A novel effect of ethyl pyruvate in Schwann cell de-differentiation and proliferation during Wallerian degeneration

Byung Sun Park†, Hyun Woo Jo†, Chan Park,a Youngbuhm Huh,a Junyang Jung*a and Na Young Jeongb*

aDepartment of Anatomy and Neurobiology, College of Medicine, Biomedical Science Institution, Kyung Hee University, Seoul 130–701, Republic of Korea; bDepartment of Anatomy and Cell Biology, College of Medicine, Dong-A University, Busan 602–714, Republic of Korea

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Ethyl pyruvate (EP), as a pyruvate derivative, has the protective effect on oxidative-stress-mediated cellular injury. In previous studies, it has been reported that EP provides protection against neuronal injury in brain, for example transient cerebral ischemia. However, the inhibition effect of EP on peripheral nerve injury is still unknown. Here, we investigated the inhibition effect of EP in Schwann cell de-differentiation and proliferation during Wallerian degeneration using ex vivo sciatic nerve explant system. We demonstrated that EP inhibits p-ERK1/2, p75NGFR, and lysosomal associated membrane protein 1 as factors implicated in Schwann cell de-differentiation during Wallerian degeneration. In addition, EP is sufficient to inhibit Schwann cell proliferation during Wallerian degeneration. Together, these findings suggest that EP has a strong protective effect on Wallerian degeneration.

Keywords: Ethyl pyruvate; Wallerian degeneration; sciatic explant; Schwann cell de-differentiation; Schwann cell proliferation

Introduction

Pyruvate, the product of glycolysis, is a scavenger of reactive oxygen species (ROS), and thus has a strong neuroprotective effect in various models of oxidative stress. In vitro, pyruvate is neuroprotective against hydrogen peroxide (H$_2$O$_2$) (Desagher et al. 1997; Wang et al. 2007), β-amyloid (Alvarez et al. 2003), and zinc (Kawahara et al. 2002; Chen & Liao 2003); in in vivo models, pyruvate protects against cerebral ischemic injury (Lee et al. 2001; Yu et al. 2005) and zinc toxicity (Lee et al. 2001). However, in the peripheral nerve, previous studies have only reported pyruvate’s involvement in energy metabolism (Miller et al. 2002; Pfeiffer-Guglielmi et al. 2007), but not in nerve injury.

After injury, the peripheral nerve shows a vigorous response, especially in the distal stump. During this morphologically and molecularly degenerative process, called Wallerian degeneration, Schwann cells, the glial cells of the peripheral nervous system, de-differentiate to an immature state, and no longer express myelin-related genes (Jessen & Mirsky 2008). These de-differentiated Schwann cells proliferate to facilitate axonal regeneration. Thus, because Schwann cell de-differentiation and proliferation are phenomena characteristic of Wallerian degeneration, these processes may be essential to treating peripheral degenerative or demyelinating diseases.

Peripheral nerve injury increases the expression of inducible nitrogen oxide synthase, which is significantly involved in Wallerian degeneration (Levy et al. 2001; Lee et al. 2007a; Campuzano et al. 2008). The present study tested the effects of pyruvate against oxidative stress in peripheral nerve injury, and defined its role in mediating Schwann cell de-differentiation and proliferation during Wallerian degeneration. Towards the latter aim, we measured its effects on signaling proteins involved in Schwann cell de-differentiation (i.e. a lysosomal protein, a neurotrophic factor, and mitogen-activated protein (MAP) kinase). However, despite pyruvate’s effectiveness as an ROS scavenger, its potential as a therapeutic agent is limited due to poor stability in solution (Vonkorff 1964). Thus, we used ethyl pyruvate (EP), a stable derivative (Sims et al. 2001), and demonstrated that its direct antioxidant effect remarkably inhibited Schwann cell de-differentiation and proliferation during Wallerian degeneration in an ex vivo sciatic nerve explant model.

Materials and methods

Animals

All the animal experiments were conducted using male C57BL/six mice (five-weeks-old, Samtako, Osan, Korea). Mice were housed with food and water available ad libitum in a temperature- (23 ± 1°C) and humidity-controlled (50%) environment on a 12 h light/dark cycle.

Materials

The primary antibodies used for immunostaining or Western blotting were commercially purchased. Antibodies

*Corresponding authors. Emails: jjung@khu.ac.kr; jnyjjy@dau.ac.kr

†Byung Sun Park and Hyun Woo Jo contributed equally to this work.

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against p75 nerve growth factor receptor (p75NGFR) and lysosomal associated membrane protein 1 (LAMP1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas those against phospho-p44/42 MAPK (p-ERK1/2) and Ki67 were obtained from Cell Signaling (Beverly, MA, USA) and Abcam (Cambridge, UK), respectively. Alexa Fluor 488- and 594-conjugated secondary antibodies were purchased from Life Technologies (Grand Island, NY, USA). All of the other antibodies and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Explant culture**

Sciatic nerve explants were cultured as described (Park et al. 2015). Briefly, connective tissues surrounding the sciatic nerves removed from mice were detached under a stereomicroscope. Sciatic nerves were cut into small explants of 3–4 mm in length. Several sciatic explants which were fixed in 4% paraformaldehyde (PFA) directly after removed from mice were used as control. Sciatic nerve explants for a model of Wallerian degeneration were cultured at 37°C, 5% CO₂ in Dulbecco’s Modified Eagle’s Medium containing 100 units/mL penicillin, 100 μg/mL streptomycin, 10% (vol/vol) heat-inactivated fetal bovine serum, and 2 mM L-glutamine with or without EP treatment. In all the experiments, nerve explants were maintained in culture for three days, after which they were fixed in 4% PFA for 6–12 h for immunostaining analysis or homogenized to obtain protein extracts.

**Western blot analysis**

Western blot analysis was carried out as described elsewhere (Lee et al. 2007b). Briefly, total protein (10 μg) from cultured sciatic explants was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrophoretically transferred onto a nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA), blocked in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at 4°C overnight, and hybridized for 1 h with primary antibodies.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** EP (10 mM) inhibits p-ERK1/2 expression. (a) Sciatic explants cross sections were double-immunostained with antibodies against p-ERK1/2 (red) and S100 (green, Schwann cell marker). Sciatic nerve explants were cultured for 3DIV in the presence or absence of EP (1 or 10 mM). Scale bar = 50 μm. (b) Percentage of p-ERK1/2-positive cells among 200 S100-positive cells. n = 3. (c) Protein lysates (10 μg) from sciatic explants were analyzed by Western blotting. EP, 10 mM. (d) Quantification of relative intensity of p-ERK1 and p-ERK2 expression levels by Western blot. n = 4. *P < .001 compared to non-treated explants at 3DIV.
diluted in the same blocking buffer. Protein loading was detected by Ponceau S staining. For quantitative analysis, X-ray films were scanned using a Hewlett-Packard company scanner and analyzed with the luminescence image analysis system (Fujifilm, Tokyo, Japan).

**Immunostaining**

Sciatic explant slides were post-fixed in 4% PFA for 20 min, washed three times with phosphate-buffered saline (PBS) for 10 min, and blocked at room temperature (RT) for 1 h in PBS containing 0.3% Triton X-100 (PBST) and 10% bovine serum albumin. Slides were incubated overnight at 4°C with the appropriate primary antibodies in blocking solution. After washing three times, slides were incubated in PBST containing Alexa 488- or 594-conjugated secondary antibodies for 2 h at RT. After further washing three times with PBS, coverslips were adhered to the slides with Gelmount (Biomed, Foster City, CA, USA) and analyzed using a laser confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany). For morphometric analysis for LAMP1 immunofluorescence, we used a software accompanied by the LSM 700 microscopy. The intensity was calculated in seven randomly selected 300 × 300 μm² areas from each sample from tree mice. For quantification of the relative intensity, we conducted to evaluate the relative number of the pixels that showed each intensity unit (Lee et al. 2009).

**Statistical analysis**

All the experiments were conducted at least three times, and values are expressed as means ± SEM. Statistically significant differences between groups were determined using the one-way analysis of variance; all of the analyses were carried out using SPSS 21.0 software (IBM, USA).

**Ethics statement**

Animals were handled in accordance with the guidelines of the Korean Academy of Medical Science, and approved by the Kyung Hee University Committee on Animal Research [KHUASP(SE)-13-027]. All of the procedures were designed to minimize animal suffering and the number of animals used.
Results

The de-differentiation of Schwann cells involves activation of MAP kinases, including JNK, ERK, and p38 MAP kinase, after peripheral nerve injury (Harrisingh et al. 2004; Monje et al. 2010; Yang et al. 2012). ERK1/2 is highly phosphorylated (p-ERK1/2) in the distal stump after nerve injury (Harrisingh et al. 2004). To determine whether Schwann cell de-differentiation is inhibited by EP during Wallerian degeneration, we examined the effect of three days in vitro (3DIV) treatment with EP (1 or 10 mM) on p-ERK1/2 by immunostaining. Levels of p-ERK1/2 were significantly increased 3DIV compared to non-degenerated nerves, and the increased p-ERK1/2 signals co-localized with S100 (a Schwann cell marker) (Figure 1(a) and 1(b)). In contrast, sciatic explants treated with EP (10 mM) showed no change in p-ERK1/2, with levels similar to control in Schwann cells, but EP (1 mM) did not show any effect on the inhibition of p-ERK1/2 expression in the denervated Schwann cells (Figure 1(a) and 1(b)). Thus, we found that 10 mM EP is suitable to inhibit Schwann cell de-differentiation. Quantitative Western blot analyses also showed that 3DIV EP treatment suppressed induction of p-ERK1/2 (Figure 1(c) and 1(d)).

Thus, exposure of sciatic nerve to EP appears to significantly inhibit p-ERK1/2 expression in Schwann cells during Wallerian degeneration.

In de-differentiated Schwann cells, lysosomal and proteasomal protein degradation are activated to remove myelin sheath debris. Previous studies have reported that expression of LAMP1, a marker of lysosomal vesicles, is dramatically increased in sciatic nerves after nerve injury (Jung et al. 2011; Shin et al. 2013). To assess the effect of EP on lysosomal activation during Wallerian degeneration, we immunostained for LAMP1 and S100. Three days of EP treatment significantly decreased LAMP1 immunoreactivity compared to non-treated explants at 3DIV (Figure 2 (a) and 2(b)). This decrease was quantitatively confirmed by analysis of relative densities on Western blots using Image J software (Khan et al. 2012; Lee et al. 2014) (Figure 2 (c) and 2 (d)). These results indicate that EP is sufficient to inhibit lysosomal protein degradation during Wallerian degeneration.

During Wallerian degeneration, de-differentiated Schwann cells induce expression of several neurotrophic factors involved in neuronal survival and axonal regeneration (Chen et al. 2007). The p75 NGFR, the low-affinity NGFR, is highly expressed in de-differentiating Schwann cells.
cells during Wallerian degeneration (Jung et al. 2011; Shin et al. 2013). To assess whether EP inhibits Schwann cell de-differentiation, we immunostained teased sciatic nerve fibers with p75NGFR antibody at 3DIV in the presence or absence of EP. Three days of EP treatment dramatically reduced p75NGFR expression compared to non-treated nerves at 3DIV (Figure 3 (a) and 3 (b)), and this effect was confirmed in Western blot analyses (Figure 3 (c) and 3 (d)). We conclude that EP downregulates p75NGFR expression in de-differentiated Schwann cells during Wallerian degeneration.

Discussion
Schwann cell de-differentiation and proliferation, characteristic of Wallerian degeneration, are also essential for axonal regeneration and effective reinnervation during peripheral nerve regeneration. However, in demyelinating diseases such as Guillain–Barré syndrome, Schwann cell de-differentiation can be lethal if patients do not receive treatment. In addition, in inherited peripheral neuropathies, including Charcot–Marie–Tooth disease, de-differentiated Schwann cells continuously promote demyelination during Wallerian-like degeneration, suppressing entry into the nerve regeneration cycle. Thus, development of
methods of inhibiting Schwann cell de-differentiation during Wallerian or Wallerian-like degeneration (Coleman & Freeman 2010) is essential for the treatment of peripheral degenerative or demyelinating disease.

In this study, we showed that EP (a simple, stable derivative of the endogenous metabolite pyruvate) inhibits Schwann cell de-differentiation during Wallerian degeneration. In previous studies, EP was reported to be effective against oxidative stress-related cellular injury (Varma et al. 1998), ischemia/reperfusion-induced epithelial injury (Sims et al. 2001), and systemic inflammation (Ulloa et al. 2002). Thus, because oxidative stress is related to Wallerian degeneration (Levy et al. 2001; Lee et al. 2007a; Campuzano et al. 2008) and EP protects against ROS and reduces inflammation, we hypothesized that EP might be effective for inhibiting Schwann cell de-differentiation.

During Wallerian degeneration, the MAP kinase pathway is activated in de-differentiated Schwann cells (Harrisingsh et al. 2004; Monje et al. 2010; Yang et al. 2012), and EP suppresses activation of p38MAPK in RAW 264.7 cells (Song et al. 2004). In the present study, EP (10 mM) directly inhibited p-ERK1/2 in sciatic nerve explants (Figure 1). Thus, inhibition of Schwann cell de-differentiation by EP may be a result of its suppression of MAP kinase. We also examined EP’s effects on p75NGFR expression because this receptor has previously been implicated in oxidative stress; ROS activates p75NGFR, leading to neurodegeneration (Shin et al. 2011; Kraemer et al. 2014). Based on our data, we infer that EP’s inhibition of p75NGFR expression in Schwann cells during Wallerian degeneration may result from its function as a ROS scavenger. Finally, oxidative stress increases lysosomal function, which supports antimicrobial activity and apoptotic cell death (Kurz et al. 2008; Yoon et al. 2011). Thus, inhibition of oxidative stress by EP may alter lysosomal function. In the present study, we found that EP, an inhibitor of oxidative stress, effectively suppresses Schwann cell de-differentiation during Wallerian degeneration. In addition, EP’s inhibition of Schwann cell de-differentiation could be related to its prevention of Schwann cell entry into the nerve regeneration process. This effect is likely upstream of EP’s suppression of Schwann cell proliferation in this ex vivo model.

In conclusion, we demonstrated that EP inhibits Schwann cell de-differentiation and proliferation in sciatic explants during Wallerian degeneration. EP-induced inhibition of Schwann cell de-differentiation and proliferation is likely a result of its reduction of oxidative stress. Thus, new therapeutic strategies related to EP may be helpful to treat peripheral degenerative or demyelinating diseases.

Disclosure
No potential conflict of interest was reported by the authors.

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References


