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RESEARCH PAPER

Neuroprotective effects of andrographolide in a rat model of permanent cerebral ischaemia

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BACKGROUND AND PURPOSE
Andrographolide is a diterpenoid lactone isolated from a traditional medicinal herb, *Andrographis paniculata*. It possesses potent anti-inflammatory activity. The present study examined potential therapeutic effects of andrographolide on cerebral ischaemia using a rat model with permanent middle cerebral artery occlusion (pMCAO).

EXPERIMENTAL APPROACH
The MCA in rats was permanently occluded (by cautery), and 24 h later neurological effects were assessed with behavioural scores. Infarct volume and microglial activation were determined histologically. The p65 form of the transcription factor, nuclear factor-κB (NF-κB), was measured by Western blot, and cytokines by immunoassay of brain extracts.

KEY RESULTS
Andrographolide, given i.p. 1 h after pMCAO, reduced infarct volume with a maximum reduction of approximately 50% obtained at 0.1 mg·kg^{-1}. Neurological deficits were also reduced by andrographolide, reflecting a correlation between infarct volume and neurological deficits. pMCAO was found to induce activation of microglia and elevate tumour necrosis factor (TNF)-α, interleukin (IL)-1β and prostaglandin (PG)E_2 in the ischaemic brain areas. Andrographolide (0.1 mg·kg^{-1}) significantly attenuated or abolished these effects. In addition, andrographolide suppressed the translocation of p65 from cytosol to nucleus, indicating reduced NF-κB activation.

CONCLUSIONS AND IMPLICATIONS
Andrographolide exhibited neuroprotective effects, with accompanying suppression of NF-κB and microglial activation, and reduction in the production of cytokines including TNF-α and IL-1β, and pro-inflammatory factors such as PGE_2. Our findings suggest that andrographolide may have therapeutic value in the treatment of stroke.

Abbreviations
ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin-1β; iNOS, inducible NOS; NF-κB, nuclear factor-kappa B; PGE_2, prostaglandin E_2; pMCAO, permanent middle cerebral artery occlusion; ROS, reactive oxygen species; TNF-α, tumour necrosis factor-α; tPA, tissue-type plasminogen activator

Introduction

Stroke is the cause of 9% of all deaths worldwide (Feigin, 2005). It is also one of the primary causes of adult long-term disability (Murray and Lopez, 1997). Currently, tissue-type plasminogen activator (tPA) is the only FDA-approved therapy for ischaemic stroke, which is the most common type of stroke (Adibhatla and Hatcher, 2008). However, tPA is a suitable therapy for only a limited number of patients, as treatment needs to be initiated within 3–5 h post-stroke (Hacke *et al.*, 2008). Moreover, use of tPA, especially delayed use, may lead to haemorrhage and oedema (Lapchak, 2002), thus increasing

mortality. Recent attempts to extend the narrow time window for safe and effective therapy with tPA have achieved only limited success in animal models (Liu *et al.*, 2009; Zhang *et al.*, 2009a). This underscores the need for new therapeutic approaches to the treatment of stroke.

Cerebral ischaemia results in the loss of blood supply followed by a cascade of events including glutamate excitotoxicity, calcium overload, oxidative stress and inflammation, leading eventually to cell death by both necrosis and apoptosis. Many of the molecules involved in this complex series of biochemical events are potential therapeutic targets for the development of effective treatment for stroke (Mehta *et al.*, 2007; Barone, 2009). Andrographolide is the major bioactive compound isolated from *Andrographis paniculata* (Chuan-Xin-Lian in Chinese), a medicinal herb that is widely used in China and other parts of Asia for the treatment of upper respiratory tract infections (Roxas and Jurenka, 2007). We now know that andrographolide exerts a wide range of therapeutic actions, including immunosuppressant (Burgos *et al.*, 2005; Iruretagoyena *et al.*, 2006), anti-thrombotic (Li *et al.*, 2009), anti-inflammatory (Wang *et al.*, 2007b; Abu-Ghefreh *et al.*, 2009; Bao *et al.*, 2009), anti-neoplastic (Varma *et al.*, 2009), anti-viral (Lin *et al.*, 2008; Chen *et al.*, 2009), anti-bacterial (Jiang *et al.*, 2009), anti-diabetic (Zhang *et al.*, 2009b), antioxidative stress (Akowuah *et al.*, 2009), anti-pyretic (Suebsasana *et al.*, 2009) and anti-oedematogenic and anti-nociceptive (Lin *et al.*, 2009; Sulaiman *et al.*, 2010) activities. Several of these pharmacological properties would appear to be beneficial in cerebral ischaemia. In addition, andrographolide has been shown to protect rat cardiomyocytes against hypoxia and reoxygenation injury (Woo *et al.*, 2008), and pancreatic β-cells in alloxan-treated diabetic mice (Zhang *et al.*, 2009b). In a clinical study, it was reported that andrographolide was able to cross the brain–blood barrier (Lu, 1995). It is therefore possible that andrographolide could provide neuroprotection against ischaemic injuries in the brain. In the present study, we have investigated if andrographolide may provide neuroprotection in stroke, and the possible mechanism(s) involved using a rat model with permanent middle cerebral artery occlusion (pMCAO).

Methods

All animal care and experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

pMCAO

Male Wistar rats (200–250 g) were randomly assigned into three groups: sham control, vehicle-treated pMCAO and andrographolide-treated pMCAO groups. Cerebral ischaemia was induced by permanent occlusion of the left middle cerebral artery (MCA) using a subtemporal approach (Tamura *et al.*, 1981; Qu *et al.*, 2006). The rats were anaesthetized with ketamine (75 $mg \cdot kg^{-1}$, i.p.; Parnell Laboratories Pty Ltd, Alexandria, NSW, Australia) and xylazine (10 $mg \cdot kg^{-1}$, i.p.; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia). The MCA was exposed through a subtemporal craniectomy and cauterized from the point proximal to its origin to the point where it intersects the inferior cerebral vein. The sham group was operated in the same way as the experimental groups without the occlusion of the MCA.

Drug administration

Andrographolide was purchased from Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA), with purity ≥98%. It was dissolved in 1% dimethyl sulphoxide and administered i.p. (0.01–1 $mg \cdot kg^{-1}$) 1 h after pMCAO. The injection volume was 2 $mL \cdot kg^{-1}$.

Evaluation of neurological deficits

Neurological deficits were assessed 24 h after pMCAO using a 4-point behavioural rating scale (Bederson *et al.*, 1986): score 0, rats extended both forelimbs towards the floor when gently suspended 1 m above the floor and with no other signs of neurological deficit; score 1, rats consistently flexed the forelimb contralateral to pMCAO; score 2, rats circled towards the contralacteral side when the tail was pulled; score 3, rats spontaneously circled towards the contralateral side when allowed to move freely; score 4, no spontaneous movement with an apparent depressed level of consciousness.

It should be noted that neurobehavioural assessment using the Bederson score has a reported sensitivity of 88.0% and that small infarctions in the area within caudate-putamen and dorsolateral cortex are not reflected in the score. Furthermore, lesions in dorsolateral frontal cortex has no effect on circling behaviour (Bederson *et al.*, 1986).

Infarct volume assessment

The rats were decapitated 24 h after pMCAO. The whole brains were cut into 2 mm coronal sections and immediately stained by 1% tetrazolium blue chloride (TTC, Sigma Chemical Co.) at 37°C for 30 min. TTC-stained brain slices were then preserved in 4% paraformaldehyde overnight. The

infarct volumes were measured using the Scion (Frederick, MD, USA) imaging software with correction for brain oedema.

Immunohistochemistry

Twenty-four hours after pMCAO, the rats were anaesthetized and perfused with Ringer solution followed by 2% paraformaldehyde through the heart. Brains were removed and immersed in 2% paraformaldehyde for 4 h at room temperature, and then dehydrated in 15% sucrose at 4°C overnight. The brain tissues were then preserved in OCT (Sakura Finetek, Torrance, CA, USA) and stored at –80°C. Coronal sections (30 μm) were cut using a cryostat and mounted onto gelatin-coated glass slides. Non-specific binding was blocked by incubating the section in 5% goat serum for 1 h. Antibodies against ED-1 (1:100, Serotec, Oxford, UK) and OX-42 (CD11b; 1:100, Serotec) were used on specific coronal brain region and incubated at 4°C overnight. This was followed by incubation of anti-mouse secondary antibody and streptavidin biotinylated horseradish peroxidase (HRP) complex (ABC kit, Vector Laboratories, Burlingame, CA, USA). Positive staining was visualized with diaminobenzidine (DAB) using DAB-enhanced liquid substrate system (Sigma Chemical Co.). Sections were then counterstained by methyl green, dehydrated, mounted and observed under a microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan). Cell counts were performed by quantifying the number of immunopositive cells in three randomly selected areas of 0.25 mm^2 in the peri-infarct and ischaemic core at +0.2, –1.8 and –2.7 mm from bregma), without knowledge of the treatments. For cell counting analysis, all positively stained cells (brown in colour) were included regardless of morphology.

Preparation of the cytoplasmic and nuclear fractions

Brain tissues were homogenized in cold lysis buffer [10 $mmol \cdot L^{-1}$ HEPES, pH 7.9 with 1.5 $mmol \cdot L^{-1}$ $MgCl_2$ and 10 $mmol \cdot L^{-1}$ KCl, containing protease inhibitor cocktails (Roche Diagnostics GmbH, Mannheim, Germany) and dichlorodiphenyltrichloroethane]. The lysates were centrifuged for 20 min at 10 000× *g*. The supernatant containing the cytoplasmic fraction was separated from the pellet, which was further resuspended and shaken gently in extraction buffer [20 $mmol \cdot L^{-1}$ HEPES, pH 7.9 with 1.5 $mmol \cdot L^{-1}$ $MgCl_2$, 0.42 $mol \cdot L^{-1}$ NaCl, 0.2 $mmol \cdot L^{-1}$ EDTA and 25% (v/v) glycerol containing protease inhibitor cocktail (Roche Diagnostics GmbH) and dichlorodiphenyltrichloroethane] for 30 min, and then centrifuged for 5 min at 20 000× *g* to obtain the supernatant containing the nuclear fraction.

Western blot analysis of p65

Cytoplasmic and nuclear fractions containing equal amounts of protein, determined by the Lowry method, were separated by 10% SDS/PAGE and transferred onto a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). After incubating at room temperature in TBST buffer (10 $mmol \cdot L^{-1}$ Tris–HCl, 120 $mmol \cdot L^{-1}$ NaCl, 0.1% Tween-20, pH 7.4) containing 10% milk for 1 h, the membrane was incubated with antibodies against the p65 protein (Cell Signaling Technologies, Beverly, MA, USA) at 4°C overnight. Membranes were then washed three times in TBST buffer, followed by incubation with 1:10 000 dilutions of HRP-conjugated anti-rabbit IgG at room temperature for 1 h, and washed three times in TBST. Visualization was carried out using the ECL (advanced chemiluminescence) kit (GE Healthcare, Buckinghamshire, UK). The density of the bands on Western blots was quantified by densitometry analysis of the scanned blots using ImageQuant software. The relative protein level was normalized to β-actin (Sigma Chemical Co.).

Measurement of tumour necrosis factor(TNF)-α, interleukin (IL)-1β and prostaglandin (PG)E_2 levels

Ipsilateral and contralateral hemispheres of the brain were homogenized in lysis buffer (celLytic MT Cell Lysis Reagent, Sigma Chemical Co.). The lysates were shaken on ice for 30 min and centrifuged at 12 000× *g* for 10 min. Supernatants were collected for total PGE_2, TNF-α and IL-1β analysis using commercial immunoassay kit (Amersham, Buckinghamshire, UK for PGE_2, and R&D Systems, Minneapolis, MN, USA for TNF-α and IL-1β). Protein band intensity was measured by a microplate reader (Sunrise, Tecan, Research Triangle Park, NC, USA) and normalized to respective protein level.

Data analysis

All data are presented as mean ± SEM. Statistical significance was assessed with one-way ANOVA followed by a *post hoc* (Bonferroni) test for multiple group comparison. Differences with *P* value less than 0.05 were considered statistically significant. For neurological scores, data were analysed by the chi-square (χ^2) method followed by Kruskal–Wallis test for multiple comparison.

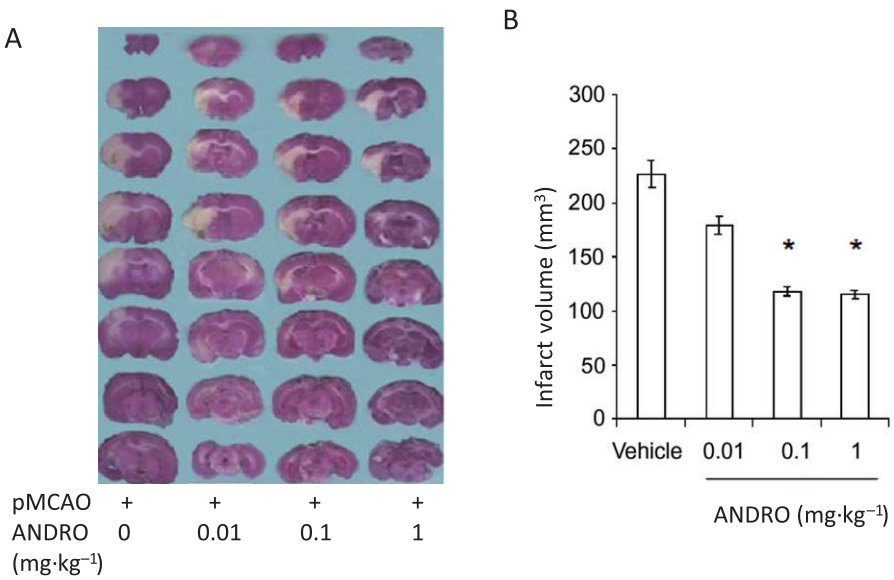

Figure 1

Effects of treatment with andrographolide (ANDRO) on infarct volume of rats 24 h after pMCAO. (A) Representative TTC-stained sections. Infarcted areas appeared white in colour. (B) Infarct volume for each treatment group. Data are presented as mean infarct volume ± SEM, $N = 6$. Analysis by one-way ANOVA, $F = 10.85$, $P = 0.000191$. *$P < 0.01$ compared to vehicle-treated group by Bonferroni's *post hoc* test.

Table 1

Effects of andrographolide treatment on neurological scores in rats 24 h after pMCAO

Neurological score	No. of vehicle-treated pMCAO rats	No. of andrographolide-treated (0.01 mg·kg^{-1}) pMCAO rats	No. of andrographolide-treated (0.1 mg·kg^{-1}) pMCAO rats	No. of andrographolide-treated (1.0 mg·kg^{-1}) pMCAO rats
1	0	0	2	0
2	0	1	4	2
3	3	5	0	4
4	3	0	0	0
Median	3.5	3	2*	3
95% CI	2.93–4.08	2.41–3.26	1.13–2.21	2.13–3.21

The number of rats with each particular neurological score in each treatment group is presented in a contingency table. $N = 6$ per treatment group. $\chi^2 = 24.7$, d.f. = 9 and $P < 0.005$. *$P < 0.01$ versus vehicle-treated (Kruskal–Wallis test).

Results

Effect of andrographolide on brain infarct volume and neurological deficits

In vehicle-treated control rats, pMCAO induced a cerebral infarct volume of 226 ± 25 mm^3 ($n = 6$). Andrographolide reduced the infarct volume with a maximum effect at ~50% reduction observed with 0.1 and 1 mg·kg^{-1} dose (Figure 1). Behavioural assessments also demonstrated reductions in neurological deficits after treatment with andrographolide (Table 1), and this was significant at the 0.1 mg·kg^{-1} dose. This dose was therefore used in all subsequent experiments. No deaths occurred in any of the groups of animals over the entire study.

Effect of andrographolide on microglia activation

OX-42-immunopositive microglial cells were observed in both the cortex and striatum. In the sham control rats, these resident microglia appeared on both ipsilateral and contralateral sides as ramified star-like cells characteristic of inactive microglia (Figure 2Aa,Dd). In the MCAO rats, OX-42-immunopositive microglial cells in the peri-infarct

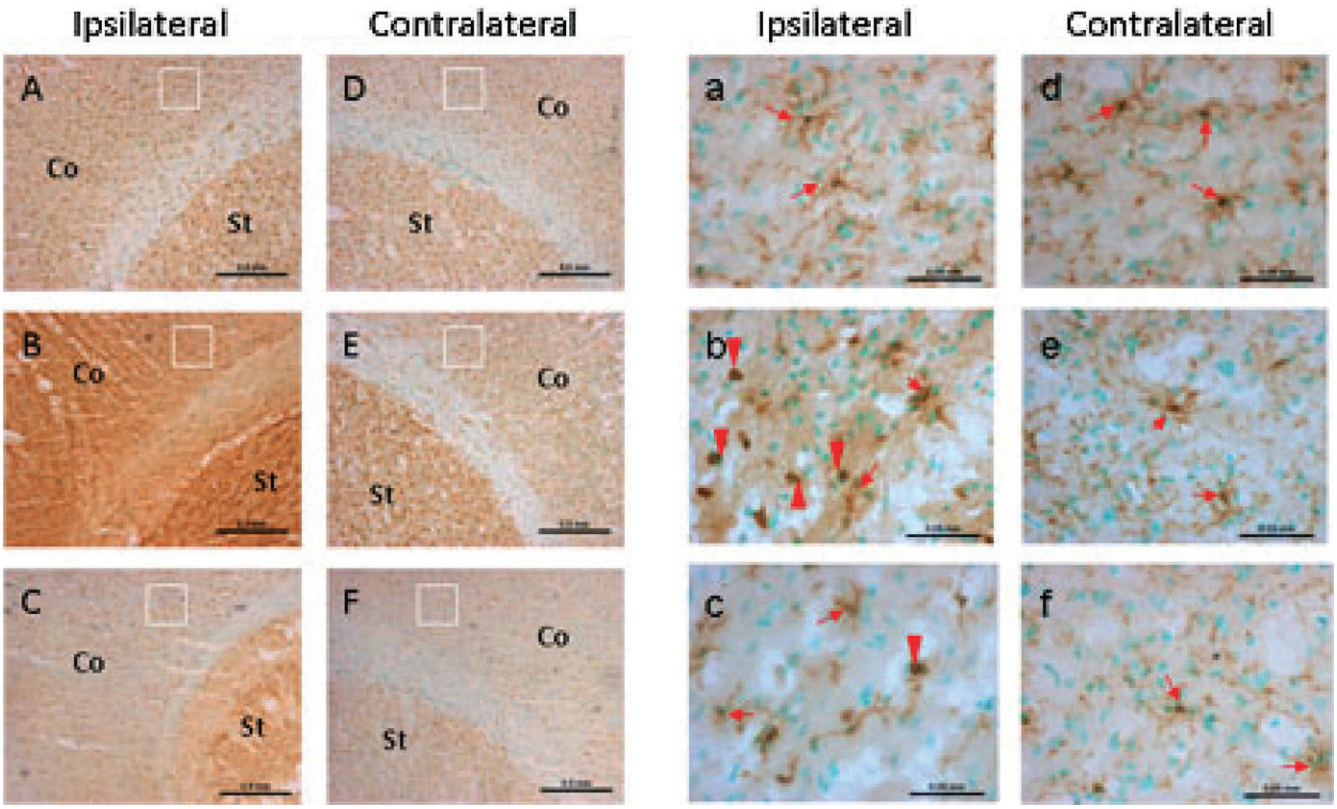

Figure 2

OX-42 immunohistochemical staining in peri-infarct regions. Photomicrographs were taken with coronal sections at –1.8 mm from bregma. Sham control rats (Aa and Dd), vehicle-treated pMCAO (Bb and Ee) and andrographolide-treated rats (Cc and Ff) 24 h after pMCAO. White squares in A–F (4× magnification) indicate the areas shown in a–f at 40× magnification; scale bar represents 0.5 or 0.05 mm respectively. Arrows indicate OX-42-immunopositive ramified star-like microglial cells, and arrowheads indicate activated microglial cells with a rounded amoeboid morphology. Co, cortex; St, striatum.

and infarct core appeared to be activated, containing phagolysosomes and characterized by a rounded amoeboid morphology with retracted or degenerated processes. In contrast, microglial activation was not observed in the contralateral side (Figure 2Bb,Ee). However, in andrographolide-treated rats, many fewer activated microglia were observed (Figure 2Cc,Ff). Andrographolide significantly reduced the total number of microglia, confirmed by quantitative cell counting in both the infarct core and peri-infarct regions (Table 2).

ED-1 stains for macrophages including phagocytic microglia. Consistent with results obtained by OX-42 immunostaining, ED-1-immunopositive cells were observed only in the ipsilateral side of MCAO rats (Figure 3Bb,Ee), and andrographolide treatment reduced the number of these cells (Figure 3Cc,Ff). Quantitative cell counts showed that the reduction was statistically significant in the peri-infarct areas, but not the infarct core (Table 3), indicating that microglial activation was significantly reduced by andrographolide only in the peri-infarct areas.

Effect of andrographolide on inflammatory markers

Figure 4 shows the expression of IL-1β (A) and TNF-α (B) in the brain tissues 24 h after pMCAO. Both cytokines increased markedly, by approximately 45- and 27-fold, respectively, but only in the ischaemic hemisphere after pMCAO. Treatment with andrographolide (0.1 $mg \cdot kg^{-1}$) markedly reduced IL-1β to a level that was only about fivefold higher than control levels. Surprisingly, andrographolide abolished the increase in TNF-α which was maintained at control levels.

Effect of andrographolide on pMCAO-induced up-regulation of PGE_2

Similarly, PGE_2 levels were significantly elevated by about 40% in the ischaemic hemisphere in pMCAO rats, and andrographolide abolished this increase in PGE_2 levels (Figure 5).

Table 2

Quantification of OX-42-immunopositive cells

ROI (from bregma)	Infarct region	MCAO only	MCAO + andrographolide
0.2	Infarct core	238 ± 18	131 ± 10*
	Peri-infarct	153 ± 2	74 ± 14*
–1.8	Infarct core	198 ± 27	111 ± 6*
	Peri-infarct	100 ± 7	75 ± 6*
–2.7	Infarct core	239 ± 4	165 ± 14*
	Peri-infarct	129 ± 13	37 ± 2*

OX-42-immunopositive cells were counted in the infarct core and peri-infarct region. In each region, three randomly selected areas of 0.25 mm^2 were counted under the microscope. Data shown are from rats with pMCAO treated with vehicle (MCAO only) or with andrographolide (0.1 $mg \cdot kg^{-1}$; MCAO + andrographolide). All the values were expressed as means ± SEM $N = 3$ per treatment group. *$P <$ 0.05 versus MCAO only (*t*-test).

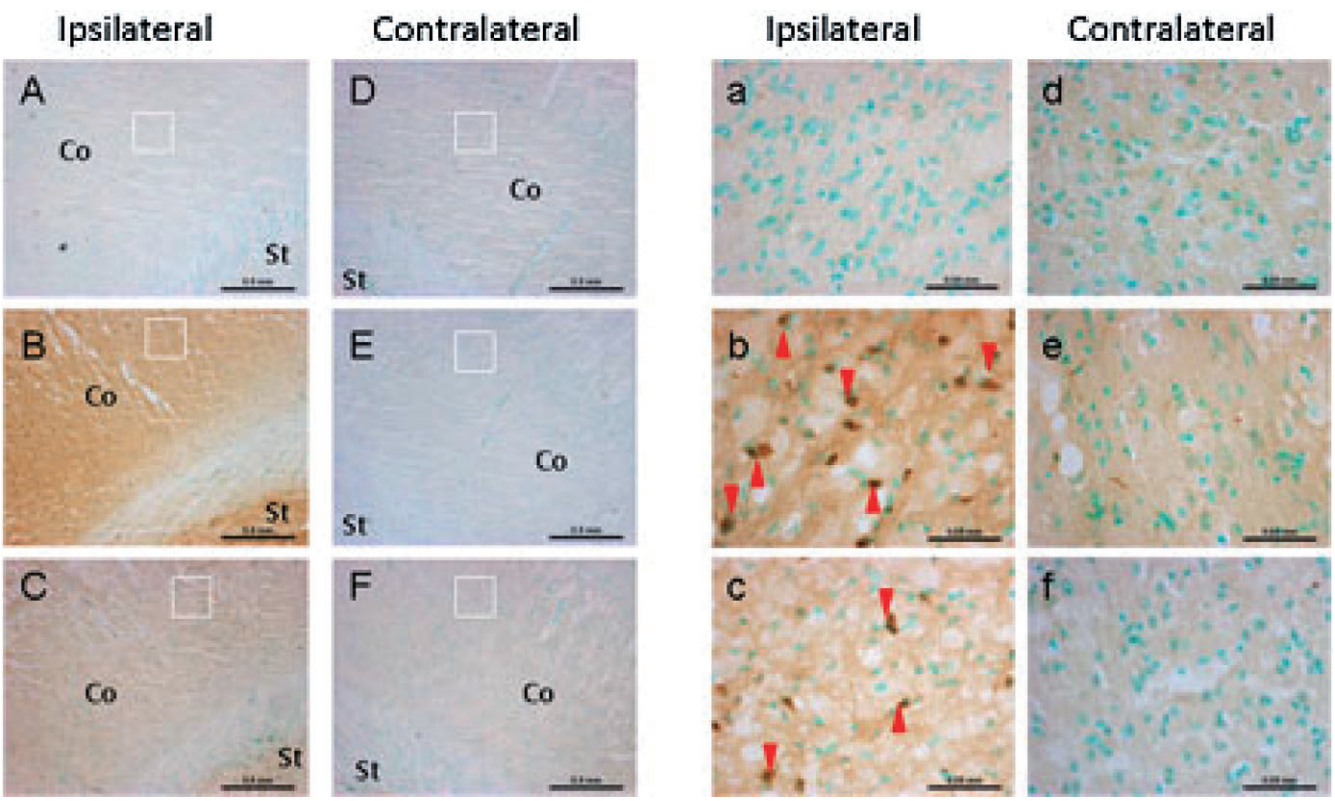

Figure 3

ED-1 immunohistochemical staining in peri-infarct region. Photomicrographs were taken with coronal sections at –1.8 mm from bregma. Sham control rats (Aa and Dd), vehicle-treated pMCAO (Bb and Ee) and andrographolide-treated rats (Cc and Ff) 24 h after pMCAO. White squares in A–F (4× magnification) indicate the areas shown in a–f at 40× magnification; scale bar represents 0.5 or 0.05 mm respectively. Arrowheads indicate ED-1-immunopositive activated microglial cells with a rounded amoeboid morphology. Co, cortex; St, striatum.

Effect of andrographolide on p65 subunit translocation

NF-κB is a transcription factor crucial for inflammatory gene expression. Activation of NF-κB promotes nuclear translocation of p50 and p65 subunits. In the present study, we observed a marked p65 subunit translocation 24 h after pMCAO, which was abolished by andrographolide treatment (Figure 6), suggesting that andrographolide suppressed the activation of NF-κB.

Table 3

Quantification of ED1-immunopositive cells

ROI (from bregma)	Infarct region	MCAO only	MCAO + andrographolide
0.2	Infarct core	72 ± 10	43 ± 8
	Peri-infarct	65 ± 3	14 ± 4*
–1.8	Infarct core	139 ± 11	113 ± 13
	Peri-infarct	155 ± 9	45 ± 13*
–2.7	Infarct core	240 ± 50	109 ± 38
	Peri-infarct	184 ± 21	37 ± 7*

ED-1-immunopositive cells were counted in the infarct core and peri-infarct region. In each region, three randomly selected areas of 0.25 mm^2 were counted under the microscope. Data shown are from rats with pMCAO treated with vehicle (MCAO only) or with andrographolide (0.1 $mg \cdot kg^{-1}$; MCAO + andrographolide). All the values were expressed as means ± SEM $N = 3$ per treatment group. *$P < 0.05$ versus MCAO only (*t*-test).

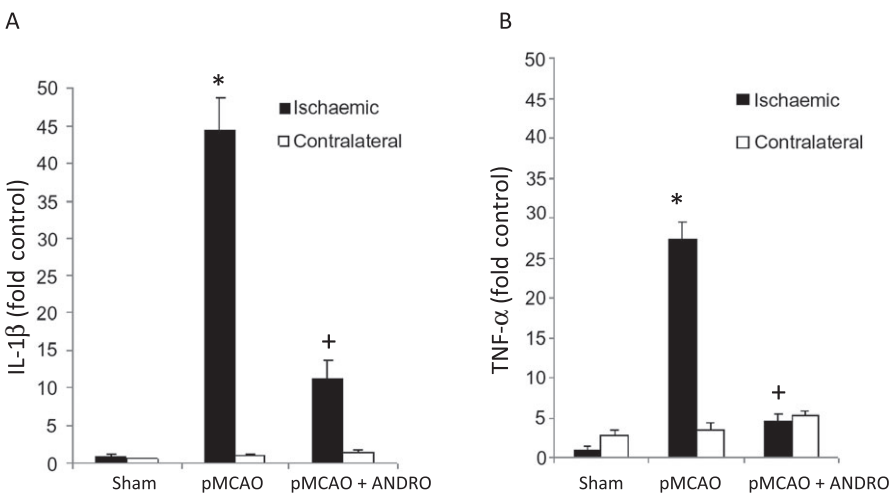

Figure 4

Effects of andrographolide (ANDRO) (0.1 $mg \cdot kg^{-1}$, i.p., 1 h after the onset of stroke) on the production of IL-1β (A) and TNF-α in the ischaemic hemisphere. Data are presented as fold changes over sham control in protein levels from brain homogenates. $N = 3–5$. *$P < 0.001$ compared with sham. +$P < 0.001$ compared with vehicle-treated group.

Discussion

Inflammation plays a key role in cerebral ischaemic injury (Barone and Feuerstein, 1999; Samson *et al.*, 2005; Chamorro and Hallenbeck, 2006; Muir *et al.*, 2007). Elevated levels of reactive oxygen species (ROS), generated by the cessation of cerebral blood flow, stimulate cells to secrete cytokines and chemokines which subsequently cause the secondary ischaemic damage (Wang *et al.*, 2007a). This is characterized by the infiltration of leucocytes and microglial activation (Dirnagl *et al.*, 1999). Therefore, drugs that have a wide spectrum of inhibitory actions on inflammation may be useful in rescuing neuronal cells, exposed to ischaemia.

Andrographolide dose-dependently reduced the production of interferon (IFN)-γ and IL-2 in T lymphocytes induced by concanavalin-A (Burgos *et al.*, 2005; Carretta *et al.*, 2009). It also inhibited the production of TNF-α and IL-12 in lipopolysaccharide-stimulated macrophages (Qin *et al.*, 2006; Abu-Ghefreh *et al.*, 2009). Current evidence suggests that andrographolide attenuates inflammation through the inhibition of NF-κB, an important transcription factor responsible for the inflammatory response (Xia *et al.*, 2004; Iruretagoyena *et al.*, 2006; Wang *et al.*, 2007b). Inhibition of NF-κB activation attenuates cytokine release (Bao *et al.*, 2009) and microglial activation (Wang *et al.*, 2004). On the other hand, NF-κB inhibition

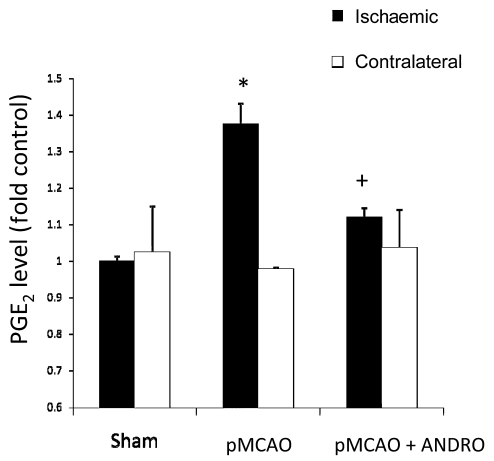

Figure 5

Effects of andrographolide (ANDRO) (0.1 mg·kg^{-1}, i.p., 1 h after the onset of MCAO) on the production of prostaglandin E_2 (PGE_2). Data are presented as fold changes over sham control in protein levels from brain homogenates. $N = 3–6$. *$P < 0.001$ compared with sham stroke. +$P < 0.01$ compared with the pMCAO group.

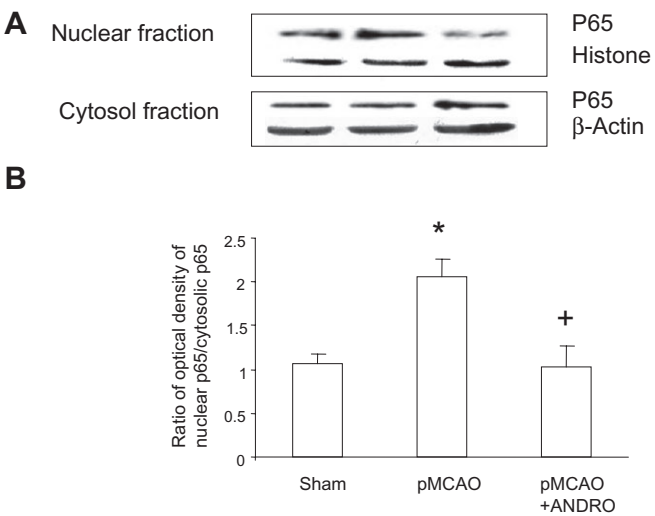

Figure 6

Effects of andrographolide (ANDRO) (0.1 mg·kg^{-1}, i.p., 1 h after the onset of MCAO) on NF-kB translocation from cytosol to nucleus. (A) Representative Western blots of p65 protein in cytosolic and nuclear fractions in sham, 24 h after pMCAO and pMCAO with 0.1 mg·kg^{-1} ANDRO treatment. (B) Quantification of p65 protein translocation level from cytosol to nucleus. Results were expressed as mean ± SEM of ischaemic hemispheres from sham, pMCAO and pMCAO with 0.1 mg·kg^{-1} ANDRO treatment respectively. $N = 4$. *$P < 0.05$ compared with the sham group. +$P < 0.05$, compared with the pMCAO group.

has been reported to increase the synthesis and secretion of macrophage migration inhibitory factor from human CD4+ T cells (Cho *et al.*, 2009).

In the present study, we demonstrated that andrographolide (0.01–1 mg·kg^{-1}) reduced infarct volume (Figure 1) and improved neurological score (Table 1) in the rat pMCAO stroke model. This is perhaps not surprising, given the recent reports of anti-inflammatory (Wang *et al.*, 2007b; Abu-Ghefreh *et al.*, 2009; Bao *et al.*, 2009), antioxidative stress (Akowuah *et al.*, 2009) and anti-oedematogenic (Lin *et al.*, 2009; Sulaiman *et al.*, 2010) activities of andrographolide. Andrographolide reduced infarct volume caused by pMCAO mainly in the peri-infarct region where energy-rich phosphates are preserved, but this region suffers from episodes of compromised energy metabolism during the passage of persistent neuronal depolarizations. Pharmacological inhibition of such depolarizations reduces infarct size in animal model (Back, 1998). Andrographolide, which possesses multiple therapeutic functions, may protect this energy-conserved region via inhibition of such depolarizations. However, there is currently no direct evidence for such inhibition.

Resident microglia express complement type 3 receptor (CR-3) and are recognized by mAb OX-42 in rats. We found in the current study that a significant increase of OX-42-stained cells (Table 2), as well as activated cells (Figure 2B and b), was observed in the ipsilateral brain, but not in contralateral side after pMCAO insult. This is consistent with previous findings that OX42 immunoreactivity on activated microglia is increased within hours after pMCAO due to the up-regulation of CR-3 receptors (Stoll *et al.*, 1998).

Macrophages and activated microglia can be recognized by mAb ED-1 as they express MHC class I and II molecules (Stoll *et al.*, 1998). By inducing photochemical infarction in macrophage-depleted rats, Schroeter *et al.* (1997) suggested that immunoreactive phagocytes are mainly derived from resident microglia, while the macrophages were recruited from the bloodstream only in the delayed stages. Immunostaining with ED-1 demonstrated activated microglia as shown in Figure 3B and b. Activation of microglia changes them from ramified into amoeboid phagocytic cells (Schroeter *et al.*, 1997), and causes the release of a variety of cytotoxic and cytoprotective substances (Wood, 1995). Activated microglia may serve as scavengers in inflammation and/or deleterious factors in ischaemic injury (Yrjanheikki *et al.*, 1999; Gunther *et al.*, 2005; Zhang *et al.*, 2005). Consistent with the findings on infarct volume and neurological score, treatment with andrographolide markedly reduced the number of microglia in both the infarct core and the peri-infarct region, while attenuating microglial activation in the peri-infarct region

(Figures 2 and 3; Tables 2 and 3). This is also consistent with a previous finding that andrographolide attenuated LPS-induced microglial activation (Wang *et al.*, 2004). The suppression of the transformation of microglia into phagocytes that exacerbate cytokine secretion implies that andrographolide may be able to reduce cytokine up-regulation after stroke. This is consistent with the finding of Wang *et al.* (2004) that andrographolide reduces the production of pro-inflammatory mediators including ROS, TNF-α, NO and PGE_2 in microglial cultures.

IL-1β and TNF-α are well-studied cytokines related to inflammatory responses in stroke, and both appear to exacerbate ischaemic damage (Yamasaki *et al.*, 1995; Barone *et al.*, 1997). Accordingly, down-regulating IL-1β and TNF-α expression appears to reduce infarcts (Garcia *et al.*, 1995; Yang *et al.*, 1998). Consistent with these earlier findings, we found here that pMCAO markedly increased the production of these cytokines which were effectively abolished by treatment with andrographolide (Figure 4). Similar results were obtained for PGE_2, although much smaller increases were observed after pMCAO (Figure 5). COX-2 inhibition has been shown to be beneficial in neurological outcome after stroke. It was reported that COX-2 is up-regulated in post-mortem specimens of ischaemic stroke patients (Iadecola *et al.*, 1999), and COX-2 exerts its deleterious actions via its downstream products including PGE_2. COX-2-deficient mice had a significant reduction in the brain injury produced by MCAO showing that COX-2-mediated inflammation has a significant role in stroke (Iadecola *et al.*, 2001).

Among numerous transcription factors that are activated in cerebral ischaemia, NF-κB is well recognized in the regulation of inflammation, and a key regulator of many genes involved in cell survival and inflammation (Clemens *et al.*, 1997; Schneider *et al.*, 1999). Pro-inflammatory NF-κB target genes, including TNF-α, IL-1α and β, iNOS, COX-2 and ICAM-1 (Wang *et al.*, 2007a), all contribute to ischaemic damage. Mice deficient in the p50 subunit of NF-κB and specific inhibition of NF-κB by pyrrolidinedithiocarbamate (PDTC) show reductions in infarct size in both permanent and transient stroke model, suggesting that inhibition of NF-κB activation could be a therapeutic target in cerebral ischaemia (Schneider *et al.*, 1999; Nurmi *et al.*, 2004). In our study, we found that andrographolide exerted its anti-inflammatory effects probably via inhibition of p65-subunit of NF-κB translocation. This may suppress the expression of the above cytokines and inflammatory factors which, in turn, suppresses microglia activation. However, there is a possibility that the reduction in inflammatory cytokines by andrographolide treatment is simply a result of diminished infarction, although this is unlikely considering the many fold changes observed.

It was reported that andrographolide remarkably decreases platelet aggregation induced by thrombin in a concentration- and time-dependent manner (Thisoda *et al.*, 2006). Wang *et al.* (2007b) found that andrographolide abolished the deposition of leucocytes (mainly CD68+ macrophages) in the injured arterial walls by reducing the up-regulation of NF-κB target genes, including tissue factor, E-selectin and VCAM-1. In addition, andrographolide was protective against deep vein thrombosis in a murine model (Li *et al.*, 2009). All of these findings suggest that the effects of andrographolide on platelet function and leucocyte infiltration may also contribute to its neuroprotective effects in stroke. Further studies on the effects of andrographolide on cerebral vascular tone are warranted.

After oral administration of andrographolide, 10 metabolites were found in blood, urine, bile and the contents of the small intestine and stomach of rats (He *et al.*, 2003). To the best of our knowledge, there is no evidence showing that any of these metabolites are active. Moreover, the more polar metabolites are less likely to cross the brain–blood barrier. In contrast, andrographolide was reported to be able to cross the brain–blood barrier (Lu, 1995). These findings argue against the notion that neuroprotective effects of andrographolide may result mainly from its metabolites.

In conclusion, we have demonstrated that andrographolide produced neuroprotective effects against cerebral ischaemia with accompanying inhibition of microglia activation, possibly caused by the suppression of NF-κB activation, leading to a reduction in the production of cytokines including TNF-α and IL-1β, and pro-inflammatory factors such as PGE_2. Our findings may have important implication in the treatment of stroke. Finally, it should be noted that the possibility of andrographolide acting through a completely different pathway cannot be ruled out.

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Conflict of interest

None.

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