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Valproic acid attenuates ischemia-reperfusion injury in the rat brain through inhibition of oxidative stress and inflammation

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ABSTRACT

Valproic acid (VPA), widely used in clinical contexts for the treatment of seizures and bipolar mood disorder, has neuroprotective properties in cellular and animal models. However, the precise mechanisms underlying its neuroprotection against stroke remain unknown. In the present study, we explored the effect of VPA on experimental ischemic stroke. Male Sprague-Dawley rats were subjected to middle cerebral artery occlusion for 90 min, followed by reperfusion. The animals received a single injection of VPA (300 mg/kg) immediately, 90, or 270 min after the induction of ischemia. Vehicle-treated animals underwent the same procedure with physiological saline. Infarct volume and neurological symptoms were evaluated 24 h after reperfusion. Immunohistochemical staining for myeloperoxidase (MPO), microglia (Iba1), 4-hydroxy-2-nonenal (4-HNE), or 8-hydroxy-deoxyguanosine (8-OHdG) was performed. Ischemic boundary zone cell death was determined by TUNEL staining. VPA injected immediately or 90 min after ischemia induction significantly reduced infarct volume and improved neurological deficit compared with vehicle ($P<0.05$). VPA was ineffective when given 270 min after ischemia induction. VPA significantly reduced TUNEL-positive cells, MPO-positive cells, Iba1-positive cells, 4-HNE-positive cells, and 8-OHdG-positive cells compared with vehicle in the ischemic boundary zone ($P<0.05$). The therapeutic time window for single injection of VPA is between 0 and 90 min in this model. Our results demonstrate that single injection of VPA may have anti-inflammatory as well as antioxidative effects, leading to reduced cell death in ischemia-reperfusion injury.

1. Introduction

Stroke is one of the leading causes of mortality and morbidity worldwide. Recently, a worldwide campaign was initiated to increase awareness in patients with the very early stages of stroke (the “Brain Attack” campaign), which is essential in view of the increasing use of acute recanalization therapies like thrombolysis and mechanical clot removal (Smith et al., 2005; Wechsler, 2011). However, the percentage of patients benefiting from acute recanalization therapies was fairly small among the entirety of cerebral infarction patients because of the short therapeutic time window for recanalization therapies. Therefore, another strategy for expanding the therapeutic time window of stroke therapy is needed (Katsura et al., 2012).

Valproate (VPA), a simple eight-carbon branched-chain fatty acid, is widely used for the treatment of epileptic seizures, migraine, and as a mood stabilizer for treating bipolar disorder. In vitro, treatment with VPA attenuates glutamate-induced excitotoxicity, inhibits ischemia-induced fast Na and high-voltage-activated calcium currents, and suppresses lipopolysaccharide-induced production of TNF-α and IL-6 (Ichiyama et al., 2000; Peng et al., 2005). A recent report showed that VPA inhibits the ischemia-induced nuclear translocation of nuclear factor-κB activation and matrix metalloproteinase 9 production in vivo. VPA also has protective effects against various types of ischemia/reperfusion injury models or inflammatory disease model in vivo (Bhavsar et al., 2008; Blanchard and Chipoy, 2005; Kim et al., 2012; Zhang et al., 2011, 2012). However, the protective effects of VPA have not been fully understood, and it remains unclear whether the protective effects of VPA on cerebral ischemia are associated with suppressing oxidative stress or neutrophil infiltration in vivo. Moreover, most studies conducted in vivo on the effects of VPA have used multiple injections. Unfortunately, clinical trials using various preclinically neuroprotective drugs for stroke have proven unsuccessful (Broderick and Hacke, 2002). Although many factors may account for the failure to develop new therapeutics for stroke, the treatment-limiting side effects of the developed drugs is thought to be one of the major reasons (Dirnagl and Macleod, 2009). Given that most of the side effects of VPA (i.e., thrombocytopenia, hyperammonemia, parkinsonism) are known to occur dose-dependently (Chateauvieux et al., 2010), a single administration would not cause severe side effects and should be more acceptable in clinical use against stroke.

The present study used a rat transient focal ischemia model to examine whether post-insult and single injection treatment with

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VPA has neuroprotective effects in reducing infarct volume and improving neurological deficits and, if so, to explore the therapeutic time window and to investigate whether the mechanisms of neuroprotection are associated with reduction of oxidative stress and inflammation in the brain.

2. Materials and methods

2.1. Stroke model

All experimental protocols were approved by our Institutional Committee on Animal Research and were carried out in accordance with the National Institutes of Health guidelines for the use and care of laboratory animals. Anesthesia for all surgical procedures was induced using 4.0% halothane and maintained with 1.0% halothane in a 70% N_2O/30% O_2 mixture using a face mask. Adult male Sprague-Dawley (SD) rats weighing 250–300 g underwent 90 min middle cerebral artery occlusion (MCAO) using an intraluminal suture technique, as described in detail previously (Katsura et al., 2008; Suda et al., 2011). Briefly, the left common and external carotid arteries were carefully exposed and a 4–0 nylon surgical thread coated with silicon rubber was transiently inserted into the left internal carotid artery for 90 min; reperfusion was achieved by withdrawal of the suture. The caudal tail artery was cannulated for continuous monitoring of mean arterial blood pressure, analysis of arterial blood gases, and blood glucose level. A heating pad (Harvard Apparatus, Germany) and heating lamp were used to maintain the rectal and head temporal muscle temperature at 37.0 ± 0.3 °C during the surgical procedures. VPA (300 mg/kg; Sigma, St. Louis, MO, USA) was intraperitoneally injected immediately, 90, or 270 min after the induction of ischemia (n=6, each). After the surgery, the animals were placed in a clean cage and allowed free access to water and food at ambient temperature (21–23 °C).

2.2. Measurement of infarct volume

Rats were euthanized 24 h after reperfusion, and the brain was cut out. Seven coronal sections at 2.0 mm intervals between the levels 4 mm anterior to bregma and 6 mm posterior to bregma, which contained the entire infarct, were stained with hematoxylin and eosin (HE) to distinguish between non-viable or viable tissue (pale areas versus non-injured colored tissue) (Shang et al., 2010). The hemispheric and infarct areas of each section were traced using Image J software (NIH, Maryland, USA) in a blinded fashion. The infarct area was determined by subtracting the intact area in the ischemic hemisphere from the total area in the contralateral hemisphere to correct for brain edema. The infarct areas from each HE-stained section were added together and multiplied by the interval thickness to yield the total infarct volume (Swanson et al., 1990).

2.3. Assessment of neurological deficits

Neurological symptoms in each rat were evaluated 24 h after reperfusion in a blind fashion using a neurological test on a 10-point scale (0=normal to 10=maximum impairment). The following three tests were performed to assess neurological symptoms: (1) To assess hemiplegia, the right hindlimb was extended gently with round-tipped forceps and the flexor response was scored as follows: 0, normal; 1, slight deficit; 2, moderate deficit; or 3, severe deficit. (2) To assess posture, the rats were suspended by the tail, and forelimb flexion and body twisting were scored as follows: 0, normal; 1, slight twisting; 2, marked twisting; or 3, marked twisting and forelimb flexion. (3) Assessment of spontaneous ability to move (normal movement=0; failure to extend the right forepaw fully=1; circling to the right=2; falling to the right=3; unable to walk spontaneously=4) was also carried out.

2.4. TUNEL staining and immunohistochemistry

Rats were transcardially perfused with heparinized saline, followed by 4% paraformaldehyde, 24 h after reperfusion (n=4, vehicle group and VPA injected immediately after ischemia induction group), and then 20-μm-thick coronal frozen sections at the level of the anterior commissure (bregma+0.70 mm) were cut on a cryostat. The adjacent sections were stained with hematoxylin and eosin (HE) to confirm the ischemic boundary area. TUNEL staining was performed with a commercially available kit (In situ apoptosis kit; Takara Biomedicals, Osaka, Japan). The anti-inflammatory effects were examined by myeloperoxidase (MPO) and ionized calcium binding adapter molecule 1 (Iba1) staining. The sections were then blocked with 3% normal goat serum in PBS, incubated once overnight with rabbit polyclonal antibodies against MPO diluted at 1:500 (DAKO, Baltimore, MD, USA) or Iba1 diluted at 1:500 (Wako, Osaka, Japan) at 4 °C, and incubated again with biotinylated goat antirabbit IgG (Vector, CA, USA) at room temperature for 1 h. The anti-oxidative effects were examined by 4-hydroxy-2-nonenal (4-HNE) and 8-hydroxy-deoxyguanosine (8-OhdG). Endogenous tissue peroxidase activity was abolished by incubation with 0.3% H_2O_2 in methanol for 20 min. After washing in Tris-buffered saline, non-specific binding was blocked with 5% normal bovine serum in Tris-buffered saline. The brain sections were incubated overnight at 4 °C with a mouse monoclonal antibody against 4-HNE diluted at 1:50 (Japan Institute for the Control of Aging, Shizuoka, Japan) to assess lipid peroxidation; a mouse monoclonal antibody against 8-OhdG diluted at 1:50 (Japan Institute for the Control of Aging, Shizuoka, Japan) was used to detect oxidative DNA damage. Binding biotinylated antibodies were visualized using diaminobenzidine (DAB). Positively stained cells for each of TUNEL, MPO, Iba-1, 4-HNE, and 8-OHdG were evaluated in the cerebral cortex of the ischemic boundary zone. The numbers of positively stained cells were counted in three randomly selected microscopic fields under 100 × magnification (1.33 mm^2) in a blind fashion.

2.5. Statistical analysis

Data are presented as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). ANOVA followed by the Student–Neuman–Keul post hoc test was used to compare differences in physiological variables, infarct volumes, edema volume, and number of stained cells. The neurological scores were analyzed with a nonparametric multiple comparison method (the Steel–Dwass method). A P value < 0.05 was considered significant.

3. Results

3.1. Physiological parameters

Physiological variables were within normal limits at all evaluated time points for mean arterial blood pressure, pH, PCO_2, PO_2, blood glucose, and rectal temperature, and showed no statistically significant differences among the vehicle- and VPA-treated groups (Table 1). No obvious adverse side effects were observed in both vehicle- and VPA-treated groups.

3.2. Infarct volume

Twenty-four hours after reperfusion, in the VPA-treated group, immediately and 90 min but not 270 min after induction of ischemia,