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Ischemic preconditioning alters cerebral microRNAs that are upstream to neuroprotective signaling pathways

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Abstract

Cerebral gene expression is known to be significantly influenced by a sublethal ischemic event (preconditioning; PC) that induces tolerance to future damaging ischemic events. Small non-coding RNAs known as microRNAs (miRNAs) were recently shown to control the mRNA translation. We currently profiled cerebral miRNAs in the cerebral cortex of rats subjected to PC. The miRNAome reacted quickly and by 6h following PC, levels of 51 miRNAs were altered (26 up- and 25 downregulated; >1.5 fold change). 20 of these stayed at the altered level even at 3 days after PC. At least 9 miRNAs showed >5 fold change at one or more time points between 6h to 3 days after PC compared to sham. Bioinformatics analysis showed 2007 common targets of the miRNAs that were up-regulated and 459 common targets of the miRNAs that were down-regulated after PC. Pathways analysis showed that MAP-kinase and mTOR signaling are the top 2 KEGG pathways targeted by the upregulated miRNAs, and Wnt and GnRH signaling are the top 2 KEGG pathways targeted by the down-regulated miRNAs after PC. We hypothesize that alterations in miRNAs and their down-stream mRNAs of signaling pathways might play a role in the induction of ischemic tolerance.

Keywords

Stroke; miRNA; Non-coding RNA; Neuroprotection, Ischemic tolerance, second messenger signaling

Ischemic preconditioning (PC) is an endogenous neuroprotective mechanism by which a sublethal ischemic event confers tolerance to a subsequent lethal ischemia (Dirnagl *et al.* 2009). The mechanisms of PC-mediated tolerance are not precisely known, but PC induces extensive temporal change in the cerebral gene expression, and a concomitant altered expression of many proteins (Dhodda *et al.* 2004, Stenzel-Poore *et al.* 2007, Feng *et al.* 2007). As inhibition of translation is known to curtail PC-induced ischemic tolerance (Barone *et al.* 1998), the neo synthesis of proteins might be a key factor in this phenomenon. The microRNAs (miRNAs) which are ~22-nt long evolutionarily conserved non-coding RNAs are currently considered as important regulators of protein translation (Bartel 2009). The miRNAs bind to complementary 8-bp seed sequences in the 3′-UTRs of target mRNAs to either arrest their translation or induce their degradation (Bartel 2009). The number of known miRNAs is much lower than the number of known mRNAs in any species. For example, despite the presence of >27,000 protein-coding mRNAs, only 325 miRNAs were reported so far in rats (miRBase Release 14; http://www.mirbase.org). As most miRNAs can bind to >200 mRNAs (Friedman *et al.* 2009), the small number of miRNAs can effectively

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control the huge number of mRNAs either sequentially or simultaneously, and thus can significantly influence the cellular homeostasis under normal and pathological conditions.

Recent studies showed that miRNA profiles alter in the blood and brain of rodents subjected to cerebral ischemia and modulating specific miRNAs can alter the post-ischemic brain damage and neurological dysfunction (Jeyaseelan *et al.* 2008, Dharap *et al.* 2009, Aebert *et al.* 1997, Liu *et al.* 2010, Yin *et al.* 2010). However, except for a paper in press (Lusardi *et al.* 2010) no studies to date evaluated the effect of PC on cerebral miRNAome. We currently profiled 265 miRNAs in the cerebral cortex of rats subjected to PC by a 10 min transient middle cerebral artery occlusion (MCAO). Using TargetScan5 (Lewis *et al.* 2005) and DNA Intelligence Analysis (DIANA) miRPath algorithm (Papadopoulos *et al.* 2009), we identified the down-stream targets and the KEGG pathways common to several miRNAs altered after PC that might play a role in mediating ischemic tolerance.

MATERIALS AND METHODS

Induction of PC

Adult male spontaneously hypertensive rats (SHR) (280-320 g; Charles River, Wilmington, MA, USA) used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services Publication number 86-23 (revised 1986). The Research Animal Resources and Care Committee of the University of Wisconsin-Madison approved all the surgical procedures. PC was induced as described earlier (Dhodda *et al.* 2004, Bowen *et al.* 2006). In brief, middle cerebral artery (MCA) was occluded for 10 min under isoflurane anesthesia with a 3-0 monofilament nylon suture. The occlusion and reperfusion were confirmed by measuring rCBF with a laser-Doppler probe and the P_aO_2 (100 to 200 mm Hg) and P_aCO_2 (30 to 40 mm Hg), body temperature (37 to 38°C) and cranial temperature (36 to 37°C) were monitored and maintained at the physiological levels. Sham-operated rats underwent the same procedure except the MCA occlusion.

miRNA profiling

miRNA profiling was conducted as described earlier (Dharap *et al.* 2009). In brief, cohorts of rats subjected to PC were sacrificed at 6h, 24h and 72h (n = 6 at each time point). Six sham-operated rats served as control. Total RNA was extracted from the ipsilateral cortex of each rat using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) as per the manufacturer's protocol. From each sample, 5 μg total RNA was size fractionated on a centrifugal filter (YM-100 Microcon; Millipore USA). To the small RNAs (<300 nt), poly-A tails were added at the 3′end mediated by poly(A) polymerase, and the nucleotide tags were ligated to the poly-A tails. Each sample was hybridized to a microarray (LC Sciences, Houston, TX) that contained 265 known rat miRNA probes (12 repeats of each probe) from the Sanger miRBase version 11.0 (http://microrna.sanger.ac.uk/sequences/). The miRNA hybridization data was corrected by subtracting the background (calculated from the median of 5% to 25% of the lowest-intensity cells) and normalized to the statistical median of all detectable transcripts using the locally-weighted regression (LOWESS) method which balances the intensities of Cy5 labeled transcripts so that the differential expression ratios can be properly calculated (Bolstad *et al.* 2003). For subtracting, the background was defined on each array as the average signal of the BKG0 spots (chemical linkers without the probes). The hybridization intensities above exp(5) (~150) were considered as significant as described earlier (Vagin *et al.* 2006) and intensities below 30,000 were considered as non-saturated, as established with titration of several synthetic 20-nt RNA oligos (external controls) spiked into each sample. In addition, on each array there were 16 sets of spatially distributed internal control probes. These include PUC2PM-20B and PUC2MM-20B which

are the perfect match and the single-base mismatch sequences, respectively. The stringency of the intensity ratio of the PUC2PM-20B and PUC2MM-20B is expected to be larger than 30 indicating proper hybridization in each case. For proper analysis of signal intensities on each chip, both the internal controls and the test miRNA probes were repeated 12 times. On a microarray, the hybridization signal was linearly obtained from 1 to ~66,000 units. A miRNA transcript was considered detectable if it met the following criteria. (a) Signal intensity higher than 3 times the maximal background signal, (b) spot CV <0.5 (CV was calculated as (standard deviation)/(signal intensity)) and (c) the signals from at least 50% of the 12 redundant repeating probes above detection level. To avoid false positives, any spot that deviated >50% from the average value of the 12 repeating spots and/or spots with CV >0.5 were eliminated. The data from different groups was normalized and analyzed statistically using ANOVA. To increase the validity of the data, we generated cross-comparison matrices. For example, the data from the 6 sham chips was cross-compared with the 6 PC chips (at each time point) to generate a matrix of 36 comparisons. A miRNA transcript was assumed altered if it showed a statistically significant change in at least 30 out of 36 cross-comparisons (83% positive). The standard deviations of the sham and PC groups for each of the miRNAs profiled were <15%.

Bioinformatics

The number of mRNA targets for each miRNA were predicted with microRNA.org that used the open-source miRanda algorithm (http://www.microrna.org/miranda.html) (Betel *et al.* 2008) by computing the optimal sequence complimentarity between mature miRNAs and mRNAs using a weighted dynamic programming algorithm (John *et al.* 2004). The miRanda uses the miRNA sequences from Sanger mirBASE database and the 5′-UTR sequences of mRNAs from the NCBI database. For predicting the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of mRNAs that are targeted by PC-altered miRNAs, we used DIANA miRPath algorithm combined with DIANA microT 4.0 and TargetScan5 (Papadopoulos *et al.* 2009, Lewis *et al.* 2005).

RESULTS

PC-induced changes in miRNA expression

Of the 265 miRNAs analyzed, 158 (59%) were expressed (>100 units on a scale of 1 to 45,000) in the cerebral cortex of sham-operated rats. Of these, 12 were expressed at a very high level (>15K units), 19 at a high level (5K to 15K units), 39 at a medium to high level (1K to 5K units), and 88 at a moderate level (100 to 1K units) (Fig. 1). In the cerebral cortex of the rats subjected to PC, 11 miRNAs showed increased expression and 9 miRNAs showed decreased expression at all the 3 time points (6h, 24h and 72h) compared to sham (Table 1). In addition, 3 miRNAs showed altered expression (2 increased and 1 decreased) at 6h and 24h, but not at 72h after PC (Table 1). 28 miRNAs also showed altered expression only at 6h after PC (13 up- and 15 down-regulated) (Table 2). For a single miRNA, mir-466c showed a maximal decrease of 27-fold and mir-21 showed a maximal increase of 13-fold at 24h after PC (Table 1). Of the 51 miRNAs altered after PC, 10 showed >5-fold change at one or the other time point between 6h to 72h (Table 1 and Table 2).

Common targets of miRNAs altered after PC

Using the web tool MicroRNA.org, we identified mRNAs targeted by multiple miRNAs increased or decreased after PC. This *in silico* analysis showed 8 mRNAs, each targeted by >5 miRNAs. The major targets of the miRNAs upregulated after PC are Fragile X mental retardation 1 (FMR1; targeted by 8 miRNAs), dishevelled-axin domain containing 1 or dixin (DIXDC1; targeted by 8 miRNAs), karyopherin subunit α-1 or importin α-1 (KPNA1; targeted by 7 miRNAs), sodium/potassium-transporting ATPase subunit β-1 (ATP1b1;

targeted by 5 miRNAs), and ubiquitin carboxyl-terminal hydrolase 1 (UCHL1; targeted by 4 miRNAs). The major targets of the miRNAs downregulated after PC are methyl CpG binding protein 2 (MeCP2; targeted by 10 miRNAs), Fas ligand 1L (Fas1L; targeted by 7 miRNAs), and protein phosphatase 1 regulatory subunit 3B (PPP1r3b; targeted by 7 miRNAs).

Convergent functional pathways downstream to the miRNAs altered after PC

Biological pathways rather than the individual genes/proteins are the major functional units that control the physiological and pathological outcomes. Furthermore, as a miRNA can act on several targets and a mRNA can be targeted by multiple miRNAs; different genes within a pathway can be influenced by combinations of altered miRNAs that can be integrated into functional pathways. Hence, we used the pathway analysis software DIANA mirPath to identify the biological pathways downstream to miRNAs altered after PC. We combined mirPath with 2 miRNA target prediction web tools (DIANA microTar 4.0 and TargetScan5) and identified pathways that are common to multiple miRNAs up- or down-regulated after PC. The major biological functional categories targeted by upregulated miRNAs are MAP-kinase signaling, mTOR signaling, TGF-β signaling, Wnt signaling, ErbB signaling, GnRH signaling, P53 signaling, insulin signaling, JAK-STAT signaling and Notch signaling (Fig. 1 top panel). The major biological functional categories targeted by down-regulated miRNAs are Wnt signaling, GnRH signaling, MAP-kinase signaling, TGF-β signaling, ErbB signaling, PI signaling and P53 signaling (Fig. 1 bottom panel).

DISCUSSION

In brief, results of the present study show that ischemic PC induces a temporal change in the cerebral miRNA profile and many of the altered miRNAs target many mRNAs that are part of the biological pathways that control cellular signaling. Although, the molecular mechanisms of the PC-induced neuroprotection are not yet completely understood, we and others showed that PC leads to extensive changes in the mRNA and protein expression in rodent brain (Dhodda *et al.* 2004, Stenzel-Poore *et al.* 2007, Feng *et al.* 2007). Many proteins reported previously to be altered after PC like heme oxygenases, metallothioneins, heat shock proteins, hypoxia-inducible factor-1, immediate early genes, growth factors, nitric oxide, ion channels, toll-like receptors and kinases were proposed as putative mediators of ischemic tolerance (Zeynalov *et al.* 2009, Tang *et al.* 2006, Dhodda et al. 2004, Trendelenburg *et al.* 2002, Truettner *et al.* 2002, Pradillo *et al.* 2009, Gonzalez-Zulueta *et al.* 2000, Bernaudin *et al.* 2002). The neo expression of genes and protein products seems to be essential for PC-induced tolerance as both actinomycin-D and cyclohexamide were shown to prevent this phenomenon (Puisieux *et al.* 2004, Barone et al. 1998, Strohm *et al.* 2002).

The miRNAs finely control the translation of mRNAs by binding to the seed sequences in their 3′-UTRs (Bartel 2009). We and others showed that miRNAs respond rapidly to focal ischemia and their targets include many mRNAs that control the post-stroke pathophysiologic processes including inflammation, ionic balance, oxidative stress and transcription (Jeyaseelan *et al.* 2008, Dharap *et al.* 2009, Liu *et al.* 2010). Recent studies also showed that miRNAs altered after focal ischemia has functional significance. Our studies showed that mir-145 upregulation after transient MCAO prevents the expression of the anti-oxidant protein SOD-2 and treating rats with antagomir-145 counters this effect leading to neuroprotection (Dharap *et al.* 2009). A recent study by Yin *et al.* (2010) showed that mir-497 upregulated after focal ischemia prevents the expression of anti-apoptotic protein Bcl-2 and antagomir-497 increased Bcl-2 levels leading to smaller infarcts. The present study extends the importance of miRNAs in ischemic pathophysiology by showing that they also respond to PC and might play a role in inducing ischemic tolerance in brain. It is interesting to note that following PC, miRNA expression alters rapidly and many miRNAs

sustain at the altered levels even at 3 days after PC. We previously showed that the expression of <5% of protein coding genes will be altered after PC (Dhodda *et al.*, 2004). Whereas, the present study show that the expression of >20% of miRNAs is altered after PC indicating that genes that transcribe miRNAs are more sensitive to a mild stroke than those that transcribe protein-coding mRNAs.

As several miRNAs can bind and silence an individual mRNA by targeting multiple sites in its 3′-UTR, using bioinformatics we identified some mRNAs that have seed sequences for miRNAs altered after PC. It is interesting to note that 8 of the miRNAs upregulated after PC target FMR1 mRNA that codes fragile X mental retardation protein which plays an important role in synaptic plasticity (Mercaldo *et al.* 2009). This might have a profound effect in promoting recovery in case of a subsequent stroke. Two other important mRNAs targeted by multiple miRNAs upregulated after PC are DIXDC1 and UCHL1. As DIXDC1 controls Wnt signaling (Wang *et al.* 2009) and UCHL1 controls ubiquitin levels (Sakurai *et al.* 2006), these two proteins modulate the neuronal differentiation. Hence, alteration of miRNAs that target these proteins might play a significant role in mediating the PC-induced neurogenesis and plasticity which was thought to be a critical factor in inducing ischemic tolerance (Maysami *et al.* 2008). It is interesting to note that a recent paper showed that UCHL1 will be released into CSF and blood following focal ischemia in rodents (Liu *et al.* 2010). We also observed that multiple miRNAs down-regulated after PC target MeCP2 and Fas Ligand mRNAs. These changes might play an important role in PC-induced neuroprotection as MeCP2 controls DNA methylation and chromatin silencing (Urdinguio *et al.* 2009), and Fas Ligand controls apoptosis (Guicciardi & Gores 2009). Interestingly, Lusardi *et al.* (2010) recently showed that MeCP2 is a major target of the miRNAs altered in mouse brain after PC.

As a single miRNA can act on hundreds of mRNAs that contain a complementary binding site, multiple miRNAs can understandably act on various mRNAs that could be part of a biological pathway. Hence, the significance of miRNAs can be understood in a better perspective by looking at the pathways they control in a synergistic manner. It is interesting to note that miRNAs altered after PC target signaling pathways including MAP-kinase signaling, TGFβ signaling, Wnt signaling, Notch signaling, insulin signaling, and mTOR signaling. Ischemia is associated with energy stress and one of the central regulators of protein synthesis, the highly energy-intensive mTOR pathway has been shown to be inhibited by hypoxia via the hypoxia inducible Redd1/RTP801 gene (Arsham *et al.* 2003; Brugarolas *et al.* 2004). Bioinformatics analysis showed that the mTOR pathway is one of the top 3 targets of the altered miRNAs. Thus, the miRNAs upregulated after PC may be involved in activating energy-conservation cascades which might allow an efficient recovery in case of a subsequent stroke. MAP-kinase signaling is another major pathway targeted by PC-responsive miRNAs. MAP-kinase family members are known to be stimulated after cerebral ischemia and were thought to regulate signal transduction, gene expression and metabolism (Johnson & Lapadat 2002). As the cellular stress following ischemia can disrupt the normal dynamics of kinase activity which in turn may lead to haphazard cellular signaling, the altered levels of miRNAs that regulate MAP-kinases might be an adaptation to counter stress in case of a future ischemic event. Another interesting indication that miRNAs are involved in PC-mediated neuroprotection is the correlation between the downregulated miRNAs and Wnt signaling pathway. In adult brain, both neurons and glia express the Wnt receptors; and the Wnt signaling pathway is thought to be involved in cell regulation and cytoprotection (Li *et al.* 2006). Wnt1 was shown to prevent apoptosis through β-catenin/Tcf transcriptional pathway, and by preventing the release of cytochrome C from mitochondria (Chen *et al.* 2001). This ties well with our observation that the Wnt signaling pathway has the highest target score against miRNAs downregulated after PC which might be an adaptation to provide an anti-apoptotic environment in case of a further ischemic event.

Future studies might decipher the precise functional significance of these pathways in the acquisition of ischemic tolerance following PC.

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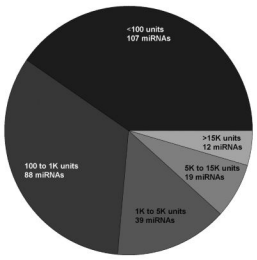

Fig. 1.

The pie-chart shows the relative expression levels of miRNAs in the normal rat cerebral cortex (each value is a mean of n = 6). Of the 265 miRNAs analyzed, 59% were observed to be expressed at >100 units on a scale of 1 to 45,000 units.

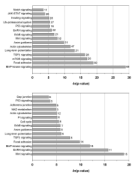

Fig. 2.

The top KEGG pathways of biological function of the targets of miRNAs altered after PC. Only miRNAs that were observed to be altered at all the 3 time points (6h, 24h and 72h) were chosen for this analysis. The top 15 KEGG pathways targeted by the upregulated and the down-regulated miRNAs were shown in the top and bottom panels, respectively. We identified the pathways only if an mRNA is a target of at least 4 of the altered miRNAs in each case. The number next to each bar is the number of mRNAs that are part of a specific KEGG pathway.

Table 1
miRNAs altered temporally after preconditioning

miRNA	6h	24h	72h	# of targets
Up-regulated				
mir-374	9.48	3.51	2.76	260
mir-98	6.98	2.95	2.91	280
mir-340 -5p	6.01	2.00	1.67	419
mir-21	5.79	13.03	5.71	195
let-7a	4.01	1.83	NC	367
mir-352	3.77	1.65	2.58	133
mir-379*	3.47	1.84	1.70	--
mir-335	3.35	1.79	1.61	332
mir-181b	2.63	1.51	1.62	396
mir-26b	2.18	1.53	1.76	231
mir-181d	2.06	2.22	NC	420
mir-15b	1.56	2.18	4.07	513
mir-146a	1.96	1.57	2.70	287
Down-regulated				
mir-466c	20.05	27.18	20.38	209
mir-292 -5p	12.37	7.11	7.24	235
mir-328	7.41	1.58	1.87	271
mir-873	7.21	8.94	9.64	328
mir-494	5.31	2.63	4.40	340
let-7d*	4.77	1.65	4.42	--
mir-345 -5p	4.41	2.04	2.01	138
mir-92b	4.06	2.13	NC	187
mir-30c-2*	3.83	2.02	3.01	--
mir-322*	1.84	2.15	2.52	--

The values are mean fold increases or decreases in the PC groups over sham group obtained from 36 cross-comparisons (n = 6 for each group) in each case. The SD was <15% in all cases. Only the fold changes that are statistically significantly different from sham (by ANOVA) were given. NC, no change compared to sham. The # of targets for each miRNA was predicted using the web tool microRNA.org. The miRNAs are transcribed as long pri-miRNAs (primirs) with several stem loop structures. The RNAse Drosha cuts a primir to release the ~70 nt stem loop structures called pre-miRNAs (premirs). Another RNAse Dicer releases a mature miRNA (~22 nt long) from a premir. In some cases, a second miRNA can be formed from the other arm of a premir. The two miRNAs formed from the same premir are usually given the same number, but the minor one will be denoted with a star. The limitation of the et prediction algorithms like microRNA.org is that they can not predict the targets of miRNAs with stars.

* Hence, the miRNAs with a were not used in the pathways analysis.

Table 2

miRNAs altered only at 6h after PC

Up -regulated	Fold increase	Dow n-regulated	Fold decrease
mir-384 -3p	8.58	mir-433	3.20
mir-30e*	3.40	mir-331	2.87
mir-153	2.35	mir-99b	2.42
mir-27a	2.26	mir-760 -3p	2.29
mir-27b	1.85	mir-324 -3p	2.18
mir-204	2.24	mir-92a	2.07
mir-539	2.16	mir-485	2.03
mir-376b -3p	2.13	mir-129*	2.01
mir-376b -5p	1.95	mir-140*	2.21
mir-30a	1.94	mir-30d	2.09
mir-137	1.85	mir-425	2.09
mir-382	1.71	mir-320	1.76
mir-384 -5p	1.61	mir-674 -3p	1.73
		mir-330*	1.54
		mir-145	1.50

The values given are mean fold changes in the PC group over sham group obtained from 36 cross-com parisons (n = 6 for each group). The SD was <15% in all cases. Only the fold changes which were statistically significantly different from sham (by ANOVA) were given.

*Please see Table 1 legend.