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SHORT COMMUNICATION

Effects of 6(5H)-phenanthridinone, an Inhibitor of Poly(ADP-ribose)Polymerase-1 Activity (PARP-1), on Locomotor Networks of the Rat Isolated Spinal Cord

Sara Ebrahimi Nasrabady · Anujaianthi Kuzhandaivel · Miranda Mladinic · Andrea Nistri

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Abstract Excitotoxicity is considered to be a major pathophysiological mechanism responsible for extensive neuronal death after acute spinal injury. The chief effector of such a neuronal death is thought to be the hyperactivation of intracellular PARP-1 that leads to cell energy depletion and DNA damage with the manifestation of non-apoptotic cell death termed parthanatos. An in vitro lesion model using the neonatal rat spinal cord has recently shown PARP-1 overactivity to be closely related to neuronal losses after an excitotoxic challenge by kainate: in this system the PARP-1 inhibitor 6(5H)-phenanthridinone (PHE) appeared to be a moderate histological neuroprotector. This article investigated whether PHE could actually preserve the function of locomotor networks in vitro from excitotoxicity. Bath-applied PHE (after a 60 min kainate application) failed to recover locomotor network function 24 h later. When the PHE administration was advanced by 30 min (during the administration of kainate), locomotor function could still not be recovered, while basic network rhythmicity persisted. Histochemical analysis showed that, even if the number of surviving neurons was improved with this protocol, it had failed to reach the threshold of minimal network membership necessary for expressing locomotor patterns. These results suggest that PARP-1 hyperactivity was a rapid onset mechanism of neuronal loss after an excitotoxic challenge and that more selective and faster-acting PARP-1 inhibitors are warranted to explore their potential neuroprotective role.

Keywords Spinal cord injury · Kainic acid · Kainate · Fictive locomotion · Motoneuron · Excitotoxicity · Parthanatos

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Introduction

During the process of neuronal excitotoxicity, hyperactivation of the intracellular enzyme poly(ADP-ribose)polymerase-1 (PARP-1) is thought to be a major step to trigger a non-apoptotic form of cell death termed parthanatos (Narasimhan et al. 2003; Wang et al. 2009; Kuzhandaivel et al. 2010).

Parthanatos is believed to be an essential mechanism to produce neuronal loss following brain ischemia/anoxia during which extensive release of glutamate is regarded as a primary cause for irreversible damage (Eliasson et al. 1997; Koh et al. 2004). A similar process has also been suggested to occur in the spinal cord after an acute lesion (Scott et al. 1999; Genovese et al. 2005; Kuzhandaivel et al. 2010). Our in vitro model using the rat-isolated spinal cord subjected to a transient excitotoxic stimulus by the glutamate receptor agonist kainate has demonstrated substantial neuronal losses attributable to parthanatos (with preservation of glia) that was attenuated by the PARP-1 inhibitor 6(5H)-phenanthridinone (PHE; Kuzhandaivel et al. 2010). Nonetheless, this observation needs validation with electrophysiological recording to prove whether locomotor network activity in vitro is preserved as well.

This article aims at investigating if fictive locomotion (Butt et al. 2002; Taccola and Nistri 2006), an alternating pattern of lumbar ventral root (VR) discharges rhythmically produced by either electrical stimulation or neurochemicals like NMDA and 5-hydroxytryptamine (5-HT), can be detected 24 h after kainate-evoked excitotoxicity and application of PHE, and how it might be related to neuronal survival.

Materials and Methods

Full details of the experimental methods have recently been published (Taccola et al. 2008; Margaryan et al. 2010). In brief, we used neonatal (age: 0–2 days) rat spinal cords with the following protocols: sham preparations were maintained in vitro for up to 24 h in standard Krebs solution (Taccola et al. 2008); injured preparations were treated with a maximally effective dose (1 mM) of the glutamate agonist kainate (60 min) followed by wash and maintenance in Krebs solution for 24 h. PHE (60 μM; Sigma, Milan, Italy)-treated preparations received this drug either at the time of kainate washout or after the first 30 min of kainate application. In all cases, PHE application continued for 24 h and was washed out immediately before electrophysiological recording. L2 and L5 pairs of lumbar ventral roots were continuously recorded to monitor reflex activity induced by stimulating either the left or the right lumbar five dorsal root (DR). We first assessed the minimal stimulus intensity to evoke a VR threshold response homolaterally (on average this was 2.0 ± 0.95 V). In accordance with previous studies (Marchetti et al. 2001), we considered this as equivalent to 1 threshold (Th) value to induce a monosynaptic reflex. Thereafter, values of Th < 2 stimuli were used to evoke monosynaptic responses, while larger Th values were used for polysynaptic responses as previously reported in detail (Baranauskas and Nistri 1995; Bracci et al. 1997). In control conditions, the peak amplitude of monosynaptic responses was 0.24 ± 0.06 mV ($n = 7$) which corresponded to 33% of the peak amplitude of the polysynaptic responses (0.73 ± 0.3 mV; $n = 7$). With pCLAMP 9 software (Molecular Devices, Sunnyvale, CA, USA), the reflex area was measured by simple summation of data points within the search region comprising the foot of the response to its decay corresponding to 20% of the peak (Baranauskas and Nistri 1995; Bracci et al. 1997; Marchetti et al. 2001). In the present experiments, the duration of polysynaptic responses was usually 20 s. Single pulses were used to elicit synaptic responses, while pulse trains were employed for inducing repeated cycles of electrical oscillation alternating homolaterally between L2 and L5 segments or from left to right in the same segment. These patterns, therefore, had all the typical properties of fictive locomotion (Butt et al. 2002). Fictive locomotion was also evoked by bath-applied NMDA (4–5 μM, Tocris, Bristol, UK), and 5-HT (10 μM; Sigma, Milan, Italy) (Butt et al. 2002). Disinhibited bursting was induced by application of bicuculline (20 μM, Fluka, Milan, Italy) plus strychnine (1 μM, Sigma, Milan, Italy) (Bracci et al. 1996). The amplitude of such bursts was measured at their plateau phase, after high-frequency discharges subsided (Bracci et al. 2006). At the end of each experiment, spinal cords were histologically fixed and sectioned to investigate the immunostaining of neurons with the selective marker NeuN (Millipore, Milan, Italy) in the three regions of interest. Motoneurons immunolabeled with the marker SMI 32 (Covance, Emeryville, CA) were counted in the ventral horn gray matter. Data were expressed as mean ± standard deviation. The statistics of data were performed by analysis of variance (ANOVA), and Tukey test with SigmaStat 3.1 (Systat Software, Chicago, USA). The accepted level for statistical significance was $P < 0.05$ where $n =$ number of spinal cords.

Results

In view of the partial protection exerted by PHE against neuronal losses evoked by kainate (Kuzhandaivel et al. 2010), we first investigated whether this treatment could produce significant improvements in electrophysiological responses. After 60-min kainate application followed by washout, and subsequent administration of PHE (24 h), the average peak amplitude of polysynaptic reflexes (induced by DR stimuli >2 Th value for eliciting monosynaptic responses) was 0.11 ± 0.04 mV ($n = 4$), a result not different from 0.16 ± 0.1 mV ($n = 6$) observed after kainate alone. Increasing the stimulus intensity fails to reinstate the initial amplitude of these reflexes (Taccola et al. 2008). Sham reflex amplitude was 0.73 ± 0.30 ($n = 7$). Previous studies have shown that kainate (1 mM) induced irreversible loss of fictive locomotion tested with trains of electrical pulses applied to a lumbar DR (Taccola et al. 2008): this result was confirmed in this study with kainate alone or kainate followed by PHE. It was, however, possible to detect disinhibited bursting, that after kainate alone, had 58 ± 25 s period (CV = 0.21 ± 0.17), a value not significantly different from the one when PHE was later applied (34 ± 16 s; CV = 0.28 ± 0.18; $n = 4$). The same findings applied also to the average burst amplitude (measured at plateau; not shown). These observations suggested that with this administration protocol, PHE was ineffective to protect spinal networks from kainate excitotoxicity manifested as loss of fictive locomotion and reflex impairment.

Our previous study has shown that 60-min application of kainate was sufficient to produce significant PARP-1 hyperactivity (Kuzhandaivel et al. 2010). Thus, we reasoned that any protective action of PHE should perhaps be tested by applying this drug before a significant onset of PARP-1 activation, but with a certain delay from the start of kainate application to enable the development of excitotoxicity. To this end, the experimental protocol was based on application of PHE 30 min after the start of kainate (always 60 min application) and kept throughout for the next 24 h. Figures 1, 2 summarize these data. Figure 1a shows examples of lack of effectiveness even of this protocol on monosynaptic reflex amplitude (see inset to Fig. 1a) or area; similar data were obtained for polysynaptic responses in the same preparation (Fig. 1a). Average results are quantified in the histograms of Fig. 1b, c.

Recovery of fictive locomotion was never found, regardless of whether trains of electrical pulses (Fig. 1d; $n = 4$) or bath-application of NMDA plus 5-HT were tested (Fig. 2c; $n = 4$). As shown in Fig. 2a, b, the period and amplitude of disinhibited bursts after kainate and PHE were similar to those obtained with kainate alone.

The different application protocol of PHE (namely, during kainate administration) with respect to our previous article (Kuzhandaivel et al. 2010) required the analysis of the histological damage in relation to the electrophysiological effects. Fig. 3a shows examples of neuronal losses evoked by kainate in the dorsal horn of the isolated spinal cord (this example refers to L5). After applying PHE (60 μM) following kainate (bottom panel of Fig. 3a), neuronal losses in the kainate's most-sensitive region (namely, the dorsal horn; cf. Mazzone et al. 2010) appeared to be decreased as quantified in the histograms of Fig. 3b depicting the number of neurons (as NeuN-positive elements) detected in the three main spinal regions of pooled L3–L5 segments. For each region investigated, the effect of PHE against kainate toxicity was significant with percent values of surviving neurons of approximately 75% with

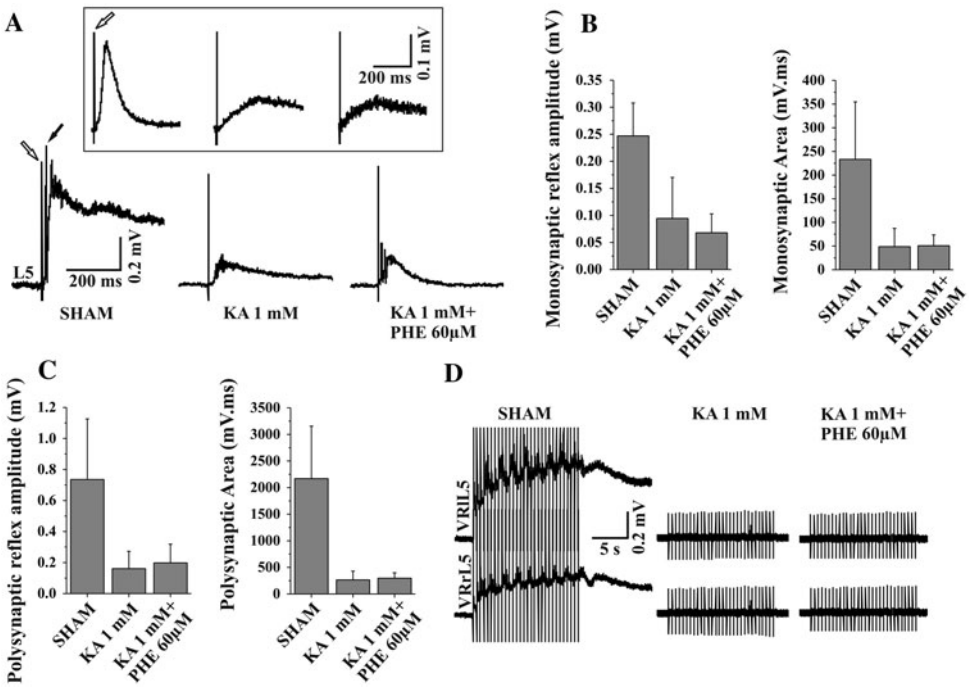

Fig. 1 Effect of PHE (60 μM) application (30 min after start of injury) on electrophysiological responses recorded after 24 h from in vitro spinal cord preparations exposed to excitotoxic injury by kainate (1 mM). **a** Examples of mono (1× Th; see *inset*) and polysynaptic VRlL5 responses evoked by single electrical stimuli (2× Th intensity for polysynaptic response) applied to the homolateral DRlL5 in sham (*left*), kainate (*middle*) or PHE treated condition (30 min after kainate application; *right*). The *open arrows* indicate the artifact while the filled *arrow* indicates the peak amplitude of the polysynaptic reflex measured from baseline. **b** *Histograms* show reflex amplitude and area of monosynaptic responses in sham, kainate, and kainate + PHE ($n = 7$, 6, and 4 respectively). No improvement was found after 24 h PHE application. Statistical analysis using ANOVA shows the significant difference between sham and two other groups (peak amplitude: $P = 0.003$ for kainate and $P = 0.002$ for kainate + PHE; area: $P = 0.01$ for kainate and $P = 0.02$ for kainate + PHE). **c** Lack of recovery of peak response amplitude and area of polysynaptic responses after application of PHE in comparison with kainate alone ($n = 7$, 6, and 4 for sham, kainate, and kainate + PHE, respectively). Significant decreases in peak amplitude and area of polysynaptic response in comparison with sham remained unchanged (peak amplitude: $P = 0.005$ for kainate and $P = 0.01$ for kainate + PHE; area: $P = 0.009$ for kainate and $P = 0.03$ for kainate + PHE). **d** Example of VR alternating cycles of a sham preparation in response to a train of DR stimuli (30 stimuli, 2× threshold intensity, 2 Hz frequency); this pattern disappears in both groups treated with kainate, and kainate + PHE (30 min after application of kainate). All data refer to the electrophysiological activity after 24 h. KA, kainate

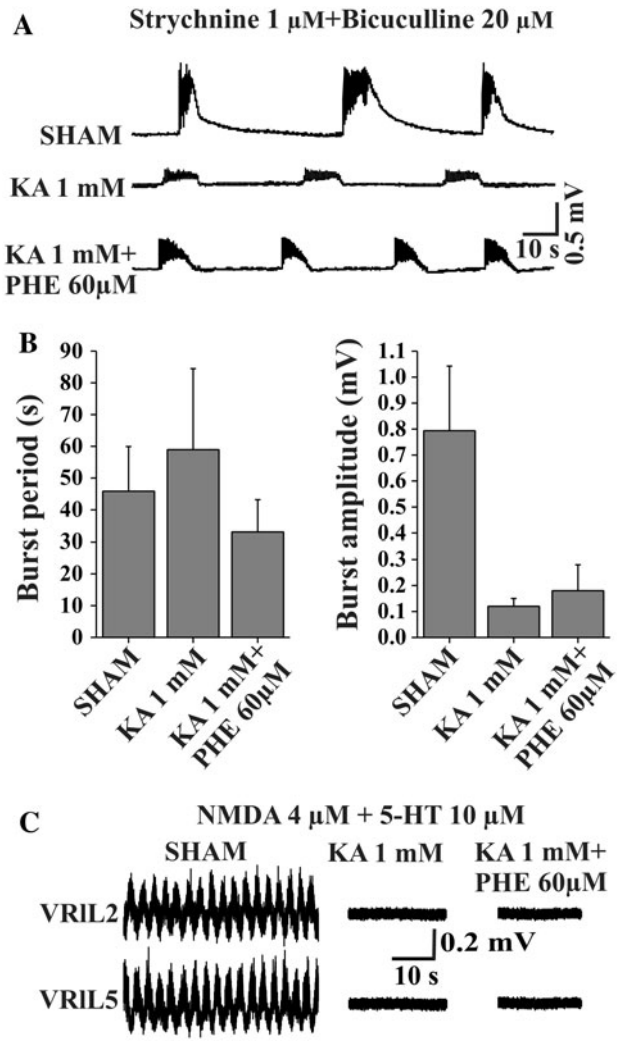

Fig. 2 Effect of PHE (60 μM) on chemically induced locomotor patterns 24 h after excitotoxic injury evoked by kainate (1 mM). **a** Examples of disinhibited bursting induced by strychnine and bicuculline in sham, kainate or kainate + PHE treated condition (started 30 min after kainate application). **b** *Histograms* show similar burst period for all the three conditions, while amplitude values for kainate, and kainate + PHE are significantly different from sham ($P = 0.005$, and 0.002, respectively; $n = 7$, 6, and 4 for sham, kainate, and kainate + PHE, respectively). Amplitude values are similar for kainate, and kainate + PHE groups. All data were analyzed with ANOVA. **c** Example of fictive locomotion induced by NMDA plus 5-HT in sham preparation: this pattern is not preserved following kainate or kainate + PHE. VRs are identified by their segmental numbering (lumbar L2 and L5 on the *left*, l, side of the spinal cord). Alternation between L2 flexor motor pools and L5 extensor motor pools is an index of fictive locomotion. All data refer to the electrophysiological activity after 24 h. KA, kainate

respect to corresponding sham values. Fig. 3c, d shows that kainate evoked, on average, strong loss of motoneurons (SMI 32 positive large cells in the ventral horn; Taccola et al. 2008) 24 h later, and that this effect could not be significantly counteracted by PHE.

Discussion

The principal finding of this article is that the PARP-1 inhibitor, PHE, could not contrast the excitotoxic damage evoked by kainate in functional terms, despite significant protection against neuronal losses, and the previous demonstration of the tight correlation between excitotoxicity and PARP-1 activity.

This observation raises several interesting issues related to the basic pathophysiology of spinal cord excitotoxicity. First, the damage to synaptic transmission induced by kainate was multifactorial because of the widespread chemical injury involving dorsal, central and ventral neurons, including motoneurons. This functional lesion was not significantly prevented by PHE since mono- and polysynaptic reflexes were strongly depressed after kainate administration, regardless of the PHE application protocol. This observation probably explains why even DR pulse trains were unable to activate fictive locomotion because afferent stimuli could not be efficiently integrated into spinal networks. Second, the persistence of disinhibited rhythmicity despite kainate being followed (or not) by PHE indicated that, in accordance with histochemical data, there was a residual circuitry still able to express a basic form of spontaneous network pattern. Improving the number of these survivors with PHE treatment had no significant consequence on disinhibited bursting presumably because this type of rhythmicity was a scale-free phenomenon generated by a relatively small subset of interneurons (Bracci et al. 1996; Taccola and Nistri 2006). The larger number of surviving neurons was, however, unable to generate locomotor patterns (which, in control conditions, are directly triggered by NMDA and 5-HT acting on intrinsic locomotor networks; Butt et al. 2002) presumably because it overall remained below the threshold (~155 neurons/central and ventral region) that constitutes the minimal membership required to produce fictive locomotion (Nistri et al. 2010).

The poor functional outcome of the PHE effect was likely to be due to the early hyperactivation of PARP-1 which presumably started even before 30 min time-point coincident with the PHE application. Furthermore, PHE is a relatively non-selective blocker of this enzymatic activity (Banasik et al. 1992; Li et al. 2001), and impairs the activity of lymphocytes (Chiarugi 2002). It is unclear whether these effects may also occur in the in vitro spinal cord, and what contribution, if any, they might have brought to the overall functional outcome. Hence, future studies with more selective inhibitors of PARP-1 are warranted to explore the early neuroprotective value of blocking this enzymatic activity.

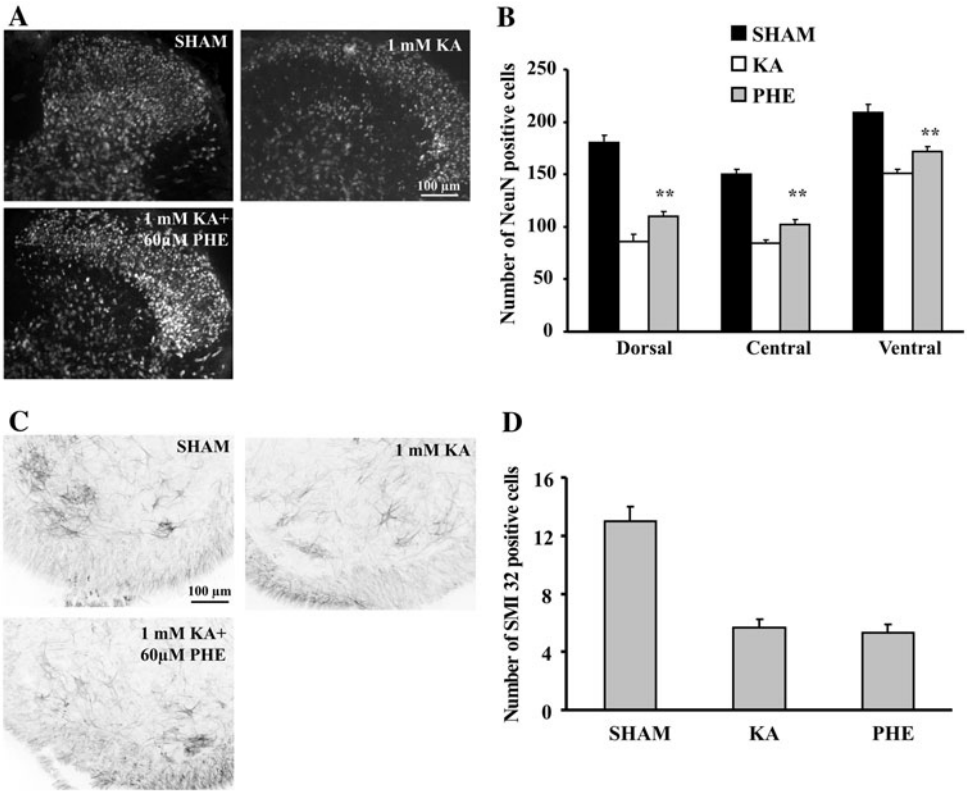

Fig. 3 Quantification of effect of kainate and PHE on neurons and motoneurons of the isolated spinal cord. **a** Examples of NeuN immunoreactivity in the L5 dorsal region of 1 mM kainate or 1 mM kainate + 60 µM PHE treated spinal cords. PHE was always applied 30 min after the start of kainate administration. **b** *Histograms* showing number of NeuN positive neurons in sham (*filled bar*)-, kainate (*open bar*)-, or kainate plus PHE (*gray bar*)-treated spinal cord for pooled L3–L5 segments ($n = 4$–7 spinal cords). **c** Representative images showing SMI 32 positive neurons (motoneurons) in L5 laminae VIII and IX of 1 mM kainate- or 1 mM kainate + 60 µM PHE-treated spinal cords. **d** *Histograms* showing number of SMI 32 positive neurons counted in 30-µm sections of 1 mM kainate- or 1 mM kainate + 60 µM PHE-treated spinal cords; $n = 4$–7 spinal cords. For **b** $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with data from kainate alone. All data were analyzed with ANOVA

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