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TARGETING THIOREDOXIN-1 WITH SIRNA EXACERBATES OXIDATIVE STRESS INJURY AFTER CEREBRAL ISCHEMIA/REPERFUSION IN RATS

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Abstract—Reactive oxygen species and their detrimental effects on the brain after transient ischemia/reperfusion (I/R) have been implicated in the pathogenesis of ischemic reperfusion injury. Thioredoxin-1 (Trx-1) is an endogenous antioxidant protein that has neuroprotective effects. We hypothesized that Trx-1 plays a crucial role in regulating cerebral I/R injury. To be able to test this, 190 Sprague–Dawley rats were subjected to transient middle cerebral artery occlusion (tMCAO) with Trx-1 siRNA (small interference RNA) injected 24 h prior to ischemia. At 24 h after tMCAO, we measured neurological deficits, infarct volume, and brain water content, and found that neurological dysfunction, brain infarct size, and brain edema were worse in the Trx-1 siRNA group than in the control group. Oxidative stress was evaluated by measuring superoxide dismutase activity and malondialdehyde level. The levels of Trx-1 and its cofactor, peroxiredoxin (Prdx), were significantly decreased after Trx-1 down-regulated. However, there is no significant difference in the Prdx mRNA level after administration of Trx-1 siRNA. In contrast, Prdx-SO_3 protein levels were significantly increased in the Trx-1 siRNA group. We also investigated the specific role of nuclear factor erythroid 2-related factor 2 (Nrf2) in Trx-1 induction by knocking down Nrf2. Nrf2 siRNA injection decreased Trx-1 mRNA and protein expression. Our results suggest that the exacerbation of brain damage was associated with enhanced cerebral peroxidation in brain tissues. Moreover, these results revealed that Trx-1, which is more likely regulated by Nrf2, exerts a neuroprotective role probably through maintaining the reduction activity of Prdx1–4. © 2014 Published by Elsevier Ltd. on behalf of IBRO.

Key words: thioredoxin-1, oxidative stress, peroxiredoxin, nuclear factor erythroid 2-related factor 2.

INTRODUCTION

Stroke is a leading cause of death and long-term disability in developing countries. Oxidative stress caused by reactive oxygen species (ROS) is a critical component of cerebral ischemic injury (Tewari et al., 2014). Oxidative damage can induce neuronal injury and necrosis in brain tissue by oxidizing intracellular molecules such as lipids, proteins, and DNA (Heo et al., 2005; Oztanir et al., 2014). Therefore, it was thought that antioxidant agents, which have the ability to scavenge ROS, could attenuate neurological damage.

Thioredoxin-1 (Trx-1) is a 12-kDa ubiquitous protein present in all living cells. It has a wide range of physiological functions, including DNA synthesis, oxidation damage repair, and regulation apoptosis (Lu and Holmgren, 2012; Chang et al., 2013; Sengupta and Holmgren, 2013). Recent study has demonstrated that exogenous rhTrx-1 attenuates post-ischemic brain damage and neuronal apoptosis by reducing oxidative/nitrative stress (Ma et al., 2012). With thiol-reducing activity at its conserved active site, Trx-1 exhibits cytoprotective effects against oxidative stress by scavenging ROS and cooperating with peroxiredoxin (Prdx) (Das and Das, 2000; Lu and Holmgren, 2014).

Prdx is a general term that refers to a family of small (22–27 kDa) non-seleno peroxidases, representing a class of important antioxidants in mammals (Park et al., 2014). In fact, Prdx, thioredoxin, and thioredoxin reductase form the mammalian thioredoxin system that plays crucial roles in defending oxidative stress injury, especially typical 2-Cys Prdxs (Prdx1–4) (Zhu et al., 2012). Therefore, elucidating the relationship between Trx-1 and Prdx1–4 in cerebral I/R injury is particularly important.

It is well acknowledged that enhanced ROS and electrophiles can evoke a series of antioxidant genes by activating nuclear factor erythroid 2-related factor 2 (Nrf2), a critical transcription factor that regulates the expression of major antioxidant enzymes and phase II detoxification enzymes (Motohashi and Yamamoto, 2004; Wang et al., 2014). Hemin has been shown to induce activation of the thioredoxin gene by regulating Nrf2 through the antioxidant responsive element (ARE) in K562 cells (Kim et al., 2001). Moreover, the expression of Nrf2 has been shown to be significantly up-regulated in the peri-infarct region and to subsequently induce Trx

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Abbreviations: ANOVA, analysis of variance; ARE, antioxidant responsive element; CV, Cresyl Violet; I/R, ischemia/reperfusion; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; MEFs, mouse embryo fibroblasts; Nrf2, nuclear factor erythroid 2-related factor 2; Prdx, peroxiredoxin; qPCR, quantitative PCR; ROS, reactive oxygen species; SOD, superoxide dismutase; Trx-1, thioredoxin-1.

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expression in male ICR mice following middle cerebral artery (MCA) occlusion (MCAO) (Tanaka et al., 2011). However, in spite of these various observations, the role of Nrf2 in regulating Trx-1 gene expression in the context of ischemic brain injury has yet to be fully investigated.

The purpose of this study was to evaluate the role of Trx-1 in ischemic injury using an experimental MCAO model with Trx-1 knock-down. We evaluated whether the Trx1 down-regulation could exacerbate brain damage. Furthermore, we elucidated the relationship between Trx-1 and Prdx1–4 in cerebral I/R injury. Because our results highlight the importance of Trx-1 in modulating the extent of brain damage, we have also investigated the influence of Nrf2 on Trx-1 expression level by interfering Nrf2 gene expression.

EXPERIMENTAL PROCEDURES

Animals and groups

Adult male Sprague–Dawley rats, weighed 270–310 g (n = 190, 36 died within 24 h) and aged 90 ± 4 d were bred and held at the Experimental Animal Center of Chongqing Medical University. All rats were allowed free access to food and water before the operation under optimal conditions (12/12-h light/dark with humidity 60 ± 5%, 22 ± 3 °C). All experimental animals were randomly allocated to the following groups: sham surgery group (n = 28, no died), untreated controls with MCAO (n = 51, 9 died), scramble siRNA of Trx-1siRNA-injected group (n = 37, 7 died), Trx-1 siRNA group (n = 41, 11 died), scramble siRNA of Nrf2siRNA-injected group (n = 15, 3 died) and Nrf2 siRNA group (n = 18, 6 died). All experimental procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University, China.

Administration of Trx-1 siRNA and Nrf2 siRNA

Trx-1 siRNA (The sense primer 5-AAGCUCGAAGCCAC UAUUATT-3 and antisense primer 5-UAAUAGUGGCUU CGAGCUUTT-3), Nrf2 siRNA (The sense primer 5-CCC UGUGUAAAGCUUUCAATT-3 and antisense primer 5-UUGAAAGCUUUACACAGGGTT-3) were designed and chemically synthesized by GenePharma Corporation, Shanghai, China. Scramble siRNA, which has the same nucleotide composition of the target gene siRNA with no sequence homology to any known rat genes was used as the control. All siRNAs were dissolved in RNase-free water, making the final concentration 2 μg/μl. Rats were anesthetized with 3.5% chloral hydrate (350 mg/kg, ip) and placed in a stereotaxic apparatus (Taimeng Software, Chengdu, China). 10 μl of siRNA or scramble siRNA was injected ipsilaterally into the left lateral cerebral ventricle at 24 h prior to the induction of MCAO, respectively. siRNA or scramble siRNA was slowly injected into the left lateral ventricle over a 20-min duration using a Hamilton microsyringe with the coordinates of 1.0 mm posterior to the bregma, 2.0 mm lateral to the midline, and 4.0 mm ventral to the surface of the skull under the guidance of a stereotaxic instrument. The injection rate was 0.5 μl/min. After injection, the needle was held in place for 5 min and then removed slowly over 2 min.

Cerebral ischemia/reperfusion (I/R) model

Transient focal cerebral ischemia was introduced into rats by left MCA occlusion technique according to our previous methods (Chen et al., 2012). In brief, a 4–0 monofilament nylon suture (Beijing Sunbio Biotech Co Ltd, Beijing, China) with a rounded tip was inserted into the left internal carotid artery through the common carotid artery stump and gently advanced to occlude the MCA. After 60 min of MCAO, the suture was removed to restore blood flow. The reperfusion was confirmed by laser Doppler (Periflux System 5000, Perimed AB, Stockholm, Sweden). The same procedure was performed on sham-operated rats, but the MCA was not occluded. Rats that did not show neurological deficits after reperfusion (neurological score < 1) were excluded from the study, as well as animals that died after ischemia induction. Rats that showed neurological deficits immediately after reperfusion (neurological score > 0) but were found to be experiencing skull base or subarachnoid hemorrhage were also excluded from the study. Core body temperatures were monitored with a rectal probe and maintained at 37 °C during the whole procedure.

Sample processing

All rats were sacrificed at 24 h after reperfusion, and the brains were quickly removed to collect the cerebral cortex for an analysis of oxidative stress assay, western blotting, and real-time quantitative PCR (qPCR). A 4-mm coronal section was taken from the area perfused by the MCA starting at 5 mm from the frontal pole. Fresh cortical tissue was collected from the MCA territory of the ischemic left hemisphere, frozen immediately in liquid nitrogen, and stored at −80 °C until needed for further processing. Tissue samples from all animals were analyzed individually.

Evaluation of neurological deficit

Neurological deficit scores were evaluated by an examiner blinded to the experimental groups after 24 h of reperfusion. The deficits were scored on a modified scoring system developed by Longa et al. (Longa et al., 1989), as follows: 0, no neurological deficits; 1, failure to extend right forepaw fully; 2, circling to right; 3, falling to right; 4, did not walk spontaneously and has depressed levels of consciousness. The higher the neurological deficit score, the more severe the impairment is of motor motion injury. Brains from these rats were analyzed for water content, infarct volume, oxidative stress analysis, western blot, and real-time qPCR.

Measurement of brain water content

Brain water content was measured using the standard wet–dry method. Six rats in each group were anesthetized with chloral hydrate and killed by decapitation at 24 h after