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Fosphenytoin Reduces Hippocampal Neuronal Damage in Rat Following Transient Global Ischemia

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Summary

Fosphenytoin, a water-soluble disodium phosphate ester of phenytoin, is a phenytoin prodrug with similar anticonvulsant properties. In this study, we evaluated its neuroprotective properties in a cardiac arrest-induced global ischemia model. After 12 minute ischemia, Long-Evans hooded rats were resuscitated, given fosphenytoin (30 mg/kg, i.m.) or saline 5 minutes after the ischemic episode, and killed on day 7. Brains were removed, fixed, and vibratome sectioned to assess the numbers of normal appearing CAI pyramidal neurons and for immunohistological staining of glial fibrillary acidic protein (GFAP). After global ischemia, the number of hippocampal CA1 pyramidal neurons decreased significantly (from 14.33 ± 1.73 to 2.19 ± 0.16 per 100 μm^2). Most hippocampal CA1 pyramidal neurons showed signs of injury and GFAP immunoreactivity of the region increased. With fosphenytoin treatment 5 min after ischemia, hippocampal CA1 pyramidal neurons remained at near control level (13.90 ± 0.92), however, GFAP staining was not significantly changed. Our data, although indicating different neuronal and glial responses following fosphenytoin treatment, nevertheless, suggest that fosphenytoin is an effective neuroprotectant against ischemia-induced damage.

Keywords: Fosphenytoin; global ischemia; GFAP; hippocampus.

Introduction

Fosphenytoin (Cerebyx) is a phosphate ester of a well known antiepileptic drug, phenytoin. In tissues and blood, fosphenytoin is rapidly converted by phosphorylase to phenytoin. The pharmacological effects of administrating these two drugs are thus nearly identical in experimental animals and in humans [3, 34]. However, better solubility and reduced tissue irritation with i.v. injection makes fosphenytoin a promising alternative to phenytoin [13]. Its rapid intramuscular absorption [4, 24, 34] also makes fosphenytoin a better parenteral antiepileptic drug.

In cerebral ischemia animal models, including rabbit [9], gerbil [8], and rat [11, 32, 33], phenytoin demonstrated protective effects in different hippocampal regions and dentate granular cells. In humans, a clinical trial in postcardiac arrest patients also indicated that phenytoin improved neurological function [1]. However, the neuroprotective properties of fosphenytoin following ischemia have not been fully explored. Recently, we reported on the neuroprotective property of a lazaroid in a transient global ischemia rat model [30]. In this study, we used the same rat model of ischemia to evaluate the protective effect of fosphenytoin on the survival of hippocampal CA1 pyramidal neurons and to assess its effect on astrocytes.

Methods and Material

Transient global ischemia was produced by chest compression in male Long-Evans hooded rats (250–350 g) [23]. Briefly, rats were ketamine anesthetized (150 mg/kg i.p.), trachea intubated, and cannulated through the left femoral artery. Pulse pressure of the supine, anesthetized rat was recorded. Brain temperature was monitored by a thermoprobe inserted in the base of the temporalis muscle and maintained at $35 \pm 0.4\,^{\circ}C$. Global ischemia was induced by lowering a padded 3 kg weighted V-shaped bar onto the rat's chest for 12 minutes. Pulse pressure disappeared within 10 sec, indicating the onset of global ischemia. After 12 min ischemia, circulation was restored by cardiopulmonary resuscitation. Animals were artifically ventilated until spontaneous breathing started, typically within 30 min after chest compression. Pulse pressure and brain temperature were continuously monitored and controlled for another hour before returning the rat to its cage.

A total of four groups of rats, including normal ($n = 2$), sham operated ($n = 2$), ischemia with saline-treated ($n = 2$), and ischemia with fosphenytoin-treated ($n = 2$), were studied. Postischemic rats in saline-treated and fosphenytoin-treated groups received a single i.m. injection of saline or fosphenytoin (30 mg/kg, a gift from Park-Davis Pharmaceutical Co., Morris Plains, NJ, USA), respectively, in the right hind limb 5 minutes after resuscitation. Sham-operated

animals were treated similarly except for chest compression. All rats were killed on the 7th postischemic day by decapitation. Brains were immediately removed, bisected longitudinally, and immersed in 4% buffered neutral formaldehyde containing 0.25% glutaraldehyde for a minimum of 2 days at 4 °C. Portions of the brain containing the dorsal hippocampus (Bregma −3.3 to −3.8 mm, Paxinos & Watson [20]) were coronally sectioned with a vibratome at 40 μm. With the aid of a dissecting microscope, rectangular blocks of about 1 mm^2 in size encompassing the mid-CA1 region from sections that approximate Bregma −3.6 [20] were dissected, postfixed in 2% osmium tetroxide, and dehydrated in ascending concentrations of ethanol before being embedded in Araldite 502. Sections of polymerized blocks 1 μm thick were cut and toluidine blue stained for light microscopic examination. Sections from all groups were blind coded before being evaluated under 40× objective. The proportion of normal appearing hippocampal CA1 neurons was determined. "Normal" pyramidal neurons had lightly stained cytoplasm and spherical nuclei. Two blocks per animal were examined. 1 μm sections obtained from each block were examined using 40× objective. Section images were projected via a digital camera on a monitor for on-screen cell counting. In each section, two to four 100 μm^2 fields of clearly aligned CA1 pyramidal cells could be observed and counted. Observed normal neurons for each animal were converted to number of normal neurons per 100 μm^2 for comparison. After all data was collected the code was broken and the differences between groups were examined statistically by ANOVA and Student's t-test.

For GFAP immunohistochemistry, a 40 μm section corresponding to Bregma −3.6 [20] from each animal was selected and stained as a single batch. Sections were washed with phosphate buffer and incubated with normal goat serum for 45 minutes before staining overnight at 4 °C in a polyclonal primary antibody against GFAP (1 : 2000 rabbit anti-bovine, Accurate Chemical & Scientific Corp., Westbury, NY, USA). After several washes with phosphate buffer, sections were incubated in biotinylated secondary antibody (biotinylated anti-rabbit, Vector, Burlingame, CA, USA) for 40 minutes at room temperature. After several washes in phosphate buffer, the sections were incubated in avidin and horseradish peroxidase (Vectastain ABC kit, Vector, Burlingame, CA, USA) for 1 hour. Lastly, sections were incubated with H_2O_2 and 3, 3′-diaminobenzidine tetrahydrochloride for 3 min to visualize the GFAP labeling sites. Mounted without counter stain, sections were examined and analyzed by an image analysis system (BioQuant PM for OS/2, Nashville, TN, USA). GFAP immunoreactivity for sections were quantified by optical density analysis according to Bullitt *et al.* [5]. Images of the hippocampal CA1 region including strata oriens, pyramidale, and radiatum were captured using a 20× objective by a CCD camera. The complete area including three different subdivisions was traced manually on the screen and the average optical densities were computed using a gray-level scale where 0 = black and 255 = white. Adjacent parietal cerebral cortex was read as reference tissue. The data of hippocampus and cortex was collected and the differences between groups were examined statistically by ANOVA and Student's t-test.

Results

Coronal sections of the mid-hippocampal CA1 region in normal, nonischemic rats showed a distinct layer of pyramidal neurons in the stratum pyramidale with parallel stacks of apical dentrites extending into the well organized stratum radiatum (Fig. 1A). The cell densities of normal-appearing pyramidal neurons for normal and sham-operated controls were 15.03 ± 2.51 and 14.33 ± 1.73 (mean ± SD) cell profiles per 100 μm^2, respectively (Fig. 2). No morphological or quantitative difference was found between normal and sham-operated animals. Severe alteration of the cytoarchitecture in the CA1 region was observed 7 days after 12 min transient global ischemia in the saline-treated group (Fig. 1B). Most pyramidal neurons in the stratum pyramidale degenerated or were pyknotic; small darkly stained cells appeared frequently in this region. Irregularly arranged apical dendrites in the stratum radiatum also were evident. One-way ANOVA analysis of the number of surviving neurons showed a significant difference between all groups ($F = 29.57$, df 3; $p < 0.005$). Compared to

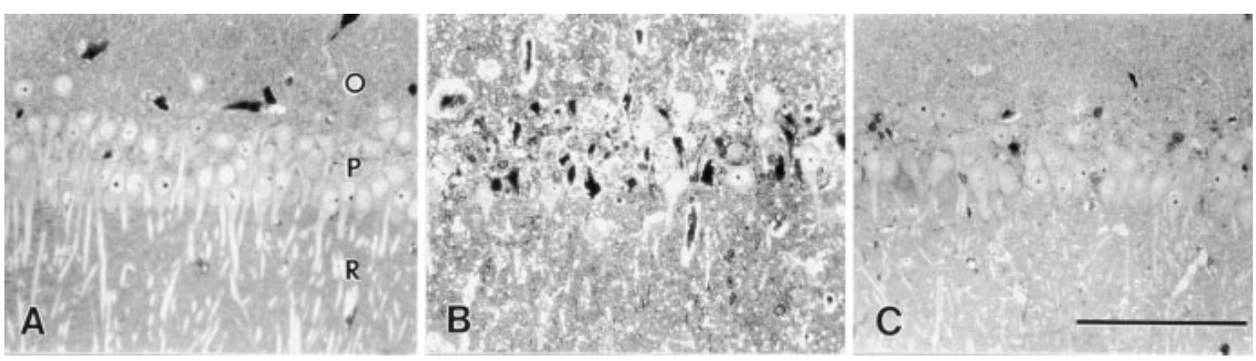

Fig. 1. Light microscopic views of the midhippocampal CA1 region, plastic-embedded 1 μm sections. (A) Sham-operated animal showing a distinct layer of pyramidal neurons and their orderly apical dendrites; (B) seven-day postischemic animal showing deformity of the stratum pyramidale and few surviving pyramidal neurons; (C) 7 day postischemic fosphenytoin treated (30 mg/kg, i.m.) rat. The stratum pyramidale shows little damage and near normal CA1 cytoarchitecture. *O* stratum oriens, *P* stratum pyramidale, *R* stratum radiatum. Bar = 100 μm

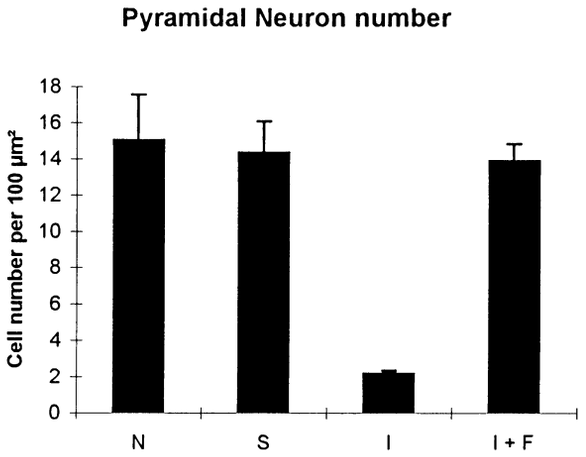

Fig. 2. The number of normal-appearing pyramidal neurons in mid-CA1 region. *N* normal group, 15.02 ± 2.51 per 100 μm^2; *S* Sham-operated group, 14.33 ± 1.73 per 100 μm^2; *I* ischemia, saline-treated group, 2.19 ± 0.16 per 100 μm^2; *I* + *F*, ischemia, 30 mg/kg i.m. fosphenytoin treated group, 13.90 ± 0.92 per 100 μm^2 ($p < 0.05$ for N vs. I and S vs. I, Student's t-test)

controls, normal-appearing neurons declined significantly to 2.19 ± 0.16 per 100 μm^2 in the saline-treated group 7 days post-ischemia ($p < 0.05$, Student's t-test). Postischemic fosphenytoin treatment resulted in almost total preservation of CA1 pyramidal neurons (Fig. 1C). While there were still a few pyknotic neurons and some deranged apical dendrites, the numbers of normal neurons for the fosphenytoin-treated group (13.90 ± 0.92 per 100 μm^2) were significantly different from those for the saline-treated group ($p < 0.005$, Student's t-test). There was no significant difference in the number of normal neurons between fosphenytoin-treated and sham-operated animals.

GFAP immunolabeling of astrocytes 7 days post-ischemia was elevated in both the saline- and fosphenytoin-treated groups as compared to the sham-operated or normal groups (Fig. 3). In the saline-treated group, there was an overall increase of labeling in the strata oriens, pyramidale, and radiatum with presence of intensely labeled GFAP positive, hypertrophic astrocytes in stratum pyramidale (Fig. 3B). Astrocytes in the strata oriens and radiatum in the fosphenytoin-treated group also showed increased GFAP labeling in comparison to the controls (Fig. 3C). However, astrocytes in fosphenytoin-treated group around the stratum pyramidale, in comparison to that in saline-treated group, appeared more uniform in size and were without prominently stained processes. Diffused increase of GFAP labeling in stratum oriens in fosphenytoin-treated group is evident comparing to that in saline-treated group.

Table 1. *GFAP Optical Density 7 Days After Transient Global Ischemia*

	Cortex	CA1
Normal	129 ± 2.83	112 ± 1.41
Sham	129 ± 7.07	112 ± 3.54
Ischemia	120.5 ± 14.85	89 ± 9.9
Ischemia + fosphenytoin	123.5 ± 3.54	93.5 ± 0.71[a]

Optical density in gray-level scale (0 = black, 255 = white). Values are means ± SD of two different slides.
[a] $p < 0.05$ (student's t-test) compare to Sham.

The optical density of GFAP immunoreactivity in the parietal cortex of all groups appeared similar (Table 1). There was no significant difference between groups (one-way ANOVA, $F = 0.56$, df 3; $P = 0.67$). There was, however, a significant difference of GFAP labeling in the CA1 region between

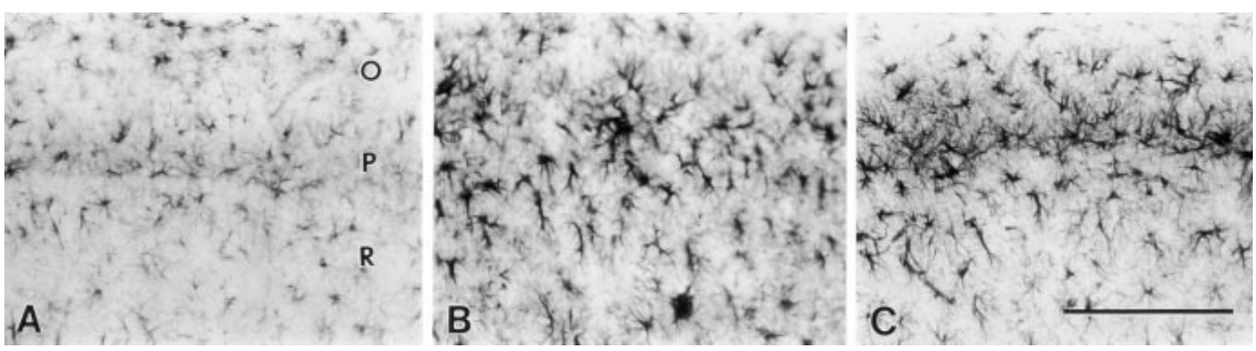

Fig. 3. GFAP stained astroglia in the midhippocampal CA1 region, 40 μm vibratome section. (A) Sham animal, GFAP positive astroglia are present in all three layers; (B) ischemia with saline; (C) fosphenytoin treated rats. *P* Stratum pyramidale; *R* stratum radiatum; *O* stratum oriens. Bar = 100 μm

groups (one-way ANOVA, $F = 10.65$, df 3; $p < 0.05$). Further analysis by Student's t-test showed no significant difference in gray level between the two ischemic groups, fosphenytoin-treated group (93.5 ± 0.71, mean $\pm$ SD) and the saline-treated group (89 ± 9.9). The difference between the sham operated (112 ± 3.54) and fosphenytoin-treated ischemic groups (93.5 ± 0.71) was significant ($p < 0.05$). Postischemic treatment with fosphenytoin effectively reduced neuronal loss in the hippocampal CA1 stratum pyramidale. However, in spite of its clear impact on pyramidal neuronal survival, fosphenytoin did not significantly alter the GFAP staining pattern induced by ischemic insult in hippocampal CA1 region.

Discussion

Data from this study of postischemically administered fosphenytoin indicate a neuroprotective potential for this antiepileptic agent. This finding is consistent with previous reports for phenytoin [11, 32, 33]. However, in clinical use, phenytoin tends to cause severe tissue irritation during intravenous injection [13] and may cause cerebral thrombosis in rats [12]. Phenytoin is also not suitable for intramuscular injection because of the high pH and propylene glycol containing solvent needed to dissolve it. In contrast, fosphenytoin is water soluble and is relatively safe for intramuscular administration, even in multiple doses [34]. In clinical trials, i.m. administration of fosphenytoin can produce the same or higher blood concentration than equivalent oral doses [31]. The i.m. administration of fosphenytoin to rats in this study did not cause significant local tissue irritation.

Although studies have shown that phenytoin alters the local vasculature [2] or free fatty acid metabolism [13] in the brain, the main mechanism of its neuroprotective property is likely to be its ability to block neuronal voltage dependent Na^+ channels [7]. After ischemia a neuronal cascade of events starts with activation of voltage dependent Na^+ channels, Ca^{2+} influx, increased glutamate release, depletion of ATP, and imbalance of ion gradients, all leading to neuronal death. Phenytoin may affect one or more of these events by blocking the initial influx of Na^+ thus protecting the neurons. Similar neuronal protective effects have also been reported with other voltage-dependent sodium channel blockers [7].

Astrocytes in hippocampus are known to be more resistant to transient ischemia than the CA1 hippocampal pyramidal neurons [16]. However, after ischemic insult, morphological changes in astrocytes can be observed within 40 min [22]. After an ischemic insult, astrocytes showed an increase in size of their perivascular processes and in the number of mitochondria [21]. These changes suggest that astrocytes may respond to an ischemic insult by increased metabolic activity. In neuronal tissue, astrocytes can regulate the glutamate-glutamine cycle by taking up excess synaptic glutamate. Normally, astrocytes remove extracellular glutamate and turn it into glutamine. The failure of astrocytic removal of excess released glutamate during ischemia can result in neuronal hyperactivity and neuronal damage [19]. In hippocampal slices, selective inhibition of astrocyte metabolism can cause an increase in neuronal excitability during an ischemic episode [15]. Astrocytes may also play a role in regulating neuronal metabolism. While brain glucose is depleted during ischemia, anaerobic glycolysis of the stored glycogen in astrocytes can generate lactate. In vitro study of hippocampal slices suggest that the lowered pH, partly contributed by lactate accumulation, during an ischemic episode can protect neurons from exitoxic damage [10, 26, 28, 29]. Lactate, mainly generated by astrocytes, can diffuse rapidly into the extracellular space and may serve as an alternative energy source for neurons during ischemia [17, 26]. Recent in vitro studies further suggest that neuronal lactate utilization after an ischemic/hypoxic episode is also required for synaptic function recovery [27]. These studies suggest that during ischemia and reperfusion, lactate production by astrocytes may be crucial for neuronal survival.

Astrocyte hypertrophy resulting in increased GFAP immunoreactivity in hippocampus is known to occur after an ischemic episode. In the hippocampus, GFAP immunoreactivity gradually increases after ischemia and peaks at day 7 [18, 25]. GFAP immunoreactivity may remain elevated for several more days [6, 18]. It is possible that the increase in GFAP immunoreactivity is directly related to neuronal damage, especially in the hippocampal CA1 region. In this study we demonstrated a sustained increase in GFAP immunoreactivity concurrent with ischemic neuronal death in the hippocampal CA1 region in the saline-treated group. Postischemic fosphenytoin administration reduced the neuronal damage, but did not significantly alter the GFAP reaction 7 days after the ischemic episode. Thus increased GFAP immunoreactivity may not always indicate neuronal damage. A study by Petito *et al.* [22] reporting that post-ischemic

GFAP increased in rat striatum without accompanying neuronal damage, support this view. The GFAP increase is more likely due to increased astrocyte activity, e.g. lactate turnover, rather than to neuronal damage.

This study shows that a single postischemic dose of fosphenytoin can significantly reduce neuronal damage in the hippocampal CA1 region without significant alteration in GFAP immunoreactivity. The reason for these divergent responses to fosphenytoin remain to be elucidated. The study clearly indicates the potential of fosphenytoin as a post-ischemia neuroprotectant.

Acknowledgement

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Comments

This is an experimental study on neuroprotective effect of a prodrug of phenytoin, disodium phosphate ester, fosphenytoin. Cerebral ischemia was produced in rats with a global ischemia model for 12 minutes using chest compression method. The selective ischemic injury of hippocampus was evaluated by assessing the CA1 pyramidal neurons and glial fibrillary acid protein (GFAP) 7 days after the ischemia. There were four study groups; controls, animals with sham operation, ischemic animals without treatment and animals treated with a single i.v. injection of fosphenytoin after ischemic insult. Pyramidal neurons were significantly reduced after ischemia, but almost normal in the treatment group. GFAP was increased in hippocampal area in both ischemia groups, what may suggest increased metabolic response of astrocytes. There were no ischemic changes in parietal area.

The results are interesting and need further strengthening. The number of animals in different groups was only two, but the results in neuropathology were already quite consistent after blinding and also statistically significant. Phenytoin has not been very attractive drug lately in the research of preventing ischemia or temporal epilepsy, both connected to the pathological mechanism of hippocampal CA1 neuronal loss and gliosis. Timing of ischemia and intensity of treatment need further experimental studies to confirm these preliminary results.

M. Vapalahti

In a well designed experimental study performed in rats, evidence is provided that phosphenytoin (a phosphate ester of phenytoin) has a protective effect against hippocampal neurons ischemia-induced damage. The experimental technique and the results are described in detail. The discussion on the possible mechanisms of the effect observed is interesting. A weak point of the study is the scarce number of animals utilized: 2 rats only, plus 6 rats for different controls, subdivided in 3 groups of 2. Should the reported findings be validated by a larger experience, their value would be remarkably increased.

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