EXPERIMENTAL STUDY

miRNA-138 regulates MLK3/JNK/MAPK pathway to protect BV-2 cells from $\text{H}_2\text{O}_2$-induced apoptosis

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ABSTRACT

BACKGROUND: miR-138 is one of the down-regulated miRNAs during acute spinal cord injury. Mixed lineage kinase 3 (MLK3), a key factor of jun N-terminal kinase (JNK)/mitogen-activated protein kinase (MAPK) pathway, is the target of miR-138. The aim of this study was to investigate the role of miR-138 in $\text{H}_2\text{O}_2$-treated BV-2 cells.

METHODS: Murine microglia BV-2 cells were treated with $\text{H}_2\text{O}_2$ and tested for cell viability and miR-138 expression. The cells were then transfected with miR-138 agomir or miR-138 antagomir, and treated with 200 $\mu\text{M}$ $\text{H}_2\text{O}_2$ for 24 h. The cellular apoptosis was detected by Annexin V/PI staining. Expression of miR-138, MLK3, and other factors of JNK/MAPK pathway was detected.

RESULTS: After treatment of various concentrations of $\text{H}_2\text{O}_2$, the cell viabilities were reduced, and miR-138 expression was down-regulated. Compared to the control cells, over-expressing miR-138 in BV-2 cells reduced apoptosis rate from 24.2 % to 11.9 %. Western blot further showed that JNK, p-JNK, c-jun, p-c-jun, p38 MAPK, and p-p38 MAPK were down-regulated. Expression of pro-apoptosis factors iNOS and COX-2 were also down-regulated. Transfection of miR-138 antagomir produced the opposite effect of the transfection of miR-138 agomir.

CONCLUSION: miR-138 was able to reduce $\text{H}_2\text{O}_2$-induced apoptosis in BV-2 cells. The protective effect was related to the down-regulation of MLK3 proteins and sequentially inhibiting JNK/MAPK signaling pathway (Fig. 3, Ref. 27).

KEY WORDS: miR-138, mixed lineage kinase 3, apoptosis, microglia, jun N-terminal kinase.

Introduction

Spinal cord injury (SCI) refers to the spinal cord damage caused by trauma, diseases, or degeneration. A recent review demonstrated that traffic accidents and fall in the elderly population are the top two causes of SCI (1). Considering currently limited treatment methods and the rising aging population, SCI not only causes serious physical and psychological harms to patients, but also brings heavy economic and social burden (2). The study of the pathological changes in SCI can play a positive role in understanding the molecular mechanism of the injury, finding new drug targets, and improving the effect of interventions.

MicroRNAs (miRNAs) are the group of endogenous small non-coding RNAs that negatively regulate gene expression (3). MiRNAs are present in all kinds of systems, including the central nervous system, and their dysregulation was related to the multiple neurotraumatic diseases (4). Recently, the role of the miRNAs in SCI development and their value in SCI diagnosis were reported in several papers (5–7). The microarray analysis revealed that miR-138 expression was significantly enriched in the adult rat spinal cord following SCI (8). Mixed-lineage kinase 3 (MLK3) contains seeding sequences of miR-138 in its 3’-untranslated region (UTR) and was proved to be a direct target of miR-138 (9). MLK3 is a member of mitogen-activated protein kinase (MAPK) family and it can activate jun N-terminal kinase (JNK) signaling pathway via phosphorylating MAP2K members (10).

JNK signaling is one of the three major MAPK pathways and is a well-known regulator under oxidative stress (11). During SCI, oxidative conditions can increase expression of inducible nitric oxide synthase (iNOS) and trigger release of nitric oxide (NO) free radicals (12). JNK/MAPK signaling pathway is involved in oxidative stress-induced NO production and contributes to cytokine-induced apoptosis (13, 14). In the central nervous system, microglia cells play a role of resident macrophages and they are activated in response to brain injury (15). Apoptosis of microglia cells is responsible for neuronal degeneration in many diseases of central nervous system (16). Based on the previous studies, we inferred that miR-138 might play a protective role via regulating JNK/MAPK signaling and we tested this hypothesis in peroxide microglia cells.

Materials and methods

Chemicals and reagents

Dulbecco’s Modified Eagles Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from HyClone (GE Healthcare, Logan, USA). Nucleotides used for
transfection and qPCR were designed and synthesized by GenePharm Co. Ltd. (Shanghai, China). The antibodies used in western blot were purchased from Abcam (Cambridge, UK).

**Cell culture**

Murine microglia BV-2 cells (ATCC, Manassas, USA) were cultured with DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured at 37 ºC in a humidified incubator with 5 % CO₂. Prior to each assay, cells were inoculated in 6-well plates at a density of 2×10⁵ cells and cultured for 2 days to reach synchronization (70–80 % confluence).

**Induction of oxidative injury**

To induce oxidative injury, BV-2 cells (80 % confluence) were treated with various concentrations of H₂O₂ (100 μM, 200μM, 400 μM) for 24 h. The cells were then tested for cell viability by Enhanced Cell Counting Kit-8 (Beyotime Biotechnology, Shanghai, China) as instructed. The harvested cells were also tested for expression of miR-138.

**Cell transfection**

For over-expression of miR-138, BV-2 cells were transfected with 100 pmol of miR-138 agomir or agomir-negative control (agomir-NC). To inhibit miR-138 expression, BV-2 cells were transfected with 100 pmol of miR-138 antagomir or antagomir-negative control (antagomir-NC). All transfections were performed using Lipofectamine™ 2000 (Thermo Fisher Scientific, Carlsbad, USA) following manufacturer’s instructions.

After 48 h of transfection, the cells were treated with 200 μM H₂O₂ and cultured for another 24 h. Meanwhile, BV-2 cells without transfection were treated with hydrogen peroxide and taken as the peroxide control; BV-2 cells cultured as normal were used as normoxia control. At the end of H₂O₂ treatment, the cells were harvested for further assays.

**Cellular apoptosis detection**

Cellular apoptosis was measured using Annexin V-FITC/PI apoptosis assay kit (Sangon Biotech, Shanghai, China). Cells were harvested as described above, washed with PBS, and suspended in binding buffer. The cells were then stained with Annexin V-FITC and propidium iodide solution. After incubation for 15 min in the dark at room temperature, the cell suspensions were analyzed on CytoFlex flow cytometer (Beckman-Couter Inc., Brea, USA).

**Real-time quantitative PCR**

Total RNA was extracted from cells using miRNeasy mini kit (Qiagen, Hilden, Germany). The quality and concentration of RNA samples were measured using the NanoDrop 1000 (ThermoFisher Scientific, Waltham, USA). Complementary DNA (cDNA) was synthesized from RNA using PrimeScript RT Master Mix reagent kit (Takara Biotechnology, Co., Ltd., Dalian, China). The primer sequences used for reverse transcription were as follows: miR-138, 5'-GTCGTATCCAGTGAGGTCGCCGAGGTATTCGACATCGACTGACCA-3'; U6, 5'-AAAATATGGAAACGCT-3'. The oligo dT primer was used for reverse transcription of MLK3. qPCR was performed using the SYBR premix EX Taq kit (Takara Biotechnology, Co., Ltd.) on GeneAmp PCR System 9700 (Applied Biosystem, Foster, USA). The primers used for qPCR were as follows: 5'-TCCGAGCTGAC-TAAGTGTGTTGTCGA-3' and 5'-GTCGAGGTCGAGGT-3' for miR-138; 5'-AGCAACTCCGACCAAGGAC-3' and 5'-GGCTAAACCAGAACTCAAGCGTG-3' for MLK3; 5'-TCCGATCGTAAGCGGTTTC-3' and 5'-GTCGACGAGGTCCGAGGT-3' for U6; 5'-GGATTGTTTCGTATTTGGG-3' and 5'-GGAAGATGGTGATGGGAT-3' for GAPDH. The relative expressions of miR-138 and MLK3 were normalized to U6 or GAPDH expression using the 2-ΔΔCt method (17).

**Western blot**

The cells were harvested and then lysed in RIPA lysis buffer (Boster Biological Technology, Wuhan, China) to prepare total protein lysates. Protein (50 μg/lane) was separated by 10 % SDS-polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and blocked with 5 % non-fat milk. The membrane was incubated with specific primary antibodies overnight at 4 ºC and incubated with appropriate secondary antibodies for 1 h at room temperature. Antibodies were used at 1:1000 dilutions as followed: rabbit monoclonal anti-JNK1/JNK2/JNK3 (ab179461), rabbit monoclonal anti-JNK1/JNK2/JNK3 (phospho T183/T182/T221, ab124956), rabbit monoclonal anti-c-jun (ab32137), rabbit polyclonal anti-c-jun (phosphor S73, ab30620), rabbit monoclonal anti-p38 (phosphor Y182, ab47363), rabbit monoclonal anti-MLK3 (ab51068), rabbit polyclonal anti-p38 (phosphor T221, ab124956), rabbit monoclonal anti-p38 (phosphor Y182, ab47363), rabbit monoclonal anti-MLK3 (ab51068), rabbit polyclonal anti-iNOS (ab3523), rabbit polyclonal anti-COX2 (ab102005), rabbit polyclonal anti-GAPDH (ab9485), goat anti-rabbit IgG (horseradish peroxidase-conjugated, ab205718). Immuno-detection was measured using the enhanced chemiluminescence kit (Beyotime Biotechnology, Shanghai, China).

**Statistics**

The statistical analyses were performed using the SPSS 21.0 software. The quantitative data were presented as the mean ± standard deviation (SD). Comparisons between two independent groups were determined by t-test. Comparisons among multiple groups were determined by one-way ANOVA with Tukey’s test. A p < 0.05 was designated as statistically significant. All the experiments were repeated at least three times and representative results were exhibited in this study.

**Results**

**Expression of miR-138 in peroxide microglia cells**

Firstly, we tested whether the expression of miR-138 was altered when BV-2 cells were treated with hydrogen peroxide. As shown in Figure 1, peroxide conditions reduced cell viability and inhibited miR-138 expression. When BV-2 cells were treated with...
286 μM H₂O₂, cell viability was reduced to 85.4 % of the control cells (p < 0.05). Meanwhile, expression of miR-138 was also significantly inhibited compared to the control (p < 0.05). Treating with 400 μM H₂O₂ further suppressed cell viability and miR-138 expression. Although treating with 100 μM H₂O₂ could also inhibit miR-138 expression, cell viability was not significantly affected (p > 0.05). Therefore, we selected 200 μM as the treatment concentration of H₂O₂ in the following assays.

**Effect of miR-138 on cellular apoptosis under peroxide conditions**

SCI-induced oxidative stress is an important cause of cellular apoptosis. Next, we tested the effect of miR-138 on apoptosis of murine microglia BV-2 cells treated with H₂O₂. As shown in Figure 2, treating with 200 μM H₂O₂ increased apoptotic rate of BV-2 cells (peroxide vs. normoxia). When over-expressing miR-138 in BV-2 cells, apoptotic rate significantly decreased from 24.2 % to 11.9 % (p < 0.01), although the rate was still higher than the rate (4.2 %) in normally cultured cells. On the other hand, inhibiting miR-138 expression in BV-2 cells could aggravate cellular apoptosis (p < 0.01). The results indicated that miR-138 had anti-apoptosis role in peroxide BV-2 cells.

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**Fig. 1. Hydrogen peroxide inhibits cell viability and reduces miR-138 expression.** BV-2 cells were treated with various concentrations of H₂O₂ for 24 h and collected for detection. Cells without H₂O₂ were taken as the control. A. Cell viability tested by CCK-8 method. B. Relative expression of miR-138 tested by RT-PCR. * p < 0.05 vs control. ** p <0.01 vs control.

**Fig. 2. miR-138 reduces cellular apoptosis under peroxide condition.** BV-2 cells were transfected with miR-138 agomir or miR-138 antagonist and then treated with 200 μM H₂O₂ for 24 h. The cell apoptotic rate was tested by Annexin V/PI staining and flow cytometry (A & B). Cells without transfection and treated with hydrogen peroxide were taken as the peroxide control; cells cultured as normal were used as normoxia control. ** p < 0.01 vs peroxide control.
Effect of miR-138 on MLK3/JNK/MAPK pathway in peroxide microglia cells

Because MLK3, the key regulator of JNK/MAPK pathway, is a confirmed target of miR-138 (9), next we investigated whether miR-138 could regulate MLK3/JNK/MAPK signaling in peroxide BV-2 cells. The results of Figure 3A and 3B showed that over-expressing miR-138 led to less expression of MLK3 and inhibiting miR-138 promoted MLK3 expression. As indicated by the Western blot (Figure 3C), peroxide induced expression of MLK3, JNK (total and active form phospho-JNK), c-jun (total and phospho-c-jun), and p38 MAPK (total and phospho-p38), indicating that MLK3/JNK/MAPK was activated under peroxide condition. The activation effect could be eased up by miR-138 agomir and further enhanced by miR-138 antagonir. In addition, we also detected the effect of miR-138 on expression of pro-apoptosis factors iNOS and COX-2. Consistent with the activation of MLK3/JNK/MAPK, peroxide condition and miR-138 antagonir could also induce expression of iNOS and COX-2, while miR-138 agomir inhibited expression of these two proteins. The results indicated that miR-138 played an anti-apoptosis role via suppressing MLK3/JNK/MAPK signaling.

Discussion

It is well known that primary and secondary injuries during SCI cause devastating health problems in thousands of patients every year (18). Secondary lesions could be reversed and modulated; hence this has been a hot research topic of SCI (19). The oxidative stress and NO production triggered by SCI is an important pathophysiological process during the secondary injury. Excessive reactive oxygen species lead to severe tissue damage and neuronal death and affect the repair and function recovery of neurons after SCI (20). In this study, we showed that miR-138 could protect microglia BV-2 cells from \( H_2O_2 \)-induced apoptosis through inhibiting MLK3/JNK/MAPK signaling pathway.

In this study, we identified MLK3 as a novel target of miR-138. MLK3 is a well-known regulator of JNK by phosphorylation; activated JNK subsequently phosphorylated transcription factor c-jun and initiated expression of apoptosis-related genes (21). Therefore, MLK3 can be considered as a pro-apoptosis kinase, which was indicated by our results (Figs 2 and 3). Similar pro-apoptosis function of MLK3 was also reported in the previous studies (see review 22). When blocking MLK3 activity by viral protein, RNAi technology, or specific inhibitor, the pro-apoptotic effect could be
prevented (23, 24). In our study, inhibiting MLK3 expression by over-expressing miR-138 also reduced cell apoptosis.

In addition to MLK3, possible targets of miR-138 also include apoptosis-related genes like caspase-3, calpain 1, calpain 2, Bcl-2, c-Myc, and apoptosis-inducing factor (AIF) (8, 25). Some of these target genes were reported to be involved in the pathological process of SCI. Activation of caspase-3 was observed during a delayed paraplegia after spinal cord ischemia (26). After contusion-derived SCI, the induced apoptosis of the spinothalamic tract cells was correlated with prompt decrease in anti-apoptotic protein Bcl-2 (27). Therefore, the regulatory effect of miR-138 during SCI is a complex network and its anti-apoptotic role may be explained by multiple mechanisms.

In conclusion, our results showed that miR-138 was able to reduce hydrogen peroxide-induced apoptosis in BV-2 microglia cells. The protective effect might be related to the down-regulation of MLK3 proteins and sequentially inhibiting JNK/MAPK signaling pathway. These findings may provide new insights for understanding and treatment of SCI.

Learning points

- miR-138 expression was down-regulated, and cell viability was inhibited when murine microglia BV-2 cells were treated with H$_2$O$_2$.
- Over-expressing miR-138 in BV-2 cells could protect the cells from H$_2$O$_2$-induced apoptosis.
- The protective role of miR-138 was related with suppressing MLK3 expression and sequentially inhibiting JNK/MAPK signaling pathway.

References