MicroRNAs Induced During Ischemic Preconditioning

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Background and Purpose—MicroRNAs (miRNA) are single-stranded short RNA molecules that regulate gene expression by either degradation or translational repression of mRNA. Although miRNAs control a number of conditions and diseases, few neuroprotective miRNAs have been described. In this study, we investigated neuroprotective miRNAs induced early in ischemic preconditioning.

Methods—Ischemic preconditioning or focal cerebral ischemia was induced in mice by transient occlusion of the middle cerebral artery for 15 or 120 minutes. We prepared RNA samples from the ischemic cortex at 3 or 24 hours after the onset of ischemia. Selective miRNAs then were synthesized and transfected into Neuro-2a cells before oxygen–glucose deprivation.

Results—We detected a total of 360 miRNAs. Two miRNA families, miR-200 and miR-182, were selectively upregulated at 3 hours after ischemic preconditioning. Transfections of some of these were neuroprotective in vitro.

Conclusion—Two miRNA families, miR-200 and miR-182, were upregulated early after ischemic preconditioning and the miR-200 family was neuroprotective mainly by downregulating prolyl hydroxylase 2 levels. These miRNAs may be useful in future research and therapeutic applications. (Stroke. 2010;41:1646-1651.)

Key Words: cerebral ischemia ■ ischemic preconditioning ■ microRNA ■ neuroprotection

MicroRNAs (miRNA) are single-stranded RNA molecules of approximately 21 to 23 nucleotides in length, and they regulate gene expression at the posttranscriptional level by either degradation or translational repression of target mRNAs. Although miRNAs control a number of physiological conditions and diseases, only a few studies have shown the implications of miRNA in neuronal death and degeneration; for example, progressive neurodegeneration occurs in the absence of Dicer, which is the key regulator of miRNA biogenesis, and miR-8 targets atrophin to prevent neurodegeneration in Drosophila. However, miRNAs regulating ischemic neuroprotection have not yet been identified.

A subthreshold ischemic insult to the brain induces cellular pathways that can reduce cerebral damage caused by subsequent ischemic insults, known as ischemic preconditioning (IPC). The IPC phenomenon has contributed to the discovery of various neuroprotective mechanisms. By analyzing the miRNA expression patterns in cerebral ischemia models, changes in miRNAs in cerebral ischemia have been revealed, and antagonizing some of them was neuroprotective. However, the analysis of IPC can reveal the miRNAs directly responsible for the neuroprotection. Thus, in this study, we investigated the miRNA profiles after IPC and associated neuroprotective mechanisms. Because the maximum downregulation of gene expression occurs at 3 hours after the cerebral ischemia, and the effect of IPC is complete within 1 day, we profiled miRNAs by focusing mainly at 3 hours and additionally at 24 hours after IPC.

Materials and Methods

Induction of Cerebral IPC and Cerebral Ischemia

This study was approved by Institutional Animal Care and Use Committee of Seoul National University Hospital, which was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Transient focal cerebral ischemia for 120 minutes or ischemic preconditioning for 15 minutes was induced in C57BL/6 mice (n=45, male, aged 7 to 8 weeks; Orient Bio, Seoul, South Korea) by intraluminal thread occlusion of the middle cerebral artery. During the ischemia, the reduction of cerebral blood flow was monitored by using laser Doppler flowmetry (PerFlux System 5000; Perimed, Järfälla, Sweden) secured to the left temporal skull surface, and only those mice with >75% flow reduction were used for miRNA analysis. Rectal temperature was...
maintained at 37 ± 0.5°C using a thermistor-controlled heating blanket.

Mice were euthanized by decapitation at 3 or 24 hours after the induction of IPC or cerebral ischemia (ISC), and the brains were immediately extracted (normal, IPC, 3 hours; IPC, 24 hours; ISC, 3 hours; and ISC, 24 hours). The tissue processing for RNA and protein isolation was performed as previously described. In brief, after removal of the olfactory bulb and cerebellum, the mice brains were placed in a preformed mouse-brain mold calibrated for 1-mm regular slices. The frontal 4-mm tissues were used for the isolation of cortical tissue. The cortex from the ischemic hemisphere was dissected from the striatum and then frozen in liquid nitrogen until RNA isolation. A previous study has shown that this cortical area is spared from ischemic injury by IPC and is thus optimal for the analysis of IPC-induced gene alterations.

From the remaining tissue, a 1-mm additional coronal slice was removed (approximately 4 to 5 mm from the rostral brain) and used for 2,3,5-triphenyltetrazolium chloride staining (Sigma-Aldrich, St Louis, Mo) to confirm the existence of cerebral ischemia in the ISC groups. Total RNA was isolated from individual cortices by using Trizol (Invitrogen, Carlsbad, Calif; n = 6 in each group). To reduce the errors from individual variance of experimental mice, RNA was pooled to generate 2 paired samples for each experimental group (normal; IPC, 3 hours; IPC, 24 hours; ISC, 3 hours; and ISC, 24 hours) by combining cortices from 3 mice. The concentration and quality of RNA were determined by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Tech, Rockland, Del) and a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, Calif). For Western blotting, homogenates of the individual cortices were serially processed using anti-prolyl hydroxylase 2 (PHD2; Abcam, Cambridge, Mass) and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) as previously described.

miRNA Microarray

miRNA expression profiling was determined by using an Agilent Mouse miRNA Microarray 8x15K kit according to the manufacturer’s protocol (Agilent Technologies). The scanning and analysis were performed on an Agilent hardware platform, and the obtained data were normalized by using GeneSpring GX software 7.3.1 (Agilent Technologies) according to the manufacturer’s protocol. Measurements <0.1 were set as 0.1. To reduce the endogenous errors of microarray analysis, we measured the miRNA profiles of 2 paired samples in each experimental group (normal; IPC, 3 hours; IPC, 24 hours; ISC, 3 hours; and ISC, 24 hours; 10 samples in total), and the mean values of the each group were used for further analysis. Selective upregulation/downregulation in the IPC 3-hour group was defined when a miRNA showed more than 2-fold changes compared with both the normal and the ISC 3-hour group. In the IPC 24-hour group, the selective upregulation and downregulation were defined in the same way by comparing the levels with those of the normal and ISC 24-hour groups. The heat map of miRNA expression was visualized by Z-score, which is (raw individual value of the miRNA expression–mean of the miRNA expressions)/SD of the miRNA expression.

Real-Time Polymerase Chain Reaction

Selectively changed miRNAs were validated by using the miRNA quantitative real-time polymerase chain reaction miRNA Detection Kit, TaqMan miRNA assays (Ambion, Applied Biosystems, Foster City, Calif). All reactions were run in triplicate on an ABI PRISM 7700 sequence detection system (Applied Biosystems). Using the comparative threshold cycle (Ct), relative expression was calculated (relative expression = 2−ΔCt) and were normalized by the expressions of control snoRNA202 measured by an endogenous snoRNA detection kit (Ambion) from the same samples. For visualization of miRNA, real-time polymerase chain reaction was performed using the same kits, and 35-cycle amplified miRNAs were visualized on 1.0% agarose gel.

Targeted Gene Prediction of miRNAs

The miRNA mature sequence database was obtained from miRBase (www.mirbase.org). To find potential miRNA target sites in the mouse gene 3′-untranslated region, we used 3 different target prediction programs: TargetScan (www.targetscan.org), PicTar (http://pictar.mdc-berlin.de), and microT (http://diana.cslab.ece.ntua.gr/microT). In Vitro Study and Western Blotting

Mouse neuroblast cells (Neuro-2a; American Type Culture Collection, Manassas, Va) were transfected with selected mature miRNAs or a negative control miRNA (100 nmol/L; Bioneer, Daejon, South Korea) using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) and subjected to 16 hours of oxygen glucose deprivation (Dulbecco modified Eagle medium without glucose and sodium pyruvate, O2 levels 1% to 1.5%) in a humidified hypoxic chamber (Bactron 1.5; Sheldon Manufacturing, Cornelius, Ore) as previously described (Supplemental Methods; available at http://stroke.ahajournals.org). Cell survival was determined by WST-1 assay agent (5 mmol/L, 1:9, Roche, Basel, Switzerland) according to the manufacturer’s instructions. For Western blotting, homogenates of cells obtained at 24 hours after the miRNA transfection were serially processed for Western blotting as described previously using anti-PHD2 (Abcam), anti-hypoxia-inducible factor-1α (HIF-1α; BD Biosciences, San Jose, Calif), and anti-β-actin antibody (Santa Cruz Biotechnology). For the ischemic preconditioning group, cells were subjected to 6 hours of oxygen glucose deprivation and maintained in glucose-containing medium for 24 hours to develop tolerance. Relative optical densities of all Western blots were calculated versus measured values of β-actin by using Image Pro-Plus (Media Cybernetics, Bethesda, Md).
Statistical Analysis
All data in this study are presented as means±SD. Student t test was used for intergroup comparisons. For comparisons among ≥3 unpaired groups, we used 1-way analysis of variances and post hoc tests (Dunnett and Tukey tests). SPSS 17.0 (SPSS Inc, Chicago, Ill) was used for the statistical analyses, and a 2-tailed probability value P<0.05 was considered to indicate significance.

Results
miRNA Signatures in Early IPC
To find miRNAs that are responsible only for the IPC-induced neuroprotection, we initially compared the miRNA of the IPC group with those of the normal and time-matched ISC groups. When determined by fold changes, 8 miRNAs were upregulated selectively in the IPC 3-hour group (Figure 1; Table); these were miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-182, miR-183, and miR-96. There were no downregulated miRNAs in the IPC 3-hour group. We also detected 2 upregulated and 1 downregulated miRNAs that were selectively changed in the IPC 24-hour group compared with the normal and ISC 24-hour groups (upregulation: miR-681 and miR-197; downregulation: miR-468; Table, see Supplemental Table I for full microarray data, available online at http://stroke.ahajournals.org).

Table. Selectively Upregulated or Downregulated miRNAs in the IPC 3-Hour and IPC 24-Hour Groups Determined by Fold Changes

<table>
<thead>
<tr>
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<th>IPC 3 Hours</th>
<th>IPC 24 Hours</th>
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<tr>
<td>Selective upregulation</td>
<td>miR-200a/miR-200b/ miR-200c/ miR-141/ miR-429, miR-182/miR-183/miR-96</td>
<td>miR-681, miR-197</td>
</tr>
<tr>
<td>Selective downregulation</td>
<td>none</td>
<td>miR-468</td>
</tr>
</tbody>
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*Selective upregulation (>2-fold) or downregulation (<0.5-fold) were determined by comparisons with the normal and time-matched ISC groups.

Two Familial miRNA Clusters in IPC
Because the downregulation of gene expression is already obvious at 3 hours after cerebral ischemia, we hypothesized that the changed miRNAs at IPC 3 hours are associated with the induction of IPC. A total of 8 miRNAs (miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-182, miR-183, and miR-96) showed selective upregulation in the IPC 3-hour group compared with the normal or the ISC 3-hour group. The miRNA–mRNA interaction needs complementarity between the miRNA 5′ end seed sequence and the 3′-untranslated region of the target mRNA. Based on the seed sequence similarity and clustered genomic location, the selectively upregulated 8 miRNAs in the IPC 3-hour group were categorized into 2 miRNA families: miR-200 (A) and miR-182 (B). Among the miR-200 family members, miR-200a, miR-200b, and miR-429 are located in chromosome 4, and miR-141 and miR-200c are located in chromosome 6 (Supplemental Figure I, available online at http://stroke.ahajournals.org).

Figure 2. Sequences and validation of the selectively upregulated miRNAs in the IPC 3-hour group. Based on the seed sequences (red and blue colors), 8 miRNAs were categorized into 2 miRNA clusters, which were the miR-200 (A) and miR-182 families (B). In measurements by real-time polymerase chain reaction, the IPC 3-hour group showed high expressions of the 8 miRNAs compared with the other groups (C). The levels of the miRNAs were normalized by the level of sno202 endogenous control RNA and expressed as ratios to those of the normal group. *P<0.05 versus the normal group.
miR-182 family comprises miR-182, miR-183, and miR-96, which are located in chromosome 6 (Supplemental Figure I). By using reverse transcriptase–polymerase chain reaction and real-time polymerase chain reaction, we could confirm the microarray results that showed the selective upregulations of the 8 miRNAs in the IPC 3-hour group (Figure 2C; Supplemental Figure II, available online at http://stroke.ahajournals.org; n/H11005 to 6 per group). The miRNAs were upregulated approximately 10- to 1000-fold compared with the normal brain, and their levels in ISC 3-hour group were not different from those of the normal group.

PHD2 as the Target of the miR-200 Family

Based on our hypothesis that some of the 8 miRNAs are neuroprotective, we transfected the miRNAs into the Neuro-2a cells and applied oxygen glucose deprivation. WST-1 assay results showed that transfections of miR-200a, miR-200b, miR-200c, miR-429, or miR-182 showed increased cell survival (high WST-1 values) compared with those transfected with the control (CTR) miRNA (A). TargetScan identified the 3'-untranslated region of PHD2 (EGLN1) as the target of miR-200b, miR-200c, and miR-429 (B, seed sequences are underlined). The transfection of each miR-200 family (miR-200a, miR-200b, miR-200c, and miR-429) downregulated the PHD2 protein and increased HIF-1α as determined by Western blotting (C). *P<0.05 versus CTR in A, versus PHD2 of CTR (nonischemia) in C, or versus normal (NOR) in D. †P<0.05 versus HIF-1α of CTR (nonischemia) in C. UTR indicates untranslated region.

miR-182 family comprises miR-182, miR-183, and miR-96, which are located in chromosome 6 (Supplemental Figure I). By using reverse transcriptase–polymerase chain reaction and real-time polymerase chain reaction, we could confirm the microarray results that showed the selective upregulations of the 8 miRNAs in the IPC 3-hour group (Figure 2C; Supplemental Figure II, available online at http://stroke.ahajournals.org; n=3 to 6 per group). The miRNAs were upregulated approximately 10- to 1000-fold compared with the normal brain, and their levels in ISC 3-hour group were not different from those of the normal group.

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Discussion

In this study, we investigated the miRNA profile during IPC and identified the selective miRNA changes early after the IPC by comparison with normal and ISC brains. The 8 miRNAs selected in the IPC 3-hour group were categorized into 2 families of miRNAs: the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and the miR-
miR-182 family (miR-182, miR-183, and miR-96). Among them, miR-200b, miR-200c, and miR-429 targeted PHD2 and had the best neuroprotective effect. Our data can be used for further researches into the IPC mechanism and can contribute to the development of neuroprotective miRNA therapeutics.

Several previous studies have investigated miRNAs as mediators of ischemic tissue damage. In the ischemia of cardiac myocytes, miR-199a is acutely downregulated as early as 30 minutes after ischemia, leading to rapid upregulation of its target HIF-1α,24 which suggests that the changes in miRNA in response to the ischemia seem to be very prompt. Given that the maximum downregulation of gene expression occurs 3 hours after cerebral ischemia,8 our analysis at 3 hours after the cerebral ischemia was within the optimal time window in this regard. miR-320 is involved in the regulation of cardiac ischemia by targeting Hsp20,25 and miR-23a, miR-326, miR-346, and miR-370 were changed in hepatic IPC.26 In cerebral ischemic models, a few studies have profiled the broad miRNA changes in the ischemic brain and blood and provided novel mechanical insights and therapeutic targets.6,7,27 In contrast, our study focused on the direct identification of neuroprotective miRNAs in the early IPC model at 3 hours after the brief ischemia and thus could suggest selective miRNAs involved in neuroprotection. Because our analysis used only cortical ischemic tissues influenced by IPC, our miRNA profiles were predesignated to the IPC study and the observed profiles in our ISC groups may be different from the previous observations that examined the entire ischemic hemisphere.6,7

Members of miR-200 family are originally associated with olfactory neurogenesis28 or the inhibition of cancer invasion.28 Interestingly, Drosophila miR-8, which has been known to target atrophin to prevent neurodegeneration,4 shares the common seed sequence (AAUACUG) with the human miR-200 family. Recently, miR-8/miR-200 have been known to regulate insulin signaling.29 Meanwhile, the miR-182 family are sensory organ-specific miRNAs, thus serving in circadian rhythm regulation,21 and are upregulated in invasive melanoma cells.30 Although miR-182 had in vitro neuroprotective effects and upregulated HIF-1α as well, we mainly identified the possible neuroprotective mechanism of the miR-200 family by focusing on PHD2. Oxygen-dependent hydroxylation of HIF-1α subunits by PHD plays a critical role in degrading HIF-1α.31 Among PHD1, PHD2, and PHD3, PHD2 is the most abundant type and hydroxylates HIF-1α most efficiently.31

However, the effects of the changed miRNAs should be interpreted given their possible pleiotropic functions. miR-200a and miR-141 have a single different seed sequence (AAACUG) compared with the other miR-200 family members (AAUACUG), and so they bind to substantially different target genes. This difference was likely to lead to the overall effect of miR-141 transfection as nonneuroprotective, although it did reduce PHD2 and increased HIF-1α. Because a single miRNA can repress the production of hundreds of proteins,32 it is likely that multiple proteins and pathways are involved in the ischemic protection mediated by our miRNAs. The identification of miRNA functions, other than the miR-200 family such as miR-182 in IPC 3 hours, and miR-681, miR-197, and miR-468 in the IPC 24-hour group, may help to resolve this issue, because miRNAs have been implicated in various ischemic diseases3 and the application of specific miRNAs or its antagonists have in vivo therapeutic effects in vascular disorders.33 Accordingly, future development of miRNA-based therapeutics for cerebral ischemia is strongly warranted.

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Disclosures
None.

References


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