Multiple effects of 2ME2 and D609 on the cortical expression of HIF-1α and apoptotic genes in a middle cerebral artery occlusion-induced focal ischemia rat model

Chunhua Chen,* Qin Hu,* Junhao Yan,* Jiliang Lei,* Lihua Qin,* Xianzhong Shi,* Liju Luan,* Lei Yang,* Ke Wang,* Jingyan Han,† Anil Nanda,‡ and Changman Zhou*‡

*Department of Anatomy and Embryology, Peking University Health Science Center, Beijing, China
†Center of Tasly Microcirculation, Peking University Health Science Center, Beijing, China
‡Department of Neurosurgery, Louisiana State University Health Science Center in Shreveport, Louisiana, USA

Abstract
Despite 2-methoxyestradiol (2ME2) and tricyclodecan-9-yl-xanthogenate (D609) having multiple effects on cancer cells, mechanistically, both of them down-regulate hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF). We hypothesize HIF-1α plays an essential role in cerebral ischemia as a pro-apoptosis regulator; 2ME2 and D609 decrease the levels of HIF-1α and VEGF, that might contribute to protecting brain from ischemia injury. A total of 102 male Sprague–Dawley rats were split into five groups: sham, middle cerebral artery occlusion (MCAO), MCAO + dimethyl sulfoxide, MCAO + 2ME2, and MCAO + D609. 2ME2 and D609 were injected intraperitoneally 1 h after reperfusion. Rats were killed at 24 h and 7 days. At 24 h, 2ME2 and D609 reduce the levels of HIF-1α and VEGF (enzyme-linked immunosorbent assay), depress the expression of HIF-1α, VEGF, BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) and cleaved caspase 3 (western blot and immunohistochemistry) in the brain infarct area. Double fluorescence labeling shows HIF-1α positive immunoreactive materials are co-localized with BNIP3 and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling inside the nuclei of neurons. At 7 days, 2ME2 and D609 reduce the infarct volume (2,3,7-triphenyltetrazolium chloride) and blood–brain barrier extravasation, decrease the mortality and improve the neurological deficits. In conclusion, 2ME2 and D609 are powerful agents to protect brain from cerebral ischemic injury by inhibiting HIF-1α expression, attenuating the superfluous expression of VEGF to avoid blood–brain barrier disruption and suppressing neuronal apoptosis via BNIP3 pathway.

Keywords: apoptosis, cerebral ischemia, hypoxia-inducible factor-1α, middle cerebral artery occlusion, rat.

Vascular endothelial growth factor as one of the target genes of HIF-1α may also have both beneficial and harmful effects on the ischemic brain (Lenzmyr et al. 2005). It is clearly indicated that VEGF is up-regulated in models of brain ischemia, but the underlying mechanisms remain unclear (Croll et al. 2004). VEGF has been shown to be neuroprotective across several experimental paradigms (Kaya et al. 2005; Sun and Guo 2005; Wang et al. 2006). However, the contrary experiments indicate VEGF increases vascular permeability and inflammation (Ponnampalam and Mayberg 2004; Althaus et al. 2006; Chi et al. 2006). The superficial increase of VEGF may enhance the permeability of blood–brain barrier (BBB) during the early brain injury period and contribute to brain edema (Lafuente et al. 2002). So, the functions of HIF-1α and its target genes such as VEGF in ischemic brain tissue remain unclear and speculative.

2-Methoxyestradiol (2ME2) is a naturally occurring metabolite of estradiol, which is known to have antiproliferative, antiangiogenic, and antiproapoptotic activities. Mechanistically, 2ME2 has been shown to inhibit basic fibroblast growth factor (bFGF) and VEGF-induced corneal neovascularization in mice (Klauber et al. 1997) and post-transcriptionally down-regulates HIF-1α expression (Mabjeesh et al. 2003). Tricyclodecan-9-yl-xanthogenate (D609) possesses antitumor and antiviral activity and inhibits hypoxia-induced HIF-1α expression via blocking the biosynthesis of phosphatidic acid (Temes et al. 2004). Our study has demonstrated both 2ME2 and D609 can reduce cerebral vasospasm by inhibiting HIF-1α activity and the expression of VEGF as its downstream in cerebral artery, suppressing endothelium and vascular smooth muscle cells apoptosis via BNIP3 pathway, and attenuating vasoproliferation in a subarachnoid hemorrhage rats model (Chi et al. 2006).

To continue this work, we attempt to clarify the mechanisms of HIF-1α in the neuronal cell death after cerebral ischemia. 2ME2 and D609 are used in a middle cerebral artery occlusion (MCAO)-induced focal ischemia rat model in the present paper. Our hypothesis is that HIF-1α is one of the important pro-apoptotic factors in the development of brain infarct and neuronal death, and 2ME2 as well as D609 can protect brain from ischemic damage by inhibiting HIF-1α-induced apoptotic pathway and VEGF activity. We will examine the expression of HIF-1α and apoptotic genes in the infarct tissues of the brain and the effect of 2ME2 and D609 on the cerebral ischemia neuronal death in the present paper.

Materials and methods

This protocol was evaluated and approved by the Animal and Ethics Review Committee at Peking University Health Science Center in Beijing, China.

Experimental groups

One hundred and two Sprague–Dawley (SD) male rats weighing 300–350 g were randomly assigned to the following five groups: control (sham surgery) (n = 6), MCAO/reperfusion (n = 24), MCAO treated with dimethyl sulfoxide (DMSO) (n = 24), 2ME2 (n = 24), and D609 (n = 24).

Experimental MCAO model

Rats were obtained from the Center of Experimental Animals of Peking University Health Science Center. Animals were kept under standard conditions with free access to food and water before and after surgery. Focal cerebral ischemia was induced by intraluminal middle cerebral artery (Badr et al. 2001) blockade with a nylon suture, as previously described by Longa et al. (1989) and modified by Kawamura et al. (1991). Briefly, animals were anesthetized using 4% isoflurane with a mixture of 60% medical air and 40% oxygen and anesthesia was maintained with 2% isoflurane. Rats were placed in the supine position on a heated operating table with body temperature maintained around 37 ± 0.5°C. Throughout the experiment, frequent checks were made to ensure that the animals were adequately anesthetized. This was performed by applying a painful stimulus to a paw and observing blood pressure responses.

Under an operating microscope, the right femoral artery was dissected and cannulated using polyethylene-50 tubing to allow continuous monitoring for mean blood pressure and sampling for analysis of blood gases. The right common carotid artery, including its bifurcation, was exposed and dissected. All branches of external carotid artery were isolated, coagulated, and transected. The external carotid artery was divided, leaving a stump of 3–4 mm. The internal carotid artery was then isolated, and the pterygopalatine artery was ligated close to its origin. The internal carotid artery was then clamped with a small vascular clip. The common carotid artery was also clamped with a small 5-mm aneurysm clip. The stump of the external carotid artery was reopened, and a 4.0 monofilament nylon suture with a slightly enlarged and round tip was inserted up through the internal carotid artery. When a small resistance was felt, insertion was stopped. The distance from bifurcation of the common carotid artery to the tip of the suture was 18–20 mm. After occlusion for 2 h, the suture was withdrawn through the internal carotid artery into the external artery, allowing reperfusion. The skin was sutured and the rats were allowed to wake up. To complete the surgery, the operator applied 0.1% lidocaine locally to the wound and allowed the rat to recover. A successful occlusion of the right middle cerebral artery is achieved when the left forelimb is paretic after filament introduction.

Drug administration

2-Methoxyestradiol and D609 were purchased from BIOMOL Inc. (Plymouth Meeting, PA, USA). 2ME2 was administered at a dose of 5 mg/kg (Mabjeesh et al. 2003) and D609 at 50 mg/kg (Temes et al. 2004), diluted in 1% DMSO to a final volume of 2 mL, and were administered by intraperitoneally injection 1 h after
reperfusion (3 h after ischemia) (Shimamura et al. 2006). The untreated group received DMSO at the same volume.

**Infarct volume**

2,3,7-Triphenyltetrazolium chloride (TTC) (Sigma Inc., St Louis, MO, USA) staining was performed at 7 days after reperfusion as described previously (Yin et al. 2002). Coronal sections of the brain (2 mm thick) were cut and immersed in 2% solution of TTC for 30 min at 37°C. The stained slices were then fixed by immersion in 4% formaldehyde solution. The infarction area and hemisphere area of each section were traced and measured using an image analysis system [Imaging-Pro-Plus (OLYMPUS, Silver Spring, MD, USA)]. The calculation of infarct volume was performed with: non-infarcted area of the ipsilateral hemisphere/total non-infarcted area (from both the ipsilateral and contralateral hemisphere) to avoid the influence of tissue edema (Swanson and Sharp 1994).

**Evaluation of BBB permeability**

The integrity of the BBB was investigated using Evans blue extravasation (Fujimura et al. 1999). Evans blue at 1% in saline (2500 μL) was injected into femoral artery and allowed to circulate for 1 h before euthanizing the animals. Brains were removed and tissue samples were dissected out from infarct areas in MCAO rats and corresponding areas in sham-operated rats. Tissue samples were then weighed, placed in 400 μL of pure formamide (Sigma) and incubated for 72 h in the dark at 50°C. The optical density of the formamide solution was measured at 620 nm. Data was expressed as μg evans blue/g tissue.

**Neurological deficits and mortality**

The neurological scores were performed in a blinded fashion at 24, 48, 72 h, and 7 days which were based on the scoring system of Garcia et al. (1995). The score given to each rat at the completion of the evaluation was the summation of all six individual test scores (Spontaneous Activity; Symmetry in the Movement of Four Limbs; Forepaw Outstretching; Climbing; Body Proprioception and Response to Vibrissa Touch). The minimum neurological score was 3 and the maximum was 18. Mortality was calculated at the same time points, as well as the immediate intra-operative mortality, but dead rats were not included in the total number of 102 rats.

**ELISA assay**

The sample of brain tissue for enzyme-linked immunosorbent assay (ELISA) was prepared as described in western blotting sample preparation. Prior to assay, samples were briefly thawed, vortexed and added in the wells and incubated at 25°C for 2 h.

**HIF-1α DuoSet IC ELISA**

The volume of HIF-1α was measured at 24 h after MCAO by DuoSet-ELISA following the protocol from the manufacturer (Biocompare IC, Costa Mesa, CA, USA). Briefly, 96-well plates were coated with anti-human HIF-1α antibody overnight, and blocked with 300 μL phosphate-buffered saline (PBS), 5% bovine serum albumin for 2 h. Hundred microliters sample in Tris-Cl, 5% bovine serum albumin, and 0.05% Tween20 (pH 7.3) were added to the wells and incubated for 2 h. Biotinylated anti-HIF-1α antibody was added for another 2 h; and the wells were then incubated with streptavidin-horseradish peroxidase for 20 min. The reaction was started by adding 100 μL H2O2 and tetramethylbenzidine for 30 min, and was stopped by adding 2 N H2SO4. The optical density of each well was detected by means of Model 680 Microplate Reader (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 450 nm. All the steps were performed at 25°C, and the well plates were washed between each step.

**ELISA of VEGF**

Flat-bottomed 96-well plates coated with rabbit polyclonal VEGF antibody were purchased from Biotec.Inc (Gleason, TN, USA). Anti-VEGF detection antibody with a colorimetric tag was allowed to bind to bound VEGF in samples for 2 h. Detection reagents and sulfuric acid were added in the sample wells, and were read by a Model 680 Microplate Reader (Bio-Rad Laboratories Inc.) at 490 nm. The readings were normalized to protein content.

**Real-time RT-polymerase chain reaction**

Animals were killed at 24 h after reperfusion for reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the brain cortex including the infarcted area in all groups using the TRIzol extraction method described previously (Mukundan et al. 2002). Briefly, total RNA was extracted with TRIzol and precipitated with isopropyl alcohol, washed in ethanol, and resuspended in RNase-free water. RNA quantity and quality were determined by spectrophotometry and agarose gel electrophoresis, respectively. Two micrograms of total RNA were used for each amplification and each experiment was repeated three times. PCR products were synthesized using the SYBR Green Realtime PCR Master Mix (Toyobo Co. Ltd, Kita-Ku Osaka, Japan) and were analyzed in real-time with the detection system (ABI Prism 7700 Sequence Detection System, PE Applied Biosystems, Foster City, CA, USA). Each 15 μL SYBR Green reaction contained 0.5 μL cDNA, 0.1 μmol/L: forward primer and 0.1 μmol/L reverse primer. For amplification of both HIF-1α and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the following PCR protocol was applied: 95°C for 60 s, 95°C for 15 s, 60°C for 60 s, 40 cycles. A forward primer, 5'-TCAAGTCACAGCACTGAAAG-3', a reverse primer, 5'-TATCGAGGCTTGTGCGACTG-3' and a forward primer, 5'-GAGGTAAGGTCGGAGTC-3', and a reverse primer 5'-GAGGTAAGGTCGGAGTC-3', were designed from rat HIF-1α and GAPDH genes, respectively. The fluorescence spectra were recorded during the elongation phase of each PCR cycle. PCR products were electrophoresed in 2% Agarose-1000 (Invitrogen, Carlsbad, CA, USA) to confirm that PCR yielded a single product of the expected size. The expected sizes were 198 bp (HIF-1α) and 226 bp (GAPDH). The results were analyzed by the ΔCt method which reflects the difference in threshold for the target gene relative to that of GAPDH in each sample. To ensure validity of our calculations, we confirmed that primers sets used in this study have the same efficiencies as ascertained by varying template concentrations. In each case, the log of the template concentration when plotted against ΔCt yielded values of <0.1 for the slope.

**Western blot**

Samples of six animals from each group were used for western blot study. Animals were killed at 24 h after reperfusion and the brains were removed for western blot. The methods for western
blot have been previously described (Calvert et al. 2002; Zhou et al. 2003). The cortex including infarction was dissected using the corpus callosum as a ventral landmark. Samples were obtained from the MCA territory cortex on the ischemic sides and from non-ischemic controls (n = 6 per group). Tissues were homogenized in ice-cold lysis buffer (0.32 mol/L sucrose, 1 mmol/L ethylenediaminetetraacetate, 5 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 μmol/L eupyepsin, 10 μmol/L pepstatin A, and 1 mmol/L β-mercaptoethanol). The protein content was determined by Bio-Rad protein assay. Equal amounts of protein per lane (50 μg) were loaded onto an 8% polyacrylamide gel and separated by electrophoresis at 90 V for 30 min and then 120 V for 1.5 h. Proteins were then transferred to nitrocellulose at 200 V for 2 h and the membrane was blocked with 5% non-fat dry milk/0.5% Tween-20 in Tris-buffered saline for 2 h. The nitrocellulose was then incubated with different antibodies overnight at 4°C: rabbit anti-HIF-1α (H206) 1 : 800; rabbit anti-VEGF (C-147) 1 : 800; goat anti-BNIP3 (C-18) 1 : 800; mouse anti-caspase 3 (E-8) 1 : 500 (Santa Cruz Inc., Santa Cruz, CA, USA). The membrane was treated with horseradish peroxidase-conjugated secondary antibody for 60 min at 37°C. Immunoblots were probed and then exposed to X-ray film. The X-ray films were scanned and the optical density was determined by Bio-Rad Image analysis. As an internal control, the same nitrocellulose was incubated with an antibody specifically for β-actin (Santa Cruz, 1 : 1000) after being stripped.

Histology and immunohistochemistry

Rats were anesthetized at 24 h after reperfusion. After transcardiac perfusion with 250 mL of 4% paraformaldehyde in 0.1 mol/L PB (pH 7.4), brain was removed and post-fixed with the same fixative solution for 48 h. The brains were cryoprotected in 30% sucrose in PBS for over 48 h at 4°C, and coronal brain sections (20 μm thick) were cut on a cryostat (Leica CM3050 S; Leica, Bensheim, Germany). A series of section was obtained from rats in all animal groups. Sections from each rat were divided into several groups for immunohistochemistry, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining and double fluorescence staining, respectively. They were observed under an OLYMPUS BX51 microscope.

Immunohistochemical staining

The methods for immunohistochemical staining have been described previously (Zhou et al. 2003). Sections were incubated in 3% hydrogen peroxide (H2O2) diluted in PBS to prevent reaction with endogenous peroxidases. After 60 min of incubation with 3% normal serum in PBS, the sections were incubated with primary antibodies overnight at 4°C. The following antibodies were used: rabbit anti-HIF-1α (H206) 1 : 200; rabbit anti-VEGF (C-147) 1 : 200; goat anti-BNIP3 (C-18) 1 : 200; and mouse anti-caspase 3 (E-8) 1 : 200 (Santa Cruz Inc.), respectively. After rinsing them with PBS, the sections were treated with a rabbit, goat or mouse ABC Kit (Santa Cruz Inc.). The sections were then incubated with goat anti-rabbit, donkey anti-goat, or rabbit anti-mouse IgG as a secondary antibody (1 : 200) for 30 min and were placed in avidin-peroxidase complex solution containing avidin-peroxidase conjugate for 30 min. Peroxidase activity was revealed by dipping the sections for 5 min in a mixture containing 3′-diaminobenzidine and H2O2 (ABC kit; Santa Cruz Inc.) at 25°C. The sections were mounted, air-dried, dehydrated, and coverslipped. Application of control serum, instead of the primary antibody, on another section of the same brain provided a negative control for each staining. Imaging-Pro Plus was used to count the number of immunohistochemistry staining of HIF-1α, VEGF, BNIP3, and cleaved caspase 3 in cortex of the ischemic sides of animals from different groups followed the methods described previously (Neese et al. 2007).

TUNEL assay

Sections were stained by TUNEL, and the TUNEL positive cells were expressed by fluorescein-dUTP with dNTP and POD with 3′-diaminobenzidine [manufacturer’s protocol for the in situ Apoptosis Detection Kit (Roche Inc., Mannheim, Germany) as described previously] (Gavrieli et al. 1992; Calvert et al. 2002; Zhou et al. 2003).

Double fluorescence labeling

The methods for double fluorescence labeling have been described previously (Yin et al. 2003; Zhou et al. 2003). Sections were used for double labeling with rabbit anti-HIF-1α (H206) 1 : 200 (Santa Cruz Inc.) and goat anti-rabbit IgG-tetramethylrhodamine isothiocyanate (TRITC) (red, fluorescein); goat anti-BNIP3 (C-18) 1 : 200 (Santa Cruz Inc.) and donkey anti-goat IgG-fluorescein isothiocyanate (FITC) (green); TUNEL fluorescence kit (green, fluorescein dUTP and dNTP Kit, Roche Inc.). Sections were coverslipped with 30% glycerin and observed under an OLYMPUS BX51 microscope with fluorescence light. The TRITC was excited by dye laser at 557 nm and emitted at 576 nm. The wavelength of FITC is 530.30 nm. The FITC was exited by an argon laser at 490 nm and emitted at 525 nm.

Data analysis

Data were expressed as mean ± SEM. Statistical significance was assessed by analysis of variance (ANOVA) performed in one-way ANOVA followed by the Tukey test for multiple comparisons. The clinical behavior scores were compared by Kruskal–Wallis one-way ANOVA followed by multiple comparison procedures by Dunn method. For statistical analysis of the mortality, the Fisher exact test was used (Ostrowski et al. 2005) in two groups’ comparisons. A probability value of p < 0.05 was considered statistically significant.

Results

Cerebral infarction

The cerebral infarction of rats’ brains from each group in 7 days is shown in Fig. 1a. The white colored areas represent the infarction regions in these sections. The ratios of cerebral infarction in five groups are shown in Fig. 1b. Severe infarction was observed in all rats of MCAO and MCAO + DMSO groups (p < 0.05 vs. sham), but the infarct ratios were significantly decreased in rats treated with 2ME2 and D609 (p < 0.05 vs. MCAO and MCAO + DMSO, respectively).
Effect on Evans blue extravasation
Evans blue content in brain tissue in sham operated was 0.58 ± 0.33 μg/g tissue (Fig. 1c). At 24 h after MCAO, Evans blue content in the area of infarction was markedly increased in the MCAO group and in DMSO (vehicle-treated group) (14.67 ± 3.14 μg/g tissue, and 13.98 ± 4.38 μg/g tissue, p < 0.001 vs. sham-operated rats). In rats treated with 2ME2 after MCAO, Evans blue content was reduced by 60% (5.82 ± 3.91 μg/g tissue, p < 0.05 vs. MCAO and vehicle-treated group). D609 also significantly reduced the Evans blue content (8.98 ± 2.64 μg/g tissue, p < 0.05 vs. MCAO and vehicle-treated group), but was not so powerful.

Mortality and neurological scores
The mortality (Fig. 2a) was 33.3% (12/36 rats) in MCAO rats, 25% (8/32 rats) in MCAO + DMSO rats, 11.11% (3/27 rats) in MCAO + 2ME2 rats, 14.3% (4/28 rats) in MCAO + D609 rats, and 0% (0/6 rats) in sham rats. Fisher exact test statistical analysis revealed that 2ME2 and D609 significantly reduced the mortality (p < 0.05, vs. MCAO and MCAO + DMSO, respectively). It was confirmed by autopsy that about two-thirds of the death was induced by subarachnoid hemorrhage.

The neurological scores revealed a poor score for all treated and untreated MCAO animals at 24 and 48 h (Fig. 2b). However, at 72 h and 7 days, both 2ME2 and D609 treated rats had a significant difference when compared to MCAO and MCAO + DMSO group (p < 0.05). In contrast, no significant effect was observed between MCAO + DMSO and MCAO (p > 0.05).

ELISA
The variation of HIF-1α protein assayed by ELISA is shown in Fig. 2c. In MCAO and MCAO + DMSO groups, it...
significantly increased when compared with sham \((p < 0.05, \text{ANOVA})\). However, the elevated expression of HIF-1\(\alpha\) protein was significantly decreased by 2ME2 and D609 \((p < 0.05, \text{vs. MCAO and MCAO + DMSO, ANOVA})\). There were no statistical differences between MCAO and MCAO + DMSO groups \((p > 0.05)\) and also between MCAO + 2ME2 and MCAO + D609 groups \((p > 0.05)\).

The variation of VEGF protein is shown in Fig. 2d. VEGF was increased in MCAO and MCAO + DMSO groups \((p < 0.05, \text{vs. sham, ANOVA})\), but it was significantly decreased in both 2ME2 and D609 treated groups \((p < 0.05, \text{vs. MCAO and MCAO + DMSO, ANOVA})\). The PCR products are demonstrated at the top of the figure. The figure below shows the melting curves of the samples from all the groups. (f) Induction of HIF-1\(\alpha\) mRNA expression after focal ischemia. The average number of HIF-1\(\alpha\) cDNA copies per 100 copies of glyceraldehyde 3-phosphate dehydrogenase cDNA in the brain samples was increased significantly in MCAO group with and without treatment when compared with the control (sham surgery) \((p < 0.05, \text{ANOVA})\). However, no significance is found between all other groups except control \((p > 0.05, \text{ANOVA})\).

**Expression of HIF-1\(\alpha\) mRNA**

To determine whether DMSO, 2ME2, and D609 treatment changed HIF-1\(\alpha\) mRNA expression at 24 h after reperfusion, real-time RT-PCR was performed. The mRNA coding for HIF-1\(\alpha\) was detected at the predicted molecular size (198 bp) in all groups verified by DNA sequencing as shown in the upper part of Fig. 2e and the melting curves for the reactions are illustrated in the lower part of Fig. 2e. Statistical results

© 2007 The Authors
of the studies, shown in Fig. 2f, demonstrated that ischemic injury increased message levels for HIF-1α approximately 15-fold after 24 h reperfusion after 2 h of MCA occlusion whereas expression of HIF-1α mRNA levels were not significantly changed by 2ME2 and D609 treatment in comparison with the MCAO and DMSO group (p > 0.05).

**Western blot**

Western blot analysis of the cerebral cortex including infarction area showed a strong up-regulation of HIF-1α after MCAO at 24 h, but it was markedly inhibited by both HIF-1α inhibitors (p < 0.05) (Fig. 3a). Although the expression of HIF-1α protein was decreased by DMSO treatment, the difference was not so significant. VEGF increased after MCAO and was attenuated by the inhibition of 2ME2 and D609 (p < 0.05) (Fig. 3b). Similar results were found for the apoptotic proteins BNIP3 (Fig. 3c) and cleaved caspase 3 (Fig. 3d).

**Immunohistochemistry**

The expression of HIF-1α, VEGF, BNIP3, and cleaved caspase 3 immunohistochemistry as well as TUNEL staining are shown in Fig. 4. No immunoreactivity and positive TUNEL staining were observed in the sham-operated rats. Massive immunoreactivity of HIF-1α was localized in infarction regions in MCAO and MCAO + DMSO group after 24 h of ischemia (Fig. 4a1 and b1). The high amplification show the HIF-1α immunoreactive material was localized in nuclei and shrunken cytoplasm (Fig. 4a2 and b2). However, the extent of HIF-1α immunostaining was decreased in D609 and 2ME2 treated rats (Fig. 4c1 and d1) and the population of immunoreactive cells as well as TUNEL positive cells (Fig. 4e and f1) were significantly diminished in treated rats compared to MCAO and DMSO group (p < 0.05, ANOVA with Tukey test).

Fig. 3 Western blot analysis of cerebral cortex tissue after 24 h of reperfusion. (a) Representative immunoblots of hypoxia-inducible factor-1α (HIF-1α). (b) Represents vascular endothelial growth factor (VEGF), (c) and (d) represents apoptotic proteins BNIP3 and cleaved caspase 3, which are measured by densitometry analysis. Values are expressed as mean ± SEM with six animals per group on three independent experiments normalized to β-actin and is expressed as a percentage of the mean value of the sham group (p < 0.05, ANOVA with Tukey test).
as the intensity of cytoplasmic immunostaining was reduced (Fig. 4c2 and d2). Similar results were found for VEGF (Fig. 4a3–d3), BNIP3 (Fig. 4a4–d4) and cleaved caspase 3 (Fig. 4a5–d5). The number of immunopositive cells and the density of cells in 1 mm² area are shown in Table 1. The number of immunopositive cells of HIF-1α, VEGF, BNIP3, and cleaved caspase 3 in cortex of the ischemic sides after 2ME2 and D609 treatment was significantly

**Fig. 4** Immunohistochemistry, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining and double fluorescence labeling. a1–d1 show the hypoxia-inducible factor-1α (HIF-1α) immunostaining area in whole brain coronal sections under low magnification. a2 through d5 show the positive immunostaining of HIF-1α, vascular endothelial growth factor (VEGF), BNIP3, and cleaved caspase 3 neurons under high magnification. The negative staining in control samples is shown in inset ‘Ctl’ of a1–a5. 2-methoxyestradiol (2ME2) and tricyclodecan-9-yl-xanthogenate (D609) treatment reduce the populations of HIF-1α, VEGF, BNIP3, and cleaved caspase 3 (c1–c5; d1–d5). TUNEL staining is showed in a6–d6. Although there is some degree of apoptotic cell death evident in the treatment groups (c6 and d6), it is mild staining. Double-fluorescence labeling shows the co-localization of HIF-1α (e1 and f1) with BNIP3 (e2) and TUNEL (f2) in the cerebral cortex after 24 h of middle cerebral artery occlusion (MCAO): HIF-1α (red) is expressed by tetramethylrhodamine isothiocyanate. BNIP3 (green) is expressed by fluorescein isothiocyanate and the apoptosis is marked by the terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end-labeling (TUNEL) assay (green). e3 shows the merges of e1 and e2 demonstrating that the HIF-1α colocalizes with BNIP3 in the nuclei of neurons (yellow); f3 shows the merges of f1 and f2 demonstrating that the HIF-1α colocalizes with TUNEL in the nuclei of neurons (yellow). The magnified images of the neurons were shown in insert panes. Scale bars: a1–d1 = 1 mm (whole brain sections); a2–f3 = 20 μm. Small white arrows indicate examples of the positive cells.

**Table 1** Immunopositive cell density in cortex of the ischemic sides (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>MCAO</th>
<th>DMSO</th>
<th>D609</th>
<th>2ME2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counted cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF</td>
<td>119.07 ± 30.08</td>
<td>87.67 ± 11.19</td>
<td>21.67 ± 2.50</td>
<td>12.93 ± 3.91</td>
</tr>
<tr>
<td>VEGF</td>
<td>37.07 ± 6.36</td>
<td>43.33 ± 5.50</td>
<td>12 ± 3.62</td>
<td>5.4 ± 1.8</td>
</tr>
<tr>
<td>BNIP3</td>
<td>329.13 ± 9.62</td>
<td>29.2 ± 9.18</td>
<td>8.93 ± 3.67</td>
<td>8.4 ± 3.75</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>58.13 ± 9.53</td>
<td>45.67 ± 10.56</td>
<td>18.6 ± 2.36</td>
<td>15.87 ± 2.66</td>
</tr>
<tr>
<td>Density of cells (cells/mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF</td>
<td>783.98 ± 198.07</td>
<td>577.23 ± 73.67</td>
<td>142.67 ± 16.47*</td>
<td>85.16 ± 25.75*</td>
</tr>
<tr>
<td>VEGF</td>
<td>244.06 ± 41.90</td>
<td>285.32 ± 36.22</td>
<td>79.01 ± 23.81*</td>
<td>35.56 ± 11.85*</td>
</tr>
<tr>
<td>BNIP3</td>
<td>191.82 ± 63.32</td>
<td>192.26 ± 60.48</td>
<td>58.82 ± 24.15*</td>
<td>55.31 ± 24.67*</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>382.77 ± 62.75</td>
<td>300.69 ± 69.55</td>
<td>122.47 ± 15.53*</td>
<td>104.47 ± 17.49*</td>
</tr>
</tbody>
</table>

*p < 0.05 (D609, 2ME2 vs. MCAO, DMSO). MCAO, middle cerebral artery occlusion; DMSO, dimethyl sulfoxide; VEGF, vascular endothelial growth factor; 2ME2, 2-methoxyestradiol; D609, tricyclodecan-9-yl-xanthogenate; HIF, hypoxia-inducible factor.
smaller than that in the MCAO and DMSO group (p < 0.05, ANOVA).

TUNEL staining and double fluorescence labeling
As a marker of apoptosis, the positive TUNEL staining was localized in the nuclei of neurons in the infarction area of cortex in MCAO and MCAO + DMSO groups after 24 h of ischemia (Fig. 4a6 and b6). Although there was some degree of apoptotic cell death evident in the treatment groups, it was mild staining (Fig. 4c6 and d6). Double fluorescence labeling of HIF-1α with BNIP3 and TUNEL in the infarction regions after 24 h of MCAO showed that HIF-1α (Fig. 4e1 and f1) was co-localized with BNIP3 (Fig. 4e2) and TUNEL staining (Fig. 4f2) in the neuronal nucleus (Fig. 4e3 and f3).

Discussion
In the present study, we evaluate the pharmacology of 2ME2 and D609 related to HIF-1α in a rat model of MCAO-induced focal ischemia in 24 h and 7 days after 2 h of occlusion. The molecular activity and protein expression of HIF-1α are increased in the infarction area and which are accompanied with enhanced expression of VEGF, BNIP3, and cleaved caspase 3. 2ME2 and D609 suppress the activity and expression of HIF-1α, depress the immunoreactive expression of VEGF, BNIP3 and cleaved caspase 3; reduce the number of TUNEL stained apoptotic cells; decrease the TTC infarction areas and Evans blue extravasation. High mortality is reduced and the neurological functional disability is improved by 2ME2 and D609. These results implicated that the elevation of HIF-1α may be harmful in cerebral ischemia after 2 h of MCA occlusion, and an abatement of HIF-1α and VEGF by 2ME2 and D609 could be neuroprotective from brain damage.

HIF-1α may be harmful in severe cerebral ischemia
It is believed that HIF-1α is a key component of the cellular response to hypoxia and ischemia under pathophysiologic conditions, such as stroke (Helton et al. 2005). When tissues are hypoxic, HIF-1α is accumulated and leads to angiogenesis, glycolysis, erythropoiesis, or cell death mediated by different target genes (Semenza et al. 2000). However, the elevation of HIF-1α being beneficial or harmful after cerebral hypoxia remains debatable. Recently, an experiment on brain-specific knock-out of HIF-1α in mice hypoxic injuries has shown that decreasing the level of HIF-1α can be neuroprotective (Helton et al. 2005). Our previous studies have also shown the elevation of HIF-1α expression in hippocampus and cortex is concurred with apoptotic neuronal loss after global cerebral ischemia (Li et al. 2005). These data support a concept that HIF-1α may initiate apoptosis immediately after cerebral ischemia (Luo et al. 2006).

BNIP3 is one of HIF-1α target genes, an E1B 19K/Bcl-2-binding protein which is a dimeric mitochondrial pro-apoptotic protein that activates apoptosis (Schmidt-Kastner et al. 2004). BNIP3 influences mitochondrial function in the early apoptotic process and can overcome Bcl-2 suppression of apoptosis (Ray et al. 2000) or cytochrome c release (Vande et al. 2000). In the present study, we have observed an increase of HIF-1α and BNIP3 proteins at 24 h after reperfusion, and double fluorescence labeling demonstrates that HIF-1α is expressed and co-localized with BNIP3 and TUNEL positive cells (Fig. 4e and f). Furthermore, HIF-1α inhibitors, 2ME2 and D609 reduce the expression of HIF-1α, BNIP3, and cleaved caspase 3 as well as TUNEL staining. It indicates that the accumulation of HIF-1α and the activation of BNIP3 may be an apoptotic pathway which occurs in the injured brain tissues after cerebral ischemia.

Vascular endothelial growth factor has been shown to be up-regulated by hypoxia in rat brain following MCAO. VEGF increases BBB permeability by a mechanism involving an increase in endothelial cell calcium influx, synthesis and release of nitric oxide, increased synthesis of platelet-activating factor, or an increased release of products via activation of the cyclooxygenase pathway (Mayhan 1999). It is shown that VEGF antagonism reduces edema and infarct size in a mouse model of cortical ischemia (Pichiule et al. 2003). At the same time, we also realized that VEGF has a neuroprotective effect (Klauber et al. 1997). In the present study, we have found that 2ME2 and D609 reduce the expression of VEGF which is co-instantaneous with the decreased Evans blue extravasation (Fig. 1c). It implies that 2ME2 and D609 protect the BBB from over extravasation. Similar observations are reported previously by others in different pathological conditions that 2ME2 decreases VEGF protein levels (Chauhan et al. 2002).

2ME2 and D609 protect brain from ischemia damage via multiple pathways
In the present paper, 2ME2 and D609 reduce cerebral infarction, decrease mortality, improve neurological scores, which imply that the neuroprotection of 2ME2 and D609 may partly be the result of blocking HIF-1α signaling pathway in the neuronal apoptosis, and down-regulating VEGF activity to protect BBB from extravasation and brain edema. As we know, 2ME2 and D609 are not specific inhibitors for HIF-1α. For example, except for antiproliferation via inhibiting HIF-1α and its effect on down-regulating BNIP3 apoptotic pathway described in the present paper, 2ME2 also has antiproliferative, anti-angiogenic, antitumor activities (Hagen et al. 2004), and anti-neovascularization induced by VEGF while D609 also has been extensively studied in biological systems and exhibits a variety of biological functions, including antiviral, antitumor, and anti-inflammatory activities. In addition, 2ME2 has been shown to interfere with the NF-kb pathway, which will affect an
inflammatory response in an injured tissue such as the ischemic brain parenchyma (Chauhan and Anderson 2003). So, the potent effect of 2ME2 and D609 on protecting brain from ischemia damage may be via different molecular pathways.

Possible mechanisms of 2ME2 and D609 attenuating hypoxia-mediated increase of HIF-1α levels may include inhibition of hypoxic stabilization of the subunit, impaired translation of HIF-1α mRNA, or alternatively, decreased HIF-1α gene expression or mRNA stability. However, 2ME2 and D609 are not found to alter HIF-1α mRNA levels at 24 h after reperfusion in our current study, which suggests that they may have function on inhibiting translation or stabilization of HIF-1α to active target genes in response to hypoxia. This finding is consistent with previous in vitro models (Mabjeesh et al. 2003). 2ME2 and D609 may down-regulate HIF-1α at the post-transcriptional level which is also verified by ELISA, western blot analysis and immunohistochemistry in the present paper. But the definite mechanisms still need further study and further experiments are necessary to clearly eliminate the effect of 2ME2 and D609 on HIF-1α expression at the mRNA level at different time points after reperfusion.

2-Methoxyestradiol was administrated by intraperitoneal in SD rats in present study. Even though the half-life of 2ME2 in male SD rats receiving 2ME2 as an intravenous bolus has been reported to be only about 20 min (Zacharia et al. 2004), a clinical study in patient shows that a preliminary pharmacokinetic evaluation of 2ME2 given orally has shown a relatively long terminal half-life of 2ME2 in plasma of approximately 1–2 days, and a combined concentration–time profile of total and unbound 2ME2 as measured in one representative patient who received a single oral dose of 1600 mg (Lakhani et al. 2006). A variety of other factors, however, may influence the prolonged circulation of 2ME2 in human or in rats. Indeed, drugs such as 2ME2 with high affinity for plasma proteins often demonstrate a relatively slow distribution and elimination of drug from the central compartment, which may prolong the apparent half-life.

In conclusion, 2ME2 and D609 can reduce mortality, improve neurological deficits and protect brain damage in part by suppressing HIF-1α and its target genes such as BNIP3 and VEGF. Prevention of the activation of BNIP3 may lead to protection of brain tissues from apoptosis. Reduction of VEGF superfluous expression may be associated with the protection of BBB and the prevention of brain edema. The therapy effects of 2ME2 and D609 may have potentials in the early treatment of severe ischemic cerebral stroke.

Acknowledgements

This work was partially supported by The Graduate Student Foundation of China (20050011123); The National Natural Science Foundation of China (30672157).

References


