http://dx.doi.org/10.5607/en.2014.23.3.238
Exp Neurobiol. 2014 Sep;23(3):238-245.
pISSN 1226-2560 • eISSN 2093-8144

Original Article

Experimental Neurobiology

Aucubin Promotes Neurite Outgrowth in Neural Stem Cells and Axonal Regeneration in Sciatic Nerves

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Aucubin is an iridoid glycoside with a wide range of biological activities, including anti-inflammatory, anti-microbial, anti-algesic as well as anti-tumor activities. Recently, it has been shown that aucubin prevents neuronal death in the hippocampal CA1 region in rats with diabetic encephalopathy. In addition, it has protective effects on H_2O_2-induced apoptosis in PC12 cells. We have shown here that aucubin promotes neuronal differentiation and neurite outgrowth in neural stem cells cultured primarily from the rat embryonic hippocampus. We also investigated whether aucubin facilitates axonal elongation in the injured peripheral nervous system. Aucubin promoted lengthening and thickness of axons and re-myelination at 3 weeks after sciatic nerve injury. These results indicate that administration of aucubin improved nerve regeneration in the rat model of sciatic nerve injury, suggesting that aucubin may be a useful therapeutic compound for the human peripheral nervous system after various nerve injuries.

Key words: aucubin, neural stem cell, neurite outgrowth, axonal regeneration, sciatic nerve

INTRODUCTION

Aucubin (1,4a,5,7a,-tetra-5-hydroxy-7-(hydroxy-methyl) cyclopenta (c) pyran-1-y1-b-D-glucopy-ranoside) has been isolated from traditional Korean medicinal herbs, such as Aucuba japonica [1], Eucommia ulmoides [2], Plantago asiatica [3] and Melitaea cinxia [4]. It shows multiple pharmacological effects, including blood pressure reduction, liver protection, as well as anti-inflammatory, anti-microbial, anti-algesic and anti-tumor activities [5-7].

There have been reports that aucubin has protective effects on H_2O_2-induced apoptosis in PC12 cells by modulating the expression of Bcl-2 family [8, 9] and natural lignans and iridoid compounds lengthen neuritis of PC12h cells, a cultured cell line of paraneuron [10, 11]. In contrast to the wide range of aucubin effects, little is known about its effects on the nervous system. Recently, Xue and his colleagues have reported that aucubin prevents cell death of hippocampal neurons by changing anti-oxidative ability and expression of Bcl-2 and Bax in in the rat model of diabetic encephalopathy [12, 13].

In this study, we examined effect of aucubin on neurite outgrowth in hippocampal neural stem cells primarily cultured from the rat embryos (E16) and then investigated whether aucubin promotes axonal regeneration in injury model of sciatic nerves. In the peripheral nervous system (PNS), axons can regrow after nerve injury but it is very slow, especially in human. During Wallerian degeneration, a process of gradual nerve degeneration in the distal stump of the injury site when the peripheral nervous

Received March 3, 2014, Revised March 6, 2014,
Accepted March 6, 2014

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system (PNS) is injured, axons and myelin sheaths are degenerated and removed for regrowing axons. When axons outgrow from the nerve ending sites toward the distal stump at the site of injury, Schwann cells remyelinate the new axons [14, 15]. Enhancing the axonal regrowth by any small compounds, it can be therapeutic for improving PNS nerve regeneration.

MATERIALS AND METHODS

Primary cultures of hippocampal neural stem cells

Hippocampal NSCs were isolated from the rat E16 embryonic brain as described previously [16, 17]. Time-pregnant Sprague-Dawley rats were purchased from Orient Co., Ltd., a branch of Charles River Laboratories (Gyunggi-do, Korea). Briefly, hippocampal eminence were dissected from the rat E16 embryonic forebrain aseptically using fine forceps under dissecting microscope. The tissues were collected and dissociated mechanically in Ca^{2+}/Mg^{2+}-free HBSS (Invitrogen) and then plated at 19,000 cells/cm^2 on 35 mm dishes pre-coated with 15 μg /ml poly-L-ornithine and 1 μg /ml fibronectin (Sigma-Aldrich). Cells were cultured in serum free N2 media supplemented with 10 ng/ml bFGF (Invitrogen) in 5% CO_2 for 3 days.

Immunostaining and immunoblotting assays

NPCs were passaged using 0.05% trypsin–EDTA on 12 mm glass coverslips (Bellco) and grown in N2 media with bFGF for an additional 1 day. They were grown for 4~6 days more without FGF and with or without aucubin (1~10 μg/ml, Sigma). NSCs were immunostained with anti-NeuN and anti-MAP2 antibodies (1:1000, Sigma-Aldrich). The effect of aucubin was detected using primary antibodies against NeuN (1:1000; Covance) and MAP2 (1:2000, Abcam) and FITC-conjugated secondary antibodies (1:500; Jackson Lab, USA), and DAPI (1 μg/ml, Sigma) as previously described with modifications [18, 19]. Immunoblotting assay was carried out as previously described [20]. The primary antibodies used were anti-neurofilament (1:1000, Covance), and anti-β tubulin (1:1000, Sigma) antibodies.

Animals

Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea. In animal experiments, male Sprague-Dawley rats (adult 250~270 g) from Orient Co., Ltd., a branch of Charles River Laboratories in Korea were housed 2 or 3 per cage, allowed access to water and food *ad libitum*, and maintained under a constant temperature (23±1°C), humidity (60±10%) and a 12 h light/dark cycle (light on 07:00~19:00 h).

Injury model of the rat sciatic nerve and aucubin treatment

After a 1-week habituation period, animals were anesthetized by an intraperitoneal injection of Equithensin (350 mM sodium pentobarbital, 250 mM chloral hydrate, 85 mM MgSO4, 40% prophylene glycol in 10% ethanol, 2 ml/kg). Left sciatic nerve between muscles was exposed with aseptic techniques 1.0 cm distal to the sciatic notch at the sciatic notch using a forcep, as described earlier [14, 19]. Under the dissection microscope, the major blood vessels were carefully separated from nerve fibers prior to the nerve transection by using #55 forceps and then the nerve fibers were transected except the artery in the sciatic nerve using iridectomy scissors. Following the surgery, animals were rested on a warm pad until recovered. Aucubin or a vehicle (physiological saline) was administrated (2.5 mg/kg) by intraperitoneal injection to the injured rats once daily for 1 week.

Immunohistochemical analysis

Nerve regeneration was assessed 2 week after aucubin injection by immunohistochemical analysis as described previously [14, 19]. After perfusion, the distal stumps of the cut sciatic nerves were post-fixed for 6 hours, frozen and dehydrated with 30% sucrose, and then cryosectioned at 7~10 μm. The nerve slices were permeabilized with 0.1% Triton-X 100 for 15 min and were blocked with 15% normal goat, horse and donkey serum and 1% BSA for 1 h. The nerve slices were incubated with the primary antibodies overnight at 4°C and then with FITC or the TRICT-conjugated secondary antibody for 1 h. Images were captured on an LSM 510 confocal microscope (Carl Zeiss, Germany). The primary antibodies used were anti-neurofilament (NF-200, 1:200, Sigma) to detect growing axons and anti-myelin basic protein (MBP, 1:1000, Sigma) as a marker for Schwann cell myelination. Images were scanned under LSM 510 confocal laser microscope (Carl Zeiss, Germany).

Measurement of axonal length and diameter and quantification of Data and Statistical Analysis

The neurite length of MAP2-immunoreactive cells were measured under 630X oil lens from 9~12 confocal microscopic fields using the LSM510 image analysis browser (Carl Zeiss). Thirty digital micrographs were taken for each nerve, from the injury sites to the fiber growing sites, of distal stumps using a confocal Laser Scanning Microscopy LSM 510, to analyze axonal growth after the injury. An image analysis program (Carl Zeiss) was used to measure the length and diameter (120 axons from 3 animals per group) of the individual axons. The statistical analyses of the data

were performed by one-way analysis of variance and $p<0.05$ was taken as significant.

RESULTS

To investigate whether aucubin facilitates differentiation of neural stem cells, we obtained neural stem cells primarily from hippocampus of the rat embryonic day 16 brain. After proliferation for 3 days, we plated cells and incubated with the differentiation media without FGF for 4~6 days to induce differentiation. In order to explore the effect of aucubin on the differentiation, we included aucubin in the differentiation media. Previously, the effect of aucubin on PC12 cells has been shown in a wide range of concentration. Cell viability in H_2O_2-treated PC12 cells was increased under concentrations of about 50~400 μg/ml (0.1 to 1 mM) aucubin [8, 9], but their neurites were elongated under 0.1 to 1 μg/ml aucubin [11]. However, these studies did not examine any differentiation markers. In our primary cultures of neural stem cells, we used 1 to 10 μg/ml. When we immunostained cells with NeuN antibodies, a mature neuron marker, aucubin increased numbers of NeuN-positive mature neurons (Fig. 1A, B) by more than 1.8 fold compared with the control. When neurons were immunostained with MAP2 antibodies, a marker for neuronal dendrites, numbers of MAP2-positive cells increased. The MAP-2-positive cells showed largely extended neurite morphology when treated with 1 and 10 μg/ml aucubin.

Aucubin increased the average length of MAP2-positive dendrites. The average length of MAP2-positive dendrites was longer (about 47 μm) in differentiated cells with 1 μg/ml aucubin, than with 10 μg/ml aucubin (about 34 μm), and in the vehicle treated cells (about 27 μm) (Fig. 1D inlet). Smaller neurites with less than 30 μm in length were observed more frequently in the vehicle treated cells, while longer neurites between 30 and 100 μm were observed more frequently in the aucubin treated cells (Fig. 1D). Next, we examined the expression of neuro-filament, an intermediate filament of mature neuron in hippocampal neuronal cells after treatment of 1 μg/ml aucubin (Fig. 1C). Aucubin up-regulated the light chain of neuro-filament without any effect on the heavy chain. Taken together, these results indicate that aucubin elevates the expression of neuronal markers and enhances the elongation of neuronal dendrites in hippocampal stem cells cultured primarily from the rat embryo.

Next we explored whether aucubin could improve elongation of injured axons in PNS. We generated the animal models with axotomized sciatic nerves. We injected aucubin (2.5 mg/kg) intraperitoneally to the injured rats, and then nerve regeneration was assessed 2 week after aucubin injection by immunohistochemical analysis. At three weeks after the sciatic nerve injury, axons were degraded in the distal stump, and myelin sheets of Schwann cells were also degraded and randomly scattered (Fig. 2A, vehicle). Newly grown axons immunostained with anti-neurofilament antibodies were very thin, and not yet myelinated in its entire length. In nerves with aucubin administration, however, axons got thicker and were immunostained with anti-MBP antibodies, suggesting they were remyelinated by Schwann cells (Fig. 2A, aucubin). Therefore, the remyelinated thick axons could be considered as regenerated axons. In the aucubin treated animals, myelination at the distal ends of growing axons were not continuous suggesting they were just undergoing wrapping of newly growing axons (Fig. 2C, aucubin).

Aucubin also increased thickness of growing axons. Thickness of growing axons were measure from 120 individual axons (n=4 animals per group) using the LSM 510 image analysis browser. The axon thickness was 4.0±1.3 μm in the aucubin-treated rats compared to 2.7±1.1 μm in the control animals (Fig. 3). There was no myelin observed at 3 weeks after nerve transection in the vehicle-injected control group, but the myelin sheets surrounded the thick axons in the aucubin-injected group. Thickness of myelinated axons (7.187±1.7318 um) increased by more than 2.5-fold compared to the vehicle-injected control group (Fig. 3A). Furthermore, the average axon diameters at the nerve cut site and the distal end of growing sites were similar in the vehicle-injected control group, while it was thicker at the nerve cut site and thinner at the distal end of growing sites in the aucubin-injected group (Fig. 2 and Fig. 3B). We next analyzed the distribution of the diameter of axon in a microscopic field at the distal stumps of the injury sites. It shows that number of the large-diameter axons (>3 μm) in the aucubin-treated group increased, while number of the small-diameter axons (<3 μm) decreased compared to the control groups (Fig. 3D). Axonal length also increased strikingly. It was 55.5±0.9 mm for the aucubin-injected group and 15.3±0.8 mm for the vehicle-injected control group. These observations indicate that administration of aucubin improved axonal re-growth and Schwann cell remyelination in the rat model of sciatic nerve injury.

DISCUSSION

After peripheral nerve injury, the nerve stump with degenerating axons and Schwann cells provides an appropriate environment for axonal regeneration [21]. The degenerated axons and myelin sheaths are removed and the microenvironment is modified to promote axonal regrowth in the nerve ending site. Neurotrophic factors in this microenvironment are known to promote nerve regeneration by enhancing axonal extension, synapse reformation,

http://dx.doi.org/10.5607/en.2014.23.3.238

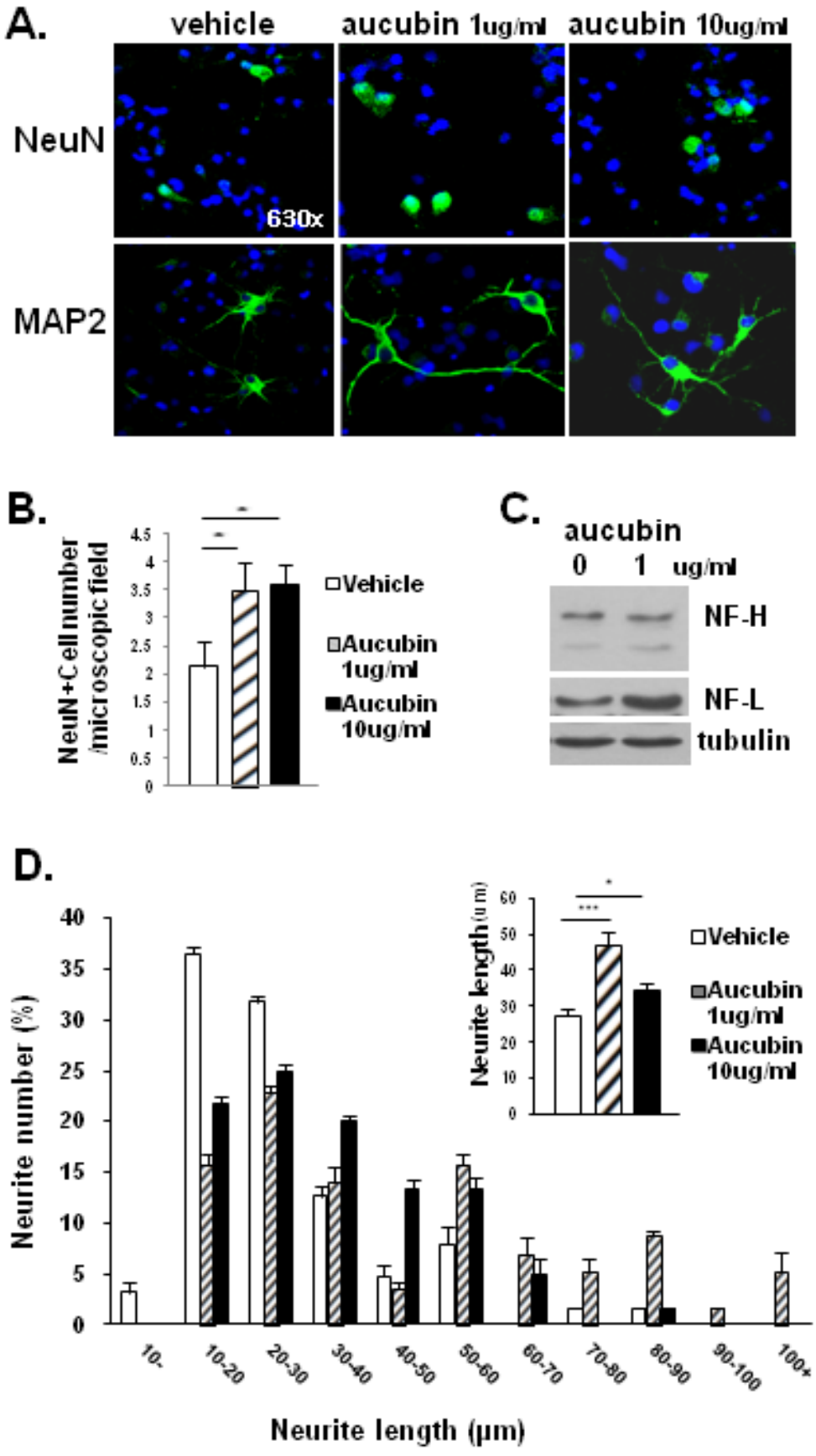

Fig. 1. Neurite extension and neuronal differentiation promoted by aucubin addition in neural stem cells cultured from the rat embryonic hippocampus. Differentiated neuron were immunostained with mature neuron marker, NeuN and neurites with MAP2 antibodies (green) and nuclei were counterstained with DAPI (blue). Numbers of NeuN positive cells and extention of MAP2 positive neurites were promoted in the presence of ACB (1 to 10 μg/mL, A and B) compared with control (vehicle). Light chain of Neurofilament protein was also increased by addition of aucubin (1 μg/mL C). (D) Quantification of MAP2 positive cells containing neurites in ranges between 0 and 100um. Inlet shows averages of neurites length in vehicle, 1 and 10 μg/mL. Data represents the means± standard error measures. **p<0.001 compared with the control, one-way ANOVA.

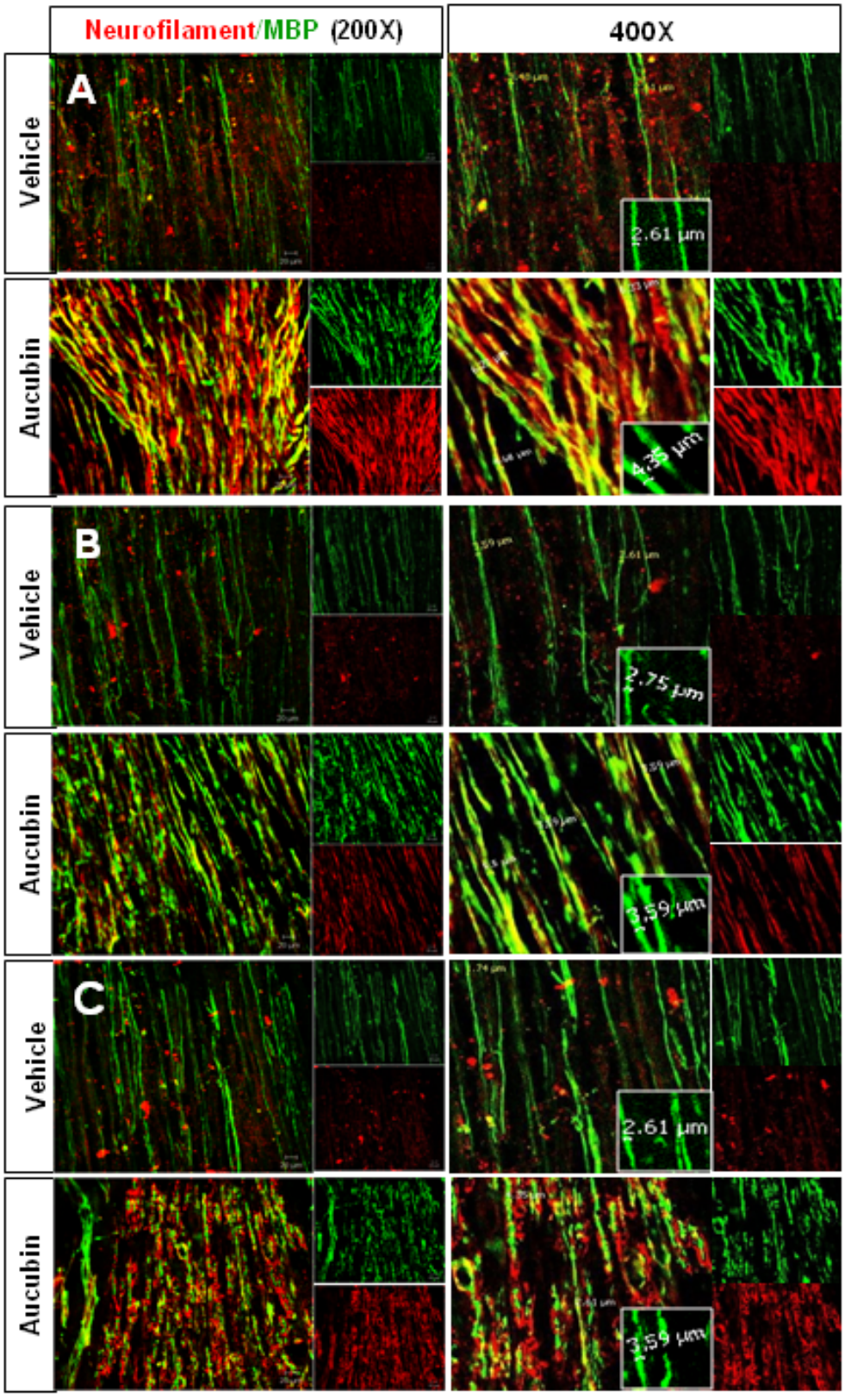

Fig. 2. Sciatic nerve regeneration improved by administration of aucubin (2.5 mg/kg) in the injured rat sciatic nerves at 3 weeks after sciatic nerve injury (A, at nerve injury site; B, in the middle; C, at the front site of growing axons). Axons were immunostained at the distal stump of the injured sciatic nerve with anti-neurofilament antibodies (green) and Schwann cells with anti-MBP antibodies (red). Inlets show diameter of axons.

http://dx.doi.org/10.5607/en.2014.23.3.238

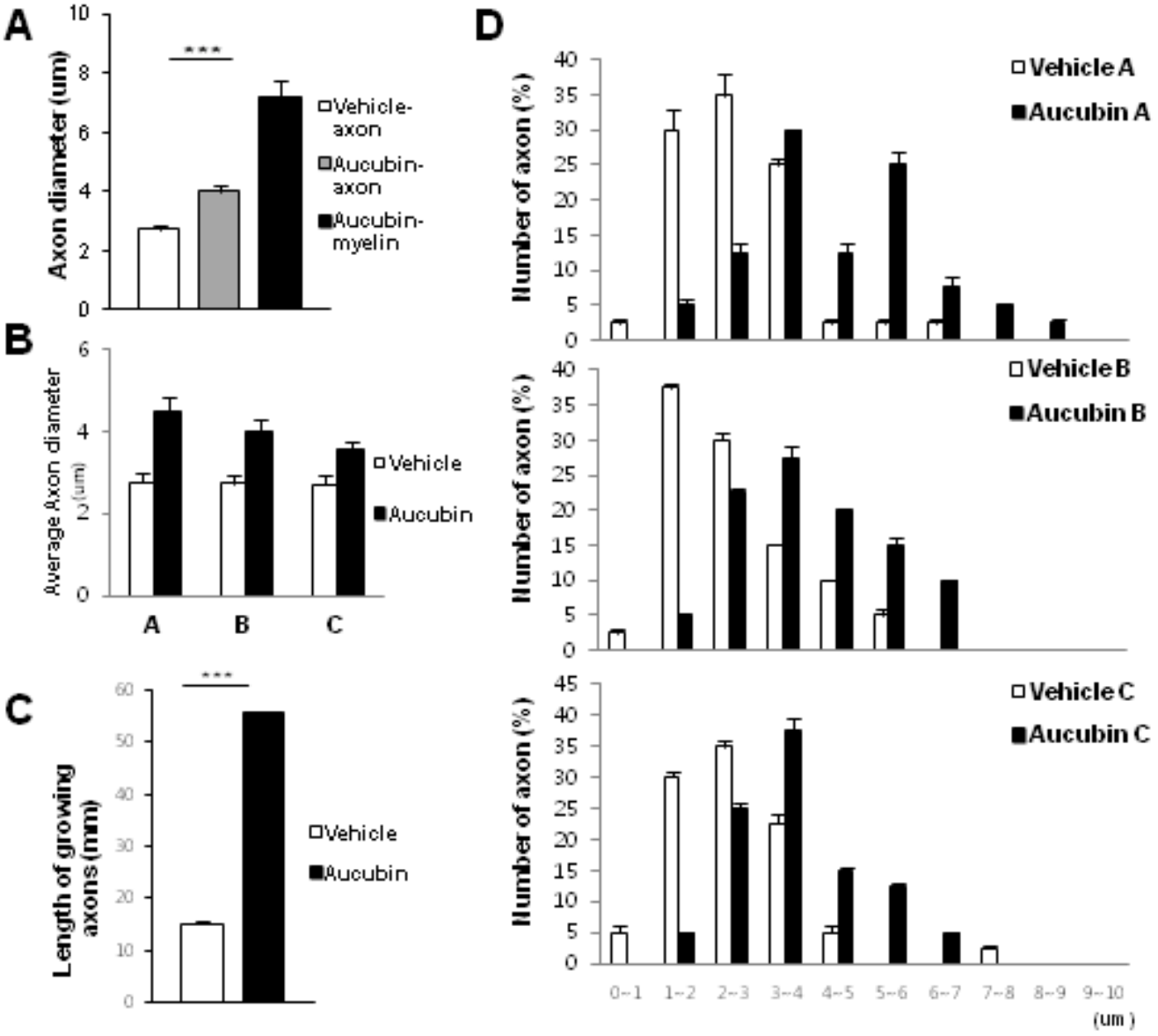

Fig. 3. Aucubin promotes axonal regeneration. The diameter of axons (A, B, D) and myelinated axons (A) increased by aucubin treatment in the distal stumps of injured sciatic nerves. At three weeks, there is no myelination observed in vehicle treated nerves (A). Quantification graph of sciatic nerve regeneration shows average of axonal diameter (A) and average diameters of 3 regrowing sites (A, the beginning, B, the middle and C, the front, Fig. 2) (C). Average length of axons in vehicle and aucubin treated animals. Data indicates means±standard error measures. (D) Numbers of growing axons show distribution of axon diameter between 10 and 100 um in 3 regrowing sites *p<0.01 and **p<0.001 compared with the vehicle injected group, one-way ANOVA.

and remyelination [22, 23].

In the present study, we showed that aucubin facilitated neurite extension in hippocampal neural stem cells and axonal regeneration in the PNS. It has been reported that relatively high concentrations of aucubin (0.1 mM to 1 mM) have protective effects on H_2O_2-induced apoptosis in PC12 cells [8, 9]. There are reports that compound VI containing natural lignans and iridoid, which includes partly glucosidase-hydrolyzed aucubin, promotes neuronal differentiation and lengthening neuritis of PC12h cells, a cultured cell line of paraneuron[10]. Yamazaki and his colleagues (1996) have also reported that 1 μg/ml aucubin hydrolysates induces neurites outgrowth of PC12h cells [11]. However, they did not examine any expression of differentiation markers. Here, we examined the effect of aucubin on neuronal differentiation and neurite outgrowth using neural stem cells cultured primarily from the rat hippocampus of E16 brain. We also examined the effect

of aucubin on expression of mature neuron marker, NeuN, and dendrite marker, MAP2.

We also observed that axonal regeneration in the injured sciatic nerves of rats was facilitated by aucubin administration. We found the improvements in axonal thickness, lengthening, axon area, and myelin thickness in the PNS injury area [24]. In addition, we observed myelination surrounding the regrowing axons in the aucubin-treated animals, while there was no myelination observed in the vehicle treated animals. After nerve injury, Schwann cells in the distal stump of the injury site de-differentiate and degrade myelin when axons degenerate. And then Schwann cells proliferate to fill the gap at the injury sites and re-myelinate the new axons. Schwann cells also release neurotrophic factors to facilitate and guide direction of axonal regrowth [25, 26]. Therefore, the remyelination of axons by Schwann cells observed in the aucubin-injected group strongly support that aucubin enhances axonal regeneration in the rat model of sciatic nerve injury. It is not clear how aucubin promotes neuronal differentiation and axonal elongation. However, our finding that aucubin affects both neurite outgrowth in neural stem cells in the rat brain and axonal regeneration in the injured sciatic nerve suggest a possibility that aucubin may enhance neurite elongation within the injured brain as well. In conclusion, our results demonstrate for the first time that aucubin treatment improves axonal regeneration in a sciatic nerve injury model. Because the axons of adult human PNS could regenerate, unlike in the central nervous system, our data imply that aucubin may be a useful therapeutic compound for the human PNS after various nerve injuries and needs to be studied for clinical correlations.

ACKNOWLEDGEMENTS

This research was supported by grants from National Research Foundation of Korea (2012M3A9C6049937)and (2012R1A2A2A01015843), funded by the Ministry of Education, Science and Technology, the Republic of Korea.

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