J Pharmacol Sci **121**, 343 – 346 (2013)

Journal of Pharmacological Sciences

Short Communication

Altered MicroRNA Expression in the Ischemic–Reperfusion Spinal Cord With Atorvastatin Therapy

Jia-Rui Hu[1,*], Guo-Hua Lv[1], and Bang-Liang Yin[2]

[1]*Department of Spine Surgery,* [2]*Department of Cardiothoracic Surgery, the Second Xiangya Hospital of Central South University, Changsha, Hunan 410011, PR China*

Received October 15, 2012; Accepted February 1, 2013

Abstract. We explored the neuroprotection by atorvastatin in the ischemia/reperfusion model of rat and its microRNA-related mechanisms. At first, we uncovered a previously unknown alteration in temporal expression of a large set of microRNAs following spinal cord ischemia–reperfusion injury (IRI). The target genes for the differentially expressed microRNAs include genes encoding components that are involved in the inflammation, apoptosis, and neural damage that are known to play important roles in IRI. Atorvastatin pretreatment restored part of the up or down regulations. These findings suggest that altered expression of microRNAs may contribute to the mechanism of neuroprotection of statins in spinal cord IRI.

Keywords: ischemia–reperfusion injury, atorvastatin, microRNA

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have pleiotropic effects that are independent of cholesterol lowering, such as anti-inflammatory effects, antioxidant effects, and endothelial function improvement. Furthermore, the beneficial effects of statins on ischemia–reperfusion injury (IRI) have been reported in various organs, including the spinal cord, brain, and heart. However, the potential mechanism for statins to promote neurologic recovery after spinal cord IRI has been studied in only a limited number of reports and is still unclear. A number of microRNAs were found in the mammalian brain and spinal cord, where they are involved in the regulation of several nervous diseases (1). Recently, several reports have revealed important roles of microRNAs in traumatic SCI (2, 3). We here describe for the first time microRNA expression profiling of ischemic rat spinal cord in the context of pre-treatment with atorvastatin using a quantitative real-time PCR based array.

Rat models of spinal cord IRI were prepared as previously described (4). The rats were anesthetized with 10% chloral hydrate (300 mg/kg, intraperitoneally). Rectal temperature was maintained between 36.5°C – 37.5°C using a heating lamp. Ischemia of lumbo-sacral segments was induced by ligation of abdominal aorta just below the left renal artery for 60 min. Recirculation was started by simple removal of the ligature. All housing, surgical procedures, analgesia, and assessments were performed in accordance with the Animal Care Guidelines and were approved by the Animal Care Committee at the Second Xiangya Hospital.

Atorvastatin (Pfizer, New York, NY, USA) was dissolved in normal saline. The rats were randomly assigned to 3 groups: the sham group (n = 6) underwent the above-mentioned surgical procedure but without aortic occlusion; the drug group (n = 6) was given a daily dose of 10 mg/kg atorvastatin by intragastric administration for 2 weeks before occlusion; and the operation group (n = 6) was administrated with an equal volume of normal saline by intragastric administration for 2 weeks before occlusion.

MicroRNA from 10-mm-long spinal cord segments containing the injury epicenter was harvested using TRIzol (Invitrogen, Carlsbad, CA, USA) and the miRNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Global microRNA expression analysis was performed using pre-spotted miRCURY™ V16.0 LNA microRNA microarrays (Exiqon, Vedbæk, Denmark). After RNA isolation from the samples, the miRCURY™ Hy3™/Hy5™ Power

*Corresponding author. yblzxh@hotmail.com
Published online in J-STAGE on March 20, 2013
doi: 10.1254/jphs.12235SC

labeling kit (Exiqon) was used according to the manufacturer's guideline for microRNA labelling. One microgram of each sample was 3′-end-labeled with Hy3™ fluorescent label. The Hy3™-labeled samples were hybridized on the miRCURY™ LNA Array according to the array manual. Following hybridization, the slides were achieved, washed several times using a Wash buffer kit (Exiqon), and finally dried by centrifugation for 5 min at 400 rpm. Then the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA).

Scanned images were then imported into GenePix Pro 6.0 software (Axon Instruments) for grid alignment and data extraction. After normalization, differentially expressed microRNAs were identified through Volcano Plot filtering. A selected subset of microRNAs modulated more than 1.5-fold was selected for further analysis by real-time qPCR. The values for the Basso–Beattie–Bresnahan (BBB) score and microRNAs fold-change are presented as the mean ± S.E.M. A one-way analysis of variance was first carried out to test for any differences in mean values between groups. If differences were established, the values of the drug group were compared with those of the operation group by the modified *t*-test. A *P*-value of < 0.05 was considered statistically significant.

The neurological function was assessed at 6, 12, 24, and 48 h after reperfusion using the BBB score. The operation group showed remarkable neurological dysfunction. Atorvastatin pretreatment induced a markedly improved BBB score at each time point compared with the operation group ($P < 0.05$ for all) (Fig. 1).

We then examined expression of all the microRNAs registered in miRBase 16.0 in the injured spinal cords at 48 h after IRI. Among the 659 microRNAs present on the array chip, 270 were detected in the adult rat spinal cord.

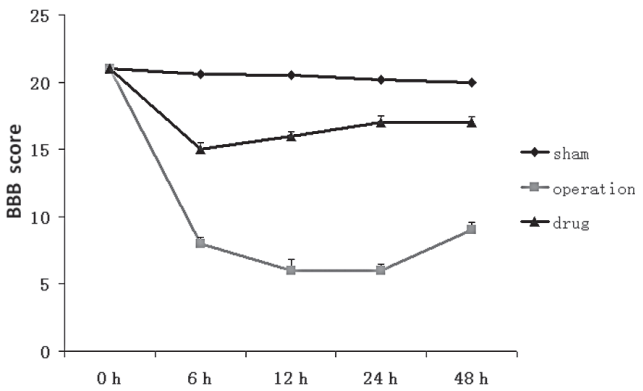

Fig. 1. Neurological function using BBB scores during 48 h after transient ischemia of the spinal cord. Data are presented as the mean ± S.E.M. (n = 6 for each group).

Table 1. Differential expression of microRNA expressed as fold change in ischemia–reperfusion samples with or without atorvastatin pretreatment

miRNA	operation group	atorvastatin group
rno-miR-204*	up 2.0 ± 0.25	
rno-miR-365	up 2.0 ± 0.48	down 2.0 ± 0.43
rno-miR-323	up 7.1 ± 0.58	down 9.1 ± 0.78
rno-miR-672*	up 2.3 ± 0.18	down 2.4 ± 0.35
rno-miR-760-5p	up 4.3 ± 0.31	down 7.8 ± 0.69
rno-miR-376b-5p	up 2.3 ± 0.30	down 2.4 ± 0.56
rno-miR-369-5p	up 2.5 ± 0.27	down 2.1 ± 0.39
rno-miR-133a	up 2.5 ± 0.45	
rno-miR-505*	up 2.5 ± 0.23	
rno-miR-466d	up 2.6 ± 0.31	
rno-miR-132*	up 2.9 ± 0.45	
rno-miR-665	up 3.1 ± 0.53	
rno-miR-463	up 4.8 ± 0.49	
rno-miR-210	down 2.2 ± 0.21	up 3.4 ± 0.68
rno-miR-146a	down 2.9 ± 0.34	
rno-miR-199a-3p	down 2.0 ± 0.17	up 1.6 ± 0.27

List of microRNAs with expression changes greater than 2-fold in the operation group after reperfusion for 48 h (n = 6). The reversed microRNAs after atorvastatin pretreatment are also listed.

A total of 48 microRNAs were significantly different after IRI. Of those, 38 microRNAs were up-regulated, with a 1.6- to 4.9-fold change, whereas 10 microRNAs were down-regulated. The expressions of all detected microRNAs with greater than 2-fold are listed in Table 1.

To study the protective effect of atorvastatin on IRI, the rats received atorvastatin (10 mg/kg) for 2 weeks before ischemia. A total of 13 microRNAs levels were significantly different after atorvastatin pretreatment (Fig. 2A, n = 6, $P < 0.05$). It has been shown that IRI samples pretreated with atorvastatin reversed the up or down regulation in the operation group in the opposite direction in 8 of the 16 microRNAs, which are listed in Table 1.

To validate the microarray platform, we assessed the expression of a subset of microRNAs by conducting real-time qRT-PCR, including the following: miR-365, miR-323, miR-760-5p, and miR-210. The relative expression measured for these microRNAs show consistency with microarray results (Fig. 2B), suggesting that the microarray data were reliable to warrant further analysis.

To explore the role of microRNAs following IRI and atorvastatin pretreatment, potential downstream targets for altered microRNAs were retrieved from the TargetScan Database (http://www.targetscan.org/). Some

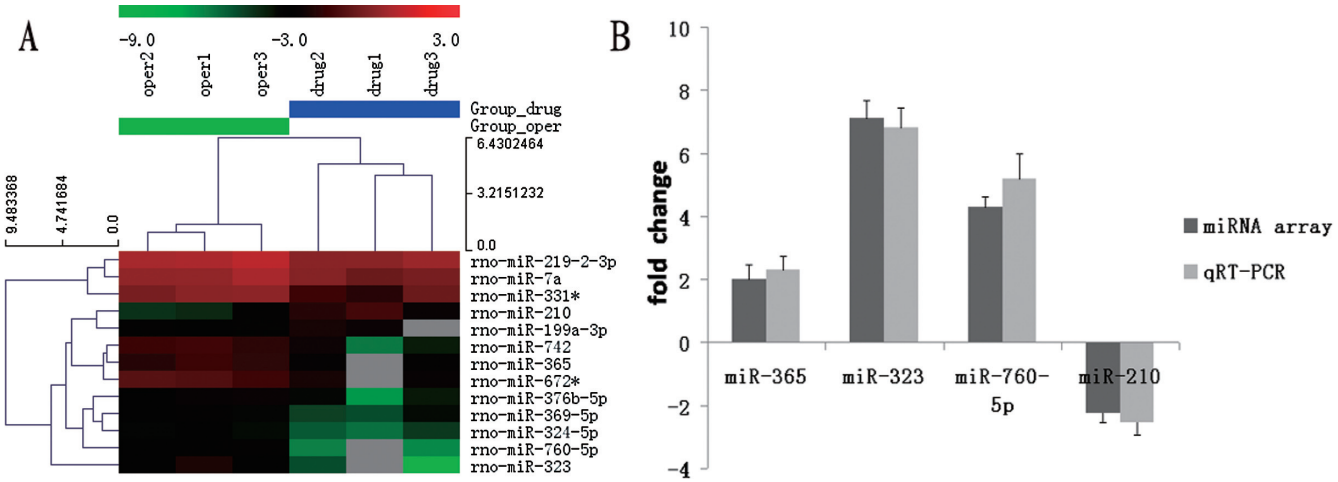

Fig. 2. Differentially expressed microRNAs and verification. A: Hierarchical clustering of differentially expressed microRNAs in drug group vs. operation group. The rows show individual genes, while the columns show individual tissue samples. Red denotes a high expression and green denotes a low expression. B: Comparison of microRNAs fold-changes between the operation group and sham group by microarray and real-time qRT-PCR. Triplicate assays were performed from each RNA sample. Data were normalized using U6 as an endogenous control for RNA input. Fold-changes for these microRNAs from array and real-time qRT-PCR are shown as the mean ± S.E.M. (n = 6 for each group).

anti-apoptotic genes such as Bcl2 and Fas apoptotic inhibitory molecule were potential targets of several microRNAs such as miR-323, miR-365, miR-204*, and miR-760-5p, which were up-regulated after SCI. Furthermore, miR-210 has been identified to mediate anti-apoptotic effects via the regulation of transcription factor E2F3. Some anti-inflammatory mRNAs such as annexin A7 mRNA were potential targets of miR-323. Conversely, some inflammatory mediator mRNAs such as integrin, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), TNF receptor-associated factor 6 (TRAF-6), interleukin-1 receptor-associated kinase 1 (IRAK-1), and CD80 mRNAs were potential targets of miR-210, miR-146a, and miR-199a-3p, which were down-regulated after SCI.

In addition, the expression of growth factors as well as neuritogenic and axonal guidance molecules has also been described in the spinal cord following nerve injury. The gene expression of many of these factors is related to different microRNAs, which are down-regulated following SCI in our analyses. Specifically, brain-derived neurotrophic factor (BDNF), ephrin-A1/A3, neurogenic differentiation 2 (NEUROD2), and semaphorin are related to miR-210; and syntaxin-3, neuroligin-1, and neuropilin 2 are related to miR-146a.

This is the first study demonstrating the microRNA expression profile after spinal cord IRI with or without atorvastatin pretreatment in adult rats. Real-time qRT-PCR analysis verified the results of the microarray study and showed that the microarray data were consistent and reliable. In this report, at first, we demonstrated that a large set of microRNAs were significantly deregulated after spinal cord IRI. The observed expression changes consist mainly of increasing numbers of up-regulated microRNAs, whereas only a few microRNAs appear to be down-regulated. Then, it has been shown that atorvastatin pretreatment can restore part of the altered microRNA expression in the spinal cord with ischemia–reperfusion injury. These altered microRNAs with a diversity of functions may affect multiple biological pathways known to be altered following spinal cord IRI.

To explore the pathophysiological relevance of deregulation of altered microRNAs after IRI, potential targets for theses microRNAs were determined by searching the TargetScan Database. The analysis indicates that the potential targets for these microRNAs include genes encoding components that are involved in many pathophysiological processes such as inflammation, apoptosis, and deregulated neuronal repairment following IRI.

In particular, among the down-regulated microRNAs, miR-146a, which has previously been described as up-regulated in renal IRI and traumatic spinal cord injury (5), was down-regulated in our analysis. Toll-like receptor (TLR) signaling plays a critical role in IRI (6, 7) and results in nuclear factor kappa B (NF-κB) activation (8). MiR-146 has been shown to target the 3′-UTR of TRAF-6 and IRAK-1, thus regulating the TLR pathways and NF-κB–dependent inflammatory responses. In addition, miR-146a expressed in neural

cells (9) targets syntaxin-3, neuroligin-1, and neuropilin 2, which involved in spinal cord injury. MiR-199a-3p targets IKKβ, resulting in its down-regulation and the down-regulation of proinflammatory environments (10). Furthermore, miR-199a-3p targets the MET proto-oncogene and ERK-2, thereby inhibiting proliferation and apoptosis (11), and targets hypoxia-inducible factor-1α, thereby reducing apoptosis of cardiac myocytes after hypoxic injury (12). Therefore, miR-199a-3p may limit spinal cord injury resulting from hypoxia and may control inflammation and ERK–MAPK signaling, which has been shown to participate in injury after IRI. Of particular note is miRNA-124a and miRNA-233, which are differentially expressed after spinal cord injury reported by Nakanishi et al. (2), showed no significant change in our study. It may be due to the difference in mouse model between our study and that in the article of Nakanishi et al.

The neuroprotective effect of statins has been suggested in several studies. Although the results in a spinal cord mechanical injury model have been inconsistent, statins have been shown to provide neuroprotection in spinal cord IRI by modulating nitric oxide synthase expression and increasing antioxidant enzyme levels (13 – 15). In our analyses, atorvastatin preconditioning significantly altered microRNA expression levels and reversed part of the up or down regulations in IRI. Among these microRNAs, miR-323, miR-365, miR-210, and miR-760-5p have been identified to participate in apoptosis and the inflammatory reaction; and miR-199a-3p and miR-210 have been shown to be involved in axonal targeting, neuronal survival, and neurite outgrowth. Since dysregulation of these microRNAs that are directly linked with ischemic injury and since atorvastatin can ameliorate spinal cord IRI through the modulation of several microRNAs, the results of the present study explains the mechanism of the complex regulatory network mediated by atorvastatin through microRNA in neuroprotection. Future detailed studies based on these analyses will pave the way for development of novel therapeutic intervention for neuroprotection in acute IRI.

References

1 Satoh J. MicroRNAs and their therapeutic potential for human diseases: aberrant microRNA expression in Alzheimer's disease brains. J Pharmacol Sci. 2010;114:269–275.

2 Nakanishi K, Nakasa T, Tanaka N, Ishikawa M, Yamada K, Yamasaki K, et al. Responses of microRNAs 124a and 223 following spinal cord injury in mice. Spinal Cord. 2010;48: 192–196.

3 Yunta M, Nieto-Díaz M, Esteban FJ, Caballero-López M, Navarro-Ruíz R, Reigada D, et al. MicroRNA dysregulation in the spinal cord following traumatic injury. PLoS One. 2012;7: e34534.

4 Taira Y, Marsala M. Effect of proximal arterial perfusion pressure on function, spinal cord blood flow, and histopathologic changes after increasing intervals of aortic occlusion in the rat. Stroke. 1996;27:1850–1858.

5 Godwin JG, Ge X, Stephan K, Jurisch A, Tullius SG, Iacomini J. Identification of a microRNA signature of renal ischemia reperfusion injury. Proc Natl Acad Sci U S A. 2010;107:14339–14344.

6 Shichita T, Sakaguchi R, Suzuki M, Yoshimura A. Post-ischemic inflammation in the brain. Front Immunol. 2012;3:132.

7 Shichita T, Hasegawa E, Kimura A, Morita R, Sakaguchi R, Takada I, et al. Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain. Nat Med. 2012;18: 911–917.

8 Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4:499–511.

9 Li X, Gibson G, Kim JS, Kroin J, Xu S, van Wijnen AJ, et al. MicroRNA-146a is linked to pain-related pathophysiology of osteoarthritis. Gene. 2011;480:34–41.

10 Chen R, Alvero AB, Silasi DA, Kelly MG, Fest S, Visintin I, et al. Regulation of IKKbeta by miR-199a affects NF-kappaB activity in ovarian cancer cells. Oncogene. 2008;27:4712–4723.

11 Kim S, Lee UJ, Kim MN, Lee EJ, Kim JY, Lee MY, et al. MicroRNA miR-199a* regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2). J Biol Chem. 2008;283:18158–18166.

12 Rane S, He M, Sayed D, Vashistha H, Malhotra A, Sadoshima J, et al. Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. Circ Res. 2009;104:879–886.

13 Die J, Wang K, Fan L, Jiang Y, Shi Z. Rosuvastatin preconditioning provides neuroprotection against spinal cord ischemia in rats through modulating nitric oxide synthase expressions. Brain Res. 2010;1346:251–261.

14 Ucak A, Onan B, Guler A, Sahin MA, Kılıçkaya O, Oztaş E, et al. Rosuvastatin, a new generation 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, reduces ischemia/reperfusion-induced spinal cord tissue injury in rats. Ann Vasc Surg. 2011; 25:686–695.

15 Saito T, Tsuchida M, Umehara S, Kohno T, Yamamoto H, Hayashi J. Reduction of spinal cord ischemia/reperfusion injury with simvastatin in rats. Anesth Analg. 2011;113:565–571.