Nano PGE_1 Enhances Phosphorylation of ERK1/2 and Akt to Promote Recovery from Motor Dysfunction and Muscle Atrophy Induced by Sciatic Nerve Injury

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Abstract

The effect of prostaglandin E_1 (PGE_1) encapsulated in nanoparticles (Nano PGE_1) on motor dysfunction and muscle atrophy induced by sciatic nerve injury (SNI) was investigated in rats, and was compared with PGE_1 encapsulated in lipid microspheres (Lipo PGE_1) or PGE_1 clathrated in cyclodextrin (PGE_1-CD). The hind limb muscle weight ratio decreased until 2 weeks after SNI. All 3 PGE_1 formulations significantly improved SNI-induced motor dysfunction. Nano PGE_1 significantly promoted recovery from muscle atrophy at 2 and 3 weeks after SNI. Lipo PGE_1 was also effective, but multiple doses were required. Compared with the SNI control group, the Nano PGE_1 group showed upregulation of vascular endothelial growth factor (VEGF) and agrin expression in the injured sciatic nerve and atrophic muscles. Nano PGE_1 accumulated prominently at the site of nerve injury and persisted for longer than Lipo PGE_1 or PGE_1-CD. Expression of all EP receptors was detected in the normal sciatic nerve, and EP2 expression increased after SNI. Finally, Nano PGE_1 promoted ERK1/2 and Akt phosphorylation. These findings suggest that PGE_1 released from nanoparticles accumulates at sites of nerve injury and increases VEGF production by augmenting ERK1/2 phosphorylation via EP receptor signaling, thus promoting tissue repair and regeneration.

Key words: drug delivery system, muscle atrophy, nanoparticle, prostaglandin E_1, sciatic nerve injury

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1. Introduction

Peripheral nerve injury can be caused by trauma (transection or contusion) or may be a consequence of tumor resection surgery. Transient sciatic nerve injury (SNI) may result in partial loss of motor, sensory, and autonomic function, as well as leading to muscle atrophy.

Many agents have been investigated for the ability to improve outcomes after peripheral nerve injury/repair. Methyl cobalamin is a vitamin B_{12} analog that is used for the treatment of peripheral nerve injury, neuropathic pain, and demyelinating diseases. Okada et al. [1] have reported that methyl cobalamin promotes neurite outgrowth and neuronal survival through activation of ERK1/2 and Akt, thus facilitating nerve regeneration in a rat model of SNI.

Neurotrophic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are proteins that are required for regeneration after nerve injury [2]. bFGF is produced by macrophages, Schwan cells, and sensory neurons, and it has an important role in nerve regeneration. Administration of nanoparticles containing bFGF was reported to improve sciatic nerve function in rats with SNI [3], but delivery of exogenous bFGF was insufficiently effective due to its short half-life and rapid metabolism *in vivo*, so a controlled-release system was developed to prolong the action and bioavailability of bFGF.

Prostaglandin E_1 (PGE_1) has multiple actions, including the promotion of vasodilation, inhibition of platelet aggregation, a cytoprotective effect, and augmentation of angiogenesis. PGE_1 clathrated in cyclodextrin (PGE_1-CD) has been employed clinically for the treatment of chronic arteriosclerosis obliterans, peripheral arterial disease (PAD), ischemic ulcers, and diabetic neuropathy. In addition, PGE_1 encapsulated

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in lipid microspheres (Lipo PGE_1) was developed by Mizushima et al. [4,5]. Lipo PGE_1 delivers the active ingredient to target sites more efficiently, and it has been used clinically in Japan [6], South Korea, and China. While Lipo PGE_1 particularly shows efficacy for PAD, it still has the disadvantage of not maintaining a stable PGE_1 concentration for a long period *in vivo* because PGE_1 escapes rapidly from lipid microspheres.

Accordingly, PGE_1 encapsulated in nanoparticles (Nano PGE_1) was developed to achieve better retention at target sites [7]. The nanoparticles consist of biodegradable block copolymers with a mean diameter of around 100 nm. Nano PGE_1 prevents inactivation of PGE_1 and the particles accumulate at target sites where PGE_1 release occurs along with degradation of the polymer. Nano PGE_1 has been demonstrated to show therapeutic potential for spinal cord injury in rats [8], and also increases the activity of rats with intermittent claudication [9]. These reports suggested to us that Nano PGE_1 could also be effective for peripheral nerve injury. Therefore, this study was performed to compare the effectiveness of Nano PGE_1 with other PGE_1 formulations in a rat model of SNI.

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2. Results

2.1. Effect of Nano PGE_1, Lipo PGE_1, and PGE_1-CD on SNI-induced motor dysfunction

The extent of nerve regeneration was evaluated by the sciatic nerve functional index (SFI). None of the rats could walk using both hind limbs until day 7 after SNI. In the SNI control group, the SFI was approximately -100 on days 8 and 11, following which it steadily returned towards normal (Fig. 1A). Rats treated with any of the 3 PGE_1 formulations showed similar SFI values to the SNI control group on days 8 and 11. On day 15, the SFI was significantly improved in the Lipo PGE_1 and PGE_1-CD groups compared with the SNI control group, while it was significantly improved on day 18 in the Nano PGE_1 group. On day 21, the SFI displayed significant improvement in all of the PGE_1 groups compared with the SNI control group.

(Fig. 1)

2.1.2. VEGF and agrin expression by the injured sciatic nerve

The anti-VEGF antibody [#18420] used in this study reacted with both the reduced and non-reduced forms of rat VEGF, showing bands of approximately 46 kDa and 28 kDa. At 1 week after SNI, the expression of VEGF (46 kDa) was increased by 2.5-fold, but it subsequently decreased again. In contrast, VEGF (28 kDa) expression was decreased by around 50% at 1 week after nerve injury and then increased again (Fig. S1A). Treatment with Nano PGE_1 had little effect on VEGF expression at 1 week after nerve injury compared with the SNI control group, but there was a significant increase of VEGF expression at 2 weeks (Fig. 1B).

The anti-agrin antibody [Agr-131] used in this study recognized all N1 isoforms of agrin and this antibody (NB110-11069, Novus Biologicals) produced bands of approximately 42 kDa and 28 kDa. Another anti-agrin antibody (ab12362, Abcam) also

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detected the same bands. Expression of agrin (42 kDa) was slightly decreased at 1 week after SNI and then returned to the basal level, while agrin (28 kDa) showed a marked decrease at 1 week and then increased again to near the basal level (Fig. S1A). All of the PGE_1 formulations increased agrin expression compared to that in the SNI control group. It was noted that agrin (28kDa) was extensively expressed by Nano PGE_1 at 2 weeks after injury (Fig. 1B).

2.2. Effect of Nano PGE_1, Lipo PGE_1, and PGE_1-CD on SNI-induced muscle atrophy

In all groups, the muscle weight ratio (L/R (%)) decreased after SNI, with the minimum value being reached at 2 weeks after nerve injury (Fig. 2A, Fig. S2A). At 2 and 3 weeks after nerve injury, all of the PGE_1 formulations improved the muscle weight ratio compared with that in the SNI control group, and the ratio showed significant recovery in the Nano PGE_1 and Lipo PGE_1 groups.

(Fig. 2)

2.2.2. VEGF and agrin expression in atrophic muscle

Expression of VEGF (46 kDa) in atrophic muscle on the injured side showed a slight decrease at 1 week after nerve injury, after which it increased by 2-3 fold from 2 to 3 weeks after injury and then gradually returned towards the basal level. VEGF (28 kDa) expression showed a marked increase from 2 weeks after injury (Fig. S1B). All of the PGE_1 formulations increased VEGF expression compared to that in the SNI control group, with the highest level being seen in the Nano PGE_1 group at 2 weeks after nerve injury (Fig. 2B).

Expression of agrin (42 kDa) in atrophic muscle decreased a little after SNI and then increased again. Normal muscle only showed a low level of agrin (28 kDa) expression,

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but it was markedly increased by more than 2-fold from 2 weeks to 3 weeks after nerve injury (Fig. S1B). Treatment with Nano PGE_1 increased agrin expression at both 1 and 2 weeks after nerve injury, as did treatment with Lipo PGE_1 and PGE_1-CD (Fig. 2B).

2.2.3. Muscle cross-sectional area, calpain 3 (CAPN3) expression, and nestin expression

At 3 weeks after SNI, the cross-sectional area (CSA) of atrophic muscle on the injured side was 698.6 ± 30.5 μm^2 in the SNI control group (Fig. 3A, Fig. S2B). In addition, the CSA was 813.1 ± 22.4 μm^2 in the Nano PGE_1 group, 873.5 ± 19.6 μm^2 in the Lipo PGE_1 group, and 801.6 ± 48.5 μm^2 in the PGE_1-CD group. Significant improvement of CSA was seen in both the Nano PGE_1 group and the Lipo PGE_1 group. Muscle expression of CAPN3 and nestin decreased after nerve injury, and then gradually increased again (Fig. S1B). At 1 week after injury, nestin expression was upregulated by all of the PGE_1 formulations compared to that in the SNI control group and nestin-positive cells were increased (Fig. 3B).

(Fig. 3)

2.3. Accumulation and persistence of Nano PGE_1 in the injured sciatic nerve

When Nano PGE_1 (10 μg/kg) was administered intravenously at 24 h after SNI, longer retention in the blood at higher levels was observed compared with the other preparations (Table 1). The PGE_1 level was 321.1 ± 38.9 ng/mL at 3 h after dosing, indicating that most Nano PGE_1 remained in the blood at this time. PGE_1 gradually decreased to 64.6 ± 9.74 ng/mL after 24 h, and was still detected on day 7 (5.33 ng/mL). On the other hand, Lipo PGE_1 and PGE_1-CD were rapidly eliminated from the blood, although the maximum PGE_1 level was detected at 0.5 min. The PGE_1 level at 3 h after

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dosing was less than 1/50 of that in the Nano PGE_1 group.

Nano PGE_1 gradually accumulated in the injured sciatic nerve, with the tissue PGE_1 level being 32.1 ± 0.46 ng/g after 3 h and 86.8 ± 0.89 ng/g after 24 h. Even after 7 days, the PGE_1 level was 6.49 ng/g in the injured sciatic nerve. Lipo PGE_1 and PGE_1-CD also accumulated at the site of injury up to 24 h, but at a much lower tissue level.

In the contralateral normal sciatic nerve, the tissue PGE_1 level was 17.2 ± 7.54 ng/g at 3 h and 7.52 ± 1.00 ng/g at 24 h, with the latter being less than one tenth of the level in the injured sciatic nerve.

(Table 1)

2.4. EP receptor expression and ERK1/2 or Akt phosphorylation in the injured sciatic nerve

Expression of all EP receptors was detected in the normal sciatic nerve (Fig. 4A). After SNI, EP2 expression was 1.5-fold higher than in the normal nerve and EP2-positive cells were increased at the site of injury (Fig. 4B).

Phosphorylation of ERK1/2 was increased by 1.4-fold in the injured sciatic nerve compared to the normal nerve (Fig. 4C). ERK1/2 phosphorylation was increased further by both Nano PGE_1 and Lipo PGE_1, with significant enhancement by Nano PGE_1 at 24 h.

At 3 h after SNI, Akt phosphorylation was reduced in the injured sciatic nerve compared to the normal nerve, while it was significantly restored by both Nano PGE_1 and Lipo PGE_1. In the control group, Akt phosphorylation was restored to baseline at 24 h after SNI, while the two PGE_1 formulations caused a further nonsignificant increase.

(Fig. 4)

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Discussion

This study demonstrated that Nano PGE_1 promoted recovery of rats with SNI-induced motor dysfunction and muscle atrophy. The beneficial effect of PGE_1 on SNI in rats has already been reported by Tang et al. [10]. They showed that intraperitoneal treatment of Lipo PGE_1 (4 μg/kg/day) increased VEGF mRNA expression and led to significant improvement of neural function evaluated by toe spacing. Milcan et al. [11] reported that a single dose of Lipo PGE_1 (0.05 μg/kg) suppressed the increase of malondialdehyde and nitric oxide after SNI. The present study provided the first evidence that PGE_1 promotes recovery of both motor dysfunction and muscle atrophy after SNI. We found that a single dose of Nano PGE_1 was effective, while multiple doses of Lipo PGE_1 and PGE_1-CD were required, suggesting that sustained release of PGE_1 from nanoparticles may have been important.

Our distribution study revealed that Nano PGE_1 accumulated persistently at the site of sciatic nerve damage. A previous investigation of Nano PGE_1 [7] showed that most PGE_1 was incorporated into the nanoparticles and little free PGE_1 was detected by an *in vitro* gel filtration assay, with complete release taking longer than 2 weeks *in vitro*. Therefore, Nano PGE_1 accumulating at the site of nerve injury might have slowly released PGE_1 that bound to EP receptors and continuously stimulated signaling to exhibit its pharmacological activity. In fact, the Nano PGE_1 level at the site of sciatic nerve injury was over 10 times higher than in the contralateral intact sciatic nerve, suggesting that accumulation of Nano PGE_1 at the site of injury was related to enhanced penetration and retention.

Transient sciatic nerve injury led to a decrease of the muscle L/R ratio and CSA, with the maximum impact being seen at 2 weeks after injury (Fig. S1AB). In the SNI control

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group, complete recovery from muscle atrophy required longer than 5 weeks. Complete paralysis of the left hind limb was noted until 1 week after nerve injury, but walking was almost completely normal by 3 weeks. Therefore, motor function did not necessarily change in parallel with muscle atrophy and rather seemed to reflect the augmented expression of VEGF and agrin in atrophic muscle.

Agrin is a heparin sulfate proteoglycan of approximately 220 kDa that plays an important role in development of the neuromuscular junction by inducing the aggregation of acetylcholine receptors during synaptogenesis [12]. Administration of Nano PGE_1 led to upregulation of agrin expression in the injured sciatic nerve and in atrophic muscle, which may have facilitated the restoration of neuromuscular transmission. Administration of multiple doses of the other PGE_1 formulations also had a similar effect, and promoted neuromuscular junction repair through upregulation of agrin expression.

CAPN3 is a skeletal muscle-specific protease that was reported to be involved in the processes of muscle atrophy and regeneration [13]. It was reported that transient nerve injury leads to early upregulation of CAPN3 that promotes satellite cell renewal, after which downregulation of CAPN3 promotes myogenic differentiation. Nestin is part of a family of cytoskeletal intermediate filament proteins, and it has been reported to regulate the initiation of myogenic differentiation [14]. In the present SNI model, nestin expression was upregulated from 2 to 3 weeks after injury as well as that of CAPN3, and the changes of expression were in parallel with those of muscle CSA. Administration of Nano PGE_1 augmented nestin expression, and this might have contributed to recovery from muscle atrophy.

VEGF is not only a key factor for angiogenesis, but also for nerve repair [15]. Haider et al. [16] reported that PGE_1 induces angiogenesis through upregulation of VEGF

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production. In rats treated with any of the PGE_1 formulations, VEGF expression was increased in the injured sciatic nerve. Upregulation of VEGF expression was slow in the Nano PGE_1 group, possibly due to slow release of PGE_1 from nanoparticles at the site of nerve injury, although Nano PGE_1 persisted for longer at this site compared with the other formulations. In the present study, it was demonstrated that all EP receptors were expressed in the sciatic nerve, with EP2 expression being particularly augmented after nerve injury. Hori et al. [17] reported that EP2 and EP4 agonists induced synthesis of VEGF in the inner ear. Hosono et al. [18] reported that PGE_2 stimulates VEGF synthesis via the EP3 receptor. Although not confirmed in this study, it is possible that PGE_1 released from nanoparticles bound to EP receptors at the site of nerve injury and activated downstream signaling pathways, including the pathway for VEGF production, thus promoting nerve repair and recovery from muscle atrophy.

ERK1/2 is involved in nerve repair and phosphorylation of ERK1/2 induces various neurotrophic factors, including VEGF [19,20]). PGE_1 and PGE_2 have been reported to promote VEGF secretion via activation of ERK1/2 [21]. We found that ERK1/2 phosphorylation was significantly increased after administration of Nano PGE_1, suggesting a role in enhancement of VEGF production. In addition, phosphorylation of Akt was reduced about 24 h after nerve injury in the saline control group, while it was significantly enhanced by both Nano PGE_1 and Lipo PGE_1. George et al. [22] reported that EP4 mediates PGE_2-dependent cell survival through the PI3 kinase/Akt pathway, so the PGE_1 formulations could also have improved cell survival.

The present study confirmed that administration of exogenous PGE_1 promoted tissue repair and functional recovery. While Lipo PGE_1 and PGE_1-CD both activated upstream kinases, less accumulation of PGE_1-CD at the target site may have reduced its activity.

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Although over 80 fold higher amounts of PGE_1 were detected, smaller amounts of Nano PGE_1 was less potent (Fig. S1C). This might be partly explained by differences of the drug release profile. PGE_1 is released slowly from nanoparticles and there is little initial burst effect, while PGE_1 is released rapidly from lipid microspheres at the target site [8]. Therefore, Lipo PGE_1 might have rapidly released PGE_1 to activate EP receptors at the site of nerve injury, unlike Nano PGE_1. This indicates that the further desired formulation of Nano PGE_1 may require some modification.

In conclusion, this study provided new evidence about the usefulness of Nano PGE_1 for the treatment of SNI. After nanoparticles accumulated at the site of nerve injury, continuous release of PGE_1 could have resulted in binding to EP receptors and induction of angiogenesis via ERK1/2 phosphorylation, thus promoting recovery from motor dysfunction and muscle atrophy.

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4. Materials & Methods

4.1. Reagents

Nano PGE_1 was prepared by the oil-in-water solvent diffusion method in the presence of iron (III) [7]. Poly-L-lactic acid (Mw: 20,000) and polyethylene glycol (Mw: 5,200)/poly-D,L-lactide block copolymers were used to create nanoparticles with a mean diameter of 125 nm (determined by the dynamic light scatter method) for the Nano PGE_1 used in this study. PGE_1, Lipo-PGE_1 (Liple®, 5 μg/mL/ampoule), and PGE_1-CD (Prostandin®, 20 μg/ampoule) were purchased from Cayman Chemical Co. (MI), Ono Pharmaceutical Company (Osaka), and Mitsubishi Tanabe Pharma Corporation (Tokyo), respectively.

4.2. Animals

Six-week-old female Sprague Dawley (SD) rats (Charles River Laboratories Japan, Yokohama) were housed in an animal room at a constant temperature (23 ± 1°C) and humidity (50~60%) with a 12 h light/dark cycle, and were allowed free access to a standard diet and water. Animal experiments were performed in accordance with the Guidelines for Animal Experimentation of St. Marianna University Graduate School of Medicine (approval Nos. 1702005 and 1802005).

4.3. SNI model and drug treatment

The rat model of SNI was prepared by crush injury [23,24]. Under anesthesia with 1.5-2.0% isoflurane (Pfizer Japan Inc., Tokyo), the left sciatic nerve was exposed and was compressed for 90 sec with a pair of forceps at a site 10 mm proximal to the bifurcation. Rats were randomly divided into groups, and intravenous administration of the drugs was

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performed at 24 h (day 1) after SNI. Nano PGE_1 and PGE_1-CD were dissolved in saline and administered to SNI rats at a volume of 1 mL/kg. For evaluation of pharmacological activity, Nano PGE_1 (20 μg/kg/mL) was administered once as described above, while Lipo PGE_1 and PGE_1-CD were given daily at 5 μg /mL/kg up to day 4 after SNI. The dose of Nano PGE_1 was selected from its effect on muscle atrophy induced by SNI (Fig. S2C).

4.4. Evaluation of sciatic nerve function

The sciatic nerve functional index (SFI) was determined as described by Bain et al. [23] and Rateb et al. [25]. The hind limbs of each rat were painted with black ink, and the rat was immediately placed at one end of a sheet of white paper. After the animal walked across the paper sheet, clear footprints from the normal (N) and experimental (E) sides were selected and the following three parameters were measured: print length (PL), toe spread (TS), and intermediary toe spread (IT). Then the SFI was calculated by using the following formula: SFI=109.5 x (ETS-NTS)/NTS-38.3 x (EPL-NPL) + 13.3 x (EIT-NIT)/NIT-8.8.

4.5. Evaluation of muscle atrophy

The muscles (including tibia and fibula) of the left (L) and right (R) hind limbs were weighed (Fig. S2A), and the muscle weight ratio (L/R (%)) was calculated. The CSA of the tibialis anterior muscle was measured after hematoxylin and eosin (HE) staining of 4-μm slices of paraffin-embedded specimens (prepared as described below). Forty fields were randomly chosen for measurement on each HE-stained section [26].

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4.6. Sciatic nerve and muscle specimens

After a blood sample was collected under deep anesthesia, animals were perfused with cold physiological saline. Then approximately 2.0 cm of the sciatic nerve with the injured region at the center and the central part of the tibialis anterior muscle were harvested. Tissue specimens were frozen rapidly and stored at -80ºC for Western blot analysis, or were fixed in 10% paraformaldehyde/phosphate-buffered saline (PBS, Fujifilm Wako Pure Chemical Corporation, Osaka) and embedded in paraffin.

For immunohistochemical staining, the sections treated with 3% hydrogen peroxide in PBS for 15 min were blocked by using 5% normal goat serum for 30 min. After incubation with a rabbit antibody for EP2 (Abcam) or a mouse antibody for nestin (Chemicon) for overnight, MAX-PO (Mulchi, Nichirei Biosciences Inc., Tokyo) was treated for 1 h, and then the substrate (3, 3'-diaminobenzidine) was applied for 15 min. After washing in PBS, sections were stained with hematoxylin for 2 min and mounted by using mount-quick aqueous (Daido Sangyo Co., Ltd, Tokyo).

4.7. In vivo distribution study

Nano PGE_1, Lipo PGE_1, or PGE_1-CD was administered intravenously at 24 h after SNI. Blood was collected under anesthesia at the designated time intervals. After perfusion of the rat with cold physiological saline, a 2.0 cm long section of the sciatic nerve with the injured region at the center was harvested and a similar section of the contralateral normal sciatic nerve was also removed.

Fifty microliters of blood was mixed with 1,4-dioxane (400 μL) and an aqueous solution of 10 mM EDTA (50 μL, pH 7) containing indomethacin (10 μg/mL Sigma-Aldrich). Each sciatic nerve was homogenized with distilled water, followed by extraction

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with 1,4-dioxane. Then the supernatant (200 μL) was dried in a vacuum centrifugal concentrator at 50ºC and was dissolved in assay buffer, after which the PGE_1 level was determined with a PGE_1 enzyme immunoassay kit (Enzo Life Sciences Inc., NY) [8].

4.8. Western blotting

Tissue lysate was prepared with 20 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl; pH 7.5), 2 M NaCl, 0.1% Tween 80, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO), or with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1(v/v)% Triton X-100, 5 mM EDTA, 1 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and Calbiochem Phosphatase Inhibitor Cocktail Set II (2 mM imidazole, 1 mM sodium fluoride, 1.15 mM sodium molybdate, 1 mM sodium orthovanadate, and 4 mM sodium tartrate anhydrous). The lysate was let stand on ice before centrifugation at 15,000 rpm for 30 min at 4ºC.

Aliquots of the lysate supernatant (10-20 μg) were separated by electrophoresis on 10% or 3-10% gradient polyacrylamide gels. The protein concentration was determined with a BCA protein assay kit (Pierce, IL). Proteins were transferred to PVDF membranes (Clearblot P™, ATTO Corporation, Tokyo) and were incubated with antibodies for the following molecules: p44/42 MAPK (Erk1/2; rabbit, Cell Signaling), phosphor-p44/42 MAP Kinase (Thr202/Tyr204; rabbit, Cell Signaling), Akt (pan) (C67E7; rabbit mAb, Cell Signaling), phosphor-Akt (Thr308) (C31E5E; rabbit mAb, Cell Signaling), VEGF (V-N, rabbit, Immuno-Biological Laboratories, Co., Ltd. Gunma, Japan), agrin (Agr-131, mouse, Novus Biologicals), CAPN3 (rabbit, LSbio), nestin (goat, Santa Cruz), EP1 (rabbit, LSBio), EP2, EP4 (rabbit, Abcam), EP3 (rabbit, Santa Cruz), , GAPDH (mouse, Santa Cruz), and β-actin (mouse, Sigma-Aldrich). Then the membranes were incubated

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with ECL HRP-labeled anti-goat IgG (Cosmo Bio, Japan), HRP-labeled anti-mouse IgG, or HRP-labeled anti-rabbit IgG (GE Healthcare Japan). Finally, each membrane was incubated with the ECL Prime Western blotting detection system (GE Healthcare Japan) and signals were quantified by densitometry.

4.9. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance followed by Tukey's test or Dunnett's test was used to evaluate differences. Statistical analyses were performed with JMP13 (SAS Institute Inc., Cary, NC), and $p<0.05$ was considered to be significant.

Author Contributions: M.T. conceived and designed the experiments, and wrote the manuscript. M.T., T.I., J.N., A.H., Y.O., T.A., and R.T. performed the experiments and analyzed the data. K.Y. and T.M. supervised the entire process. All authors approved the final version of the manuscript.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Fig. 1 Effect of Nano PGE_1 and the other PGE_1 formulations on SNI-induced motor dysfunction and the injured sciatic nerve.

(A) Sciatic nerve functional index (SFI). Nano PGE_1, Lipo PGE_1, PGE_1-CD, or saline (SNI CTL) was administered intravenously after sciatic nerve injury, as described in "Materials and Methods". Mean ± SEM, N=8 *P<0.05 **p<0.01 vs. SNI CTL

(B) Expression of VEGF and agrin in the injured sciatic nerve at 1 and 2 weeks after injury. The intensity of expression is shown relative to that of β-actin in normal tissue, which was defined as 1.0. *P<0.05 vs. SNI CTL.

Fig. 2 Effect of Nano PGE_1 and the other PGE_1 formulations on SNI-induced muscle atrophy (1)

(A) Muscle weight ratio (L/R (%)) at 1, 2, and 3 weeks after SNI. Mean ± SEM, N=8 *P<0.05, **p<0.01 vs. the saline-treated control group (SNI CTL).

(B) Expression of VEGF and agrin in atrophic muscle at 1 and 2 weeks after injury. The intensity of expression is shown relative to that of GAPDH in normal tissue, which was defined as 1.0. *P<0.05, **p<0.01 vs. SNI CTL

Fig. 3 Effect of Nano PGE_1 and the other PGE_1 formulations on SNI-induced muscle atrophy (2)

(A) Muscle CSA at 3 weeks after injury and representative HE-stained sections. (a): Normal sciatic nerve, (b): Saline-treated SNI control (SNI CTL), (c): SNI + Nano PGE_1, (d): SNI + Lipo PGE_1, (e): SNI + PGE_1-CD. Scale bar = 50 μm. Mean ± SEM, N=8, *P<0.05, **p<0.01 vs. SNI CTL

(B) Expression of CAPN3 and nestin in atrophic muscle at 1 week after SNI.

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Immunohistochemistry for nestin. (a) Normal, (b) SNI CTL, (c) SNI + Nano PGE_1, Scale bar = 30 μm.

Fig. 4 EP receptor expression and ERK1/2 or Akt phosphorylation in the sciatic nerve

(A) EP receptor expression in the injured sciatic nerve. EP2 expression was normalized for that of β-actin.

(B) HE staining and immunohistochemistry for EP2. Scale bar = 50 μm.

(C) Phosphorylation of ERK1/2 and Akt in the injured sciatic nerve. Nano PGE_1 (20 μg/kg) or Lipo PGE_1 (5 μg/kg) was administered intravenously at 24 h after injury. Tissues were harvested at 3 h or 24 h after dosing for analysis. *$p<0.05$, **$p<0.01$ vs. saline-treated SNI control (SNI CTL).

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Supplemental Fig. 1 (Fig. S1)

Time course of VEGF, agrin, CAPN3, and nestin expression

(A) VEGF and agrin expression in the injured sciatic nerve

(B) VEGF, agrin, CAPN3, and nestin expression in atrophic muscle

The intensity of expression is shown relative to that of β-actin (sciatic nerve) or GAPDH (muscle) in normal tissue, which was defined as 1.0.

Supplemental Fig. 2 (Fig. S2)

SNI-induced muscle atrophy and effect of Nano PGE_1.

(A)Muscle weight ratio (L/R (%)) at 1, 2, 3, and 5 weeks after injury. The muscle (including tibia and fibula) of the left (L) and right (R) hind limbs was weighed (photo).

(B) CSA of atrophic muscle.

(C) Muscle weight ratio (L/R (%)) at 3 weeks after injury. A single dose of Nano PGE_1, Lipo PGE_1, or PGE_1-CD was administered intravenously at 24 h after injury. Mean ± SEM, N=4-6, *P<0.05 vs. saline-treated SNI control (SNI CTL).

Table 1 Distribution of Nano PGE_1, Lipo PGE_1, and PGE_1-CD in rats with SNI.

	0	0.5 min	2 min	4 min	8 min	3 h	24 h	48 h	day 7
Blood (ng/mL)									
Nano PGE_1		323.8 ± 15.8	303.7 ± 23.6	271.8 ± 30.4	318.0 ± 18.8	321.1 ± 38.9	64.6 ± 9.74	7.94 ± 0.84	5.33 ± 1.76
Lipo PGE_1	1.42 ± 0.15	57.0 ± 4.52	17.7 ± 1.72	9.45 ± 1.19	4.24 ± 0.68	6.07 ± 1.17	2.45 ± 0.58	1.43 ± 0.56	1.28 ± 0.21
PGE_1-CD		36.5 ± 9.23	15.6 ± 3.19	5.95 ± 1.55	4.97 ± 0.56	3.31 ± 0.93	2.12 ± 0.43	1.15 ± 0.45	0.83 ± 0.22
Sciatic nerve (injured) ng/g									
Nano PGE_1						32.1 ± 0.46	86.8 ± 0.89	18.5 ± 0.60	6.49 ± 1.26
Lipo PGE_1	0.65 ± 0.04					0.49 ± 0.01	1.08 ± 0.21	0.67 ± 0.15	0.69 ± 0.25
PGE_1-CD						0.42 ± 0.04	0.81 ± 0.19	0.55 ± 0.21	0.66 ± 0.31
Sciatic nerve (non-injured) ng/g									
Nano PGE_1						17.2 ± 7.54	7.52 ± 1.00	7.06 ± 1.45	1.67 ± 1.43

Nano PGE_1, Lipo PGE_1, or PGE_1-CD (10 μg/kg as PGE_1) was administered intravenously at 24 h after injury. The PGE_1 level in blood or sciatic nerve tissue (injured and non-injured) was determined by enzyme immunoassay after extraction. Mean ± SEM, N=3

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