Roles of the miR-137-3p/CAPN-2 gene pair in ischemia-reperfusion-induced neuronal apoptosis through modulation of p35 cleavage and subsequent caspase-8 overactivation

He Wang
Department of Anesthesiology, First affiliated Hospital, China Medical University
Qian Yu
Department of Thoracic surgery, Fourth Affiliated Hospital , China Medical University
Zai-Li Zhang
Department of Anesthesiology, First Affiliated Hospital, China Medical University
Hong Ma
Department of Anesthesiology, First Affiliated Hospital,China Medical University
Xiao-Qian Li (shirley037305@hotmail.com)
China Medical University First Hospital https://orcid.org/0000-0001-9852-8345

Research

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Abstract

Background: Neuron survival after ischemia-reperfusion (IR) injury is the primary determinant of motor function prognosis. MicroRNA (miR)-based gene therapy has gained attention. Our previous work explored the mechanisms by which miR-137-3p modulates neuronal apoptosis in both *in vivo* and *in vitro* IR models.

Methods: IR-induced motor dysfunction and spinal calpain (CAPN) subtype expression and subcellular distribution were detected within 12 h post IR. Dysregulated miRs, including miR-137-3p, were identified by miR microarray analysis and confirmed by PCR. Luciferase assay confirmed that CAPN-2 is a corresponding target of miR-137-3p, and their modulation of motor function was evaluated by intrathecal infection with synthetic miRs. CAPN-2 activity was measured by the intracellular Ca^{2+} concentration and mean fluorescence intensity *in vitro*. Neuronal apoptosis was detected by flow cytometry and lactate dehydrogenase (LDH) release. The activities of p35, p25, Cdk5 and caspase-8 were evaluated by ELISA and Western blotting after transfection with specific inhibitors and miRs.

Results: The IR-induced motor dysfunction time course was closely associated with CAPN-2 protein upregulation, which was mainly distributed in neurons. The miR-137-3p/CAPN-2 gene pair was confirmed by luciferase assay. miR-137-3p mimic significantly improved IR-induced motor dysfunction and decreased CAPN-2 expression, even in combination with recombinant rat calpain-2 (rr-CALP2) injection, whereas miR-137-3p inhibitor reversed these effects. Similar changes were observed in the intracellular Ca^{2+} concentration and CAPN-2 expression and activity when cells were exposed to OGD/R and transfected with synthetic miRs *in vitro*. Moreover, double fluorescence revealed that CAPN-2, p35, p25 and caspase-8 were all identically distributed in neurons. The decrease in CAPN-2 expression and activity was accompanied by the opposite changes in p35 activity and protein expression in cells transfected with miR-137-3p mimic, roscovitine (a Cdk5 inhibitor) or Z-IETD-FMK (a caspase-8 inhibitor). Correspondingly, more surviving neurons were observed with the abovementioned treatments, indicated by a decrease in apoptotic cell percentage, LDH release and p25, Cdk5, caspase-8 and caspase-3 protein expression.

Conclusions: The miR-137-3p/CAPN-2 gene pair functions to modulate neuronal apoptosis during IR injury, possibly through CAPN-2 inhibition leading to p35 cleavage and inhibition of subsequent p25/Cdk5 and caspase-8 overactivation.

Background

Spinal cord ischemia-reperfusion (IR) injury occurs during operations that require a transient block of blood flow to the spinal cord [1, 2]. Usually, reperfusion cannot prevent ischemia-induced neurological impairment (known as primary insults); on the contrary, reperfusion will further aggravate neurological function (known as secondary insults) during the initial period [3]. Apart from the high incidence of sensory deficits, IR injury is reported as a major cause of permanent motor dysfunction due to wide

neuronal death after recovery of blood flow [4–6]. Due to the limited proliferation capacity of adult neurons, exploring the underlying mechanisms to prevent neuronal death is of particular importance[7]. Various types of death have been found in neurons, including apoptosis, necroptosis, and ferroptosis [5, 7, 8]. We previously identified which types of cell death are involved in a specific type of spinal cord IR injury and found that blocking the process of apoptosis was effective in preserving hindlimb motor function in rodent models [5, 9]. Recently, some studies have shown that disturbed ionic homeostasis, such as ischemic or mechanical injury-induced excessive intracellular calcium ion concentration ($[Ca^{2+}]$) in neurons, could eventually trigger neuronal apoptosis by influencing vital biological functions and metabolism [10–12]. Thus, preserving intracellular calcium homeostasis may represent a promising treatment for attenuating neuronal apoptosis after IR insult.

Increased intracellular Ca^{2+} can activate a verity of proteases [13]. Belonging to a family of calcium-dependent neutral proteases, calcium-activated neutral proteinases (CANPs, also called calpains) are the most well-known effector that reacts to intracellular Ca^{2+} dysregulations through calcium-binding subunits [14, 15]. Eleven types of calpain isoforms have been identified in humans so far, of which calpain-1 (μ-calpain, CAPN-1) and calpain-2 (m-calpain, CAPN-2) are the most widely ubiquitous isoforms in the central nervous system (CNS) [13]. Being distributed in the same subcellular localization (cytoplasm) and sharing a common small subunit (known as CAPN-4) upon activation, CAPN-1 and CAPN-2 seem to have similar biochemical properties [13, 16]. CAPN-1 and CAPN-2 have previously been demonstrated to be overactivated in various models of neurodegenerative diseases and injury, although they require micromolar and millimolar calcium levels for activation, respectively [13, 14–16]. However, in contrast to traditional views, some studies have recently suggested that CAPN-1 activation plays prosurvival roles whereas CAPN-2 plays neurodegenerative roles, based on their opposite functions in promoting neuronal plasticity following CNS injury [17–19]. The most notable characteristic of calpains is their ability to perform partial truncation, which is a proteolytic cleavage of protein substrates, such as cytoskeleton proteins, membrane-bound proteins and protein kinases, at specific sites [13]. Commonly, the downstream products of CAPN-mediated truncation are bioactive, which can further amplify neurotoxic insults or oxidative stress by activating a subsequent signaling pathway [13, 20]. For example, the membrane-bound protein p35, known as a specific neuronal activator for cyclin-dependent kinase-5 (Cdk5), has been demonstrated to be a major substrate exclusively regulated by CAPNs in the pathogenesis of neurodegenerative disease [13, 19, 20]. Further exploration of downstream targets in rodent in vivo and in vitro experiments revealed that Cdk5 overactivation induced by cleaved p35 occurred exclusively in the presence of CAPN-2 [19–21]. In those studies, overexpressed CAPN-2 precisely cleaved the normally membrane-bound p35 into the more stable p25 form, which finally led to inappropriate increases in p25/Cdk5 activation and protein levels of caspase-3, a final executioner of neuronal apoptosis [19, 22, 23]. Acting as an upstream activator of caspase-3, caspase-8 is implicated in various models of CNS diseases and is critical for neuronal apoptosis [24, 25]. Thus, preventing caspase-8 proteolysis is especially important for controlling a series of broad caspase activations [24, 25]. Consistently, the p35 protein from baculovirus effectively blocked the apoptosis cascade by forming a p35-caspase-8 complex via a thioester bond [24, 26]. The structural experiments further identified the N

terminus of p35 as the major element necessary to preserve the intact covalent bond within the p35-caspase-8 crystal structure [26]. Thus, an increase in CAPN-2-mediated p35 cleavage may be reasonably inferred to also lead to conformational changes in p35 to further initiate caspase-8 and downstream caspase activation. Collectively, strong evidence suggests that the destructive functions of CAPN-2 during CNS injury are greatly attributed to its catalyzed substrates and downstream signaling pathways [17, 19, 20, 27]. However, no study has explored the abovementioned hypothesis in spinal cord IR injury using methods specifically targeting CAPN-2.

MicroRNAs (miRs) are a group of small, endogenous, noncoding RNAs [28]. miRs are widely expressed in the CNS and able to negatively regulate target genes by either degradation or posttranscriptional repression [5, 6, 28]. In our previous studies, we identified hundreds of aberrant miRs in injured spinal cords by microarray analysis [5, 6, 29]. Intrathecal pretreatment with synthetic miR mimic resulted in significant improvement in neurological deficits by recovering the altered miR expression [5, 6, 29]. These findings suggest promising miR-based gene therapy targeting CAPN-2. In this context, we first searched bioinformatical databases, identify potential miRs that may have binding sites with CAPN-2 among all dysregulated miRs detected in microarray analysis. Our present study suggested that miR-137-3p and miR-124-3p had target interactions with CAPN-2, which is supported by another study that explored the roles of miR-137-3p in rescuing motoneuron degeneration after brachial plexus root avulsion injury [30]. Then, we studied the functions and mechanisms by which the miR-137-3p/CAPN-2 gene pair regulates neuronal apoptosis by pretreatment of in vivo and in vitro models with synthetic miRs, a selective CAPN-2 inhibitor, recombinant rat calpain-2 (rr-CALP2) or a specific caspase-8 inhibitor.

Materials And Methods

Experimental animals

Sprague-Dawley rats weighting 200-to 250g were obtained from the Animal Center of China Medical University (Shenyang, China). All rats were preacclimatized 7 days before surgery. They were housed in standard cages under a 12-h light/dark cycle with the temperature at 23-24°C, humidity at 40-50%. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (United States National Institutes of Health publication number 85-23, National Academy Press, Washington DC, revised 1996).

Rat IR model establishment and experimental groups

The rat IR model was preformed by occluding the aortic arch for 14 minutes [4, 29]. Briefly, after being anesthetized, the rats were catheterized at the left carotid artery and the tail artery to measure proximal and distal blood pressure (BP), respectively. Following exposing the aortic arch, the clamp was placed between the left common carotid artery and the left subclavian artery for 14 min to induce ischemia. The ischemia was confirmed as a 90% decrease in distal BP. Then the clamp was removed to induce the reperfusion for 12h. The sham-operated rats were preformed the same procedures without inducing ischemia.

MiR microarray analysis

As we previously reported, the rat miRNA microarray analysis was performed with the miRNeasy mini kit (Qiagen, West Sussex, United Kingdom) [29,31]. The L4–6 segments of the spinal cord were collected at 4h after reperfusion. According to the manufacturers' instructions, 2.5μg total RNA samples were firstly labeled with the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) and hybridized on a miRCURY™ LNA Array (version 18.0, Exiqon, Vedbaek, Denmark).

After removing the nonspecific bindings, the fluorescent images of microarray slides were scanned by an Axon GenePix 4000B microarray scanner (Axon Instruments, CA, USA). The fluorescent intensity of the scanned images were loaded into the GenePix Pro 6.0 program (Axon Instruments) for feature extraction. The average of the replicated miRs with intensities of 50 or more were used to calculate a normalization factor. After normalized by the median normalization method, the significantly different miRs were identified by Volcano Plot filtering. Finally, the hierarchical clustering was performed to determine the differences of the miR expression by MEV software (version 4.6, TIGR).

Intrathecal injection and drug delivery

All treatments *in vivo* including the synthetic miRs (Dharmacon,Chicago, IL, USA) and recombinant rat calpain-2 (rr-CALP2,B71107, 150U/L, Calbiochem, China) were diluted into 20 μl in total volume and intrathecally injected, as we previously described [5,6]. Briefly, the needle of a 25μ microsyringe was inserted into the $L_{5\text{-}6}$ segment of spinal cords by the sign of a tail flick. Then, the concentration of 100 μmol/L of miR-137-3p mimic, 125 μmol/L miR-137-3p inhibitor or 100 μmol/L negative control (NC) was co-administered with Lipofectamine 3000 (Invitrogen, USA) at a 24h-interval for five consecutive days before surgery. Likewise, the rr-CALP was dissolved into a final concentration of 75 U/L immediately before injection. The number of days and the dosage used in this stud were evaluated by the overall effects *in vivo* by PCR and Western blotting in preliminary experiments. Only the rats displayed normal motor function were included for further study.

Motor function assessment

All being fully preacclimatized to the testing environment, the hind-limb motor functions were scored with Tarlov scores by two observers by the double-blind method [5].

Luciferase reporter assay

The target interaction between miR-137-3p and CAPN-2 was verified by the luciferase reporter assay[5]. Briefly, 293T cells were seeded in a 96-well plate at 4×10^4 cells/well. Using Lipofectamine 3000, the cells were co-transfected with 100 nM miR-137-3p mimic or 100 nM NC and 180 ng luciferase reporter vector containing the wild-type (WT) 3'UTR (5'-ACATCGTCTCTCAT*AGCAATA*T-3') or mutant (MT) 3'UTR (5'-ACATCGTCTCTCAT*CAUGGCA*T-3'). After 48h after transfection, the relative activity was determined with a Dual-Luciferase Reporter Assay Kit (Promega Corp.,WI, USA).

Oxygen-glucose deprivation and reperfusion (OGD/R) model

As we previously performed, the OGD/R model was established in 70–80% confluent VSC4.1 neurons to mimic the IR insult *in vivo*[5]. After twice washes and replacement the medium with glucose-free Hank's Balanced Salt Solution (HBSS), the neurons were kept in an anaerobic chamber (95% N2 and 5% CO2) at 37 °C for 6h. Then the medium was changed into initial medium and air condition for another 18h to induce reoxygenation. The control neurons were cultured in normal and atmosphere for 24h without depriving oxygen and glucose.

VSC4.1 motor neuron cultureand treatments

VSC4.1 motor neurons were purchased from Huatuo Biotechnology Co., Ltd (Shanghai, China). According to the manufacturer's instructions, the cells were grown in 75-cm^2 flasks containing 6ml culture medium (89% Eagle"s minimum essential medium (EMEM) with supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin) at 37 °C with 5% CO_2 in humidified air. The culture medium was replaced twice weekly.

For *in vitro* experiment, the neurons were pretreated with the synthetic miR and specific inhibitors 24h before underwent OGD/R insults [5]. As we previously, after seeded at a concentration of 4×10^5 per well, the miR-137-3p mimic (50 nmol/L) or NC (50 nmol/L) was cotransfected with 5 μL Lipofectamine 3000, whereas for inhibitor experiments, the Roscovitine (10 μM, Cdk5 inhibitor, Sigma-Aldrich Co., China) or Z-IETD-FMK (50 μM, caspase-8 inhibitor, R&D Systems,United States) was added into culture medium alone. The concentration of each treatment and the *in vitro* effects were determined by PCR in preliminary experiments.

Detection of CAPN-2 activity

The tensin homolog (PTEN) is a selective substrate for CAPN-2. PTEN is degraded as a result of CAPN-2 activation and is widely used for quantitative analysis of neuronal CAPN-2 activity *in vivo* and *in vitro* [19, 32]. As previously described, CAPN-2 enzymatic activity was assessed by the fold change in the mean fluorescence intensity (MFI) of PTEN (Santa Cruz, CA, USA). The increase in CAPN-2 activity was defined as the MFI in the treated group subtracted from the MFI in the control group. Total CAPN-2 activity was the MFI in the treated group summed with the MFI in the control group.

Detection of cytosolic [Ca^{2+}]

The intracellular [Ca^{2+}] in VSC4.1 neurons was measured with the Ca^{2+}-sensitive indicator fura-2/-acetoxymethyl ester (AM) (Molecular Probes, CA, USA) [14]. After each treatment, the neurons were loaded with 5 μM fura-2-AM for 30 min at 37°C in the dark. After being diluted to 1×10^6 cells/ml with the same Ca^{2+} buffer solution, Fura-2-AM was excited at wavelengths of 340 and 380 nm. The relative changes in intracellular [Ca^{2+}] were determined by the fluorescence ratio (R) at 340/380 with the

following formula: $[Ca^{2+}]=Kd\times\beta\times(R-R_{min})/(R_{max}-R)$[14]. We used the Calcium Calibration Buffer Kit with Magnesium (Molecular Probes, CA, USA) and determined that the Kd, a cell-specific constant, for VSC4.1 neurons was 0.264 μM.

Lactate dehydrogenase (LDH) assay

The LDH released from VSC4.1 neurons was detected by a commercial LDH Assay Kit (Abcam, CA, USA). According to the manufacturer's instructions, 50 μL medium was collected at 24h after each treatment and measured at the absorbance of 450 nm.

Detection of Caspase-8 activity

The caspase-8 activity was detected by the caspase-8 assay kit (Abcam, CA, USA), which is based on the spectrophotometric detection of p-nitroaniline (*p*NA) moiety after it is cleaved from the labeled substrate Ac-IETD by caspase-8. The sample were measured in triplicate at the absorbance at 405 nm.

Detection of p25/Cdk5and p35/Cdk5 activities by ELISA

The commercialized ELISA kits (Runyu Biological Technology Co., Shanghai,China) were used to measure the p25/Cdk5 and p35/Cdk5 activities in VSC 4.1 neurons. According to the manufactures instructions, the activities in supernatants were measured at 450 nm after each treatment. Each sample were performed in triplicate and the average was presented as ng/L.

Detection of neuronal apoptosis by flow cytometry

The apoptotic neurons were by detected by BD FACSCalibur flow cytometry (BD Bioscience, MA, USA) with excitation at 488 nm and emission at 530 nm [5]. Briefly, the 1×10^5 neurons were first stained with 10 μl Annexin V-fluorescein isothiocyanate (FITC) at 37 ℃ for 15 min and then counterstained with 5 μl propidium iodide (PI) for 30 min in the dark. The fluorescence was excitation at 488 nm and emission at 530 nm. Each sample was prepared in triplicate.

Quantitative RT-PCR

Total RNA was extracted from L_{4-6} segments of spinal cords or VSC4.1 neurons by the TRIzol/chloroform method or the miRNeasy FFPE kit (Qiagen, Hilden, Germany) [5]. RNA (500 ng) was reversely transcribed to cDNA by cDNA SuperMix (TaKaRa, China) or a MicroRNA Reverse Transcription Kit (Applied Biosystems, USA). The quantification of miR-137-3p and CAPN-2 were carried out with TaqMan MicroRNA Assays Kit or power SYBR green PCR master mix (Takara, China) on an Applied Biosystems 7500 RT-PCR System (Applied Biosystems, CA, USA). β-actin or U6 were used as an internal control. Each sample was measured in triplicate by the $2^{-\Delta\Delta CT}$ method.. The primers used in this study were as follows: miR-137-3p (forward: 5'-ACACTCATTATTGCTTA-3'; reverse: 5'-CTACGCGTATTGAGAGTAC-3'); CAPN-1 (forward: 5'-CTCCGGGGCAGGAGTAGGCA-3'; reverse: 5'-

CTCCGGGGCAGGAGTAGGCA-3'); CAPN-2 (forward: 5'-CTCCGGGGCAGGAGTAGGCA-3'; reverse: 5'-AACTGGCTGTGGGGCTCCCA-3'); U6 (forward: 5'-CTCGCTTCGGCAGCACA-3'; reverse: 5'-AACGCTTCACGAATTTGCGT-3') and β-actin (forward: 5'-GGAGATTACTGCCCTGGCTCCTA-3'; reverse: 5'-GACTCATCGTACTCCTGCTTGCTG-3').

Double immunofluorescence (IF)

As previously described, for *in vivo* samples, the 20-μm-thick sectioned spinal cord were blocked with 10% bovine serum albumin (BSA) for 1 h. Then, the sections were incubated with the primary mouse anti-calpain-2 antibody (SantaCruz, sc-373967, 1:300, Dallas, USA) and the antibodies specific marker for neurons (rabbit anti-NeuN, Abcam, ab177487, 1:500), for astrocytes (rabbit anti-GFAP, Abcam, ab7260, 1:500) and for microglial cells (rabbit anti-Iba-1, Abcam, ab178847,1:400) overnight at 4 °C. Then the sections were incubated with Alexa 594-conjugated donkey anti-mouse IgG (1:500, Life Technologies, CA, USA) and Alexa 488-conjugated donkey anti-rabbit IgG (1:500, Life Technologies, CA, USA) for 2 h at room temperature.

For *in vitro* samples, after be fixed with 4% formaldehyde for 20 min at 4 °C, the neurons were permeabilized with 0.1% TritonX-100 for 10 min and blocked by 3% donkey serum for 1 h at room temperature. Then the neurons were incubated with primary rabbit anti-p35 antibody (Abcam, ab64960, 1:300, CA, USA), primary rabbit anti-TPPP/p25 antibody (Abcam, ab92305, 1:300, CA, USA), mouse anti-calpain-2 antibody, mouse anti-caspase-8 p18 antibody (SantaCruz, sc-393776,1:400, Dallas, USA) overnight at 4 °C, followed by Alexa-conjugated secondary antibodies (1:500, Life Technologies, CA, USA) for 1h at room temperature in the dark. For cell counting, the nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, China) for 10 min at room temperature. The image were captured with a Leica TCS SP2 fluorescence microscope (Leica Microsystems, IL, USA). The integrated fluorescent density was measured with LEICA IM50 software (Nussloch, Germany)

Western blotting

The total proteins from L_{4-6} spinal cords or VSC4.1 neurons were extracted and purified with a protein extraction kit (KangChen, China) [4,6]. After determined by a BCA protein assay kit (Beyotime, China), equal protein concentration were loaded onto 10% SDS-PAGE gel and transferred to PVDF membranes. The membrane was incubated with 5% skim milk for 1 h to avoid nonspecific binding and probed with anti-calpain-1 antibody (SantaCruz, sc-271313, 1:400, Dallas, USA) , anti-calpain-2 antibody (1:500), anti-p35 (1:400), anti-TPPP/p25 antibody (1:500), anti-PTEN antibody (SantaCruz, sc-7974, 1:400, Dallas, USA), anti-Cdk5-antibody (SantaCruz, sc-6247, 1:300, Dallas, USA), anti-caspase-8 p18 antibody, anti-caspase-3 antibody (Abcam, ab184787, 1:500, CA, USA) or β-actin (SantaCruz, sc-47778, 1:2000, Dallas, USA) overnight at 4 °C. After washes, the membrane were incubated with peroxidase-conjugated secondary antibodies (Beyotime, A0192, 1:10,00, China) for 2 h at room temperature. The blots were detected by an ECL kit (Beyotime, China) and quantified by Quantity One software (Bio-Rad Laboratories, Italy).

Statistical analysis

The data were expressed as the mean±standard deviation (SD) and analyzed using SPSS 19.0 software (SPSS, Chicago,USA). Statistical comparisons between two groups were assessed by *t* tests or Mann-Whitney tests, whereas comparisons among three or more groups were performed one- or two-way ANOVA followed by the Tukey-Kramer test. A *P* value <0.05 was considered statistically significant.

Results

Temporal changes in motor dysfunction and spinal CAPN subtype expression post IR

All rats exhibited normal motor function before undergoing IR surgery. As shown in Figure 1A, compared with sham-operated rats, the rats in the IR groups displayed obvious hindlimb motor dysfunction, indicated by significant decreases in average Tarlov score throughout the reperfusion period ($P<0.05$). Likewise, the protein levels of spinal CAPN-1 and CAPN-2 were measured at 4-h intervals. In contrast to the decrease in CAPN-1 protein expression over time, CAPN-2 protein expression increased unimodally, reaching a maximum at 4 h post surgery (Figure 1B, C, $P<0.05$). Notably, there were no significant differences in CAPN-1 protein expression among the IR groups ($P>0.05$). Thus, the specific cellular distribution of CAPN-2 in injured spinal cords was further identified by double immunofluorescence at the time point with the highest CAPN-2 expression. Colocalization was indicated by a yellow fluorescent signal, revealing that the majority of CAPN-2 fluorescent signals overlapped with neurons, not astrocytes or microglia, at 4 h post surgery (Figure 1D). Similarly, the quantification of the number of CAPN-2- and NeuN-double-positive cells confirmed that the IR insult-induced increase in CAPN-2 expression was primarily distributed in neurons (Figure 1E, F, $P< 0.05$).

IR-induced aberrant spinal miR-137-3p expression and negative regulation of CAPN-2 expression *in vivo*

Microarray analysis showed that several aberrant miRs were greatly dysregulated in injured spinal cords at 4 h post IR (Figure 2A). Among these miRs, miR-137-3p has been indicated to be closely associated with neurodevelopment and CNS disease and to be highly expressed in the brain [30,33]. Thus, we hypothesized that miR-137-3p was also widely expressed in spinal cord tissues and confirmed that it showed an abnormal decrease in expression at 4 h post IR by RT-PCR (Figure 2B, $P< 0.05$). Moreover, analysis with TargetScan (Release 7.2, http://www.targetscan.org/vert_72/) showed that the binding site of miR-137-3p has 7 base pairs that matched the 3¢- untranslated region (UTR) of the CAPN-2 mRNA. This negative target interaction was further confirmed by a luciferase reporter assay, in which the miR-137-3p mimic significantly decreased the luciferase activity in cells containing the wild-type (WT) 3¢-UTR but not the mutated (MT) 3¢-UTR (Figure 2C, $P< 0.05$). As we previously reported, we assessed the potential *in vivo* interactions by intrathecal pretreatment with synthetic miRs [5,6]. Consistently, compared with the IR group, the group given intrathecal injection of miR-137-3p mimic had significantly lower CAPN-2 protein and mRNA expression, whereas the group pretreated with miR-137-3p inhibitor injection had significantly higher CAPN-2 expression (Figure 2D-E, $P<0.05$). As expected, the synergistic upregulation in CAPN-2 expression post IR that occurred with injection of rr-CALP2, a recombinant CAPN-2 that

specifically upregulates CAPN-2 expression, was partly reversed by miR-137-3p mimic injection (P<0.05). No significant changes were detected when injected with miR-137-3p NC injection had no significant effects on CAPN-2 expression (P>0.05).

Effects of the miR-137-3p/CAPN-2 pair on IR-induced hindlimb motor dysfunction

To further clarify the regulatory roles of the miR-137-3p/CAPN-2 pair *in vivo*, the hindlimb motor function was assessed (Figure 2F). As expected, compared with baseline and sham-operated rats, all IR-injured rats showed obvious hindlimb motor dysfunction during the reperfusion period (P<0.05). Compared with the time-matched injured rats in the IR group, the rats injected with the miR-137-3p mimic exhibited higher average Tarlov scores, whereas the rats injected with the miR-137-3p inhibitor showed lower Tarlov scores (P<0.05). Likewise, in conjunction with the mRNA and protein levels of CAPN-2, rr-CALP2 injection reversed the improvement in motor function, indicated by Tarlov scores comparable to those for the IR group (P>0.05). There were no detectable differences between the IR-injured rats with or without miR-137-3p NC treatment at any observed time point (P>0.05).

Modulation of CAPN-2 expression and activity by miR-137-3p in VSC4.1 neurons after OGD/R

Given that increased intracellular Ca^{2+} can activate CAPN-2 [13], we measured the free intracellular [Ca^{2+}] in each treatment group at 24 h post OGD/R. As expected, compared to control cells, the VSC 4.1 neurons exposed to OGD/R for 24 h exhibited a significant increase in intracellular free [Ca^{2+}] (Figure 3A, P< 0.05). In addition, miR-137 mimic pretreatment effectively prevented the OGD/R-induced [Ca^{2+}] increase, whereas miR-137 inhibitor aggravated the [Ca^{2+}] increase (P< 0.05). No differences were detected between cells with or without miR-137 NC treatment (P>0.05).

PTEN is a selective substrate for CAPN-2 [32]. Therefore, the OGD/R-induced changes in CAPN-2 expression and activity were further confirmed by assessment of PTEN at the same observed time points. As shown by representative images of double fluorescent staining, both PTEN and p35 fluorescent labels were predominantly distributed in the cytoplasm and nucleus of VSC 4.1 neurons (Figure 3B). Consistent with previous studies [19,32], the mean PTEN and CAPN-2 immunoreactivity exhibited opposite changes in all treatment groups, confirming that the net and total CAPN-2 activity were changed in accordance with the intracellular [Ca^{2+}] (Figure 3C and D, P<0.05). Similar to the Western blotting results *in vivo* and fluorescent quantification in Figure 3C, CAPN-2 protein levels were significantly decreased in the presence of miR-137 mimic treatment but were increased with miR-137 inhibitor treatment (Figure 3E, P<0.05). Conversely, PTEN protein levels were increased in miR-137 mimic-transfected cells and decreased in miR-137 inhibitor-transfected cells (P<0.05). No such changes were detected upon pretreatment with miR-137 NC (P>0.05).

Modulation of p35 cleavage and p25/Cdk5 activation by the miR-137-3p/CAPN-2 gene pair after OGD/R

Then, we tested whether p35 cleavage and subsequent p25/Cdk5 activation was regulated by the miR-137-3p/CAPN-2 gene pair in VSC4.1 neurons by double immunofluorescent staining and Western blotting,

as previously published [5, 19]. As shown in Figure 4A, representative fluorescent images showed CAPN-2, p35 and p25 and were all predominantly distributed in the cytoplasm and nucleus of VSC4.1 neurons. OGD/R injury induced significant increases in CAPN-2 and p25 immunoreactivity but a decrease in p35 immunoreactivity in neurons at 24 h post injury, consistent with the Western blotting results shown in Figure 4D, E (P<0.05). Furthermore, the ELISA and Western blotting results showed that, in contrast to the decrease in CAPN-2 expression caused by mimic transfection, transfection with miR-137-3p mimic significantly reversed the OGD/R-induced decrease in p35 activity and protein level (Figure 4B-E, P<0.05), whereas no differences were detected in the presence of miR-137 NC (P>0.05). As expected, following p35 cleavage, the activity and protein expression profiles of p25 and Cdk5 were changed in parallel to the CAPN-2 protein level detected in each treated group (P<0.05).

Additionally, transfection with roscovitine, a specific Cdk5 inhibitor, regulated p35, p25 and Cdk5 protein levels and activity in a similar manner as transfection with miR-137-3p mimic, just as roscovitine and mimic had comparable effects on CAPN-2 expression (Figure 4B-E, P>0.05).

Modulation of caspase-8 activation by the miR-137-3p/CAPN-2 gene pair after OGD/R

Likewise, we also tested the regulatory effects of the miR-137-3p/CAPN-2 gene pair on the caspase-8-mediated apoptotic network. As shown in representative fluorescent images, p35 and caspase-8 were identically distributed in cytoplasm VSC4.1 neurons. OGD/R injury induced opposite changes in p35 and caspase-8, a decrease in immunoreactivity and protein expression of p35 but an increase in immunoreactivity and protein expression of caspase-8 at 24 h postinjury (Figure 5A, C, D, P<0.05). In contrast to the effects of mimic transfection of p35 expression, miR-137-3p mimic transfection significantly decreased caspase-8 activity and protein levels (Figure 5B-D, P<0.05), whereas miR-137 NC had no effect (P>0.05). However, compared with the miR-137-3p mimic, transfection with Z-IETD-FMK, a specific caspase-8 inhibitor produced greater inhibitory effects on caspase-8 activity and protein levels at 24 h post transfection (Figure 5B-D, P<0.05).

Additionally, as the final executor of the apoptotic network, caspase-3 protein expression was measured in each treatment group at the same time point. The Western blotting results showed that the protein expression of caspase-3 changed in accordance with the change in the protein expression of caspase-8 (Figure 5C, D).

Modulation of VSC4.1 neuronal apoptosis by the miR-137-3p/CAPN-2 gene pair after OGD/R

Finally, the effects of the miR-137-3p/CAPN-2 gene pair on neuronal apoptosis were assessed by flow cytometry *in vitro*. Consistent with the assessment of motor function *in vivo*, the ODG/R insult obviously increased the percentage of apoptotic neurons (A2+A4 quadrant) at 24 h of reperfusion (Figure 6A, B, P<0.05). Treatment with the miR-137-3p mimic, roscovitine or Z-IETD-FMK produced comparable and significantly inhibitory effects on ODG/R-induced neuronal apoptosis (P<0.05), whereas miR-137 NC treatment had no such inhibitory effects (P>0.05).

In addition, changes in LDH release from injured neurons exhibited the same pattern as changes in flow cytometry among the treatment groups, suggesting an important role of the miR-137-3p/CAPN-2 pair in regulating subsequent Cdk5 and caspase-8 overactivation and neuronal apoptosis (Figure 6C, $P<0.05$). No significant differences were observed between the injured neurons with or without miR-137 NC treatment ($P>0.05$).

Discussion

Our previous studies revealed that IR-induced dysregulated miRs in spinal cords played important roles in driving pathogenesis during the reperfusion period and finally caused severe motor and sensory dysfunction [5, 6, 29]. Recently, an increasing number of studies have suggested that miR-based gene therapy is a promising treatment for neurological recovery by effectively preventing neuronal apoptosis. In the present study, we investigated the function and mechanisms of miR-137-3p and its target CAPN-2 in both in vivo and in vitro IR models to better understand the pathophysiological mechanisms and find better treatments in the clinic.

Previous studies have explored the crucial roles of CAPNs in determining neuronal survival during CNS injury [16, 17–19, 27, 30]. Some studies have observed prosurvival roles for CAPN-1 activation but destructive roles for CAPN-2 activation in retinal ganglion cell degeneration [17, 19]. However, in a recent rat contusive spinal cord injury model, CAPN-1 was activated and contributed to impaired locomotor function [16]. These differences might be explained by the preferential participation of CAPN isoforms to different cellular functions even in different substructures of the same cells [14, 35]. Thus, the contradictory roles of the activation of these two isoforms provide great challenges for our detailed understanding of the pathophysiological mechanisms of IR injury. In this context, we examined CAPN-1 and CAPN-2 protein expression and assessed motor function using Tarlov scores at several time points post IR. Our results showed that only the temporal expression patterns of CAPN-2 were negatively correlated with IR-induced motor dysfunction, with initial significant differences detected at 0.5 h post IR and reaching the maximum at 4 h post IR (Fig. 1). This finding was consistent with a previous study on spinal cord injury, in which the progressively increased calpain content in the lesion was first detected as early at 30 min after trauma and reached a 91% increase at 4 h after trauma [36]. We further explored the cellular distribution of CAPN-2 in major spinal cord cell types by double immunofluorescence at 4 h post IR when CAPN-2 expression reached its peak. Representative images and quantification showed that CAPN-2 was primarily expressed in spinal neurons, indicating that neuronal CAPN-2 might be the major effector during the reperfusion period.

MiRs are small RNA molecules that negatively regulate gene expression by binding with the 3'-UTR of targets via complementary base pairs [28]. MiRs are widely expressed in the CNS and have been implicated in multiple pathological processes, including IR [29]. We have suggested that some miRs, including miR-187-3p, miR-27 and miR-125b, that are highly expressed in spinal cords may provide new insights for research and clinical treatment [5, 6, 29]. Likewise, in this study, using miR microarray analysis and luciferase assay, we found that miR-137-3p expression was greatly changed at 4 h post IR

and had a target interaction with CAPN-2 (Fig. 2). Continuous intrathecal injection of synthetic miRs before IR was previously reported to effectively regulate miR expression and corresponding target gene expression in vivo [5, 6, 29]. Given the complicated cellular crosstalk in vivo, we defined the overall effects of the miR-137-3p/CAPN-2 gene pair by assessing motor function in a rat model. As expected, in contrast to the decrease in CAPN-2 protein expression, intrathecal injection of miR-137-3p mimic greatly increased Tarlov scores, indicating decreased motor dysfunction, whereas treatment with miR-137-3p inhibitor or NC did not have these effects. Moreover, to further confirm the above interaction, a direct delivery of exogenous CAPN-2 (rr-CALP2) was intrathecally performed. Consistent with the ability of exogenous CAPN-2 to activate the intrinsic CAPN-2 in uninjured nerves [35, 37], the synergistic increase in CAPN-2 expression caused by exogenous rr-CALP2 injection was significantly prevented by miR-137-3p mimic, as comparable CAPN-2 protein levels and similar behavioral assessments were observed throughout the reperfusion period in injured rats with or without combined injection with rr-CALP2 (Fig. 2). These findings suggested that miR-137-3p acted as a functional regulator of CAPN-2 in the spinal cord.

As a trigger, CAPN-2 requires millimolar (0.250–0.750 mM) calcium concentrations for its activation [13]. Thus, parallel in vitro experiments were performed to better define the possible mechanisms by which the miR-137-3p/CAPN-2 gene pair is implicated in VSC4.1 neuronal apoptosis. Consistent with the in vivo results and VSC4.1 neuron glutamate-related neurotoxicity [14], overactivation of CAPN-2 was accompanied by an increase in the intracellular free [Ca^{2+}] in OGD/R-stressed VSC4.1 neurons; both the MFI of CAPN-2 and total and net CAPN-2 activity were significantly increased by OGD/R treatment (Fig. 3). Previous reports have indicated that both peptide and nonpeptide CAPN inhibitors downregulate CAPN expression by preventing increases in the intracellular free [Ca^{2+}] [14, 38]. Consistently, our current data showed that the synthetic miR-137 mimic also prevented the increase in the intracellular [Ca^{2+}] and decreased CAPN-2 expression and activity in stressed neurons. In addition, PTEN is a selective substrate for CAPN-2 [32] and is thus widely used to quantitatively measure neuronal CAPN-2 activity in vivo and in vitro [19, 32]. As expected, PTEN and CAPN-2 were identically distributed in the cytoplasm of neurons, but their protein levels and immunoreactivities changed in opposite directions in response to transfection with the different treatments. Decreased PTEN expression indicates an increase in CAPN-2 activation; therefore, these results suggest that OGD/R-induced CAPN-2 overactivation was regulated by synthetic miR.

Previous in vitro and in vivo studies have shown that CAPN-2 upregulation triggers neuronal apoptosis [20, 39]. These studies found that activated CAPN-2 directly and precisely cleaves its substrate, membrane-bound protein p35 into p25, which consequently results in Cdk5 activation in cultured primary neurons and retinal ganglion cells [13, 20, 39]. Similarly, our in vitro immunofluorescent staining (Fig. 4) revealed that the cytoplasmic and nuclear labels for p35 and p25 fluorescence completely overlapped with the CAPN-2 labels in VSC4.1 neurons. In a previous study, during polybrominated diphenyl ether-153-induced neuronal apoptosis, p35 was found to accumulate in the perinuclear region and plasma membrane, and p25 was localized in both the cytoplasm and nucleus [20]. Given that the p35/Cdk5 complex mainly exerts its function in the nucleus, these mislocalizations of p35 and p25 might be a sign

of the formation of the p25/Cdk5 complex [20, 40, 41]. Additionally, our Western blotting results showed that the expression pattern of Cdk5 protein in each group changed in agreement with the levels of the p25 and CAPN-2 protein and opposite to the levels of the p35 protein (Fig. 4). On the other hand, the changes in p35 and p25 activities in accordance with the protein level of CAPN-2 in neurons transfected with miR-137-3p mimic or NC also demonstrated that the conversion of p35 into p25 requires the presence of CAPN-2. Additionally, selective inhibition of CAPN-2 expression has been shown to preserve both the structure and function of vulnerable neurons [14, 17, 19]. In this study, pretreatment with miR-137-3p and Cdk5 inhibitor comparably inhibited the number of neurons located in the A4 and A2 quadrants of the flow cytometry dot-plot graphs and LDH leakage in culture medium after OGD/R insult (Fig. 6). These findings all support the hypothesis that the dynamic localization of p35 and p25 are considered a signal for p25/Cdk5 activation-induced apoptosis.

Caspase-cascade activation has been suggested to be central for neuronal apoptosis during IR injury [5, 9]. Acting as the apical member of the caspase family, caspase-8 overactivation has been shown to hold special importance in controlling a series of broad caspase-cascade networks by initiating downstream caspase activation, such as activation of caspase-3 [24, 42]. Meanwhile, as a cysteine protease, caspase-8 requires proteolytic cleavage before activation. Under normal conditions, caspase-8 forms the p35-caspase-8 complex through a covalent bond with the N terminus of p35 [26]. The N terminus of p35 is known to be a major element necessary for preserving the p35-caspase-8 complex, and the baculovirus p35 protein has been demonstrated to effectively block the apoptosis cascade by preventing caspase-8 proteolysis and activation [24, 26]. In contrast, CAPN-2 overexpression-mediated p35 cleavage may cause conformational changes in p35 and consequently initiate aberrant caspase-8 activation. In agreement with the above hypothesis, a previous study showed that CAPN-2 was required to initiate endoplasmic reticulum stress-induced apoptosis mediated by a caspase-8-dependent pathway [25]. Similar in our in vitro immunofluorescence and Western blotting results, the fluorescent labels for p35 in the cytoplasm were identically distributed with the caspase-8 labels in neurons, and the protein expression of p35 and caspase-8 were changed in opposing directions when CAPN-2 expression was downregulated by pretreatment with miR-137-3p mimic (Fig. 5). In addition, our hypothesis that CAPN-2 initiates caspase-8-mediated apoptosis was additionally supported by the similar results observed in neurons transfected with miR-137-3p mimic and those transfected with the caspase-8-specific inhibitor Z-IETD-FMK. Both treatments exhibited comparable inhibitory effects on the protein level and activity of caspase-8, the number of injured neurons in the A4 and A2 quadrants of the dot-plot graphs and the amount of LDH leakage (Figs. 5 and 6). Acting as the final executor of the caspase family, caspase-3 expression was equally decreased in neurons transfected with miR-137-3p mimic and Z-IETD-FMK, supporting the assumption that CAPN-2-induced caspase-8 activation may simultaneously lead to caspase-3 activation. Indeed, similar observations were made in a study of hydrogen peroxide-induced apoptotic pathways [25].

Of note, transfection with the Cdk5 inhibitor, roscovitine, had no significant effects on the expression of CAPN-2 or p35, possibly due to the eventual modulation of the CAPN-2/p35-p25/Cdk5 pathway [20]. Roscovitine is known to be highly specific for Cdk5 and is therefore unlikely to influence the activity of the apical members of the signaling pathway [20]. Undoubtedly, whether caspase-8 directly activates

caspase-3 by proteolytic cleavage or by activating caspase-3 activators needs to be elucidated in further study.

Conclusion

This study highlights the roles of CAPN-2 in triggering motor dysfunction after spinal cord IR injury and investigates the target interactions with miR-137-3p in both in vivo and in vitro models. The effects of the miR-137-3p/CAPN-2 gene pair on neuronal apoptosis may be attributed to CAPN-2 inhibition resulting in an inhibition of the cleavage of the substrate p35, consequently preventing the overactivation of p25/Cdk5 and the initiation of the caspase-8-mediated caspase cascade.

Declarations

Ethics approval and consent to participate

The animal experiments in this study were approved by the Ethics Committee of China Medical University and were performed in compliance with the Care and Use of Laboratory Animals under approved protocols.

Consent for publication

Not applicable.

Availability of data and materials

The materials supporting the conclusions of this article are included within the article.

Competing interests

All authors in this study declare they have no conflicts of interest.

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Authors' contributions

H.W. and Z.-L.Z. participated in animal care and made the animal models. H.W.and Q.Y. performed the cell models and prepared samples for RT-PCR and immunohistochemistry assays; H.W., Q.Y. and Z.-L.Z, performed experiments and statistical analysis; X.-Q.L. conducted luciferase assays and H.M. guided the study design and gave important suggestions of manuscript writing.

Acknowledgments

Not applicable.

Abbreviations

BP: blood pressure; BSA: bovine serum albumin; Ca^{2+}: calcium ion; [Ca^{2+}]: Ca^{2+} concentration; CANPs: calcium-activated neutral proteinases; Cdk5: cyclin-dependent kinase-5; CNS: central nervous system; EMEM: Eagle"s minimum essential medium; FBS: foetal bovine serum; HBSS: Hank's Balanced Salt Solution; IF: immunofluorescence; IR: ischemia-reperfusion; LDH: lactate dehydrogenase; miR: microRNA; MFI:mean fluorescence intensity; MT:mutant; NC:negative control; *p*NA:p-nitroaniline; PTEN: tensin homolog; R:ratio; SD: standard deviation; VSC: ventral spinal cord; UTR: untranslated region; WT: wild-type

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Figures

Figure 1

Temporal changes in motor dysfunction and spinal CAPN subtype expression post IR. A, Temporal changes in hindlimb motor function were evaluated using Tarlov scores at 0.5, 1, 4, 8 and 12 h after IR. n=6 per group. B, Representative Western blots of CAPN-1 and CAPN-2 in injured spinal cord samples after IR. C, Protein quantification of CAPN-1 and CAPN-2 levels after IR. The relative protein levels were normalized to the those in the sham group. Data are expressed as the mean± standard deviation (SD). n=6 per group. *P < 0.05 versus the sham group. D, Representative double immunofluorescence of CAPN-2

with spinal neurons (NeuN), microglia (Iba-1) and astrocytes (glial fibrillary acidic protein (GFAP)) in anterior horns of spinal cords at 4 h after IR. The yellow labels with white arrows indicate colocalization. Scale bars = 50 μm. n=6 per group. E-F, Quantification of cells double-labeled with CAPN-2 immunoreactivity and markers of specific cell types. Data are presented as the average of three independent images of laminae and in the gray matter and expressed as the mean±SD. *P < 0.05 versus the sham group.

Figure 2

IR-induced aberrant spinal miR-137-3p expression and negative regulation of CAPN-2 expression in vivo. A, A heat map representation of miRs differentially expressed in spinal cord samples at 4 h post IR. Three independent replicates were performed. The red signals indicate the upregulated miRs, and the green signals indicate the downregulated miRs. B, Quantification of miR-137-3p expression post IR. n=4 per group. Data are expressed as the mean±SD. *P<0.05 versus the sham group. C, The putative target binding site of miR-137-3p in the rat 3-UTR of CAPN-2 was predicted by the TargetScan database and confirmed by a luciferase reporter assay. *P < 0.05 versus the WT 3-UTR cells transfected with miR-137-3p mimic. D, Representative Western blots and protein quantification of CAPN-2 in the spinal cord after different treatments. β-actin was used as a loading control. E, Quantification of CAPN-2 mRNA expression after different treatments. F, Hindlimb motor function was assessed by Tarlov scores after different treatments. *P<0.05 versus the sham group, #P<0.05 versus the IR group.

Figure 3

Modulation of CAPN-2 expression and activity by miR-137-3p in VSC4.1 neurons after OGD/R. A, Percentage of the intracellular free [Ca2+] in each condition after OGD/R. B, Representative double immunofluorescence staining showing that PTEN (green) and CAPN-2 (red) are predominantly distributed in the cytoplasm of VSC 4.1 neurons. Scale bar = 50 μm. C, Quantification of the MFI of PTEN and CAPN-2 in neurons of each treatment group. D, Statistical analysis of total CAPN-2 and increased CAPN-2 activities at 24 h post OGD/R. E, Representative Western blots and protein quantification of CAPN-2 and PTEN in neurons. All samples were analyzed in triplicate and expressed as the mean±SD. *P < 0.05 versus the control group, #P < 0.05 versus the OGD/R group.

Figure 4

Modulation of p35 cleavage and p25/Cdk5 activation by the miR-137-3p/CAPN-2 gene pair after OGD/R. A, Representative double immunofluorescence staining showing that p35 (green) and CAPN-2 (red) (left panel) and p25 (green) and CAPN-2 (red) (light panel) are predominantly distributed in the cytoplasm of VSC 4.1 neurons. Scale bar = 50 μm. B-C, Quantification of p35/Cdk5 and p25/Cdk5 activities in VSC 4.1

neurons of each treatment group by ELISA. D, Representative Western blots and protein quantification of CAPN-2, p35, p25 and Cdk5 in neurons. All samples were analyzed in triplicate and expressed as the mean±SD. *P < 0.05 versus the control group, #P < 0.05 versus the OGD/R group, &P < 0.05 versus the OGD/R+miR-137 mimic group.

Figure 5

Modulation of caspase-8 activation by the miR-137-3p/CAPN-2 gene pair after OGD/R. A, Representative double immunofluorescence staining showing that p35 (green) and caspase-8 are predominantly distributed in the cytoplasm of VSC 4.1 neurons. Scale bar = 50 μm. B, Quantitative analysis of caspase-8 activity in VSC 4.1 neurons of each treatment group. C-D, Representative Western blots and protein quantification of p35, caspase-8 and caspase-3 in neurons. All samples were analyzed in triplicate and expressed as the mean±SD. *P < 0.05 versus the control group, #P < 0.05 versus the OGD/R group, &P < 0.05 versus the OGD/R+miR-137 mimic group.

Figure 6

Modulation of VSC4.1 neuronal apoptosis by the miR-137-3p/CAPN-2 gene pair after OGD/R. A, The percentage of OGD/R-induced neuronal apoptosis in each treatment group determined by flow cytometry. B, Quantification of the percentage of apoptotic neurons. C, The percentage of OGD/R-induced neuronal apoptosis in each treatment group determined by LDH release. All samples were analyzed in triplicate and expressed as the mean±SD. *P < 0.05 versus the control group, #P < 0.05 versus the OGD/R group.