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Extracellular ATP inhibits Schwann cell dedifferentiation and proliferation in an *ex vivo* model of Wallerian degeneration

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ABSTRACT

After nerve injury, Schwann cells proliferate and revert to a phenotype that supports nerve regeneration. This phenotype-changing process can be viewed as Schwann cell dedifferentiation. Here, we investigated the role of extracellular ATP in Schwann cell dedifferentiation and proliferation during Wallerian degeneration. Using several markers of Schwann cell dedifferentiation and proliferation in sciatic explants, we found that extracellular ATP inhibits Schwann cell dedifferentiation and proliferation during Wallerian degeneration. Furthermore, the blockage of lysosomal exocytosis in ATP-treated sciatic explants is sufficient to induce Schwann cell dedifferentiation. Together, these findings suggest that ATP-induced lysosomal exocytosis may be involved in Schwann cell dedifferentiation.

1. Introduction

Peripheral nerve injury induces Schwann cells distal to the injury to dedifferentiate and re-enter the cell cycle. These proliferative Schwann cells, similar to their immature state, facilitate nerve regeneration. Schwann cell dedifferentiation and proliferation occurs when Schwann cells are detached from axons following nerve injury. The molecular mechanism of the transition from myelinating Schwann cells to immature cells is still unknown. However, it is becoming increasingly clear that several regulatory proteins are involved in the initiation of Schwann cell dedifferentiation and proliferation. A growing body of evidence suggests that members of the mitogen-activated protein kinase (MAPK) family, such as ERK (extracellular signal-regulated kinase), c-jun and p38 MAPK, have been shown to mediate the initiation of Schwann cell dedifferentiation [1–3]. These molecules also function as negative regulators of myelin differentiation of Schwann cells [3,4]. The p75 neurotrophin receptor (NGFR) is the low affinity nerve growth factor receptor. The p75NGFR is normally expressed in non-myelinated Schwann cells in uninjured nerves and demyelinating myelinated Schwann cells after nerve injury [5,6]. The expression of p75NGFR after nerve injury also implies the involvement of the Schwann cell dedifferentiation process during Wallerian degeneration (WD) [7,8]. In addition, it is reported that lysosomal activation plays an important role in p75NGFR induction in demyelinating Schwann cells during WD [8]. This previous study showed the possibility of the role of lysosomal vesicles in Schwann cell dedifferentiation [8].

Adenosine triphosphate (ATP) is an essential neurotransmitter that interacts with both neurons and Schwann cells [9–11]. Previous studies have shown that ATP is released from peripheral nerves and Schwann cells in response to stimuli [9,12]. Such extracelluar ATP inhibited the proliferation and differentiation of Schwann cells [11,13]. However, in WD, the physiological function of ATP in Schwann cell dedifferentiation and proliferation has not been fully addressed. In this study, we address functional consequences of extracellular ATP during WD, and demonstrate, using *ex vivo* culture system, that extracellular ATP inhibits Schwann cell dedifferentiation and proliferation during WD.

2. Materials and methods

2.1. Materials

The primary antibodies used for immunostaining or western blotting were raised against p75NGFR and LAMP1 (Santa Cruz Biotechnology, Santa Cruz, USA). Phospho-p38MAPK and phospho-p44/42 MAPK (p-ERK1/2) were obtained from Cell signaling (Beverly, USA). Alexa Fluor 488- and 594-conjugated secondary antibodies were purchased from Life Technologies (Grand Island, USA). Adenosine triphosphate (ATP), Metformin (Met), and S100 were obtained from Sigma (St. Louis, USA). Ki67 was obtained from Abcam (Cambridge, UK).

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2.2. Animals

All efforts were made to minimize animal's suffering and to reduce the number of animals used. Male Sprague–Dawley rats (6-weeks old) were housed with food and water available *ad libitum* in a temperature- (23 ± 1 °C) and humidity- (50%) controlled environment on a 12 h light/dark cycle. All of the procedures were performed according to protocols approved by the Kyung Hee University Committee on Animal Research that followed the guidelines of animal experimentation established by The Korean Academy of Medical Science.

2.3. Explant culture

The sciatic nerve explant cultures were performed as described previously [14]. The sciatic nerves from adult Sprague–Dawley rats (Samtako, Osan, Korea) were removed, and the connective tissues surrounding the nerves were detached under a stereomicroscope. The sciatic nerves were divided into three or four explants of 3–4 mm in length. The explants were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 units/mL penicillin, 100 μg/mL streptomycin, 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) and 2 mmol/L L-glutamine. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. After culture, the sciatic explants or pieces of sciatic nerve removed after axotomy were fixed with 4% paraformaldehyde (PFA) for 6–12 h and teased into single- or several-nerve fibers under a stereomicroscope.

2.4. Western blot analysis

For the western blot analysis, cultured sciatic explants were prepared with a modified radioimmunoprecipitation assay buffer [RIPA; 50 mm/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 0.5% deoxycholic acid, 0.5% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium *o*-vanadate, and 1X protease inhibitor mixture (Roche Molecular Biochemicals, Nutley, USA)]. Thirty-five micrograms of the total protein was separated using 10% sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose membrane (Amersham Bioscience, Piscataway, USA). The blotted membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature (RT) for 1 h, and the membranes were incubated overnight at 4 °C with appropriate primary antibodies diluted (1:500–1000) in prepared TBST containing 3% non-fat milk. After three washes in TBST, the blots were reacted with horseradish peroxidase-conjugated secondary antibodies (1:3000, Cell signaling, Beverly, USA) for 1 h at RT, and then washed again with TBST. Detection was performed using an enhanced chemiluminescence-Western blot system (Amersham, Piscataway, USA). For quantification, the X-ray films were then scanned using a Samsung scanner and analyzed with LAS image

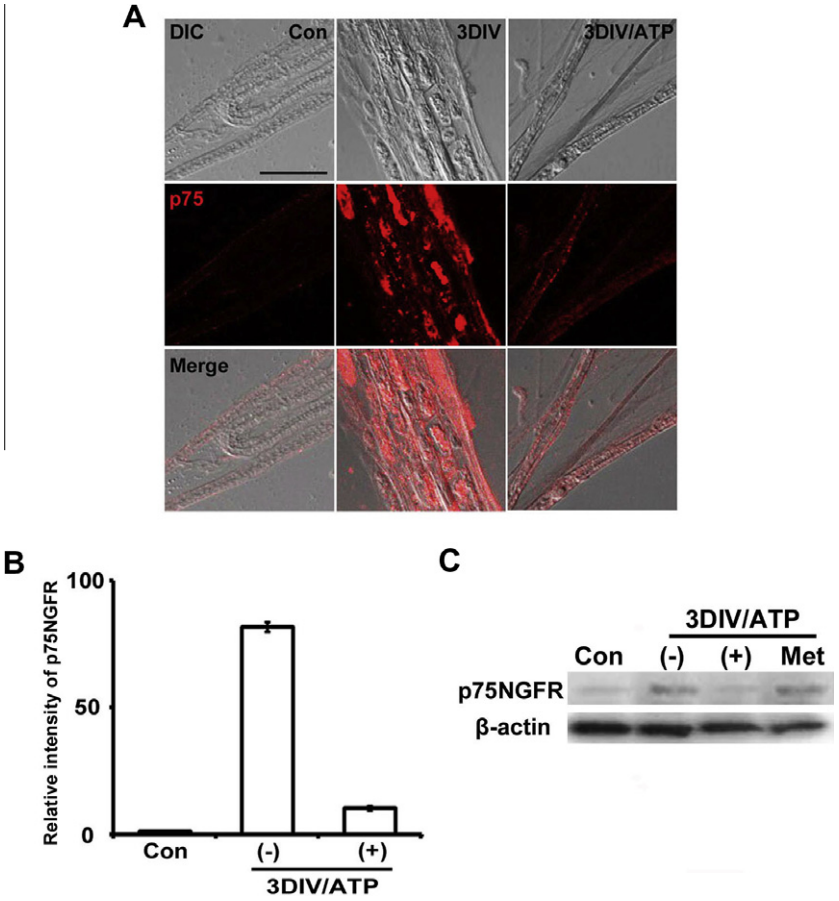

Fig. 1. Extracellular ATP inhibits p75NGFR expression. (A) The sciatic nerve sections of cultured explants were immunolabeled with an antibody against p75NGFR (p75). Sciatic nerve explants were cultured for 3 days *in vitro* (3DIV) in the absence or presence of ATP (2 mM). DIC (differential interference contrast microscopy). Size bar = 50 μm. (B) Quantitative analysis of the fluorescent intensity of p75 immunoreactivity using the software that accompanied the LSM510 laser confocal microscope. A total of 7 sections from each group were measured and (C) protein lysates from the *ex vivo* explants were analyzed by western blotting. Met (Metformin, 500 μM).

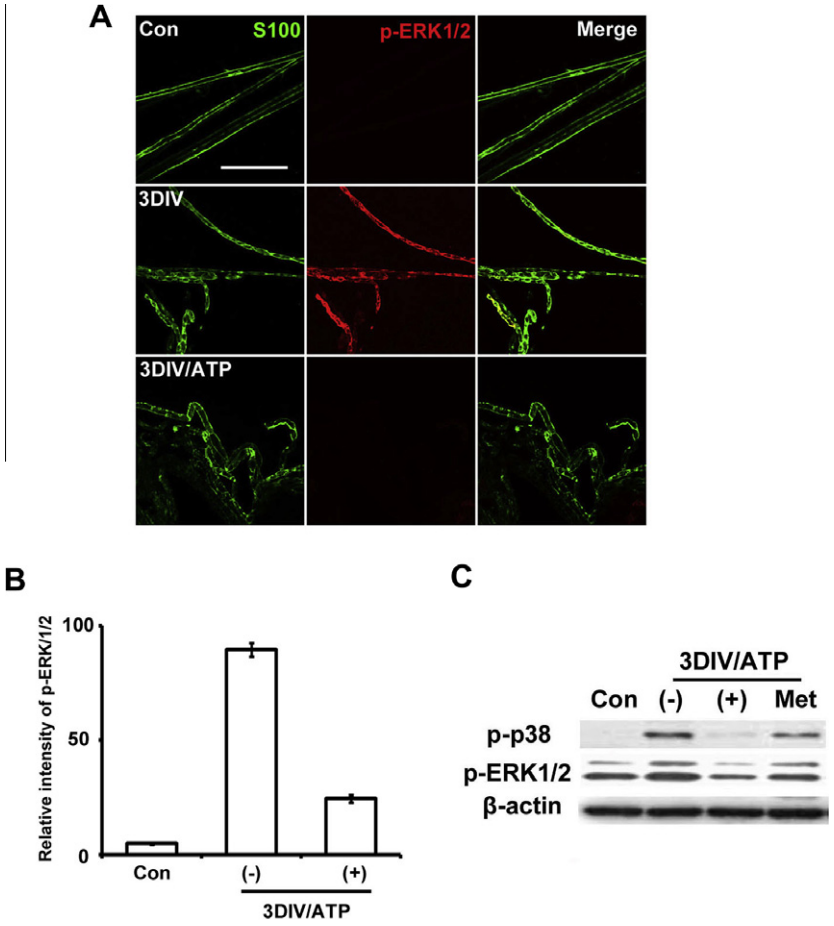

Fig. 2. Schwann cell dedifferentiation markers are suppressed by extracellular ATP. (A) Rat sciatic nerve fibers were double-immunostained with S100 (green, Schwann cell marker) and p-ERK1/2 (red, dedifferentiation marker). Size bar = 200 μm. (B) Quantitiative results showing the fluorescent intensity of p-ERK1/2 immunostaining from sections and (C) protein extracts (20 μg) from sciatic explants in the absence or presence of several chemicals were analyzed by western blotting. Actin is a loading control. Metformin (Met, 500 μM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analysis system (Fujifilm, Tokyo, Japan). All experiments were repeated a minimum of three times.

2.5. Immunofluorescent labeling

The teased nerve fibers or frozen nerve sections on slides were fixed in 4% PFA for 10 min. After washing three times with phosphate-buffered saline (PBS), samples were permeabilized in ice-cold methanol for 10 min and were blocked with PBS containing 0.3% Triton X-100 and 10% bovine serum albumin (BSA) for 1 h at RT. Samples were then incubated overnight with primary antibodies (1:1000) in PBS containing 0.3% Triton X-100 (PBST) at 4 °C, and washed three times with PBS. Samples were incubated with Alexa 488- or 594-conjugated anti-goat (1:1000) or anti-rabbit IgG (1:1000) for 2 h at RT. The slides were washed three times with PBS, and coverslips were adhered to the slides with Gelmount (Biomeda, Foster City, USA). The samples were analyzed using a laser confocal microscope (LSM510, Carl Zeiss, Oberkochen, Germany).

2.6. Statistical analysis

The means of the collected data were determined for each experimental group. Statistically significant differences between the groups were determined using Student's *t*-test.

3. Results

To determine whether extracellular ATP is involved in Schwann cell dedifferentiation during WD, we first immunostained teased nerve fibers with p75NGFR antibody at 3 days *in vitro* (3DIV) in the presence or absence of ATP (2 mM). The induction of p75NGFR in denervated nerves had definitely occurred at 3DIV, but ATP-treatment of teased nerve fibers significantly reduced p75NGFR induction (Fig. 1A and B). Because ATP is known to induce lysosomal exocytosis, we investigated the involvement of this lysosomal exocytosis in Schwann cell dedifferentiation during WD, using the lysosomal exocytosis inhibitor, metformin (Met), which acidifies the lysosomal compartment [15]. Western blot analyses revealed that extracellular ATP suppressed p75NGFR at 3DIV, and a blockage of lysosomal exocytosis with Met (500 μM) in the presence of ATP showed a reversal of the p75NGFR pattern at 3DIV (Fig. 1C).

The expression of phospho-ERK1/2 (p-ERK1/2) rises highly in the distal stump after nerve injury [1,16]. This induction of p-ERK1/2 means that nerve injury is involved in Schwann cell dedifferentiation [1,4]. Next, we examined whether extracellular ATP inhibits the expression of p-ERK1/2 using double-immunostaining for p-ERK1/2 (a marker of Schwann cell dedifferentiation) and S100 (a Schwann cell marker). The expression of p-ERK1/2 in teased nerve fibers rises dramatically at 3DIV compared with the control, but the induction of p-ERK1/2 decreases when sciatic explants are

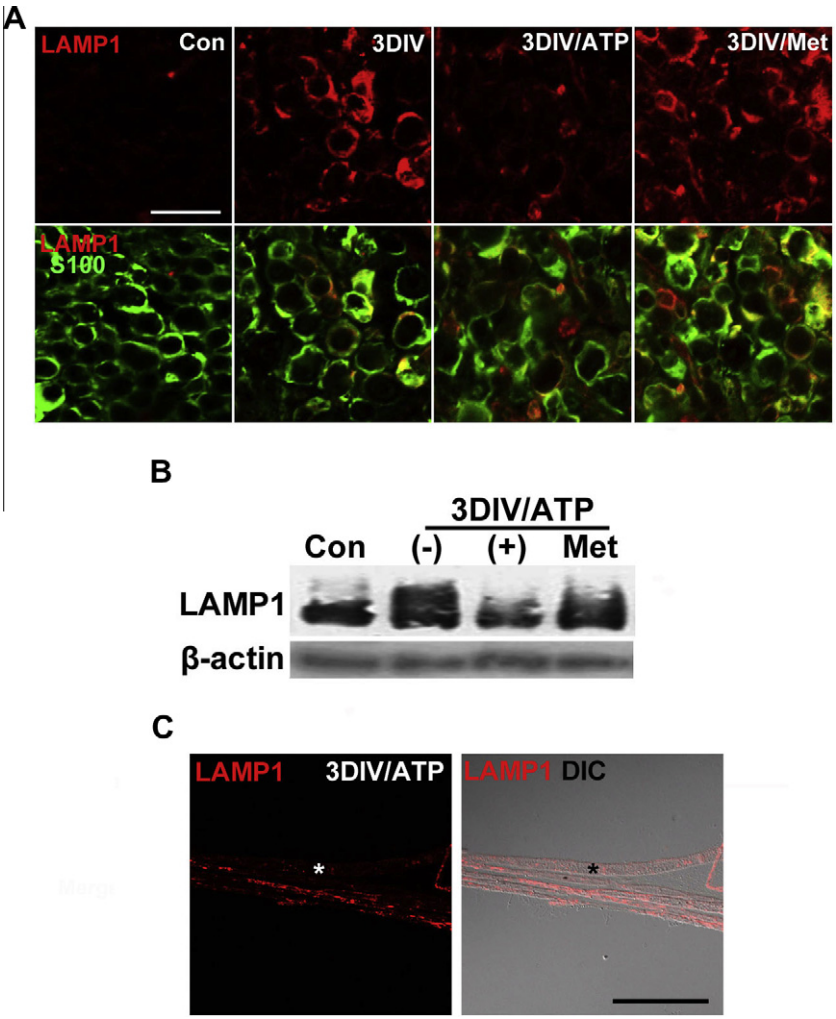

Fig. 3. Schwann cell lysosomal activation is regulated by extracellular ATP. (A) Cryostat sections of sciatic explants were immunostained with antibodies S100 (green) and lysosomal associated membrane protein 1 (LAMP1, red). Size bar = 100 μm. (B) Western blot analysis showing the levels of LAMP1 and (C) the teased nerve preparations of cultured explants were immunostained for LAMP1 expression. An asterisk indicates conserved myelinated Schwann cells. Size bar = 200 μm.

exposed to extracellular ATP (Fig. 2A and B). The immunoblot in Fig. 2C shows that extracellular ATP suppressed the induction of p-ERK1/2 at 3DIV. However, the co-addition of ATP and Met to explant cultures restored the induction of p-ERK1/2 at 3DIV.

Previous studies have shown that expression of LAMP1 (a marker of lysosomal vesicles) increased in sciatic nerves after nerve injury [7,8]. We assessed the effect of extracellular ATP on lysosomal activation by immunostaining for LAMP1. In ATP-treated explants at 3DIV, there was a drastic decrease in LAMP1 immunoreactivity compared with the control, but lysosomal activation in the sciatic explants was increased again by lysosomal exocytosis inhibitors Met at 3DIV (Fig. 3A and B).

Lastly, we conducted an experiment to determine the effect of extracellular ATP on Schwann cell proliferation by immunostaining for Ki67 (a cell proliferation marker) during WD. We found that the number of Ki67-positive cells was increased at 3DIV in cross-sections of sciatic explants, but extracellular ATP blocked Schwann cell proliferation at 3DIV, with population similar to the number of Ki67-positive cells in the control sciatic nerve (Fig. 4A and B). Altogether, our data suggest the possibility that extracellular ATP is sufficient to inhibit Schwann cell dedifferentiation and proliferation during WD.

4. Discussion

After nerve injury, Schwann cell dedifferentiation and proliferation is essential for nerve regeneration in the peripheral nervous system (PNS). However, the molecular mechanisms of these processes are largely unknown. In this study, we showed that extracellular ATP in sciatic explants inhibits Schwann cell dedifferentiation and proliferation during WD. We also showed that ATP-induced lysosomal exocytosis in the cytoplasm of Schwann cells blocks Schwann cells dedifferentiation. Thus, this study confirms that extracellular ATP in the PNS is essential for nerve degeneration and nerve repair.

Previous studies have shown that sciatic nerve injury induces the expression of members of the MAPK family in Schwann cells [1–3]. We found that a western blot analysis showed increases in p-p38MAPK and p-ERK1/2 at 3DIV in explants, and extracellular ATP induced the downregulation of p-p38MAPK and p-ERK1/2 at 3DIV (Fig. 2C). In other cells, ATP induced the activation of p-p38MAPK and p-ERK1/2 [17–19], but, in primary Schwann cell cultures, adenosine, not ATP, activated p-ERK1/2 [20]. Thus, the effect of ATP on the activation of p-ERK1/2 and p-p38MAPK is especially dependent on culture conditions. In this study, we used *ex vivo*

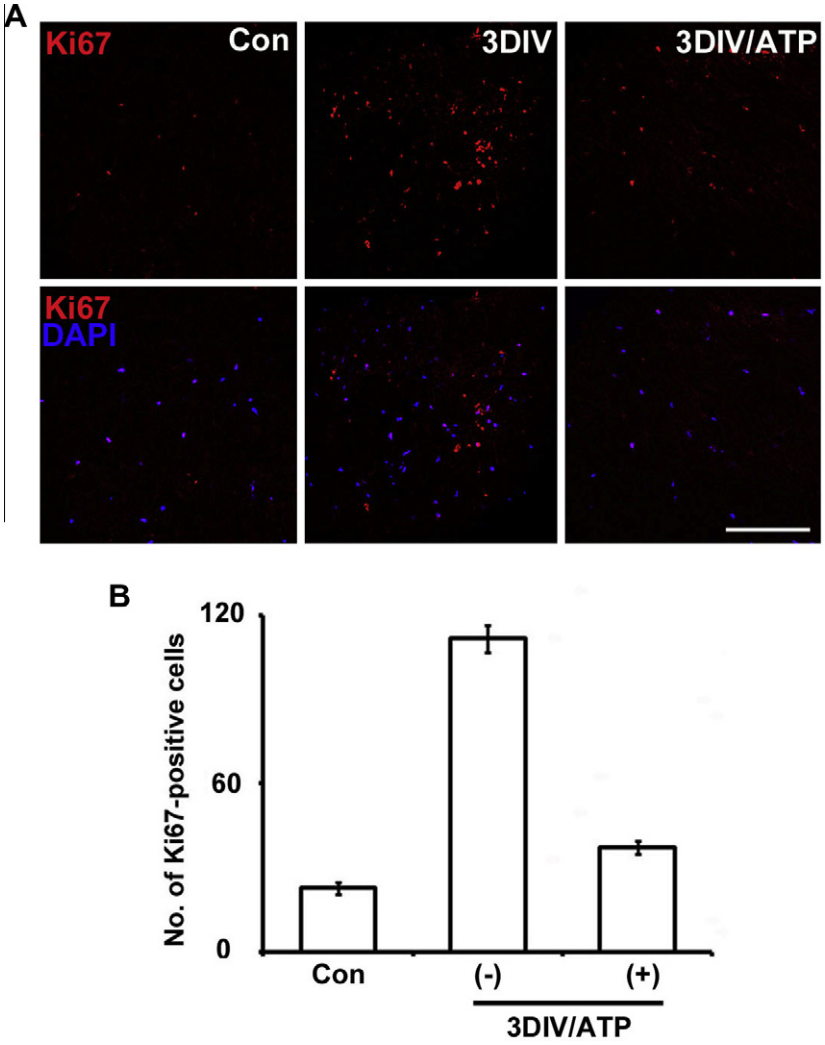

Fig. 4. Extracellular ATP inhibits Schwann cell proliferation in sciatic explants. (A) Cross-sections of rat sciatic explants were immunostained for Ki67 (red, cell proliferation marker). Con; uninjured control nerve. Size bar = 300 μm. (B) Quantification of the result shown in A. Four independent experiments were performed, and the mean number of Ki67-positive cells was measured from randomly selected areas (700 μm × 700 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sciatic nerve explants, not Schwann cells primary cultures, as a model for WD. In *ex vivo* explants, ATP was sufficient to decrease the activation of p-p38MAPK and p-ERK1/2 (Fig. 2A–C). It is known that ATP is responsible for the Schwann cell's Ca^{2+} response and induces Ca^{2+}-dependent lysosomal exocytosis [21,22]. We showed that the activation of p-p38MAPK and p-ERK1/2 was reversed by lysosomal exocytosis inhibitor in ATP-treated Schwann cell explants (Fig. 2C). We also found that extracellular ATP blocked Schwann cell proliferation (Fig. 4A and B). Thus, these findings suggest that ATP induces the inhibition of Schwann cell dedifferentiation and proliferation, and, especially, ATP-induced inhibition of Schwann cell dedifferentiation may be occurred by lysosomal-exocytosis-dependent mechanisms.

Our data also support the notion that extracellular ATP plays a role in Schwann cell dedifferentiation through a nerve growth receptor and lysosomal vesicles. We showed that extracellular ATP inhibits the expression of lysosomal activation and p75NGFR in the denervated state of Schwann cells (Figs. 1 and 3). In injured nerves, p75NGFR and LAMP1 expression can be activated [8,23,24]. Interestingly, extracellular ATP inhibited the induction of lysosomal activation, and the inhibitor of lysosomal exocytosis converted lysosomes in sciatic explants to a highly activated state (Fig. 3A and B). The reason for the decreased lysosomal activation in ATP-treated explants may be like that myelin degradation is inhibited by extracellular ATP, and thus conserved Schwann cells is able to delay lysosomal activation (Fig. 3C). However, further studies are needed to explain why lysosomal activation is decreased after ATP treatment in nerve explants.

In conclusion, we found that extracellular ATP inhibits Schwann cell dedifferentiation and proliferation during WD. Especially, ATP-induced inhibition of Schwann cell dedifferentiation in the state of denerved Schwann cells is involved in lysosomal exocytosis. Thus, elucidating the roles of extracellular ATP during WD may provide insights into understanding degenerative and regenerative functions of peripheral nerves.

Acknowledgments

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