RETRACTED ARTICLE: Long non-coding RNA SNHG16 reduces hydrogen peroxide-induced cell injury in PC-12 cells by up-regulating microRNA-423-5p

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ABSTRACT

Functions of long non-coding RNAs (lncRNAs) have been widely probed in spinal cord injury (SCI). But, the influences of lncRNA-small nucleolar RNA host gene 16 (lncRNA-SNHG16) is still not well documented in SCI. The study explored the impacts of SNHG16 on H2O2-injured PC-12 cells. PC-12 cells were disposed with H2O2, cell viability, apoptosis, autophagy and ROS level were detected. RT-qPCR was executed to explore SNHG16 or miR-423-5p expression in H2O2-stimulated cells. After transfection with pc-SNHG16 or miR-423-5p inhibitor, the functions of SNHG16 and miR-423-5p in H2O2-injured cells were studied. AMPK and ERK1/2 pathways were finally assessed by western blot. We found that H2O2 evoked cell injury in PC-12 cells, and repressed SNHG16 was observed in H2O2-disposed cells. Overexpressed SNHG16 prominently alleviated H2O2-induced cell injury as indicated by repressing cell apoptosis, autophagy and ROS level. Additionally, SNHG16 enhanced miR-423-5p expression, and miR-423-5p inhibition abrogated the protective effect of SNHG16 in H2O2-injured PC-12 cells. SNHG16 mediated AMPK and ERK1/2 pathways via up-regulating miR-423-5p in H2O2-injured PC-12 cells. In conclusion, these findings indicated that SNHG16 reduced H2O2-evoked cell injury by mediating miR-423-5p in PC-12 cells. The findings might uncover the effect of SNHG16 on SCI, which provide a new reference for remediying SCI.

HIGHLIGHTS

1. H2O2 evokes cell injury in PC-12 cells;
2. SNHG16 reduces H2O2-induced cell injury in PC-12 cells;
3. SNHG16 enhances miR-423-5p expression in H2O2-stimulated PC-12 cells;
4. MiR-423-5p inhibition abrogates the protective effect of SNHG16 in PC-12 cells;
5. SNHG16 mediates AMPK and ERK1/2 pathways by up-regulating miR-423-5p.

Introduction

The morbidity and mortality of trauma are gradually on the rise in recent years, and one of the most common traumas is spinal cord injury (SCI) [1]. SCI is a neurological disorder resulting in motor dysfunction even permanent paralysis, which not only difficult to treat but also expensive to rehabilitation therapy [2]. In the most instances, SCI is mainly caused by physical traumas, such as traffic accidents, falls, assaults and sport injuries [3,4]. Nevertheless, several non-traumatic diseases, such as insufficient blood flow, tumor and inflammatory injury can also induce the occurrence of SCI [5]. Oxidative stress is a series of adaptive responses caused by imbalance between reactive oxygen species (ROS) and antioxidant system [6]. It has reported that oxidative stress evoked by free radicals plays a vital role in the pathophysiology of SCI [7]. Therefore, take effective measures to prevent oxidative stress might be a potential strategy for the treatment of SCI.

Long non-coding RNA (IncRNA) is a kind of non-coding protein RNA, which functions to mediate the biological processes by regulating genes or competitive targeting miRNAs [8,9]. Study from Shi et al. summed up the emerging roles of lncRNAs in SCI [10]. Additionally, a recent publication reported the regulatory effect of lncRNA on inflammation after SCI [11]. Small nucleolar RNA host gene 16 (SNHG16) is a new discovered lncRNA, which is first found in neuroblastoma in 2009 [12]. Recently, a substantial amount of studies have been reported that SNHG16 could regulate cell proliferation, migration, invasion in various cancer cells, including breast cancer, esophagus cancer and gastric cancer [13–15]. However, the impacts of SNHG16 on SCI are still not well documented and need to be investigated.

MicroRNA (miRNA) is a class of small non-coding RNAs, which regulates the gene expression at the post-transcriptional level [16]. It is intriguing that miRNAs function as gene expression switches in the pivotal processes of the SCI [17]. MiR-423-5p is a verify miRNA, which has been reported to
promote cell autophagy in hepatocellular carcinoma cells [18]. Moreover, miR-423-5p is involved in mediating hypoxia/reoxygenation-induced apoptosis in renal proximal tubular epithelial cells [19]. Whereas, it is still unclear whether miR-423-5p is associated with the pathogenesis of SCI. In this study, we attempted to probe the protective effect of SNHG16 on H2O2-injured PC-12 cells, meanwhile to investigate the role of miR-423-5p in this process. The underlying mechanisms were uncovered by studying AMP-activated protein kinase (AMPK) and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways.

Materials and methods

Cell culture and treatment

PC-12 cells derived from a transplantable rat pheochromocytoma were purchased from American Type Culture Collection (ATCC® CRL-1721™, Rockville, MD, USA). The cells were cultured in ATCC-formulated RPMI-1640 medium (ATCC® 30–2001™) and supplemented with the heat-inactivated horse serum to a final concentration of 10% and fetal bovine serum (FBS) to a final concentration of 5% (ATCC® 30–2001™). These cells were maintained at 37 °C in a humidified incubator containing 5% CO2 and 95% air. Cells were grown in culture multi-well plates (Thermo Scientific, Nunc™, Denmark) and then subjected to H2O2 disposition. H2O2 at the concentrations of 1.25, 25, 50, 100 and 200 μM were utilized to administrate PC-12 cells for 24 h. The control group was stimulated with the culture medium without H2O2.

Detection of cell viability

After administration and incubation for 24 h, 1 × 10⁵ cells seeded in 24-wells plates were washed with phosphate buffered saline (PBS, GibcoBRL, Grand Island, NY, USA) and stained with trypan blue dye (Sigma-Aldrich, St. Louis, USA). After staining for 5 min, cells were observed under a microscope and viable cells were counted using cell counting chamber (Superior, Lausa-Koenigshofen, Germany). The formula for calculating cell viability is number of unstained cells/total number of cells observed × 100.

Cell transfection

The full-length of SNHG16 sequences were constructed in pcDNA3.1, which was named as pc-SNHG16. The empty pcDNA3.1 plasmid served as control group. Additionally, the expression plasmids of miR-423-5p inhibitor and the negative control (NC) were synthesized by GenePharma Co (Shanghai, China). These plasmids were transfected into PC-12 cells using lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA). Subsequently, the cells were collected after 48 h transfection for utilizing in the next experiments.

Cell apoptosis assay

For detection of cell apoptosis, the Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) was implemented. The treated or transfected cells were rinsed twice with PBS and re-suspended in buffer. Afterward, these cells were stained with 5 μL Annexin V-FITC for 15 min at room temperature in the dark. Thereafter, 5 μL PI was added to the cell suspension. Flow cytometry analysis was done using FACScan (Beckman Coulter, Fullerton, CA, USA). The data were analyzed using FlowJo software (Tree Star Software, San Carlos, CA, USA).

ROS assay

The intracellular ROS level was measured by flow cytometry using 2, 7-dichlorofluorescein diacetate (DCFH-DA, Nanjing Jiancheng, Nanjing, China). After treatment and transfection, PC-12 cells were washed twice with PBS and co-incubated with the serum-free culture medium containing 10 μM DCFH-DA in the dark for 20 min at 37 °C. Then, cells were washed with PBS again and were centrifuged to remove the supernatants. After this, the fluorescent intensities were measured using flow cytometer in 488 nm excitation and 521 nm emission.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

PC-12 cells were disposed with H2O2 or transfected with pc-SNHG16, miR-423-5p inhibitor and the relevant control plasmids. The total RNA from these cells was isolated by utilizing TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For detection of SNHG16 expression, One Step SYBR1 PrimeScript1-PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China) was employed for RT-qPCR analysis. For detection of miR-423-5p expression, Taqman MicroRNA Reverse Transcription Kit (Takara, Tokyo, Japan) and TaqMan® Universal Master Mix II (Applied Biosystems, Foster City, CA) were performed. The relative expression of SNHG16 or miR-423-5p was normalized to β-actin or U6. The data were calculated using 2⁻^ΔΔCt method [20].

Western blot assay

After transfection and stimulation, PC-12 cells were collected and lysed in RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with protease cocktail (Sigma-Aldrich). The total protein was quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Following electrophoresis, the separated proteins were transferred to nitrocellulose membranes. Afterward, the membranes were blocked in 5% BSA and were incubated with the special primary antibodies of Bax (ab32503), Pro-Caspase-3 (ab32150), Cleaved-Caspase-3 (ab32042), p62 (ab109012), LC3-II (ab51520), t-AMPK (ab32047), p-AMPK (ab32047), p-ERK1/2 (ab184699), p-ERK1/2 (ab214362) and β-actin (ab8227, Abcam, Cambridge, MA) at 4 °C overnight. After incubation, the membranes were rinsed and were incubated with the secondary antibody (ab205718, Abcam, Cambridge, MA) at 1:2000, Abcam) for 1 h at room temperature. The blots of immunoreactive proteins were visualized by exploiting enhanced chemiluminescence (ECL) reagents (Super Signal Enhanced...
Dura kit, Pierce, IL, USA). The image was scanned using the UMAX Vista S6E Flatbed Scanner (UMAX Data Systems Inc., Hsinchu, Taiwan). The intensity of the signals was analyzed using Image LabTM Software (Bio-Rad).

**Statistical analysis**

The results from multiple experiments were analyzed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL), as well as were shown as the mean ± standard deviation (SD). These data were calculated using Student's t-test and a one-way analysis of variance (ANOVA) followed by Tukey post-test for comparison between two or more groups. The level of significance was indicated as $p < .05$, $p < .01$ and $p < .001$.

**Results**

**H$_2$O$_2$ induced cell injury in PC-12 cells**

PC-12 cells were exposed to H$_2$O$_2$ for 12 h at the concentration range of 0–200 μM. Treatment of PC-12 cells with H$_2$O$_2$ resulted in a dose-dependent manner of cell viability (25 μM, $p < .05$; 50 and 100 μM, $p < .01$; 200 μM, $p < .001$), as indicated in Figure 1(A). Considering that 100 μM H$_2$O$_2$ administration significantly restrained cell viability to approximately 50%, therefore, this concentration of H$_2$O$_2$ was selected as the optimum concentration for treating PC-12 cells in the following experiments. We then measured cell apoptosis and the related factors (Bax and Pro/Cleaved-Caspase-3) in H$_2$O$_2$-treated cells. As shown in Figure 1(B–D), we observed that cell apoptosis and the protein levels of Bax and Cleaved-caspase-3 were all increased by H$_2$O$_2$ treatment as relative to control group (all $p < .001$). Additionally, H$_2$O$_2$ treatment slightly inhibited the protein level of p62 as well as significantly increased the ratio of LC3-II/LC3-I compared to control group ($p < .001$, Figure 1(E)). Further, the intracellular ROS level was also increased in H$_2$O$_2$-treated cells as compared to that in non-treated cells ($p < .01$, Figure 1(F)). Results indicated that H$_2$O$_2$ evoked cell injury by induction of cell apoptosis and autophagy in PC-12 cells.

**SNGH16 attenuated H$_2$O$_2$-induced cell injury in PC-12 cells**

The expression level of SNGH16 was determined in PC-12 cells after administration with 100 μM H$_2$O$_2$. The results...
revealed that H2O2 administration remarkably declined the expression level of SNGH16 as relative to control group (p < .01, Figure 2). Thereafter, we explored whether SNGH16 can protect PC-12 cells against H2O2-induced cell injury. After transfection with pc-SNGH16 and pcDNA3.1 plasmids, we observed that the expression level of SNGH16 was evidently up-regulated in pc-SNGH16-transfected cells compared to that in pcDNA3.1-transfected cells (p < .001, Figure 3(A)). In the subsequent experiments, 100 μM H2O2 was utilized to dispose above cells to further explore the alteration of cell injury after transfection. Cell viability detected by CCK-8 assay was apparently increased in H2O2+pc-SNGH16 group compared to that in H2O2+pcDNA3.1 group (p < .05, Figure 3(B)). Whereas, cell apoptosis and the protein levels of Bax and Cleaved-Caspase-3 were all decreased in H2O2+pc-SNGH16

Figure 2. H2O2 administration down-regulated SNGH16 expression in PC-12 cells. After stimulation with 100 μM H2O2 for 12 h, PC-12 cells were collected, and SNGH16 expression in H2O2-treated cells was determined with RT-qPCR assay. Data were presented as the mean ± SD of three independent experiments. **p < .01 versus untreated control cells.

Figure 3. SNGH16 alleviated H2O2-induced cell injury in PC-12 cells. (A) PC-12 cells were transfected with pcDNA3.1 and pc-SNGH16, after 48 h transfection, the transfection efficiency was detected via utilizing RT-qPCR assay. The transfected cells were subsequently disposed with 100 μM H2O2 for 12 h, (B) cell viability, (C) cell apoptosis, (D) Bax and Pro/Cleaved-Caspase-3, (E and F) p62, LC3-I and LC3-II protein levels and (F) the intracellular ROS level was examined by CCK-8, flow cytometry, western blot and DCFH-DA dye assays. Data were presented as the mean ± SD of three independent experiments. *p < .05, **p < .01, ***p < .001 versus pcDNA3.1, H2O2 or H2O2+pcDNA3.1 group; ns: no significant difference.
Results suggested that SNGH16 could attenuate H2O2-induced cell injury in PC-12 cells (p < .01 or p < .001, Figure 3(C,D)). Western blot analytical results showed that SNGH16 overexpression restored the expression level of p62, while decreased the ratio of LC3-II/LC3-I in H2O2-treated cells (p < .001, Figure 3(E,F)). More importantly, we observed that SNGH16 overexpression inhibited H2O2-triggered ROS burst in PC-12 cells (p < .05, Figure 3(G)). These results suggested that SNGH16 could attenuate H2O2-induced cell injury in PC-12 cells.

**SNGH16 up-regulated miR-423-5p expression in H2O2-treated PC-12 cells**

Next, we tried to explore whether miR-423-5p was involved in the protective effect of SNGH16 on H2O2-induced cell injury, the expression level of miR-423-5p in PC-12 cells disposed with H2O2 alone or transfected with pc-SNGH16 and pcDNA3.1 plasmids following H2O2 treatment was determined. The results showed that H2O2 administration significantly decreased miR-423-5p expression compared to control group (p < .01). However, SNGH16 overexpression apparently increased miR-423-5p expression in H2O2-treated PC-12 cells (p < .01, Figure 4). These results indicated that SNGH16 could enhance miR-423-5p expression in H2O2-injured PC-12 cells.

**MiR-423-5p inhibition abrogated the protective effect of SNGH16 against H2O2-induced cell injury**

MiR-423-5p inhibitor was transfected into PC-12 cells to alter miR-423-5p expression. NC plasmid transfected into PC-12 cells served as a control group. As expected, we found that the expression level of miR-423-5p was remarkably down-regulated in miR-423-5p inhibitor-transfected cells compared to that in NC-transfected cells (p < .001, Figure 5(A)). PC-12 cells were then co-transfected with pc-SNGH16 and miR-423-5p inhibitor and treated with H2O2 to further investigate the effect of miR-423-5p inhibition on H2O2-induced cell injury. Results in Figure 5(B) showed that miR-423-5p inhibition significantly reversed the increased effect of SNGH16 on cell viability in H2O2-treated PC-12 cells (p < .001). Moreover, the restrained effects of SNGH16 on cell apoptosis as well as the protein levels of Bax and Cleaved-Caspase-3 were all reversed by miR-423-5p inhibition in H2O2-treated PC-12 cells (p < .01 or p < .001, Figure 5(C,D)). Similarly, the inhibitory effects of SNGH16 on cell autophagy and the intracellular ROS level were also reversed by miR-423-5p inhibition in H2O2-treated PC-12 cells (p < .01 or p < .001, Figure 5(E-G)). Above results indicated that SNGH16 attenuated H2O2-induced cell injury in PC-12 cells might by regulation of miR-423-5p expression.

**SNGH16 regulated AMPK and ERK1/2 signaling pathways by up-regulating miR-423-5p**

Western blot assay was finally implemented to examine the regulatory impacts of SNGH16 on AMPK and ERK1/2 signaling pathways in H2O2-injured PC-12 cells. After stimulation with H2O2, we observed that the phosphorylation of AMPK was decreased, but the phosphorylation of ERK1/2 was increased in PC-12 cells (p < .05 or p < .001). However, overexpressed SNGH16 notably promoted the protein level of p-AMPK, simultaneously declined the protein level of p-ERK1/2 in H2O2-treated cells (p < .05 or p < .001). Interestingly enough, miR-423-5p inhibition observably reversed the effects of SNGH16 on the phosphorylation of AMPK and ERK1/2 (p < .05 or p < .01, Figure 6(A-D)). These results hinted that SNGH16 regulated AMPK and ERK1/2 signaling pathways by up-regulation of miR-423-5p in H2O2-injured PC-12 cells.

**Discussion**

SCI is a serious trauma that results in devastating physiological consequences [21]. Increasing interest has been brought to bear on the prevention, treatment and rehabilitation of SCI [22]. Recently, a larger number of IncRNAs are discovered in the spinal cord and their expression level can be changed following SCI, which indicates that IncRNAs might be key regulators in SCI [10]. In our study, we constructed a cell injury model induced by H2O2 to imitate the process of SCI and the effect of SNGH16 on H2O2-injured PC-12 cells was investigated. The results demonstrated that SNGH16 lightened H2O2-induced cell injury in PC-12 cells. More interestingly, we found that miR-423-5p expression was increased by SNGH16 overexpression in H2O2-treated cells. Meanwhile, miR-423-5p suppression significantly abrogated the protective effect of SNGH16 against H2O2-induced cell injury. Finally, we observed that AMPK and ERK1/2 signaling pathways were mediated by SNGH16 through up-regulation of miR-423-5p expression.

Currently, the biological roles of IncRNAs in SCI have received considerable critical attention. After SCI, IncRNAs have been found to be involved in regulating the processes of inflammation and angiogenesis [23,24]. Utilization of IncRNAs might provide an attractive proposal for the development of spinal cord repair. SNGH16 as an oncogene has been widely reported in various cancers, which is associated with poor prognosis and regulates the cancer cells growth.
As far as we know, most of the studies about SNGH16 are focused on cancers; however, the effect of SNGH16 on SCI has not been reported. Cell apoptosis and autophagy are two important biological processes, which can be enhanced by oxidative stress [27]. H2O2 has been widely utilized to construct oxidative stress injury model in many diseases [28,29]. In the present study, we also construct H2O2-induced cell injury model as the foundation for exploration of the effect of SNGH16 on the pathogenesis of SCI. The findings demonstrated that SNGH16 expression was down-regulated in H2O2-stimulated PC-12 cells. Moreover, SNGH16 overexpression could alleviate H2O2-evoked cell injury in PC-12 cells by repression of cell apoptosis and autophagy.

Microarray data demonstrated that SCI could alter miRNAs expression patterns in the murine models [30]. Additionally, evidence from Hu et al. found the anti-apoptotic effect of miR-21 after contusion SCI in rats [31]. Likewise, miR-126 has reported to promote angiogenesis and alleviate inflammation after SCI [32]. MiR-423-5p has been confirmed to be a biomarker for heart failure [33]. Moreover, detection of miR-423-5p expression in serum can be used for diagnosis of colorectal cancer [34]. However, whether miR-423-5p is implicated in the pathogenesis of SCI remains unclear. In the study, we found that miR-423-5p expression was up-regulated by SNGH16 overexpression in H2O2-disposed PC-12 cells. To further probe the impacts of miR-423-5p on H2O2-induced cell injury in PC-12 cells, we utilized miR-423-5p inhibitor to repress miR-423-5p expression in PC-12 cells. The results stated that miR-423-5p inhibition obviously abolished the protective impacts of SNGH16 in PC-12 cells. This seemed to indicate the important role of miR-423-5p in the development of SCI.

AMPK is a key regulator of cellular and organismal metabolism in eukaryotes, which can coordinate cell growth, autophagy and cell polarity [35]. One study reported that resveratrol exerted the protective effect against SCI by mediating cell autophagy and apoptosis regulated by activation of AMPK pathway [36]. ERK1/2 is a momentus member of the mitogen-activated protein kinase (MAPK) family, which can be activated after SCI [37]. The study of Cao et al. displayed that activation of ERK1/2 signaling could impair neural cell...
migration, neurogenesis, synapse formation, and dendritic spine development in spinal cord [38]. Evidence from Tiziana et al. confirmed that PD98059, an inhibitor of MAPK3/MAPK1 reduced the development of SCI through repression of ERK1/2 activation [37]. On the basis of these researches, we explored whether SNGH16 exerted the protective activity against SCI via regulating AMPK and ERK1/2 pathways. The results in the present study revealed that SNGH16 activated AMPK signaling pathway but blocked ERK1/2 signaling pathways in H2O2-treated PC-12 cells. Moreover, the process was mediated through enhancement of miR-423-5p. These data indicated that AMPK and ERK1/2 pathways might participate to mediate the development of SCI. Nonetheless, more underlying mechanisms of AMPK and ERK1/2 pathways in SCI are worth further investigated.

Taken together, this study testified that SNGH16 exerted the protective effect against H2O2-evoked cell injury in PC-12 cells. AMPK and ERK1/2 pathways were regulated by SNGH16 through enhancement of miR-423-5p in H2O2-injured PC-12 cells. These observations might provide a novel management for the treatment of SCI. The clinical and in vivo experiments are still need to further explore the effect of SNGH16 on SCI in the future.

Disclosure statement
No potential conflict of interest was reported by the authors.

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