

Neuroprotection by Valproic Acid in Mouse Models of Permanent and Transient Focal Cerebral Ischemia

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Valproic acid (VPA) is a well-known anti-epileptic and mood stabilizing drug. A growing number of reports demonstrate that VPA is neuroprotective against various insults. Despite intensive efforts to develop new therapeutics for stroke over the past two decades, all treatments have thus far failed to show clinical effect because of treatment-limiting side effects of the drugs. Therefore, a safety-validated drug like VPA would be an attractive candidate if it has neuroprotective effects against ischemic insults. The present study was undertaken to examine whether pre- and post-insult treatments with VPA protect against brain infarct and neurological deficits in mouse transient (tMCAO) and permanent middle cerebral artery occlusion (pMCAO) models. In the tMCAO (2 hr MCAO and 22 hr reperfusion) model, intraperitoneal injection of VPA (300 mg/kg, i.p.) 30 min prior to MCAO significantly reduced the infarct size and the neurological deficit. VPA treatment immediately after reperfusion significantly reduced the infarct size. The administration of VPA at 4 hr after reperfusion failed to reduce the infarct size and the neurological deficit. In the pMCAO model, treatment with VPA (300 mg/kg, i.p.) 30 min prior to MCAO significantly attenuated the infarct size, but did not affect the neurological deficit. Western blot analysis of acetylated H3 and H4 protein levels in extracts from the ischemic cortical area showed that treatment with VPA increased the expression of acetylated H3 and H4 at 2 hrs after MCAO. These results demonstrated that treatment with VPA prior to ischemia attenuated ischemic brain damage in both mice tMCAO and pMCAO models and treatment with VPA immediately after reperfusion reduced the infarct area in the tMCAO model. VPA could therefore be evaluated for clinical use in stroke patients.

Key Words: Ischemic stroke, Therapeutic time window, Drug development, Histone deacetylase inhibitor

INTRODUCTION

Valproic acid (VPA), a short-chain fatty acid, is widely used as an anticonvulsant and mood stabilizing drug, primarily in the treatment of epilepsy and bipolar disorder [1].

VPA has neuroprotective effects on various *in vitro* and *in vivo* experiments. In cellular models, treatment with VPA attenuates glutamate-induced excitotoxicity in rat cultured neurons [2,3], inhibits the neuronal death induced by oxygen-glucose deprivation in hippocampal slice cultures [4], and prevents cultured rat cortical neurons from spontaneous neuronal death [5]. In animal models, treatment with VPA decreases brain infarct volume and neurological deficits in a permanent middle cerebral artery occlusion (pMCAO) model [6] and a transient (tMCAO) model in rats [7]. VPA

also reduced hemorrhage volume and hemispheric atrophy and promoted functional recovery in rat intracerebral hemorrhage model [8]. These results suggest that VPA could be used as a neuroprotective agent for ischemic stroke.

Ischemic stroke is the second most common cause of death worldwide and a major cause of disability. Despite intensive efforts to develop new therapeutics for stroke over the past two decades, all treatments have so far failed to show clinical effects, except thrombolysis with tissue plasminogen activator [9]. Although many reasons may account for the failure to develop new therapeutics for stroke, the treatment-limiting side effects of the developing drugs is one of the major reasons [10]. In this respect, VPA could be an attractive candidate as a stroke therapeutic because VPA has an established safety record in humans at antiepileptic doses.

The present study was undertaken to examine whether pre- and post-insult treatments with VPA protect against brain infarct and neurological deficits in mouse pMCAO and tMCAO models.

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ABBREVIATIONS: VPA, Valproic acid; tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion; MCA, middle cerebral artery; CCA, common carotid artery; ICA, internal carotid artery; ECA, external carotid artery; CBF, cerebral blood flow; TTC, 2, 3, 5-triphenyl tetrazolium chloride; HDAC, histone deacetylase.

METHODS

Animals

The institutional animal care and use committee at Chonnam National University approved all experimental methods and animal care procedures, in accordance with the criteria described in the *NIH Guide for the Care and Use of Laboratory Animals*. Male ICR mice (Daehan Biolink Co, Chungbuk, Korea), weighing 25~30 g, were allowed free access to food and water and kept under 12 : 12 light/dark cycle in a temperature (21~25°C) and humidity (45~60%) controlled room.

Transient and permanent MCAO models

Anesthesia was induced with 4% enflurane and maintained at 2% in 100% O₂ using rodent mask (Stoelting, USA). The right middle cerebral artery (MCA) was occluded using the intraluminal suture technique as described previously [11]. Briefly, the right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were exposed through midline cervical incision. MCA occlusion was achieved by introducing a silicon-coated 7-0 nylon monofilament (Ethicon, NJ, USA) into the CCA through ECA and advancing it 9±1 mm via ICA to the origin of MCA in the circle of Willis. For the tMCAO model, animals were subjected to 2-hr MCAO, followed by 22-hr reperfusion. Reperfusion was performed by withdrawal of the intraluminal suture. The interruption and reperfusion of blood flow to the MCA was confirmed using transcranial laser Doppler (DRK4, Moor, Devon, UK). For pMCAO, the intraluminal suture was placed permanently. During all surgical procedures, core body temperatures were monitored by a rectal probe and maintained at 37±0.5°C using a homeothermic blanket control unit (Harvard, UK); animals were kept in a warmed incubator until recovered from anesthesia and fully ambulatory.

Blood flow measurement

Cortical cerebral blood flow (CBF) was measured transcranially in all animals in this study during and after MCAO using a laser Doppler flow device. In brief, relative changes in CBF were measured through the skull in the core region of the MCA territory (2 mm posterior to the bregma and 5~6 mm lateral to midline). In the animals subjected to tMCAO, measurements were made for 5 min each before ischemia, at 5 min after the start of MCAO, at the end of the 2-h MCAO period, and at 5 min after start of reperfusion. The surgical procedure was considered successful if suture placement resulted in a sustained 80% or greater reduction in relative CBF from baseline and early reperfusion CBF was 70% of baseline. Animals not meeting both of these criteria were excluded from the study. In the animals subjected to pMCAO, CBF was measured before ischemia, 5 min after MCAO, and again 2 hr after MCAO to confirm the maintenance of the occlusion.

Neurological deficit evaluation

Neurological deficit was assessed using a neurological score as previously described [12]. Neurological examination was performed by a blinded observer for each animal subjected to MCAO. Each mouse was assigned a score of

0~4, wherein 0 is no observable neurological deficit, 1 is failure to extend the left forepaw, 2 is circling to the left, 3 is falling/rolling to the left, and 4 is inability to walk spontaneously and/or maintain upright posture. The scores were obtained immediately before MCAO and 24 hr after MCAO just before sacrifice.

Measurement of infarct volume

The animals were euthanized by overdose of enflurane at 24 hr after MCAO (2 hr ischemia/22 hr reperfusion in tMCAO) and decapitated. Brains were rapidly removed and cooled on ice for 5 min, and five coronal sections 2 mm apart were cut beginning 2 mm posterior to the anterior pole using a mouse brain matrix (Zivic-Miller Lab. Inc., U.S.A.). The brain slices were incubated in phosphate-buffered saline (PBS; pH 7.4) containing 2% 2, 3, 5-triphenyl tetrazolium chloride (TTC, Sigma, MO, U.S.A.) at 37°C for 30 min and were fixed by immersion in 10% neutral buffered formalin solution [13]. The caudal face of each section was scanned using a flatbed color scanner and the images were stored. Ischemic and non-ischemic hemisphere infarct areas were measured using Image-Pro Plus (Image & Graphics, U.S.A.).

Western blot analysis

To investigate the effects of VPA on histone deacetylase (HDAC) activity, western blot analysis of acetylated histone3 (H3) or histone4 (H4) protein expression was performed. The ischemic hemispheres were homogenized in Nonidet p-40 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM benzamidine, 1% Nonidet P-40, 5 mM EDTA, pH 8.0, 1 µg/ml trypsin inhibitor, 1 mM PMSF) on ice using a Teflon glass homogenizer. After centrifugation (14,000 g) for 15 min at 4°C, the supernatant was collected and aliquoted into cryovials. Total protein concentration of each sample was determined using the Bradford assay (Bio-Rad Laboratories, CA, U.S.A.). The protein samples were separated in 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 10% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T). Acetyl-H3 and acetyl-H4 were detected with rabbit polyclonal antibody (Upstate Biotechnology, MA, U.S.A.) at a dilution rate of 1 : 2000. After washing with PBS-T, the membrane was incubated with peroxidase-conjugated secondary antibody at room temperature for 1 hr. Finally, acetyl-H3 and acetyl-H4 were detected using chemical luminescence (ECL; Amersham Pharmacia Biotech, NJ, U.S.A.).

Drug used

VPA (2-propylpentanoic acid) was purchased from Sigma (MO, U.S.A.) and dissolved in physiologic saline.

Statistical analysis

The data are expressed as mean±standard error of the mean (SEM). To compare multiple means of infarct volume, one-way ANOVA followed by post-hoc Tukey's multiple comparison test was used. For neurological deficit score, Kruskal Wallis test followed by post-hoc Dunn's multiple comparison test was performed. All analyses were performed using InStat (GraphPad Software, CA, USA), and differences were considered statistically significant when p

value was less than 0.05.

RESULTS

Effect of VPA on the infarct volume and neurological deficit score in tMCAO model

In the saline-treated group, the cerebral infarction induced by 2-hr ischemia/22-hr reperfusion was found on most of 5 coronal sections and the percent of infarction was $17.2 \pm 0.9\%$ ($n=12$). The neurological score was 1.92 ± 0.16 . Treatment with VPA (300 mg/kg, i.p.) immediately after reperfusion and 30 min before MCAO significantly reduced the cerebral infarction (30-min pretreatment: $10.4 \pm 1.1\%$, $p < 0.01$, $n=9$; at reperfusion: $11 \pm 1.0\%$, $p < 0.01$, $n=9$) (Fig. 1, 2). The neurological score in pre-treatment with VPA was significantly reduced (1.22 ± 0.16 , $p < 0.05$), whereas the score of animals treated immediately after reperfusion was reduced but not statistically significant (1.33 ± 0.18). However, treatment with VPA 4 hr after reperfusion failed not only to reduce the infarction ($15.2 \pm 1.7\%$, $n=9$) but also to affect the neurological score (1.77 ± 0.16) (Fig. 1, 2).

Effect of VPA on the infarct volume and neurological deficit score in pMCAO model

In the saline-treated control group, the cerebral infarction was found on all of 5 coronal sections and the percent of infarction and the neurological score were $27.4 \pm 1.1\%$ ($n=9$) and 2.22 ± 0.16 , respectively. Pretreatment of animals

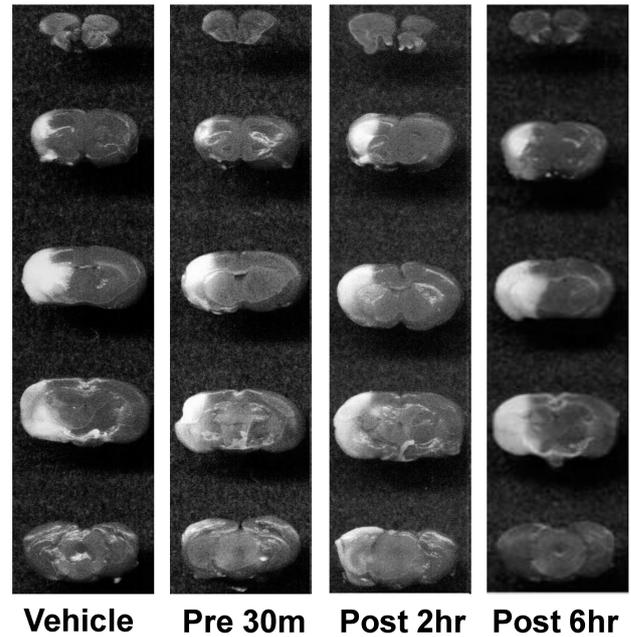


Fig. 1. Representative infarcted brain slices subjected to 2 hr ischemia and 22 hr reperfusion showing effect of treatment with valproic acid (VPA, 300 mg/kg, i.p.) 30 min prior to MCAO (Pre 30 m), immediately after reperfusion (Post 2 hr) and 4 hr after reperfusion (Post 6 hr) in mice. The slices were stained with 2% TTC.

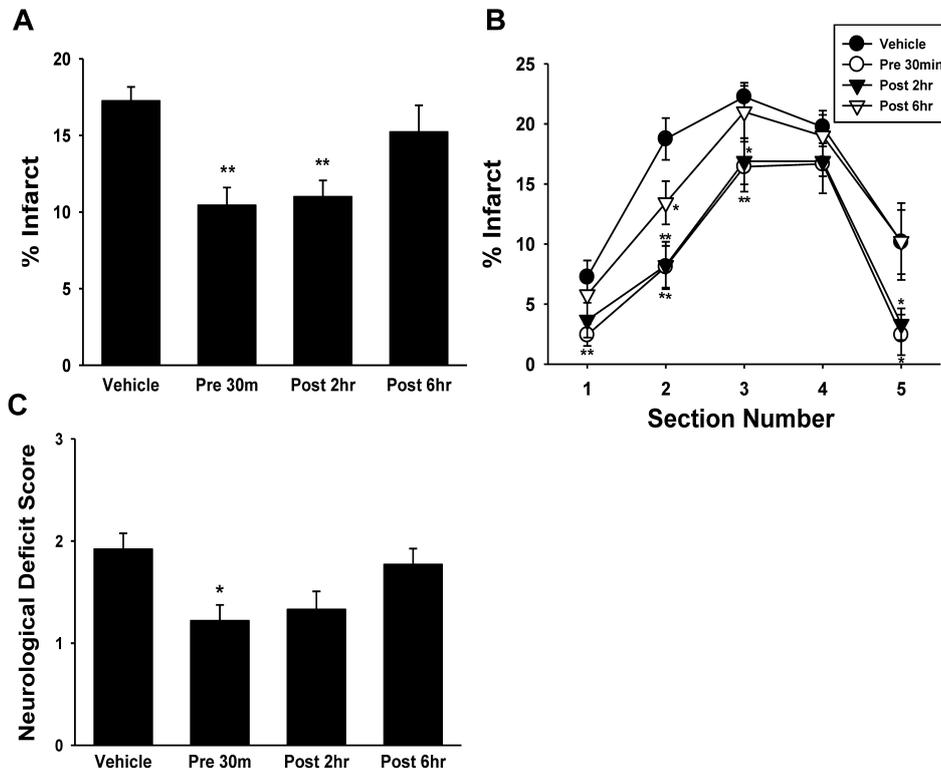


Fig. 2. Effect of treatments with valproic acid (VPA, 300 mg/kg, i.p.) at various time-points on % cerebral infarct of total brain (A), % cerebral infarct of each section (B) and neurological deficit score (C) in the tMCAO model. Each column/point and the vertical bars represent mean \pm SEM from 9~12 animals. Asterisks represent significant difference vs. vehicle-treated group (* $p < 0.05$, ** $p < 0.01$).

with VPA (300 mg/kg, i.p.) significantly reduced the cerebral infarction (23.5±1.1% infarction, n=9) ($p < 0.05$) but did not affect the neurological score (2.33±0.18). When the effects of pretreatment with VPA on the infarct area of each coronal section was compared, only the infarct area of 5th section was significantly reduced (Fig. 3).

Effect of VPA on expression of acetylated histone

To examine the effect of VPA on HDAC activity, the expression of acetylated histone H3 and H4 was analyzed by western blot. The expression of acetylated H3 and H4 at

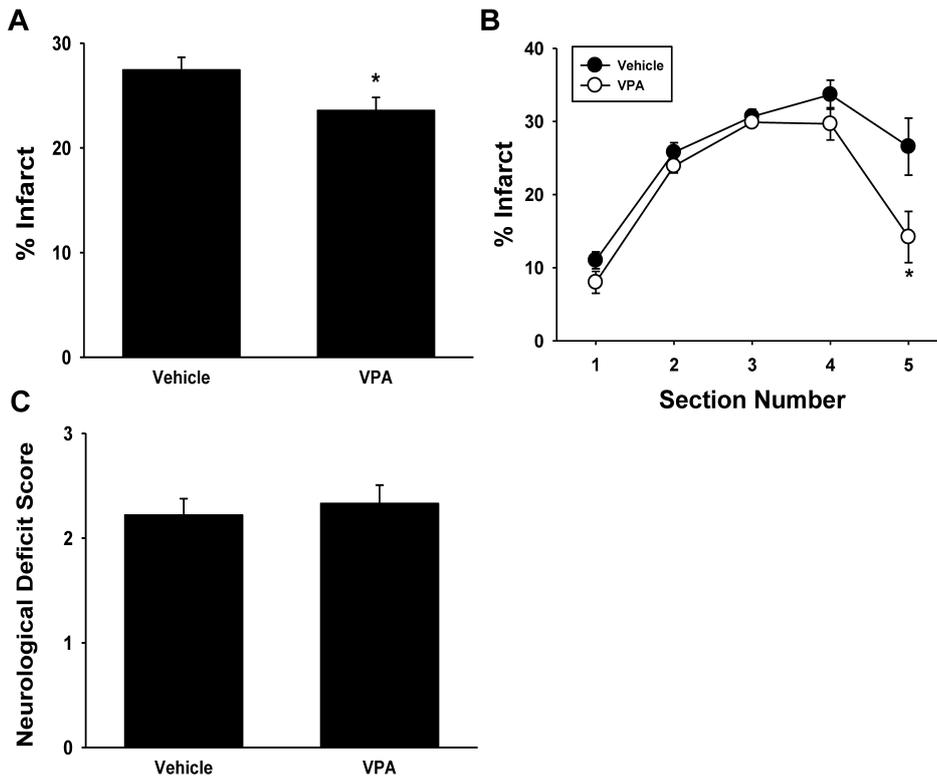


Fig. 3. Effect of pretreatment with valproic acid (VPA, 300 mg/kg, i.p.) on % cerebral infarct of total brain (A), % cerebral infarct of each section (B) and neurological deficit score (C) in the pMCAO model. Each column/point and the vertical bars represent mean±SEM from 9 animals. Asterisk represents significant difference from vehicle-treated group (* $p < 0.05$).

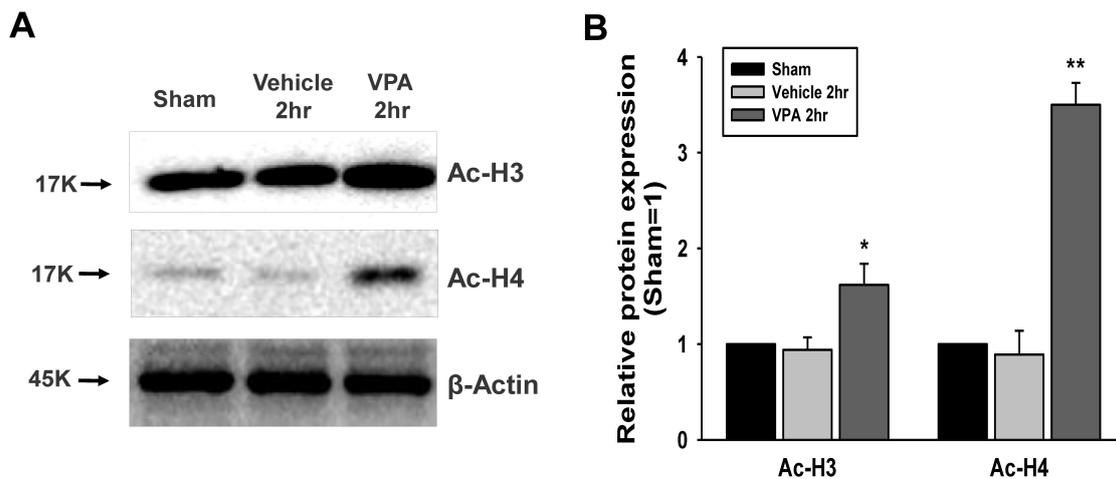


Fig. 4. Effect of valproic acid on the expression of acetylated histone H3 and H4 in the cortices of mouse brain. (A) Western blot analysis of acetylated H3 (Ac-H3), acetylated H4 (Ac-H4) and β -actin in extracts from cortical areas in sham-operated animals (sham), and ischemic cortical area at 2 hr after MCAO in mice treated with saline (vehicle) and valproic acid (VPA). (B) Quantified results of Ac-H3 and Ac-H4. Each column and vertical bar represents mean±SEM from 3~4 animals. Asterisks represent significant difference vs. vehicle-treated group (* $p < 0.05$, ** $p < 0.01$).

2 hrs after MCAO was markedly increased by pretreatment with VPA but not affected by vehicle pretreatment (Fig. 4).

DISCUSSION

In present study, we used the tMCAO model in which 2 hr MCAO was followed by 22 hr reperfusion and the pMCAO model in which MCA was occluded for 24 hr without reperfusion. The infarct volume and neurological score of tMCAO were $17.2 \pm 0.9\%$ and 1.92 ± 0.16 , and those of pMCAO were $27.4 \pm 1.1\%$ and 2.22 ± 0.16 , respectively. The infarct volume of pMCAO is significantly larger than that of tMCAO, whereas the neurological deficit scores between the two models do not show statistical significance. These results suggest that the neurological deficit score in this study may not be sensitive enough to represent changes in the infarct area, and differ from the report that the infarct volume of 2 hr/22 hr tMCAO and 24 hr pMCAO are similar in CD-1 mouse [14]. In general, pMCAO produces a more severe and rapid brain infarction with a smaller and more short-lived penumbra than tMCAO does. The process of pMCAO also favors the mechanisms of necrotic-type cell death, presumably due to the rapid loss of glucose utilization and protein synthesis following permanent ischemia [15]. The VPA dose used in the present study, 300 mg/kg, is close to that used in animal studies to control seizures induced by maximal electroshock [16].

In the tMCAO model, treatment with VPA prior to MCAO or immediately after reperfusion reduced brain infarct size when measured at 24 hr after the onset of MCAO-induced ischemia. VPA also significantly reduced neurological deficits when treated before MCAO. These results are consistent with the results using the rat tMCAO model [7] in which VPA was subcutaneously administered immediately after MCAO and repeatedly every 12 hr for 24 or 48 hr. However, the treatment 4 hr after reperfusion did not reduce the infarct and the neurological deficits. These results suggest that the therapeutic time window for VPA may be earlier than 4 hr after reperfusion.

In the pMCAO model, pre-treatment with VPA significantly reduced the infarct size but did not affect the neurological deficits. These results suggest that pretreatment may not be neuroprotective in our mouse pMCAO model and differ from those reported by Kim et al. [6] using the rat pMCAO model. They reported that the neuroprotective effects were evident even when VPA was administered at least 3 h after MCAO in rat pMCAO model. The differences in neuroprotective effects of VPA between the pMCAO model of present study and that of the previous study [6] may be responsible for the different results. The differences between the two studies are the animal species employed in the study (mouse vs. rat) and the number of VPA treatments (single vs. repeated).

Interestingly, when the infarct area of each section was compared, only the 5th section of brain slices exhibited a reduction of the infarct area in the present study. This result suggests that the area corresponding to the 5th section receives more collateral circulation than the other sections, thus increasing the penumbra area.

Although the underlying mechanisms of the therapeutic effect of VPA are still unclear, recent experiments suggest that VPA directly inhibits HDAC, causing histone hyperacetylation [17]. The status of histone acetylation allows chromatin remodeling and regulates gene expression [18].

In the present study, treatment with VPA significantly increased expression of acetylated H3 and H4 at 2 hrs after MCAO. These findings demonstrate that treatment with VPA inhibits the HDAC activity, and suggest that the HDAC inhibition by VPA may contribute to the neuroprotective effect against ischemic insults in our study.

Taken together, our findings demonstrate that single treatment with VPA prior to ischemia attenuates ischemic brain damage in both mouse tMCAO and pMCAO models and that single treatment with VPA immediately after reperfusion could reduce the infarct area in the tMCAO model. Thus, VPA could be evaluated for its clinical use in stroke patients, even though pMCAO is more ideal than tMCAO as a model for human stroke [19].

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