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RESEARCH PAPER

Suppressing inflammation by inhibiting the NF-κB pathway contributes to the neuroprotective effect of angiotensin-(1-7) in rats with permanent cerebral ischaemia

Teng Jiang[1], Li Gao[2], Jun Guo[3], Jie Lu[2], Yao Wang[2] and Yingdong Zhang[1]

[1]*Department of Neurology, Nanjing First Hospital, Nanjing Medical University, Nanjing, China,* [2]*Department of Neurology, Nanjing Brain Hospital, Nanjing Medical University, Nanjing, China, and* [3]*Department of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing, China*

Correspondence
Dr Yingdong Zhang, Department of Neurology, Nanjing First Hospital, Nanjing Medical University, PO Box 210006, No. 68 Changle Road, Nanjing 210006, China. E-mail: jt870918@163.com; zhangyingdong@yahoo.com.cn

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BACKGROUND AND PURPOSE

Angiotensin-(1-7) [Ang-(1-7)] has anti-inflammatory effects in peripheral organs, but its effects in ischaemic stroke are unclear as yet. We investigated whether its anti-inflammatory effect contributes to the neuroprotection induced by Ang-(1-7) in a rat model of permanent middle cerebral artery occlusion (pMCAO).

EXPERIMENTAL APPROACH

We infused Ang-(1-7), Mas receptor antagonist A-779, angiotensin II type 2 receptor antagonist PD123319 or artificial CSF into the right lateral ventricle of male Sprague-Dawley rats from 48 h before onset of pMCAO until the rats were killed. Twenty-four hours after pMCAO, the neuroprotective effect of Ang-(1-7) was analysed by evaluating infarct volume and neurological deficits. The levels of oxidative stress were detected by spectrophotometric assay. The activation of NF-κB was assessed by Western blot and immunohistochemistry analysis. The level of COX-2 was tested by Western blot analysis and concentrations of pro-inflammatory cytokines were measured by ELISA.

KEY RESULTS

Infusion of Ang-(1-7), i.c.v., significantly reduced infarct volume and improved neurological deficits. It decreased the levels of oxidative stress and suppressed NF-κB activity, which was accompanied by a reduction of pro-inflammatory cytokines and COX-2 in the peri-infarct regions. These effects of Ang-(1-7) were reversed by A-779 but not by PD123319. Additionally, infusion of A-779 alone increased oxidative stress levels and enhanced NF-κB activity, which was accompanied by an up-regulation of pro-inflammatory cytokines and COX-2.

CONCLUSION AND IMPLICATIONS

Our findings indicate that suppressing NF-κB dependent pathway via Mas receptor may represent one mechanism that contributes to the anti-inflammatory effects of Ang-(1-7) in rats with pMCAO.

Abbreviations

aCSF, artificial CSF; Ang II, angiotensin II; Ang-(1-7), angiotensin-(1-7); CBF, cerebral blood flow; MDA, malondialdehyde; pMCAO, permanent middle cerebral artery occlusion; RAS, renin-angiotensin system; ROS, reactive oxygen species; SOD, superoxide dismutase

Introduction

Ischaemic stroke, usually caused by a temporary or permanent reduction of local cerebral blood flow (CBF), is one of the leading causes of disability and death in humans (Lopez *et al.*, 2006). Among several molecular mechanisms contributing to ischaemia-induced brain injury, inflammation plays a crucial role not only being invloved in the pathophysiology of cerebral ischaemia but is also recognized as a risk factor for ischaemic stroke (Sieber *et al.*, 2011). The inflammatory reaction after cerebral ischaemia involves activation of transcriptional regulators and expression of cytokines by inflammatory cells (Brouns and De Deyn, 2009). NF-κB, a well-characterized transcriptional regulator involved in inflammation, normally exists as heterodimers composed of p50 and p65 subunits in the cytoplasm, binding to its inhibitory proteins IκB-α, and remains inactive. Upon stimulation, IκB-α is phosphorylated and subjected to degradation by the 26S proteasome, which facilitates p50/p65 heterodimers to translocate into the nucleus and regulate gene transcription (Ridder and Schwaninger, 2009). Numerous studies have revealed that NF-κB is activated after cerebral ischaemia and contributes to infarction in models of permanent middle cerebral artery occlusion (pMCAO) (Nurmi *et al.*, 2004; Chan *et al.*, 2010; Zhang *et al.*, 2011). Meanwhile, as target genes of NF-κB, several pro-inflammatory cytokines and enzymes including TNF-α (Zhu *et al.*, 2012), IL-1β (Chan *et al.*, 2010) and COX-2 (Shwari *et al.*, 2011) are up-regulated and lead to neuronal damage after cerebral ischaemia. In addition, angiotensin II (Ang II), the main renin-angiotensin system (RAS) product, also participates in the inflammatory reactions that contribute to brain damage after ischaemic stroke (Saavedra, 2005).

There is increasing evidence that angiotensin-(1-7) [Ang-(1-7)], as another product of the RAS, could counteract numerous harmful actions of Ang II and provide protective effects against cardiovascular disease (Ferreira *et al.*, 2010). Ang-(1-7) is generated predominately from Ang II by ACE2, and exerts physiological effects by binding to its receptor, Mas (Xu *et al.*, 2011). However, to date, few studies have focused on the anti-inflammatory effects of Ang-(1-7). Souza and Costa-Neto (2011) found that Ang-(1-7) decreased LPS-induced inflammatory responses in macrophages while exogenously administered Ang-(1-7) was shown to exert anti-inflammatory effects in cardiac ischaemia-induced dysfunction (Al-Maghrebi *et al.*, 2009) and antigen-induced arthritis (da Silveira *et al.*, 2010). Moreover, a recent study revealed that Ang-(1-7) inhibited allergic inflammation by suppressing the NF-κB dependent pathway (El-Hashim *et al.*, 2012).

In addition to adrenal glands and plasma, Ang-(1-7) is also present as an endogenous constituent of the brain in areas that include the hypothalamus, medulla oblongata and amygdala (Chappell *et al.*, 1989). Due to the presence of the blood-brain barrier, Ang-(1-7) in brain is distinct from that in peripheral organs. Based on the evidence that Ang-(1-7) has anti-inflammatory effects in peripheral organs, we investigated whether Ang-(1-7) exerts anti-inflammatory effects and contributes to the neuroprotection following cerebral ischaemia. In view of the fact that only approximately 2% of stroke patients are eligible for thrombolytic treatment with recombinant tissue plasminogen activator within the first 3 h of ischaemia stroke onset (Schulz and Heusch, 2006), and in most stroke patients there is no reperfusion of ischaemic tissue during the first 24 h (Nurmi *et al.*, 2004), we adopted the pMCAO model in this study.

Methods

Reagents

Ang-(1-7) and PD123319 were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. A-779, the antagonist of GPCR Mas (nomenclature follows Alexander *et al.*, 2011), was purchased from Abbiotec Inc., San Diego, CA, USA. They were dissolved in an artificial CSF (aCSF, composition in mmol·L^{-1}: NaCl 130, KCl 2.99, $CaCl_2$ 0.98, $MgCl_2{\cdot}6H_20$ 0.80, $NaHC0_3$ 25, $Na_2HP0_4{\cdot}12H_20$ 0.039, $NaH_2P0_4{\cdot}2H_20$ 0.46).

Animals

Male Sprague-Dawley rats (250–280 g) were purchased from the Experimental Animals Center of Nanjing Medical University. They were housed in a standard animal room with a 12 h light/dark cycle and given free access to food and water. The protocol was approved by the Nanjing Medical University Experimental Animal Care and Use Committee and all efforts were made to minimize the number of animals used and their suffering. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Drug administration

Rats were anaesthetized with 10% chloral hydrate (0.35 mL·100 g^{-1}, i.p.) and placed in a stereotactic frame (David Kopf Instrument Inc., Tujunga, CA, USA). The scalp was reflected under sterile surgical conditions. The depth of anaesthesia was monitored by assessing the withdrawal reflex to footpad pinching. A brain-infusion cannula (Brain Infusion Kit 2; ALZET Inc., Cupertino, CA, USA) coupled via vinyl tubing to an osmotic pump (Model 1003D; ALZET Inc.) was implanted into the right cerebral ventricle (0.8 mm posterior and 1.4 mm lateral to bregma, 4.5 mm below the surface of the cranium) by surgeons who were blinded to the experimental groups. Osmotic pumps were placed s.c. between the scapulae and used to infuse Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}), A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}), PD123319 (6.5 nmol·L^{-1}; 1 μL·h^{-1}) or aCSF (1 μL·h^{-1}) into the right lateral cerebral ventricle from 48 h before the onset of pMCAO and lasting until the animals were killed. Following this surgery, the wounds were carefully closed with sutures. The dose and route of administration for Ang-(1-7) and A-779 were chosen based on previous studies by our group and Mecca *et al.* (2011). The dose and route of administration for PD123319 were chosen according to a previous study (Blume *et al.*, 2005).

Experimental groups

In total, 128 rats were included in this study. They were randomly allocated to seven groups using a random number

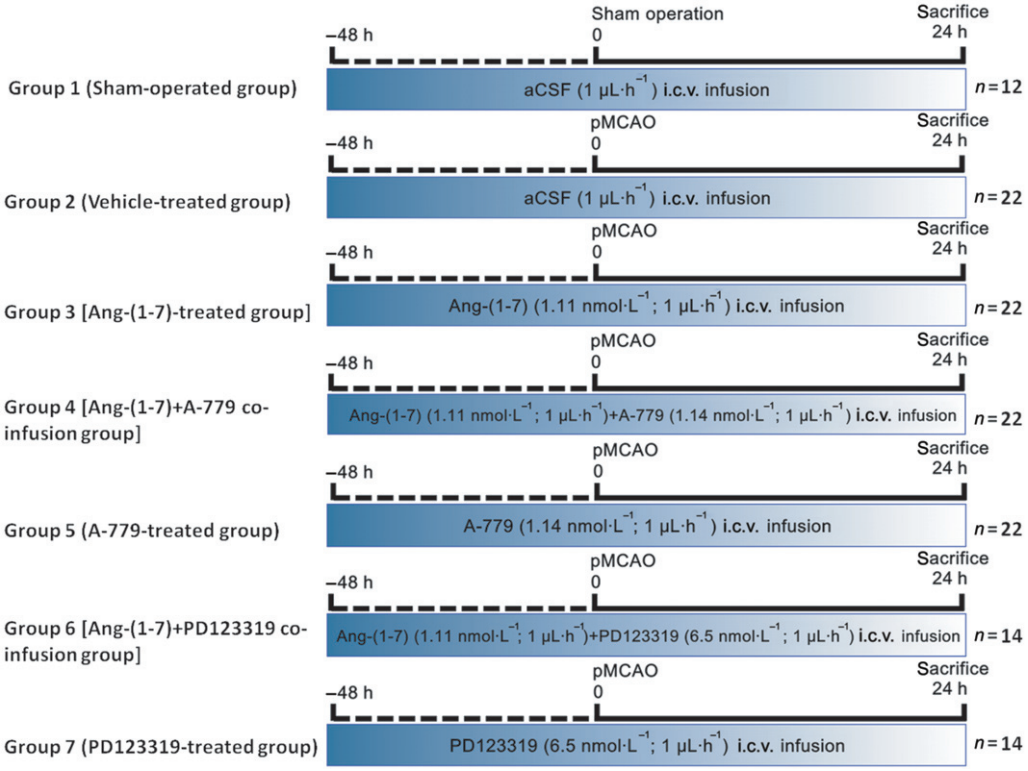

Figure 1
Scheme of the experimental protocol.

table generated by SPSS software 13.0 (IBM Inc., Armonk, NY, USA) and received treatment as shown in Figure 1.

pMCAO

Rats were subjected to pMCAO by an intraluminal filament according to the methods of Longa *et al.* (1989). They were anaesthetized with 10% chloral hydrate (0.35 mL·100 g^{-1}, i.p.). The right common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA) were isolated through a ventral midline incision. A 3.0-cm-length of monofilament nylon suture (Φ 0.26 mm), with its tip rounded by heating near a flame, was inserted from the right ECA into the lumen of ICA, then advanced until resistance was felt (1.8–2.0 cm from the bifurcation). The reduction of the CBF was confirmed by a laser-Doppler flowmetre (moorVMS-LDF-1; Moor Instruments Inc., Axminster, UK) and the procedure was considered successful if a > 80% drop in CBF was observed after occlusion. The filament remained there until the rat was killed. Throughout the procedure, body temperature was closely monitored with a rectal probe and maintained in the range of 37.0 ± 0.5°C with a heating pad. Rats in the sham-operated group were subjected to the filament insertion into the ICA but with no reduction in blood flow.

CBF monitoring and 2, 3, 5-triphenyltetrazolium chloride (TTC) staining

CBF monitoring (n = 6 per group) was performed at the beginning of occlusion and 24 h after pMCAO with a probe attached to the skull above the supply territory of the middle cerebral artery (MCA) (2 mm caudal to bregma and 6 mm lateral to midline). Changes in CBF after MCA occlusion are expressed as percentage of the baseline value of laser-Doppler flowmetry.

TTC staining was performed at 24 h after pMCAO (n = 6 per group) by investigators who were blinded to the experimental groups. The rats were killed under deep anaesthesia and the brains were carefully removed, then sectioned coronally into five 3-mm-thick slices using a rat brain matrix. The slices were immersed in the dark staining with 2% TTC for 30 min and then fixed with 4% paraformaldehyde. The infarct volume was evaluated by Image Pro-Plus 5.1 analysis system (Media Cybernetics Inc., Silver Spring, MD, USA) using Swanson's method which corrects for oedema (Swanson *et al.*, 1990). It should be noted that the infarct volumes of Ang-(1-7) + A-779 co-infusion group, A-779-treated group, Ang-(1-7) + PD123319 co-infusion group and PD123319-treated group were generating from an additional experiment, which was not done at the same time as the experiment generating the infarct volumes of vehicle-treated group and Ang-(1-7)-treated group.

Neurobehavioral testing

Neurobehavioral tests were performed at 24 h after pMCAO (n = 18 per group) using a 5-point scale (Bederson *et al.*, 1986) by an investigator who was blinded to the experimental groups: 0, rats extended both forelimbs towards the floor when gently suspended 1 m above the floor and with no

other signs of neurological deficit; 1, rats consistently flexed the forelimb contralateral to pMCAO; 2, rats circled towards the contralateral side when the tail was pulled; 3, rats spontaneously circled towards the contralateral side when allowed to move freely; 4, no spontaneous movement with an apparent depressed level of consciousness.

Brain tissue preparation

Rats were killed under deep anaesthesia at 24 h after pMCAO. For Western blot analysis, spectrophotometric assays for the malondialdehyde (MDA) level and superoxide dismutase (SOD) activity in brain and ELISA, rats (n = 6 per group) were perfused transcardially with 0.9% saline (pH 7.4), brains were removed and stored in liquid nitrogen until use. For cresyl violet staining and immunohistochemistry analysis, rats (n = 6 per group) were perfused transcardially with 0.9% saline (pH 7.4) followed by 4% paraformaldehyde in 0.9% saline (pH 7.4). The brains were removed and fixed in the same fixative for an additional 6–12 h at 4°C until use.

Western blot analysis

For Western blot analysis, the brain tissues from the peri-infarct regions (Ashwal *et al.*, 1998) and the corresponding area of sham-operated rats were homogenized and the total proteins were extracted by RIPA lysis buffer (Beyotime Inc., Shanghai, China). The protein concentrations were determined using a BCA kit (Beyotime Inc.). Different samples with an equal amount of protein (60 μg) were separated on 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and blocked in 5% BSA powder in 1 × tris-buffered saline (TBS) with 0.1% Tween 20 (1 × TBST) at room temperature for 2 h. Membranes were incubated overnight at 4°C with a mouse monoclonal antibody against serine 32 phosphorylated IκB-α (1:500, SC-8404; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), a rabbit polyclonal antibody against serine 536 phosphorylated NF-κB p65 (1:500, SC-101752; Santa Cruz Inc.), a mouse monoclonal antibody against NF-κB p65 (1:1000, #6956; Cell Signaling Technology Inc., Beverly, MA, USA), or a mouse monoclonal antibody against COX-2 (1:500, SC-166475; Santa Cruz Inc.), then washed with 1 × TBST, and incubated with HRP-coupled secondary antibody for 2 h at room temperature. After washing, protein bands were detected with chemiluminescent HRP substrate (SuperSignal West Pico; Thermo Scientific Inc., Rockford, IL, USA) for 5 min at room temperature and exposed to X-ray film (Fujifilm Inc., Tokyo, Japan). The signal intensity of primary antibody binding was analysed using Quantity One software 4.6.2 (Bio-Rad Laboratories Inc., Hercules, CA, USA) and normalized to a loading control β-actin.

Spectrophotometric assays for the MDA level and SOD activity

Brain homogenates were obtained from the peri-infarct tissues and centrifuged at 1000 x g and 4°C for 15 min to remove cellular debris. The supernatant was collected and stored at –80°C until used. Measurement of MDA level and SOD activity was performed according to the technical manuals of the detection kits (Beyotime Inc.). MDA level was measured with the thiobarbituric acid (TBA) reaction. The colour produced during the reaction of TBA with MDA was detected by a spectrophotometer (NanoVue Plus; GE Healthcare, Piscataway, NJ, USA) at 535 nm and the estimated MDA level is expressed as nmol·mg^{-1} protein. SOD activity was measured following the reduction of nitrite by a xanthine-xanthine oxidase system, which is a superoxide anion generator. One unit of SOD is defined as the amount that shows 50% inhibition, which was detected by the spectrophotometer at 560 nm. The activity of SOD is expressed as U·mg^{-1} protein.

Cresyl violet staining and immunohistochemistry analysis

The brains were embedded in paraffin. For cresyl violet staining, the paraffin-embedded sections were dewaxed and rehydrated according to standard protocols, and then stained in 1% cresyl violet at 50°C for 5 min. After being rinsed with double distilled water, the sections were dehydrated in increasing concentrations of ethanol, mounted on the slides and examined with a light microscope. For immunohistochemistry analysis, the paraffin-embedded sections received deparaffinization and rehydration treatments while endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min. After being washed in PBS, the sections were blocked with 5% normal goat serum in 1% BSA for 30 min, incubated with a mouse monoclonal antibody against NF-κB p65 (1:400, #6956; Cell Signaling Technology Inc.) overnight at 4°C and then treated with biotinylated goat anti-mouse IgG (Zhongshan Inc., Beijing, China) for 60 min. Immunoreactivity was tested with the avidin-biotin-peroxidase technique, using diaminobenzidine as the chromogen. In order to visualize cell nuclei, sections were then counterstained with Mayer's Hematoxylin (Sigma-Aldrich Inc., St. Louis, MO, USA), dehydrated, mounted on the slides and examined with a microscope equipped with a CCD camera. Cells with positive NF-κB p65 immunoreactivity in the nucleus were counted as NF-κB p65 nuclear positive cells. Cell counting was performed on five randomly selected non-overlapping fields in peri-infarct regions per slide by three independent observers who were blinded to the experimental groups. Data obtained in each field were added together to make a final data count for each slide and expressed as percentage of total cell number within the relevant fields.

ELISA

Brain homogenates were obtained from the peri-infarct tissues and centrifuged at 1000 x g and 4°C for 15 min to remove cellular debris. The supernatant was collected and stored at –80°C until use. The concentrations of TNF-α and IL-1β were measured by specific ELISA kits (R&D Systems Inc., Minneapolis, MN, USA). The change in absorbance in every well at 450 nm was detected with the spectrophotometer.

Statistical analysis

Statistical analysis was carried out by the SPSS software 13.0. Statistically significant differences were evaluated by an independent sample t-test and one-way ANOVA followed by least significant difference *post hoc* test. For neurological deficits, Mann–Whitney U-test was used for comparisons between two groups. The mortality of rats subjected to pMCAO was assessed with the chi-squared (χ) method. With the exception of neurological deficit, the data are expressed as mean ± SD. $P < 0.05$ was considered significant.

Results

Effect of Ang-(1-7) and A-779 on mortality and CBF after pMCAO

Twelve rats died before completion of the whole experiment and were excluded from the study: two rats (9.1%) in the vehicle-treated group, one rat (4.5%) in the Ang-(1-7)-treated group, two rats (9.1%) in the Ang-(1-7) + A-779 co-infusion group, four rats (18.2%) in the A-779-treated group, one rat (7.1%) in the Ang-(1-7) + PD123319 co-infusion group, and two rats (14.3%) in the PD123319 group. Post-mortem examinations did not reveal the occurrence of intracerebral or subarachnoid haemorrhage in any of these animals. No significant differences among mortality of each group were found ($P = 0.743$).

CBF was measured in the core region of the MCA immediately and at 24 h after pMCAO by laser-Doppler flowmetry as described in Methods. CBF dropped immediately after pMCAO to <20% of the baseline and this remained at this level for 24 h in the supply region of the MCA in all groups. The decrease in CBF was not significantly different between each group at the two time points (Table 1, $n = 6$ per group).

Effect of Ang-(1-7) and A-779 on brain infarct volume and neurological deficits

In vehicle-treated rats, pMCAO induced extensive infarction in the cerebral cortical and subcortical areas over a series of brain sections (23.9 ± 2.1% of the whole brain). Infusion of Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) significantly reduced infarct volume when compared with that in the vehicle-treated rats (18.1 ± 1.9 vs. 23.9 ± 2.1% of the whole brain; $n = 6$, $P < 0.05$). This reduction was reversed by co-treatment with A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) (22.5 ± 3.2 vs. 18.1 ± 1.9% of the whole brain; $n = 6$, $P < 0.05$). Compared with the vehicle-treated rats, infusion of A-779 alone (1.14 nmol·L^{-1}; 1 μL·h^{-1}) slightly increased infarct volume, but not significantly (25.9 ± 2.3 vs. 23.9 ± 2.1% of the whole brain; $n = 6$, $P = 0.181$) (Figure 2A and 2C). Additionally, representative brain slices stained with cresyl violet also illustrated the effects of Ang-(1-7) and A-779 on infarct volume (Figure 2B).

Table 1

Effect of Ang-(1-7) and A-779 on cerebral blood flow after pMCAO

Group	*n*	Cerebral blood flow (% of the baseline) 0 h	24 h
Vehicle	6	14.4 ± 2.8	15.8 ± 3.5
Ang-(1-7)	6	15.8 ± 2.5	17.6 ± 4.1
Ang-(1-7) + A-779	6	14.1 ± 3.2	16.2 ± 4.5
A-779	6	13.9 ± 2.6	15.5 ± 3.7

Surface cerebral blood flow was determined immediately and at 24 h after pMCAO by laser-Doppler flowmetry as described in Methods. Blood flow change is expressed as a percentage of basal flow rates. Data are expressed as mean ± SD ($n = 6$ per group); Analysis by one-way ANOVA followed by least significant difference *post hoc* test.

Rats subjected to pMCAO manifested obvious neurological deficits (median of the vehicle-treated group: 2, $n = 18$). Infusion of Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) showed a significant reduction in neurological deficits [median of the Ang-(1-7)-treated group: 1, $n = 18$, $P < 0.05$]. The Ang-(1-7)-induced reduction in neurological deficits was abolished by A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) [median of the Ang-(1-7) + A-779 co-infusion group: 2, $n = 18$, $P < 0.05$]. Infusion of A-779 alone (1.14 nmol·L^{-1}; 1 μL·h^{-1}) showed a small increase in neurological deficits but this did not reach statistical significance (median of the A-779-treated group: 2, $n = 18$, $P = 0.559$) (Figure 2D).

Effect of Ang-(1-7) and A-779 on MDA level and total SOD activity

The effect of Ang-(1-7) and A-779 on MDA level and total SOD activity is shown in Table 2. Vehicle-treated rats had a higher MDA level ($n = 6$, $P < 0.05$) and lower total SOD activity ($n = 6$, $P < 0.05$) when compared with those of sham-operated rats. Infusion of Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) significantly attenuated the increase in MDA level ($n = 6$, $P < 0.05$). The Ang-(1-7)-induced reduction in the level of MDA was abolished by A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) ($n = 6$, $P < 0.05$). Infusion of A-779 alone (1.14 nmol·L^{-1}; 1 μL·h^{-1}) markedly increased the MDA level ($n = 6$, $P < 0.05$) when compared with that in vehicle-treated rats. Meanwhile, infusion of Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) enhanced total SOD activity ($n = 6$, $P < 0.05$). However, this increase in total SOD activity was inhibited by A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) ($n = 6$, $P < 0.05$). Infusion of A-779 alone (1.14 nmol·L^{-1}; 1 μL·h^{-1}) significantly reduced total SOD activity ($n = 6$, $P < 0.05$) when compared with that in vehicle-treated rats.

Effect of Ang-(1-7) and A-779 on NF-κB activation

In order to investigate the effect of Ang-(1-7) and A-779 on NF-κB activation, the expressions of the proteins of IκB-α phosphorylated at serine 32, NF-κB p65 phosphorylated at serine 536 and total NF-κB p65 were detected by Western blot analysis. Phosphorylated IκB-α and phosphorylated NF-κB p65 were markedly elevated in the vehicle-treated rats by 4.3-fold ($n = 6$, $P < 0.05$) and 3.3-fold ($n = 6$, $P < 0.05$), respectively, at 24 h after pMCAO. Infusion of Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) inhibited the increase in phosphorylated IκB-α and phosphorylated NF-κB p65 by 58% ($n = 6$, $P < 0.05$) and 41% ($n = 6$, $P < 0.05$), respectively, while co-treatment with A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) abolished the Ang-(1-7)-induced reduction in the level of phosphorylated IκB-α ($n = 6$, $P < 0.05$) and phosphorylated NF-κB p65 ($n = 6$, $P < 0.05$). Infusion of A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) alone significantly increased the levels of phosphorylated IκB-α ($n = 6$, $P < 0.05$) and phosphorylated NF-κB p65 ($n = 6$, $P < 0.05$) by 15 and 16%, respectively, when compared with those in the vehicle-treated rats (Figure 3A, B, D and E). Western blot analysis also indicated that the expression of total NF-κB p65 was significantly increased, by twofold ($n = 6$, $P < 0.05$), in the vehicle-treated rats at 24 h after pMCAO when compared with that in the sham-operated rats. Infusion of Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) markedly attenuated this increase by 33% ($n = 6$, $P < 0.05$) while co-treatment with

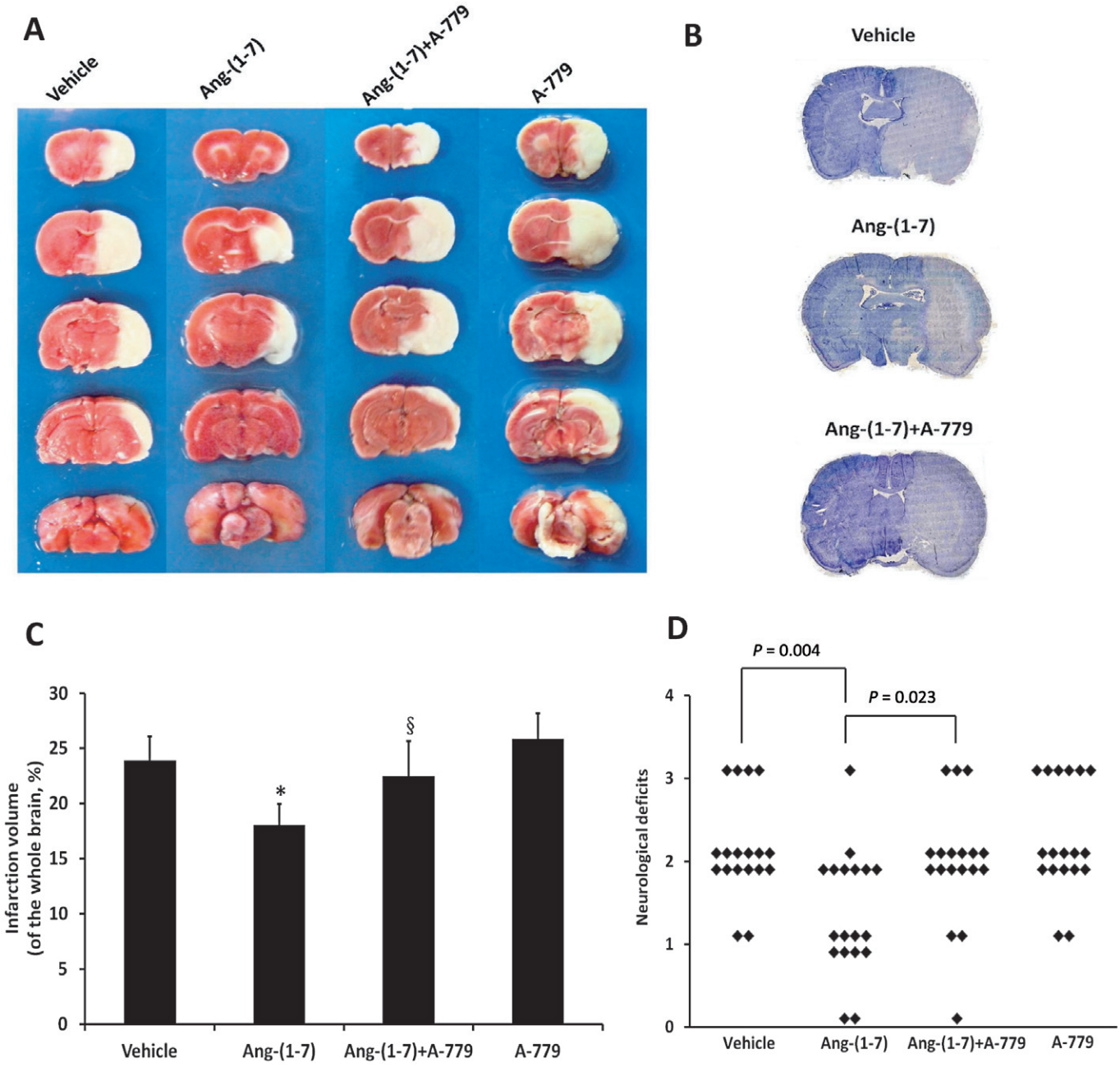

Figure 2

Effect of Ang-(1-7) and A-779 on brain infarct volume. (A) TTC staining of representative coronal sections at 24 h after pMCAO. White is infarct area and red is normal area. (B) Cresyl violet staining of representative coronal sections at 24 h after pMCAO. Infarct tissues are shown as unstained regions. (C) Infarct volume was determined at 24 h after pMCAO (n = 6 per group). Columns represent mean ± SD. *P < 0.05 versus vehicle-treated group, §P < 0.05 versus Ang-(1-7)-treated group. (D) The distribution of neurological deficit score at 24 h after pMCAO (n = 18 per group).

A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) reversed this reduction in the level of total NF-κB p65 (n = 6, P < 0.05). Infusion of A-779 alone (1.14 nmol·L^{-1}; 1 μL·h^{-1}) did not significantly change the expression of total NF-κB p65 (n = 6, P = 0.341) (Figure 3C and F).

In addition, the nuclear translocation of NF-κB p65 in the peri-infarct regions at 24 h after pMCAO in rats was determined using immunohistochemical analysis, which showed that the NF-κB p65 immunostaining was predominantly located in the cytoplasm of cells and only a few NF-κB p65 positive cells were detected in the cortex of sham-operated rats. The location of NF-κB p65 was changed to the nuclei instead of cytoplasm and the percentage of the NF-κB p65 positive cells was significantly increased by approximately 2.9-fold (n = 6, P < 0.05) in the vehicle-treated rats when compared with that in the sham-operated rats. Infusion of

Table 2

Effect of Ang-(1-7) and A-779 on MDA level and total SOD activity in brain of rats at 24 h after pMCAO

Group	*n*	MDA (nmol·mg^{-1}, protein)	SOD (U·mg^{-1}, protein)
Sham	6	4.52 ± 0.84	136.24 ± 15.39
Vehicle	6	10.48 ± 1.86*	96.53 ± 10.2*
Ang-(1-7)	6	7.68 ± 1.66#	116.21 ± 20.3#
Ang-(1-7) + A-779	6	12.86 ± 2.18#§	97.22 ± 13.23§
A-779	6	13.35 ± 2.41#	76.89 ± 14.82#

Data are expressed as mean ± SD ($n = 6$ per group); Analysis by one-way ANOVA followed by least significant difference *post hoc* test; *$P < 0.05$ compared with sham-operated group, §$P < 0.05$ compared with Ang-(1-7)-treated group, #$P < 0.05$ compared with vehicle-treated group.

Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) markedly reduced the percentage of NF-κB p65 positive cells by 34% ($n = 6$, $P < 0.05$) when compared with that in the vehicle-treated rats. The Ang-(1-7)-induced reduction was abolished by co-treatment with A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) ($n = 6$, $P < 0.05$). Infusion of A-779 alone (1.14 nmol·L^{-1}; 1 μL·h^{-1}) modestly increased the percentage of NF-κB p65 positive cells by 23% and this difference was statistically significant ($n = 6$, $P < 0.05$) (Figure 4A and B).

Effect of Ang-(1-7) and A-779 on the protein expression of COX-2

The protein levels of COX-2 in the peri-infarct tissues were measured using Western blot analysis. In the vehicle-treated rats, the expression of COX-2 was significantly higher ($n = 6$, $P < 0.05$) than that in the sham-operated group at 24 h after pMCAO. Compared with the vehicle-treated rats, infusion of Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) markedly attenuated this increase by approximately 28% ($n = 6$, $P < 0.05$). However, co-treatment with A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) significantly prevented the Ang-(1-7)-induced reduction in the expression of COX-2 ($n = 6$, $P < 0.05$). Infusion of A-779 alone (1.14 nmol·L^{-1}; 1 μL·h^{-1}) modestly increased COX-2 protein expression by approximately 22% and this was statistically significant ($n = 6$, $P < 0.05$) when compared with that in the vehicle-treated rats (Figure 5A and B).

Effect of Ang-(1-7) and A-779 on the protein levels of TNF-α and IL-1β

In the vehicle-treated rats, TNF-α and IL-1β levels were significantly elevated in the peri-infarct tissues by approximately 4.9-fold ($n = 6$, $P < 0.05$) and 1.9-fold ($n = 6$, $P < 0.05$), respectively, at 24 h after pMCAO when compared with those in the sham-operated rats. Compared with the vehicle-treated rats, infusion of Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) significantly inhibited the increase in TNF-α and IL-1β levels by 54% ($n = 6$, $P < 0.05$) and 24% ($n = 6$, $P < 0.05$) respectively. Co-treatment with A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}) significantly reversed the Ang-(1-7)-induced decrease in TNF-α ($n = 6$, $P < 0.05$) and IL-1β ($n = 6$, $P < 0.05$) levels. Meanwhile, infusion of A-779 alone (1.14 nmol·L^{-1}; 1 μL·h^{-1}) significantly increased the protein expression of TNF-α and IL-1β by 18% ($n = 6$, $P < 0.05$) and 19% ($n = 6$, $P < 0.05$), respectively, when compared with those in the vehicle-treated rats (Figure 5C and 5D).

Effect of PD123319 on Ang-(1-7)-induced neuroprotection

Co-treatment with PD123319 (6.5 nmol·L^{-1}; 1 μL·h^{-1}) did not significantly reverse the Ang-(1-7)-induced reduction in infarct volume (18.6 ± 2.3 vs. 18.1 ± 1.9% of the whole brain; $n = 6$, $P = 0.709$) while infusion of PD123319 alone (6.5 nmol·L^{-1}; 1 μL·h^{-1}) had no effect on infarct volume when compared with that in the vehicle-treated rats (22.6 ± 2.8 vs. 23.9 ± 2.1% of the whole brain; $n = 6$, $P = 0.325$) (Figure 6A and C). Of note, the data of infarct volume from vehicle- and Ang-(1-7)-treated groups presented here are the same data presented in Figure 2C. The attenuation in the level of phosphorylated IκB-α and phosphorylated NF-κB p65 caused by Ang-(1-7) was unaffected by PD123319 (6.5 nmol·L^{-1}; 1 μL·h^{-1}) ($n = 6$; $P = 0.798$ for phosphorylated IκB-α, $P = 0.344$ for phosphorylated NF-κB p65) while PD123319 itself did not alter the level of phosphorylated IκB-α ($n = 6$, $P = 0.507$) and phosphorylated NF-κB p65 ($n = 6$, $P = 0.496$) when compared with those in the vehicle-treated rats (Figure 6B, D and E). The Ang-(1-7)-induced decrease in the level of TNF-α and IL-1β was not abolished by co-treatment with PD123319 (6.5 nmol·L^{-1}; 1 μL·h^{-1}) ($n = 6$; $P = 0.763$ for TNF-α, $P = 651$ for IL-1β) while PD123319 alone (6.5 nmol·L^{-1}; 1 μL·h^{-1}) had no effect on the level of TNF-α ($n = 6$, $P = 0.516$) and IL-1β ($n = 6$, $P = 0.742$) (Figure 6F and G).

Discussion

The major finding of this study is that Ang-(1-7) has anti-inflammatory actions and contributes to neuroprotection by interacting with the Mas receptors in a rat model of pMCAO. Treatment with Ang-(1-7) caused a significant decrease in the infarct volume and ameliorated the ischaemia-induced neurological deficits, which was consistent with the findings obtained in a recent study using a transient middle cerebral artery occlusion (MCAO) model of rats (Mecca *et al.*, 2011). Ang-(1-7) treatment significantly suppressed the activation of NF-κB in the peri-infarct regions after pMCAO. Ang-(1-7) not only significantly decreased the protein expression of total NF-κB p65, but also reduced the phosphorylated IκB-α at serine 32, phosphorylated NF-κB p65 at serine 536, and inhibited the ischaemia-induced nuclear translocation of NF-κB p65, which are obvious signals of NF-κB activation (Perkins, 2006). Meanwhile, a reduction in TNF-α, IL-1β and COX-2 levels was also observed in the peri-infarct regions. These beneficial effects were abolished by A-779, indicating that the Mas receptors were involved in these anti-inflammatory actions of Ang-(1-7).

In view of the fact that Ang-(1-7) exhibits potential affinity at angiotensin II type 2 receptors (AT_2 receptors) (Bosnyak *et al.*, 2011) and provides beneficial effects in peripheral organs (Walters *et al.*, 2005; Tesanovic *et al.*, 2010), the role of AT_2 receptors in Ang-(1-7)-induced neuroprotection was

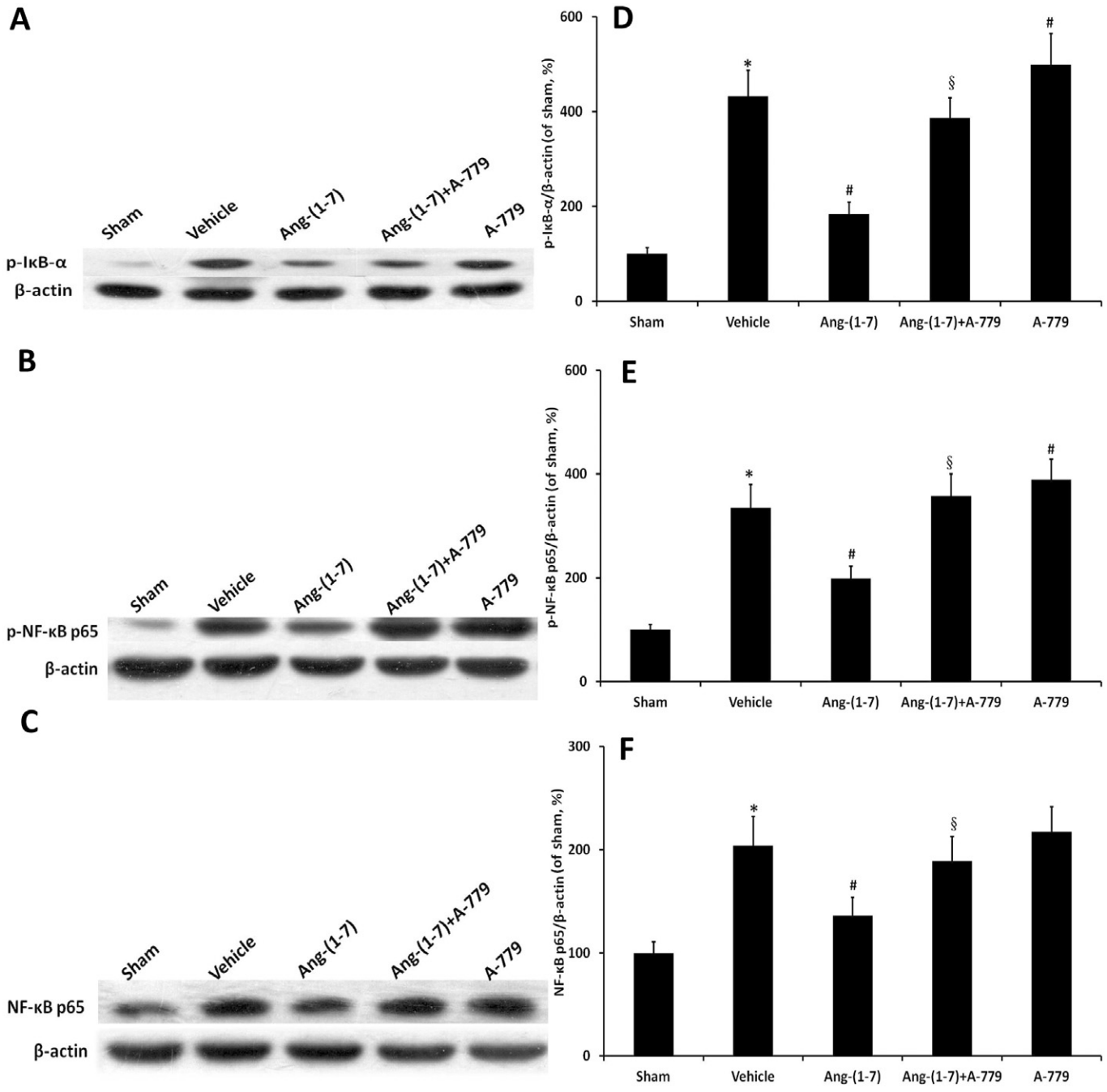

Figure 3

Effect of Ang-(1-7) and A-779 on the activation of NF-κB. Protein levels of phosphorylated IκB-α, and phosphorylated and total NF-κB p65 in peri-infarct regions were determined at 24 h after pMCAO by Western blot analysis. (A) Protein expression of phosphorylated IκB-α using β-actin as the loading control. (B) Protein expression of phosphorylated NF-κB p65 using β-actin as the loading control. (C) Protein expression of total NF-κB p65 using β-actin as the loading control. (D) Quantitative analysis of changes in the protein level of phosphorylated IκB-α ($n = 6$ per group). (E) Quantitative analysis of changes in the protein level of phosphorylated NF-κB p65 ($n = 6$ per group). (F) Quantitative analysis of changes in the protein level of total NF-κB p65 ($n = 6$ per group). Columns represent mean ± SD. *$P < 0.05$ versus sham-operated group, §$P < 0.05$ versus Ang-(1-7)-treated group, #$P < 0.05$ versus vehicle-treated group.

verified in this study using PD123319. However, co-treatment with PD123319 did not affect the anti-inflammatory and neuroprotective actions of Ang-(1-7), suggesting that the beneficial effects of Ang-(1-7) after pMCAO are not mediated by AT_2 receptors.

Additionally, the results obtained after infusing A-779 alone revealed that endogenous Ang-(1-7) may exert anti-inflammatory effects and contribute to neuroprotection after permanent cerebral ischaemia. Although A-779 itself did not significantly enlarge infarct volume or exacerbate neurologi-

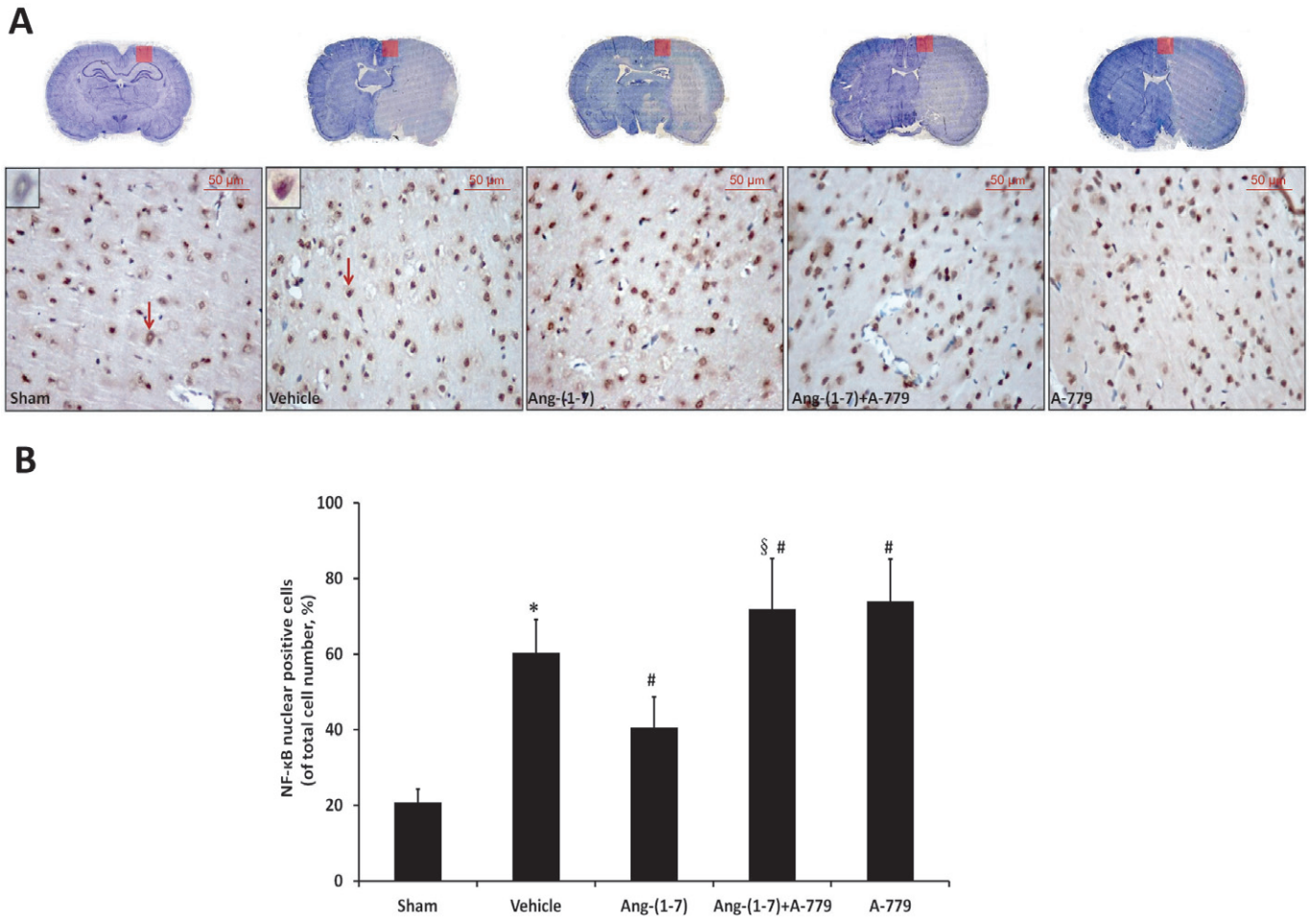

Figure 4

Effect of Ang-(1-7) and A-779 on the nuclear translocation of NF-κB p65. (A) Representative photos of immunohistochemistry staining with anti-NF-κB p65 antibody (×400). The red boxes in cresyl violet stained sections showed the peri-infarct regions selected for immunohistochemistry analysis in each group. In the cortex of the sham-operated rats, the p65 immunostaining was predominantly located in the cytoplasm of cells (red arrow in the sham-operated group). In the peri-infarct regions of the vehicle-treated rats, increased nuclear p65 immunostaining (red arrow in the vehicle-treated group) was detected at 24 h after pMCAO. Infusion of Ang-(1-7) (1.11 nmol·L^{-1}; 1 μL·h^{-1}) markedly blocked the nuclear translocation of NF-κB p65. This effect was abolished by co-treatment with A-779 (1.14 nmol·L^{-1}; 1 μL·h^{-1}). Infusion of A-779 alone (1.14 nmol·L^{-1}; 1 μL·h^{-1}) modestly promoted the nuclear translocation of NF-κB p65. (B) Quantitative analysis of NF-κB p65 nuclear positive cells. The data are expressed as percentage of total cell number ($n = 6$ per group) and columns represent mean ± SD. *$P < 0.05$ versus sham-operated group, §$P < 0.05$ versus Ang-(1-7)-treated group, #$P < 0.05$ versus vehicle-treated group.

cal deficits, it markedly increased oxidative stress levels and enhanced NF-κB activity which was accompanied by up-regulated expression of TNF-α, IL-1β and COX-2 in the peri-infarct regions, providing evidence that Ang-(1-7) as an endogenous heptapeptide is involved in regulating the magnitude of the inflammatory reaction after cerebral ischaemia.

Previous studies have indicated that inflammation is an important pathological mechanism in cerebral ischaemia (Sieber *et al.*, 2011; Zhu *et al.*, 2012). As a pivot regulator of inflammation, NF-κB is activated after pMCAO and contributes to ischaemia-induced neurological injury as shown in numerous research studies (Nurmi *et al.*, 2004; Chan *et al.*, 2010; Zhang *et al.*, 2011). Inhibiting the activation of NF-κB by pyrrolidine dithiocarbamate or other non-specific inhibitors is neuroprotective and leads to smaller infarcts in the acute stage of cerebral ischaemia (Ridder and Schwaninger, 2009). Activation of NF-κB after cerebral ischaemia induces expression of pro-inflammatory genes including TNF-α, IL-1β and COX-2. TNF-α and IL-1β are two well-studied cytokines involved in inflammatory responses after stroke and appear to aggravate ischaemic damage (Chan *et al.*, 2010). On the other hand, both TNF-α and IL-1β are possible inducers of NF-κB activity in the ischaemic brain and form a positive feedback loop, thus perpetuating the inflammatory response and worsening the outcome. COX-2 is a key enzyme in inflammation. Its main product, PGE_2, an inflammatory mediator, was found to be increased and lead to histopathological changes in the hippocampus following cerebral ischaemia (Candelario-Jalil *et al.*, 2003), while selective COX-2 inhibitors as well as COX-2 gene disruption reduced neuronal damage after cerebral ischaemia (Sasaki *et al.*, 2004).

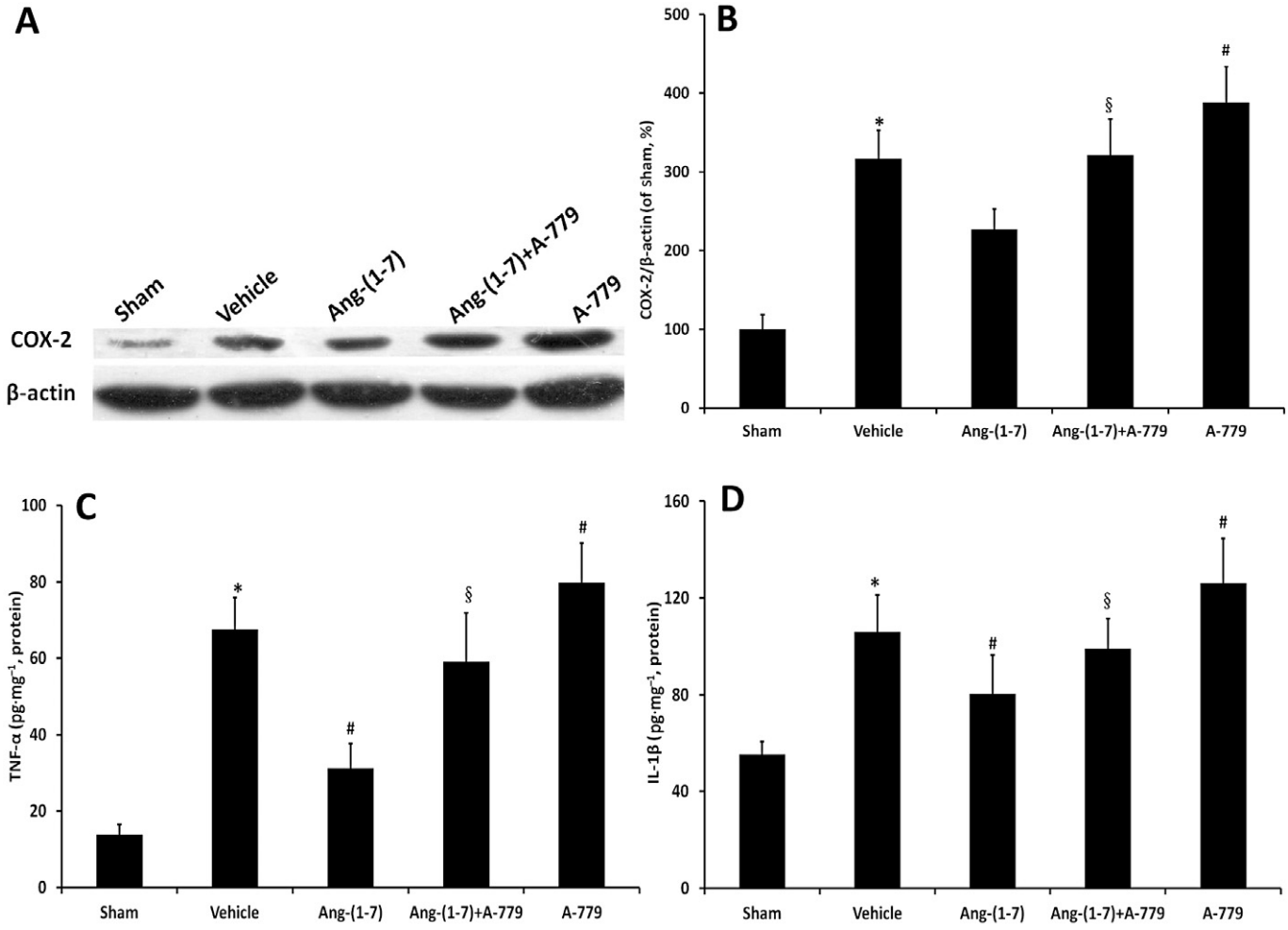

Figure 5

Effect of Ang-(1-7) and A-779 on the protein expression of COX-2, TNF-α and IL-1β. Protein level of COX-2 in the peri-infarct regions was determined at 24 h after pMCAO by Western blot analysis and protein levels of TNF-α and IL-1β in the peri-infarct regions were determined at 24 h after pMCAO using ELISA. (A) Expression of COX-2 using β-actin as the loading control. (B) Quantitative analysis of changes in the protein levels of COX-2 ($n = 6$ per group). (C) Protein level of TNF-α measured at 24 h after pMCAO ($n = 6$ per group). (D) Protein level of IL-1β measured at 24h after pMCAO ($n = 6$ per group). Columns represent mean ± SD. *$P < 0.05$ versus sham-operated group, §$P < 0.05$ versus Ang-(1-7)-treated group, #$P < 0.05$ versus vehicle-treated group.

However, the mechanisms for Ang-(1-7)-induced inhibition of NF-κB activation are at present still not clear. Reducing Ang II signalling may be a possible mechanism, and Fu *et al.* (2011) observed that the levels of brain Ang II are transiently increased in MCAO rats, suggesting the involvement of Ang II in the pathogenesis of cerebral ischaemia. As a primary source of free radicals, Ang II could increase the formation of superoxide in the normal brain and impair the blood-brain barrier after acute infusion (Kim-Mitsuyama *et al.*, 2005). The formation of reactive oxygen species (ROS), which has been implicated in the pathophysiological mechanisms of cerebral ischaemia, was attenuated by AT_1 receptor blockers (Iwai *et al.*, 2004). When taken together these results indicate that Ang II is involved in the oxidative stress reaction following cerebral ischaemia. In addition, as a strong promoter of vascular contraction, Ang II also participates in the vasoconstriction response following cerebral ischaemia, further reducing perfusion and leading to local hypoxic conditions in the peri-infarct regions (Stenman and Edvinsson, 2004). Both increased oxidative stress levels and a reduced oxygen supply are strong promoters of NF-κB activation (Ridder and Schwaninger, 2009). Activation of NF-κB up-regulates its downstream pro-inflammatory factors, increases inflammatory responses and worsens stroke prognosis. As a newly established pathway of RAS, the ACE2-Ang-(1-7)-Mas axis counteracts a number of pathophysiological effects of the ACE-Ang II-AT_1 receptor axis, providing protective effects in peripheral systems. It has been shown that Ang-(1-7) can down-regulate AT_1 receptors in cell culture studies (Clark *et al.*, 2001) and Roks *et al.* (1999) revealed that Ang-(1-7) modulated the RAS by inhibiting ACE activity in human atrial tissue. A more recent study showed that infusion of Ang-(1-7) reduces heart Ang II levels in rats (Mendes *et al.*, 2005). Together, these finidings indicate that Ang-(1-7) may suppress the activity of

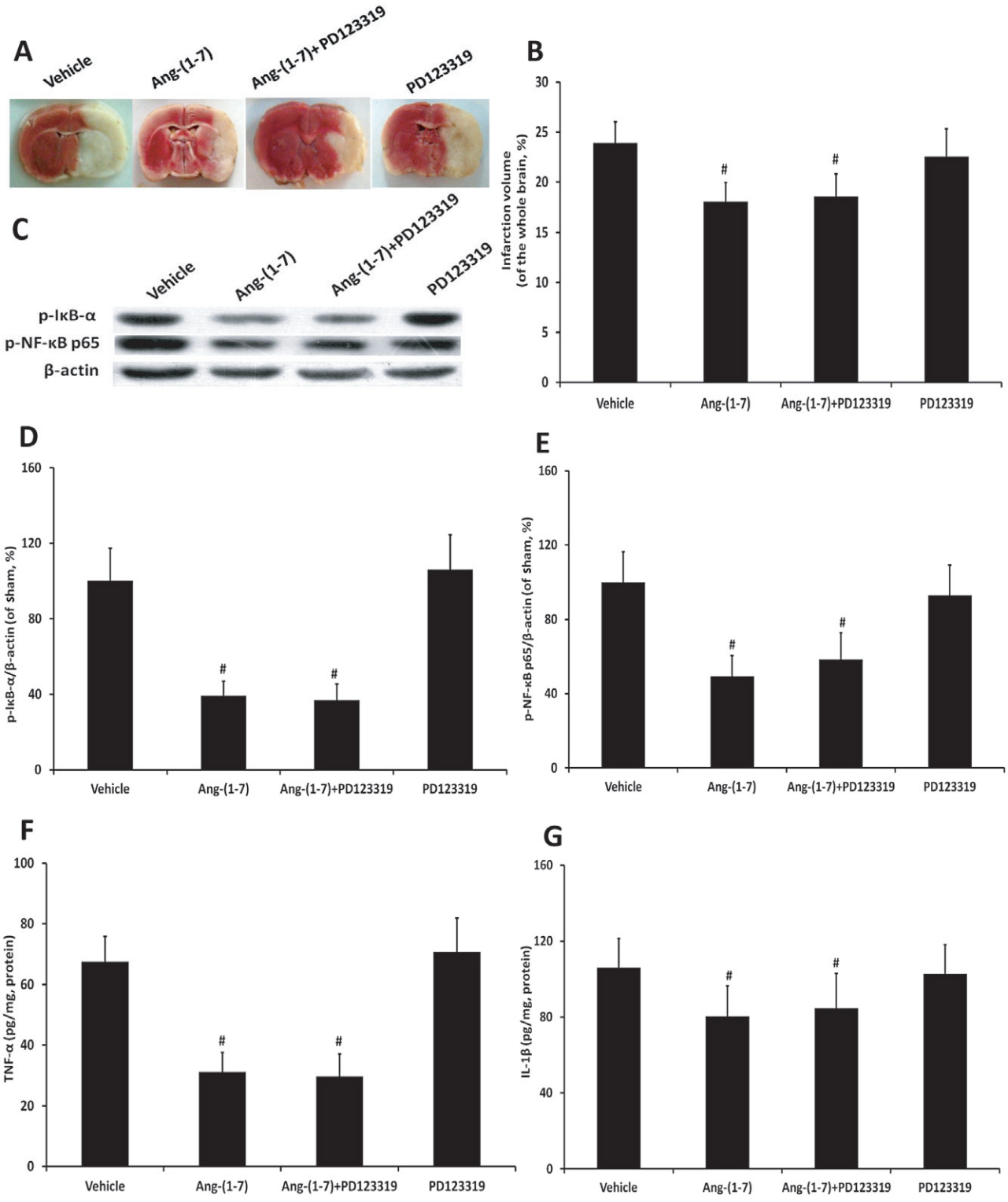

Figure 6

Effect of PD123319 on the Ang-(1-7)-induced neuroprotection. (A) TTC staining of representative coronal sections at 24 h after pMCAO. White is infarct area and red is normal area. (B) Infarct volume was determined at 24 h after pMCAO ($n = 6$ per group). Of note, the data for infarct volume from vehicle- and Ang-(1-7)-treated groups are the same data presented in Figure 2C. (C) Protein expression of phosphorylated IκB-α and phosphorylated NF-κB p65 using β-actin as the loading control. (D) Quantitative analysis of changes in the protein level of phosphorylated IκB-α ($n = 6$ per group). (E) Quantitative analysis of changes in the protein level of phosphorylated NF-κB p65 ($n = 6$ per group). (F) Protein level of TNF-α measured at 24 h after pMCAO ($n = 6$ per group). (G) Protein level of IL-1β measured at 24 h after pMCAO ($n = 6$ per group). Columns represent mean ± SD. #$P < 0.05$ versus vehicle-treated group.

NF-κB by directly affecting Ang II signalling after cerebral ischaemia.

Additionally, either overexpression of ACE2 (Xia *et al.*, 2011) or administration of Ang-(1-7) results in a reduction in ROS formation and reduced oxidative stress damage in animal and cell culture studies (Liao *et al.*, 2011; Raffai *et al.*, 2011). In the current study, Ang-(1-7) caused a reduction in oxidative stress levels after cerebral ischaemia which was mediated by Mas receptors, providing further evidence that Ang-(1-7) exerts positive effects in attenuating oxidative stress. Moreover, we also demonstrated that Ang-(1-7) at a dose of 1.11 $nmol \cdot L^{-1}$ (1 $\mu L \cdot h^{-1}$) increased bradykinin (BK) levels (Lu *et al.*, 2008) and stimulated NO release (Zhang *et al.*, 2008), which was accompanied by up-regulated endothelial NOS expression following cerebral ischaemia in previous studies. Both NO and BK are strong vasodilator agents that contribute to the vasodilator effects of Ang-(1-7), and may restore blood flow in the peri-infarct regions and ameliorate local hypoxic conditions in this tissue following cerebral ischaemia. A reduction in oxidative stress and improvement in oxygen supply could inhibit the activity of NF-κB, thus suppressing inflammatory responses and protecting neurons from ischaemic damage.

In conclusion, the current study demonstrated that Ang-(1-7) has anti-inflammatory effects and contributes to neuroprotection after permanent cerebral ischaemia. The beneficial effects of Ang-(1-7) were reversed by A-779, but not by PD123319. An interaction with Mas receptors to suppress the NF-κB dependent pathway may represent one of the underlying mechanisms for the anti-inflammatory effects of Ang-(1-7). These results indicate that Ang-(1-7) may be a viable strategy for treating ischaemic stroke and more studies should be performed to investigate other possible mechanisms involved in Ang-(1-7)-induced neuroprotection.

Acknowledgements

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Conflict of interest

None.

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