Silencing of Long Noncoding RNA Nespas Aggravates Microglial Cell Death and Neuroinflammation in Ischemic Stroke

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Background and Purpose—Ischemic stroke is one of the leading causes of morbidity and mortality worldwide and a major cause of long-term disability. Recently, long noncoding RNAs have been revealed, which are tightly associated with several human diseases. However, the functions of long noncoding RNAs in ischemic stroke still remain largely unknown.

Methods—We used in vivo models of middle cerebral artery occlusion and in vitro models of oxygen-glucose deprivation to illustrate the effect of long noncoding RNA Nespas on ischemic stroke.

Results—We found expression of Nespas was significantly increased in ischemic cerebral tissues and oxygen-glucose deprivation–treated BV2 cells in a time-dependent manner. Silencing of Nespas aggravated middle cerebral artery occlusion operation–induced IR injury and cell death. In addition, proinflammatory cytokine production and NF-κB (nuclear factor-κB) signaling activation were inhibited by Nespas overexpression. TAK1 (transforming growth factor-β–activated kinase 1) was found to directly interact with Nespas, and TAK1 activation was significantly suppressed by Nespas. At last, we found Nespas-inhibited TRIM8 (tripartite motif 8)-induced K63-linked polyubiquitination of TAK1.

Conclusions—We showed that Nespas played anti-inflammatory and antiapoptotic roles in cultured microglial cells after oxygen-glucose deprivation stimulation and in mice after ischemic stroke by inhibiting TRIM8-related K63-linked polyubiquitination of TAK1.

Visual Overview—An online visual overview is available for this article. (Stroke. 2019;50:1850-1858. DOI: 10.1161/STROKEAHA.118.023376.)

Key Words: cell death ■ lncRNA ■ neuroinflammation ■ stroke ■ TAK1

Stroke is one of the 3 major causes of human death, and ischemic stroke accounts for 80% to 85% of all strokes.1 Although many clinical trials of stroke treatment have been completed, up to date, thrombolysis by intravenous recombinant tPA (tissue-type plasminogen activator) is the only clinically effective therapeutic strategy for ischemic stroke.2 However, only 20% of patients with stroke receive this treatment because of its narrow therapeutic window (<4.5 hours after onset of stroke symptoms) and its association with an increased risk of symptomatic intracerebral hemorrhage.3 Therefore, development of effective therapies is urgently required.

Ischemic stroke initiates complex processes including neuroinflammation, oxidative stress, excitotoxicity, and apoptosis, therefore, leading to the neuronal death.4 Microglial cells, which are rapidly and time dependently activated after ischemia, are considered as the major cellular contributors to neuroinflammation in ischemic stroke,5 and cytokines such as TNF-α (tumor necrosis factor-α), IL (interleukin)-1β, IL-6, and reactive oxygen species produced by microglial cells have been reported as mainly inflammatory factors in ischemic stroke–induced brain injury.6,7 Numerous studies investigating the underlying mechanisms of microglial cells induced neuroinflammation to...
demonstrate a critical role of NF-κB (nuclear factor-xB) in the induction of these inflammatory cytokines.\textsuperscript{8,9} Long noncoding RNAs (lncRNAs) are an important class of noncoding RNA, whose transcription sequence is >200 nucleotides.\textsuperscript{10} Because they cannot be translated into proteins, lncRNAs were originally viewed as the noise of translational process. But recently, lncRNAs have been reported to participate in chromatin modification, transcriptional regulation, post-transcriptional regulation, X chromat inactivation, genomic imprinting, splicing, transcription interference, cell cycle regulation, epigenetic regulation, and play an important role in numerous pathological and physiological processes.\textsuperscript{11,12} However, whether lncRNAs are involved in the neuroinflammation and ischemic stroke still remains largely unknown.

LncRNA-Nespas is known to be mainly expressed in the progress zone, menenchyme and ectoderm of the limb, and the neural tube, and it has been reported to play essential roles in osteoarthritis progression and serves as a potential new prognostic indicator for various cancers.\textsuperscript{13} But recently, lncRNAs have been reported to participate in chromatin modification, transcriptional regulation, post-transcriptional regulation, X chromat inactivation, genomic imprinting, splicing, transcription interference, cell cycle regulation, epigenetic regulation, and play an important role in numerous pathological and physiological processes.\textsuperscript{11,12} However, whether lncRNAs are involved in the neuroinflammation and ischemic stroke still remains largely unknown.

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LncRNA-Nespas as a potential target for related medical research.

Methods

Ethics Approval

All animal experimental procedures and animal care were approved by the Ethics Committee of Capital Medical University and were conducted in accordance with the guidelines of the National Institutes of Health on the care and use of animals. The data that support the findings of this study are available from the corresponding author on reasonable request.

Cerebral Ischemia/Reperfusion Induced by Surgical Middle Cerebral Artery Occlusion

Focal cerebral ischemia was induced in mice by intraluminal middle cerebral artery occlusion (MCAO) as described previously.\textsuperscript{14} Briefly, male mice (8–10 weeks, 23–25 g) were anesthetized with 1.5% to 3% isoflurane (Henry Schein Animal Health). A 2-cm length of a 7 μm silicon guide tube was inserted into the right common carotid artery up to the internal carotid artery until regional cerebral blood flow was reduced to <16% of baseline. After 60 minutes of MCAO, blood flow was restored by removing the suture, and the mice were allowed to recover for 1 to 7 days. Changes in cerebral blood flow, arterial blood gases, mean arterial pressure, and heart rate were monitored in animals 30 minutes before, during, and after MCAO. The rectal temperature was controlled at 37 ±0.5°C during surgery.

Stereotaxic Injection of siRNA in Mice

Mice were anesthetized and fixed to a stereotaxic apparatus (Stoelting, Kiel, WI). A total of 5 μL siRNA-negative control or siRNA-Nespas (100 μmol/L) and lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) was mixed gently, incubated at room temperature for 20 minutes, and injected (intracerebroventricular) into the right cerebral ventricle of mice. One day post-injection, MCAO operation was established.

Cell Cultures and Oxygen-Glucose Deprivation

Microglial cells from mouse brain were isolated and confirmed by immunofluorescence labeling according to the previous study.\textsuperscript{15} Mouse BV2 microglial cells were purchased from ATCC (Gaithersburg, MD). The cells were cultured in normal culture medium containing DMEM solution mixed with 10% FBS and incubated in a humidified incubator at 37°C in 5% CO₂. Cells were grown to 85% to 95% confluence before use. BV2 cells were treated with siRNA-negative control or siRNA-Nespas for 48 hours or treated with control or Nespas plasmid for 48 hours before oxygen-glucose deprivation (OGD) exposure. To mimic ischemia-like conditions in vitro, mouse BV2 cultures were exposed to OGD for 24 hours.

Neurological Deficit Scores

Neurological deficits were evaluated as previously reported\textsuperscript{1} by using a 9-point scale at 24 and 72 hours after MCAO induction on the basis of the following criteria: (1) absence of neurological deficits (0 points); (2) either left forelimb flexion after suspension by the tail or failure to fully extend the right forepaw (1 point); (3) left shoulder adduction after suspension by the tail (2 points); (4) decreased resistance to a lateral pull toward the left (3 points); (5) spontaneous movement in all directions with circling to the left only if pulled by the tail (4 points); (6) spontaneously circling or walking only to the left (5 points); (7) walking only when stimulated (6 points); (8) no response to stimulation (7 points); and (9) stroke-related death (8 points).

Infarct Volume Determination

At the indicated times (24 and 72 hours) after MCAO, the brains were removed and sliced into 71-mm coronal sections, which were immersed in a 2% 2,3,5-triphenyl-2H-tetrazolium chloride solution for 15 minutes at 37°C. Normal brain tissue was stained a red color, whereas injured tissue stained a pale gray color. The sections were photographed and analyzed using Image-Pro Plus 6.0 (Media Cybernetics), and the infarct volume (percentage) of the 7 slices was calculated after correcting for edema as described previously.\textsuperscript{3}

Western Blot Analysis and Ubiquitination Assay

Microglial cells were harvested using radio immunoprecipitation assay buffer (MDL Biotech, Beijing, China) after MCAO or OGD treatment. The protein concentration was measured using Bio-Rad quantification assay (Bio-Rad Laboratories, Hercules, CA). Proteins were separated using 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica). The membrane was blocked with 3% nonfat dry milk for 2 hours. The antibodies for TAK1 or K63-linked ubiquitin (Abcam, Cambridge), p65, phospho-p65, IKKβ (IκB kinase β), phospho-IKKβ, IkBα (inhibitor of NF-xB α), phospho-IκB (Cell Signaling Technology, Inc, Beverly), TRIM8, GAPDH, hemagglutinin, Flag (Santa Cruz Biotechnology, Santa Cruz) were added and incubated overnight at 4°C. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), the target protein was visualized by enhanced chemiluminescence (Thermo Fisher Scientific, Bremen, Germany). The ubiquitination of TAK1 was detected as described.\textsuperscript{17}

RNA Isolation and Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted with TRizol reagent according to the manufacturer’s instructions. A LightCycler (ABI PRISM 7000; Applied Biosciences) and the SYBR RT-PCR kit (Takara Biotechnology, Dalian, China) were used for real-time polymerase chain reaction analysis. GAPDH was used as the internal control, and the 2^-ΔΔCT method was used to evaluate the relative quantities of each amplified product in the samples. Primer sequences used in quantitative polymerase chain reaction were shown in Table I in the online-only Data Supplement.

RNA-Binding Protein Immunoprecipitation Assays

The RNA-binding protein immunoprecipitation (RIP) assay was performed as described previously.\textsuperscript{18} After treatment of OGD, BV2 cells were lysed in RIP lysis buffer, after incubation with RIP buffer consisting of 150 mmol/L KCl, 25 mmol/L Tris pH 7.4, 5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.5% NP40, and 100 U/mL Protein A/G agarose beads (Santcapture, Beijing, China). The RIP pellets were washed twice with lysis buffer, and the bound RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) as described.
RNAse inhibitor SUPERASin. The RIP buffer contained magnetic beads conjugated with anti-TAK1 antibody (Millipore) or negative control IgG. The samples were incubated with proteinase K and then immunoprecipitated RNA was isolated. Purified RNAs subjected to real-time polymerase chain reaction to determine the presence of binding targets using respective primers for mouse Nespas as follows: forward: 5′-AGATGCTGAACCCTGCACAA-3′; reverse: 5′-AGCTAGGATCACATCGGGGT-3′.

Dual-Luciferase Reporter Gene Assays
Luciferase reporter constructs containing the NF-κB promoter region were cloned into pGL3-based vectors, then temporarily transfected with 1 μg of the promoter reporter plasmid into siRNA-negative control–treated or siRNA-Nespas–treated BV2 cells using jetPEI (polyplus-transfection), and 50 ng of the phRL-TK plasmid was cotransfected into the cells to verify transfection efficiency. After 48 hours of transfection, the luciferase activities were measured on a spectraMax M5 reader (Molecular Devices, CA) using the dual-luciferase reporter assay system (Promega, Madison, WI).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End Labeling Assay (TUNEL) Assay
TUNEL assay was performed using DeadEnd Fluorometric TUNEL system (Promega) according to the manufacturer’s instructions. In brief, microglial cells were fixed with 4% formaldehyde in PBS and then incubated in TUNEL reaction mixture in the dark for 1 hour at 37°C. Labeled samples were visualized with a fluorescence microscope (OLYMPUS IX71; Shinjuku-ku, Tokyo, Japan) and counted using Image J software (National Institutes of Health).

Statistical Analysis
All data are presented as mean±SD of 3 experiments. Statistical significance was determined with the 2-tailed Student t test, P<0.05 considered statistically significant. Differences among ≥3 groups were statistically analyzed by 1-way ANOVA followed by Bonferroni post hoc test.

Results
Expression of Nespas Is Upregulated After Ischemia In Vivo and In Vitro
To investigate the role of IncRNA-Nespas after ischemic stroke, at first, we established the mouse MCAO model and detected Nespas expression in the brain tissue at 6, 12, 24, and 72 hours after ischemia. As shown in Figure 1A, expression of Nespas was greatly increased in the hippocampal region, striatal, cerebral cortical, and plasma in MCAO mice compared with the sham group in a time-dependent manner. We next established OGD model in BV2 mouse microglia cells and HT22 mouse hippocampal cells. Interestingly, we observed
that expression of Nespas has significantly increased in BV2 cells in a time-dependent manner but not HT22 cells after OGD treatment (Figure 1B and 1C). These data suggested that Nespas may have a critical role in microglial cells during the progression of ischemic stroke.

Silencing of Nespas Aggravates I/R-Induced Ischemic Brain Damage

To determine whether upregulation of Nespas participates in the pathophysiology of cerebral ischemic stroke, intraventricular injection of Nespas siRNA was used to knock down the expression of Nespas in the brain of MCAO mice. The efficiency of siRNA injection in brain was detected (Figure 2A). As shown in Figure 2B and 2C, silencing of Nespas showed a larger cerebral infarct volume and a significantly more severe neurological deficit (Figure 2D) in response to ischemic insults. These data suggested that Nespas could alleviate I/R-induced ischemic brain damage.

Silencing of Nespas Exacerbates I/R-Induced Microglial Cell Death

Because of the high expression of Nespas in microglial cells, we speculated that Nespas might impact the viability of microglia and then affect cerebral ischemia injury. Therefore, we next examined whether Nespas could affect I/R-induced microglial cell death. The isolated microglial cells from mouse brain were confirmed by immunofluorescence labeling of CD11b, which is a widely used microglial marker (Figure IA in the online-only Data Supplement). We found in an MCAO model that silencing of Nespas markedly increased the number of apoptotic microglial cells (Figure 3A). Consistently, we observed that the expression of proapoptotic protein cleaved caspase3 and Bax (BCL2-associated X) was significantly enhanced, but antiapoptotic protein Bcl-2 (B-cell lymphoma-2) expression was decreased in microglial cells of siRNA-Nespas–treated mice after I/R (Figure 3B; Figure IB in the online-only Data Supplement). To further confirm this phenomenon, we
evaluated the antiapoptotic effect of Nespas in cultured BV2 cells in vitro after OGD stimulation. As shown in Figure 3C, we found cell viability was decreased and LDH (lactate dehydrogenase) release, which is an indicator of cell injury, was markedly increased in siRNA-Nespas–treated BV2 cells after OGD. Consistent with the in vivo results, we observed that after OGD treatment, cleaved caspase3 and Bax expression was increased and Bcl-2 expression was decreased in Nespas silenced BV2 cells (Figure 3D; Figure IC in the online-only Data Supplement). Taken together, these data suggested that Nespas alleviated I/R-induced microglial cell death.

**Nespas Alleviates I/R-Induced Neuroinflammation Through Suppressing NF-κB Activation**

Microglial cells have been considered as the major cellular contributors to neuroinflammation in ischemic stroke, and NF-κB signaling pathway has been proved to be the main regulator of neuroinflammation1,2; therefore, we examined the effect of Nespas on I/R-induced neuroinflammation in microglial cells and the effect of Nespas on NF-κB activation. As shown in Figure 4A, we found that siRNA-Nespas treatment significantly increased the mRNA expression level of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, MCP-1, VCAM-1, and ICAM-1 in BV2 microglial cells after OGD treatment (Figure 4C; Figure ICD in the online-only Data Supplement). Consistently, we observed that the expression of TNF-α, IL-1β, IL-6, MCP-1, VCAM-1, and ICAM-1 was inhibited in Nespas-overexpressed BV2 cells after OGD treatment (Figure 4D).
Nespas Interacts With TAK1

It has been reported that TAK1 played critical roles in the regulation of NF-κB activation; however, the relationship between lncRNA and TAK1 has not been well studied. Therefore, we detected the role of Nespas in TAK1-induced NF-κB activation. As shown in Figure 5A, interestingly, we observed that Nespas obviously suppressed NF-κB activation induced by TAK1. In addition, we performed RIP assay, and we found that Nespas could directly interact with TAK1 (Figure 5B). We also detected the effect of Nespas on TAK1 expression, and the data showed that mRNA level of TAK1 was not affected by Nespas overexpression (Figure 5C). Taken together, these data suggested that lncRNA-Nespas could directly interact with TAK1.

Nespas Inhibits TRIM8-Induced K63-Linked Polyubiquitination of TAK1

Because of the invalid effect of Nespas on TAK1 mRNA expression, we suspected whether Nespas could affect the modulation level of TAK1. K63-linked polyubiquitination has been shown to play great roles in TAK1 activation. Therefore, we performed the immunoprecipitation experiment to examine the effect of Nespas on K63-linked polyubiquitination of TAK1. We observed that after OGD treatment, the K63-ub level of TAK1 was greatly enhanced in siRNA-Nespas-treated BV2 cells (Figure 6A). Because of the lack of ubiquitin ligase activity, we suspect that this effect may be because of the participation of another TAK1-related E3 ubiquitin ligase or deubiquitinating enzyme. TRIM8 has been reported to directly interact with TAK1 and promote K63-linked polyubiquitination of TAK1. Therefore,
we examined the effect of Nespas on the interaction between TRIM8 and TAK1 after OGD stimulation. We found that after OGD treatment, the interaction between TRIM8 and TAK1 was greatly enhanced. However, silencing of Nespas significantly promotes the interaction, and overexpression of Nespas obviously suppressed OGD-induced interaction between TRIM8 and TAK1 (Figure III in the online-only Data Supplement; Figure 6B). We constructed several deletion mutants of TAK1 during immunoprecipitation experiment, and we found that TRIM8 interacts with region 200–291, located in the internal kinase domain of TAK1 (Figure 6C). Most importantly, we observed that the interaction between TRIM8 and TAK1 deletion mutants was obviously suppressed in Nespas-overexpressed BV2 cells after OGD stimulation (Figure 6D). Taken together, these findings indicated that Nespas inhibits TRIM8-induced K63-linked polyubiquitination of TAK1 through suppressing the interaction between TRIM8 and TAK1.

Discussion
To the best of our knowledge, the current study is the first article to demonstrate the role of IncRNA-Nespas in ischemic stroke, and it is also the first report to reveal that Nespas could inhibit the interaction between TRIM8 and TAK1, therefore suppressed TRIM8-induced K63-linked polyubiquitination of TAK1 and NF-κB activation. These findings suggested Nespas as a potential therapeutic target for the treatment of cerebral ischemia.

Ischemic stroke is one of the major causes of adult morbidity and mortality, representing a large health challenge around the world, and there is an urgent need for better understanding of the molecular pathogenesis during stroke and developing novel strategy against neuronal cerebral ischemia damages.8,19 Recently, more and more researchers revealed that different kinds of proteins or RNAs played great roles in molecular pathogenesis of ischemic stroke.20–23 In the current study, we found that Nespas expression was significantly increased in ischemic cerebral tissues and plasma of acute ischemic stroke as well as in the cultured BV2 microglial cells after OGD stimulation but not HT22 hippocampal cells. In addition, silencing of Nespas showed a larger cerebral infarct volume and a significantly more severe neurological deficit in MCAO mice, which indicated that Nespas could alleviate I/R-induced ischemic brain damage.
LncRNAs have been reported to participate in lots of complicated biological processes including ischemic stroke. LncRNA-H19 has been shown to promote neuroinflammation in ischemic stroke through driving HDAC1 (histone deacetylase 1)-dependent M1 microglial polarization.\(^2\) LncRNA-Fos downstream transcript (FosDT) was found to promote ischemic brain injury by interacting with RE-1-silencing transcription factor (REST)-associated chromatin-modifying proteins.\(^3\) Zhang et al\(^4\) suggested that LncRNA-Malat1 played antiapoptotic and anti-inflammatory roles in ischemic stroke by physically binding to Bim and E-selectin. In the present study, we observed that LncRNA-Nespas significantly suppressed microglial cell death after MCAO or OGD treatment; in addition, the expression of proinflammatory cytokines was also suppressed by Nespas overexpression, which suggested that Nespas played critical protective roles in ischemic stroke through alleviating cell apoptosis and neuroinflammation; in addition, we also found the regulation is not relevant with sexual dimorphism; the expression of Nespas and the effect of neuroinflammation regulation in female or male mice were consistent (female data not shown), which suggested that Nespas is a basic regulator of this process and there is no sexual dimorphism in Nespas-regulated immune responses.

TAK1 is one of the most crucial regulators of the innate immunity and the proinflammatory signaling pathway. Inhibition of TAK1 was shown to be an efficient way to interfere with multiple mechanisms in ischemic stroke, and TAK1 inhibitors were proved as efficient therapeutic method for the treatment of ischemic stroke.\(^5\) It has been reported that K63-linked polyubiquitination of TAK1 is necessary for NF-κB activation, and TRIM8 was reported as the main E3 ligase 1). In the current study, we observed that LncRNA-Nespas significantly suppressed microglial cell death after MCAO or OGD treatment; in addition, the expression of proinflammatory cytokines was also suppressed by Nespas overexpression, which suggested that Nespas played critical protective roles in ischemic stroke through alleviating cell apoptosis and neuroinflammation; in addition, we also found the regulation is not relevant with sexual dimorphism; the expression of Nespas and the effect of neuroinflammation regulation in female or male mice were consistent (female data not shown), which suggested that Nespas is a basic regulator of this process and there is no sexual dimorphism in Nespas-regulated immune responses.

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Drs Deng and Chen performed the experiments and drafted the manuscript. Dr Wang analyzed the data. Drs Gao and Jin performed the neurological evaluation and contributed to the data interpretation. Drs Lv and Zhang performed the immunofluorescence image analysis. Drs Sun and Liu assisted with the cell culture and Western blot. Drs Mo, Ma, and Song performed the experiments and collected the data. Dr Huo assisted with the experiment design. Drs Yan and Miao conceived and designed the study, analyzed and interpreted the data, and critically revised the manuscript.

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DISCLOSURES

None.

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