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ORIGINAL PAPER

Inhibition of Neuronal Nitric Oxide Synthase by Ethyl Pyruvate in Schwann Cells Protects Against Peripheral Nerve Degeneration

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Abstract

Schwann cells are essential glial cells in the peripheral nervous system (PNS), and dysfunction of Schwann cells can induce various peripheral neurodegenerative diseases. Oxidative stress has been implicated as a causative factor in degenerative nerve diseases; however, there no effective molecules are available to inhibit nerve degeneration in peripheral neurodegenerative diseases. Ethyl pyruvate (EP) is a candidate regulator of oxidative stress, targeting Schwann cells during peripheral nerve degeneration. Here, we investigated the effects of EP on axonal degradation, demyelination, transcriptional regulation, and macrophage recruitment during Wallerian degeneration of the sciatic nerve, ex vivo and in vivo. EP prevented the expression of neuronal nitric oxide synthase (NOS1), but not that of inducible nitric oxide synthase (NOS2), during Wallerian degeneration. These results suggest that effect of EP on Schwann cells may protect against peripheral nerve degeneration through its NOS1-specific regulation.

Keywords Schwann cells · Demyelination · Ethyl pyruvate · Neuronal nitric oxide synthase · Axonal degradation · Oxidative stress

Introduction

Wallerian degeneration is a multistep process required for nerve regeneration that involves axonal degradation and demyelination in the distal part of injured peripheral nerves after nerve injury, and depends upon the alteration of signals between axons and Schwann cells. During Wallerian degeneration, Schwann cells have an essential role in axonal degradation and demyelination. In the early period (24 h to 3 days) of Wallerian degeneration, compact myelin sheaths within Schwann cells collapses. At this time, Schwann cells initiate myelin sheath fragmentation and axonal degradation via actin polymerization around Schmidt–Lanterman incisures [1, 2]. However, no clinically effective chemicals have been discovered that regulate peripheral nerve degeneration (to support effective nerve regeneration) or delay the progression of peripheral neurodegenerative diseases (e.g., diabetic neuropathy and Charcot–Marie–Tooth disease).

The reactive oxygen species (ROS)-scavenging property of pyruvate is well supported by in vivo and in vitro oxidative stress models. For example, in in vitro models, pyruvate shows neuroprotective effects against ROS and β-amyloid in neuronal cells [3–5]. In in vivo models, pyruvate protects the brain against abnormal conditions, such as cerebral ischemia and zinc toxicity [6, 7]. Recently, protective effects of pyruvate were reported in peripheral nerves during Wallerian degeneration. For instance, in an ex vivo model, a derivative of pyruvate inhibited Schwann cell proliferation and dedifferentiation, which are observed during abnormal interactions between Schwann cells and axons [8]. Such results suggest that pyruvate may regulate demyelination and axonal degeneration, as well as Schwann cell proliferation and dedifferentiation in Wallerian degeneration.

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Here, we found that, by targeting Schwann cells, pyruvate could attenuate demyelination, macrophage recruitment, and axonal degradation in ex vivo and in vivo sciatic nerve degeneration models. In addition, we demonstrated that pyruvate regulated the expression of the myelin-related transcription factor, c-Jun, during Wallerian degeneration. Moreover, we showed that neuronal nitric oxide synthase (NOS1) was a molecular target of pyruvate in the regulation of Wallerian degeneration in Schwann cells. NOS1 is related to signal transmission [9] and is regulated in a calcium-dependent manner [10] in various cell types. It is also related to oxidative stress and is distributed in both the central nervous system (CNS) and the peripheral nervous system (PNS) [11].

Method and Materials

Animals

Five-week-old male C57BL/6 mice (Samtako, Osan, Korea) were used. The mice were housed with food and water at the Kyung Hee University Animal Experimental Study Facility (temperature, 23 ± 1 °C; humidity, 50%). Approval of the study protocol was obtained from the Kung Hee University Committee on Animal Research [KHUASP(SE)-16-043-1], following the guidelines of animal experimentation established by The Korean Academy of Medical Science. For in vivo experiments, sciatic nerve axotomy was performed as previously described [12]. Briefly, under sterile surgical condition, sciatic nerves was cut after induction of anesthesia by intraperitoneal injection of pentobarbital (35 mg/kg). Then, we used a 10-mm blind PVC tube packed with gel foam presoaked in EP to treat the distal stumps of axotomized sciatic nerves with EP for 3 days. At 3 days after axotomy, mice was sacrificed by CO_2 asphyxiation and the distal part of sciatic nerves was harvested. Control was an intact sciatic nerve.

Explant Culture

Ex vivo sciatic nerve explant cultures were performed as previously described [12]. Briefly, connective tissues around the sciatic nerves were removed and detached under a stereomicroscope. Extracted sciatic nerves were divided into 3–4 mm small size pieces in length. For construction of Wallerian degeneration ex vivo model, sciatic nerve pieces were cultured at 37 °C and 5% CO_2 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 in the presence or absence of ethyl pyruvate (EP) treatment (1, 10 and 100 mM). The explant cultures were maintained in culture for 3 or 5 days and the explants were used for immunostaining analysis or Western blot analysis. Control was an intact sciatic nerve.

Nerve Fiber Teasing

The detached sciatic nerve pieces from ex vivo culture or the nerve pieces from in vivo animals were incubated for 1 day in 4% para-formaldehyde (PFA) at 4 °C. After removing the nerve sheath, the nerve segments separated into one single fibers and straightened on the slides using fine forceps under a stereoscope.

Morphometrical Indices

To identify morphologically the degree of Wallerian degeneration, we used strip index, ovoid index and myelin index. Strip index is the number of white transverse stripe within 500 µm of a sciatic nerve explant under a stereoscope. Ovoid index is the number of myelin ovoids within 200 µm of a teased nerve fiber under a differential interference contrast (DIC)-filtered microscope. Myelin index is the number of nerve fibers which have intact myelin sheaths with longer than 50 µm among 100 teased nerve fibers under a microscopic field.

Immunohistochemistry

Each section or teased nerve fiber slide was post-fixed in 4% PFA for 15 min, washed three times with phosphate-buffered saline (PBS) for 10 min, blocked with 10% bovine serum albumin at room temperature (RT) for 1 h and then, reacted with a primary antibodies. Anti-myelin basic protein (MBP, Santa Cruz Biotechnology, Santa Cruz, Ca, USA) and anti-p75 NTR (Santa Cruz Biotechnology, Santa Cruz, Ca, USA), anti-c-Jun (BD Biosciences, San Jose, CA, USA), anti-S100 (Sigma-Aldrich, St. Louis, MO, USA) and anti-neurofilament (Sigma-Aldrich, St. Louis, MO, USA), anti-CD68 (Millipore, Billerica, MA, USA), anti-NOS1 (BD Biosciences, San Jose, CA, USA) and anti-3-nitrotyrosine (NT; BD Biosciences, San Jose, CA, USA) were used as the primary antibodies. Each primary antibody was reacted at 4 °C for 12 h. As secondary antibodies, Alexa Fluor 488- and 594-conjugated antibodies were reacted at RT for 2 h. After washing three times with PBS, coverslips were adhered to the section slides with Gelmount (Biomeda, Foster City, CA, USA) and analyzed under a laser confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using the acid guanidine thiocyanate–phenol–chloroform extract method and the cDNA was

synthesized using SuperScriptII (Invitrogen, Calsbad, CA, USA) and the amplification of cDNA is used by reverse transcription-polymerase chain reaction (RT-PCR). The PCR primers were as follows: mouse NOS1 (forward 5′-GCCTGT GATGTGTTCTGTGTG-3′ and reverse 5′-GTGTTCCTT TCCAGCATT-3′), mouse NOS2 (forward 5′-TGATGTGCT GCCGGTCT-3′ and reverse 5′-ACTTCCTCCAGGATGTTG TA-3′) and mouse GAPDH (forward 5′-CTACATGGTCTA CATGTTCCAGTATG-3′ and reverse 5′-AGTTGTCATGGA TGACCTTGG-3′). RT-PCR was performed in 25–33 cycles for GAPDH, NOS1 and NOS2 and then, gel electrophoresis was performed to confirm the correct size of the amplified cDNA and the absence of nonspecific bands.

Western Blot Analysis

At 3 days in vitro (3DIV) cultures, the sciatic explants were washed in ice-cold PBS and lysed in 2% SDS containing 1% protease inhibitor cocktail (Roche Molecular Biochemicals, Nutley, NJ, USA). 30 µg of sample was separated electrophoretically on 10% SDS-polyacrylamide gel and electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The blots were blocked with 5% non-fat milk in Tri-buffered saline (TBS) containing 0.05% Tween 20 (TBST) at 4 °C for 12 h and incubated with primary antibodies prepared in 2% non-fat milk containing TBST at RT for 1 h. Blots were probed with secondary antibodies and visualization was performed with an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, England).

Statistical Analysis

Differences in the means between groups were statistically determined using the one-way analysis of variance. All of the analyses were performed using SPSS 21.0 software (IBM, Armonk, NY, USA). All the experiments were conducted at least four times.

Results

Ethyl Pyruvate Prevents Demyelination During Wallerian Degeneration

To determine the effects of pyruvate on peripheral nerves during Wallerian degeneration, we used ethyl pyruvate (EP), due to the poor stability of pyruvate in solution [13], and an ex vivo sciatic nerve explant culture system, as described previously [1, 8, 12]. On day 3 of in vitro (3DIV) sciatic nerve explant culture, the transverse stripes (bands of Fontana) disappeared due to Wallerian degeneration [12]. The border between the white and the black could be disappeared due to the loss of myelinated portions during Wallerian degeneration. Using this peripheral nerve phenotype, we dose-dependently tested whether EP inhibited the disappearance of stripes during Wallerian degeneration. On 3DIV, explants treated with 10 mM EP showed intact stripes, similar to control explants (Fig. 1a). Meanwhile, explants treated with 1, 100, or 200 mM EP showed disappearance of stripes, similar to EP-untreated explants on 3DIV

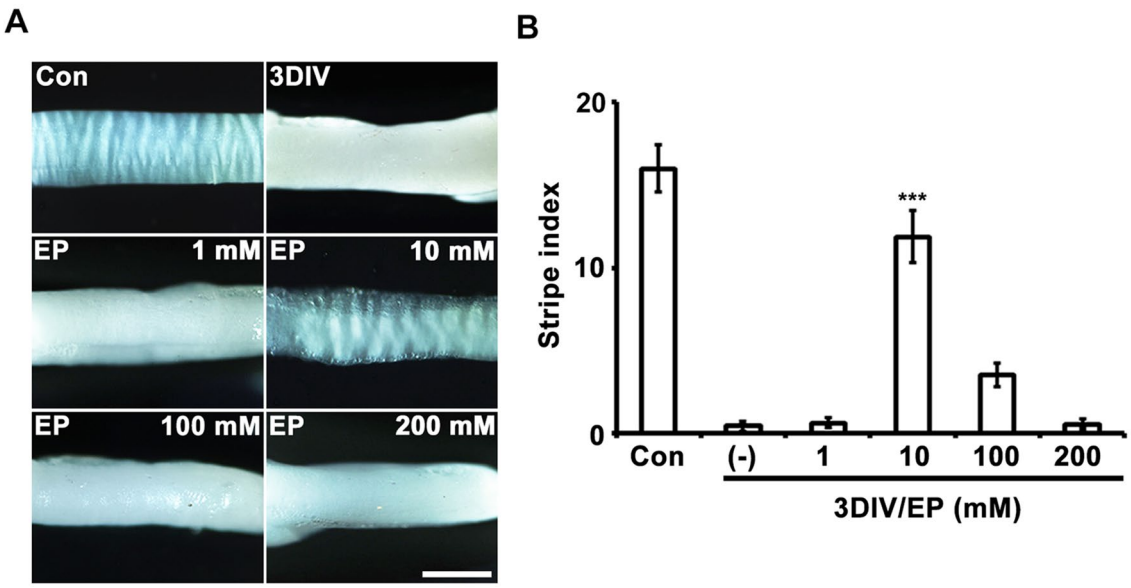

Fig. 1 Ethyl pyruvate (EP) inhibits morphologically ex vivo sciatic nerve degeneration. **a** Dose-dependent treatment of altered transverse stripe patterns in sciatic nerve explants. 3DIV, 3 days in vitro. Scale bar = 600 µm. **b** Quantitative strip index shows the number of white transverse stripe under a stereoscope (n = 3 mice). $P < 0.0001$

(Fig. 1a). Moreover, treatment with ≧ 100 mM EP induced toxic effects in Schwann cells, and individual Schwann cells were scattered throughout the culture medium. Overall, the quantitative stripe index indicated that 10 mM EP, which we used in the subsequent ex vivo experiments, was the most effective concentration against peripheral nerve degeneration (Fig. 1b).

Next, we quantified the effects of EP on demyelination during Wallerian degeneration. During Wallerian degeneration, myelin fragmentation occurs, and the processes take on an ovoid-like shape [1, 12]. Thus, we quantified demyelination based on ovoid process formation in sciatic explants during Wallerian degeneration, using the ovoid index and myelin index. Control teased nerve fibers showed no ovoid-like shape under a differential interference contrast (DIC)-filtered microscope (Fig. 2a). By contrast, the fibers of 3DIV explants showed numerous ovoid fragments, whereas explants treated with 10 mM EP showed only a small degree of ovoid fragmentation (Fig. 2a). Next, we immunostained myelin basic protein (MBP) as a marker of myelin sheath in sciatic nerve explants. Consecutive MBP-stained lines were observed in teased nerve fibers (Fig. 2a). By contrast, 3DIV explants showed immunostaining of MBP with irregular margins and cleaved lines, whereas explants treated with 10 mM EP maintained continuous MBP-stained double lines (Fig. 2a). Similarly, Western blot analysis showed that EP inhibited the disappearance of MBP and protein zero (P0), another myelin marker, during Wallerian degeneration in

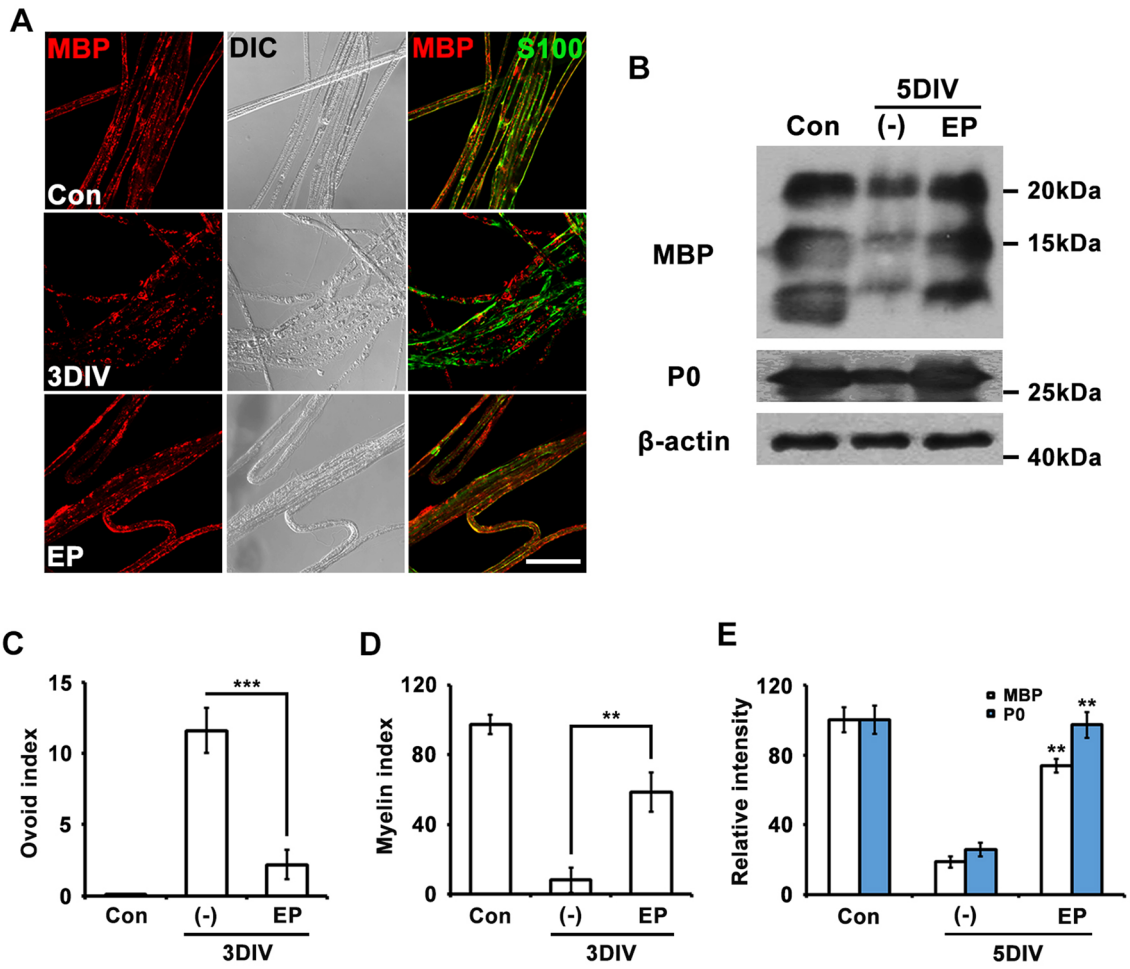

Fig. 2 EP protects myelin sheath fragmentation during Wallerian degeneration. **a** Sciatic nerve explants were cultured for 3DIV in absence or presence of EP (10 mM). Confocal images show immunolabeling for MBP (red) and S100 (green) as a marker for Schwann cells. Scale bar = 100 µm. **b** Protein lysates (30 µg) from sciatic explants cultured for 5DIV were analyzed by Western blotting (n = 4 mice). **c** Ovoid index is the number of myelin ovoids in Fig. 2a under a differential interference contrast (DIC)-filtered microscope. In y-axis, index 1 = 1 ovoid on a teased nerve fiber. $P < 0.0001$. **d** Myelin index shows the number of nerve fibers which have intact myelin sheaths under a microscopic field (n = 4 mice). In y-axis, index 1 = 1 nerve fibers including 100 µm-long intact myelin. $P < 0.001$. **e** Quantitative analysis shows the relative intensities of MBP and P0 in EP-treated explants (n = 4 mice) as compared with control explants. $P < 0.001$ (Color figure online)

5DIV sciatic explant cultures (Fig. 2b). While morphological phenotypes of ovoid fragmentation and breaking down the myelin lines are optimal at 3DIV, myelin-related protein level is not disappeared at 3DIV. Thus, we selected 5DIV to verify decreased expressions of myelin-related proteins during Wallerian degeneration. In addition, morphometric quantification based on DIC and MBP immunostaining images revealed that EP protected against myelin sheath fragmentation (Fig. 2c, d). Finally, the relative intensity of MBP protein expression showed that EP quantitatively inhibited the decrease in MBP and P0 protein levels during Wallerian degeneration (Fig. 2e). Taken together, the results suggest that EP treatment effectively protects against demyelination during Wallerian degeneration in degenerated sciatic explants.

EP Regulates c-Jun Expression During Wallerian Degeneration

Transcriptional events occur during Wallerian degeneration that promote a balance between negative and positive regulation. Among transcription factors, c-Jun is a negative regulator of myelination [14]. To identify whether EP influenced transcriptional regulation during Wallerian degeneration, we immunostained c-Jun in longitudinal sciatic nerve explants. In 3DIV explants, increased nuclear localization of c-Jun was observed compared with the control (Fig. 3a). By contrast, explants treated with 10 mM EP showed inhibited nuclear translocation of c-Jun on 3DIV (Fig. 3a). The counts of DAPI/c-Jun double-positive cells supported an effect of EP on the inhibition of c-Jun expression in Schwann cell nuclei on 3DIV (Fig. 3b). Overall, these results suggest the EP inhibits demyelination via transcriptional regulation during Wallerian degeneration.

EP Regulates Axonal Degradation During Wallerian Degeneration

After nerve injury, axons can undergo either degradation via a self-destruction pathway or indirect mechanical reconstruction by Schwann cells, where change their characteristics to support axonal regeneration during Wallerian degeneration. To determine whether EP regulates axonal degradation during Wallerian degeneration, we performed neurofilament (NF) immunostaining of teased sciatic nerve fibers. In 3DIV explants, NF immunostaining revealed increased breakage of consecutive lines in the longitudinal middle portion of the nerve fiber compared with the control (Fig. 4a). By contrast, EP-treated explants showed single consecutive NF-immunostained lines similar to the control (Fig. 4a). The quantitative analysis supported the inhibitory effect of EP on axonal degradation during Wallerian degeneration (Fig. 4b). Finally, Western blot analysis revealed that EP treatment preserved NF protein levels (Fig. 4c, d). Taken together, these results suggest that EP effectively protects against axonal degradation during Wallerian degeneration.

EP Inhibits Demyelination and Axonal Degradation During Wallerian Degeneration In Vivo

To confirm the neuroprotective effects of EP on Wallerian degeneration in vivo, we performed morphometric analyses using the ovoid, myelin, and NF indices. First, we used a 10-mm blind PVC tube packed with gel foam presoaked in 1, 10, or 100 mM EP to treat the distal stumps of axotomized sciatic nerves with EP for 3 days [12, 15]. In in vivo sciatic nerves treated with EP for 3 days after axotomy, treatment with 100 mM EP was most effective for inhibition of transverse stripes, indicating that it was the most effective concentration for the in vivo system (Fig. 5a). In addition, 100 mM EP inhibited myelin fragmentation during Wallerian degeneration in vivo after 3 days based on the number of ovoid fragments (Fig. 5b). Thus, we used 100 mM EP for all subsequent in vivo experiments.

Next, we performed a quantitative analysis of the degree of myelin sheath and axon preservation after EP treatment in vivo during Wallerian degeneration, using MBP and NF immunostaining (Fig. 5c). Treatment with EP effectively inhibited myelin destruction and axonal degradation during Wallerian degeneration (Fig. 5d, e), suggesting that EP regulates demyelination and axonal degradation during Wallerian degeneration in vivo.

EP Regulates Macrophage Recruitment to Injured Sciatic Nerves During Wallerian Degeneration

During myelin destruction in peripheral nerves, blood-derived macrophages are recruited to degenerating nerves to remove myelin debris and support the growth of newly regenerated axons. To confirm whether EP affected macrophage recruitment to peripheral nerves during Wallerian degeneration, we immunostained for CD68, a marker of macrophages, in longitudinal in vivo sciatic nerves. Three days after axotomy, the intensity of CD68 immunostaining increased in sciatic nerves in vivo (Fig. 6a). By contrast, treatment with 100 mM EP inhibited CD68 expression after 3 days (Fig. 6a). The counts of DAPI/CD68 double-positive cells supported the inhibitory effect of EP on CD68 expression at 3 days after axotomy. Although the 1 and 10 mM EP treatments reduced CD68 expression, the differences were nonsignificant. These results suggest that EP inhibits macrophage recruitment to peripheral nerves during Wallerian degeneration.

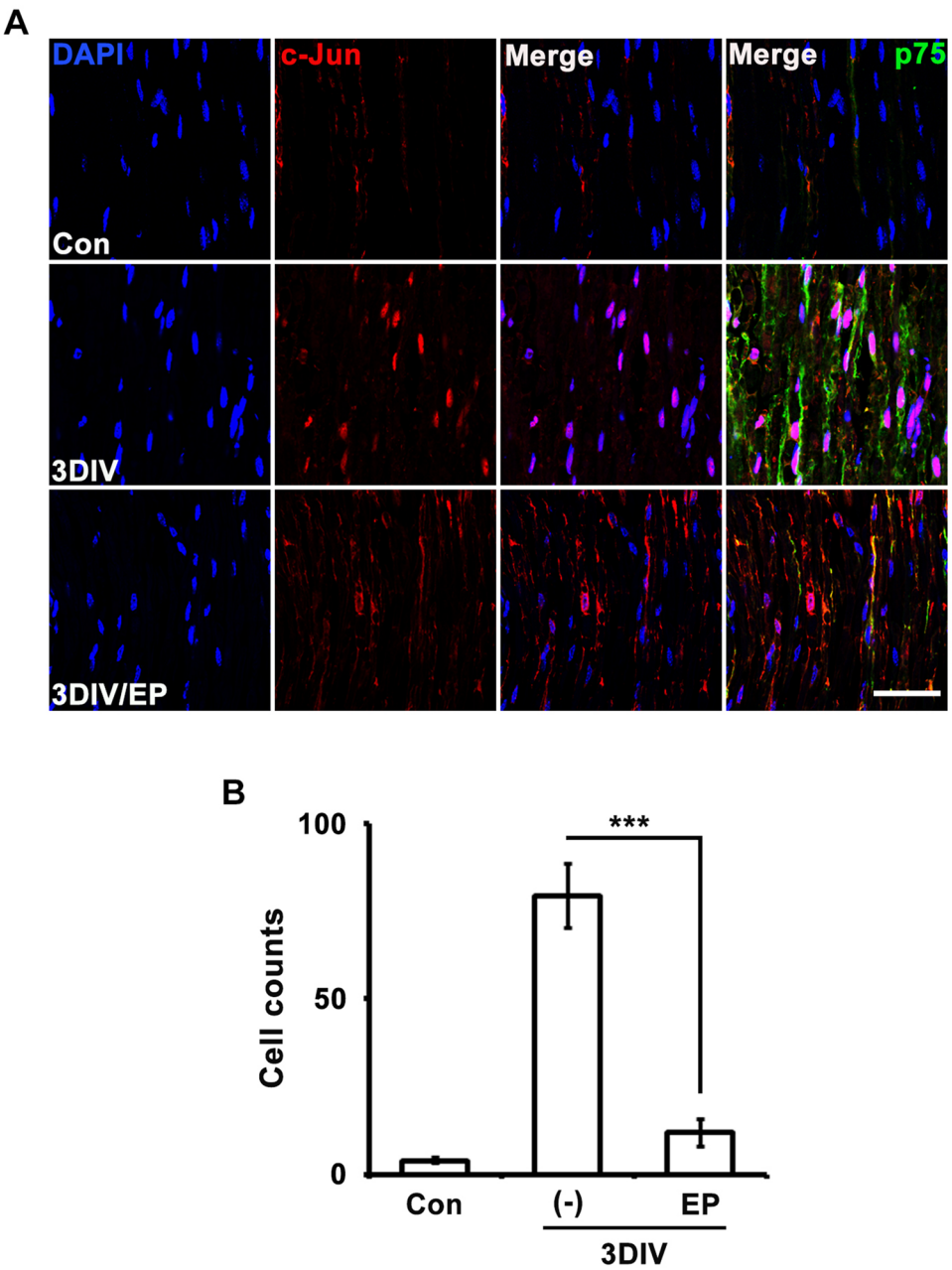

Fig. 3 EP controls the translocation of c-jun in Schwann cells during Wallerian degeneration. **a** longitudinal sciatic nerve sections were immunolabeled for c-jun (red) as a negative transcription factor, p75 NTR (green) as a marker of injured Schwann cells and DAPI (blue). Scale bar = 50 µm. **b** The number of cells was counted with c-jun immunolabeling out of 200 DAPI-(+) nuclei in teased sciatic nerve fibers as compared with that of the control (n = 4 mice). In y-axis, count 1 = 1 c-jun/DAPI double-positive Schwann cell. $P < 0.0001$ (Color figure online)

NOS1 is a Major Target of EP in the Regulation of Wallerian Degeneration

The previous results supported the neuroprotective effect of EP against demyelination, axonal degradation, and macrophage recruitment during Wallerian degeneration. Pyruvate is also a known ROS scavenger; however, its role in nerve degeneration remains unclear. Thus, we performed experiments to determine the main targets of EP in the regulation of Wallerian degeneration. To determine the molecular targets of EP during Wallerian degeneration, we examined mRNA expression patterns of representative oxidative-stress-related molecules expressed in the PNS, NOS1 and inducible nitric oxide synthase (NOS2) [16–19]. Reverse transcription PCR revealed highly upregulated NOS1 mRNA expression, but unchanged NOS2 mRNA expression, at the site of injury in in vivo *s*ciatic nerve samples after axotomy, suggesting that NOS1 is a major oxidative-stress-related molecule during Wallerian degeneration (Fig. 7a).

Next, we investigated whether EP regulated NOS1 protein expression during Wallerian degeneration using Western blot analysis. NOS1 and NT, as markers of nitrogen-free radical species, were highly expressed on 3DIV (Fig. 7b). By contrast, EP treatment inhibited the increases in NOS1 and NT on 3DIV, with levels similar to the control (Fig. 7b). In addition, NOS2 protein expression was not found in the

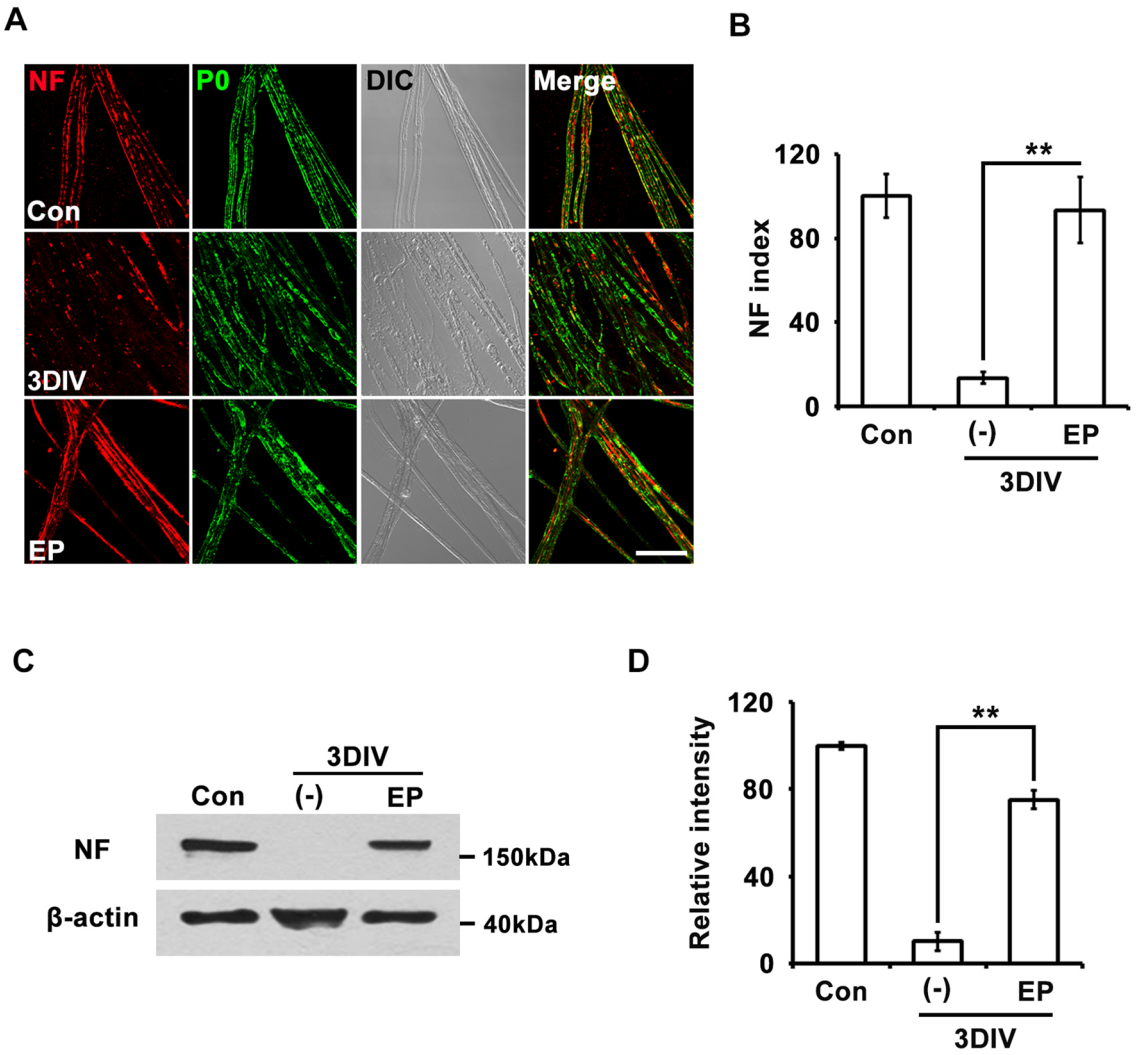

Fig. 4 EP protects axonal degradation during Wallerian degeneration. **a** Teased sciatic nerve fibers were immunolabeled for neurofilament (NF, red) and protein zero (P0, green) as makers for axons and myelin sheath, respectively. Size bar = 100 μm. **b** Quantification shows the number of nerve fibers which have intact NF with longer than 50 μm among 100 teased nerve fibers (NF index) under a microscopic field (n = 4 mice). In y-axis, index 1 = 1 nerve fibers including 100 μm-long intact axon. $P < 0.001$. **c** Protein lysates from sciatic nerve explants were analyzed by Western blotting using NF and β-actin antibodies (n = 4 mice). **d** Relative intensity is shown using quantitative western blotting analysis for NF intensity (n = 4 mice). $P < 0.001$ (Color figure online)

Western blot analysis (data not shown). Similarly, the quantitative analysis indicated that EP treatment inhibited NOS1 and NT expression during Wallerian degeneration (Fig. 7d).

Finally, we assessed the localization of NOS1 expression and the main cellular targets of EP in peripheral nerves during Wallerian degeneration. To identify NOS1 localization, we performed NOS1 immunostaining of sciatic nerve explant cross-sections for markers of Schwann cells (S100) and axons (NF). Confocal images of the sciatic nerve explant cultures revealed high NOS1 expression on 3DIV without EP treatment, where NOS1 immunostaining overlapped with S100, but not NF, staining (Fig. 7c). In addition, on 3DIV, EP treatment reduced NOS1 expression in S100-immunostained locations (Fig. 7c). The counts of NOS1/S100 and NOS1/NF double-positive cells showed that NOS1 was mainly located in S100-stained areas (Fig. 7e). Taken together, these results suggest that EP inhibits Wallerian degeneration via the regulation of NOS1 expression in Schwann cells.

Discussion

During Wallerian degeneration, peripheral nerves experience various morphological, functional, and molecular changes. Among these, in the distal part of injured nerves, interaction between an axon and Schwann cells is altered and then Schwann cells show dramatic intracellular changes.

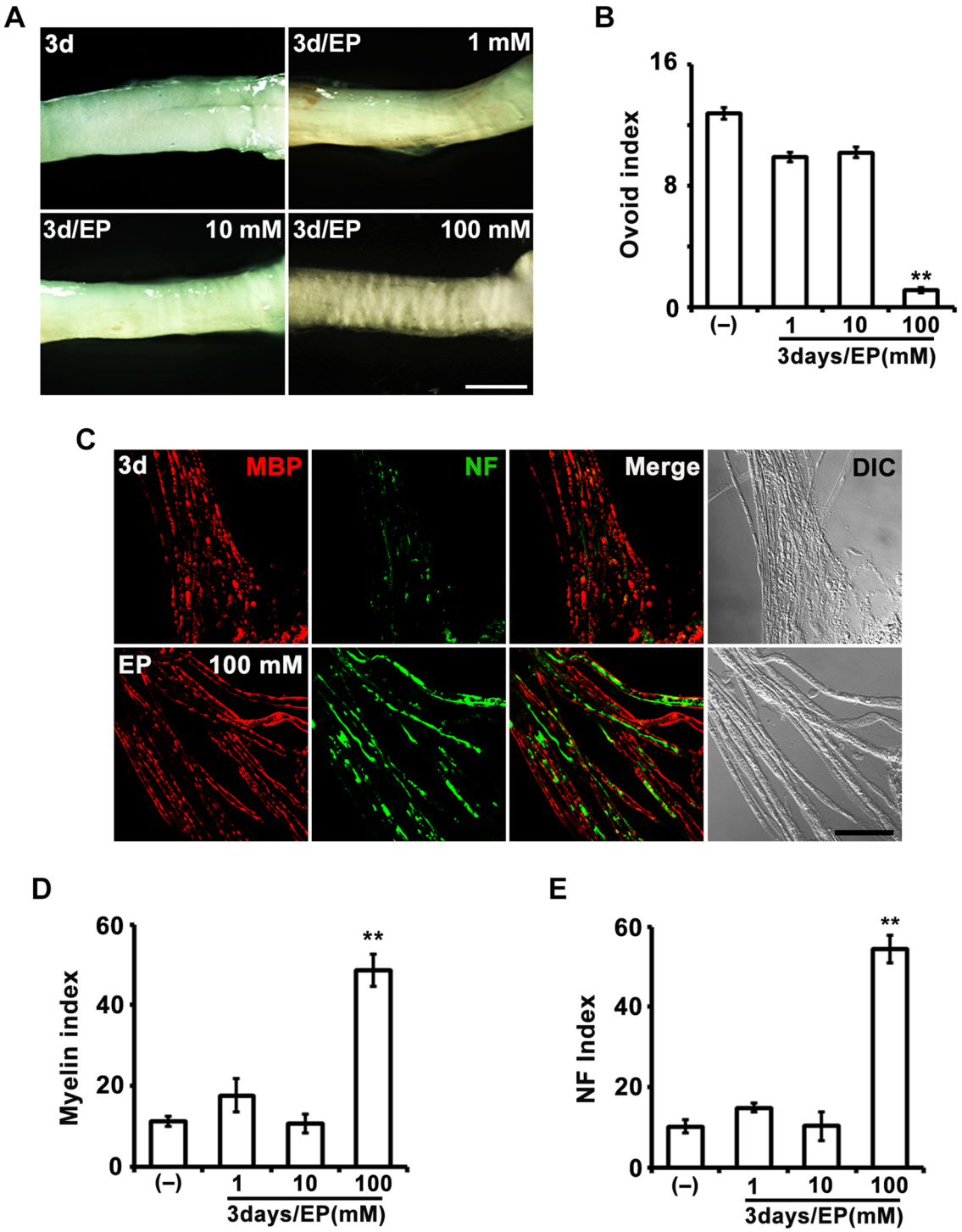

Fig. 5 EP inhibits Wallerian degeneration in in vivo animal model after nerve axotomy. **a** Explants with the dose-dependent treatments of EP were photographed under the stereoscope. Scale bar = 600 µm. **b** Quantification shows ovoid index (n = 4 mice). In in vivo sciatic nerves, 100 mM EP was effective to inhibition of nerve degeneration. $P < 0.001$. **c** Teased sciatic nerve fibers were immunolabeled fo NF (green) and MBP (red). Size bar = 100 µm. **d** Morphometric analyses were performed by myelin index and NF index (n = 4 mice). $P < 0.001$ (Color figure online)

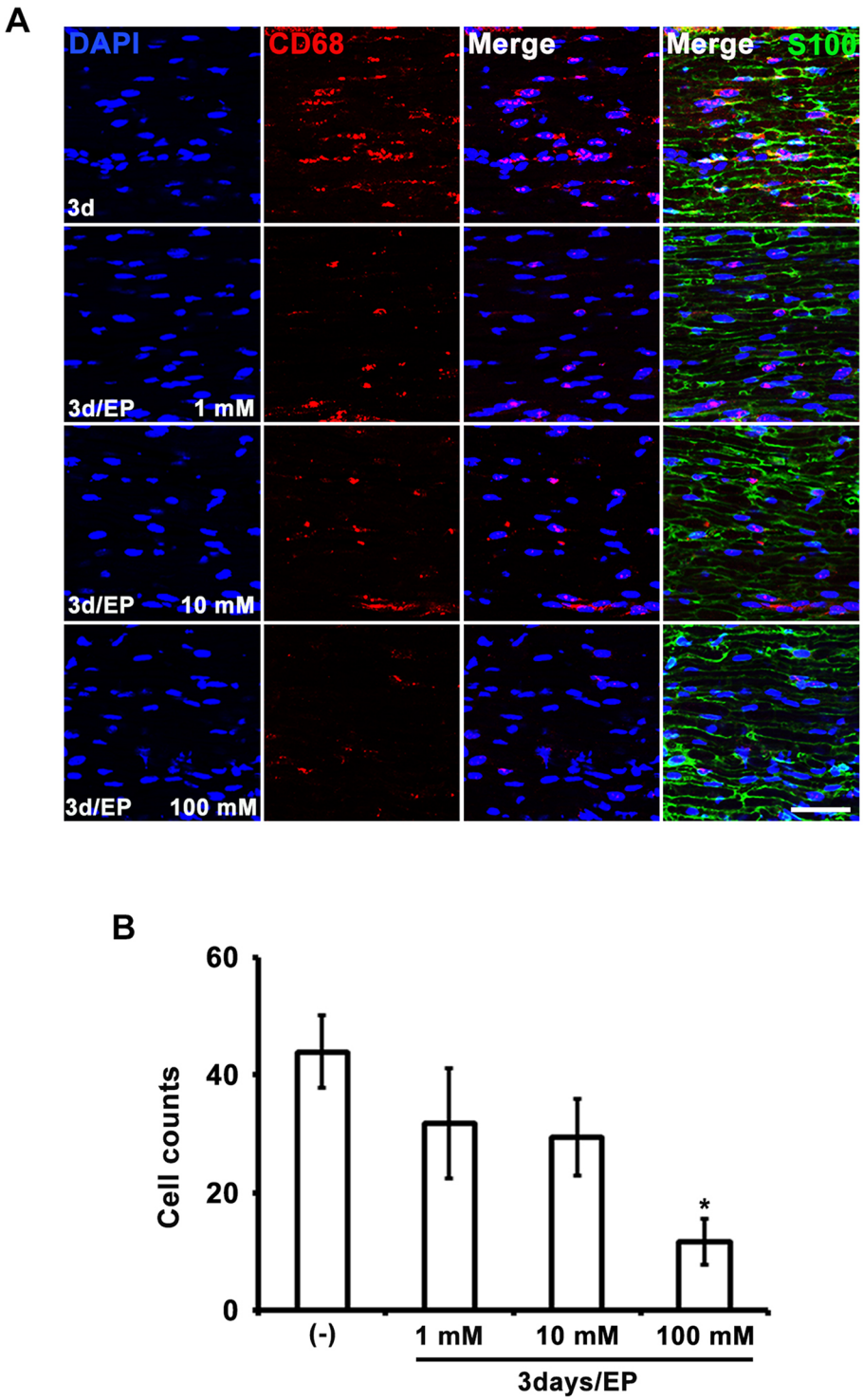

Fig. 6 EP inhibits macrophage recruitments into sciatic nerves during Wallerian degeneration. **a** Longitudinal sections of in vivo sciatic nerves were immunostained with CD68 (red) as a marker of macrophages and S100 (green) and stained with the nuclear dye DAPI (blue). Size bar = 00 μm. **b** Cell counts show the numbers of DAPI/CD68 double-positive cells under a microscopic field (300 × 300 μm). $P < 0.01$. n = 4 mice (Color figure online)

As a result, Schwann cells exhibit new phenotypes, such as dedifferentiation, proliferation, and demyelination. In the early period of Wallerian degeneration, the mechanical actions of Schwann cells affect axonal degradation [1, 2]. Thus, the regulation of Schwann cell dynamics during Wallerian degeneration is critical to the search for therapeutic measures against peripheral neurodegenerative diseases.

Oxidative stress has been studied extensively as a cause of neurodegeneration. Although researchers have searched for efficient therapeutic methods targeting oxidative stress, there is currently no treatment that efficiently regulates oxidative stress in peripheral neurodegenerative diseases. As a candidate therapeutic agent, EP is a simple derivative of the endogenous metabolite pyruvate. It has been shown to

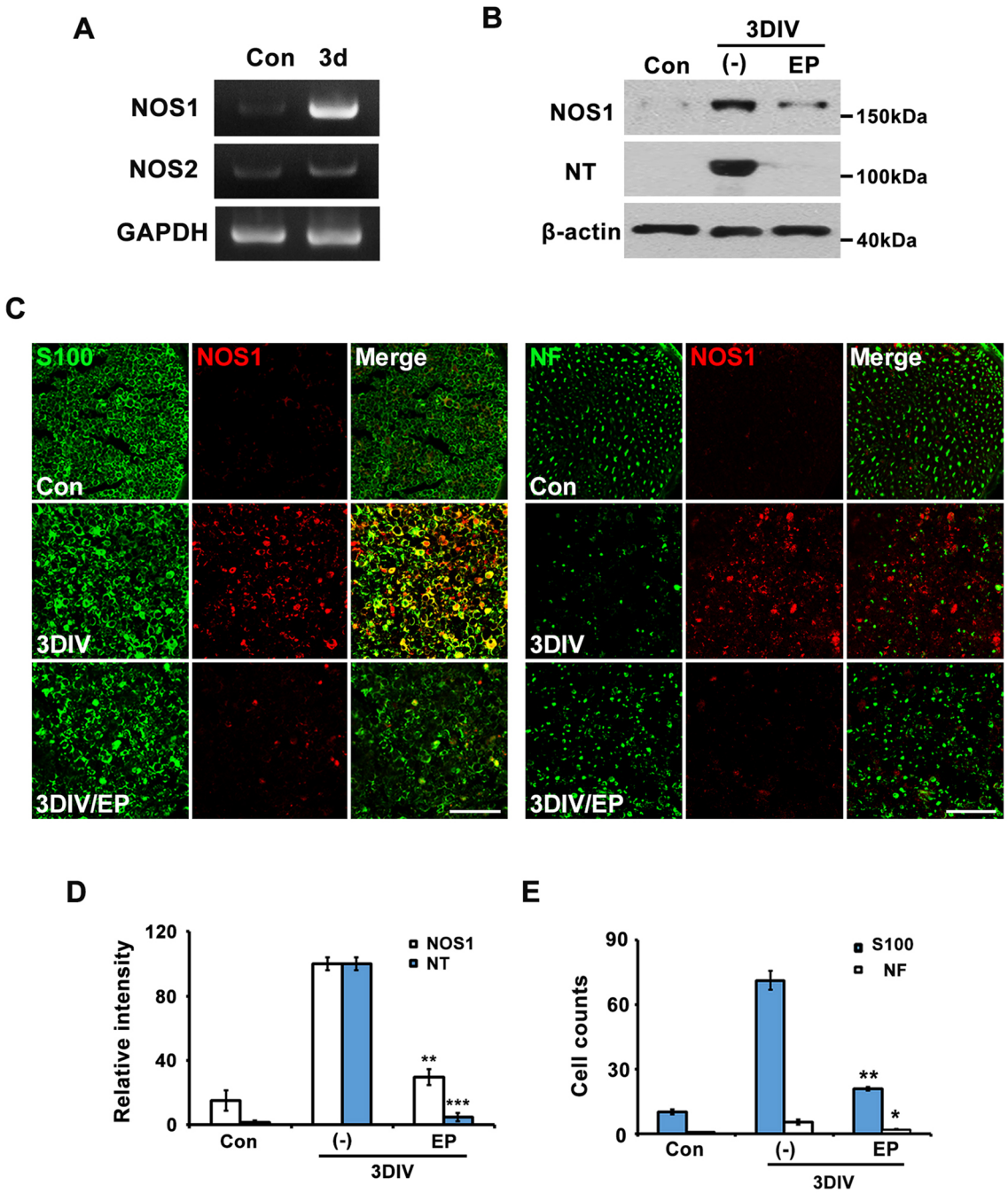

Fig. 7 Molecular target of EP in Schwann cells is oxidative stress during Wallerian degeneration. **a** mRNA expression patterns show neuronal nitric oxide synthase (NOS1) and inducible nitric oxide synthase (NOS2) levels. **b** Western blot for NOS1 and 3-nitrotyrosine (NT) as makers for oxidative stress (n = 4 mice). **c** Cross sections of sciatic explants were immunostained with NOS1 (red) and NF or S100 (green). Size bar = 100 μm. **d** Quantification of **b** shows relative intensities of NOS1 and NT protein expressions (n = 4 mice). $^{**}P < 0.001$, $^{***}P < 0.0001$. **e** Cell counts in c show the numbers of NOS1/S100 or NF double-positive cells under a microscopic field (300 × 300 μm). $^{**}P < 0.001$, $^{*}P < 0.01$. n = 4 mice (Color figure online)

regulate oxidative stress and thus improve the survival and recovery of dysfunctional organs in preclinical models, such as hemorrhagic shock [20], digestive organ dysfunction [21], diabetes-induced cataract [22], systemic inflammation [23], and stroke [6].

Recently, the protective effect of EP against peripheral nerve degeneration in an ex vivo Wallerian degeneration model was revealed [8]. Because EP targets oxidative stress, oxidative events that occur during peripheral nerve degeneration are also likely targeted by EP in the PNS. For example,

oxidative stress can modify lipid profiles and induce myelin disruption in peripheral nerves [24, 25]. Thus, EP may reduce oxidative stress and protect against myelin destruction during Wallerian degeneration. In addition, MAPK signaling pathways have an important role in transcriptional regulation of injured Schwann cells during Wallerian degeneration [26]. Among the MAPK signaling pathways, c-Jun is a critical transcription factor related to peripheral nerve repair [27]. Meanwhile, since oxidative stress activates JNK in several neural disease models [28, 29], EP may regulate the influence of oxidative stress on JNK activation and, in subsequent transcriptional regulation of myelination, protect against c-Jun activation, which occurs downstream of JNK during Wallerian degeneration. During Wallerian degeneration, macrophages enter the sites of injury in peripheral nerves and engulf myelin debris, which is triggered by the expression of cytokines and chemokines, such as MCP-1, MIP-α, IL-1β, and M-CSF in the injured nerve [30]. Therefore, as an anti-inflammatory agent [23], EP likely inhibits cytokine and chemokine expression in peripheral nerve tissue and prevents macrophage entry into the site of injury.

Moreover, we considered the possible cellular and molecular targets of EP in peripheral nerves during Wallerian degeneration. Pyruvate is an effective ROS scavenger [31] and its derivative, EP, shows even greater effectiveness against ROS [32]; therefore, we hypothesized that oxidative stress was the main target of EP in injured peripheral nerve. Nitric oxide (NO) is endogenously biosynthesized from amino acid L-arginine by the members of the NOSs. If NO is overproduced in a cell, NO can change to toxic compounds (e.g., reactive nitrogen species, RNS) by oxidative-reductive reactions. NO and RNS has been known as the pathogenesis of neurodegenerative diseases [33, 34]. Thus, main causes of oxidative stress during Wallerian degeneration are likely NO and RNS as well as ROS in PNS. To test this hypothesis, we examined NOS1 and NOS2 expression in ex vivo and in vivo sciatic nerve models. The results revealed that NOS1, but not NOS2, was highly expressed in Schwann cells compared with peripheral axons (Fig. 7). These results suggest that EP targets NOS1 in Schwann cells and protects against nerve degeneration via the inhibition of oxidative stress, which is controlled by the regulation of NOS1 expression. Similarly, Kikuchi et al. [19] showed that NOS1 was highly expressed, while NOS2 was not expressed, in Schwann cells during Wallerian degeneration, supporting our results that EP targets NOS1 during Wallerian degeneration. Interestingly, several studies have reported that NOS2 has a critical effect on Schwann cells during peripheral nerve degeneration [16, 17]. In vivo, NOS2 likely has a critical role in Wallerian degeneration, especially in macrophages recruited to the site of injury, as observed in artificially induced in vivo disease models using conventional NOS2 knock-out mice [16], and in inflammation-induced demyelination [17]. In those studies, because the interactions between Schwann cells and macrophages in injured nerves were ignored during Wallerian degeneration, NOS2 appeared to be a more critical molecule than NOS1 in peripheral nerves. By contrast, in Schwann cells, NOS1 is likely the main oxidative-stress-related molecule during Wallerian degeneration, because the ex vivo model excluded the effects of blood-derived macrophages and only NOS1 was highly expressed in Schwann cells, not NOS2 (Fig. 7). Thus, EP showed effective regulation of NOS1 in Schwann cells and conferred a neuroprotective effect in injured nerves by regulating oxidative stress, especially NO and RNS. Overall, the cellular and molecular targets of EP may be Schwann cells and NOS1, respectively, during Wallerian degeneration.

Finally, EP treatment protected against axonal degradation in both ex vivo and in vivo sciatic nerve models (Figs. 4, 5). Previous studies have shown that oxidative stress directly affects peripheral axonal degradation [35, 36]. However, an indirect mechanism of axonal degradation may be involved during Wallerian degeneration via Schwann cells. Because NOS1 is not found in peripheral axons during Wallerian degeneration (Fig. 7c–e), EP likely inhibits Schwann cell myelin fragments, subsequently inhibiting mechanical axonal destruction via demyelination [1, 2]. On the other hands, EP could affect directly to inhibit axonal degradation during Wallerian degeneration. In transected axons, nicotinamide adenine dinucleotide (NAD) and adenosine triphosphate concentrations decreased, and supplementation of NAD and pyruvate conserved axonal integrity during Wallerian degeneration [37, 38]. Because pyruvate is an important intermediate in energy metabolic pathway in the neuronal cell, EP (a derivative of pyruvate) treatment to degenerating nerves could reduce depletion of energy substrates, conserve axonal integrity and then maintain interaction between axon and Schwann cells. The continuous interaction may inhibit Schwann cell denervation and persevere normal characteristics of Schwann cells.

In summary, EP has a wide variety of effects on peripheral nerve degeneration phenotypes, such as demyelination, axonal degradation, transcriptional regulation, and macrophage recruitment, as well as on Schwann cell proliferation and dedifferentiation. Our findings indicate that pyruvate can regulate Schwann cell dynamics (e.g., demyelination, dedifferentiation, proliferation, and transcriptional regulation) during Wallerian degeneration, offering a potential explanation for its molecular targeting of NOS1 expression. Thus, we hope that EP could be effective therapeutic choice to stop or delay the progression of peripheral neurodegenerative diseases (e.g., diabetic neuropathy and Charcot–Marie–Tooth disease). Future studies should assess the potential for treating human peripheral neurodegenerative disease with EP.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no conflict of interests.

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