Int J Physiol Pathophysiol Pharmacol 2015;7(2):107-114
www.ijppp.org /ISSN:1944-8171/IJPPP0006988

Original Article

Aralar plays a significant role in maintaining the survival and mitochondrial membrane potential of BV2 microglia

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Received February 11, 2015; Accepted February 28, 2015; Epub August 15, 2015; Published August 30, 2015

Abstract: NADH shuttles mediate the transfer of the reducing equivalents of cytosolic NADH into mitochondria. Increasing evidence has suggested that malate-aspartate shuttle (MAS), one of the two types of NADH shuttles, plays important roles in certain biological processes. Aralar/AGC1, a Ca^{2+}-dependent aspartate-glutamate carrier on mitochondrial membrane, is a component of MAS. It has been reported that Aralar plays crucial roles in linking increased cytosolic Ca^{2+} concentrations to enhanced mitochondrial energy metabolism of neurons under certain conditions, while the role of the carrier in cell survival remains unknown. In the current study, we tested our hypothesis that Aralar plays an important role in cell survival, using BV2 microglia as a cellular model. Our study showed that Aralar siRNA-produced decrease in the Aralar level led to a significant reduction of the cell survival. Our FACS-based Annexin V/7-AAD assays also showed that the Aralar siRNA treatment led to a significant increase in apoptosis of the cells. Moreover, the Aralar siRNA treatment led to both mitochondrial depolarization and decreases in the intracellular ATP level of the cells. Collectively, our study has provided the first evidence suggesting that Aralar plays a significant role in cell survival, at least for such cell types as BV2 microglia, possibly by producing mitochondrial depolarization. These observations have also provided novel information for understanding the roles of NADH shuttles in cell survival.

Keywords: Aralar, NADH, malate-aspartate shuttle, microglia, apoptosis

Introduction

NAD^+ and NADH (reduced form of NAD^+) are major coenzymes for the electron transfer in numerous biochemical reactions, which play crucial roles in a number of biological functions including energy metabolism, mitochondrial functions, and gene expression [1]. While cumulative evidence has indicated a key role of NAD^+ in cell survival [2], there has been distinct deficiency in the information regarding the roles of NADH in cell survival and cellular functions.

Previous studies have suggested that NADH may be used to decrease the symptoms of Parkinson's disease (PD) [3]. However, the mechanisms underlying the effects of NADH remain unclear. NADH shuttles mediate the transfer of the reducing equivalents of cytosolic NADH into mitochondria [4]. Malate-aspartate shuttle (MAS) and glycerol-3-phosphate shuttle are two major types of NADH shuttles [1]. Increasing evidence has suggested that MAS plays significant roles in certain biological processes, including synthesis of glutamate and glutamine in neurons [5], insulin secretion from -islet cells [6], and growth of breast cancer cells [7]. However, there have been few studies regarding the roles of NADH shuttles in cell survival and inflammation.

Aralar/AGC1, a Ca^{2+}-dependent aspartate-glutamate carrier on mitochondrial membrane, is a component of MAS [4, 8, 9]. It has been reported that Aralar plays a significant role in neuronal energy metabolism under certain condi-

tions [8-10]. However, there has been no report regarding the role of the carrier in cell survival. Since energy metabolism is a significant factor in cell survival, we hypothesized that Aralar plays an important role in cell survival. In current study we tested this hypothesis using BV2 microglia as a cellular model, considering that microglia plays critical roles in the brain injury in multiple neurological disorders [11, 12]. Our study has shown that decreased Aralar by Aralar siRNA led to a significant increase in apoptosis of the cells, thus suggesting a novel biological function of Aralar.

Methods and materials

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except where noted.

Cell cultures

BV2 microglia were plated into 24-well cell culture plates at the initial density of 1×10^5 cells/mL in Dulbecco's Modified Eagle Medium containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine (Thermo Scientific, Waltham, MA, USA), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), supplemented with heat-inactivated (56°C for 30 min) 5% fetal bovine serum (GIBCO, Melbourne, Australia). The cells were maintained at 37°C in a 5% CO_2 incubator.

RNA interference

BV2 microglia were transfected with either Aralar siRNA oligonucleotides (5' GCACGAGGAAAGAUAUUGATT 3') against mitochondrial carrier Aralar (Slc25a12) (Genepharma, Shanghai, China) or Control siRNA oligonucleotides (5' UUCUCCGAACGUGUCACGUTT 3') (Genepharma, Shanghai, China), when the cells were approximately 40% confluent. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for the transfection according to the manufacturer's instructions. For each well of a 24-well plate, 100 µl Opti-MEM containing 0.06 nmol of the siRNA oligonucleotides and 2 µl lipofectamine 2000 was added into 500 µl culture media of the cells. The Aralar level of BV2 microglia was determined by Western Blot 24 hrs after the transfection.

Intracellular lactate dehydrogenase (LDH) assay and extracellular LDH assay

Cell survival was quantified by intracellular LDH assay [13]. Briefly, cells were lysed for 20 min in lysing buffer containing 0.04% Triton X-100, 2 mM HEPES, 0.01% bovine serum albumin (pH 7.5). Fifty µl cell lysates were mixed with 150 µL 500 mM potassium phosphate buffer (pH 7.5) containing 0.3 mM NADH and 2.5 mM sodium pyruvate. The A_{340nm} change was monitored over 90 sec by a plate reader. Percentage of cell survival was calculated by normalizing the LDH values of the samples to the LDH activity measured in the lysates of the control (wash only) culture wells.

Cell death was determined by extracellular LDH assay [13]. In brief, 100 µl of extracellular media was mixed with 150 µl potassium phosphate buffer (500 mM, pH 7.5) containing 0.3 mM NADH and 2.5 mM sodium pyruvate. Subsequently changes of the A_{340nm} of the samples were monitored over 90 sec by a plate reader.

Flow cytometry-based Annexin V/7-AAD assay

The flow cytometry assay was performed to determine the levels of early-stage apoptosis, late-stage apoptosis, and necrosis using ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) according to the manufacturer's protocol [13]. Briefly, BV2 microglia were digested by 0.25% trypsin-EDTA, washed by cold PBS one time and resuspended in cold binding buffer at concentrations between 1×10^6 and 1×10^7 cells/ml. Five µL of labeled Annexin V was added into 100 µL of the cell suspension. After incubation on ice for 15 min, 200 µL binding buffer and 5 µL 7-AAD solution were added into the cell suspensions. The number of stained cells was assessed immediately by a flow cytometer (FACSAria II, BD Biosciences).

Flow cytometry-based JC-1 assay

Mitochondrial membrane potential ($\Delta\psi_m$) was measured by flow cytometry-based JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide) assay. BV2 microglia were harvested by 0.25% trypsin-EDTA, and incubated in cell media containing 10 µg/mL JC-1 (Enzo Life Sciences, Farmingdale,

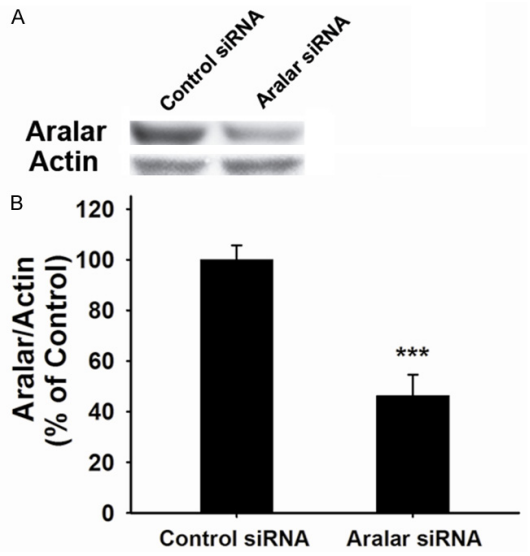

Figure 1. Aralar siRNA treatment led to decreased Aralar levels of BV2 microglia. A. Western blot assays on Aralar showed that the Aralar level in the cells treated with Aralar siRNA was lower than that of the cells treated with Control siRNA. B. Quantifications of the Western Blots indicated that the Aralar level in the cells treated with Aralar siRNA was significantly lower than that of the cells treated with Control siRNA. BV2 microglia were treated with Control siRNA or Aralar siRNA. Twenty four hrs after the treatment, the Aralar levels in the cells were determined by Western Blot. *N*=10-11. Data were collected from five independent experiments. ***P<0.001.

NY, USA) for 20 min in 37°C. After washed once with PBS, BV2 microglia was analyzed by a flow cytometer (FACSAria II, BD Biosciences), which detected emission fluorescence at the wavelength of 575 nm (PE-A) and the wavelength of 518 nm (FITC-A) with excitation wavelength of 488 nm. The $\Delta\psi_m$ of each cell was calculated by the ratio of the fluorescence at wavelength of 575 nm/the fluorescence at 518 nm. For each sample, the percentage of the cells with low $\Delta\psi_m$ in total cell population was reported by the flow cytometer (FACSAria II, BD Biosciences).

Western blot

BV2 microglia was harvested and lysed in RIPA buffer (Millipore, Temecula, CA, USA) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) and 1 mM PMSF. After quantifications of the protein samples using BCA Protein Assay Kit (Pierce Biotechonology, Rockford, IL, USA), 45 μg of total protein was electrophoresed through a 10% SDS-polyacrylamide gel, and then transferred to 0.45 μm nitrocellulose membranes. The blots were incubated overnight at 4°C with a rabbit monoclonal anti-Aralar antibody (1:2000 dilution) (Abcam, Cambridge, MA, USA), then incubated with HRP-conjugated secondary antibody (Epitomics, Hangzhou, Zhejiang Province, China). Protein signals were detected using the ECL detection system (Pierce Biotechonology, Rockford, IL, USA). An anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to normalize the sample loading and transfer. The intensities of the bands were quantified by densitometry using Image J.

ATP assay

Intracellular ATP levels were determined using an ATP Bioluminescence Assay Kit (Roche Applied Science, Mannheim, Germany), according to the protocol provided by the vendor. Briefly, after washed once with PBS, the cells were lysed with the Cell Lysis Reagent, which was mixed with 50 μL of the Luciferase Reagent. A plate reader (Biotek Synergy 2) was used to detect the chemiluminescence of the samples. The ATP concentrations of the samples were calculated using an ATP standard, and normalized to the protein concentrations of the samples, which were determined by BCA assay.

Statistical analyses

All data are presented as mean + SE. Data were assessed by one-way ANOVA, followed by Student-Newman-Keuls post hoc test. *P* values less than 0.05 were considered statistically significant.

Results

Aralar siRNA treatment led to decreased survival of BV2 microglia

In order to study the roles of Aralar in the survival of BV2 microglia, we applied Aralar siRNA to decrease the Aralar level of the cells. Our Western blot assay showed that the Aralar siRNA treatment was capable of producing a significant decrease in the Aralar level of the cells (**Figure 1A** and **1B**).

By assessing intracellular LDH activity and extracellular LDH activity, we determined the effects of the decreased Aralar on the survival and death of the cells, respectively. Our intra-

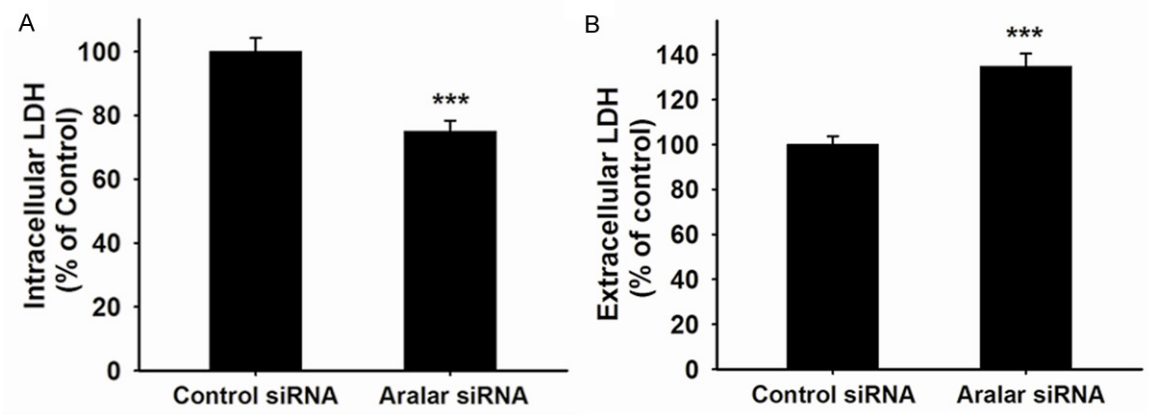

Figure 2. Aralar siRNA treatment can lead to a significant decrease in the intracellular LDH activity and a significant increase in the extracellular LDH activity of BV2 microglia. A. Treatment of the cells with Aralar siRNA led to a significant decrease in the intracellular LDH activity of the cells, assessed 24 hrs after the Aralar siRNA treatment. *N*=11. Data were collected from three independent experiments. ***P<0.001. B. Treatment of the cells with Aralar siRNA led to a significant increase in the extracellular LDH activity of the cells, assessed 24 hrs after the Aralar siRNA treatment. *N*=12. Data were collected from three independent experiments. ***P<0.001.

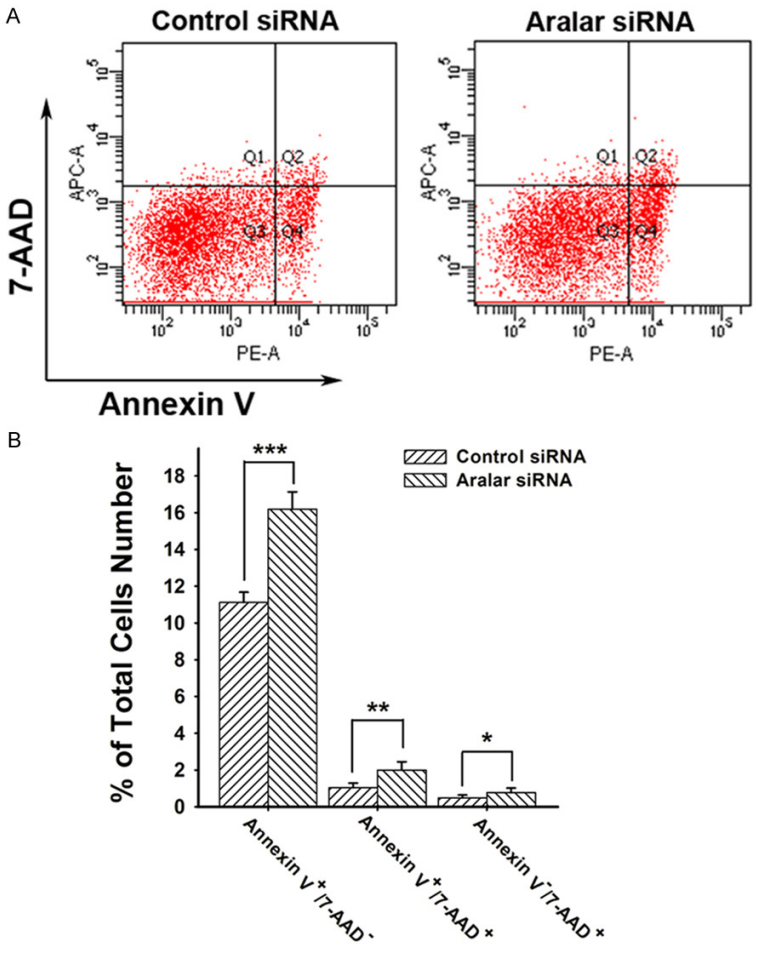

Figure 3. Treatment of BV2 microglia with Aralar siRNA led to significant increases in both early-stage and late-stage apoptosis of the cells. A. FACS-based Annexin V/7-AAD assay showed that treatment of the cells with the Aralar siRNA induced increases in the number of both Annexin V^+/7-AAD^- (early-stage apoptosis) and Annexin V^+/7-AAD^+ (late-stage apoptosis) cells, assessed 24 hrs after treatment. In the four fields of the original images from the FACS-based study, the number of the dots in the bottom-left field, the bottom-right field, the top-left field and the top-right field indicates the number of Annexin V^-/7-AAD^-, Annexin V^+/7-AAD^-, Annexin V^-/7-AAD^+, and Annexin V^+/7-AAD^+ cells, respectively. B. Quantifications of the results from the FACS-based Annexin V/7-AAD assay showed that the Aralar siRNA induced significant increases in the number of both early-stage apoptosis and late-stage apoptosis of the cells. BV2 microglial were treated with Control siRNA or Aralar siRNA. Twenty-four hrs after the treatment, the levels of early-stage apoptosis, late-stage apoptosis and necrosis of the cells were determined by FACS-based Annexin V/7-AAD assay. *N*=9. Data were collected from three independent experiments. *P<0.05; **P<0.01; ***P<0.001.

cellular LDH assays showed that the Aralar siRNA treatment led to a significant reduction of the intracellular LDH activity of the cells (**Figure 2A**), suggesting that decreased Aralar led to a reduc-

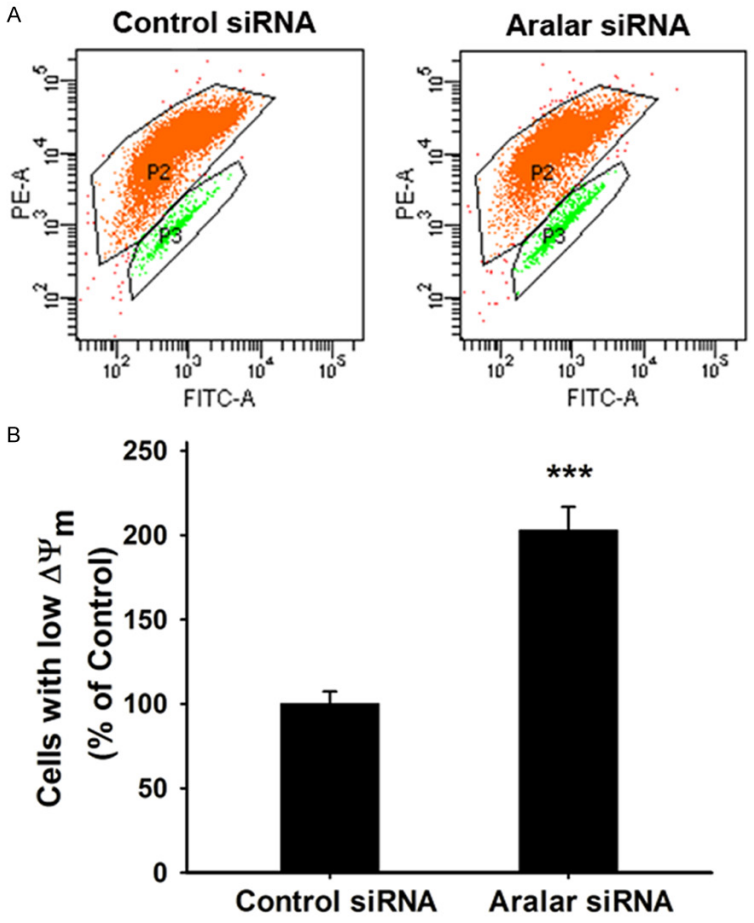

Figure 4. Aralar siRNA treatment led to decreased mitochondria membrane potential ($\Delta\psi_m$) of BV2 microglia. A. FACS-based JC-1 assay showed that the Aralar siRNA treatment led to an increase in the number of the cells with low $\Delta\psi_m$ (green dots). The number of the cells with high $\Delta\psi_m$ were indicated by the red dots. B. Quantifications of the results from the FACS-based JC-1 assay showed that the Aralar siRNA treatment led to a significant increase in the number of the cells with low $\Delta\psi_m$. BV2 microglial were treated with Control siRNA or Aralar siRNA. Twenty-four hrs after the treatment, the $\Delta\psi_m$ of the cells were determined by FACS-based JC-1 assay. N=9. Data were collected from three independent experiments. ***P<0.001.

tion of the cell survival. Consistent with our intracellular LDH assays, our extracellular LDH assays also showed that the Aralar siRNA treatment led to a significant increase in the extracellular LDH activity of the cells (**Figure 2B**), suggesting that decreased Aralar led to increased cell death.

Aralar siRNA treatment led to both early-stage and late-stage apoptosis of BV2 microglia

We found that treatment of the cells with the Aralar siRNA led to increases in both Annexin V^+/7-AAD^- (early-stage apoptosis) cells and Annexin V^+/7-AAD^+ (late-stage apoptosis) cells (**Figure 3A**). Quantifications of these results indicated that the Aralar siRNA treatment led to significant increases in both early-stage apoptosis and late-stage apoptosis of BV2 microglia (**Figure 3B**).

Aralar siRNA led to decreases in both $\Delta\psi_m$ and the intracellular ATP level of BV2 microglia

We used FACS-based JC-1 assay to determine the effect of Aralar siRNA treatment on the $\Delta\psi_m$ of the cells, which plays a significant role in cell survival [14-16]. Twenty four hrs after the Aralar siRNA treatment, the percentage of the BV2 microglial cells with low $\Delta\psi_m$ was significantly increased (**Figure 4A** and **4B**), suggesting that the Aralar siRNA treatment was capable of producing decreased $\Delta\psi_m$ of the cells. We also found that the Aralar siRNA treatment led to a significant decrease in the intracellular ATP level of the cells (**Figure 5**).

Discussion

The major findings of our study include: First, Aralar siRNA-produced decrease in the Aralar level led to a significant decrease in the survival of BV2 microglia; second, the Aralar siRNA treatment led to significant increases in both early-stage and last-stage apoptosis of the cells; third, the Aralar siRNA treatment led to mitochondrial depolarization of the cells; and fourth, the Aralar siRNA treatment led to a significant decrease in the intracellular ATP level of the cells. In summary, our study has provided the first evidence suggesting that Aralar plays a significant role in cell survival, at least for such cellular models as BV2 microglia. Our study has also suggested that Aralar plays significant

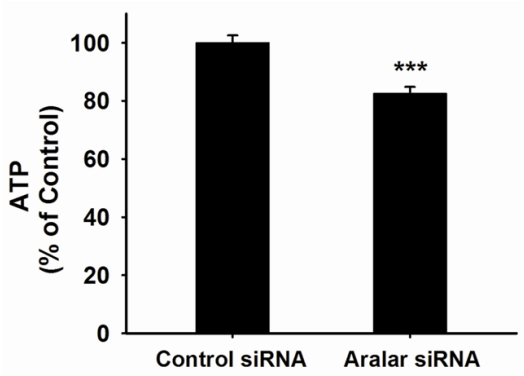

Figure 5. Treatment of BV2 microglia with Aralar siRNA led to a significant decrease in the intracellular ATP level of the cells, assessed at 24 hrs after the Aralar siRNA treatment. N=12. Data were collected from four independent experiments. ***P<0.001.

roles in maintaining both the mitochondrial membrane potential and the intracellular ATP level of BV2 microglia.

Aralar, a aspartate-glutamate carrier on mitochondrial membrane, is a component of MAS [4, 8, 9]. Several studies have suggested a critical role of the carrier in linking the cytosolic Ca^{2+} concentrations and the mitochondrial energy metabolism in neurons [8-10]. In appears that increased cytosolic Ca^{2+} levels can enhance mitochondrial energy metabolism by at least two pathways [8-10]: First, to activate the three mitochondrial matrix dehydrogenases and F1F0-ATP synthase via producing mitochondrial calcium uniporter-dependent increases in mitochondrial Ca^{2+} concentration; and second, by activating Ca^{2+}-dependent Aralar and ATP-Mg/Pi carriers on mitochondrial membranes. Aralar knockout mice have been found to have hyperactivity, motor discoordination, and short life span [10]. However, the mechanisms underlying the short life span of Aralar knockout remain unclear. There has been no study on cellular levels to determine the roles of Aralar in cell survival. Moreover, there has been no information regarding the biological functions of Aralar in immune cells.

Based on the information stated above, our current study was conducted to investigate the roles of Aralar in the survival and energy metabolism of BV2 microglia-a widely used model of microglia. Our study has provided several lines of evidence suggesting that Aralar plays an important role in the basal survival of BV2 microglia: Our intracellular LDH assays and extracellular LDH assays have shown that decreased Aralar led to decreased cell survival and increased cell death, respectively. Our FACS-based Annexin V/AAD assays have further indicated that decreased Aralar led to significant increases in both early-stage and late-stage apoptosis of the cells.

It is noteworthy that Aralar is a Ca^{2+}-dependent protein [17, 18]. Previous studies have suggested that Aralar is required for transmission of cytosolic Ca^{2+} signals to mitochondria by increasing mitochondrial NADH levels [8-10]. Both previous studies [8-10] and our current study have also suggested that Aralar plays significant roles in energy metabolism at least in certain types of cells. In addition to its role in shuttling of the reducing equivalents of NADH from cytosol to mitochondria, it has also been suggested that Aralar plays an important role in enhancing pyruvate supply to mitochondria [9]. Collectively, Aralar appears to be an important protein that links calcium signaling with energy metabolism. Because both Ca^{2+} and energy metabolism are important factors in cell death under many conditions [19-21], it is tempting to speculate that Aralar might also be a significant factor that links $[Ca^{2+}]_i$, energy metabolism, and cell death. Many future studies are warranted to further investigate the roles of Aralar in cell survival and various biological functions.

Our study using FACS-based JC-1 assay has also shown that decreased Aralar can produce mitochondrial depolarization of the cells. This observation has suggested that the decreased Aralar may lead to cellular apoptosis at least partially by producing mitochondrial depolarization, due to the following reasons: Mitochondrial alterations play critical roles in apoptosis [22, 23], which are also important pathological factors in the brain injury of both cerebral ischemia and PD [24-26]. Previous studies have suggested that mitochondrial depolarization can promote mitochondrial permeability transition (MPT) that can initiate apoptotic cascades under many conditions [14-16]. Future studies are warranted to further determine the relationships among Aralar, mitochondrial energy metabolism, MPT and apoptosis.

Because Aralar is an important component of MAS, it is expected that the decreased Aralar may lead to decreased MAS activity, thus

decreasing the shuttling of the reducing equivalents of cytosolic NADH into mitochondria. Because mitochondrial NADH plays a critical role in the generation of mitochondrial membrane potential [4], the decreased Aralar may lead to mitochondrial depolarization of the cells by decreasing shuttling of the reducing equivalents of cytosolic NADH into the mitochondria. In other words, our current study has also suggested that MAS may play a significant role in the basal survival of BV2 microglia. Microglia plays key roles in multiple major neurological diseases including brain ischemia and PD [11, 12]. BV2 microglia is a widely used model of microglia, which is a valid substitute of primary microglia in many experimental settings [27]. Our current study has provided novel evidence suggesting that Aralar plays an important role in the basal survival of microglia. Therefore, Aralar might become a new target for modulating the survival and functions of microglia in certain neurological diseases.

In summary, our current observations have provided valuable information for not only elucidating the biological functions of Aralar and NADH shuttles, but also understanding the mechanisms of basal survival of microglia. Considering the significant roles of Aralar in linking calcium homeostasis and mitochondrial energy metabolism, it is reasonable to expect that many future studies are needed to fully understand the mechanisms underlying the roles of Aralar in cell death. It is also warranted to determine if Aralar plays significant role in the survival of other types of cells, and to further expose the mechanisms underlying the roles of Aralar and NADH shuttles in cell death both *in vitro* and *in vivo*.

Acknowledgements

This study was supported by Chinese National Science Foundation Grants #81171098 and #81271305 (to W.Y.), and a National Key Basic Research '973 Program' Grant #2010CB834306 (to W.Y.). The authors report no conflicts of interest.

Disclosure of conflict of interest

None.

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