Notoginsenoside R1 alleviates lipopolysaccharide-triggered PC-12 inflammatory damage via elevating microRNA-132

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**ABSTRACT**

**Background:** Delayed inflammatory response is closely associated with the severity of Spinal cord injury (SCI). Herein, the function and molecular mechanism of notoginsenoside R1 (NGR1) in the in vitro model of SCI inflammation injury were explored.

**Methods:** PC-12 neuronal cells were subjected with LPS to construct a cell-based model of SCI inflammatory injury. NGR1 was applied in this cell model. miR-132 was silenced by transfection with miR-132 inhibitor. Cell viability and apoptosis were assessed, respectively. Then, the expression changes of pro-inflammatory cytokines and JNK pathway were examined.

**Results:** In this model, LPS was neurotoxic, with inhibiting PC-12 cell viability, inducing apoptosis, and enhancing concentrations of IL-6, IL-8 and TNF-α. However, NGR1 weakened the influence of LPS on PC-12 cells via elevating cell viability, decreasing apoptosis, decreasing pro-inflammatory cytokines expression, and suppressing activation of JNK signalling pathway. miR-132 was up-regulated by NGR1 treatment. Silence of miR-132 eliminated the influence of NGR1 on LPS-stimulated PC-12 cells.

**Conclusion:** NGR1 relieved PC-12 cells from LPS-triggered inflammatory damage via elevating miR-132 and hereafter suppressing JNK pathway.

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**INTRODUCTION**

Spinal cord injury (SCI) usually includes primary injury and secondary injury [1]. Primary injury occurs at initial impact, which includes mechanical compression of the spine [1]. Secondary injury develops soon after the primary injury, which includes oxidative stress, postrauamatic inflammation, motor neuron apoptosis and necrosis, which can cause extra damage to the spine and result in a more serious consequence [2]. Many studies reported that secondary injury of SCI could trigger inflammatory response in spine [3,4] and further lead to the damage of neuronal [3,5]. Thus, we believe that inhibition of inflammatory injury may improve treatment and recovery of SCI.

Panax notoginseng (Burkill) F. H. Chen is a common and widely used herbal medicine in China [6]. Notoginsenoside R1 (NGR1), chemically named C\(_{47}H\(_{80}\)O\(_{18}\)), is the main active ingredient extracted from the root of P. notoginseng. Some recent studies have demonstrated that NGR1 has antioxidant, anti-inflammatory, anti-angiogenic and anti-apoptotic activities [7–9]. For example, it was effective in treating cardiovascular and cerebrovascular disease via exert anti-inflammatory and anti-apoptotic activities [10]. Moreover, it was found to have anti-metastatic properties against human colorectal cancer [11]. Studies also indicated that NGR1 attenuated high glucose-triggered apoptosis of podocyte via activating cell autophagy [12]. Besides, NGR1 attenuated amyloid-β-induced neurons damage via enhancement of cell viability, reduction of oxidative damage, and restoration of mitochondrial membrane potential [13]. However, until now, the influence of NGR1 on inflammatory damage in SCI injury has not been explored.

MicroRNAs (miRNAs) are found to exert key roles in post-transcriptional regulation [14]. A number of miRNAs have been shown to be closely related to the inflammatory response in SCI [15]. Besides, dysregulation of some miRNAs have been proved to result in uncontrolled production of inflammatory cytokines in some diseases [16]. miRNA-132 (miR-132) has been demonstrated to be closely associated with inflammation regulation and play multiple roles in neuronal plasticity, gene expression and cognition [17,18]. Previous studies reported that resveratrol could relieve lipopolysaccharide (LPS)-triggered inflammatory damage in vitro (PC-12 cells) and in vivo (rat model) through modulating miR-132 [19]. More studies are needed to test whether miR-132 joins in the influence of NGR1 on LPS-stimulated PC-12 cells.

Herein, we evaluated the influence of NGR1 on LPS-triggered PC-12 cell inflammatory damage. The molecular mechanism...
involving in miR-132 was emphatically studied. We want to investigate the relationship between NGR1 and miR-132 in terms of inflammation.

**Materials and methods**

**Cell culture and LPS treatment**

PC-12 cells were received from Kunming Institute of Zoology (Kunming, China) and cultivated at 37 °C with 5% CO2 in DMEM containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin solution. Cells were exposed to 0.1–10 μg/ml LPS for 12 h, or 1–100 μM NGR1 for 24 h in this research.

**Cell viability assay**

Cell Counting Kit-8 assay (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD) provides a method for detecting PC-12 cell viability. 5 × 10³ PC-12 cells were cultivated in 96-well plate overnight and stimulated by LPS and/or NGR1 or miR-132 inhibitor transfection. 10 μl CCK-8 solution was mixed into the culture medium. Followed by placing the 96-well plate for 1 h at 37 °C, the absorbance of each well was measured at 450 nm using a Microplate Reader (Molecular Device, CA, USA). Cell viability (%) was represented as the percentage of control.

**Cell apoptosis assay**

PI/FITC-Annexin V apoptosis kit provides a method for measuring PC-12 cell apoptosis. After different exposure, cells were harvested in line with experimental group and rinsed with kit buffer. Then, 200 μl of cells in kit buffer was mixed with PI/FITC-Annexin V solution for 30 min at room temperature in the dark. Afterwards, the percentage of apoptotic PC-12 cells in each group was measured using flow cytometry analysis (Beckman Coulter, Fullerton, CA, USA).

**miRNA transfection experiment**

The miR-132 inhibitor and inhibitor negative control (inhibitor NC) were received from GenePharma Co. (Shanghai, China). miR-132 inhibitor was synthesized by following sequence: 5'-AGUAAACAUGGAAAGCCAGGGU-3'. Inhibitor NC was synthesized by following sequence: 5'-UCACAAACCUCUAGAAGAUGAGA-3'. Cell transfection experiment was conducted using Lipofectamine 3000 reagent (Invitrogen, Barcelona, Spain).

**qRT-PCR**

Total RNAs were extracted from cells with RNA isolation kit (Takara Biomedical Corporation, Beijing, China). cDNA was synthetized using Super M-MLV reverse transcriptase (Takara Biomedical Corporation). Subsequently, SYBR Green Master Mix was used to detect miRNA or mRNA expression. U6 and β-actin acted as internal controls, respectively. Primers for miR-132 were 5'-GCCGCTAACAGTCTACACCCA-3' (F) and 5'-GTGAGGGTCCAGGT-3' (R). Primers for IL-6 were 5'-CGTGGAAATGAGAAAAGAGTTTG-3' (F) and 5'-ATGCTAGGCAATACGCTAG-3' (R). Primers for IL-8 were 5'-ATGCTCCAAACTGCGTG-3' (F) and 5'-TTATGATTCGAGCGCTT-3' (R). Primers for TNF-α were 5'-GACAAGTCTGAAGGGTCCAGG-3' (F) and 5'-TGAGTGCTACGAGGTTG-3' (R). Primers for β-actin were 5'-GACACACCTCGTCAAGTG-3' (F) and 5'-TGCTGCTGATCCACATCTG-3' (R). Data were calculated using the 2⁻ΔΔCT method.

**Western blot**

For immunoblotting, total proteins were isolated from PC-12 cells with the help of RIPA buffer containing protease inhibitors. Protein sample was tested using Bradford assay. Equal concentration of proteins was separated using SDS-PAGE system and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, USA). Then, membranes were covered with following antibodies: anti-p53 (ab1311442, 1:1000), anti-pro caspase-3 (ab90437, 1:1000), anti-cleaved caspase-3 (ab13847, 1:500), anti-cytochrome-C (ab90529, 1:1000), anti-TCF-β (ab179461, 1:1000), anti-p-JNK (ab124956, 1:1000), anti-c-Jun (ab7747, 1:10), anti-TNF-α (ab90529, 1:1000), anti-IL-6 (ab9324, 1:1000), anti-IL-8 (ab7747, 1:10), anti-TNF-α (ab6671, 1:1000), anti-JNK (ab179461, 1:1000), anti-p-JNK (ab124956, 1:1000), anti-β-actin (ab8227, 1:1000; Abcam, Cambridge, MA) overnight. After that, the membranes were covered with horseradish peroxidase-conjugated secondary antibodies (1:2000; Abcam) for 1 h. Followed by washing, the signals of proteins in membranes were visualized via enhanced chemiluminescence and band intensity was measured.

**Statistical analysis**

Data were presented as the mean ± SD from three independent experiments. One-way analysis of variance (ANOVA) with post hoc Tukey t-tests was carried out to measure statistical significance using SPSS 19.0 statistical software (SPSS, Chicago, IL, USA). A p value of < .05 was considered statistically significant.

**Results**

**LPS triggered inflammatory damage of PC-12 cells**

Cell viability was detected after cells were treated with various doses of LPS for 12 h. Data in Figure 1(A) showed that LPS stimulation significantly decreased the PC-12 cell viability in a dosage-dependent pathway (2 μg/ml, p < .05; 5 μg/ml, p < .01; 10 μg/ml, p < .001). Apoptotic cell rate was determined after 5 or 10 μg/ml LPS treatment. Data in Figure 1(B) showed that LPS at 5 μg/ml and 10 μg/ml both significantly promoted cell apoptosis (both p < .01). LPS at 5 μg/ml obviously enhanced p53, cleaved caspase-3, and cyto-c expressions, which were dramatically enhanced by 10 μg/ml LPS.
Subsequently, the influence of LPS on pro-inflammatory cytokines expression in PC-12 cells was analyzed. The mRNA and protein levels of IL-6, IL-8, and TNF-α were all markedly increased after LPS incubation (5 μg/ml, \( p < .05 \) or \( p < .01 \); 10 μg/ml, \( p < .01 \) or \( p < .001 \) in mRNA levels; Figure 1(D,E)). Overall, these above outcomes indicated that LPS triggered inflammatory damage of PC-12 cells. LPS with 2 μg/ml induced too little injury but LPS with 10 μg/ml induced excessive serious injury. Thus, the middle concentration of LPS at 5 μg/ml was picked to further experiments.

NGR1 inhibited LPS-induced inflammatory damage of PC-12 cells

Then, NGR1 was introduced to test the possible protective effects on PC-12 cell inflammatory damage caused by LPS. NGR1 single incubation had no obvious influence on viability of PC-12 cells (Figure 2(A)). 5–100 μM NGR1 treatment all significantly mitigated the PC-12 cell viability reduction caused by LPS (5, 10 and 100 μM, \( p < .05 \); 50 μM, \( p < .01 \), Figure 2(B)). 50 μM NGR1 had the best protective effect. Thus, 50 μM was selected for subsequent experiments. Results of flow cytometry analysis displayed that 50 μM NGR1 treatment notably alleviated the apoptosis of PC-12 cells stimulated by LPS (\( p < .05 \), Figure 2(C)). The p53, cleaved caspase-3, and cyto-c expressions were all decreased after 50 μM NGR1 co-treatment, compared to LPS single stimulation (Figure 2(D)). Effects of NGR1 on LPS-induced pro-inflammatory cytokine productions were also investigated. As shown in Figure 2(E), in LPS-stimulated PC-12 cells, mRNA levels of IL-6 (\( p < .01 \)), IL-8 (\( p < .01 \)) and TNF-α (\( p < .001 \)) were all reduced in the presence of NGR1, in comparison to absence of NGR1. Protein expression levels showed the same changes (Figure 2(F)). These data verified that NGR1 protected PC-12 cells from LPS-triggered inflammatory damage.

NGR1 reduced LPS-triggered inflammatory damage of PC-12 cells possibly via elevating miR-132

Lower expression of miR-132 was found in PC-12 cells after LPS treatment (\( p < .05 \)), but miR-132 level was elevated by NGR1 treatment (\( p < .05 \)), indicating an association between miR-132 and LPS-triggered cell injury (Figure 3(A)). miR-132 inhibitor was introduced into PC-12 cells to explore whether miR-132 was implicated in the influence of NGR1 in LPS-damaged PC-12 cells. miR-132 was successfully silenced after miR-132 inhibitor transfection (\( p < .01 \), Figure 3(B)). Cell viability assay showed that the protective influence of NGR1 on PC-12 cell viability decrease triggered by LPS was abolished by miR-132 silence (\( p < .05 \), Figure 3(C)). Moreover, the apoptotic-inhibiting effect of NGR1 on LPS-injured PC-12 cells was also impaired by miR-132 knockdown (\( p < .01 \) for apoptotic cell rate, Figure 3(D,E)). Changes of pro-inflammatory factor expressions were also observed. The IL-6, IL-8 and TNF-α levels in NGR1-incubated group were all largely up-regulated after down-regulation of miR-132 (\( p < .01 \) in mRNA level, Figure 3(F,G)). These data demonstrated that NGR1 attenuated LPS-triggered PC-12 cell inflammatory damage might be via elevating miR-132.

NGR1 inhibited activation of JNK signalling pathway in LPS-stimulated PC-12 cells by elevating miR-132

JNK signalling pathway can be activated in cellular inflammatory response, and activation of this pathway has been found to be associated with overproduction of inflammatory cytokines [20,21]. Therefore, expression of main factors in this pathway was determined to explore whether JNK pathway was implicated in the influence of NGR1 on LPS-triggered PC-12 cell inflammatory damage. As shown in Figure 4, the p-JNK and p-c-Jun levels were both up-regulated after LPS.
treatment while were remarkably down-regulated after NGR1 co-treatment. Moreover, knockdown of miR-132 both enhanced the p-JNK and p-c-Jun levels. These data verified that NGR1 inhibited LPS-triggered inflammatory responses might be by elevating miR-132 and then suppressing JNK pathway.

Discussion

Secondary injury of SCI has been demonstrated to be closely associated with cellular inflammatory response and apoptosis [22]. Thus, this research focused on the possible protective activity of NGR1 on inflammatory injury in secondary injury of SCI. PC-12 cells stimulated by LPS were used to establish an in vitro inflammatory damage model of SCI.

LPS-induced cell inflammatory injury model has been widely used for SCI research [23,24]. Our data showed that LPS could inhibit PC-12 cell viability, promote cell apoptosis, and enhance pro-inflammatory cytokines productions, indicating that inflammatory injury occurred in PC-12 cells. Recent studies have reported that NGR1 has multiple pharmacological activities, including neuro-protective, cardioprotective, anti-inflammatory, and even anti-cancer [25–27]. The anti-inflammatory effect of NGR1 was also confirmed in this study. Our findings displayed that NGR1 effectively increased cell viability, lowered cell apoptosis, inhibited pro-apoptotic factors levels, as well as inhibited productions of...
pro-inflammatory cytokines in LPS-injured cells. Previous study has been reported that NGR1 protected neuronal cells against injury [13,25]. The findings of our research were also consistent with the previous researches, which proved that NGR1 could relieve oxidative, glutamate, LPS and Aβ triggered damages of nerve cells *in vitro* [28–30].

Subsequently, the underlying action mechanism was studied. According to our results, miR-132 was lowered by LPS, whereas, it was boosted by NGR1. Therefore, we suspected that miR-132 exerted indispensable influence on the anti-inflammatory property of NGR1. We found that the anti-inflammatory activity of NGR1 was completely abolished by miR-132 silence, which hinted that NGR1 unleashed its anti-inflammatory activity at least by elevating miR-132. Luikart et al. confirmed that miR-132 knockdown could lead to hundreds of genes expression increases in PC-12 cells and most of them were joined in inflammatory/immune signalling [31]. Moreover, miR-132 and a number of miR-132 targets have been considered as potential mediators of neuronal phenotype, which are involved in a number of important processes, including spine formation, synaptic maturation, and synapse function [32–34].

Finally, we also demonstrated that NGR1 protected neuron cells from inflammatory damage by inactivating the JNK pathway. The JNK pathway is an important regulatory pathway in cellular inflammatory response. JNK pathway can regulate the maturation and activity of T cells, and also affect productions of a number of pro-inflammatory cytokines, such as IL-2, IL-6 and TNF-α [20,21,35]. Moreover, miR-132 also has been discovered to be related to the regulation of JNK pathway [36]. Fu et al. reported that NGR1 protected human umbilical vascular endothelial cells (HUVEC) against oxidized low density lipoprotein (Ox-LDL)-caused atherogenic response via modulating miR-132 and inactivating JNK

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**Figure 3.** NGR1 weakened LPS-triggered PC-12 cell inflammation damage via elevating miR-132. (A) Followed by LPS and/or NGR1 stimulation, miR-132 expression was measured. (B) Transfection efficiency of miR-132 inhibitor was tested. Followed by LPS and/or NGR1 stimulation or miR-132 inhibitor transfection, (C) cell viability, (D) apoptosis, (E) p53, Caspase 3, Cytochrome-C protein levels, (F) IL-6, IL-8, TNF-α mRNA levels, and (G) IL-6, IL-8, TNF-α protein levels were analyzed. *p < .05, **p < .01.
pathway [36]. Herein, we discovered that miR-132 could activated JNK pathway in PC-12 cells, which further indicated that NGR1 exerted protective activity on LPS-damaged PC-12 cells was achieved by elevating miR-132 and then suppressing JNK pathway.

Conclusion

In conclusion, we affirmed that NGR1 attenuated LPS-triggered PC-12 cell inflammatory injury via enhancement of cell viability, inhibition of apoptosis, and suppression of pro-inflammatory cytokines production through up-regulating miR-132. Moreover, NGR1 exerted the anti-inflammatory property by inactivation of JNK signalling pathway, which was also mediated by miR-132. As the anti-inflammatory effect was believed to be one of the contributing mechanisms of SCI medications, NGR1 might provide a new therapeutic option for the treatment of SCI.

Disclosure statement

No potential conflict of interest was reported by the authors.

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