Role of Neuronal Nrdp1-USP8-Hypoxia Inducible Factor-1 Alpha Signal Axis in Hypoxic Injury after Cerebral Ischemia

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To investigate the role of neuromodulin receptor degradation protein-1-ubiquitin-specific protease 8-hypoxia inducible factor alpha signal axis in neuronal hypoxic injury after cerebral ischemia. 20 clean-grade healthy male Sprague-Dawley rats and 2 pregnant rats about 17 d of gestation were randomly selected. The rat model of cerebral ischemia was established and the rats were divided into control group (n=10) and model group (n=10). The pregnant mice were killed, the primary cortical neurons were obtained and cultured, and the target gene was connected to the shuttle plasmid to construct adenovirus vector. Western blotting and real-time quantitative fluorescence polymerase chain reaction were used to detect the expression of neuromodulin receptor degradation protein-1 in each group and the expression of neuromodulin receptor degradation protein-1 in cortical neurons induced by hypoxia at different time. Apoptosis of neurons in each group was detected by *in situ* terminal deoxynucleotidyl transferase dUTP nick end labeling. The expression levels of neuromodulin receptor degradation protein-1, apoptosis-related proteins poly (ADP-ribose) polymerase, Bcl-2-associated X protein, B-cell lymphoma 2, ubiquitin-specific protease 8 and hypoxia-inducible factor alpha were measured by Western blotting. Compared with the control group, the expression of neuromodulin receptor degradation protein-1 in the model group was raised. After hypoxia treatment, the expression of neuromodulin receptor degradation protein-1 in cerebral cortical neurons increased. Compared with neuromodulin receptor degradation protein-1 interference control group, the expression level of neuromodulin receptor degradation protein-1, proteins poly (ADP-ribose) polymerase and Bcl-2-associated X protein in neuromodulin receptor degradation protein-1 interference group were decreased, while the expression level of B-cell lymphoma 2, ubiquitin-specific protease 8 and hypoxia-inducible factor alpha were increased.

Key words: Cerebral ischemia, neuronal neuromodulin receptor degradation protein-1, ubiquitin-specific protease 8, hypoxia-inducible factor alpha, neuronal hypoxic injury

Cerebral ischemia, also known as ischemic stroke, refers to a variety of diseases with neurological deficit caused by ischemia and hypoxia after the disturbance of blood supply of local brain tissue caused by various reasons[1]. According to statistics, at present, the number of new stroke cases worldwide is more than 8 million, and ischemic stroke accounts for more than 85 % of them, which ranks 2nd in the diseases that cause human death, and is also the leading cause of disability, which has a serious impact on patients and their families, and even the society[2]. Because the brain tissue is extremely dependent on the oxygen and energy substances given by the cerebral blood flow, the brain tissue is particularly sensitive to ischemic stroke. A very short stroke time can cause complex reactions of brain cells and eventually lead to neuronal death. Patients have varying degrees of neurological impairment. Once the disease occurs, neuronal cells will lose at a very rapid rate, resulting in irreversible brain damage[3]. Therefore, early detection, timely treatment and restoration of blood supply of brain tissue is the key to the treatment of patients with

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Accepted 14 March 2024
Revised 18 October 2023
Received 10 April 2022
Indian J Pharm Sci 2024;86(2):717-721

ischemic stroke. Neuromodulin receptor degradation protein-1 (Nrdp1) is an E3 ubiquitin ligase with obvious ubiquitin ligase activity. It is mainly expressed in adult brain, heart, prostate and skeletal muscle tissues. It can specifically recognize and degrade some substrate proteins[4]. It has been found that Ubiquitin-Specific Protease 8 (USP8), Epidermal Growth Factor Receptor 3 (EGFR3), Parkin protein and other signal proteins are regulated by Nrdp1[5]. Nrdp1 can significantly reduce the expression of Bruce in neurons and plays an important role in neuronal apoptosis, but its role and mechanism in neuronal hypoxic injury is not clear[6,7]. In order to explore its mechanism, the following studies are made. 20 clean healthy male Sprague-Dawley (SD) rats (body weight 215±15 g) and 2 % rats about 17 d of pregnancy (Guangdong Medical Experimental Animal Center (production license: SCXK (Guangdong) 2017-0029) were randomly selected. All rats were fed adaptively at laboratory temperature of 24°±3° and humidity of 45 %±5 %. Nrdp1 polyclonal antibody (Baude Biotechnology Co., Ltd.); USP8 polyclonal antibody (Shanghai Kanglang Biotechnology Co., Ltd.); Hypoxia-Inducible Factor Alpha (HIF-1α) polyclonal antibody (Shanghai Hengfei Biotechnology Co., Ltd.); Poly (ADP-Ribose) Polymerase (PARP) polyclonal antibody (Shenzhen Haodi Huatuo Biotechnology Co., Ltd.) and penicillin-streptomycin mixture (Shanghai Junrui Biotechnology Co., Ltd.). Fetal bovine serum (Shanghai Laichuang Biotechnology Co., Ltd., No: A6904FBS); real-time quantitative Polymerase Chain Reaction (PCR) detection kit (Tianjin Biochip Technology Co., Ltd.); ultra-low temperature refrigerator (Beijing Aizexin Technology Co., Ltd., model: DW-86L288); constant temperature water bath pot (Beijing Changfeng instrument Co., Ltd., model: HW. SY11-KP1); electronic balance (Shanghai Precision instrument Co., Ltd., model: FA1004B). Biological microscope (Shanghai Batuo instrument Co., Ltd., model: XSP-19C); cell incubator (Shanghai Rundu Biotechnology Co., Ltd. model: Herocell180); flow cytometry (Beckman Coulter Trading (China) Co., Ltd., model: DxFLEX); low temperature and high-speed centrifuge (Shandong Boko Scientific instrument Co., Ltd., model: TG-16M). The rat model of cerebral ischemia was established including the after successful anesthesia, the rats were taken supine position, skin preparation, all mouth in the middle of the neck, blunt separation of skin and subcutaneous tissue, right common carotid artery, internal/external carotid artery and its branches. A dead knot and a living knot were tied at the distal and proximal ends of the external carotid artery, respectively. A small cut was made between the two knots, and a silicone-coated nylon thread bolt was inserted to block the opening of the middle cerebral artery along the internal carotid artery with about 10 cm. The rats were killed 3 h later, and the ischemic brain tissue was cryopreserved. Rats were divided into control group (n=10) and model group (n=10). In primary cortical neuron culture, the pregnant rats were killed, the embryos were removed from the uterine cavity, the fetal rat head was cut off after disinfection, the cerebral cortex was removed, the cerebral cortex was transferred to a fresh precooled phosphate buffer, the cortical tissue was chopped, trypsin digestion, centrifugation, and the supernatant was removed to obtain neuronal precipitation, which was suspended in the neuronal basic culture medium containing 2 % B27 and 0.5 ml L-glutamine. Polylysine (20 μg/ml) was coated overnight at room temperature. After gas depression, polylysine was washed with phosphate buffer. The cells were inoculated at a certain density and cultured in the incubator for 7 d. The culture medium was changed in time during the culture process. The target gene was connected to the shuttle plasmid to construct Adenovirus (Ad) vector. Before hypoxia treatment, Ad-control, Ad-Nrdp1, Ad-si-control and Ad-si-Nrdp1 were infected for 72 h to obtain Nrdp1 control group, Nrdp1 overexpression group, Nrdp1 interference control group and Nrdp1 interference group, with 4 complex groups in each group. Preparation of hypoxia model including the oxygen in Dulbecco's Modified Eagle Medium (DMEM) glucose-free medium was removed by 95 Nitrogen (N_2) and 5 % Carbon dioxide (CO_2) and the cells were cultured in the medium for 1 h, 3 h and 6 h, respectively. When the oxygen concentration was 0.1, it was replaced by the normal medium without fetal bovine serum. Western blotting and real-time quantitative fluorescence PCR were used to detect the expression of Nrdp1 in each group and the expression of Nrdp1 in cortical neurons induced by hypoxia at different time. Apoptosis of neurons in each group was detected by *in situ* Terminal Deoxynucleotidyl Transferase Mediated dUTP

Nick end Labeling (TUNEL). The expression of Nrdp1, apoptosis-related proteins PARP, Bcl-2 Associated X protein (BAX), B-cell lymphoma-2 (Bcl-2), USP8 and HIF-1α were measured by Western blotting. In this study, the counting data were compared by Chi-square (χ^2) comparison, expressed by n (%). The measurement data were compared by independent sample t-test between the two groups, and single-factor multi-sample mean comparison was used in multi-group comparison, all of which were expressed by (regression x±s). Western blotting and real-time quantitative fluorescence PCR were used to detect the expression of Nrdp1 in each group and the expression of Nrdp1 in cortical neurons induced by hypoxia at different time. Neuronal apoptosis in each group was measured by TUNEL method. The expression levels of Nrdp1, apoptosis-related proteins PARP, BAX, Bcl-2, USP8 and HIF-1α were measured by Western blotting. In this study, Statistical Package for the Social Sciences (SPSS) 18.0 software was used to analyze the statistical data, and the difference was regarded as statistically significant. Compared with the control group, the expression of Nrdp1 in the model group was raised ($p<0.05$) as shown in Table 1. After hypoxia treatment, the expression of Nrdp1 in cerebral cortical neurons was increased as shown in Table 2. Compared with Nrdp1 control group, the positive rate of cell staining in Nrdp1 overexpression group was increased, while that in Nrdp1 interference group was reduced than that in Nrdp1 interference control group. Western blotting showed that the expression of Nrdp1, PARP and BAX in Nrdp1 overexpression group was raised than that in Nrdp1 control group, while Bcl-2 expression was decreased in Nrdp1 overexpression group, while Nrdp1, PARP, BAX expression in Nrdp1 interference group was reduced than that in Nrdp1 interference control group, while Bcl-2 expression was raised in Nrdp1 interference group than in Nrdp1 interference control group as shown in Table 3.The results of Western blotting showed that the expression of USP8 and HIF-1α in Nrdp1 overexpression group was reduced than that in Nrdp1 control group, and that in Nrdp1 interference group was raised than that in Nrdp1 interference control group ($p<0.05$) as shown in Table 4. Ischemic stroke is caused by insufficiency or interruption of intracranial blood supply caused by various reasons, resulting in cerebral ischemia, hypoxia, edema, necrosis, etc., and finally manifests as a kind of cerebrovascular disease characterized by neurological damage in the corresponding part. It is the second leading cause of death among human beings in the world, and it is also the leading cause of death in China. Ischemic stroke, coronary heart disease and cancer constitute the top three fatal diseases in most countries and regions. Most of the patients with acute ischemic stroke have progressive neurological function, which significantly aggravates the morbidity and mortality of the patients, and causes a serious burden on the quality of life and safety of the patients. At present, the only drug for the treatment of ischemic stroke, but because of the narrow treatment time window and the risk of cerebral hemorrhage after thrombolysis, there are obvious use restrictions[8]. Therefore, to clarify the pathogenesis of ischemic stroke and find new therapeutic targets has become the focus of medical researchers. Some studies have found that cerebral ischemia can initiate a series of cytotoxic molecules, resulting in neuronal death and blood-brain barrier destruction, inflammatory injury, apoptosis and protein degradation disorders can lead to cerebral ischemic injury[9]. Among them, the main pathway of intracellular protein degradation is ubiquitin-proteasome system, which can participate in signal transduction, cell cycle progression, apoptosis and many other physiological functions. The results of this study found that Nrdp1 can significantly promote neuronal apoptosis[10]. Nrdp1, also known as Ring Finger Protein 41 (RNF41), was first found in mouse grandmother cells and hematopoietic stem cells. It has obvious ubiquitin ligase activity and can specifically recognize and degrade some substrate proteins[11]. Some studies have found that Nrdp1 plays an important role in regulating cell growth, apoptosis, oxidative stress and inflammation[12]. In addition, many signal proteins such as USP8, EGFR3, EGFR4, Myeloid Differentiation Primary Response 88 (MYD88), Parkin and so on are regulated by Nrdp1. Nrdp1 can participate in apoptosis, cell growth and inflammation by regulating target proteins. Some scholars have confirmed that there is an important relationship between Nrdp1 and ischemic cardiomyocyte death. Nrdp1 can significantly promote cardiomyocyte apoptosis induced by ischemia-reperfusion[13]. Nrdp1 can induce cells to

participate in different stimuli, and its stability mainly depends on the substrate USP8. Nrdp1 and USP8 restrict each other. When the expression of Nrdp1 increases, most of the USP8 will be degraded by it, while a small part of USP8 will make Nrdp1 unstable, resulting in the decrease of Nrdp1 and the increase of USP8 in cells[14]. The results are the same as those of this group of studies. HIF-1α is a nuclear transcription factor, which is produced by tumor cells under hypoxia. It can regulate the expression of target genes such as vascular endothelial growth factor and glycolytic enzyme[15]. Many studies have found that HIF-1α is not only an important transcriptional regulator, but also an important downstream effector molecule of Nrdp1-mediated apoptosis in the process of ischemic neuronal injury, which can participate in physiological processes such as adaptation to anoxic environment, cell proliferation and apoptosis. Some scholars have found that HIF-1α can participate in apoptosis caused by cerebral ischemia, resulting in the occurrence of ischemic brain injury[16]. The activity of USP8 can protect HIF-1α from the degradation mediated by ubiquitin ligase von Hippel–Lindau protein (pVHL) and stabilize the expression of HIF-1α protein. When cerebral ischemia occurs, the expression of Nrdp1 increases and USP8 is degraded rapidly, which affects the adaptive response of nerve cells to hypoxia/ischemia[6]. The results of this study showed that Nrdp1 could inhibit the expression of USP8 and HIF-1α. To sum up, the expression of Nrdp1 in neurons was increased after cerebral ischemia, and Nrdp1 may aggravate the hypoxic injury of neurons by inhibiting the expression of USP8 and HIF-1α.

TABLE 1: EXPRESSION OF NRDP1 (x̄±s)

Group	Nrdp1
Control	1.01±0.04
Model	1.84±0.05
t	40.91
p	<0.001

TABLE 2: EXPRESSION OF NRDP1 IN CORTICAL NEURONS INDUCED BY HYPOXIA AT DIFFERENT TIME (x̄±s)

Group	Nrdp1
0 h	1.01±0.06
1 h	1.34±0.17
3 h	1.95±0.23[a]
6 h	2.21±0.25[a]
F	32.72
p	<0.001

Note: Compared with 0 h, [a]p<0.05

TABLE 3: EXPRESSION OF NRDP1 AND APOPTOSIS-RELATED PROTEINS PARP, BAX AND BCL-2 IN EACH GROUP (x̄±s)

Group	Nrdp1	PARP	BAX	Bcl-2
Nrdp1 control	1.27±0.03	1.67±0.06	1.39±0.03	1.02±0.09
Nrdp1 overexpression	2.32±0.06	3.54±0.59	2.59±0.16	2.81±0.19
Nrdp1 interference	1.35±0.06	2.65±0.02	1.58±0.07	1.13±0.08
Nrdp1 interference	3.27±0.34	1.33±0.08	3.37±0.21	3.87±0.73
F	114.67	44.60	180.65	52.08
p	<0.001	<0.001	<0.001	<0.001

TABLE 4: CHANGES OF USP8 AND HIF-1α EXPRESSION IN EACH GROUP (x̄±s)

Group	USP8	HIF-1α
Nrdp1 control	2.15±0.64	1.61±0.12
Nrdp1 overexpression	1.96±0.21	1.23±0.05
Nrdp1 interference control	1.02±0.15	1.02±0.08
Nrdp1 interference	2.36±0.76	1.45±0.12
F	9.59	28.13
p	<0.001	<0.001

Conflict of interests:

The authors declared no conflict of interests.

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