Inhibition of Arginyl-tRNA Synthetase Promotes the Protection of Neuronal Ischemic Tolerance in Vitro

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Research Article

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Abstract

Backgrounds

Studies have shown that the metabolic rate of mammals decreases during hibernation, suggesting that effective reduction of the energy consumption of ischemic cells may be the basis of the protective effect of ischemic tolerance. Anderson et al. reported after inhibition of the gene encoding arginyl-tRNA synthetase, the protein translation and mortality in C. elegans under anoxic conditions decreased significantly.

Purposes

Whether inhibition of arginyl-tRNA synthetase (RARS), in addition to combating hypoxic injury in C. elegans, protects rat neurons from ischemic damage remains unknown. The aim of this study is to determine whether knockdown of arginyl-tRNA synthetase improves the tolerance of primary cultured rat neurons to ischemic anoxia.

Methods

For the primary neuronal culture, cerebral cortex tissues were collected from newborn 24 hours Sprague-Dawley rats. Different viral vectors were transfected into cultured primary neurons, and the optimal viral vector and time points for gene silencing were determined by detecting the expression of RARS gene and protein. The adenovirus vector expressing shRNA-RARS with the highest silencing efficiency was transfected into rat primary cultured cortical neurons. The time point at which the viral vector exhibits optimal gene silencing efficiency was selected as the detection time point for subsequent experiments. The neurons after OGD treatment were divided into 2 groups: the experimental group and the control group. Each group was divided into 3 subgroups: the normal group, the control shRNA group and the shRNA-RARS group. After 3 hours of OGD treatment, cell survival, ATP levels and RARS protein expression were evaluated, and the data was analyzed.

Results

Primary cultured neurons were identified and purified. The adenoviral vector expressing RARS-RNAi (27394-1) exhibited the highest silencing efficiency of the three adenoviral vectors, and 3 days after transfection of the adenoviral vector was selected as the optimal time point for gene silencing. Transfection of adenoviral vectors expressing shRNA-RARS reduced RARS protein expression, ATP consumption and neuronal death, and increased CCK8 activity in neurons after OGD insult.

Conclusions

Our work revealed neurons transfected with adenoviral vectors expressing shRNA-RARS exhibited stronger tolerance to ischemia and hypoxia, which was due to the inhibition of RARS activity and...
cell energy metabolism rate. These results suggested that RARS inhibition reduced protein translation and energy consumption, and played a protective role in ischemic tolerance.

1. Introduction

Stroke is responsible for significant mortality and long-term disability, and it is a major public health burden worldwide.[1, 2] According to the World Health Organization (WHO), stroke caused more than 6 million deaths in 2015, making it the second largest global killer only behind ischemic heart diseases and the third leading cause of disability-adjusted life-years lost globally.[3] In particular, the American Heart Association/American Stroke Association (AHA/ASA) forecasts a 20.5% increase in stroke prevalence by 2030.[4] Approximately 80% of strokes occur after cerebral artery occlusion, so it is classified as ischemic stroke, which is one of the various subtypes of stroke.[5] Ischemic stroke is characterized by high morbidity, high mortality, high disability, and low cure rate, and it is one of the most debilitating diseases worldwide.[6] Unfortunately, there are currently no approved drug-based therapies for preventing ischemic stroke or restoring the lost functions in patients with ischemic stroke.[7] Therefore, alleviating the “ischemic stroke pandemic” and elucidating the mechanism of cerebral ischemia tolerance are necessary to ensure a high quality of life for stroke patients.

The brain is the most sensitive organ to hypoxic and ischemic treatment. Glucose delivery is decreased when ischemic stroke occurs, directly leading to severe consequences, including defects in the citrate cycle and mitochondrial function, which reduces the ATP production of the infarct core to below 35% of the normal level and leads to neuronal death. Increased and irreversible brain damage.[8–10]

Previous studies showed that the metabolic rate of mammals decreases during hibernation. During winter hibernation, small mammals fall into long periods of deep cold torpor, during which the metabolic rate decreases by > 90%.[11] In recent years, some cellular mechanisms that cause insufficient metabolism have been recognized. For example, activities like transcription, translation, and protein synthesis are largely suppressed, the production of ATP from glucose is reduced, and lipids serve as the major substrate for the remaining energy required.[12] Ischemic-like treatments decrease the viability and increase the death of cultured human neuronal progenitor cells. However, these treatments have less effect on cell death and promote the proliferation of cultured arctic ground squirrel neuronal progenitor cells.[13]

Hochachka et al.[14] proposed a unifying theory of hypoxia tolerance. The first line of defense against hypoxia includes the balanced suppression of the ATP demand and ATP supply pathways. The demand for ATP for protein synthesis is decreased under hypoxic conditions, likely due to translational arrest. This translational arrest in hypoxia-tolerant cells seems irreversible, but the cascade of processes underlying hypoxia rescue and defense begins with an oxygen sensor (a heme protein) and a signal transduction pathway and leads to significant gene-based metabolic reprogramming (the rescue process) with a persistent downregulation of energy demand and energy supply pathways related to metabolism.
throughout the hypoxic period. Therefore, effectively reducing the energy consumption of ischemic cells may underlie the protective effect of ischemic tolerance.

Aminoacyl-tRNA synthetase (AARS) is a key enzyme in the activation of amino acids that catalyzes the attachment of an amino acid to its cognate tRNA for protein biosynthesis. The aminoacylation reaction occurs in two steps. First, a specific AARS recognizes its cognate amino acid in the presence of ATP and forms an enzyme-amino acid-AMP complex. Second, the aminoacyl moiety is transferred to a specific tRNA via the release of AMP to form aminoacyl-tRNA (aatRNA). As the primary source of energy for cells, ATP is consumed during the process of protein biosynthesis. Experimental evidence shows that AARS not only plays a role in protein translation but also participates in gene transcription, RNA splicing, signal transduction pathways and other biological activities, as well as autoimmune dysfunction, cancer and cell regulation pathways that are unrelated to protein synthesis. Anderson et al. studied the survival of Caenorhabditis elegans in an anoxic state using shRNA technology and found that the level of protein translation and mortality rate of C. elegans were significantly reduced after the inhibition of the gene that encodes arginyl-tRNA synthetase (RARS). These findings indicate that there is a strong negative correlation between hypoxia tolerance and protein translation levels, and a decrease in protein translation leads to a reduction in energy metabolism, such as a reduction in ATP consumption.

Whether the inhibition of RARS exerts a protective effect on ischemic stroke in rats, and if yes, the mechanism underlying this effect are unknown. To answer these important questions, we directly inhibited the RARS gene expression in rat cortic neurons in vitro by transfecting adenoviral vectors expressing shRNA-RARS, and evaluated the ATP levels and the survival rate of cortical neurons after oxygen and glucose deprivation (OGD) treatment.

2. Materials And Methods

2.1. Animals and Materials

The laboratory animals used in this study were provided by the Animal Center of Huazhong University of Science and Technology and were born within 24 hours Sprague-Dawley (SD) rats. All rats were housed in an environmentally controlled room under a 12-h light/dark cycle with ad libitum access to food and water. All animal experiments were approved by the Medical Ethics Committee and the Medical Faculty Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. All experiments were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). All methods in the experiments are in accordance with ARRIVE guidelines.

An antibody against MAP2 (bm1243) was purchased from Boster (Wuhan, China). An antibody against RARS (ab128956) was purchased from Abcam (Cambridge, MA, USA). The ATP Assay Kit and Apoptosis and Necrosis Assay Kit were purchased from Beyotime (Nantong, China). The CCK8 Assay Kit was purchased from Beyotime (Nantong, China), and the shRNA-RARS (GGACAATGGACAAAGCTACA) and
con190 (control shRNA, TTCTCCGAACGTGTCACGT)-expressing adenovirus vectors were purchased from GeneChem (Shanghai, China).

2.2. Cell culture, cell identification and virus transfection

The entire cortex was dissected from the brains of postnatal SD rat pups in sterile Hank's solution, and culture medium (98% neurobasal-A, 2% B27) was used to suspend the cells. The cell suspensions were added to 6-well plates containing 0.01% poly-L-lysine hydrobromide, placed in a cell incubator (37°C, 5% CO₂) and cultured for 7 days. For staining the cell culture medium was removed and the cells were washed with PBS (1X) 3 times. A 4% paraformaldehyde solution was added for 30 minutes to fix the cells, followed by PBS (1X) 3 times and 5 minutes each time. A 0.5% Triton X-100 solution was added for 15 minutes. After the supernatant was discarded, the cells were blocked with 2% goat serum (37°C, 30 min), then the supernatant was discarded and the cells were incubated with antibodies against MAP2 overnight at 4°C. After washed with PBS (1X) 3 times and 10 minutes each time, the cells were incubated with secondary antibodies for 1 h at room temperature. DAPI was added in the dark for 3 min. After washed again, the plates were observed under an inverted fluorescence microscope.

The number of positive cells was evaluated by counting cells in five randomly chosen fields (40×) from five sections. The proportion of neurons (double labeled with DAPI and MAP2) relative to cell nuclei (labeled with DAPI) was evaluated, and cultures in which the proportion of neurons exceeded 95% were considered successful.

For transfection experiments, neurons in good condition were used, and the medium was changed once before transfection. 2x10^6TU of virus (10^9TU/ml concentration) was added to the medium. After 8 to 16 h later, the culture medium was replaced again if the cells were in poor condition.

Three single-stranded DNA-coding short hairpin RNA sequences were designed based on shRNA design principles as follows[19–21]: RARS-RNAi (27392-1): target sequence: GCAAAGGAGCAGAAGGTTA, GC%: 47.37%; RARS-RNAi (27394-1): target sequence: GGACAATGGACAAGCTACA, GC%: 47.37%; RARS-RNAi (27395-1): target sequence: GGAACAGTTACGAGAAGAA, GC%: 42.11%; con190 (control shRNA): target sequence: TTCTCCGAACGTGTCACGT. The RARS-RNAi (sequence 27392-1), RARS-RNAi (sequence 27394-1), and RARS-RNAi (sequence 27395-1); the con190 negative control sequences were designed based on the Rattus norvegicus RARS mRNA sequence. RARS gene and protein expression in neurons was measured after transfection with the three RARS-RNAi-expressing adenoviral vectors.

2.3. Determination of the optimal viral vector and optimal time point for gene silencing

2.3.1. RARS gene expression in primary neurons transfected with different viral vectors was detected using qRT-PCR
For the RNA isolation, the culture medium was removed from the primary neurons, and the neurons were washed once with PBS. One milliliter of RNAiso reagent was added to each well for cell lysis. The solutions were repetitively pipetted and transferred to 1.5 ml EP tubes. Two hundred microliters of chloroform was added to each tube and centrifuged. The supernatants were transferred to another EP tube. An equal volume of isopropanol was added, the samples were centrifuged, and the supernatants were discarded. After centrifugation, the ethanol solution was discarded, and the RNA precipitates were dried for 5–10 min and dissolved in 400 µl of RNase-free water. The RNA concentration and purity were detected using UV spectrophotometry.

RNA was reverse transcribed to cDNA using a PrimeScriptTM RT MasterMix Kit (Takara Corporation).

The following primers were designed and synthesized: internal control: β-actin (240 bp), upper strand (F): 5’-CACGGATGGAGGGCCGGACTCATC-3’, lower strand (R): 5’-TAAAGACCTCTATGCCCAACACAGT-3’; target gene: RARS (233 bp), upper strand (F): 5’-CATCAAATACGCGACCGACCTTT-3’, lower strand (R): 5’-GTAAATGCACCGTCCCAGT-3’. Real-time quantitative PCR was performed using a SYBR® Premix Ex Taq™ Kit (TaKaRa Corporation). The two-step method was performed according to the manufacturer’s instructions. The following reaction conditions were used: stage 1: 1 cycle of 95°C for 30 s; stage 2: 40 cycles of 95°C for 5 s and 60°C for 30 s; and stage 3: melting curve analysis.

The mRNA expression in primary neurons was measured using RT-PCR on day 2.5 after transfection with one of the three adenoviral vectors expressing shRNA-RARSs. The adenoviral vector with optimal gene silencing efficiency was selected for subsequent experiments.

2.3.2 The RARS gene expression in primary neurons was detected at different time points using qRT-PCR

Expression of the RARS gene with the optimal gene silencing efficiency in primary neurons was detected at different time points, i.e., 24 h, 48 h, and 72 h, after transfection. The time point at which the adenoviral vector exhibited optimal gene silencing efficiency was selected as the detection time point for the subsequent experiments.

2.4. Western blot analysis of RARS protein expression in primary neurons transfected with different viral vectors

For total protein extraction, the culture medium was removed from the cultured plate, and the cells were washed with PBS. An appropriate amount of lysate was added to each well. The cells were gently scraped from the plate, transferred to a 1.5-ml EP tube, lysed for 30 min on ice, and then centrifuged for approximately 10 min at 12000 rpm and 4°C. The supernatant was transferred to a new EP tube. The samples were boiled for approximately 5 min at 95°C after the addition of protein loading buffer, and then used for subsequent experiments. The total protein (20 µg) was added to each lane, and electrophoresed at a constant voltage of 80 V. When the protein markers began to separate, the voltage was increased to 120 V, and electrophoresis was continued until the markers were distinctly separated or bromophenol blue
reached the bottom of the separating gel. The proteins were transferred to a PVDF membrane at a constant current of 200 mA for approximately 1-1.5 hour, and the membrane was removed and blocked with 5% nonfat milk at room temperature for 1 hour. The PVDF membrane was incubated in a primary antibody solution overnight at 4°C and washed 3 times with TBST for 10 minutes each wash. The PVDF membrane was immersed in a secondary antibody working solution, incubated with shaking at room temperature for 1 hour and washed with TBST. For exposure, a hypersensitive ECL working solution was added to the PVDF membrane, and the membrane was exposed in a UVP chemiluminescence imaging apparatus. Photoshop software was used to analyze the gray values of the bands.

2.5. Oxygen-glucose deprivation (OGD) model establishment

Cells were divided into the OGD model group and the control group. In the OGD model group, the culture medium was removed and washed with PBS, and then glucose-free DMEM was added to the culture plate. The plates were placed into a tri-gas incubator (37°C, 95% N₂, 5% CO₂; O₂ concentration below 1%) to simulate ischemia and hypoxia. In the control group, the culture medium was removed and washed with PBS, and then DMEM was added followed by being placed in a normal incubator. Both groups of cells were further divided into the following 3 subgroups: the normal group, the con190 (control blank vector) group and the shRNA-RARS (shRNA-RARS-expressing vector) group. Each group was analyzed in triplicate. The cells were observed 3 h after OGD treatment, and the data were measured and analyzed.

2.6. ATP detection

To prepare the samples, the cell lysis buffer was used to replace the medium after transfection. The neuronal cells were homogenized in cell lysis buffer containing protease inhibitors. After centrifugation at 12000 rpm and 4°C for approximately 5 min, the supernatant was collected for subsequent analysis. The entire process was performed on ice. The signal of the supernatant in relative light units (RLU) was determined using a multifunction enzyme labeling instrument, and the signals in RLU were plotted on the x axis. The ATP concentration (µM/L) was plotted on the y axis. An ATP Assay Kit was used to generate a standard ATP concentration curve, and the ATP concentration in each group was calculated based on the standard curve. Taking into account the changes in ATP concentration caused by the difference in protein content in different samples, we detected the protein concentration in the sample. For analysis the ATP concentration was divided by the protein concentration, and the values are expressed as nmol/mg protein.

2.7. PI staining

To prepare the detection working solution, 2 µl of Hoechst and 2 µl of PI were added to 1 ml of diluent. The culture medium was removed followed by washing with PBS, and then the working solution was added. The cells were incubated at 4°C for 20–30 min and washed 3 times with PBS. The plate was observed under a fluorescence microscope (within 20 min).

2.8. RARS protein expression
The same procedures as described in Sect. 2.4 were performed.

2.9. CCK8 detection

For the test, a 100 µl of suspended cell sample was added to each well of a 96-well plate, and a 10% volume enhanced CCK-8 test solution was added to each well. A well containing no cells was used as a blank control. After incubation for 30–60 minutes, the absorbance at a wavelength of 450 nm was detected using an enzyme labeling instrument.

2.10. Statistical analysis

SPSS 19 professional statistical software was used to analyze the data, and the results were expressed as the means ± SEMs. Comparisons between the control group and experimental group at the same time points were performed using two-sample independent t - tests, and rates were compared using the χ² test. P < 0.05 was considered significant.

3. Results

3.1. Identification of primary neurons

Primary cultured neurons were identified after approximately 7 days by randomly counting the number of cell nuclei (DAPI-positive) and the number of neurons (DAPI and MAP2 double-positive) (Fig. 1) in 5 fields of view and calculating the percentage of neurons relative to all cells. When the proportion of neurons was above 95%, the culture was considered successful, and the purified neurons were used in subsequent experiments.

3.2. Optimal viral vector and optimal time point for gene silencing

3.2.1 Gene expression of RARS in primary neurons after transfection with the three adenoviral vectors at different time points

Expression of the ArgRS gene in the primary neurons 2.5 days after adenoviral vector transection was as follows (Table 1). The target gene amplification of RARS genes transfected with different vectors was determined (Fig. 2A). The adenoviral vector expressing Rars-RNAi (27394-1) (RARS-RNAi (27394-1)-group) had the best silencing efficiency, which is 74%, among the adenoviral vectors expressing Rars-RNAi. Expression of the RARS gene at different time points after adenoviral vector expressing Rars-RNAi (27394-1) transfection was as follows (Table 2). Target gene amplification of the RARS gene in the RARS-RNAi (27394-1) group at different time points was shown in Fig. 2B. RARS-RNAi (27394-1) exhibited the best silencing efficiency 3 d after the adenoviral vector transfection.
Table 1
The expression of the RARS gene in primary neurons 2.5 d after adenoviral vector transfection

| Sample   | CT value | ΔΔCT | Δ ΔΔCT |
|----------|----------|------|--------|
| normal   | 1.00     | 14.97| 14.83  | 19.96  | 14.83 | 19.84 | 5.01 | 0.00 |
| normal   | 1.00     | 14.97| 14.83  | 19.63  | 14.83 | 19.84 | 5.01 | 0.00 |
| normal   | 1.00     | 14.57| 14.83  | 19.93  | 14.83 | 19.84 | 5.01 | 0.00 |
| con190   | 0.99     | 15.33| 15.40  | 20.39  | 15.40 | 20.42 | 5.02 | 0.01 |
| con190   | 0.99     | 15.58| 15.40  | 20.40  | 15.40 | 20.42 | 5.02 | 0.01 |
| con190   | 0.99     | 15.28| 15.40  | 20.47  | 15.40 | 20.42 | 5.02 | 0.01 |
| 2 - 1    | 0.64     | 16.33| 16.04  | 21.76  | 16.04 | 21.69 | 5.65 | 0.64 |
| 2 - 1    | 0.64     | 15.88| 16.04  | 21.50  | 16.04 | 21.69 | 5.65 | 0.64 |
| 2 - 1    | 0.64     | 15.92| 16.04  | 21.80  | 16.04 | 21.69 | 5.65 | 0.64 |
| 4 - 1    | 0.26     | 14.99| 14.86  | 22.05  | 14.86 | 21.84 | 6.98 | 1.97 |
| 4 - 1    | 0.26     | 14.65| 14.86  | 21.65  | 14.86 | 21.84 | 6.98 | 1.97 |
| 4 - 1    | 0.26     | 14.95| 14.86  | 21.82  | 14.86 | 21.84 | 6.98 | 1.97 |
| 5 - 1    | 0.68     | 14.18| 14.99  | 20.62  | 14.99 | 20.54 | 5.56 | 0.55 |
| 5 - 1    | 0.68     | 15.25| 14.99  | 20.90  | 14.99 | 20.54 | 5.56 | 0.55 |
| 5 - 1    | 0.68     | 15.53| 14.99  | 20.11  | 14.99 | 20.54 | 5.56 | 0.55 |

Note: normal is the normal control group
con190 is the blank vector group
Rars-RNAi (27392-1) (2 - 1) is the RAR-RNAi (27392-1)-expressing adenoviral vector-transfected group
Rars-RNAi (27394-1) (4 - 1) is the RARS-RNAi (27394-1)-expressing adenoviral vector-transfected group
Rars-RNAi (27395-1) (5 - 1) is the RARS-RNAi (27395-1)-expressing adenoviral vector-transfected group
3.2.2. Protein expression of RARS in primary neurons after transfection with three adenoviral vectors at different time point

Protein expression of RARS in primary neurons 72 hours after transfection with the different vectors is shown in Fig. 2C,2D. Protein expression of RARS in the RARS-RNAi (27394-1)-group at different time points is shown in Fig. 2E,2F. From the above data and analyses of the gene silencing and protein expression levels, the RARS-RNAi (27394-1)-group was selected for subsequent experiments. The optimal time point for gene silencing was 3 days after adenovirus transfection, and this time point was used in subsequent experiments.

3.3. shRNA-RARS-expressing adenoviral vector transfection decreased ATP consumption and RARS protein expression in neurons after OGDtreatment
After 3 days of transfection and 3 hours of OGD treatment, the ATP content in each group was measured (Fig. 3A,3B). The results showed that the cells in the shRNA-RARS group consumed less energy than cells in the other groups, and the residual energy in these cells maintained survival for a longer duration than that in the cells in the other groups. RARS protein expression was measured in each group (Fig. 3C,3D), and the results showed that RARS activity was increased after OGD treatment, and that the energy demand was increased. However, RARS activity was limited following the OGD treatment in the shRNA-RARS group, and the energy demand was lower than that in the normal group and the blank vector group.

3.4. shRNA- RARS-expressing adenoviral vector transfection decreased neuronal death and increased CCK8 activity in neurons after OGD treatment

After OGD treatment, the apoptosis and necrosis of each group of cells (Fig. 4A and 4C) and CCK8 activity (Fig. 4B and 4D) were assessed. The results showed that the cellular activity in the experimental group was higher than that in the normal and blank vector groups, indicating that the shRNA-RARS group had better tolerance to ischemia and hypoxia stimulation than the other groups due to inhibition of RARS activity and decrease in the energy metabolism rate of the cells. Therefore, inhibition of RARS had a protective effect on ischemia.

4. Discussion

The present study showed that knockdown of ArgRS significantly inhibited protein synthetases, decreased ATP assumption, inhibited neuronal apoptosis and necrosis and increased neuronal activity, thereby alleviated neuronal injury. Indeed, neurons transfected with shRNA-RARS exhibited better tolerance to ischemia and hypoxia due to the inhibition of RARS activity and decrease in the energy metabolism rate of cells. Therefore, knockdown of RARS had a protective effect on cerebral ischemia.

The current findings extend previous reports concerning the role of RARS in C. elegans following hypoxic injury.

We induced ischemia and hypoxia in this experiment by subjecting cells to OGD treatment. Chemical ischemia models induced by drugs such as CoCl₂, 3-NP, and Na₂S₂O₄, have also been established.[19–23] However, it is unclear whether these drugs affect the properties of the solution and whether they have other effects on cells. The onset of ischemia and duration of drug treatment are not easy to control. Therefore, we did not use such models in this study.

RARS is a key enzyme in the amino acid activation process. In addition to reducing energy consumption, RARS inhibition leads to the inhibition of arginine activation, which prevents its binding to the corresponding tRNA and leads to a reduction in protein translation. Whether a significant reduction in protein levels affects certain cell functions or causes cell death is unknown. Viruses themselves have a certain toxic effect on cells, and the amount of virus (multiplicity of infection) that should be transfected into cells should be determined according to the extent of RARS inhibition required.[24].
The AARS family includes 20 enzymes (one per amino acid), and each enzyme performs a different function. Anderson et al.\textsuperscript{18} reported that protein translation was significantly reduced after the inhibition of the RARS gene, and the death of \textit{C. elegans} in the RARS inhibition group was significantly lower than that in the control group in a hypoxic environment. Crean et al.\textsuperscript{25} reported that inhibition of protein synthesis using the AARSs inhibitor halofuginone or cycloheximide prevented OGD-induced injury, while inhibition of the CREB/CBP interaction prevented OGD-induced isoleucyl-AARS expression, reduced protein synthesis and protected against OGD-induced cellular injury in renal epithelial HK-2 cells in rats. Kamphuis et al.\textsuperscript{26} performed ischemic preconditioning on rat retina and found that after continuous ischemia in the experimental group, the activity of AARS was lower than that of the control group. These findings suggest that ischemic preconditioning decreases protein synthesis and energy consumption related to the translation process, thereby protecting cells from hypoxic-ischemic injury. Our previous studies showed that RARS mRNA and protein expression increased after focal cerebral ischemia,\textsuperscript{27} but its expression decreased when ischemic preconditioning was performed before the subsequent ischemic treatment.\textsuperscript{28} These data support the importance of RARS in the pathogenesis of cerebral ischemia and the necessity of basic research on it.

Our results showed that adenoviral vector expressing Rars-RNAi (27394-1) exhibited the best silencing efficiency of the target gene, with a silencing rate of 74%, and had the most obvious inhibitory effect on target protein expression. This adenovirus vector worked best regardless of whether its effect was due to gene silencing or protein expression inhibition. To understand the temporal changes in RARS protein expression in the RARS-RNAi (27394-1) group, the protein expression of RARS in the RARS-RNAi (27394-1) group was measured using Western blot analysis at different time points after adenoviral vector transfection. The results showed that RARS protein expression was markedly decreased 3 days after transfection and slightly enhanced 4 days after transfection (P < 0.01). Therefore, the adenoviral vector expressing RARS-RNAi (27394-1) and 3 d after transfection were selected for gene silencing in this study.

After OGD treatment, the apoptosis and necrosis of each group were observed, and the ATP level, cell survival and CCK8 cell activity were detected. Our results showed that the cell activity in the the RARS-RNAi (27394-1)-group was higher than that in the normal and blank adenovirus vector groups, and inhibition of RARS activity decreased the energy metabolism rate of the cells. The notion that preventing energy consumption by preserving cellular metabolism is neuroprotective against ischemia is well known. Many studies in literature have proposed several therapeutic strategies with similar mechanisms. For example, the inhibition of poly(ADP)-ribose polymerase (PARP) by TIQ-A(a PARP-1 / PARP-2 inhibitor) leads to neuroprotection in mixed cortical cells as well as in organotypic hippocampal slices exposed to OGD by reducing ATP depletion \textsuperscript{32, 33}, but also natural or chemical compound able to increase mitochondrial ATP production in spite of reduced oxygen consumption \textsuperscript{34}. Our work does not directly indicate that the reduction in energy metabolism is due to a reduction in protein translation because it is difficult to directly measure protein translation or energy metabolism in neurons \textit{in vitro}. Positron emission tomography (PET) with 2-deoxy-2-[fluorine-18]fluoro-D-glucose (\textsuperscript{18}F-FDG PET) has been used to assess glucose metabolism after ischemia and infarction.\textsuperscript{35} Using \textsuperscript{18}F-FDG PET in small animals
allows the visualization of glucose metabolism throughout the brain. Our previous study measured \(^{18}\)F-FDG uptake in the ischemic cortex and contralateral cortex after stereotaxic injection of an adenoviral vector expressing Rars-RNAi into middle cerebral artery occlusion (MCAO) rats.[36] The \(^{18}\)F-FDG microPET imaging showed that RARS knockdown significantly increased standardized uptake values in the ischemic cortex, which may be due to the preservation of glucose utilization and improved neuron survival.

The reduction in energy metabolism \textit{in vivo} and \textit{in vitro} suggests that knockdown of RARS leads to protein synthesis inhibition, because ATP is the main energy source for protein biosynthesis. The protein synthesis inhibitor cycloheximide reduced the infarct volume in rats even when treatment was delayed for up to 6 hours after the onset of ischemia.[37] Cycloheximide exerted a synergistic effect in a rodent model of spinal cord ischemia-reperfusion injury.[38] Compared with the control group, rats treated with dextromethorphan and cycloheximide had less severe paraplegia and markedly reduced numbers of necrotic and apoptotic neurons on the first and second days after ischemia. [39] Further examination of amino acid metabolism by using other imaging techniques such as \(^{11}\)C-methionine (MET) PET in a focal ischemic model \textit{in vivo} may be helpful.

Collectively, our data showed that the RARS-RNAi (27394-1)-group exhibited stronger tolerance to ischemia and hypoxia due to inhibition of RARS activity and reduced cell energy metabolism rate. Our work also suggested that RARS inhibition can reduce protein translation, decrease energy consumption, and play a protective role via ischemic tolerance. Whether RARS-specific inhibitors or other AARSs also have protective effects remains unclear. However, there are only very few studies have investigated the ischemic protective effects of AARS and RARS inhibitors. Further \textit{in vivo} and \textit{in vitro} evidence regarding this mechanism should be obtained and will provide a theoretical basis for the development of new drugs.

5. Conclusions

Our work revealed neurons transfected with adenoviral vectors expressing shRNA-RARS exhibited stronger tolerance to ischemia and hypoxia, which was due to the inhibition of RARS activity and reduced cell energy metabolism rate. These results suggested that RARS inhibition reduced protein translation and energy consumption, and played a protective role in ischemic tolerance.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Medical Ethics Committee and and the Medical Faculty Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Consent for publication
This research is consent for publication by both authors

Availability of data and material

Article’s supporting data and materials can be accessed from Medical Records and Statistics Room, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, wuhan 430022, china

Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Authors’ contributions

Zhang Lizhi, Master’s Degree in Medicine, conducted experiments; analyzed data; drafted the manuscript

Fu Rong, MD, PhD, conception and design; analyzed data; revised the manuscript

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**Figures**
Figure 1

Primary cultured rat cortical neurons, identified by MAP2 (red) and DAPI (blue) double staining.

Figure 2

2A

RQ vs Sample

2B

RQ vs Sample

2C

ArgRS

β-actin

2D

ArgRS

2E

ArgRS

2F

ArgRS

Sample

24 h

48 h

72 h

Con190

Normal

Sample

Normal con190 1 d 2 d 3 d 4 d

Sample

Normal Con190 1 d 2 d 3 d 4 d
Analysis of RARS gene expression in primary cultured cortical neurons (7 days) transfected with adenoviral vectors expressing RARS-RNAi by using semi-quantitative RT-PCR and western-blotting. 2A. RARS mRNA levels in neurons transfected with the adenoviral vectors expressing RARS-RNAi (27392-1) and (27394-1), but not (27395-1) was lower than the normal group and blank vector group, and the difference was statistically significant (P < 0.05). 2B. RARS mRNA levels in the adenoviral vectors expressing RARS-RNAi (27394-1) did not differ (P > 0.05) at 24 hours, but started to decline at 48 hours, and significantly decreased at 72 hours (P < 0.01) after transfection at which point the target gene activity was only 0.16 as compared to the blank vector group. 2C-D. RARS protein levels levels in neurons transfected with the adenoviral vectors expressing RARS-RNAi (27392-1) and (27394-1), but not (27395-1) was lower than the normal group and blank vector group, and the difference was statistically significant (P < 0.05). 2C, A representative gel photograph of western-blotting of 2D. 2E-F. RARS protein expression in neurons transfected with the adenoviral vectors expressing RARS-RNAi (27394-1) was comparable to that in the normal and blank vector groups at 1 day (P > 0.05), decreased at 2 days, and significantly declined 3 and 4 days (P < 0.01) after transfection. There was no significant difference in RARS protein expression between 3 days and 4 days after transfection (P < 0.05). 2E, A representative gel photograph of western-blotting of 2F. In 2C-F, the gray values of the protein bands were analyzed using Photoshop software, and the data were plotted as a histogram using GraphPad software. N = 3/group.

**Figure 3**

Evaluation of ATP levels and RARS protein expression in neurons transfected with adenoviral vectors expressing RARS-RNAi after OGD treatment. 3A-B. ATP levels were not significantly different between the groups before OGD treatment. ATP levels were lower in the OGD model groups than that in the control groups. Although the ATP level in each group decreased after OGD treatment, the ATP content in the shRNA-RARS group was greater than that in the control group. The bars represent the means ± SEMs of 3
independent experiments. 3B, the data sheet of that showed in 3A. *Significantly different from the control group (* p < 0.05, ** p < 0.01, compared to the control group; # p < 0.05, compared to the OGD group). 3C-D. RARS protein expression was higher in the OGD model groups than that in the control groups. Although the protein expression of RARS increased after OGD, it was lower in the shRNA-RARS group than that in the control group. 3D, bar graphs represent the means ± SEMs of 3 independent experiments (gray value of RARS/β-actin). *Significantly different from the control group (* p < 0.05, compared to the control group; # p < 0.05, compared to the OGD group).

Figure 4

Evaluation of cell viability of neurons transfected with adenoviral vectors expressing RARS-RNAi after OGD treatment. Primary cultured cortical neurons (7 days) were challenged with OGD stimulation for 3 hours and then the cell viability was assessed. The adenovirus vectors were transfected 3 days prior to OGD stimulation. (A) Analysis of PI staining in OGD-challenged primary neurons. The results are expressed as the percentage of necrotic neurons relative to the total number of cells. The bars represent the means ± SEMs of 3 independent experiments. (B) Analysis of CCK8 activity in OGD-challenged primary neurons. The results are expressed as the OD450 values of the wells of multi-well plates. The bars represent the means ± SEMs of 3 independent experiments. (C) Representative images of the data shown in A. (D) Table of the data shown in B. *Significantly different from the control group (* p < 0.05, compared to the control group; # p < 0.05, compared to the OGD group).

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