Quercetin alleviates high glucose-induced Schwann cell damage by autophagy

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Abstract

Quercetin can reverse high glucose-induced inhibition of neural cell proliferation, and therefore may have a neuroprotective effect in diabetic peripheral neuropathy. It is difficult to obtain primary Schwann cells and RSC96 cells could replace primary Schwann cells in studies of the role of autophagy in the mechanism underlying diabetic peripheral neuropathy. Here, we show that under high glucose conditions, there are fewer autophagosomes in immortalized rat RSC96 cells and primary rat Schwann cells than under control conditions, the proliferative activity of both cell types is significantly impaired, and the expression of Beclin-1 and LC3, the molecular markers for autophagy, is significantly lower. After intervention with quercetin, the autophagic and proliferative activity of both cell types is rescued. These results suggest that quercetin can alleviate high glucose-induced damage to Schwann cells by autophagy.

Introduction

Diabetic peripheral neuropathy (DPN) is one of the major chronic complications of diabetes (Tsapas et al., 2014). The pathogenesis of DPN has not been elucidated, and effective therapies for the condition are still lacking (Zychowska et al., 2013). Autophagy has recently been found to play important roles in the maintenance of islet β cell structure and function, improve insulin resistance, and delay the onset of complications of chronic diabetes such as diabetic nephropathy, retinopathy, cardiomyopathy and atherosclerosis (Marsh et al., 2007; Ebato et al., 2008; Fujimoto et al., 2009; Masini et al., 2009; Lo et al., 2010; Ost et al., 2010; Younce et al., 2010; Kitada et al., 2011; Mellor et al., 2011; Peng et al., 2011; Wu et al., 2011; Xie et al., 2011; He et al., 2012; Hu et al., 2012; Miranda et al., 2012; Quan et al., 2012). However, little is understood about the relationship between autophagy and DPN.

Schwann cells are specialized glial cells in the peripheral nervous system, playing important roles in maintaining neuronal structure and function and repairing damaged nerves. Schwann cell dysfunction is one of the important pathogenic mechanisms underlying diabetes-associated abnormalities in neural regeneration and repair (Kennedy et al., 2005). It is believed that hyperglycemia leads to increased aldose reductase activity and polyol metabolism in Schwann cells, and the resultant abnormal metabolites cause the organelle's damage and morphological changes such as swelling and vacuolation (Mizisin et al., 1997; Kalichman et al., 1998; Murakawa et al., 2002). Furthermore, clearance of defective organelles is closely related to the autophagy process (Jung and Lee, 2010; Wang et al., 2011; Grimaldi et al., 2012). Autophagy is an important physiological process and a major defensive mechanism of the body under stresses such as nutrient or energy deprivation (Gonzalez et al., 2011). Autophagy can remove the damaged organelles, but also provide the materials for cell survival under stressful conditions. The Beclin1 gene (also called Becn1), is homologous to yeast Atg6/Vps30 and is involved in the initiation of autophagosome formation. The expression of Beclin-1 protein is positively correlated to the occurrence of autophagy, and is therefore used to monitor and determine autophagy dynamics when combined with other biochemical parameters (Yamahara et al., 2013). LC3 is the analog of the Atg8 gene product in yeast. LC3 precursor (ProLC3) was first processed to form soluble LC3-I, and then activated by Atg7 and modified by Atg3 to create the membrane binding form LC3-II. LC3-II is located on pre-autophagosomes and autophagosomes, and its abundance is positively proportional to the number of autophagic

vacuoles. Therefore, it serves as a molecular marker for autophagy (Klionsky et al., 2012).

Quercetin is widely present in traditional Chinese medicine and food, and is known to have antioxidant, anticancer, anti-inflammatory, antidiabetic, antihypertensive, neuroprotective and immunoregulatory effects (Olaleye et al., 2013; Bądziul et al., 2014; Liao and Lin, 2014; Milackova et al., 2014). Recently, it was reported that quercetin increased the proliferation of neurons or glial cells that were inhibited by high concentrations of glucose, and reduced oxidative stress-mediated damage (Shi et al., 2013; Wu et al., 2014). Quercetin was also implicated in the mechanism underlying the reduction of apoptosis through autophagy induction (Wang et al., 2011; Kim et al., 2013). Whether or not quercetin protects Schwann cells through autophagy pathways remains unclear.

Most cultured Schwann cells are primary cultures of peripheral nerve tissue from normal experimental animals. Primary cultured cells exhibit more accurate biological characteristics and fewer differences in proliferation and cytokine expression than cells from other sources (Qu et al., 2008). However, it is difficult to obtain primary Schwann cells because of the complex techniques involved, low cell yield, poor cell activity and high costs (Pan et al., 2005; Fu et al., 2012). RSC96 cells are an immortalized cell line derived from the long-term culture of rat primary Schwann cells. Although proteome differences have been found between primary Schwann cells and RSC96 cells that highlight several differentially expressed proteins with potential biological significance (Ji et al., 2012), RSC96 cells have been used in many studies of peripheral nerve injury and regeneration (Hai et al., 2002; Chang et al., 2011; Yin et al., 2012; Gui et al., 2013; Huang et al., 2014). However, whether RSC96 cells can be used as a substitute for primary rat Schwann cells in the study of autophagy is unclear. To the best of our knowledge, the differences in autophagy between primary Schwann cells and RSC96 cells have not been investigated under high glucose conditions.

In the present study, we observed the effects of quercetin on the morphology, proliferation and autophagy activity of primary Schwann cells and RSC96 cells cultured in high concentrations of glucose, with the aim of providing information for future studies on the relationship between autophagy and DPN and the search for effective therapies for DPN.

Materials and Methods

Animals
Male Sprague-Dawley rats, aged 3 days, were purchased from the Institute of Laboratory Animals of the Chinese Medical Science Academy, Beijing, China (license No. SCXK (Jing) 2005-0013). The study was approved by the Animal Ethics Committee of Peking Union Medical College Hospital in China.

Cells
RSC96 cells were purchased from the Cell Bank, Chinese Academy of Sciences, Shanghai, China.

Preparation of quercetin
Quercetin ($C_{15}H_{10}O_7$, molecular weight 302.23) was purchased from Sigma, St. Louis, MO, USA. Stock solution of quercetin was dissolved in deionized water at a concentration of 25 μmol/L and filtered through a 0.22 μm filter before use.

Primary culture of Schwann cells
Rats were sacrificed under anesthesia and bilateral sciatic nerves dissected out and subjected to enzymatic digestion. Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) was supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mmol/L L-glutamate, 1 mmol/L sodium pyruvate and 12.5 mmol/L hydroxyethyl piperazine ethanesulfonic acid (Sigma). Schwann cells were purified by differential speed adherence after 5 days and treated with G-418 (Sigma; 100 μg/mL) 7 days later.

Culture of RSC96 cells
RSC96 cells were revived. The medium was the same as that used for primary culture of Schwann cells and was changed once every 2 or 3 days.

Grouping
Six groups were designated as follows: primary Schwann cell cultures treated with DMEM (PC group), DMEM + 125 mmol/L glucose (PG group), or DMEM + 125 mmol/L glucose + 25 μmol/L quercetin (PQ group); RSC96 cells were treated with DMEM (RC group), DMEM + 125 mmol/L glucose (RG group), or DMEM + 125 mmol/L glucose + 25 μmol/L quercetin (RQ group).

Immunofluorescence identification of Schwann cells
Cells were seeded and incubated under each condition for 24 hours before being treated with acetone for 15 minutes at 4°C and 0.1% Triton-X 100 for 10 minutes at 4°C. Thereafter, they were treated with mouse anti-S-100 antibody (1:50 dilution; Boster, Wuhan, China) at 4°C overnight (PBS as negative control). Following PBS washes, the cells were incubated with FITC-conjugated Affinipure goat anti-mouse IgG (Beijing Xiya Jinqiao Biotechnology Co., Ltd., Beijing, China) at 37°C for 20 minutes and stained with fresh DAPI for 10 minutes. The fluorescent intensity of different groups of cells was analyzed using an Olympus FluoView FV 1000 (Olympus Corporation, Tokyo, Japan; excitation 364 nm, emission 488 nm for DAPI; excitation 488 nm, emission 525 nm for FITC).

Ultrastructure of Schwann cells
Primary Schwann cells and RSC96 cells were fixed in 2.5% glutaraldehyde, treated with 1% osmium tetroxide and embedded in Epon812 (Shell Chemical, Houston, TX, USA), and dehydrated with ethanol and acetone. Ultrathin sections (60–80 nm) were made and stained with uranyl acetate and lead citrate (Alfa Aesar (China), Shanghai, China). Transmission electron microscopy (JEM1010, JEOL (Beijing), Beijing, China) was used to observe the ultrastructure of Schwann cells.
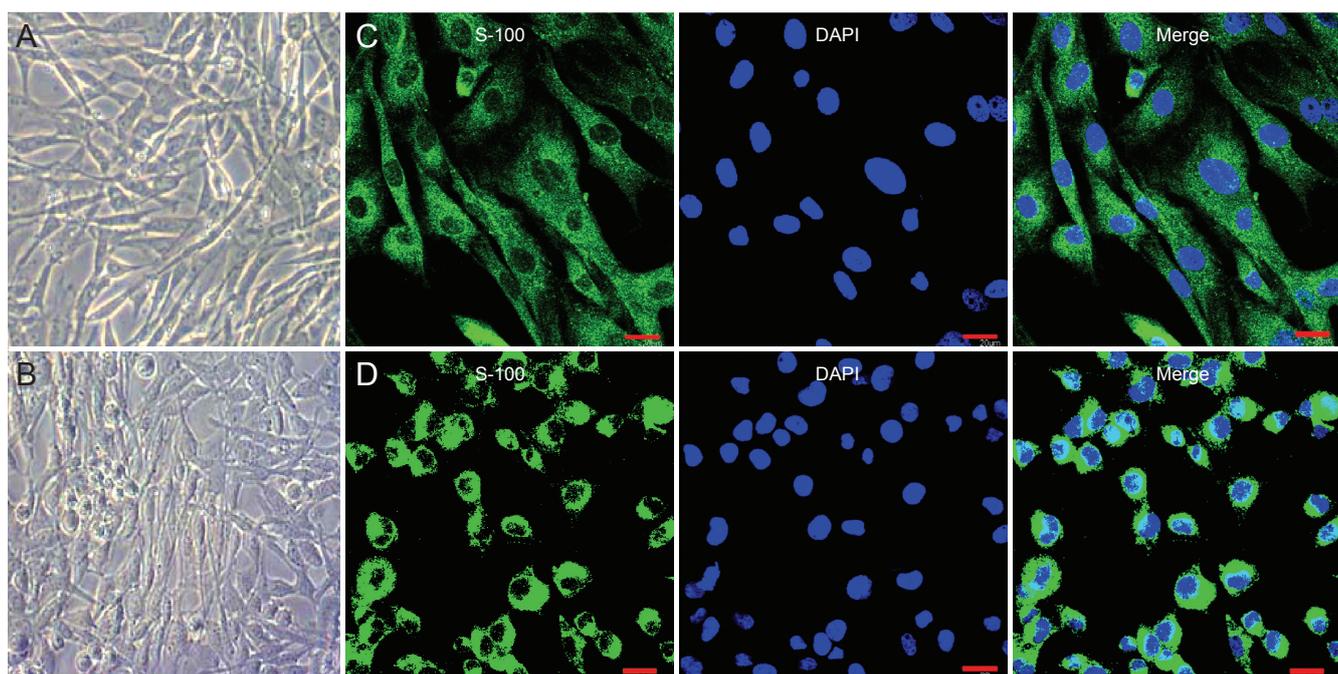
Figure 1 Identification and morphology of primary Schwann cells and RSC96 cells.
(A) Primary Schwann cells and (B) RSC96 cells under an inverted phase contrast microscope (× 200). (C) S-100 immunofluorescence in primary Schwann cells and (D) RSC96 cells. S-100 protein is labeled green and nuclei are labeled blue (DAPI). Scale bars: 20 μm. Cells were seeded and incubated for 24 hours. The last image in each panel is the merged one of the adjacent two images before it.
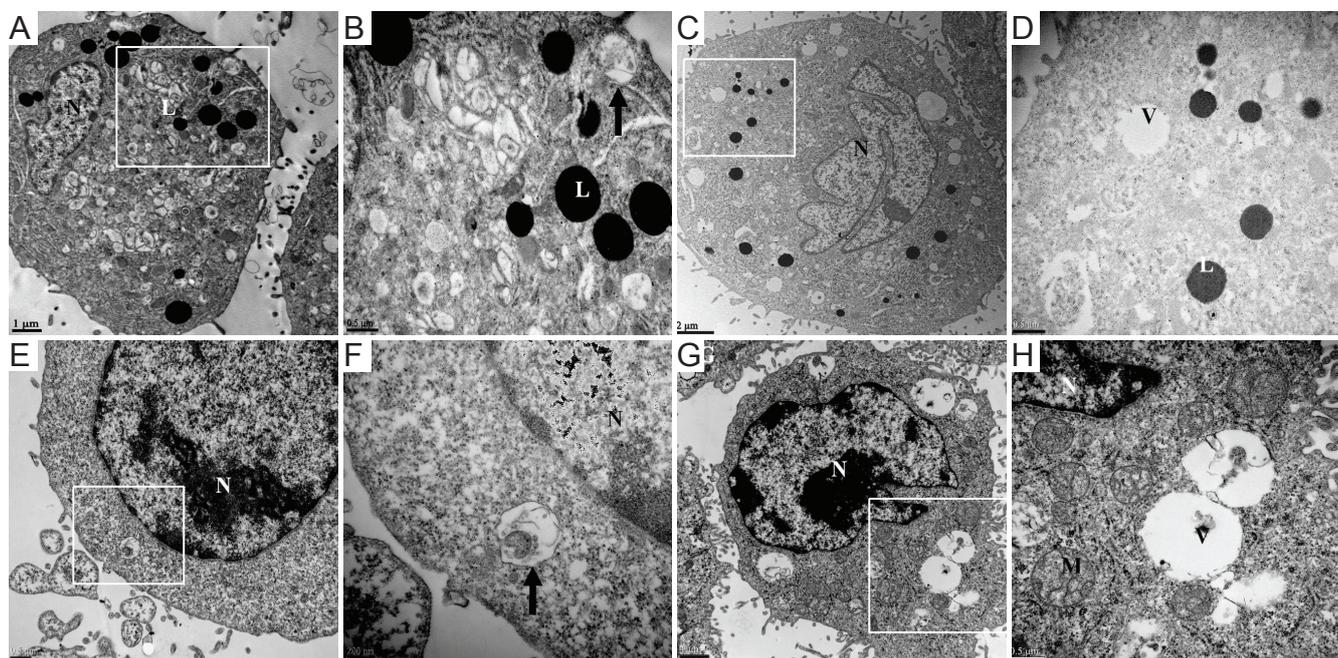
Figure 2 Effect of high glucose concentration on the ultrastructure of primary Schwann cells and RSC96 cells..
(A–D) Primary cultured Schwann cells treated with DMEM (PC (control) group; A, B) or DMEM + 125 mmol/L glucose (PG group; C, D). (E–H) RSC96 cells treated with DMEM (RC (control) group; E, F) or DMEM + 125 mmol/L glucose (RG group; G, H). In the PG and RG groups, the number of vacuoles is increased but autophagosomes are less visible, compared with respective controls. Boxed areas in A, C, E, G are magnified in B, D, F, H. Scale bars: A, 1 μm; B, 0.5 μm; C, 2 μm; D, 0.5 μm; E, 0.5 μm; F, 0.22 μm; G, 1 μm; H, 0.5 μm. L: Lysosome; M: mitochondrion; N: nucleus; V: vacuolar structure; ↑: autophagosome.

Determination of Schwann cell proliferation and viability by MTT assay

MTT (Sigma) was used to evaluate the proliferation of both types of Schwann cells after seeding. 200 μL of 10% MTT was added to each well for 2–4 hours at 37°C and mixed with 200 μL of dimethyl sulfoxide for 30–60 minutes. Absorbance values were measured at 570 nm by multi-mode readers (BioTek Instruments Inc, Vermont, USA). Five wells
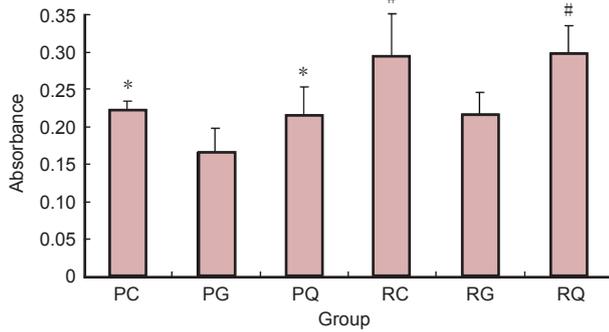
Figure 3 Effect of quercetin on the proliferative ability of primary Schwann cells and RSC96 cells.

Proliferative activity was detected by MTT assay. Data are expressed as mean ± SD and analyzed by one-way analysis of variance and the least significant difference test. *P < 0.05, *vs.* PG group; #P < 0.05, *vs.* RG group. Five wells from each group were selected for analysis. The test was performed twice. PC: Primary cultured Schwann cells treated with Dulbecco's modified Eagle's medium (DMEM); PG: primary cultured Schwann cells treated with DMEM + 125 mmol/L glucose; PQ: primary cultured Schwann cells treated with DMEM + 125 mmol/L glucose + 25 μmol/L quercetin; RC: RSC96 cells treated with DMEM; RG: RSC96 cells treated with DMEM + 125 mmol/L glucose; RQ: RSC96 cells treated with DMEM + 125 mmol/L glucose + 25 μmol/L quercetin.

from each group were selected for detection. The test was performed twice.

Determination of Beclin-1 and LC3 expression by immunofluorescence staining

Schwann cells were seeded at 2×10^5/mL on poly-l-ly-sine-coated coverslips. When they reached 70% confluence, the cells were starved overnight and observed in duplicate under the three conditions described above (control, or high glucose with or without quercetin). The cells were treated with 5% bovine serum albumin, followed by primary antibody (dilution 1:100; rabbit anti-Beclin-1 antibody and rabbit anti-LC3 A/B antibody; Abcam, Cambridge, UK) at 4°C overnight. PBS was used in place of primary antibody as the negative control.

Following PBS washes, the cells were incubated with secondary antibody (TRITC-conjugated Affinipure goat anti-rabbit IgG; Beijing Xiya Jinqiao Biotechnology Co., Ltd.) at 37°C for 20 minutes, and stained with fresh DAPI for 10 minutes. The fluorescent intensity of different groups of cells was analyzed using an Olympus FluoView FV 1000 (Olympus Corporation; excitation 364 nm, emission 488 nm for DAPI; excitation 547 nm, emission 620 nm for TRITC). Average absorbance values of each cell were measured by FV10-ASW 3.0 analysis software (Olympus Corporation). Five fields of view were randomly chosen from each group for analysis. The experiment was performed twice.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). One-sample Kolmogorov-Smirnov Z test was used to determine whether the data fit the normal distribution before analysis. Normally-distributed data were expressed as mean ± standard deviation. The inter-group differences were compared by one-way analysis of variance and the least significant difference test. A level of *P* < 0.05 was considered statistically significant.

Results

Identification and morphology of primary Schwann cells and RSC96 cells

Primary Schwann cells were long and thin, spindle-shaped or bipolar-like, with bright edges. Their nuclei were oval or spindle-shaped and contained little nucleoplasm. The cells appeared interconnected and fasciculated (**Figure 1A**). RSC96 cells were also oval, spindle-shaped or bipolar-like, but smaller than primary Schwann cells. Their nuclei were oval and full (**Figure 1B**). Both primary Schwann cells and RSC96 cells were stained green by the marker S-100 (**Figure 1C, D**).

Effect of high concentrations of glucose on the ultrastructure of primary Schwann cells and RSC96 cells

In the PC group (**Figure 2A, B**), there were a few microvilli on the surface of the primary Schwann cells. Nuclei were ovoid and located to one side of the cells. Mitochondria, autophagosomes and other organelles were identifiable. In the PG group (**Figure 2C, D**), Schwann cells were of different sizes. Some had lobulated nuclei. Mitochondria and numerous lysosomes were seen in the cell matrix. Vacuolar structures were seen, but not autophagosomes. In the RC group (**Figure 2E, F**), cells and their nuclei were ovoid and possessed distinct nucleoli and uniform chromatin. Cellular organelles including mitochondria, autophagosomes and autolysosomes were visible. In the RG group (**Figure 2G, H**), most nucleoli were distinct, and chromatin was less uniform. Mitochondria were swollen with an increased number of vacuoles and autophagosomes, and autolysosomes were less visible, compared with the other groups.

Effect of quercetin on the viability of primary Schwann cells and RSC96 cells

MTT assay showed that at 72 hours, proliferative ability of cells in the PG and RG groups was significantly lower than that in the PC and RC groups, respectively (*P* < 0.05). However, in the PQ and RQ groups, proliferative ability was significantly greater than that in the PG and RG groups, respectively (*P* < 0.05), and not significantly different from their respective controls (*P* > 0.05; **Figure 3**).

Effect of quercetin on Beclin-1 expression in primary Schwann cells and RSC96 cells

Immunofluorescent analysis showed that in the PC and RC groups, Beclin-1 expression was patchy in distribution. In the PG and RG groups, Beclin-1 expression was noticeably weaker than in the control groups, whereas the signal in the PQ and RQ groups was stronger than that in the high glucose groups (**Figure 4A**).

Semi-quantitative analysis (**Figure 4B**) revealed that expression levels of Beclin-1 in the PG and RG groups were
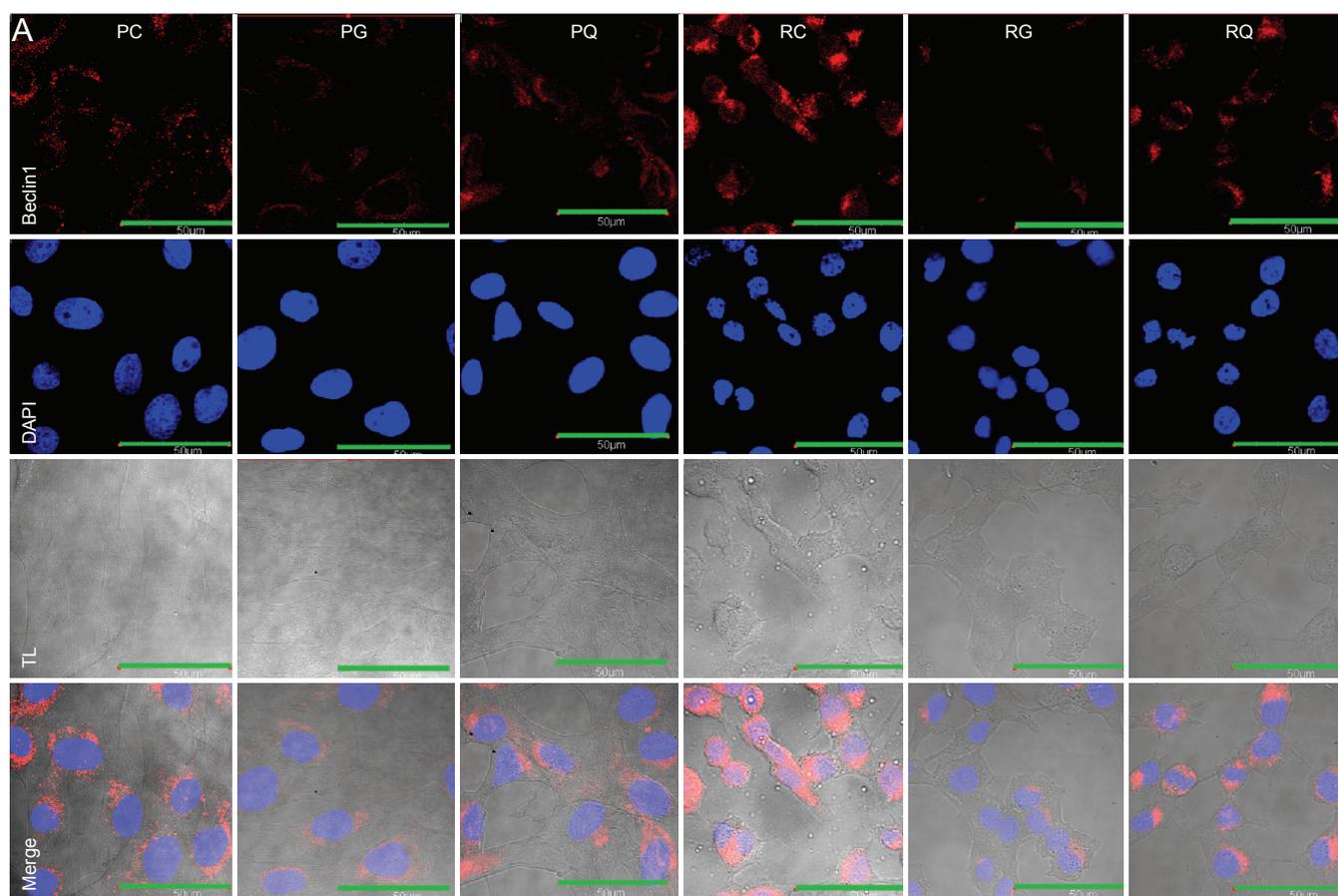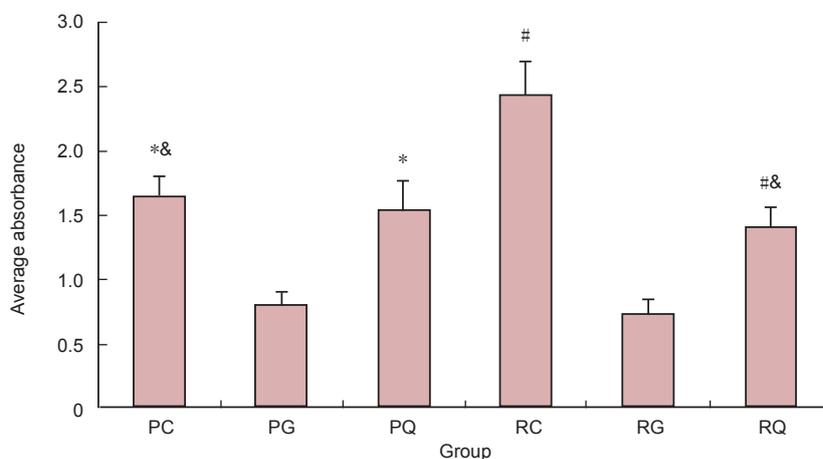
Figure 4 Effect of quercetin on Beclin-1 expression in primary Schwann cells and RSC96 cells.
(A) Immunofluorescent analysis. Beclin-1 protein (red) and nuclei (blue) in primary Schwann cells and RSC96 cells. Scale bars: 50 μm. (B) Semi-quantitative analysis. Data are expressed as mean ± SD and analyzed by one-way analysis of variance and the least significant difference test. *$P < 0.01$, *vs.* PG; #$P < 0.01$, *vs.* RG; &$P < 0.01$, *vs.* RC. The experiment was performed twice. PC: Primary cultured Schwann cells treated with Dulbecco's modified Eagle's medium (DMEM); PG: primary cultured Schwann cells treated with DMEM + 125 mmol/L glucose; PQ: primary cultured Schwann cells treated with DMEM + 125 mmol/L glucose + 25 μmol/L quercetin; RC: RSC96 cells treated with DMEM; RG: RSC96 cells treated with DMEM + 125 mmol/L glucose; RQ: RSC96 cells treated with DMEM + 125 mmol/L glucose + 25 μmol/L quercetin.

significantly lower than those in the PC and RC groups ($P < 0.01$), while the expression of Beclin-1 in the PQ and RQ groups was significantly higher than in the PG and RG groups, respectively ($P < 0.01$), with no difference detected between the control and quercetin-treated groups in either cell type ($P > 0.05$). The results indicate that Beclin-1 expression is similar in the two kinds of Schwann cells cultured with high glucose and quercetin.

Expression levels of Beclin-1 in the RC group were significantly greater than in the PC group ($P < 0.01$; **Figure 4B**), but no differences in Beclin-1 expression were observed between the two cell types under test conditions (RG or RQ *vs.*
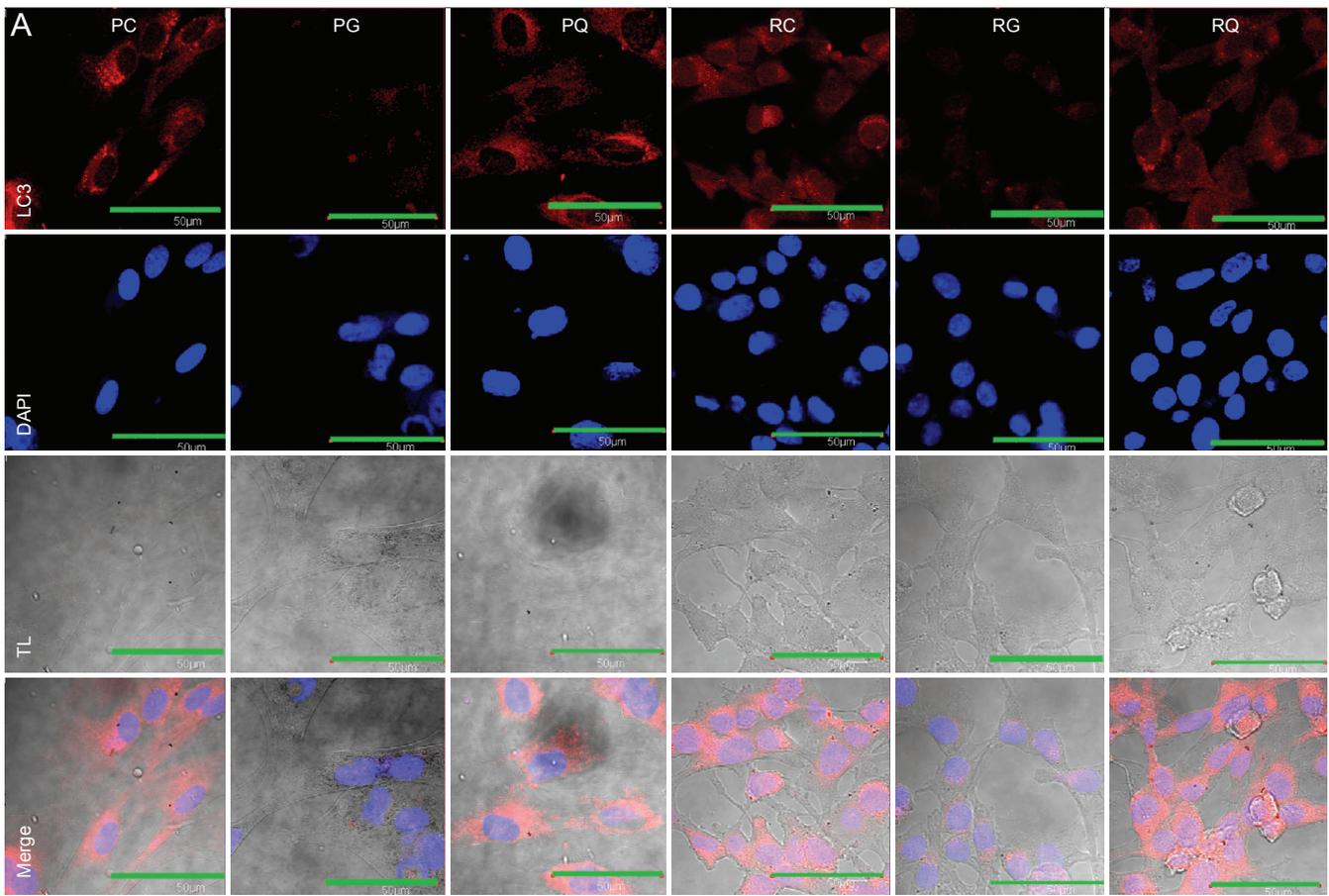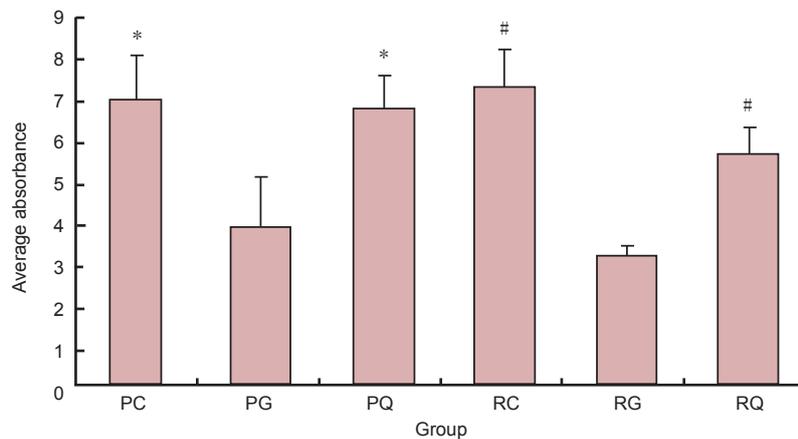
Figure 5 Effect of quercetin on LC3 expression in primary Schwann cells and RSC96 cells.
(A) Immunofluorescent analysis. LC3 protein (red) and nuclei (blue) in primary Schwann cells and RSC96 cells. Scale bars: 50 μm. (B) Semi-quantitative analysis. Data are expressed as mean ± SD and analyzed by one-way analysis of variance and the least significant difference test. *$P < 0.05$, vs. PG; #$P < 0.01$, vs. RG. The experiment was performed twice. PC: Primary cultured Schwann cells treated with Dulbecco's modified Eagle's medium (DMEM); PG: primary cultured Schwann cells treated with DMEM + 125 mmol/L glucose; PQ: primary cultured Schwann cells treated with DMEM + 125 mmol/L glucose + 25 μmol/L quercetin; RC: RSC96 cells treated with DMEM; RG: RSC96 cells treated with DMEM + 125 mmol/L glucose; RQ: RSC96 cells treated with DMEM + 125 mmol/L glucose + 25 μmol/L quercetin.

PG or PQ, respectively).

Effect of quercetin on LC3 expression in primary Schwann cells and RSC96 cells

Immunofluorescent analysis revealed that in the PC and RC groups, LC3 expression was patchy in distribution. In the PG and RG groups, LC3 expression was significantly weaker than in the corresponding control groups, while the signal in the PQ and RQ groups was significantly stronger than in the respective high glucose groups (**Figure 5A**).

Semi-quantitative analysis of LC3 expression (**Figure 5B**) in the same cell types under different conditions showed that

the expression level of LC3 in the PG group was significantly lower than that in the PC and PQ groups ($P < 0.05$), with no significant difference between the PC and PQ groups ($P > 0.05$). The expression level of LC3 in the RG group was significantly lower than that in the RC group ($P < 0.01$), and LC3 expression in the RQ group was significantly higher than that in the RG group ($P < 0.01$), but there was no significant difference between LC3 expression in the RQ and RC groups ($P > 0.05$). LC3 expression levels were not significantly different between cell types under any condition ($P > 0.05$; **Figure 5B**).

Discussion

Schwann cell dysfunction caused by diabetes mellitus leads to reduced regeneration and repair capability in peripheral nerves (Eckersley, 2002; Kennedy et al., 2005). It is believed that demyelination of nerve fibers may be caused by metabolic disorders in Schwann cells. *In vivo* studies revealed myelin breakdown, lamella disorganization, demyelination, and swollen Schwann cell mitochondria in the sciatic nerve tissues of diabetic rats (Chopra et al., 1969; Bestetti et al., 1981; Zemp et al., 1981; Mizisin and Powell, 1997; Kalichman et al., 1998; Murakawa et al., 2002; Shi et al., 2013). The damage to cell ultrastructure seen in the presence of high concentrations of glucose correlates with that observed with cellular metabolic disorders (Kalichman et al., 1998; Ishibashi et al., 2002; Liu et al., 2013; Prasath and Subramanian, 2013). Energy metabolism disorders induce autophagy pathways to degrade self-phospholipids and cytoplasmic proteins to maintain energy supply and anabolism (Uchiyama et al., 2008; Yamahara et al., 2013; Fierabracci, 2014; Mizukami et al., 2014).

In the present study, we found that double-membrane autophagosomes 300–900 nm in diameter (average 500 nm) were seen in both RSC96 cells and primary Schwann cells in the basic medium. However, in the presence of high concentrations of glucose, these two kinds of Schwann cells were irregular in shape and had swollen organelles and nuclei, and many vacuoles. Most of the vacuoles were 1,000 nm in diameter and did not have the double membrane structure, differing from autophagosomes described in the literature (Marsh et al., 2007; Ebato et al., 2008; Masini et al., 2009; Fujimoto et al., 2009; Klionsky et al., 2012). Furthermore, we also found that protein expression of Beclin-1 and LC3, used to monitor autophagy (Klionsky et al., 2012; Yamahara et al., 2013), was downregulated under high glucose conditions. Together, our results show that high concentration glucose suppresses autophagy in Schwann cells.

We also found that quercetin not only protected the proliferative activity of RSC96 cells and primary Schwann cells from high glucose-mediated damage, but also upregulated protein expression levels of Beclin-1 and LC3. Quercetin is widely used in traditional Chinese medicine and food and can reduce the inhibition of nerve cell proliferation caused by high concentrations of glucose. Reductions in oxidative stress-mediated damage and apoptosis have also been shown by autophagy induction (Wang et al., 2011; Kim et al., 2013; Shi et al., 2013; Wu et al., 2014). On the basis of these results,

we postulated that quercetin alleviates the impairment of proliferation induced by high glucose, and that this might correlate with autophagy in Schwann cells. We therefore compared the morphology of RSC96 cells and primary rat Schwann cells, and the effects of quercetin on the proliferative and autophagy ability in these two cell types when cultured in high glucose medium. We found that the morphology of both kinds of Schwann cell was similar under optical and transmission electron microscopes, and stained positive for the marker S-100. We also found that RSC96 cells and primary cultured rat sciatic nerve Schwann cells are shaped differently according to protein expression. Furthermore, changes in ultrastructure, proliferative activity and autophagy were similar between cell types in high glucose and after quercetin treatment. These results indicate that RSC96 cells could be used as a substitute for primary rat Schwann cells in studies of autophagy.

In summary, the present study reports that quercetin increases proliferation and upregulates autophagy in two different cell models in the presence of high concentrations of glucose. RSC96 cells and primary rat Schwann cells were similar both in morphology and in their responses to high glucose and quercetin. The use of RSC96 cells would avoid problems frequently arising in primary culture, such as a long cycle, complicated technique and limited cell yield. RSC96 cells could replace primary Schwann cells in studies of the role of autophagy in the mechanism underlying DPN and related drug screening models. In future studies, we will address the regulation of autophagy under high glucose conditions and evaluate the differences in biological activity between RSC96 cells and primary Schwann cells in the field of autophagy.

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