Journal of the Neurological Sciences 274 (2008) 57–63

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Phenytoin protects central axons in experimental autoimmune encephalomyelitis

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ARTICLE INFO

Article history:
Received 14 February 2008
Received in revised form 20 March 2008
Accepted 2 April 2008
Available online 15 May 2008

Keywords:
Axon protection
EAE
Inflammatory cell
Multiple sclerosis
Phenytoin
Sodium channel

ABSTRACT

Axon degeneration is a major contributor to non-remitting deficits in multiple sclerosis (MS). Thus the development of therapies to provide protection of axons has elicited considerable interest. Voltage-gated sodium channels have been implicated in the injury cascade leading to axonal damage, and sodium-channel blockers have shown efficacy in ameliorating axonal damage in disease models following anoxia, trauma and damaging levels of nitric oxide (NO). Here we discuss studies in our laboratory that examined the protective effects of phenytoin, a well-characterized sodium-channel blocker, in the inflammatory/demyelinating disorder experimental autoimmune encephalomyelitis (EAE), a model of MS. Administration of phenytoin to C57/Bl6 mice inoculated with rat myelin oligodendrocyte glycoprotein (MOG) provides improved clinical status, preservation of axons, enhanced action potential conduction and reduced immune infiltrates compared to untreated mice with EAE. Moreover, continuous treatment with phenytoin provides these protective actions for at least 180 days post-MOG injection. The withdrawal of phenytoin from mice inoculated with MOG, however, is accompanied by acute exacerbation of EAE, with significant mortality and infiltration of immune cells in the CNS. Our studies demonstrate the efficacy of phenytoin as a neuroprotectant in EAE. Our results also, however, indicate that we need to learn more about the long-term effects of sodium-channel blockers, and of their withdrawal, in neuroinflammatory disorders.

1. Introduction

Axonal degeneration has been recognized as a major contributor to non-remitting disability in multiple sclerosis [1,2] and this has elicited considerable interest in identifying neuroprotective therapies that can ameliorate axonal injury in neuroinflammatory disorders. Early *in vitro* work provided evidence that voltage-gated sodium channels can participate in calcium-mediated degeneration of central white matter axons following anoxic injury [3,4]. These studies demonstrated that persistent sodium current can drive reverse operation of the Na^+/Ca^{2+} exchanger (NCX), leading to importation of high levels of Ca^{2+} that lead to irreversible axonal injury. Consistent with a role for sodium channels in the pathogenesis of axonal injury during anoxia, sodium-channel blockers, including tetrodotoxin (TTX), phenytoin, carbamazepine and quartenary anesthetics, were shown to prevent the development of irreversible dysfunction of axons in isolated white matter *in vitro* following injury [3,5,6]. Sodium-channel blockers also exert protective effects on axons exposed to trauma [7,8] and to injurious levels of nitric oxide (NO) [9], which is present at increased levels within MS lesions [10–12].

The present chapter presents work in our laboratory that examined the effects of phenytoin on C57/Bl6 mice in which experimental autoimmune encephalomyelitis (EAE) was induced by inoculation with rat myelin oligodendrocyte glycoprotein (MOG). The results demonstrate that phenytoin treatment provides robust protection of spinal cord axons, preservation of action potential conduction, significantly diminished immune cell infiltrate, and amelioration of neurological deficits in mice with EAE for at least 180 days post-MOG injection. Withdrawal of phenytoin, however, can result in acute exacerbation of EAE in these mice, which is accompanied by substantial immune infiltrate in the spinal cord and a high mortality rate.

2. Phenytoin treatment improves EAE

2.1. Clinical status

Untreated C57/Bl6 mice injected with MOG developed a disease onset that was evident 7–10 days post-inoculation and a progression of disability that plateaued around 19–22 days post-injection (Fig. 1A [13,14]). In contrast, when the C57/Bl6 mice were administered phenytoin-supplemented chow commencing on day 10 following MOG injection (using a regimen that achieved phenytoin blood levels within the human therapeutic range, 10–20 μg/ml), there was a significantly less severe clinical course in the treated mice compared to untreated mice from day 13 until the end of the study at day 28 (Fig. 1A).

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doi:10.1016/j.jns.2008.04.001

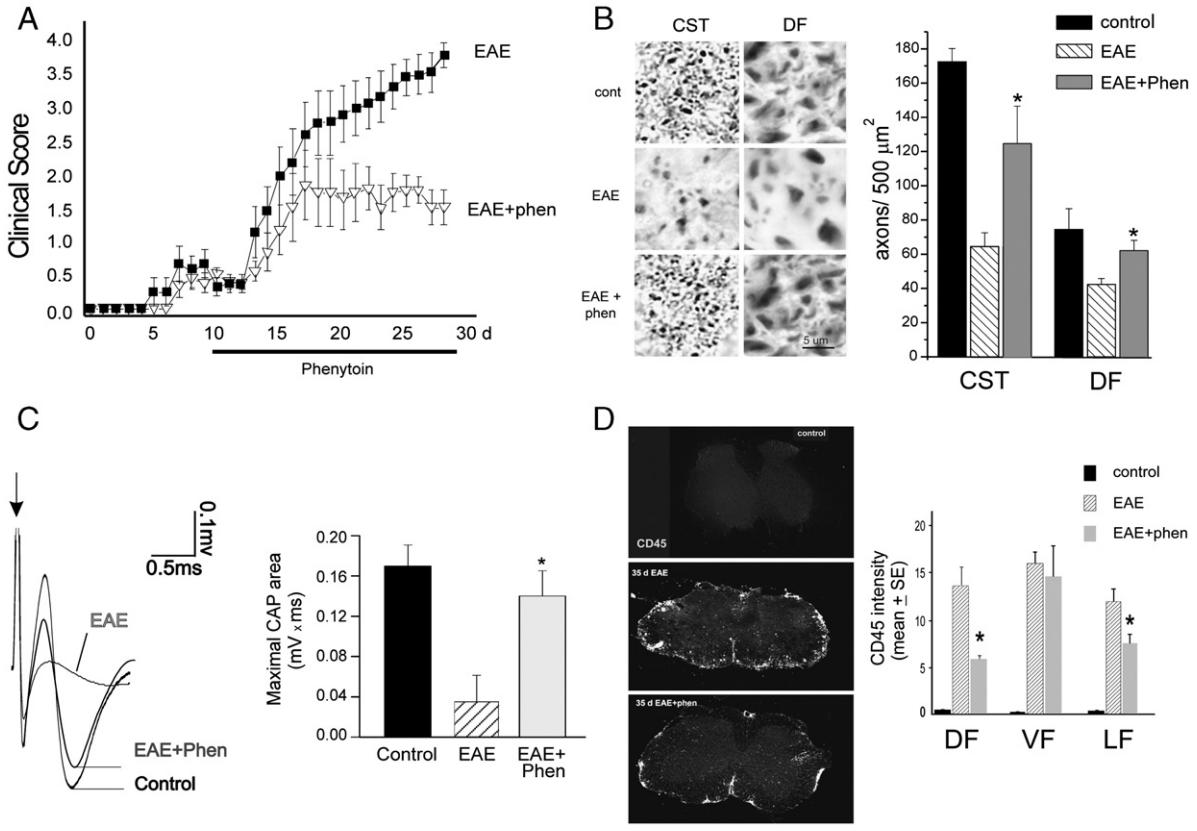

Fig. 1. Phenytoin treatment improves EAE. A. Clinical scores (mean ± SEM) are shown for untreated EAE (filled squares) and for phenytoin-treated EAE (open triangles). Phenytoin-supplemented chow was administered to mice commencing on day 10, as indicated by the horizontal bar. B. Representative sections demonstrate that the number of neurofilament immunostained axons is reduced in untreated mice with EAE in both the corticospinal tract (CST) and dorsal funiculus (DF) in comparison to control mice, but phenytoin-treated mice with EAE exhibit protection of axons compared to untreated mice with EAE. Quantification of CST and DF axons from control, untreated EAE mice, and phenytoin-treated EAE mice demonstrates a significant reduction of axons in untreated EAE mice compared to control mice. Phenytoin treatment of mice EAE results in a significant (*, $p<0.05$) protection of axons within the CST and dorsal columns compared to untreated mice with EAE. C. Superimposed compound action potential (CAP) traces from representative control, untreated EAE, and phenytoin-treated EAE mice (arrow indicates stimulus artifact). The typical biphasic wave exhibited by control mice is highly attenuated in untreated EAE mice, but is restored in phenytoin-treated EAE mice. Average supramaximal CAP area (±SEM, mV×ms) in control, untreated EAE, and phenytoin-treated EAE mice (*, $p<0.05$, phenytoin-treated EAE compared to untreated EAE). D. Low magnification images of cross-sections through lumbar spinal cords labeled for CD45 are shown for control, EAE 35 days post-injection (35 d EAE), and EAE + phenytoin 35 days post-injection (35 d EAE + phen). Few $CD45^+$ cells are present in control spinal cords. Phenytoin-treated mice with EAE exhibit reduced numbers of $CD45^+$ cells in comparison to untreated mice with EAE. Quantification of CD45 intensity in dorsal (DF), ventral (VF) and lateral (LF) funiculi of control, untreated EAE, and phenytoin-treated EAE mice. CD45 intensity is significantly (*, $p<0.05$) reduced in the DF and LF of phenytoin-treated mice with EAE compared to untreated mice with EAE [modified and reproduced with permission from Refs. [13,14]].

2.2. Axon counts

To determine whether the increased clinical disability in untreated mice with EAE was accompanied by a loss of spinal cord axons, we quantified the number of axons within the dorsal corticospinal tract (a descending tract) and the dorsal funiculus (cuneate fasciculus; an ascending tract). Sections were reacted with antibodies against both phosphorylated (SMI-31) and non-phosphorylated (SMI-32) neurofilaments, to identify all axons within these tracts [15,16]. In parallel with the increasing disability in untreated mice with EAE, there was a significant loss of axons in these mice in both the corticospinal tract and the dorsal funiculus compared to control mice, with an approximately 60% loss of axons in the corticospinal tract and a 40% loss in the dorsal funiculus compared to control mice, as assessed at 28–30 days post-MOG inoculation (Fig. 1B) [13]. The substantial loss of spinal cord axons in these mice with EAE is consistent with previous studies that reported axonal damage in rats with EAE [17,18].

In contrast to untreated mice with EAE, treatment with phenytoin at clinically-relevant levels significantly attenuated the loss of axons in mice with EAE, such that there was only a 28% loss of axons in the corticospinal tract and a 17% loss in the dorsal funiculus compared to control values (Fig. 1B). The protective effects of phenytoin in spinal cords of mice with EAE are similar to those reported in a rat model of chronic-relapsing EAE in which the rats were administered the sodium-channel blockers flecainide [19] and lamotrigine [20].

2.3. Spinal cord electrophysiology

To functionally assess axonal conduction in untreated and phenytoin-treated mice with EAE, we also performed compound action potential (CAP) recordings on the surface of dorsal spinal cords. The maximal CAP area in untreated mice with EAE was significantly attenuated compared to control mice (0.038 mV ms vs 0.16 mV ms; Fig. 1C) [13], consistent with substantial axon damage in mice with EAE. In contrast, phenytoin-treated mice with EAE exhibited maximal CAP areas (0.13 mV·msmV ms) that were significantly greater than that of untreated mice with EAE and that approached control values (0.13 mV·msmV ms vs 0.16 mV·msmV ms). Mean conduction velocities were determined in the three groups of mice (control: 11.6 ± 2.1 m/s; EAE: 2.3 ± 1.7 m/s; EAE + phenytoin: 13.2 ± 1.1 m/s) and further supported the functional protection of axons with phenytoin treatment [13].

2.4. Inflammatory infiltrates

The onset and progression of EAE is associated with infiltration of immune cells into the CNS [21,22]. To examine whether phenytoin