Free Radical Biology and Medicine 108 (2017) 345–353

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original article

Sirt3 confers protection against neuronal ischemia by inducing autophagy: Involvement of the AMPK-mTOR pathway

Shu-Hui Dai[a,1], Tao Chen[a,b,c,1], Xia Li[a,1], Kang-Yi Yue[a], Peng Luo[a], Li-Kun Yang[b], Jie Zhu[b], Yu-Hai Wang[b], Zhou Fei[a,*], Xiao-Fan Jiang[a,*]

[a] Department of Neurosurgery, Xijing Institute of Clinical Neuroscience, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China
[b] Department of Neurosurgery, The 101th Hospital of PLA, Rescue Center of Craniocerebral Injuries of PLA, Wuxi, Jiangsu 214044, China
[c] Department of Neurosurgery, The 123th Hospital of PLA, Bengbu, Anhui 233000, China

ARTICLE INFO

Keywords:
Ischemic stroke
Sirt3
Autophagy
AMPK
mTOR

ABSTRACT

Sirtuin3 (Sirt3) is a member of the silent information regulator 2 (Sir2) family of proteins located in mitochondria that influences almost every major aspect of mitochondrial biology, including ATP generation and reactive oxygen species (ROS) production. Our previous study showed that Sirt3 exerts protective effects against oxidative stress in neuronal cells. In this study, we investigated the role of Sirt3 in neuronal ischemia using an oxygen and glucose deprivation (OGD) model. Sirt3 was up-regulated by OGD and overexpression of Sirt3 through lentivirus transfection significantly reduced OGD-induced lactate dehydrogenase (LDH) release and neuronal apoptosis. These effects were accompanied by reduced hydrogen dioxide (H_2O_2) production, enhanced ATP generation and preserved mitochondrial membrane potential (MMP). The results of immunocytochemistry and electron microscopy showed that Sirt3 increased autophagy in OGD-injured neurons, which was also confirmed by the increased expression of Beclin-1 as well as LC3-I to LC3-II conversion. In addition, the autophagy inhibitor 3-MA and bafilomycin A1 partially prevented the effects of Sirt3 on LDH release and apoptosis after OGD. The results of western blotting showed that overexpression of Sirt3 in cortical neurons markedly increased the phosphorylation of AMPK, whereas the phosphor-mTOR (*p*-mTOR) levels decreased both in the presence and absence of OGD insult. Furthermore, pre-treatment with the AMPK inhibitor compound C partially reversed the protective effects of Sirt3. Taken together, these findings demonstrate that Sirt3 protects against OGD insult by inducing autophagy through regulation of the AMPK-mTOR pathway and that Sirt3 may have therapeutic value for protecting neurons from cerebral ischemia.

1. Introduction

Ischemic stroke induced by hypoxic ischemic encephalopathy or cerebrovascular accident is still one of the leading causes of death and permanent disability in the world [1]. Although ischemic stroke has been demonstrated to be associated with many devastating cascades, such as intracellular calcium overload, reactive oxygen species (ROS) generation, excitotoxicity mediated apoptosis and inflammation related necrosis, the exact molecular mechanism underlying ischemic neuronal injury has not been fully elucidated [2–4].

The sirtuins (or Sir2-like proteins) comprise a family of NAD^+-dependent protein deacetylases and ADP-ribosyltransferases that belong to class III histone deacetylases (HDACs) [5]. Mammals express seven homologues of sirtuins, Sirt1-7. Of those homologues, Sirt3 resides primarily in the mitochondria and has been identified as a responsive deacetylase that regulates metabolism and oxidative stress [6]. Several studies have shown that Sirt3 plays an important role in regulating cell defence and mediating cell survival [7–9]. Our previous study showed that Sirt3 attenuates hydrogen peroxide-induced oxidative stress through preservation of mitochondrial function in HT22 cells [10]. We also found that overexpression of Sirt3 by lentivirus transfection protects cortical neurons against oxidative stress through regulating mitochondrial Ca^{2+} and mitochondrial biogenesis [11]. In addition, Sirt3 was shown to act as a pro-survival factor that plays an essential role in protecting neurons experiencing excitotoxicity [12]. However, the exact role of Sirt3 in ischemic neuronal cell injury and the associated molecular mechanisms have not yet been investigated.

Autophagy is an evolutionarily conserved process for the bulk degradation and recycling of cytosolic proteins and organelles [13]. Mammalian autophagy exists in three different forms: macroautophagy,

* Corresponding authors.
E-mail addresses: fmmuzhoufei@163.com (Z. Fei), fmmujiangxiaofan@163.com (X.-F. Jiang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.freeradbiomed.2017.04.005
Received 30 August 2016; Received in revised form 1 April 2017; Accepted 4 April 2017
Available online 07 April 2017

microautophagy, and chaperone-mediated autophagy. These forms differ in their mechanisms and functions, and macroautophagy has been widely referred to as autophagy [14]. As a degradation/recirculation system, autophagy is believed to play an important role in pathological conditions in many organs, including brain ischemia [15,16]. Although the existence and activation of autophagy after ischemic stroke is undisputable, whether autophagy is a protective or detrimental mechanism for ischemic neuronal injury is still a topic of debate. Previous studies have shown that autophagy is one of several morphological features that occur during cell death after brain ischemia, and inhibition of autophagy exerts protective effects under hypoxic-exicitotoxic conditions [17,18]. In contrast, increasing amounts of evidence have indicated that autophagy may promote neuronal survival through removing damaged organelles to delay apoptosis or preserving energy to prevent necrosis, and the protective role of autophagy has also been demonstrated using chemical inhibitors or inducers under both in vitro and in vivo conditions [19,20].

In the present study, we measured the dynamic changes of Sirt3 expression after neuronal ischemia, and investigated the potential protective effects of Sirt3 against oxygen and glucose deprivation (OGD) in cortical neurons. We also determined the effect of Sirt3 overexpression on neuronal autophagy regulation, and confirmed the involvement of the AMPK-mTOR pathway in autophagy and Sirt3-induced protection.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies against Sirt3, LC3, and Beclin-1 were obtained from Cell Signaling (Danvers, MA, USA). Antibodies against p-AMPK (Thr172), AMPK, p-mTOR (Ser2448), mTOR and β-actin were obtained from Santa Cruz (Dallas, TX, USA). The secondary antibodies, Rh123, and MitoSox Red were purchased from Sigma (St. Louis, MO, USA). Rapamycin, compound C, 3-MA and bafilomycin A1 were purchased from Tocris Bioscience (Bristol, UK). The TUNEL staining kit was obtained from Promega (Madison, WI, USA). The lactate dehydrogenase (LDH) assay kit was obtained from Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Primary culture of cortical neurons

All experimental protocols and animal handling procedures were performed according to the National Institutes of Health (NIH) guidelines for the use of experimental animals, and the study was approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University (No. 2014–81371447). Cortical neurons were cultured from Sprague-Dawley rats using a modified method. Briefly, cerebral cortices were removed from embryos at 16–18 days, stripped of meninges and blood vessels and minced. The tissues were dissociated through 0.25% trypsin digestion for 15 min at 37 °C and gentle trituration. Neurons were resuspended in neurobasal medium containing 2% B27 supplement and 0.5 mM L-Glutamine, and then the neurons were plated at a density of 3×10^5 cells/cm^2. Before seeding, the culture vessels, consisting of 96-well plates, 1.5-cm glass slides or 6-cm dishes were coated with PLL (50 μg/ml) at room temperature overnight. Neurons were maintained at 37 °C in a humidified 5% CO_2 incubator and half of the culture medium was changed every other day.

2.3. Lentivirus construction and transfection

The coding sequence of Sirt3 was amplified by RT-PCR. The primer sequences were forward, 5′-TACTTCCTTCGGCTGCTTCA-3′; reverse, 5′-AAGGCGAAATCAGCCACA −3′. The PCR fragments and the pGC-FU plasmid (GeneChem, Shanghai, China) were digested with Age I and then ligated with T4 DNA ligase to produce pGC-FU-Sirt3. To generate the recombinant Lentivirus LV-Sirt3, 293 T cells were co-transfected with the pGC-FU plasmid (20 μg) with a cDNA encoding Sirt3, a pHelper 1.0 plasmid (15 μg) and a pHelper 2.0 plasmid (10 μg) using Lipofectamine 2000 (100 μl). The supernatant was harvested and the viral titre was calculated by transducing 293 T cells. As a control, we generated a lentiviral vector that expressed GFP alone (LV-control). Cortical neurons were transfected with lentivirus vectors for 72 h and subjected to various treatments.

2.4. Oxygen and glucose deprivation (OGD)

To initiate OGD, the culture medium was removed and rinsed with phosphate buffered saline (PBS) three times. The cultured neurons were placed into a specialized, humidified chamber containing 5% CO_2, 95% N_2 at 37 °C with glucose-free DMEM, which was pre-gassed with N_2/CO_2 (95%/5%) to remove residual oxygen. After 2 h, the neurons were removed from the anaerobic chamber, and the culture medium was replaced with neurobasal medium containing 2% B27 supplement and 0.5 mM L-Glutamine. The neurons were maintained for 24 h at 37 °C in a humidified 5% CO_2 incubator to generate the reperfusion insult.

2.5. Cytotoxicity assay

Cytotoxicity was determined by measuring the release of LDH with a diagnostic kit according to the manufacturer's instructions. Briefly, 50 μl of supernatant from each well was collected to assay LDH release. The samples were incubated with a reduced form of nicotinamide-adenine dinucleotide (NADH) and pyruvate for 15 min at 37 °C and the reaction was stopped by adding 0.4 M NaOH. The activity of LDH was calculated from the absorbance at 440 nm, and the background absorbance from culture medium that was not used for any cell cultures was subtracted from all of the absorbance measurements. The results are presented as fold increase of the control.

2.6. TUNEL staining

Apoptosis in primary cortical neurons subjected to various treatments was detected using TUNEL staining, which is a method to observe DNA strand breaks in nuclei. For TUNEL staining, cortical neurons were seeded on 1.5-cm glass slides at a density of 3×10^5 cells/cm^2. Twenty-four hours after OGD, the cells were fixed by immersing the slides in freshly prepared 4% methanol-free formaldehyde solution in PBS for 20 min at room temperature and permeabilized with 0.2% Triton X-100 for 5 min. Neurons were labelled with fluorescein TUNEL reagent mixture for 60 min at 37 °C according to the manufacturer's suggested protocol. Then, the slides were examined by fluorescence microscopy, and the TUNEL-positive (apoptotic) cells were counted. DAPI (10 μg/ml) was used to stain nuclei.

2.7. Caspase-3 activity

Briefly, after being harvested and lysed, 10^6 cells were mixed with 32 μl of assay buffer and 2 μl of 10 mM Ac-DEVD-pNA substrate. Absorbance at 405 nm was measured after incubation at 37 °C for 4 h. The absorbance of each sample was determined by subtraction of the mean absorbance of the blank and corrected by the protein concentration of the cell lysate.

2.8. Measuring intracellular H_2O_2 by aminotriazole-mediated inactivation of catalase

The intracellular steady-state levels of H_2O_2 were estimated using a sensitive assay based on 3-aminotriazole inhibition of catalase [21]. After various treatments, neurons were scrape-harvested and the protein concentrations were quantified with the BCA method. The assay was initiated through addition of 100 μl of a 30 mM H_2O_2 stock