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Neuroprotective effects of breviscapine against apoptosis induced by transient focal cerebral ischaemia in rats

Liu Yiming, He Wei, Lin Aihua and Zeng Fandian

Abstract

Breviscapine, a flavonoid isolated from the traditional Chinese medicinal herb *Erigerin breviscapus*, has been proved to be effective in protecting the brain against ischaemic damage, but the mechanisms remain unknown. In this study, we have demonstrated the effects of breviscapine on neuronal apoptosis in a rat model of transient focal cerebral ischaemia. Rats were administered with breviscapine (50 or 100 mg kg^{-1}/day) intragastrically for seven successive days, then subjected to 2-h brain ischaemia induced by middle cerebral artery occlusion, followed by 24-h reperfusion. After reperfusion, the rats were killed and the brain samples were collected. Haematoxylin-eosin staining was used to evaluate the histopathological changes. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) and flow cytometry (FCM) analysis were used to detect the level of apoptosis. The expressions of bcl-2 and caspase-3 in the cortex were determined by Western blot. Significant increases in the number of haematoxylin–eosin- and TUNEL-positive staining cells and DNA fragmentation were observed at 24 h after reperfusion, and the increases were inhibited by breviscapine (50 and 100 mg kg^{-1}). Breviscapine at both doses markedly inhibited the expression and activation of caspase-3 and up-regulated the expression of bcl-2. These findings suggested that breviscapine attenuated neuroapoptosis and regulated the protein expression related to apoptosis after transient focal cerebral ischaemia, which may have contributed, in part, to the protective effects of breviscapine on cerebral ischaemic damage.

Introduction

Breviscapine is the flavonoid constituent isolated from a traditional Chinese herb *Erigerin breviscapus* (Vant.) Hand-Mazz. The main active ingredient is scutellarin (4′, 5, 6-tetrahydroxyflavone-7-O-glucuronide). Previous studies (Chen & He 1997; He & Zeng 2002) have shown that breviscapine could dilate blood vessels, improve microcirculation, increase cerebral blood flow, diminish the aggregation of platelets and scavenge oxygen free radicals. Moreover, in China breviscapine has been extensively used clinically to treat cerebral infarction and the sequelae. Breviscapine injection (20 mg, i.v., gtt., once a day, 14 days) and breviscapine tablet (40 mg, p.o., 3-times a day, 2 months) have been reported to be effective in the treatment of cerebral infarction (Bu 1999; Yang & Li 2007). Another clinical observation (Zhang 1998) demonstrated the curative effect of breviscapine tablet (40 mg, p.o., 3-times a day, 1 month) on the sequelae after stroke.

Most evidence has shown that cerebral ischaemia initiates a cascade of detrimental events, including the accumulation of intracellular calcium, the disorder of energy metabolism, the formation of free radicals and cytokines, and mitochondrial injury and so on, which result in serious neuronal injury (Hou & MacManus 2002). Although damaged neurons often die from necrosis, a significant amount of neurons particularly at the inner boundary of the ischaemic lesion (penumbra) may die from apoptosis (Ginsberg 2003). After focal ischaemia in mice, the predominant localization of apoptotic cells at the inner boundary of the ischaemic lesion suggests that the apoptotic process largely contributes to the expansion of ischaemic damage (Murakami et al 1997). Inhibition of apoptosis may

Central Laboratory of Guangdong Provincial Hospital of Traditional Chinese Medicine, Guangzhou University of Traditional Chinese Medicine, Guangzhou 510120, China

Liu Yiming, Lin Aihua

Institute of Clinical Pharmacology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Liu Yiming, He Wei, Zeng Fandian

Correspondence: Liu Yiming, Central Laboratory of Guangdong Provincial Hospital of TCM, Guangzhou University of Traditional Chinese Medicine, Guangzhou 510120, China.
E-mail: lyming2000@163.com

reduce neuronal death and infarct size, which plays an important role in cerebral ischaemic injury and the treatment of ischaemic stroke (Padosch & Bottiqer 2003). Apoptosis in ischaemic brain injury is regulated by many factors. The bcl-2 protein has an important role in the inhibition of cell death through decreasing the generation of reactive oxygen species, binding to pro-apoptotic proteins (e.g. Bax and Bid), blocking cytochrome c release and inhibiting caspases activation (Kane et al 1993; Krajewski et al 1999; Soane & Fiskum 2005). Caspases have been demonstrated to be involved in the initiation and execution phases of apoptosis (Zhao et al 2005). As an effector, caspase-3 can be activated by distinct pathways of initial caspase activation and results in apoptotic death. Inhibition of apoptotic pathways with caspase inhibitors reduces ischaemic damage after transient focal cerebral ischaemia (Li et al 2000; Li et al 2001).

The neuroprotective effects of breviscapine on cerebral ischaemia have been reported (He & Zeng 2002; He et al 2003; Xiong et al 2006), but the underlying mechanism has not been fully elucidated. In this study, we have investigated the effects of breviscapine on neuronal apoptosis and the changes of bcl-2 and caspase-3 in the penumbra using a rat model of transient focal cerebral ischaemia.

Materials and Methods

Drugs and reagents

Breviscapine (purity > 95%) was supplied by Yunnan Yuxi Pharmaceutical Co., Ltd. Propidium iodide (PI) and RNase A were purchased from Sigma Company (USA). Mouse monoclonal antibody against bcl-2 (sc-7382) and rabbit polyclonal antibody against caspase-3 (sc-7148) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). In-situ cell death detection kit (POD) for terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labelling (TUNEL) study was from Boehringer Mannheim (Germany). Caspase-3 activity assay kit was from R&D Company. Other reagents were of analytical grade.

Transient focal cerebral ischaemia

The experimental protocol was approved by the Committee of Tongji Medical College of Huazhong University of Science and Technology on Ethics of Animal Experimentation.

Adult male Sprague–Dawley rats (200 ~ 250 g; Experimental Animal Center of Tongji Medical College, Grade II, Certificate N0.19-053) were subjected to transient focal ischaemia by intraluminal middle cerebral artery occlusion (MCAO) with a nylon suture (Longa et al 1989). Briefly, rats were anaesthetized with 10% chloral hydrate (350 mg kg^{-1}, i.p.), the bifurcation of the right common carotid artery (CCA) was exposed, the external carotid artery (ECA) was dissected and ligated distally and the internal carotid artery (ICA) was isolated and separated from the vagus nerve. A 50-mm length monofilament nylon suture (φ 0.23 mm), whose tip had been rounded by heating near a flame, was introduced into the ICA through the stump of the ECA and advanced carefully for a distance of 20 ± 2 mm from the CCA bifurcation to block the origin of the MCA. The blood flow to the MCA was restored by withdrawal of the nylon suture 2 h after occlusion. Sham operations were performed using the same surgical procedures except that no suture was inserted. Animals were randomized into four groups (n = 5 for each): sham-operated group; vehicle-treated group in which only normal saline was administered; breviscapine 50 mg kg^{-1} group (Bre-L group); and breviscapine 100 mg kg^{-1} group (Bre-H group). Breviscapine was administered intragastrically once daily for seven successive days before the operation. Room temperature was maintained in the range of 25–27°C throughout the experiment.

Haematoxylin–eosin staining and TUNEL assay

At 24 h of reperfusion after 2 h of MCA occlusion, rats were anaesthetized with 10% chloral hydrate and transcardially perfused with 100 mL normal saline, followed by 250 mL 4% paraformaldehyde in 0.1 mol L^{-1} phosphate buffer solution (PBS, pH 7.4, 4°C). The brains were removed and post-fixed overnight in the same buffer at 4°C. Each was embedded in paraffin and 6-μm coronal sections between 3 and 4 mm posterior of the bregma were prepared for neuropathological studies. Two sections from each rat were stained with haematoxylin–eosin for histopathological examination.

The TUNEL assay was performed according to a reported method (Gavrieli et al 1992; Sobrado et al 2003) to detect DNA fragmentation and apoptotic bodies in cortex penumbra cells. Briefly, de-paraffined and rehydrated sections were pretreated with proteinase K and placed in TUNEL reaction buffer for 60 min at 37°C, followed by incubation with fluorescein–POD complex for 30 min at 37°C. The staining was visualized by diaminobenzidine (DAB) (Fluka). For quantitative analysis of TUNEL-positive cells, five microscopic fields on the ischaemic cortex of each stained section were observed under high-power microscopic fields (×200) and analysed by HPIAS-1000 image analysis system (Champion Imagine). For each animal five sections were analysed.

DNA fragmentation assay by flow cytometry (FCM)

The DNA fragmentation in this study was detected by FCM with the Sub-G1 method (Tao et al 1999). After treatment with phosphate-citric acid (PC) buffer, the degraded and low-molecular-weight DNA was extracted from apoptotic cells, which led to the decrease of DNA content in cells. In the DNA histogram, the apoptotic cells were distinguished by an extra Sub-G1 peak ahead of the G1 peak. At 24 h after reperfusion, the brains were quickly removed and divided into ipsilateral (infarct) and contralateral (non-infarct) hemispheres. Cell suspensions were prepared from ipsilateral cortex tissues in PBS according to the method of reference (Chen et al 2002) with some modifications. Briefly, the tissues were mechanically dissected with eye scissors and placed into PBS containing 0.125% trypsin. Trypsinization was performed at 37°C for 20 min, after which time it was discontinued by adding RPMI 1640 medium containing 0.5% bovine serum. The isolated cells were filtered through nylon mesh (200 meshes), were washed with PBS, and fixed in 75% ethanol at −20°C. Before analysis, cells were washed again with PBS, resuspended, treated with PC buffer, and then treated with

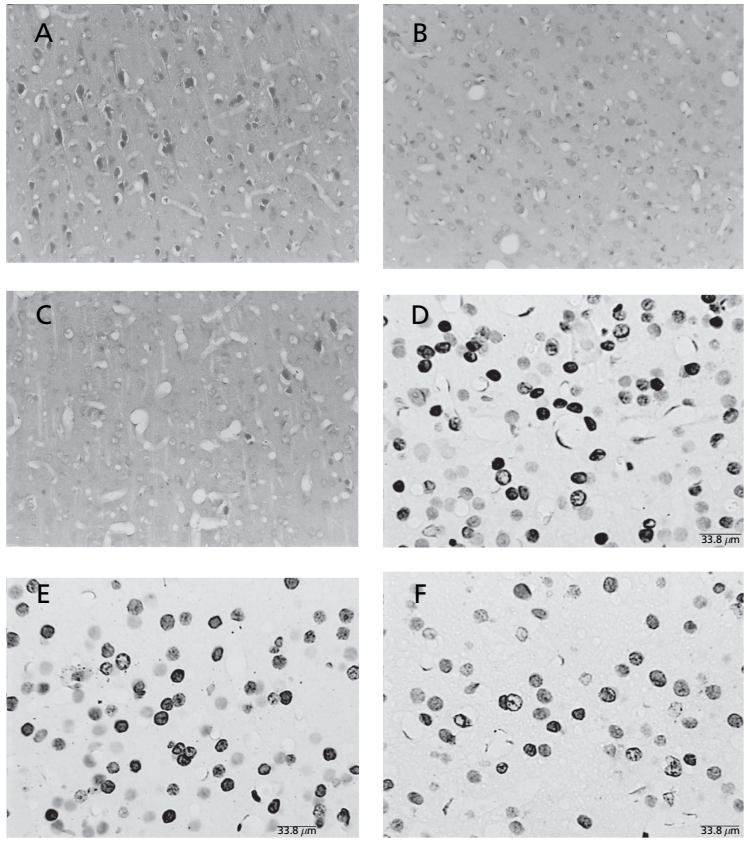

Figure 1 Representative photomicrographs of haematoxylin–eosin staining (A–C) and TUNEL analysis (D–F) of cells in cortex penumbra in coronal cerebral sections between 3 and 4 mm posterior of the bregma after 2 h of the MCA occlusion followed by 24 h of reperfusion in rats pretreated with or without breviscapine. A, D: animals administered with normal saline intragastrically once daily for 7 days before MCAO (vehicle-treated); B, E: animals administered with breviscapine 50 mg kg^{-1} (Bre-L); C, F: animals administered with breviscapine 100 mg kg^{-1} (Bre-H). The magnification of the photographs was 200 ×.

20 μL RNase A (1 g $\cdot$ L^{-1}) for 30 min at 37°C. The cells were then incubated with 200 μL PI solution (100 mg $\cdot$ L^{-1}) in darkness for 30 min at 4°C. DNA content was determined with a Becton-Dichison FACS flow cytometer. The cell population in Sub-G1 area (apoptotic percentage) was quantified from a standard count of 10 000 cells using CELLQUEST. ModFIT LT for mac V1.01, BD.

Western blot analysis

After 24 h of reperfusion, the samples corresponding to the penumbra zone were collected for Western blot assays. The tissues were homogenized in ice-cold buffer (Tris 50 mmol L^{-1}, pH 7.4, NaCl 150 mmol L^{-1}, 0.5% TritonX-100, edetic acid 1 mmol L^{-1}, phenylmethylsulfony fluoride 1 mol L^{-1}, and aprotinin 5 mg L^{-1}), and centrifuged at 14 000 *g* for 30 min at 4°C. The supernatants were then collected as total proteins. Protein concentration was determined by the Lowry method. Protein samples (50 μg) were denatured in 15% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrically transferred to a nitrocellulose membrane. The membrane was incubated in 5% milk TBST at 4°C overnight, and subsequently incubated with primary antibodies: the mouse monoclonal antibody against bcl-2 (1:200) and the rabbit polyclonal antibody against caspase-3 (1:200). After washing with TBST, the membranes were incubated with the secondary antibodies (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG) at room temperature for 1 h. The gels were scanned and quantified by Image Quant V1.19 (Molecular Dynamics, Sunnyvale, CA). β-Actin as an internal control was also analysed by Western blot. The levels of bcl-2, caspase-3 and β-actin were quantified by Image Quant programmer, and the relative amount was obtained after normalization with β-actin values in the same lane.

Analysis of caspase-3 activity

The enzymatic activity of the caspase-3 in apoptotic cells was determined by fluorometric reaction (Yin et al 2002). When activated, the caspase-3 cleaves the specific substrate DEVD-AFC, and the fluorochrome AFC that emits fluorescence at 505 nm wavelength when excited by light at 400 nm is released. The level of caspase-3 enzymatic activity is directly proportional to the fluorescence signal detected with a fluorimeter. After 2-h ischaemia and 24-h reperfusion, brains were quickly removed and ischaemic cortex tissues were

dissected. Caspase-3 activity was measured according to the instructions of the enzyme activity assay kit. Protein extracts were prepared on ice by homogenization of tissues in a lysis buffer. Following centrifugation at 14 000 rev min^{-1} for 20 min (4°C), the supernatant was separated and the protein concentration was adjusted to 3 g L^{-1}. Samples (50 μL) were incubated for 1.5 h at 37°C with 5 μL DEVD-AFC and 50 μL reaction buffer. The release of the fluorogenic group AFC was measured by a fluorescence spectrometer (PerkinElmer Life Sciences LS50-B) at 400/505 nm (excitation/emission). The results were expressed as ratio of values to those in the sham-operated group.

Statistical analysis

Data were presented as mean ± s.d., and analysed using analysis of variance followed by Student–Newman–Keul's test. $P < 0.05$ was accepted to be statistically significant.

Results

Anti-apoptotic effect of breviscapine

In the vehicle-treated group, the neurons in the cortex of ischaemic penumbra showed the following features: a decrease of cell size, condensation of cytoplasma, and aggregation of chromatin into dense staining. A large number of cells were scored as apoptosis when they were TUNEL-positive (brown staining) and showed nuclear chromatin condensation (Figure 1A, D), whereas there were only very few in the sham-operated group and the contralateral hemisphere of ischaemic rats. Treatment with breviscapine (50 or 100 mg kg^{-1}) obviously reduced the damage of the neurons (Figure 1B, C, E, F). Compared with the sham-operated group (1.4 ± 1.1 mm^{-2}), the number of TUNEL-positive cells in the cortex of ischaemic hemisphere was significantly increased in the vehicle group (24.8 ± 5.1 mm^{-2}, $P < 0.01$). Compared with the vehicle group, the increased number was significantly reduced to 19 ± 3.2 mm^{-2} ($P < 0.05$) and 14 ± 3.4 mm^{-2} ($P < 0.01$) in 50 and 100 mg kg^{-1} breviscapine-treated groups (Figure 2), respectively. There was a significant difference between Bre-H and Bre-L groups ($P < 0.05$).

Besides TUNEL analysis, FCM detection also indicated the anti-apoptotic effect of breviscapine. After 2-h MCAO and 24-h reperfusion, the percentage of apoptotic cells in the cortex was $29.53 \pm 6.98\%$. Breviscapine at the doses of 50 and 100 mg kg^{-1} resulted in reduction of apoptotic percentage to 20.74 ± 4.3 and $16.64 \pm 3.13\%$, respectively (Figure 3, $P < 0.05$ and $P < 0.01$ compared with vehicle group). Breviscapine 100 mg kg^{-1} had greater effect than 50 mg kg^{-1}.

Effects of breviscapine on the expression of caspase-3 and bcl-2

Normally, the caspase-3 protease in cells exists as an inactive precursor ($M_r = 32\,000$), which is cleaved into two subunits of p17 ($M_r = 17\,000$) and p12 ($M_r = 12\,000$) when activated. The antibody against caspase-3 we used recognized both the precursor and the p17 in the brain protein extracts. The sample

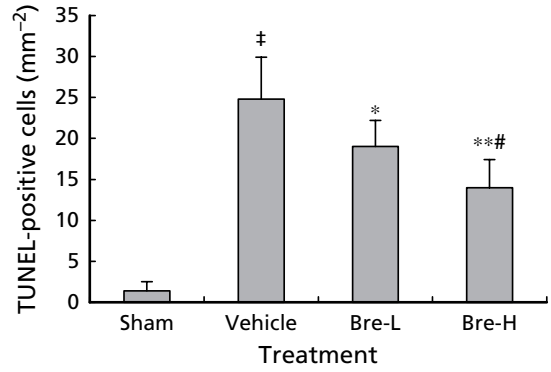

Figure 2 Quantitative analysis of the effects of breviscapine on the TUNEL-positive cells in the cortex penumbra after 2-h ischaemia and 24-h reperfusion in rats. Five microscopic fields per region on each section were analysed. There were a few TUNEL-positive cells in the ischaemic penumbra of vehicle group and significantly fewer in breviscapine-pretreated group. Vehicle: vehicle-operated group; Bre-L: breviscapine-treated group (50 mg kg^{-1}); Bre-H: breviscapine-treated group (100 mg kg^{-1}). Data are mean ± s.d., n = 5 rats. $^{‡}P < 0.01$ vs sham; $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle; $^{\#}P < 0.05$ vs Bre-L.

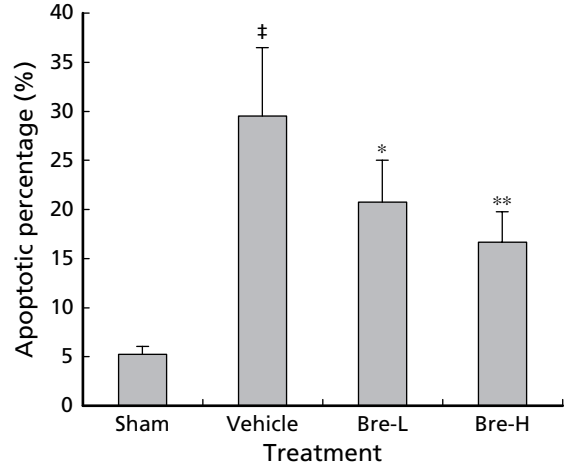

Figure 3 Effects of breviscapine on percentage of apoptotic cells in the cortex penumbra after MCAO (2 h ischaemia and 24-h reperfusion) in rats. The apoptotic percentage was determined by FCM. Breviscapine was given to Bre-L (50 mg kg^{-1}) and Bre-H (100 mg kg^{-1}) groups intragastrically once daily for seven successive days before MCAO, respectively. Rats in vehicle group were pretreated with normal saline. Data are mean ± s.d., n = 5 rats. $^{‡}P < 0.01$ vs sham; $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle.

obtained from the sham-operated rat brain cortex showed high level of the precursor expression but very low level of the p17 subunit expression (Figure 4A, lane 1); whereas the level of the p17 subunit significantly increased in the ischaemic cortex at 24 h after reperfusion (vs sham-operated group, $P < 0.01$) (Figure 4A, lane 2). The protein expression of bcl-2 in the ischaemic cortex was observed weakly or diffusely in the vehicle-treated rat brain (Figure 4B, lane 2), whereas the expression in sham-operated rat brain showed a considerable

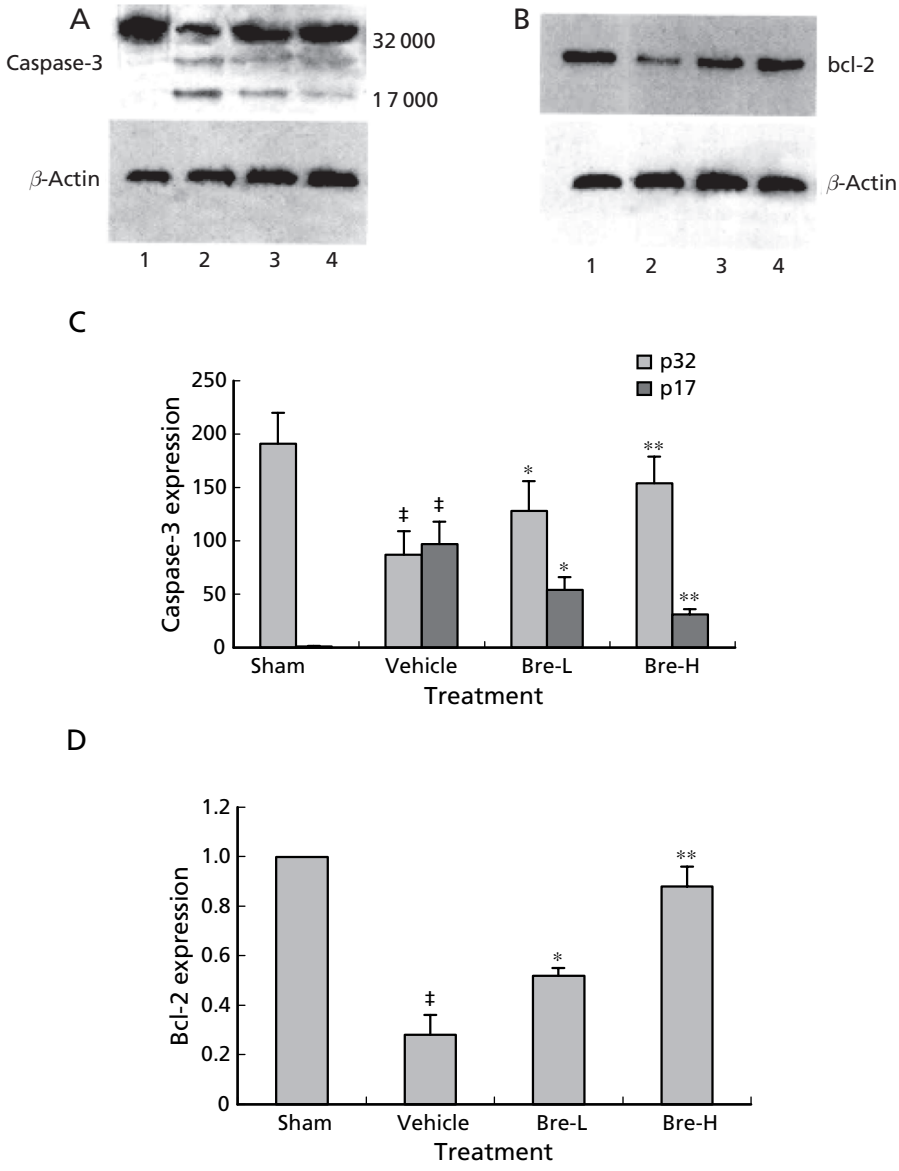

Figure 4 Western blot analysis of breviscapine on the expression of caspase-3 (A) and bcl-2 (B) after 2-h ischaemia and 24-h reperfusion in rats. Lane 1: sham; lane 2: vehicle; lane 3 and 4: breviscapine 50, 100 mg kg^{-1}; β-actin as an internal control. Activation of procaspase-3 ($M_r = 32\,000$) was indicated by the appearance of the p17 subunit ($M_r = 17\,000$). Semiquantitative analysis of breviscapine on the expression of caspase-3 (C) and bcl-2 (D) (the relative abundance of the immunostaining) determined by Image Quant programmer. The levels of caspase-3 and bcl-2 are expressed as % of absorbance. Breviscapine was given to Bre-L (50 mg kg^{-1}) and Bre-H (100 mg kg^{-1}) groups intragastrically once daily for seven successive days before MCAO, respectively. Rats in vehicle group were pretreated with normal saline. Data are mean ± s.d., n = 5 rats. $^{\ddagger}P < 0.01$ vs sham; $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle.

amount of bcl-2 protein (Figure 4B, lane 1). However, in the rats pretreated with breviscapine (50 or 100 mg kg^{-1}), the immunoreactivity of the p17 subunit was markedly decreased (Figure 4A, lanes 3 and 4), while the protein expression of bcl-2 was increased in the ischaemic cortex (Figure 4B, lanes 3 and 4) (vs vehicle group, $P < 0.05$ and $P < 0.01$). Quantitative analysis of breviscapine on the expression of caspase-3 and bcl-2 was described in Figure 4 C and D, respectively. In this Western blot analysis, a consistent amount of β-actin immunostaining was detected at the bottom panel, which suggested that the amount of loaded protein in each lane was consistent.

Effects of breviscapine on the activity of caspase-3

The enzymatic activity of caspase-3 in ischaemic cortex was measured using DEVD-AFC as the substrate, and the data were expressed as a ratio of values to those in the sham-operated group. In the vehicle group, the ratio was 1.86 ± 0.08, which suggested the activity of caspase-3 was

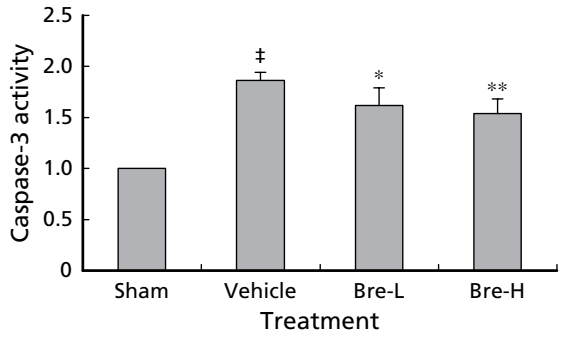

Figure 5 Effects of breviscapine on caspase-3 activity in the cortex after 2-h ischaemia and 24-h reperfusion in rats. Breviscapine was given to Bre-L (50 mg kg^{-1}) and Bre-H (100 mg kg^{-1}) groups intragastrically once daily for seven successive days before MCAO, respectively. Rats in vehicle group were pretreated with normal saline. Data are expressed as ratio of values to those in sham-operated group (mean ± s.d.), n = 5 rats. $^{‡}P < 0.01$ vs sham; $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle.

elevated after cerebral ischaemia–reperfusion. However, the elevation of caspase-3 activity was significantly suppressed by administration of breviscapine (Figure 5). In 50 and 100 mg kg^{-1} breviscapine-treated groups, the ratio was significantly decreased to 1.618 ± 0.174 and 1.538 ± 0.144 (vs vehicle group, $P < 0.05$ and $P < 0.01$), respectively.

Discussion

We have demonstrated the anti-apoptotic results of breviscapine from the rat cerebral cortex subjected to 2 h of MCAO followed by 24 h of reperfusion. Additionally, this compound preserved profoundly increased expression of bcl-2 protein with a significant decrease in caspase-3 protein and in the activity of caspase-3 enzyme. Clinical observations have indicated that the effective dosage of breviscapine is 40 mg (3-times a day) by oral administration for 1–2 months (Zhang 1998; Bu 1999). To shorten the administration time and simplify the experiment, we designed a protocol in which the rats were administered breviscapine (50 mg kg^{-1}, i.e., once a day, 7 days) before MCAO, and found that breviscapine significantly reduced the infarct size and brain oedema. Based on these findings, we continued to study the effect of breviscapine on apoptosis at 50 and 100 mg kg^{-1}.

Most of the evidence supporting a role of apoptosis in the progression of neuronal cell death comes from studies using animal models of cerebral ischaemia. Li et al (1995) documented that apoptosis contributed to the development of ischaemic infarction with abundant TUNEL-positive cells and DNA fragmentation, which prominently occurred in the penumbral zone. In this study, a number of TUNEL-positive cells were detected dominantly in the penumbral region at 24 h after reperfusion and a significant amount of DNA fragmentation was also detected by FCM, which were consistent with previous reports (Li et al 1995). Breviscapine, as a well-known traditional Chinese medicinal composition, has been proved to be effective on brain ischaemia (Chen & He 1997; He et al 2003). It is mainly composed of scutellarin, some plantaginin and pyromeconic acid (He & Zeng 2002). Lin et al (2007) demonstrated that there were no significant differences among rats treated with three different dosages of breviscapine (5–50 mg kg^{-1}), and the protective effects of scutellarin on cerebral ischaemia were better than breviscapine. However, breviscapine instead of scutellarin has long been used clinically to treat cerebral ischaemia (Zhang 1998; He & Zeng 2002; Yang & Li 2007). Therefore, we studied the effects of breviscapine on apoptosis induced by cerebral ischaemia, to compare the results with some different results from Lin et al (2007). In rats administered breviscapine 50 and 100 mg kg^{-1}, especially the latter, the level of DNA fragmentation in the cortex of the ischaemic hemisphere was significantly decreased. The above results suggested that cerebral ischaemia resulted in cell apoptosis in selectively vulnerable brain regions, and that breviscapine may have markedly decreased the number of TUNEL-positive cells and inhibited DNA fragmentation induced by focal cerebral ischaemia–reperfusion. Therefore, this study implicated that the protective effect of breviscapine on brain ischaemia-induced injury was partially due to the inhibition of neuroapoptosis.

In the process of apoptosis, the bcl-2 gene plays an important role in preventing apoptosis. By Western blot analysis, bcl-2 protein level was found to be significantly decreased in ischaemic brain subjected to 2-h MCAO and 24-h reperfusion (Hong et al 2002). Zhao et al (2003) and Tanaka et al (2005) suggested that overexpression of bcl-2 protein may protect neurons from ischaemia-induced cell death. On the contrary, increased levels of either expression or activity of effector caspase-3 are detected in ischaemic neurons (Han et al 2000; Chang & Wang 2003; Tang et al 2006) and caspases play the key role in cerebral ischaemia-induced apoptosis (Nicholson & Thornberry 1997; Pape et al 2006). Therefore, in this study, the expression of bcl-2 and the expression and activity of caspase-3 in the process of cerebral ischemia-induced apoptosis were detected with or without breviscapine pretreatment after 2-h MCAO and 24-h reperfusion. The results showed that the activation of caspase-3 and the reduction of bcl-2 expression were observed in the cortex of ischaemic hemisphere after reperfusion, which was consistent with Chang & Wang (2003) and Hong et al (2002). Breviscapine treatment markedly reduced the activation of caspase-3 and significantly up-regulated the expression of bcl-2 at the same time. Although it did not prove the hypothesis that the inhibition of breviscapine on cerebral ischaemia-induced apoptosis directly contributed to a decrease in the activation of caspase-3 and increase in the expression of bcl-2, these results at least implicated the possibility that breviscapine may have suppressed apoptosis partially due to the inhibition of caspase-3 expression, and activation and the increase of bcl-2 expression. These data may provide important information for more detailed study on the mechanism of breviscapine on cerebral ischaemia-induced apoptosis.

Conclusion

Treatment with breviscapine (50 or 100 mg kg^{-1}) may have suppressed the activation of caspase-3 protein, up-regulated

bcl-2 expression and reduced DNA fragmentation after transient focal cerebral ischaemia and reperfusion in rats. These findings on the inhibitory effects of breviscapine on brain ischaemic injury-induced apoptosis have provided important insight into the treatment of ischaemic cerebrovascular disease.

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