MicroRNA-223 alleviates lipopolysaccharide-induced PC-12 cells apoptosis and autophagy by targeting RPH1 in spinal cord injury

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Abstract: Spinal cord injury (SCI) is one of the most devastating diseases. MicroRNAs (miRNAs) are recognized as key regulators in SCI; however, the role of miR-223 in SCI remains unclear. Herein, our study aimed to explore the effect of miR-223 on lipopolysaccharide (LPS)-induced injury to PC-12 cells. PC-12 cells were treated with different concentrations of LPS, and then cell viability, apoptosis, apoptosis-related factors and autophagy-related factors were analyzed by CCK-8, flow cytometry and western blot. Subsequently, miR-223 mimic, miR-223 inhibitor, pEX-RPH1, sh-RPH1 and corresponding controls were transfected into PC-12 cells followed by 5 μg/ml of LPS treatment. Cell viability, apoptosis, apoptosis-related and autophagy-related factors were analyzed again. A target gene of miR-223 was validated by dual-luciferase assay. Besides, the main factors expressions of mTOR and NF-κB signal pathways were measured by western blot. LPS reduced cell viability but increased apoptotic cells rate, up-regulated Bax, cleaved-caspase-3, cleaved-caspase-9, LC-II and Beclin-1, and down-regulated Bcl-2 and p62 expressions in a dose-dependent way. Additionally, miR-223 overexpression promoted cell viability but inhibited apoptosis, and autophagy in LPS-stimulated PC-12 cells. RPH1 was a direct target of miR-223, and RPH1 exhibited contrary impacts to miR-223 on LPS-induced cell apoptosis and autophagy. Besides, the promoting effects of miR-223 suppression on cell apoptosis and autophagy were relieved by RPH1 silence. Furthermore, miR-223 blocked LPS-induced mTOR and NF-κB pathways by down-regulation of RPH1. MiR-223 improved cell viability but declined apoptosis and autophagy by targeting RPH1 and blocked mTOR and NF-κB pathways in LPS challenged PC-12 cells.

Keywords: MicroRNA-223, spinal cord injury, cell viability, apoptosis, autophagy, RPH1

Introduction

Spinal cord injury (SCI) is one of the most serious diseases which causes by various pathogenic factors, such as trauma, inflammation and tumors and leads to progressive damage and motordysfunction [1, 2]. With the development of economy in the world, the incidence of SCI is increasing year by year. From 1950 to 2012, the global annual incidence of SCI varied from 8 to 246 cases per million people [3, 4]. More than 180,000 patients of SCI suffer permanent disability [5]. The prevention, treatment and rehabilitation of SCI have become a major issue in the medical field [6]. Although many promising therapies have been explored, the efficacy and safety treatment methods of SCI are still under discussion.

MicroRNAs (miRNAs) are endogenous, non-coding, short (18-22 nt), single-stranded RNA molecules that able to regulate gene expression at post-transcriptional level [7]. The ability of miRNAs to regulate cell state and function by silencing hundreds of genes are being recognized as key actors in the pathophysiology of SCI [5]. Plenty of researches indicated that dysregulations of miRNAs have been found and involved in cell biological process in SCI. As Deng et al. reported that miR-34a was one of the most dysregulated miRNAs in SCI, and down-regulation of miR-34a inhibited cell apoptosis [8]. In a similar way, Hu et al. demonstrated that miR-21 alleviated the functional of tissue damage and reduced apoptotic cells death in SCI [9]. In terms of miR-223, previous studies indicated that miR-223 might regulate neutrophils in the
Potential effects of miR-223 on SCI and autophagy remains elusive.

In the present study, we aimed to explore the roles of miR-223 in SCI and to find their possible therapeutic application. This study began with lipopolysaccharide (LPS) stimulation in PC-12 cells. Then miR-223 mimic, miR-223 inhibitor, pEX-RPH1 and the short hairpin RNA RPH1 (sh-RPH1) were transfected into PC-12 cells for analyzing cell viability, apoptosis and autophagy in vitro detected by CCK-8, flow cytometry and western blot. Related factors of apoptosis, autophagy and factors in mammalian target of rapamycin (mTOR) and nuclear factor (NF)-κB signal pathways were examined by western blot. Our findings will provide a new insight into the molecular effects of miR-223 on SCI and suggest the potential value of miR-223 for the treatment of SCI.

Materials and methods

Cell culture and LPS treatment

The PC-12 cells were purchased from Kunming Institute of Zoology (Kunming, China) and used throughout the study. The cells were seeded onto flasks at a density of 1 × 10^4 cells/ml in Dulbecco’s Modified Eagle’s medium (DMEM, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS, Gibco). It was maintained at 37°C in a humidified incubator containing 5% CO₂. Culture medium was changed every 2-3 days. Cells were treated by LPS in a series of concentration (0, 1, 5 and 10 μg/ml) for 12 h.

Transfection

MiR-223 mimic, inhibitor and their respective controls were synthesized by GenePharma (Shanghai, China) and were transfected into PC-12 cells in the study. To analyze the functions of RPH1, the full-length RPH1 sequences and shRNA directed against RPH1 were constructed into pEX-2 and U6/GFP/Neo plasmids (GenePharma) respectively. And they were referred as to pEX-RPH1 and sh-RPH1. The highest transfection efficiency was occurred at 48 h, thus 72 h post-transfection was considered as the harvest time in the subsequent experiments. The lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) was used for the cells transfection according to the manufacturer’s instructions.

Cell viability

Cell viability was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD) as described in previous study [12]. Briefly, PC-12 cells or the transfected PC-12 cells were seeded in 96-well plate with 5000 cells per well. After LPS stimulation, 20 μl CCK-8 was added to the culture medium, and the cultures were incubated for another 4 h at 37°C in humidified 95% air and 5% CO₂. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The cells (1 × 10⁵ cells/well) were seeded in 6-well plates. Treated cells were washed twice with ice-cold phosphate buffer saline (PBS) and re-suspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer’s instruction and measured with flow cytometer (Beckman Coulter, USA) to differentiate apoptotic cells from necrotic cells (10 μl Annexin-V positive and 5 μl PI-negative).

Dual luciferase activity assay

The 3’ untranslated region (3’UTR) target site was generated by PCR and the luciferase reporter constructs with the RPH1 3’UTR carrying a putative miR-223-binding site into pMiR-report vector were amplified by PCR. These vectors were co-transfected with miR-223 mimic or mimic control into PC-12 cells using lipofectamine 3000 (Life Technologies). After transfection, reporter assays were done using the dual-luciferase assay system (Promega, Fitchburg, WI, USA) following to the manufacturer’s information [13].

Quantitative RT-PCR (qRT-PCR)

Total RNAs of PC-12 cells were extracted by using Trizol reagent (Life Technologies Cor-
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... according to the manufacturer’s instructions. miRNAs were converted to complementary DNAs (cDNAs) using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and the PrimeScript RT Master Mix Kit (Takara, Dalian, China). The qRT-PCR was performed by Applied Biosystems 7500 Real-Time PCR System, using TaqMan MicroRNA Assay (Applied Biosystems). U6 were used for normalization to the expression levels of miR-223 in PC-12 cells. Data were calculated by the 2–ΔΔCt method [14].

**Western blot**

The cellular protein used for western blot was extracted using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Basle, Switzerland). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer’s instructions. After blocking at 5% skimmed milk for 1 h at room temperature, the membranes were incubated at 4°C overnight with primary antibodies: B-cell lymphoma 2 (Bcl-2; ab32124), Bcl-2-associated X (Bax, ab32503), pro-caspase-3 (ab32150), cleaved caspase-3 (ab13865), pro-caspase-9 (ab32068), Beclin-1 (ab62557), p62 (ab56416), RPH1 (ab187124), phosphorylated (p)-mTOR (ab84400), mTOR (ab-2732); p-p65 (ab86299), p65 (ab16502), p-IκBα (ab92700), IκBα (ab32518) GAPDH (ab8245, Abcam, Cambridge, UK) and cleaved caspase-9 (#9501, Cell signaling Technology) (dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then membrane followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the Polyvinylidene Difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and

**Figure 1.** LPS inhibited cell viability but promoted apoptosis and autophagy in SCI. PC-12 cells were treated with different concentration of LPS (0, 1, 5 and 10 μg/ml) for 12 h. (A) Cell viability was determined by CCK-8 assay; (B) Cell apoptosis was analyzed by flow cytometry assay; Levels of (C) apoptosis-related factors and (D) autophagy-related factors were examined by western blot. LPS: Lipopolysaccharide; SCI: spinal cord injury; CCK-8: Cell Counting Kit-8. *P < 0.05. **P < 0.01. ***P < 0.001.
then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± standard derivations (SD). Statistical analyses were performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). The P-values were calculated using a one-way analysis of variance (ANOVA). A P-value of < 0.05 was considered to indicate a statistically significant result.

Results

LPS inhibited cell viability but promoted apoptosis and autophagy

To explore the effects of LPS on SCI, PC-12 cells were treated with different concentration of LPS (0, 1, 5 and 10 μg/ml), and then cell viability and apoptosis were analyzed by CCK-8 and flow cytometry. The results showed in Figure 1A and 1B that, LPS significantly reduced cell viability but increased apoptosis in a dose-dependent way (P < 0.05, P < 0.01 or P < 0.001). Further, the protein levels of apoptosis-associated factors were measured by western blot. As results showed in Figure 1C, LPS down-regulated Bcl-2, up-regulated Bax, and activated cleaved caspase-3 and cleaved caspase-9 expressions. However, the protein levels of pro-caspase-3 and pro-caspase-9 were not activated by LPS.

As we all known that microtubule-associated protein 1 light-chain (LC)-I/II are essential for autophagosome formation and are widely used to monitor autophagic activity [15]. Moreover Beclin1 and p62 are important autophagy-related genes [16, 17]. To understand the effect of LPS on cell autophagy, western blot were used to evaluate the levels of LC-1, LC-II, Beclin1 and p62 in PC-12 cells at different concentration of LPS. As results displayed in Figure 1D, LPS remarkably up-regulated LC-II, Beclin1, but down-regulated p62 expression in a dose-dependent way. LC-1 expression was not significantly altered by LPS. Taken together, LPS could inhibit cell viability as well as promoted apoptosis and autophagy. LPS at the concentration of 5 μg/ml was used in the further study.

Figure 2. MiR-223 promoted LPS-induced cell viability but inhibited apoptosis and autophagy. PC-12 cells were transfected with miR-223 mimic, miR-223 inhibitor and corresponding controls and treated with 5 μg/ml of LPS. (A) MiR-223 level was examined by qRT-PCR; (B) Cell viability was determined by CCK-8 assay; (C) Cell apoptosis was analyzed by flow cytometry assay; Levels of (D) apoptosis-related factors and (E) autophagy-related factors were examined by western blot. MiR-223: microRNA-223; LPS: Lipopolysaccharide; qRT-PCR: quantitative RT-PCR; CCK-8: Cell Counting Kit-8. *P < 0.05. **P < 0.01. ***P < 0.001.
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MiR-223 promoted cell viability but inhibited apoptosis and autophagy induced by LPS

To explore the effect of miR-223 on LPS-induced cell viability, apoptosis and autophagy, PC-12 cells were transfected with miR-223 mimic, miR-223 inhibitor and their corresponding controls and then stimulated with 5 μg/ml LPS. The results displayed that miR-223 expression was obviously enhanced by miR-223 mimic, but reduced by miR-223 inhibitor (Figure 2A, $P < 0.01$) indicating that the transfection efficiency was high and could be used for further study. Thereafter, cell viability, apoptosis and autophagy were analyzed again. As results showed in Figure 2B and 2C, miR-223 overexpression treated with LPS prominently elevated cell viability, but inhibited apoptosis compared with their controls ($P < 0.05$). However, the effects were reversed by simultaneous transfection with miR-223 suppression.

Western blot results showed that miR-223 overexpression combined with 5 μg/ml LPS up-regulated Bcl-2, down-regulated Bax, and blocked cleaved caspase-3 and cleaved caspase-9 expressions. Meanwhile, miR-223 suppression down-regulated Bcl-2, up-regulated Bax and activated cleaved caspase-3 and cleaved caspase-9 expressions. The protein levels of pro-caspase-3 and pro-caspase-9 were not significantly regulated by LPS (Figure 2D). In terms of cell autophagy, miR-223 overexpression combined with 5 μg/ml LPS down-regulated LC-III, Beclin-1 and up-regulated p62 expressions. Suppression of miR-223 led to the opposite results. LC-1 was not significantly regulated by LPS (Figure 2E).

RPH1 was a direct target of miR-223

We detected RPH1 expression at mRNA and protein levels when the PC-12 cells transfected with miR-223 mimic and their controls. As exhibited in Figure 3A, miR-223 overexpression significantly decreased RPH1 mRNA and protein expressions. Conversely, suppression of miR-223 obviously increased the RPH1 expression ($P < 0.05$ or $P < 0.01$). Further, dual-luciferase reporter assay was performed and results in Figure 3B showed that miR-223 mimic reduced the activity of the luciferase reporter fused to the RPH1-Wt ($P < 0.05$), while did not suppress the reporter fused to the RPH1-Mt version. These results suggested that RPH1 might be a direct target of miR-223, and miR-223 was negatively related to RPH1.

RPH1 inhibited cell viability but promoted apoptosis and autophagy induced by LPS

To further explore the roles of RPH1 in cell viability, apoptosis and autophagy, pEX-RPH1 and sh-RPH1 were transfected into PC-12 cells to overexpress or suppress RPH1 expression. Then the transfected cells were treated by 5 μg/ml LPS for 12 h. Results showed that pEX-
RPH1 remarkably up-regulated RPH1 expression, while sh-RPH1 down-regulated RPH1 expression (Figure 4A, \( P < 0.01 \)). Besides, pEX-RPH1 reduced cell viability as well as facilitated apoptosis induced by LPS, while the adverse results were showed by sh-RPH1 (Figure 4B and 4C, \( P < 0.05 \) or \( P < 0.01 \)). The apoptosis-related factors expressions results showed that pEX-RPH1 down-regulated Bcl-2, up-regulated Bax and activated cleaved caspase-3 and cleaved caspase-9 expressions induced by LPS. But sh-RPH1 displayed the inverse impacts on these factors expressions (Figure 4D). No significant effects on pro-caspase-3 and pro-caspase-9 expressions. Cell autophagy results showed in Figure 4E, pEX-RPH1 promoted LC-II, Beclin-1 expressions, but inhibited p62 expression. Simultaneously, sh-RPH1 suppressed LC-II, Beclin-1 expressions, but promoted p62 expression. Based on these data, we confirmed that RPH1 inhibited cell viability but promoted apoptosis and autophagy induced by LPS.

**MiR-223 suppression enhanced LPS-induced cell viability, apoptosis and autophagy by regulating RPH1**

To explore the relationship between miR-223 and RPH1 on cell viability, apoptosis and autophagy in SCI, miR-223 inhibitor and sh-RPH1 were transfected into PC-12 cells to suppress miR-223 and RPH1 expression. Cell growth in vitro results showed in Figure 5A and 5B that, miR-223 suppression together with sh-RPH1 inhibited LPS-induced cell viability and apoptosis compared with miR-223 suppression (\( P < 0.05 \)). Western blot results showed that miR-223 suppression together with sh-RPH1 up-regulated apoptosis-related factors and autophagy-related factors of Bax, cleaved caspase-3, cleaved caspase-9 and p62 expressions, down-regulated Bcl-2, LC-II, and Beclin-1 expressions induced by LPS. There is no significant change in pro-caspase-3, pro-caspase-9 and LC-1 expressions (Figure 5C and 5D). These results evidenced that miR-223 suppression promoted LPS-induced cell viability, apoptosis and autophagy by targeting RPH1 in PC-12 cells.

**MiR-223 blocked mTOR and NF-κB pathways by down-regulation of RPH1**

The mTOR and NF-κB signaling pathways are vital in regulated cell biological processes [18, 19]. Herein, they were suspected to be an important mechanism of miR-223 and RPH1 in...
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PC-12 cells. To confirm this possibility, expression changes of key factors in mTOR and NF-κB were analyzed. The protein levels of RPH1, p-mTOR, p-p65 and p-IκBα were notably down-regulated by miR-223 overexpression, and up-regulated by miR-223 suppression. The factors of mTOR, p65, IκBα have no signal changes by miR-223 overexpression or suppression (Figure 6A and 6B). Thus, miR-223 was likely to block mTOR and NF-κB pathways by down-regulation of RPH1.

Discussion

In this study, we observed that LPS significantly suppressed cell viability but promoted apoptosis and autophagy. The related factors of apoptosis and autophagy were also regulated by LPS in a dose-dependent way. Additionally, overexpression of miR-223 promoted cell viability but inhibited apoptosis, autophagy and regulated their related factors expression in LPS challenged cells. RPH1 was a direct target of miR-223 and RPH1 exhibited contrary effects to miR-223 on LPS-induced cell apoptosis and autophagy. Besides, miR-223 blocked mTOR and NF-κB pathways by down-regulation of RPH1.

LPS is the major component of the outer membrane of gram-negative bacteria and have been identified as the vital factor in various diseases [20]. Several evidences indicated that LPS-induced preconditioning protects neurons against traumatic spinal cord injury (TSCI) [21]. Moreover, LPS was participated in regul-
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lated cell proliferation and death [22]. Similar with previous studies, we demonstrated that LPS promoted PC-12 cells viability as well as induced apoptosis and autophagy. Moreover, several studies demonstrated that miRNAs regulated biological processes were induced by LPS [23]. For example, miR-199a was down-expressed in mice with LPS-induced acute lung injury and promoted cell metabolism and cell cycle [24]. MIR-Let7A modulated autophagy induction in LPS-activated microglia during CNS inflammation [25]. However, the relationship between miR-223 and LPS in SCI remain unclear. In the study, we showed that miR-223 promoted cell viability but inhibited apoptosis and autophagy induced by LPS in PC-12 cells. Taken together, miR-223 alleviated LPS-induced cell apoptosis and autophagy in SCI.

RPH1 is a Cys2-His2 zinc finger protein, binds to an upstream repressing sequence of the photolyase gene PHR1 [26]. Pervious study showed that RPH1 as a negative regulator of the transcription of several ATG genes and a repressor of autophagy induction. Additionally, preventing RPH1 phosphorylation or overexpressing the protein causes a severe block in autophagy induction [27, 28]. However, whether RPH1 participated in cell growth and death in SCI remains unclear. Our study first confirmed that RPH1 was a target gene of miR-223 and was negatively regulated by miR-223. In addition, RPH1 overexpression inhibited cell viability as well as promoted apoptosis and regulated the relative factor expressions of apoptosis and autophagy stimulated by LPS. In sum up, these results suggested that RPH1 inhibited cell viability but promoted apoptosis and autophagy induced by LPS.

The mTOR and NF-κB signal pathways play important role in pathophysiological processes including cancer, metabolic diseases, and inflammation which also multiple cellular functions, such as cell proliferation, metabolism and survival [29]. In terms of SCI, previous study showed that inhibition of mTOR reduced cell death in damaged neural tissue following SCI [30]. MiR-17 activated mTOR signal pathway by targeting PTEN in SCI [31]. In terms of miR-223, one study displayed that miR-223 could alter cellular viability in breast cancer via affecting NF-κB and mTOR pathways [32]. Besides, miR-223 promoted tumor progression in lung cancer A549 cells via activation of the NF-κB signaling pathway [33]. Similar with above research, our study confirmed that miR-223 blocked mTOR and NF-κB by down-regulation of RPH1 thereby effecting cell viability, apoptosis and autophagy.

In conclusion, our results showed that miR-223 alleviated LPS-induced cell apoptosis and autophagy by targeting RPH1 in PC-12 cells, and miR-223 blocked LPS-induced mTOR and NF-κB pathways by down-regulation of RPH1. These findings provided basic information for the potential effects of miR-223 in PC-12 cells. However, the exact role of miR-223 in SCI needs further to be explored.

Disclosure of conflict of interest

None.
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