecha-nisms are still unidentified. The present study was undertaken to fillthis void. Based on a MCAO model that mimics clinically focal transientischemic stroke, in conjunction with a neural substrate that is intimatelyrelated to brain stem cardiovascular regulation but is not part of the ce-bral ischemic core or penumbra, we demonstrated that upregulation ofAng II, followed by activation of AT1R and AT2R that leads to infarctvolatility, has been reported (Min et al., 2014) to improve the outcome ofstroke by amelioration of ischemic brain damage. On the other hand,itis not unreasonable to stipulate that both AT1R and AT2R signaling in thebrain stem are more promising against stroke-induced pressor response.Nevertheless, those findings are seemingly at variance with the contemporaryview (Luoh and Chan, 2001) that AT2R in the brain counteracts AT1R-mediated hypertension. We noted, however, that Du et al. (2013) reported recently that application of the AT2R antagonist PD123319 into the RVLM increases BP only in control rats but not during stress-induced hypertension. As such, it is not unreasonable to stipulate that both AT1R and AT2R signaling in theRVLM are involved in the pressor response after stroke. More impor-tantly, the demonstration of a critical role for AT2R at the RVLM in stroke-induced pressor response suggested that an alternative explana-tion for the controversial effect of contemporary ARBs is that theseagents are directed only against AT1R but not AT2R.

The physiological and pathological functions of Ang-(1–7)/MasR andAng IV/AT4R signaling have attracted recent attention (McKinley et al.,2003). With reference to stroke, MasR activation by i.c.v. infusion ofAng-(1–7) (Mecca et al., 2011) or AT4R activation by internal carotidinfusion of Ang IV (Faure et al., 2006) is protective against mortality,neurological deficit and cerebral infarct size after acute cerebral isch-e mia. Mechanistically, the present study revealed a reduction of Ang-(1–7), MasR, Ang IV and AT4R level in the RVLM on day 1 after MCAOthat is concurrent with the activation of AT1R and AT2R signaling. It fol-lows that the resultant decrease in the capacity to lower BP and HR in effect exacerbates stroke-induced pressor response and tachycardia. However, the inability of pretreatment with A779 and AT4R antiserum to affect pressor response and tachycardia after stroke implies that MasR or AT4R at the RVLM must be activated by Ang-(1–7) or Ang IV to exert its depressive effects on BP and HR.

The present study also implicated an important role for inflammation at the RVLM in stroke-induced pressor response. The pro-inflam-matory chemokine MCP-1 and its receptor CCR2 play an

**Fig. 6.** Effects of Cand, PD, A779 or AT4R antiserum on concentration of Ang II, Ang-(1–7), Ang IV or ATG in RVLM after stroke. Changes in concentration of Ang II, ATG, Ang-(1–7) or Ang IV in the RVLM of stroked rats that received pretreatments with Cand, PD, A779, AT4R antiserum or Veh, 120 min after MCAO. Values are mean ± SEM, n = 5–7 animals per experimental group. *P < 0.05 versus SC group in the post hoc Scheffé multiple-range test.
Fig. 7. Effects of AK against stroke-induced neuronal activation and neuroinflammation in the RVLM. A: Representative laser scanning confocal microscopic images showing cells in the RVLM that were immunoreactive to a activation of astrocyte and microglia stained positively by GFAP and OX-42 in SC or MCAO group one day after stroke. Scale bar, 200 μm. B and C: Representative laser scanning confocal microscopic images showing cells in the RVLM that were immunoreactive to a neuronal activation (B) double-stained positively by c-Fos and NeuN or to a neuroinflammation (C) stained by GFAP or OX-42 in sham control (SC), aCSF + MCAO (MCAO) or aliskiren + MCAO (AK + MCAO) group one day after stroke. Scale bar, 10 μm. Note the position of individual nucleus of neuron or glia is denoted by + or white arrow. D–F: Effects of AK produce efficacious antagonism of the protein expressions of c-Fos (D), GFAP (E) and OX-42 (F) in SC, aCSF + MCAO, AK + MCAO or AK alone group one day after stroke. Values are mean ± SEM of triplicate analyses from 3 to 6 animals per experimental group and are presented in folds relative to SC group. *P<0.05 versus SC group, and †P<0.05 versus vehicle (aCSF) + MCAO group in the post hoc Scheffé multiple-range test.
Fig. 8. Schematic illustration of signaling pathways and cellular responses in RVLM that are differentially involved in eliciting pressor response and tachycardia after stroke. Activation of AT1R and AT2R by the augmented Ang II concentration in the RVLM after stroke promotes pressor response and tachycardia by upregulation of the MCP-1/CCR2 signaling pathway. This process is exacerbated by the concurrent reduction in Ang-(1–7) and Ang IV, both of which downregulate the MCP-1/CCR2 signaling pathway via MasR and AT4R in the RVLM. At the cellular level, elevation of BP and HR may result from activation of RVLM neurons and neuroinflammation because of activation of astrocytes and microglia in the RVLM. Inhibition of renin in the RVLM by aliskiren reduces the concentration of primarily Ang II and secondarily Ang-(1–7) and Ang IV, with an overall net suppressive effect on the MCP-1/CCR2 signaling. In addition, aliskiren may reduce activation of neurons, astrocytes and microglia in the RVLM. It follows that combined treatment with aliskiren and Ang-(1–7) or Ang IV provides more effective amelioration of stroke-induced pressor response and tachycardia. AC1E, angiotensin converting enzyme 1; ACE2, angiotensin converting enzyme 2; Ang-(1–7), angiotensin(1–7); Ang I, angiotensin I; Ang II, angiotensin II; Ang IV, angiotensin IV; APN, aminopeptidase N; AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; AT4R, angiotensin type 4 receptor; BP, blood pressure; CCR2, C-C chemokine receptor 2; HR, heart rate; MasR, Mas receptor; MCP-1, monocyte chemoattractant protein-1; RVLM, rostral ventrolateral medulla. Solid line arrows denote activation, and dashed line arrows denote depression.

Important role in inflammatory responses following cerebral ischemia (Tsukuda et al., 2011), and MCP-1/CCR2 signaling mediates macrophage recruitment and neutrophil infiltration to the injury site, resulting in an increase of infarct volume and brain damage (Schilling et al., 2009). Thus, an intriguing finding of the present study is that in addition to the infarct area, there is an increase in concentration of MCP-1, accompanied by inflammation in the brain stem RVLM that is responsible for the pressor response after stroke. This notion is commensurate with a newly emerged concept proposes that inflammatory responses underlie Ang II-mediated hypertension (Pan et al., 2009). Our results demonstrated that augmentation of the pro-inflammatory mediator MCP-1 and subsequent activation of CCR2 in the RVLM underlie the pressor response induced by AT1R and AT2R signaling after stroke. Another intriguing finding in the present study is that the depressor effects of Ang-(1–7)/Mas or Ang IV/AT4R in the RVLM after stroke, which antagonizes the pressor response induced after AT1R and AT2R activation, are exerted by depressing the MCP-1/CCR2 signaling. Inhibition of pro-inflammatory cytokines in the cerebral cortex, accompanied by a reduction of the infarct area, is induced by Ang-(1–7) administration and MasR activation (Regenhardt et al., 2013).

Our results suggest that aliskiren (a renin inhibitor) presents itself as a promising agent to reduce pressor response after stroke. Renin is the first and rate-limiting enzyme for the production primarily of Ang II, and secondarily of Ang-(1–7) and Ang IV (Schroten et al., 2012). In aliskiren-treated animals, the serum levels of Ang II and Ang-(1–7) are reduced (Rusai et al., 2011). We found that, in addition to inhibiting renin, aliskiren affects a reduction of pressor response after stroke via an anti-inflammatory action by decreasing MCP-1 level and inhibiting the activation of neuron, microglia and astrocyte in the RVLM. Aliskiren depresses MCP-1 concentration in serum of hypertensive peritoneal dialysis patients (Makówka et al., 2012) or in gentamicin-treated kidney and human proximal tubular epithelial cell HK-2 lines (Bae et al., 2013; Singh et al., 2013); and inhibits glial activation in white matter in a mouse model of chronic cerebral hyperperfusion (Dong et al., 2011). O’Brien et al. (2007) reported that combination of lower doses of aliskiren and MasR agonist AVE 0991, diuretics, angiotensin-converting enzyme inhibitor or ARB significantly reduces experimental hypertension in deoxycortesterone acetate-treated rats or provides better end-organ protection in patients with mild-to-moderate hypertension. We also observed that combined treatment with half doses of aliskiren and Ang-(1–7) or Ang IV produced significant antagonism of the stroke-induced increase in BP and HR to a degree that was comparable to or more than that elicited by those test agents when delivered alone at full dose, with an overall net suppressive effect on the MCP-1/CCR2 signaling. It follows that combined treatment with aliskiren and Ang-(1–7) or Ang IV provides more effective amelioration of stroke-induced pressor response and tachycardia.

Conclusions

Based on results obtained from a MCAO model of transient focal ischemic stroke, we concluded that AT1R- and AT2R-mediated activation of MCP-1/CCR2 signaling induced by an elevated level of Ang II in the RVLM, a critical brain stem neural substrate for the maintenance of blood pressure, accompanied by concurrent reduction in Ang-(1–7), MasR, Ang IV or AT4R expressions, account for the pressor response observed after stroke. We further showed that the novel renin inhibitor aliskiren antagonizes the stroke-induced pressor response by reducing Ang II, Ang-(1–7) and Ang IV levels, with an overall net suppression of MCP-1/CCR2 signaling and an inhibition of neuroinflammation in the RVLM. Finally, we showed that combined application of aliskiren and Ang-(1–7) or Ang IV is more efficacious in ameliorating stroke-induced pressor response and tachycardia.

Perspectives

For obvious reasons, timely and effective management of pressor response is beneficial to patients presented to the intensive care unit (ICU) after stroke. However, the remedial efficacy of systemic administration of ARBs on stroke-induced pressor response is still controversial. By providing further insights into the cellular and molecular mechanisms that underlie pressor response and tachycardia after stroke, our present findings offer several new directions for future therapeutic developments. We demonstrated that both AT1R and AT2R signaling in the brain stem, particularly the RVLM, are engaged in the pressor response after stroke. This demonstration offers a reasonable explanation for the controversy surrounding the efficacy of currently available ARBs that are based on peripheral AT1R antagonism. More importantly, it implies that targeting both AT1R and AT2R may hold more promise against stroke-induced pressor response. Our results also suggested that therapy using aliskiren, alone or in combination with Ang-(1–7) or Ang IV, offers a new remedial direction. Finally, anti-inflammation by MCP-1 antagonism, particularly in conjunction with the two above-mentioned directions, is another vista for the development of effective therapeutic management against stroke-induced pressor response. One primary requirement, however, for these strategies to be operative is that the new therapeutic agents must cross the BBB if the ultimate target is the ATRs or MCP-1 in the RVLM.

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