Lipin1 Alleviates Autophagy Disorder in Sciatic Nerve and Improves Diabetic Peripheral Neuropathy

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Received: 24 April 2021 / Accepted: 17 August 2021 / Published online: 26 August 2021
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Abstract
Diabetic peripheral neuropathy (DPN) is a chronic complication of diabetes, and its neural mechanisms underlying the pathogenesis remain unclear. Autophagy plays an important role in neurodegenerative diseases and nerve tissue injury. Lipin1 is a phosphatidic acid phosphatase enzyme that converts phosphatidic acid (PA) into diacylglycerol (DAG), a precursor of triacylglycerol and phospholipids which plays an important role in maintaining normal peripheral nerve conduction function. However, whether Lipin1 involved in the pathogenesis of DPN via regulation of autophagy is not elucidated. Here, we show that the Lipin1 expression was downregulated in streptozotocin (STZ)-induced DPN rat model. Interestingly, STZ prevented DAG synthesis, and resulted in autophagic hyperactivity, effects which may increase the apoptosis of Schwann cells and lead to demyelination in sciatic nerve in DPN rats. More importantly, upregulation of lipin1 in the DPN rats ameliorated autophagy disorders and pathological changes of the sciatic nerve, which associated with the increase of the motor nerve conductive velocity (MNCV) in DPN rats. In contrast, knockdown of lipin1 exacerbates neuronal abnormalities and facilitates the genesis of DPN phenotypes in rats. In addition, overexpression of lipin1 in RSC96 cells also significantly decreased the autophagic hyperactivity and apoptosis induced by hyperglycemia. These results suggest that lipin1 may exert neuroprotection within the sciatic nerve anomalies and may serve as a potential therapeutic target for the treatment of DPN.

Keywords Lipin1 · Diabetic peripheral neuropathy · Autophagy · Sciatic nerve · RCS96 cell

Abbreviations

7-AAD 7-Aminoactinomycin D
ADV Adenovirus
ADVs Adenoviral vectors
BCA Bicinchoninic acid
CCK-8 Cell counting kit-8
DAG Diacylglycerol
DAPI 4′,6-Diamidine-2-phenyldirole dihydrochloride
DM Diabetes mellitus
DMEM Dulbecco’s modified Eagle’s medium
DPN Diabetic peripheral neuropathy
ELISA Enzyme-linked immunosorbent assay
EM Electron microscopy
FBS Fetal bovine serum
FBG Fasting-blood-glucose
FITC Fluorescein isothiocyanate
LV Lentivirus
LVs Lentiviral vectors
MNCV Motor nerve conduction velocity
MOI Multiplicity of infection
NC  Negative control
PA  Phosphatidic acid
PAP  Phosphatidic acid phosphatase
PBS  Phosphate-buffered saline
PKD  Protein kinase D
PMWT  Paw mechanical withdrawal threshold
PVDF  Polyvinylidene fluoride
qPCR  Quantitative real-time polymerase chain reaction
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STZ  Streptozotocin
TBS-T  Tris-buffered saline Tween

Introduction

With the development of economy and the progress of society, the incidence of diabetes is increasing worldwide [1, 2]. The incidence of chronic complications of diabetes increases with prolonged human life and improved medical conditions [3, 4]. Diabetic peripheral neuropathy (DPN) is a chronic complication of diabetes that is knotty and ineffective in clinical treatment [5]. Patients may have spontaneous pain, hyperalgesia, acupuncture-like pain, and other symptoms, which may lead to diabetic foot ulcer, gangrene, or amputation. This condition severely affects the quality of life of patients [6, 7]. At present, the mechanisms underlying the pathogenesis of DPN remains unclear [8, 9]. Therefore, no specific etiology treatment has been established up to now, which made the underlying mechanisms and thus the potential for development of corresponding therapeutic measures of DPN was eager to be investigated in the medical field.

Autophagy plays an important role in neurodegenerative diseases, ischemia–reperfusion injury of nerve tissues, and repair of nerve tissue injury. In patients with DPN, autophagy disorder occurs in nerve tissues due to long-term hypoxia, lack of nerve factors, and oxidative stress [10]. In 1966, Duve first reported that the number of autophagy lysosomes in neurons was increased under various conditions, such as axonal injury, toxic exposure, and genetic degenerative disease [11, 12]. Wang et al. confirmed that autophagy is induced in mouse Purkinje cells during axonal atrophy and degeneration; hence, autophagy dysfunction may be one of the potential mechanisms of axonal diseases [13]. Previous study has reported that p62 localizes in membrane-confined autophagosomal and lysosomal bodies within the brain and p62 knockout mice exhibit a phenotype of neurodegeneration [14]. However, the relationship between autophagy and peripheral nerve injury, especially in diabetic peripheral neuropathy, has been rarely studied.

Lipin1 is an enzyme closely related to glycolipid metabolism produced by the expression of LIPIN [15]. Previous study showed that Lipin1 played an important role in maintaining normal peripheral nerve conduction function [16]. In our previous study, we found that cognitive impairments were present in Lipin1+/− mice via DAG-PKD-ERK pathway [17]. Furthermore, the expression of lipin1 in the hippocampal CA1 region was also decreased, while upregulation of lipin1 expression could improve diabetic encephalopathy in rats [18]. However, whether Lipin1 influences peripheral nerve function in DPN by regulating autophagy remains unclear. Therefore, in the present study, we constructed DPN rat models and explored the mechanism of Lipin1 in the pathogenesis of diabetic autophagy disorder.

Materials and Methods

Animals

Adult male Wistar rats, weighing 150–185 g (or 5–6 weeks-old), obtained from the Experimental Animal Center of Shandong University, were used throughout the study. All rats were housed in a temperature and humidity-controlled environment under a 12-h light/dark cycle with free access to water and a standard rodent chow diet. For handling and care of animals, all procedures were performed according to the International Guiding Principles for Animal Research provided by the World Health Organization.

Cells

For the in vitro diabetes model experiments, the RSC96 (rat Schwann cell) cells (CRL-2765) were obtained from the American Type Culture Collection (ATCC, NO. CRL-2765) and were cultured in DMEM modified to contain 4 mM L-glutamine, 25 mM glucose, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, 10% FBS, 100 IU penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2 and were passaged once every 3–4 days.

Virus Packaging

Lipin1 overexpression and knock-down adenovirus (pADV5-mCMV-MCS-3xFLAG) and lentivirus (pLenti-CMV-EGFP-3xFLAG-WPRE) were purchased from GeneChem, Shanghai, China. Adenovirus and lentivirus were carried eGFP. The Lipin1 overexpression and knock-down adenovirus (ADV-Lipin1/ ADV-Lipin1-RNAi) and corresponding adenovirus empty shell control (ADV-Lipin1-Con/ADV-Lipin1-RNAi-Con) were separately packaged for in vivo experiments. Meanwhile, the Lipin1 overexpression and knock-down Lentivirus (LV-Lipin1/ LV-Lipin1-RNAi) and the corresponding Lentivirus empty shell control (LV-Lipin1-Con/LV-Lipin1-RNAi-Con) were
packaged for in vitro experiments. The titers of ADV-Lipin1, ADV-Lipin1-RNAi, ADV-Lipin1-Con, and ADV-Lipin1-RNAi-Con were $4.0 \times 10^9$PFU/ml, $1.5 \times 10^9$PFU/ml, $5.0 \times 10^9$PFU/ml, and $1.0 \times 10^9$PFU/ml separately. The titers of LV-Lipin1, LV-Lipin1-RNAi, LV-Lipin1-Con, and LV-Lipin1-RNAi-Con were $1.0 \times 10^9$TU/ml, $7 \times 10^8$TU/ml, $2.0 \times 10^8$TU/ml, and $1.0 \times 10^9$ T U/ml separately. The transfection and expression efficiency of both overexpression and knock-down viruses were up to standard. The sequence of Lipin1-RNAi was caGCGAGTCCTCAGACAACCTTTT. The sequence of Lipin1-RNAi-Con was TTCTCCGAACGT GTCACG.

**Animal Model of DPN and Virus Intrathecal Injection**

Rats were first divided into negative control (NC) and diabetes mellitus (DM) group. All rats were subjected to adaptive feeding for one week in the rat room. After 1 week, DM was induced by intraperitoneal injection of streptozotocin (STZ, 55 mg/kg in 0.1 M citric acid buffer, pH 4.5) [19]. STZ is a highly selective pancreatic islet β cell-cytotoxic agent which could cause complete necrosis of β-cell within 48 h and thus induced hyperglycemia in rats [20]. In the present study, the rats with a fasting blood glucose level above 16.7 mM 3 days after STZ injection were considered diabetic and were continued feeding for 8 weeks. Fasting blood glucose levels were measured 3-day, 1-week, 2-week, 4-week, 6-week, and 8-week after STZ injection to monitor the persistence of diabetes and paw mechanical withdrawal threshold (PMWT) were measured every 4 weeks to track the occurrence of peripheral neuropathy.

Eight weeks after STZ injected, DM rats were divided into two groups, Lipin1 overexpression group (ADV-Lipin1) and adenovirus empty shell group (ADV-Lipin1-Con). Meanwhile, NC rats were also divided into two groups, Lipin1 knock-down group (ADV-Lipin1-RNAi) and adenovirus empty shell group (ADV-Lipin1-RNAi-Con). Rats were anesthetized with sodium pentobarbital (40 mg/kg), and then ADV was intrathecally injected. Briefly, the lumbar hair of rats was shaved to expose the skin, and then disinfected with 75% alcohol. The rats were punctured into the subarachnoid space between the lumbar 3–4 or lumbar 4–5 with a special 25ul micro syringe (Feige, Nanjing, China) [21, 22]. The tail flick reflex of the rats was used as a sign of the micro syringe entering the sheath. The ADV-Lipin1-RNAi and empty vector (20ul per rat) was then injected into NC rats (ADV-Lipin1-RNAi and ADV-Lipin1-RNAi-Con groups). The ADV-Lipin1 and empty vector was injected into DM rats (ADV-Lipin1 and ADV-Lipin1-Con groups). The virus was injected at a flow rate of 0.5ul/min. The micro-injection needle remained in the injection site for at least 2 min after infusion and was then slowly withdrawn. Rats were disinfected and moved into the ordinary feeding box after recovery from surgery in the incubator. Behavioral tests or biochemical assays were performed after 2 weeks of intrathecal injection.

**Cell Transfection**

RSC96 cells plated at a density of $5.0 \times 10^4$cells/well in 6-well plates and were allowed to adhere overnight. The cells then transfected with four kinds of lentivirus according to the manufacturer’s instructions. According to the MOI value of RSC96 cells, the corresponding virus amount was added to the cultured cells (Virus volume = (MOI×cell number) / virus titer). The culture solution was changed after 12 h to keep cell viability during 2 days. Seventy-two hours after infection, cells were aspirated and cultured in 25 mM or 100 mM glucose growth medium for 48 h respectively. Lipin1 knock-down LV (LV-Lipin1-RNAi) and empty shell control (LV-Lipin1-RNAi-Con) group were treated with 25 mM glucose, while Lipin1 overexpression LV (LV-Lipin1) and empty shell control (LV-Lipin1-Con) group were treated with 100 mM glucose.

**Measurement of Paw Mechanical Withdrawal Threshold (PMWT)**

In a quiet environment, rats were placed in a transparent plexiglass cover with a mesh pad made of metal wire at the bottom. After adapting to the environment for 15 min until they were in a quiet state, the rats’ PMWT was measured with the acupuncture pain test kit (vonfrey, Aesthesio, damnic Global, USA, measuring range of 0.008–300 g stimulation) [23]. The skin between the third and fourth toes of the rats was pressed vertically with nylon wires using different stimulating forces (the nylon wires were bent each time). When the rats exhibited symptoms of rapid retraction, hind foot lifting, quick swing, licking, and hissing after shaking their feet, the pressure was stopped. Then the stimulation force was taken as the PMWT. Each trial was repeated 3 times and the interval was more than 5 min. Each trial included both feet and the average value of both feet in each trial was taken as the PMWT.

**Measurement Motor Nerve Conduction Velocity (MNCV) of Sciatic Nerve**

Nerve conduction velocity was assessed using Functional Experiment System (BL-420 s, Techman, China) as reported previously. Rats were anesthetized with isoflurane, then the left lower leg hair was shaved to expose the skin. The skin of the sciatic node and the passing part of the sciatic nerve of the ankle joint were cut, then the sciatic nerve was carefully separated [24]. The stimulation electrode (S1) is located at the ischial notch, and the recording electrode (S2)
is located at the ischial nerve passing through the ipsilateral ankle joint. The reference electrode (E) is located between S1 and S2, 1 cm away from S2 at least. Sciatic nerve was stimulated with single square wave pulses (1.2 V in intensity, 1 ms in width), and a biphasic compound action potential was recorded from S2. The stimulus was repeated 5 times with an interval of more than 3 min. MNCV (m/s) = sciatic nerve length (between S1 and S2) / nerve conduction time.

**Electron Microscopy (EM) Analysis**

Electron microscopy (EM) analysis was performed to assess the ultrastructure of sciatic nerve and RSC96 cell. The tissues or cell sedimentation were placed in 2.5% glutaraldehyde at 4 °C for 24 h, followed by fixation with 1% osmium tetroxide for 2 h. After a series of graded ethanol dehydrations, the tissues or cell sedimentation were infiltrated with a mixture of one-half propylene oxide overnight and embedded in resin. The tissues or cell sedimentation were then cut into ultrathin Sects. (70 nm) and stained with 4% uranyl acetate for 20 min followed by 0.5% lead citrate for 5 min. Sciatic nerve and RSC96 cell ultrastructure were observed using EM (Philips Tecnai 20 U-Twin, Holland).

**Quantitative Real-Time PCR (qPCR)**

Total RNA was isolated and extracted from sciatic nerve and RSC96 cell samples using the Trizol Reagent (Invitrogen, USA). Total RNA (1 μg) was reverse-transcribed into cDNA using the PrimeScript™ RT reagent Kit with a gDNA Eraser (TaKaRa, Japan) according to the manufacturer’s instructions. The reverse transcription reaction was amplified using a Bio-Rad CFX96 Detection System (Bio-Rad, USA). Quantitative Real-time PCR was performed with use of the ChamQ SYBR qPCR Master Mix (TaKaRa, Japan) on the Bio-rad IQ5 Real Time PCR System (Bio-Rad, USA). Reaction conditions were as follows: 95°C for 2 min and 40 cycles of the amplification step (denaturation at 95°C for 5 s, annealing at 55°C for 5 s, and extension at 72°C for 25 s). The following primers were used for qPCR: Lipin1-forward TATGACACGGCTTTTCC; reverse GTGGCT GCCCTGTATTTCC; β-actin-forward CCTAGACTTCGA GCAAGAGA; reverse GGAAGAAAGGCTGGAAGA. β-Actin served as a loading control in each sample, and targeted gene expression levels were evaluated using the 2−ΔΔCt method [25].

**Western Blot**

Sciatic nerves and RSC96 cells were lysed on ice in RIPA buffer with protease inhibitor cocktail and phosphatase inhibitor cocktail for 30 min, and then centrifuged at 1000×g for 15 min at 4°C to extract total protein. The proteins were analyzed with a bicinchoninic acid (BCA) protein assay kit (Pierce Bio-technology, Inc., US). Equal amounts of protein (30 μg) were subjected to SDS-PAGE analysis. The resolved proteins were transferred to PVDF membranes (Millipore, Bedford, MA). PVDF membranes were blocked in 5% nonfat milk for 1 h and then incubated overnight at 4 °C with the appropriate primary antibodies. The primary antibodies were as follows: rabbit anti-Lipin1 (1:250, Cell Signaling Technology, Beverly, MA), rabbit anti-P62 (1:500, Cell Signaling Technology, Beverly, MA), rabbit anti-LC3B (1:500, Cell Signaling Technology, Beverly, MA), rabbit anti-β-actin (1:5000, Cell Signaling Technology, Beverly, MA). After washing in TBS-T, blots were exposed to the appropriate secondary antibodies (1:5000, Abcam Co., UK) in TBS-T for 1 h at room temperature. Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA01821, US) was used to form image. Blots were developed using ChemiDocTM Imager from Bio-Rad. The protein bands were quantitated with Image J software and normalized to β-actin.

**Measurement of Diacylglycerol (DAG)**

An enzyme-linked immunosorbent assay (ELISA) kit (IL20977-96 T, Shanghai Jianglai industrial Limited by Share Ltd, China) was used to measure rat diacylglycerol (DAG) according to the manufacturer’s instructions. The sciatic nerves were rinsed by precooling PBS to remove the residual blood. After weighing, the tissues were cut into pieces. The cut tissues and PBS of the corresponding volume (1 mg: 9ul) were added into the glass homogenizer and fully ground on ice. If necessary, ultrasonic breaking or repeated freezing and thawing were carried out. After centrifugation at 5000 rpm for 5 min, the supernatant was taken for test.

**Apoptosis Assay**

An Annexin-FITC Apoptosis Detection Kit (556547, BD Biosciences, USA) was used to examine apoptosis according to the manufacturer’s instructions. In brief, cells were added with 300μL binding buffer followed by staining with 5μL of FITC-labeled annexin V and 5μL of propidium iodide (PI) and incubated at room temperature for 20 min in the dark. After lentivirus transfection, an Annexin-APC Apoptosis Detection Kit (E-CK-A218, Elabscience, China) was used to examine apoptosis of RSC96 cells according to the manufacturer’s instructions. Cells were added to 300μL of binding buffer followed by staining with 5μL of APC-labeled annexin V and 5μL of 7-Aminoactinomycin (7-AAD) and incubated at room temperature for 20 min in the dark. BD LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA, USA) was used for analysis.
Cell Counting Kit-8 (CCK8)

Cell Counting Kit-8 (CK04-500 T, Dojindo, Japan) was used to examine the activity of RSC96 cells according to the manufacturer’s instructions. RSC96 cell suspension (100ul/well, about 3000–5000 cells/well) was inoculated in 96-well plate and cultured for 48 h (37 °C, 5% CO2). Then, 10ul CCK-8 solution was added to each hole (be careful not to generate bubbles in the hole, they will affect the reading of O.D value), followed by incubating for 2–4 h. The absorbance at 450 nm was determined by enzyme scale.

Statistical Analysis

Data were expressed as means ± SEM. Statistical analysis was performed by two-tailed Student’s t-test or one-way ANOVA using SPSS 17.0. A p value < 0.05 was considered statistically significant.

Results

Decreased Lipin1 Expression Was Accompanied with Dysfunction of Motor Never in DPN Rats

To firstly investigated whether the Lipin1 expression would alter in DPN model, we intraperitoneally injected STZ (55 mg/kg) to induce DPN in the rat. The results showed that from the third day after STZ injection, the body weight of rats was significantly decreased (P < 0.01), and the fasting blood glucose (FBG) levels was significantly increased (P < 0.01) in the DPN group as compared with that of the negative control (NC) group (Fig. 1A). The motor nerve conductive velocity (MNCV) of the sciatic nerve and paw mechanical withdrawal threshold (PMWT) of DPN rats decreased compared with that of NC group (P < 0.01, respectively) (Fig. 1B, C). The mRNA (P < 0.05, Fig. 1D) expression levels of Lipin1 within the sciatic nerve of DPN group was significantly decreased. Meanwhile, the levels of diacylglycerol (DAG) were decreased within the sciatic nerve of DPN group as compared with that of NC group (P < 0.01, Fig. 1E). Moreover, sciatic nerve showed degenerative and demyelinating changes in DPN rats. Results from the transmission electron microscope showed that the demyelination of the sciatic nerve was occurred as well as the lamella was obviously loose and broken (Fig. 1F). Furthermore, the protein (P < 0.01, Fig. 1G) expression levels of Lipin1 in the sciatic nerve of DPN group was also significantly decreased than that of NC group, which consistent with our previous study [26]. In addition, the expression of the autophagy-related protein P62 was decreased (P < 0.01) in the sciatic nerve of DPN rats as compared with that in NC group (Fig. 1G), whereas the LC3II expression level was increased (P < 0.01) in the sciatic nerve of DPN group than NC group (Fig. 1G). These results suggested that the decreased Lipin1 level may associated with the impairment of the motor nerve function in DPN rats.

Overexpression of Lipin1 in DPN Rats Ameliorated Autophagy Disorders and Pathological Changes

To verify whether increasing Lipin1 expression can improve behavior and autophagy disorders in the sciatic nerve of DPN rats, we intrathecally injected ADV-Lipin1 to construct a Lipin1 high-expression DPN rat model (Fig. 2A).

After the overexpression of lipin1, PMWT and MNCV in ADV-Lipin1 rats were slightly improved than those in ADV-Lipin1-Con group (Fig. 2B, C). After intrathecally injected of corresponding ADV viruses, the mRNA (P < 0.05, Fig. 2D) expression levels of Lipin1 in the sciatic nerve of ADV-Lipin1 group were all significantly increased than that of ADV-Lipin1-Con group. More important, after overexpression of Lipin1, the structure of myelin sheath of sciatic nerve fibers and the myelin sheath separation was improved significantly and the lamella was dense (Fig. 3A). Meanwhile, the expression of DAG (P < 0.01, Fig. 3B) and Lipin1 (P < 0.05, Fig. 3C) significantly increased, as well as the expression of P62 (P < 0.05) was increased, and the expression of LC3II (P < 0.05) was decreased (Fig. 3D, E). These results demonstrated that upregulation of Lipin1 could ameliorate autophagy disorders and pathological changes of the sciatic nerve in DPN rat.

Downregulation of Lipin1 in Normal Rats Induced Phenotypes of DPN

To explore the role of Lipin1 in the sciatic nerves of normal rats, we intrathecally injected the small interference RNA sequence form of lipin1 in the ADV virus (ADV-Lipin1-RNAi) to knock-down the expression of Lipin1 in the sciatic nerve of normal rats and observed the changes in the behavior and sciatic nerves in rats. In ADV-Lipin1-RNAi rats, PMWT and MNCV were decreased than that in ADV-Lipin1-RNAi-Con rats (Fig. 2B, C). After virus intrathecally injected, the mRNA (P < 0.01, Fig. 2D) expression levels of Lipin1 in the sciatic nerve of ADV-Lipin1-RNAi group was all significantly decreased than that of ADV-Lipin1-RNAi-Con group. Meanwhile, the sciatic nerve showed mild swelling in ADV-Lipin1-RNAi rats and the lamella was more loose compared with ADV-Lipin1-RNAi-Con rats (Fig. 3A). The DAG levels (P < 0.01, Fig. 3B) of sciatic nerve significantly decreased in ADV-Lipin1-RNAi rats than that in ADV-Lipin1-RNAi-Con rats. The protein expression levels of Lipin1 in the sciatic nerve of ADV-Lipin1-RNAi group were decreased (P < 0.05, Fig. 3C, E). After knock-down of lipin1, P62 (P < 0.05) level was decreased, while LC3II (P < 0.01) level was significantly decreased.
Fig. 1 Decreased Lipin1 and demyelination of sciatic nerve in DPN rats. (a) Changes in body weights and blood glucose levels in rats after STZ injection (n = 6 animals/group). (b) Changes of the motor nerve conductive velocity (MNCV) between diabetic rats (DM) vs negative control rats (NC) (n = 6 animals/group). (c) Changes of the paw mechanical withdrawal threshold (PMWT) between DM and NC group (n = 6 animals/group). (d) Lipin1 mRNA expression as determined using qPCR between DM and NC group (n = 6 animals/group). (e) The levels of diacylglycerol (DAG) between DM and NC group (n = 6 animals/group). (f) Demyelination of sciatic nerve in DM rats occur, the lamella is loose and broken (scale bar = 2 μm and 1 μm). Arrows indicate the lamella. (g) Lipin1, LC3II and P62 protein expressions as determined using Western blot (n = 3 animals/group). Data are presented as the means ± SEMs. *P < 0.05, **P < 0.01 versus NC group.
increased (Fig. 3D, E). These results demonstrated that knock-down of Lipin1 induced neuronal abnormalities and facilitates the genesis of DPN phenotypes in rats.

**Hyperglycemia Reduced the Expression of Lipin1 in RSC96 Cells**

To further verify the role and underlying mechanisms of Lipin1 in DPN, we cultured Schwann cells (RSC96 cells) in vitro with normal glucose (25 Mm/48 h) and high glucose (100 Mm/48 h) to imitate DPN [27]. Based on observations using an electron microscope, the number of autophagic bodies in RSC96 cells were increased in 100 mM more than 25 mM (Fig. 4A). We found that cell viability was decreased ($P < 0.05$, Fig. 4B) in 100 mM than 25 mM. The levels of DAG also decreased in 100 mM more than 25 mM ($P < 0.01$, Fig. 4C). Meanwhile, cell apoptosis increased ($P < 0.05$, Fig. 4D) in 100 mM higher than 25 mM. The mRNA ($P < 0.01$, Fig. 4E) and protein ($P < 0.01$, Fig. 4F) expression levels of Lipin1 were significantly decreased in 100 mM than 25 mM. We also found that the P62 level was decreased ($P < 0.01$) in 100 mM more than 25 mM, whereas the LC3II expression level was increased ($P < 0.01$) in 100 mM more than 25 mM (Fig. 4G). These results suggested that hyperglycemia induced downregulation of Lipin1, autophagic hyperactivity and increased apoptosis in RSC96 cells.

**Overexpression of Lipin1 in RSC96 Cells Ameliorated the Injury Induced by Hyperglycemia**

Hyperglycemia can reduce Lipin1 expression, decrease cell viability, and increase apoptosis in RSC96 cells. However, whether increasing the expression of Lipin1 can alleviate these changes remains unknown. We then transfected RSC96...
cells with LV-Lipin1 overexpressing virus to enhance the expression of Lipin1. In LV-Lipin1 group, autophagy bodies were decreased in the cytoplasm in RSC96 cells (Fig. 5A). Cell viability was increased in LV-Lipin1 group than LV-Lipin1-Con group \((P < 0.01, \text{Fig. 5B})\). The DAG expression levels \((P < 0.01, \text{Fig. 5C})\) was increased, while cell apoptosis slightly decreased in LV-Lipin1 group than LV-Lipin1-Con group (Fig. 5D). The mRNA \((P < 0.01, \text{Fig. 5E})\) and protein \((P < 0.01, \text{Fig. 5F, G})\) expression levels of Lipin1 in RSC96 cells of LV-Lipin1 group were significantly increased than that of LV-Lipin1-Con group. Besides, P62 slightly increased and the LC3II expression level was decreased in LV-Lipin1 group than LV-Lipin1-Con group (Fig. 5F, G).

These results demonstrated that overexpression of lipin1 in RSC96 cells significantly decreased the autophagic hyperactivity and apoptosis induced by hyperglycemia.

**Downregulation of Lipin1 Induced Injury of RSC96 Cells**

Whether RSC96 cells show similar changes in normal glucose (25 mM/48 h) after decreasing the expression of Lipin1 remains unclear. We then transfected RSC96 cells with LV-Lipin1-RNAi low expression virus to reduce the expression of Lipin1 in normal glucose. In LV-Lipin1-RNAi group, autophagy bodies were increased in the
cytoplasm in RSC96 cells (Fig. 5A). Cell viability was decreased in LV-Lipin1-RNAi group than LV-Lipin1-RNAi-Con group ($P < 0.01$, Fig. 5B). The DAG expression levels ($P < 0.01$, Fig. 5C) was decreased, while cell apoptosis slightly increased in LV-Lipin1-RNAi group than LV-Lipin1-RNAi-Con group ($P < 0.01$, Fig. 5D). We found the mRNA ($P < 0.01$, Fig. 5E) and protein ($P < 0.01$, Fig. 5F, G) expression levels of Lipin1 in RSC96 cells.
LV-Lipin1-RNAi group were significantly decreased than that of LV-Lipin1-RNAi-Con group. Moreover, P62 significantly decreased and the LC3II expression level significantly increased in LV-Lipin1-RNAi group than LV-Lipin1-RNAi-Con group ($P < 0.05$, respectively, Fig. 5F, G). These results demonstrated that knock-down of Lipin1 induced abnormalities in RSC96 cells.

**Discussion**

DPN is a degenerative disease, which may be related to oxidative stress, glycosylation end products, polyol pathway, lacking of neurotrophic factors, and microcirculation disorders [28–32]. Typical changes in neuropathology include axonal injury, focal segmental deletion of myelin,
and neuronal damage [33]. Hyperglycemia, advanced glycation end products, polyols, oxidative stress, and other stimuli usually act as noxious stimulus signals, which can damage sensory neurons and axons. In normal nerves, Schwann cells surround axons to form the myelin sheath and provide nutrition for the nerves. However, under the harmful stimulation of hyperglycemia, Schwann cells would proliferate and migrate again, through which resulting in axonal degeneration and demyelination.

Autophagy is common in eukaryotic cells and plays a crucial role in maintaining cell homeostasis and body function [34]. Autophagy is the major process to clear large targets for degradation, such as protein aggregates and dysfunctional organelles. Neurodevelopment and neuronal health require effective removal of aggregated proteins and aged or defective organelles. Therefore, normal autophagy is essential for the survival, differentiation, growth of cell, and thus the maintenance of homeostasis of the body, through which can cope with the adverse environment. However, when over-activated during sustained harmful conditions, the autophagy disorder could lead to self-killing of cell via triggering apoptotic signaling pathway. Neurodegeneration is one of the many different afflictions that autophagy impairment can cause to human [35].

Lipin1 is a phosphatidic acid phosphatase (PAP) enzyme that converts phosphatidic acid (PA) to DAG, a precursor of triacylglycerol and phospholipids [36, 37]. The PAP activity of Lipin1 is required for the generation of DAG and activation of the PKD signaling pathway in autophagy clearance. Lipin1 deficiency reduces DAG levels and impairs the activation of PKD–Vps34, which result in preventing the maturation of autolysosomes and destroying autophagy homeostasis [38]. In some previous studies, autophagy in the sciatic nerve of diabetic rats was reported weakened [39], whereas other studies reported enhanced autophagy in diabetic rats [13]. These results indicated that the changes of autophagy in diabetic animal models is complicated and needs further investigation. In Lipin1-deficient mice, a disorder of peripheral nerve myelination, including demyelination and axonal degeneration, has been observed [16]. In the present study, we found that hyperglycemia reduced Lipin1 expression in the sciatic nerve of rats, as well as reduced DAG levels. Meanwhile, the MNCV of DPN rats are lower than that in NC rats. Morphological changes in the sciatic nerve are observed in DPN rats, such as demyelination and degenerative changes.

RSC96 cells, a cell line of rat Schwann cells, has been widely used in neurophysiological studies in vitro [40–42]. Schwann cell dysfunction directly affects neuronal function due to myelin disruption and demyelination [34]. Peripheral nervous system (PNS) includes all nerve structures except brain and spinal cord, which is composed of nerve trunk, nerve plexus, ganglion, and nerve terminal apparatus. Schwann cells are the glial cells responsible for producing the myelin sheath in the PNS and are highly susceptible to autophagy [43]. Therefore, in the present study, high glucose-treated Schwann cells were used for investigation. In vitro experiments, results showed that hyperglycemia reduces Schwann cell activity and increases apoptosis rate, as well as increases the number of autophagic bodies.

LC3 and P62 are the main proteins regulating cell autophagy [44]. In the process of autophagy, LC3 in cytoplasm hydrolyzes a small part of polypeptide to form LC3I, and then LC3I is transformed into LC3-II. When autophagy is activated, LC3II would increase and P62 would decrease [45]. In our study, we observed that autophagy was over-activated both in the sciatic nerves of diabetic rats and high glucose–treated RSC96 cells compared with the normal control group. Hyperglycemia enhances autophagy both in vivo and in vitro because of increased LC3II and decreased P62. Moreover, hyperglycemia decreases the level of Lipin1, which obstructs DAG synthesis, and enhances autophagy. Finally, the excessive enhancement of autophagy impairs the sciatic nerve and damages Schwann cells.

After the overexpression of Lipin1 in the sciatic nerve of DPN rats through the intrathecal injection of ADV-Lipin1, we observed that the sciatic nerve morphology of DPN rats (Fig. 3A) and MNCV (Fig. 2C) was significantly ameliorated. Decreased LC3II and increased P62 levels indicate that autophagy has been decreased and autophagy disorder has been reduced. In vitro experiments showed that Schwann cells transfected with LV-Lipin1 increased the cell viability, decreased apoptosis rate, improved cell morphology, and decreased autophagy.

When we used ADV-Lipin1-RNAi to knock-down the expression of Lipin1 in the sciatic nerve of NC rats, we were surprised to find that the sciatic nerve of NC rats had similar changes to DPN rats (Fig. 3A). MNCV is slightly lower than that in NC rats. Autophagy is slightly enhanced in ADV-Lipin1-RNAi group than NC rats. When we transfected Schwann cells with LV-Lipin1-RNAi virus under normal glucose concentration, they showed similar changes as well as in high glucose environment.

Hyperglycemia and dyslipidemia can lead to apoptosis [46]. In vitro, the autophagy and apoptosis of Schwann cells were increased in hyperglycemia. According to our results, hyperglycemia decreases the expression of Lipin1 in the peripheral nerves, which further leads a disorder of lipid metabolism and then enhances cell autophagy. Excessive autophagy can increase apoptosis and aggravate diabetic neuropathy.

In the current study of autophagy in diabetic peripheral neuropathy, the researchers have reached different conclusions [39, 47, 48]. In vivo and in vitro experiments, Qu Ling and Du W. et al. observed that in hyperglycemia, autophagy has been decreased, and Schwann cell damage
is aggravated. However, Towns R. reported that autophagy in the spinal dorsal root neurons of STZ-induced diabetic mice has been increased significantly, and neurons activate excessive autophagy, which may lead to neuronal loss and neuropathic pain. In our study, a similar result to Towns R. was observed, that is, hyperglycemia increases autophagy in sciatic nerve and Schwann cells, and then aggravates the damage of Schwann cells and sciatic nerves. Since autophagy is a continuous dynamic process including autophagy formation and autophagy degradation, which known as autophagy flow, so different intervention times and intensities may affect the conclusion. Therefore, it is necessary to further clarify whether the autophagy disorder is caused by increased autophagy formation or decreased degradation. Whether hyperglycemia increases or decreases autophagy in peripheral nerve tissue will also needs further studies. In the present final analysis, hyperglycemia leads to the disorder of normal autophagy, resulting in the increased apoptosis of Schwann cells and aggravated DPN.

In summary, we demonstrated that Lipin1 plays an important role in the neuropathy of DPN rats. The loss of Lipin1 decreases DAG expression, which leads to lipid metabolism disorders. This condition may induce over-activity of autophagy and promotes DPN. In contrast, overexpression of Lipin1 can reduce autophagy disorders and alleviate DPN. Hence, Lipin1 may be a potential target for DPN treatment via improved autophagy disorder in the future.

Conclusions

Hyperglycemia reduces the expression of Lipin1, prevents DAG synthesis, and over-activates autophagy, leading to increased apoptosis of Schwann cells. These factors cause demyelination of peripheral nerves and induce DPN. Lipin1 overexpression can alleviate autophagy disorders and ameliorate DPN.

Acknowledgements Thanks for the guidance of the teachers from the Institute of basic medicine of Shandong University and the provision of the rat sciatic nerve conduction velocity meter. We also thank the teachers of the basic laboratory of the Second Hospital of Shandong University for their guidance in the research of apoptosis.

Author Contribution S.C. conceived the study, X.Z., S.Y., L.C., and S.C. designed the study, M.W., M.X., P.S., C.Z., X.H., and C.F. performed the experiments and interpreted data analyses. M.W. wrote the first version of the paper. All authors critically reviewed, revised, and approved the final version of the manuscript.

Funding This study was supported by the National Natural Science Foundation of China (NNSFC) (NO.81670753 and NO. 82070847) to S.C., and NNSFC (NO.81800722) to X.Z., the grants from the Key R & D programs of Shandong Province (No.2018GSF118108) to S.C., and the Hospital Youth Foundation of Qilu Hospital of Shandong University, Qingdao (QDKY2017QN12) to M.W.

Data Availability All data generated or analyzed during this study are included in this published article and are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval and Consent to Participate This article does not contain any studies with human participants performed by any of the authors. Animal experiments were performed in accordance with the International Guiding Principles for Animal Research as stipulated by the World Health Organization were followed.

Consent for Publication All authors agree to publish the article in this magazine and the manuscript contains no any individual person’s data in any form.

Competing Interests The authors declare no competing interests.

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