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ScienceDirect
Pharmacology, Biochemistry and Behavior 85 (2006) 658–668
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Effect of the flavonoid, oroxylin A, on transient cerebral hypoperfusion-induced memory impairment in mice

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Received 23 June 2006; received in revised form 26 October 2006; accepted 30 October 2006
Available online 14 December 2006

Abstract

Oroxylin A is a flavonoid compound that is found in the root of *Scutellaria baicalensis* Georgi. The aim of this study was to determine the effects of oroxylin A on memory impairment induced by transient bilateral common carotid artery occlusion (2VO) in mice. The ameliorating effect of oroxylin A on memory impairment was investigated using a passive avoidance task, the Y-maze task, and the Morris water maze task in mice. Oroxylin A was found to significantly reverse 2VO-induced cognitive impairments in the passive avoidance and Y-maze tasks in a dose dependant manner ($P<0.05$). Moreover, oroxylin A (5 mg/kg, p.o.) shortened the escape-latency and prolonged swimming times in the target quadrant during the probe trial in the Morris water maze task ($P<0.05$). Histochemical and immunohistochemical studies showed that the number of Nissl bodies and OX-42 positive cells in the hippocampal CA1 and dentate gyrus regions were attenuated by oroxylin A. Moreover, phosphorylated cAMP response element-binding protein (CREB) and brain derived neurotrophic factor (BDNF) positive cell numbers were markedly increased in animals treated with oroxylin A than in untreated 2VO controls. These results suggest that oroxylin A dramatically attenuates the memory impairment induced by 2VO, and that this effect may be mediated by the neuroprotective effects of oroxylin A as supported oroxylin A induced reductions in activated microglia and increases in BDNF expression and CREB phosphorylation.

Keywords: Oroxylin A; Memory; Passive avoidance task; Y-maze task; Morris water maze task; Carotid artery occlusion

1. Introduction

The hippocampus is highly vulnerable to transient cerebral ischemia (Pulsinelli et al., 1982), and much interest has been focused on the effects of global forebrain ischemia on the hippocampal formation, and in particular on CA1 pyramidal cells, which are extremely vulnerable to ischemic insults and die in response to only a few minutes of blood flow reduction (Pulsinelli et al., 1982; Smith et al., 1984). Ischemic cell death in the hippocampus is selective. Most CA1 pyramidal neurons die after 15 min of ischemia, with maximal cell death occurring one week after reperfusion. Ischemia-induced neuronal degeneration is also observed in other structures, such as, the striatum, cerebral cortex, and thalamus (Pulsinelli et al., 1982; Smith et al., 1984; Johansen and O'Hare, 1989; Freund et al., 1990). Many reports have shown learning and memory deficits in rat models of permanent or transient bilateral common carotid artery occlusion (2VO), an accepted model of vascular dementia (Masada et al., 1997; Pazos et al., 1999; Hartman et al., 2005).

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0091-3057/$ - see front matter © 2006 Published by Elsevier Inc.
doi:10.1016/j.pbb.2006.10.025

In addition, several studies have shown mild neuronal damage in mice administered transient 2VO (Sheng et al., 1999; Wellons et al., 2000; Urayama et al., 2002). However, it is unclear whether memory dysfunction is caused by transient hypoperfusion in mice administered 2VO.

Oroxylin A (5, 7-dihydroxy-6-methoxyflavone) is a flavonoid isolated from the root of *Scutellaria baicalensis* Georgi that is widely used in traditional Chinese medicine (Tomimori et al., 1982). Several previous reports have revealed that oroxylin A is anti-oxidative and anti-inflammatory. For example, it suppresses superoxide and nitric oxide generation (Jiwajinda et al., 2002) and inhibits lipopolysaccharide-induced iNOS and COX-2 gene expression by suppressing nuclear factor-kappaB activation (Chen et al., 2000). Polyphenolic fractions from *S. baicalensis* attenuate the memory dysfunctions caused by chronic global ischemia in rats, however, they did not identify the compound involved (Shang et al., 2005). *S. baicalensis* contains many flavonoid compounds, such as, baicalein, wogonin, and oroxylin A. We have found that baicalein and wogonin do not affect or worsen memory functions (unpublished data). Recently, it was reported that oroxylin A possesses antagonistic properties at the $GABA_A$ receptor (Huen et al., 2003). $GABA_A$ antagonists have been reported to ameliorate the memory dysfunction induced by scopolamine (Lal et al., 1988; Sharma and Kulkarni, 1990; Diez-Ariza et al., 2003). Thus, we reasoned that oroxylin A, a $GABA_A$ antagonist, may ameliorate memory dysfunction induced by hypoperfusion in mouse 2VO model.

The purpose of this study was to investigate whether mice that have experienced cerebral hypoperfusion induced by transient 2VO show learning and memory impairments, and if so, whether these impairments can be attenuated by oroxylin A. Cognitive function was evaluated using the passive avoidance task, the Y-maze task, and the Morris water maze task. In addition, morphological changes in the 2VO mouse hippocampus with or without oroxylin A treatment were investigated using histochemical and immunohistochemical methods.

2. Materials and methods

2.1. Animals

Male ICR mice (25–30 g) were purchased from the Orient Co., Ltd, a branch of Charles River Laboratories (Seoul). Animals were housed 5 or 6 per cage, allowed access to water and food ad libitum, and maintained under a constant temperature (23 ± 1 °C) and humidity (60 ± 10%) under a 12-h light/dark cycle (light on 07.30–19.30 h). Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea.

2.2. Materials

Oroxylin A was donated by one of the author (K.H. Son), and its purity was 99.9%. Oroxylin A was suspended in a 10% Tween 80 solution. Anti-CD11b antibody (OX-42), anti-phosphorylated cAMP response element-binding protein (pCREB) antibody, and anti-brain derived neurotrophic factor (BDNF) antibody were purchased from Serotec Ltd. (UK), Upstate Lake Placid (USA), or Santa Cruz Biotech (USA), respectively. All other materials were of the highest grade available and were obtained from normal commercial sources.

2.3. Surgeries and drug administration

ICR mice were anesthetized with 1.0% isoflurane and 70% nitrous oxide in oxygen. Mice were subjected to a transient cerebral hypoperfusion as described by Zhao et al. (2005), with minor modifications. Transient cerebral hypoperfusion was induced by occluding both common carotid arteries with aneurysm clips for 7 min. Circulation was restored by removing clips. Mice that received the same surgical operation without clipping of the carotid arteries served as sham-operated controls.

Mice were treated orally with 1.25, 2.5, or 5 mg/kg of oroxylin A 60 min after reperfusion, and then once a day for a week. The sham operated vehicle treatment group and the untreated 2VO control group received 10% Tween 80 solution at the same volumes and times. The last treatment was completed 1 h prior to each test.

2.4. The passive avoidance task

Testing was carried out in identical illuminated and non-illuminated boxes (20 × 20 × 20 cm), separated by a guillotine door (5 × 5 cm) (Gemini Avoidance System, San Diego). The illuminated compartment contained a 50 W bulb, and the floor of the non-illuminated compartment (20 × 20 × 20 cm) was composed of 2 mm stainless steel rods spaced 1 cm apart. For the acquisition trial, mice were initially placed in the illuminated compartment and the door between the two compartments was opened 10 s later. When mice entered the dark compartment, the door automatically closed and an electrical foot shock (0.5 mA) of 3 s duration was delivered through the stainless steel rods. One hour before the acquisition trial, mice were administered vehicle or oroxylin A (1.25, 2.5, or 5 mg/kg). Twenty-four hours after the acquisition trial, mice were replaced in the illuminated compartment for the retention trial. The time taken for a mouse to enter the dark compartment after door opening was defined as latency for both acquisition and retention trials. Latency to enter the dark compartment was recorded up to 300 s.

2.5. The Y-maze task

The Y-maze was a horizontal maze (40 cm long and 3 cm wide with walls 12 cm high) with three arms (labeled A, B and C) at 120° angles from each other (Sarter et al., 1988). The maze floor and walls were constructed from dark opaque polyvinyl plastic. Mice were initially placed within one arm, and the sequence (i.e., ABCCAB) and number of arm entries were recorded manually for each mouse over an 8 min period. An alternation was defined as entry into all three arms consecutively (i.e., ABC, CAB, or BCA but not BAB). One hour before this test, mice were treated with vehicle or oroxylin A (1.25, 2.5, or 5 mg/kg). Maze arms

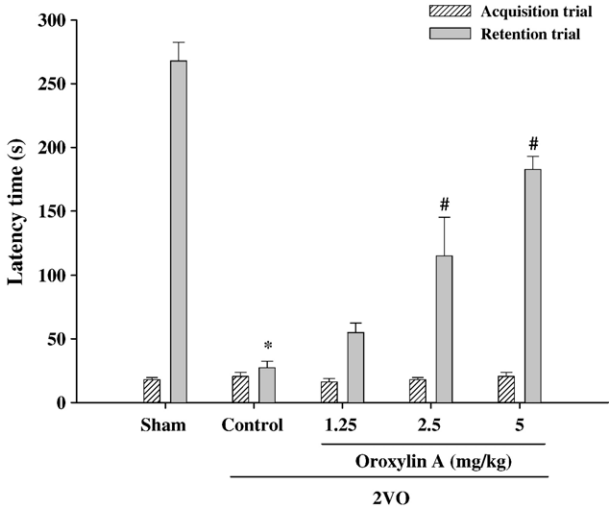

Fig. 1. Effect of oroxylin A on transient bilateral common carotid artery occlusion (2VO)-induced memory deficits on passive avoidance task. Oroxylin A (1.25, 2.5, or 5 mg/kg, p.o.) or the same volume of 10% Tween 80 solution was administered to mice for 7 days, and the final treatment was administered 60 min before the acquisition trials. The retention trials were carried out 24 h after acquisition trials. Eight different animals were used per each treatment group. Data represent means±S.E.M. *$P<0.05$, compared with the sham group. #$P<0.05$, compared with the untreated 2VO control group.

were thoroughly cleaned between tests to remove residual odors. Percentage alternation was determined by dividing the total number of alternations by the total number of arm entries subtracting 2 and then multiplying the result by 100 according to the following equation: % Alternation=[(Number of alternations)/(Total arm entries−2)]×100. The total number of arm entries served as an indicator of locomotor activity.

2.6. The Morris water maze task

Morris water maze learning task adapted for mice was conducted to assess spatial learning ability (Morris, 1984). The Morris water maze is a circular pool (90 cm in diameter and 45 cm in height) with a featureless inner surface, which is filled to a depth of 30 cm with water containing 500 ml of milk (20±1 °C). This tank was placed in a dimly lit, soundproof test room with various visual cues. A submerged white platform (6 cm in diameter, 1 cm below the surface of the water) was then placed in one of the pool quadrants. The first experimental day was dedicated to swimming training for 60 s in the absence of the platform. During the following four days mice were given two trial sessions per day with the platform in place. For each training trial, mice were placed in the water facing the pool wall at one of the pool quadrants in a different order each day. The time interval between each trial sessions was 30 min (Kim et al., 2003). When a mouse located the platform, it was permitted to remain on it for 10 s. If a mouse did not locate the platform within 120 s, it was placed on the platform for 10 s. After each trial sessions animals were returned to their home cages and allowed to dry under an infrared lamp. During each trial session, the time taken to find the submerged platform (latency) was recorded using a video camera-based Ethovision System (Nodulus, Wageningen, The Netherlands). One day after the final training trial sessions, mice were subjected to a probe trial session in which the platform was removed from the pool, and were allowed to swim for 120 s to search for it. A record was kept of the swimming time in the pool quadrant where the platform had previously been placed. Vehicle or oroxylin A (5 mg/kg) was administered 1 h before the first trial session at every consecutive day. Control group animals received 10% Tween 80 solution only.

2.7. Tissue preparation

The mice were immediately anesthetized with pentobarbital sodium (60 mg/kg, i.p.) after the Morris water maze task and

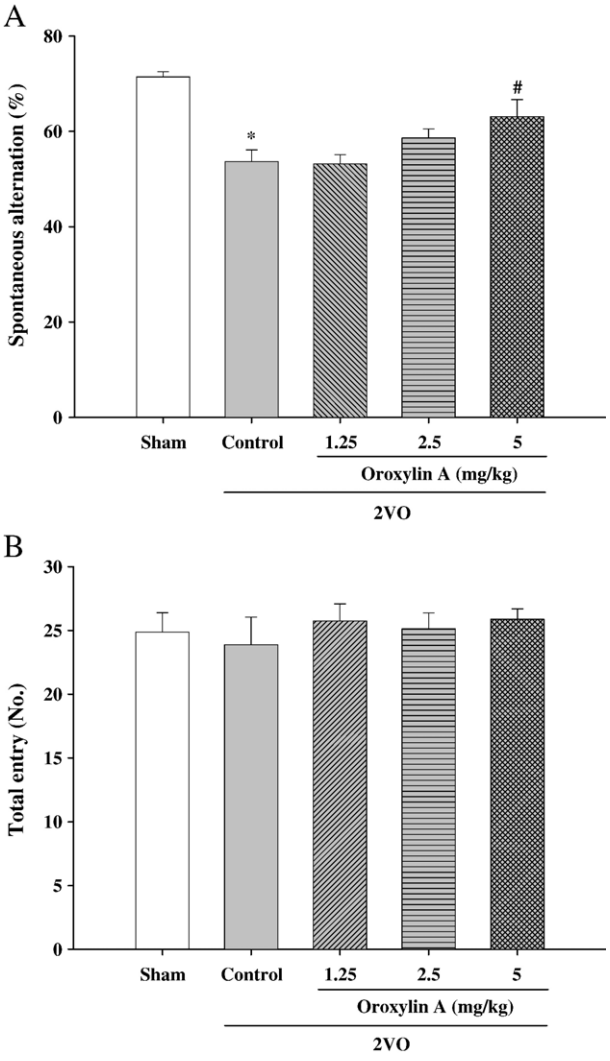

Fig. 2. Effect of oroxylin A on transient bilateral common carotid artery occlusion (2VO)-induced memory deficits as determined by the Y-maze task. Oroxylin A (1.25, 2.5 or 5 mg/kg, p.o.) or the same volume of 10% Tween 80 solution was administered to mice for 7 days, and the final treatment was administered 60 min before the test. Spontaneous alternation behavior (A) and the number of arm entries (B) during an 8-min session were measured as described in Materials and methods. Eight different animals were used per each treatment group. Data represent means±S.E.M. *$P<0.05$, compared with the sham group. #$P<0.05$, compared with the untreated 2VO control group.

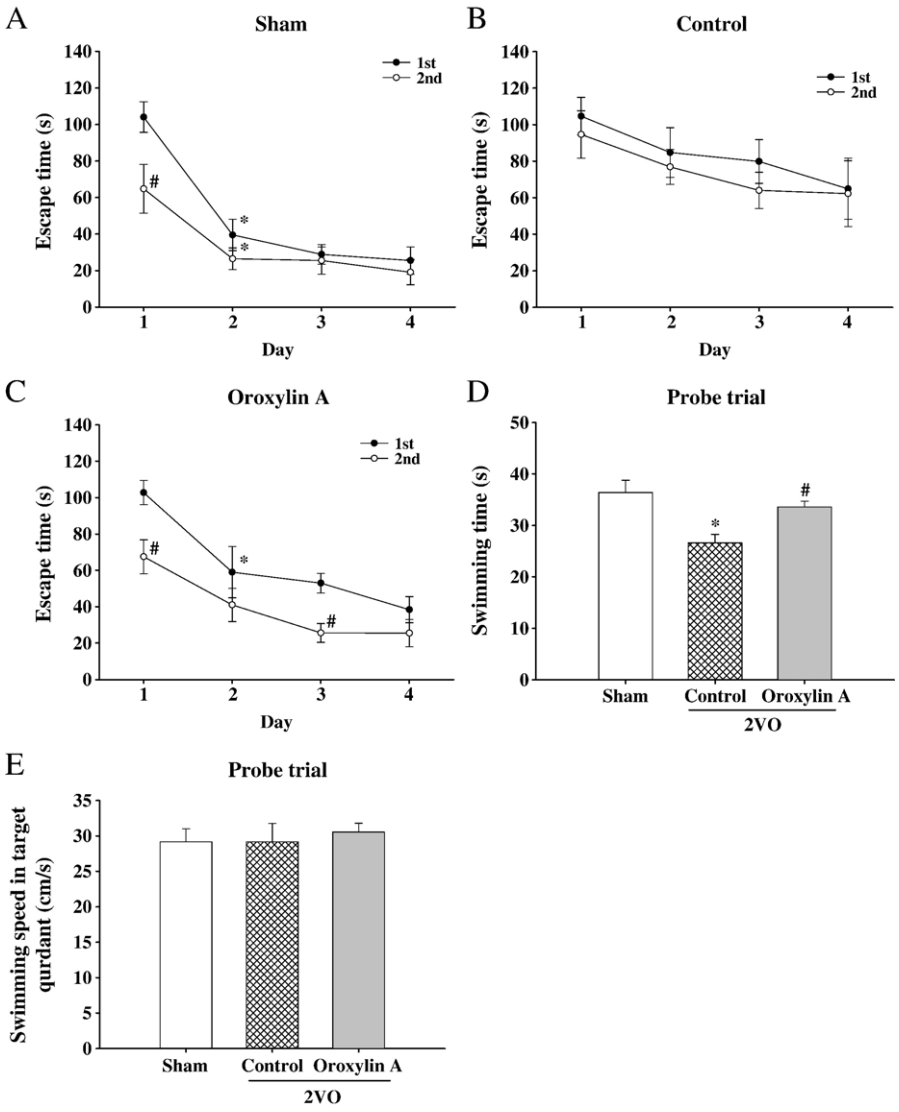

Fig. 3. Effect of oroxylin A on the performance in the training trials (A, B and C) and in the probe trial (D) of the Morris water maze task in transient bilateral common carotid artery occlusion (2VO)-induced memory deficits mice. Oroxylin A (5 mg/kg, p.o.) or the same volume of 10% Tween 80 solution was administered to mice for 7 days, and each administration was performed 60 min before first training trial session at every consecutive day. In the training trial sessions, each trial was carried out twice a day for 4 consecutive days. The second trial was carried out 30 min after the first trial. Escape latency was measured during a 2-min session. Eight different animals were used per each treatment group. Data represent means ± S.E.M. *$P<0.05$, compared with previous day; #$P<0.05$, compared with 1st trial in A–C. *$P<0.05$, compared with sham group; #$P<0.05$, compared with the untreated 2VO control group in D, E.

perfused transcardially with 0.1 M phosphate buffer (pH 7.4) followed by ice-cold 4% paraformaldehyde. Brains were removed and postfixed in phosphate buffer (0.05 M, pH 7.4) containing 4% paraformaldehyde overnight and then immersed in 30% sucrose solution (in 0.05 M phosphate-buffered saline), and stored at 4 °C until sectioned. Frozen brains were coronally sectioned on a cryostat at 30 μm and then stored in storage solution at 4 °C.

2.8. Cresyl violet staining

After mounting sections onto gelatin-coated slides, they were stained with 0.5% cresyl violet, dehydrated through graded alcohols (70, 80, 90, and 100% × 2), placed in xylene, and coverslipped using Histomount medium.

2.9. Immunohistochemistry

Free floating sections were incubated for 24 h in PBS (4 °C) containing monoclonal anti-CD11b antibody (OX-42, 1:1000 dilution; Serotec Ltd., Oxford, UK) or anti-pCREB antibody (1:1000 dilution; Upstate, Lake Placid, NY) or anti-BDNF antibody (1:500, Santa Cruz Biotech, CA), 0.3% Triton X-100, 0.5 mg/ml of bovine serum albumin and 1.5% normal horse serum as described elsewhere (Lee et al., 2002; Park et al., 2004). Sections were then incubated for 90 min with biotinylated secondary antibody (1:200 dilution, Vector, Burlingame, CA), and then with avidin–biotin–peroxidase complex (1:100 dilution, Vector, Burlingame, CA) for 1 h at room temperature. Thereafter, they were reacted with 0.02% 3, 3′-diaminobenzidine and 0.01% H_2O_2 for about 3 min. After

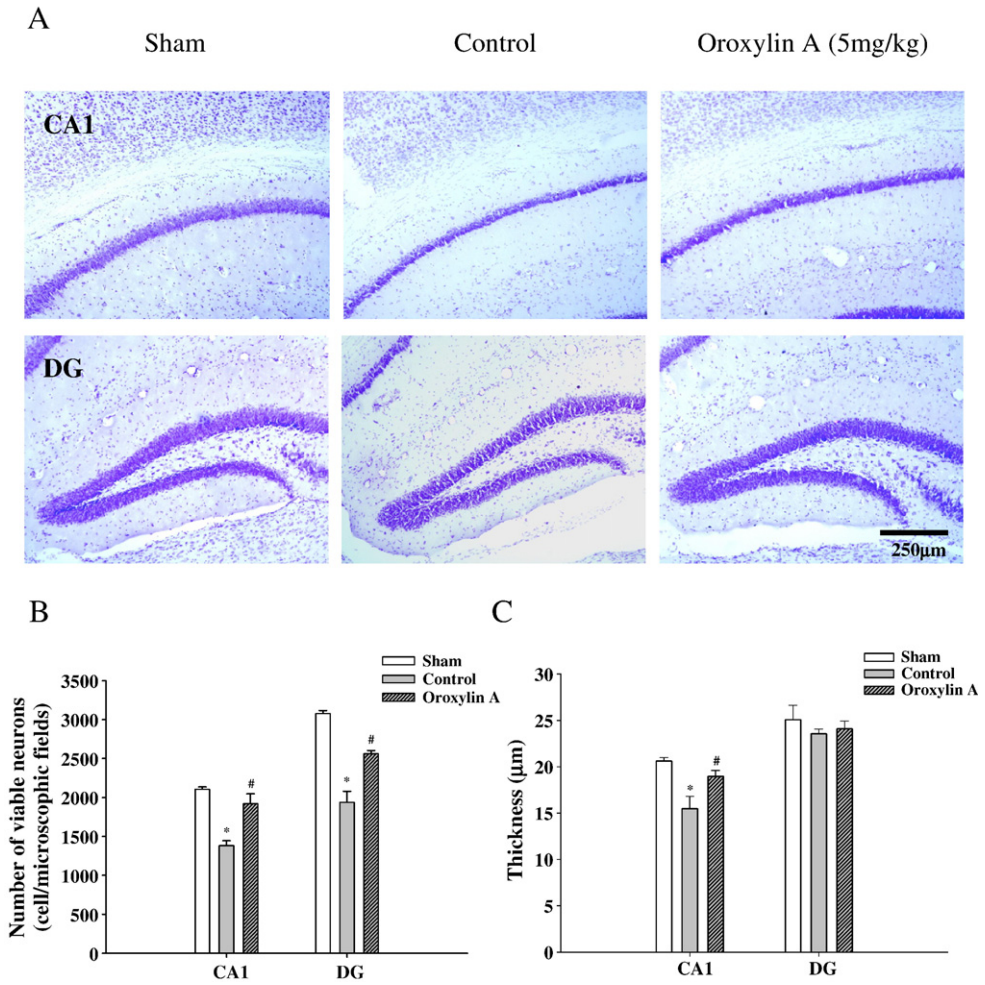

Fig. 4. A. Photomicrographs of Nissl bodies in the CA1 region (CA1) and dentate gyrus (DG) in the hippocampus. B. Numbers of viable neurons in the CA1 region (CA1) and the dentate gyrus (DG). C. Thickness of the layer in the CA1 region (CA1) and the dentate gyrus (DG). Data are presented as means±S.E.M for 6 determinations of each region in 6 animals. *$P<0.05$, compared with the sham group. #$P<0.05$, compared with the untreated 2VO control group. Magnification: 100×. Bar=250 μm. 2VO; transient bilateral common carotid artery occlusion.

each incubation step, the sections were washed three times with PBS. Finally, they were mounted on gelatin-coated slides, dehydrated in ascending alcohol concentrations, and cleared in xylene.

2.10. Quantitation and statistical analysis

Cell counts and thicknesses of the CA1 and dentate gyrus layers were measured using a computerized image analysis system (Leica Microsystems AG, Wetzlar, Germany). Cells were counted in the CA1 and dentate gyrus regions in the hippocampal area in six mice per group by one person blind to treatment group identities.

2.11. Statistics

Values are expressed as means±S.E.M. Results of passive avoidance, Y-maze, and of the probe trial of the Morris water maze task, and histological study results were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons. Morris water maze latencies were analyzed by two-way ANOVA followed by Tukey analysis with day as one variable and treatment as a second. For the comparison of the results of day to day and each trial session in the Morris water maze test, data were analyzed using the Student's *t*-test. Statistical significance was set at $P<0.05$.

3. Results

3.1. Effect of oroxylin A on the memory impairment induced by 2VO in passive avoidance test

A significant group effect was observed in the step-through latency in retention trial [$F(4, 35)=36.09$, $P<0.05$] (Fig. 1). The step-through latency of the 2VO control group was significantly shorter than that of the sham control group ($P<0.05$). Moreover, the shorter step-through latency induced by 2VO was significantly reversed by oroxylin A treatment for 7 days (2.5 and 5 mg/kg, p.o., $P<0.05$). In the acquisition trial, no significant differences were observed in step-through latency among all groups [$F(4, 35)=0.61$, $P>0.05$].

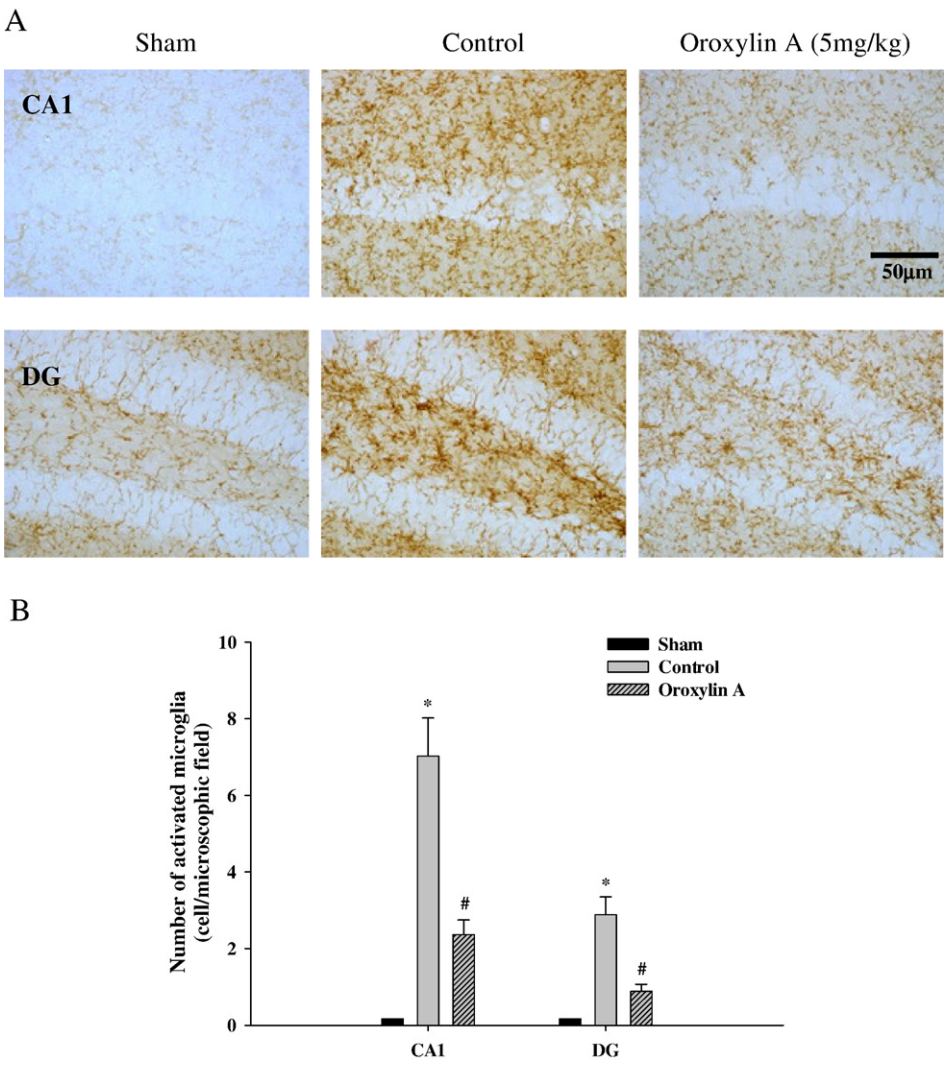

Fig. 5. A. Photomicrographs of OX-42 positive cells in the CA1 region (CA1) and dentate gyrus (DG) in the hippocampus. B. Numbers of OX-42 positive cells in the CA1 region (CA1) and the dentate gyrus (DG). Data represent means±S.E.M for 6 determinations of each region in 6 animals. *$P<0.05$, compared with the sham group. #$P<0.05$, compared with the untreated 2VO control group. Magnification: 400×. Bar=50 μm. 2VO; transient bilateral common carotid artery occlusion.

3.2. Effect of oroxylin A on the memory impairment induced by 2VO in the Y-maze test

There was a significant group effect in terms of spontaneous alternation behavior [$F(4, 35)=10.35$, $P<0.05$] (Fig. 2A). Spontaneous alternation shown by the 2VO control group was significantly less than that shown by the sham group ($P<0.05$). Lowered levels of spontaneous alternation induced by 2VO were significantly attenuated by oroxylin A (5 mg/kg, p.o.) treatment for 7 days (Fig. 2A, $P<0.05$). The number of arm entries was similar across all experimental groups [$F(4, 35)=0.29$, $P>0.05$] (Fig. 2B), demonstrating that general locomotor activity was not affected by oroxylin A.

3.3. Effect of oroxylin A on the memory impairment induced by 2VO in the Morris water maze test

All treatment groups revealed significant effects of Day ([trial 1]: $F(3, 84)=20.16$, $P<0.001$; [trial 2]: $F(3, 84)=9.54$, $P<0.001$) and Treatment ([trial 1]: $F(2, 84)=11.11$, $P<0.001$; [trial 2]: $F(2, 84)=18.22$, $P<0.001$), but no significant effect of day by treatment interactions ([trial 1]: $F(6, 84)=1.25$, $P>0.05$; [trial 2]: $F(6, 84)=0.97$, $P>0.05$). The sham control group showed a rapid reduction in escape latency from the first to the second trial session on day 1 (Fig. 3A, $P<0.05$). In contrast, the 2VO control group spent more time searching for the platform and showed no improvement in escape latencies between the first and the second trials during the 4-day training sessions (Fig. 3B). Daily treatment with oroxylin A (5 mg/kg), however, significantly shortened escape latencies in 2VO mice between the first to second trial sessions on days 1 and 2 (Fig. 3C, $P<0.05$). Stable latencies were reached after 6 trial sessions in oroxylin A treated group, exhibiting the formation of long-term memory during the 3-day testing period. On the day following the last day of training, a significant group effect was observed on time spent in the target quadrant (quadrant in which the platform had been located during training) [$F(3, 28)=6.23$, $P<0.05$]. Post hoc analysis revealed swimming time within the

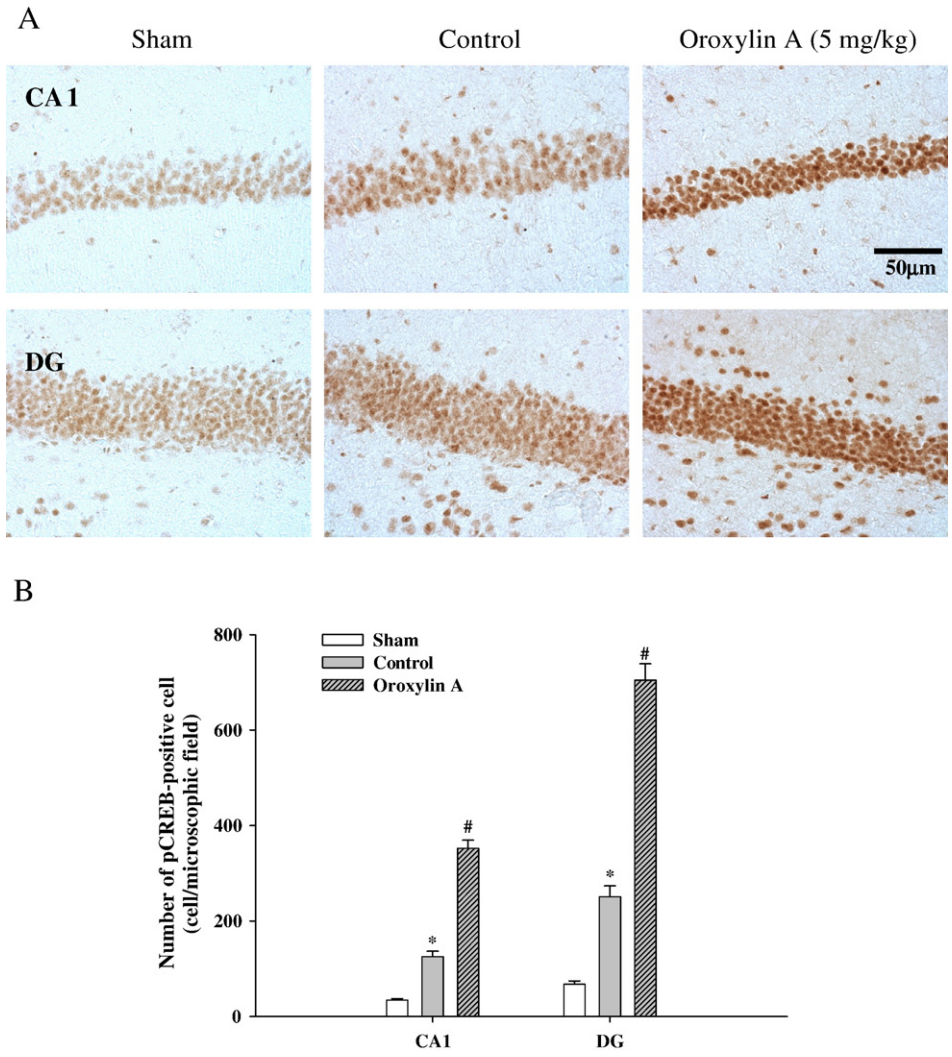

Fig. 6. A. Photomicrographs of pCREB positive cells in the CA1 region (CA1) and the dentate gyrus (DG) in the hippocampus. B. Numbers of pCREB positive cells in the CA1 region (CA1) and dentate gyrus (DG). Data represent means±S.E.M for 6 determinations of each region in 6 animals. *$P<0.05$, compared with the sham group. #$P<0.05$, compared with the untreated 2VO control group. Magnification: 400×. Bar=50 μm. 2VO; transient bilateral common carotid artery occlusion.

target quadrant in the 2VO control group was significantly lower than that in the sham control group (Fig. 3D, $P<0.05$). This reduced swimming time in the target quadrant induced by 2VO was significantly attenuated by oroxylin A treatment ($P<0.05$). Swimming speed within the target quadrant was similar for all the experimental groups [$F(3, 28)=0.53$, $P>0.05$] (Fig. 3E).

3.4. Cresyl violet staining

Representative photomicrographs of the CA1 and dentate gyrus regions in the hippocampal formation are shown in Fig. 4A. Marked cell losses in these subfields of the hippocampal formation were observed in the 2VO control group. Significant group effects on surviving cell numbers were observed [$F(3, 30)=4820.52$, $P<0.05$, Fig. 4B]. The mean number of surviving cells in the CA1 and dentate gyrus regions in the 2VO control group were significantly lower than in the sham group ($P<0.05$), and a significant increase in surviving cell numbers were observed in oroxylin A treated group compared with the 2VO control group ($P<0.05$). Moreover, significant group effects on the thickness of the CA1 and dentate gyrus layer were observed [$F(3, 30)=13.94$, $P<0.05$, Fig. 4C]. The thickness of the CA1 pyramidal cell layer in the 2VO control group was significantly lower than that of the sham group ($P<0.05$), and a significant increase was observed in oroxylin A treated group compared with the 2VO control group ($P<0.05$). However, although the thickness of the granule cell layer in the dentate gyrus was reduced by 2VO, this was not significantly affected by oroxylin A treatment.

3.5. Immunohistochemistry

As shown in Fig. 5A, in the sham control group, microglial cells immunostained with OX-42 antibody were observed in the ramified form, an inactivated form. However, cells in CA1 and dentate gyrus regions in the 2VO control group were condensed and amoeboid, an activated form. As shown in Fig. 5B,

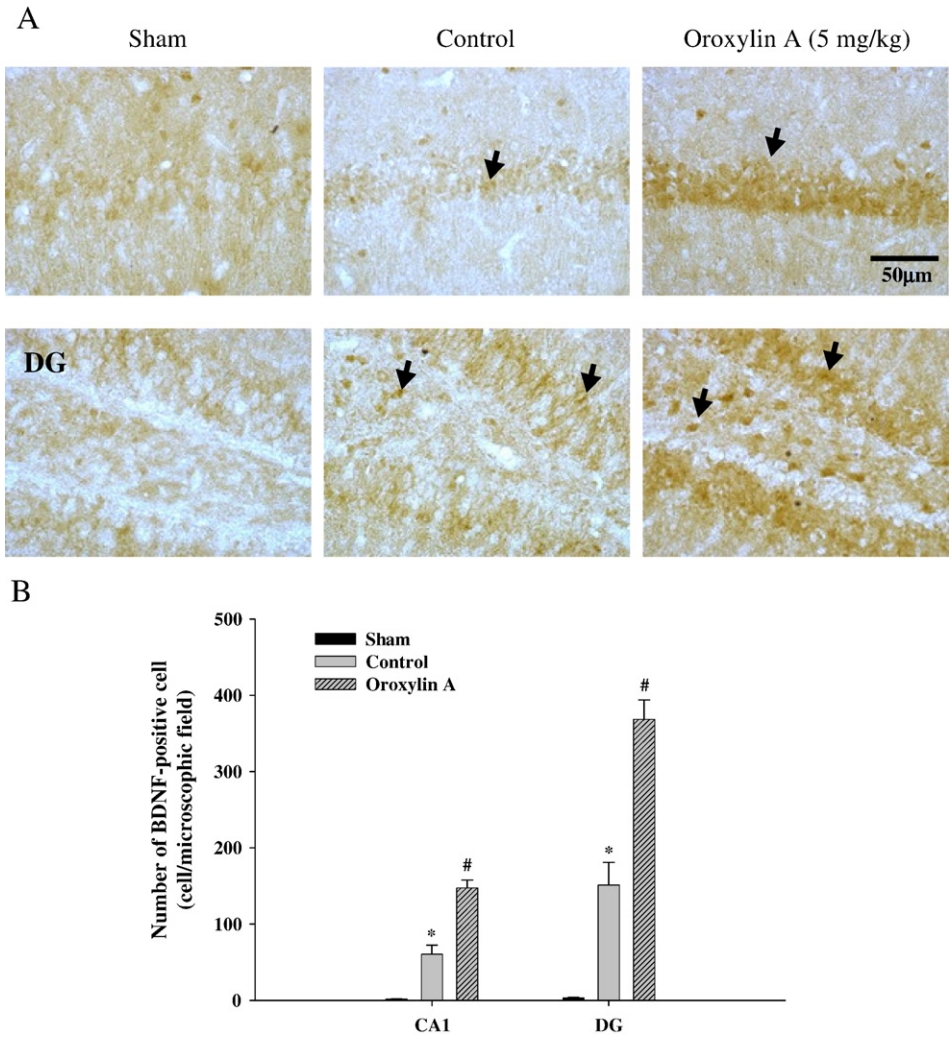

Fig. 7. A. Photomicrographs of BDNF positive cells in the CA1 region (CA1) and dentate gyrus (DG) in the hippocampus. B. Numbers of BDNF positive cells in the CA1 region (CA1) and dentate gyrus (DG). Data represent means±S.E.M for 6 determinations of each region in 6 animals. *$P<0.05$, compared with the sham group. #$P<0.05$, compared with the untreated 2VO control group. Magnification: 400×. Bar=50 μm. 2VO; transient bilateral common carotid artery occlusion.

significant increases in activated microglial cells were detected in the hippocampal CA1 and dentate gyrus regions in the 2VO control group. Moreover, microglial cell activation induced by 2VO was significantly reversed by oroxylin A treatment ($P<0.05$).

pCREB-positive cells in the sham control group were observed, but to a negligible extent (Fig. 6A). pCREB-positive cells were significantly increased in the CA1 and dentate gyrus regions in the 2VO control group ($P<0.05$, Fig. 6A,B), and BDNF positive cells were also significantly increased in the CA1 and dentate gyrus regions in the 2VO control group ($P<0.05$, Fig. 7A,B). Unlike that observed for activated microglial cells, these increases in pCREB and BDNF-positive cells were further markedly increased by oroxylin A treatment (Figs. 6 and 7).

4. Discussion

The present study demonstrates that mice with transient cerebral hypoperfusion induced by 2VO show marked hippocampal injuries and memory impairments as evaluated immunohistochemically, and by passive avoidance, the Y-maze, and the Morris water maze task. Moreover, some of these physiological and behavioral impairments were ameliorated by oroxylin A treatment, which may be associated with a reduction in the number of activated microglia and increased BDNF expression via CREB phosphorylation.

Several reports claim that transient ischemia causes cognitive impairment (Hagan and Beaughard, 1990; Green et al., 1995; Shuaib et al., 1995). The hippocampus is highly vulnerable to transient cerebral ischemia, which causes cognitive deficits and damage in the CA1 and dentate gyrus regions (Pulsinelli et al., 1982). Sheng et al. (1999) reported that 40% of CA1 neurons died after transient cerebral hypoperfusion with 10 min of 2VO in mice. In addition, Murakami et al. (2005) reported that mice with chronic cerebral hypoperfusion caused by 2VO exhibit learning impairments in a water maze task without marked histological alterations in the hippocampus. Furthermore, Xavier et al. (1999) reported that dentate gyrus granule cell damage could cause memory impairments. Taken together, mild

damage in the CA1 region of the hippocampus can cause learning and memory impairment, but only one other report has found that transient cerebral hypoperfusion with occlusion of bilateral common carotid arteries (2VO) in mice causes spatial cognitive impairment (Zhao et al., 2005). In the present study, we observed that mice with transient cerebral hypoperfusion induced by 2VO exhibited serious memory impairment. Behaviors related to short and long term memory and spatial memory were severely impaired according to passive avoidance, Y-maze task, and Morris water maze tests. Moreover, histological changes were also observed in 2VO mice versus sham controls. The mean number of surviving cells in the CA1 and dentate gyrus regions were decreased by about 30% and 40% in the vehicle-treated 2VO control group, respectively. These neuronal changes in the CA1 and dentate gyrus regions might explain the memory impairments observed in 2VO mice. Treatment with oroxylin A significantly attenuated these memory impairments in 2VO mice across all 3 tests used (passive avoidance, Y-maze, and the Morris water maze). Moreover, the number of surviving Nissl stained CA1 neurons were also increased by oroxylin A treatment. These results suggest that oroxylin A may attenuate the memory dysfunction caused by 2VO in mice by sparing neuronal cell loss.

Increasing evidence suggests that inflammation is an important cause of the neuronal damage induced by ischemic conditions. Microglia, a macrophage precursor in brain, acts as a sensor of pathological events in the central nervous system including inflammatory events, and it has been shown that microglia is rapidly activated when brain damage occurs, such as, during ischemia or trauma (Kreutzberg, 1996). Several studies have found that increased microglial activation may represent the spatiotemporal pattern and severity of neuronal damage in rats with middle cerebral artery occlusion (Morioka et al., 1991, 1993; Korematsu et al., 1994). In addition, activated microglia is the principal source of nitric oxide (NO) and mediates neuronal cell damage via NO (Chao et al., 1992; Boje and Arora, 1992). In the present study, the number of activated microglial cells immunostained by OX-42 antibody in the CA1 and dentate gyrus regions in the hippocampus were significantly higher in 2VO control mice than that of sham controls. Accordingly, it might be expected that inflammation derived by microglial activation plays an important causative role in the neuronal damage resulting from transient cerebral hypoperfusion with 2VO in mice. Moreover, these increase in the activated microglial cells were dramatically reduced by oroxylin A treatment, suggesting that oroxylin A has neuroprotective activities through its anti-inflammatory properties (Chen et al., 2000; Jiwajinda et al., 2002).

BDNF is one of the best characterized neurotrophic factors, and plays a pivotal role in neuronal survival, growth (Lindsay et al., 1994), and synaptic plasticity (Thoenen, 1995; Schinder and Poo, 2000). Some studies have shown that hippocampal BDNF expression increases during spatial learning (Gooney et al., 2002; Mizuno et al., 2000), whereas BDNF knockout mice show deficits in spatial learning (Linnarsson et al., 1997) and synaptic plasticity (Korte et al., 1995). Transient forebrain or global ischemia is reported to lead to the rapid enhancement of BDNF gene expression in a wide area of the brain including the hippocampus and the cerebral cortex, to protect the neurons from ischemic damage (Tsukahara et al., 1998; Kiprianova et al., 1999; Shirakura et al., 2004). The present results concerning increased BDNF-immunoreactivities in 2VO mice describe the self-protecting properties of hypoperfused neurons. In addition, these properties were found to be further enhanced in 2VO mice treated with oroxylin A. The reasons for increased BDNF-immunoreactivies in the CA1 and dentate gyrus regions in 2VO mice are unclear. However, it could be speculated that the upstream regulator of BDNF expression might be activated by oroxylin A resulting in increased BDNF-immunoreactivity. BDNF expression can be regulated by CREB, one of the families of DNA-binding proteins that mediates the effect of cAMP on the transcriptional regulation of a large number of peptides and proteins (Yamamoto et al., 1988; Gonzalez et al., 1989). Moreover, pCREB, the activated form of CREB, regulates many aspects of neuronal function, including long-term memory formation (Struthers et al., 1991). Furthermore, pCREB mediates expression of several genes, including BDNF and nerve growth factor (Beck et al., 1994; Walton et al., 1996; Tao et al., 1998). Jin et al. (2001) reported that global ischemia induced the expression of pCREB in vulnerable neurons of the hippocampus. In the present study, we observed that pCREB expression was increased in the hippocampal CA1 and dentate gyrus regions in 2VO control mice compared with sham controls. Moreover, pCREB-positive cells were markedly increased by oroxylin A treatment in 2VO mice. Taken together, our findings suggest that hypoperfusion induces BDNF expression in a process that is mediated by CREB phosphorylation, perhaps to facilitate recovery healing, and that these increases in BDNF and pCREB are potentiated by oroxylin A. However, it is unclear whether oroxylin A alone can regulate the expressions of pCREB or BDNF to protect hippocampal neuronal cells.

Oroxylin A was also reported to be a $GABA_A$ antagonist (Huen et al., 2003) and identified to inhibit GABA-induced Cl^- current in hippocampal slices (unpublished data). Moreover, it was reported that $GABA_A$ receptor, especially its antagonist, modulates cAMP-mediated long-term potentiation and long-term depression at hippocampal slices (Yu et al., 2001). If oroxylin A can induce those memory-related phenomena via cAMP at the hippocampus, although we have not yet studied signal mechanism, the increases of pCREB and BDNF immunoreactivities in the hippocampus by oroxylin A are likely to be associated with the result of the intracellular signaling cascade including adenylyl cyclase and cAMP-dependent protein kinase A. However, extensive investigation is needed to confirm these speculations.

In summary, oroxylin A was found to ameliorate memory impairment and the neuronal damage caused by 2VO in a dose-dependent manner in a mouse model. Moreover, we found that activated microglia levels were lower in oroxylin A treated groups than in the 2VO vehicle group. Furthermore, we found that the expressions of pCREB and BDNF are increased by oroxylin A treatment in the hippocampus. These results suggest that oroxylin A has an anti-inflammatory effect that reduces the number of activated microglial cells, and that

it has a neuroprotective effect derived by increasing the expressions of pCREB and BDNF, which results in the amelioration of the memory impairments induced by 2VO in mice.

Acknowledgements

This research was supported by a grant funded by the Korean Food and Drug Administration (2005; L-05-03-2-CHM-628-B). We thank Dr. Mark A. Geyer and Dr. Victoria B. Risbrough at University of California, San Diego for their helpful discussions and editorial assistance.

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