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Contribution of microRNA-203 to the isoflurane preconditioning-induced neuroprotection

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Abstract

A prior exposure to isoflurane, a common volatile anesthetic, provides neuroprotection (isoflurane preconditioning). To determine the role of microRNAs in this protection, we performed microRNA array assay on cerebral cortex harvested from rats exposed to isoflurane or isoflurane-exposed rat B35 neuron-like cells. We showed that isoflurane significantly increased microRNA-203 expression in B35 neuron-like cells. The microRNA-203 expression in rat cerebral cortex also trended to increase after isoflurane exposure. Over-expression of microRNA-203 increased the tolerance of B35 cells to oxygen-glucose deprivation and the expression of phospho-Akt, a protein kinase that promotes cell survival. Isoflurane preconditioning also reduced the injury of these cells after oxygen-glucose deprivation. These results suggest that isoflurane preconditioning-induced neuroprotection may involve increased expression of microRNA-203. This finding provides the initial evidence that microRNA-203 is a target for isoflurane in the brain.

Keywords

Akt; isoflurane; microRNA; microRNA-203; neuroprotection

1. Introduction

Ischemic preconditioning describes a phenomenon in which short episodes of ischemia induce protection against subsequent prolonged ischemia. We and others have shown that a prior exposure to isoflurane, a commonly used volatile anesthetic, also can reduce ischemic brain injury [5,19]. This phenomenon is called isoflurane preconditioning-induced neuroprotection and may be mediated by increased expression of activated proteins, such as Akt, that promote cell survival [3,10]. Although the mechanisms of this protection have been actively investigated in recent years, it is not known yet whether this protection involves microRNA (miRNA).

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miRNAs are small, non-coding RNAs that are often 19 – 25 nucleotides [1]. They can bind to 3' untranslated region of mRNAs to inhibit the translation of the mRNAs. Interestingly, one miRNA has multiple mRNA targets [1]. It is known that this form of regulation is a fundamental process to regulate the expression of genes that are involved in various functions.

It is proposed that the expression of every protein-coding gene may be subjected to miRNA-mediated regulation. Also, miRNA has been shown to regulate the expression of proteins that can cause cell death [15]. Thus, we hypothesize that isoflurane preconditioning-induced neuroprotection may involve miRNA regulation of gene expression. To test this hypothesis, we first performed miRNA array study to identify the miRNAs whose expression was altered by isoflurane exposure. The possible role of miRNA-203, a 22 nucleotide RNA, in the isoflurane preconditioning-induced neuroprotection was then tested.

2. Materials and methods

2.1. Exposure of rats to isoflurane and harvest of cerebral cortex

As we described before [11], two-month old male Fisher 344 (200 - 230 g) rats were induced by isoflurane and intubated. They were then ventilated with isoflurane carried by 100% O₂ to maintain end-tidal isoflurane concentration at 2%. The inhaled and exhaled gas concentrations were monitored continuously with a Datex™ infrared analyzer (Capnomac, Helsinki, Finland). The ventilator settings were adjusted to maintain the end-tidal CO₂ at ~32 mmHg. Rectal temperature was maintained at 37°C ± 0.5°C. Heart rate and pulse oximeter oxygen saturation (SpO₂) were measured continuously during anesthesia with a MouseOx™ Pulse Oximeter (Harvard Apparatus, Holliston, MA). This isoflurane exposure was for 1 h. Control animals were exposed to 100% O₂ for 1 h. Their cerebral cortices at bregma level were harvested at 6 h after the exposure.

2.2. Cell culture

Rat B35 cells from American Type Culture Collection (ATCC, Manassas, VA) were maintained in the ATCC-formulated Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in air containing 5% CO₂.

2.3. Isoflurane exposure in cells

Rat B35 cells were plated at 1.5×10⁶ cells per 100 mm dish or 3.0×10⁵ cells per well in 6-well plate. Twenty-four hours later, complete culture medium was changed into basal DMEM. Cells were cultured under this condition for 3 days for cell differentiation. Next day, isoflurane exposure was performed in an airtight chamber with 2% isoflurane for 1 h in air containing 5% CO₂ at 37°C. Control cells were treated identically but without isoflurane. After this exposure, cells were cultured for 6 h in normal cell culture condition before they were harvested for preparing miRNAs or cultured for 24 h before they were subjected to oxygen-glucose deprivation (OGD).

2.4. MicroRNA isolation, profiling and real-time PCR assay

miRNAs from the rat cerebral cortex or the differentiated B35 cells were isolated using mirVana miRNA isolation kit (Ambion, Carlsbad, CA). They were labeled using FlashTag Biotin HSR RNA Labeling Kit (Genisepere Inc., Hatfield, PA). miRNA profiling assay was conducted using Affymetrix GeneChip miRNA Arrays (Affymetrix, Santa Clara, CA) according to the manufacture's procedure. Briefly, enrichment of low molecular weight RNA was quantitated by NaoDrop Spectrophotometer. After PolyA tailing, RNA was ligated with Biotin-labeled 3DNA. The arrays were hybridized at 48°C for 16 h, washed, stained and scanned. The data was analyzed by the miRNA QC Tool software.

MicroRNA real-time PCR was conducted according to the procedure of TaqMan microRNA rno-miR-203 assay kit (Applied Biosystems, Carlsbad, CA). Quantitative PCR was carried out in triplicates using U6 snRNA as control as we described before [4]. Amplifying PCR and monitoring of the fluorescent emission in real time were performed in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The data collected from these quantitative PCRs defined a threshold cycle number (Ct) of detection for the microRNA or U6 snRNA in each sample.

2.5. Cell transfection

Before cell transfection, 1.5×10^5 cells per well were plated on 6-well plate and deprived of fetal bovine serum for 3 days for differentiation. Cells were transfected with 30 pmol mimic miRNA-203 duplex (top: GUGAAAUGUUUAGGACCACUAG; bottom: CUAGUGGUCCUAAACAUUUCAC) or Stealth RNAi medium GC negative control duplex (miRNA-N, 5'-GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCA GTACATTT-3') (Invitrogen, Carlsbad, CA) by 5 μ l of the transfection reagent lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The sequence of miRNA-N is not similar to those of any known miRNAs. miRNA-N is recommended as a control miRNA or small interference RNA by Invitrogen and is used for this purpose in the literature [4]. Twenty-four hours later, cells were subjected to OGD or harvested for Western analysis.

2.6. OGD and LDH release assay

OGD was applied by replacing the medium with glucose-free medium. This medium was pre-gassed with 100% N₂ for 5 min. The cells were placed in an airtight chamber that was gassed with 100% N₂ for 10 min. After confirmation of hypoxia (< 2% oxygen content) in the chamber gas, the chamber was closed and kept at 37°C for different times. After the OGD, D-glucose was added to the medium to make its final concentration in the medium at 25 mM that was the glucose concentration in the culture medium recommended by ATCC for culturing these cells. The cultures were maintained under their normal culture conditions for 24 h before they were used for lactate dehydrogenase (LDH) release assay.

LDH assay was conducted as we described before [6] using LDH cytotoxicity detection kit. Briefly, the amount of LDH in the culture medium (released LDH) and in the cells (intracellular LDH that was measured after the cells were lysed by 0.5% Triton X-100™ solution) was quantified. The percentage of released LDH in the total LDH (released LDH plus intracellular LDH) was calculated and used to reflect the cell injury.

2.7. Western Blotting

After washed with 1×Dulbecco's phosphate-buffered saline, cells were lysed with the M-PER Mammalian Protein Extraction Reagent (Promega Corp., Madison, WI) containing protease inhibitor cocktail (Sigma, St Louis, MO) and PhosSTOP Phosphatase inhibitor (Roche Applied Science, Indianapolis, IL). Protein concentration was determined by Bradford assay. About 30 μ g proteins per lane were separated by SDS-PAGE and then transferred to nitrocellulose. After being blocked with the Protein-Free T20 Blocking Buffer (Thermo Scientific, Rockford, IL), membranes were incubated with each of the following primary antibodies: anti-phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA), anti-Akt (Cell Signaling Technology, Danvers, MA), anti-actin (Sigma, St Louis, MO) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sigma, St Louis, MO). Appropriate secondary antibodies were used and protein bands were visualized using a Genomic and Proteomic Gel Documentation (Gel Doc) Systems from Syngene (Frederick,

MD). The protein band intensities were normalized by the corresponding band intensities of actin or GAPDH from the same samples.

2.8. Statistical analysis

All data in this study were parametric. They are presented as means $\pm$ S.D. (n = 5) and analyzed by Student's paired t test, Student's t test, Rank Sum test or one way analysis of variance followed by the Tukey test. A $P < 0.05$ was accepted as significant. All statistical analyses were performed with the SigmaStat (Systat Software, Inc., Point Richmond, CA, USA).

3. Results

As shown in Table 1, the expression of two miRNAs (miR-350 and miR-674-3p) in rat cerebral cortex as assessed by miRNA array was significantly increased after isoflurane exposure. The amount of miRNA-203 in the cerebral cortex of rats exposed to isoflurane was 1.47-fold of that in the cortex of control rats. However, this increase did not reach statistic significance (Table 1). The miRNA array assay showed that the expression of miRNA-203 was significantly increased and miRNA-331 was significantly decreased in the B35 cells after isoflurane exposure (Table 2). Among the 4 miRNAs whose expression was statistically significantly changed by isoflurane in either rat cerebral cortex or B35 cells, there was only one miRNA (miRNA-203) whose expression had a change that was in the same direction and relatively large magnitude. Thus, we decided to determine the possible role of this miRNA in isoflurane preconditioning-induced neuroprotection.

Similar to the array results, the expression of miRNA-203 in the B35 cells as assessed by real-time PCR was significantly increased after isoflurane exposure (Fig. 1A). OGD, an *in vitro* simulation of ischemia, significantly increased the LDH release from B35 cells, suggesting that OGD causes cell injury. Isoflurane preconditioning significantly reduced this OGD-induced LDH release (Fig. 1B), indicating that isoflurane preconditioning induces protection in these cells. Cells over-expressing miRNA-203 had an increased basal LDH release (Fig. 1C), although the total LDH (the sum of intracellular and extracellular LDH) at this time-point was not different among the cultures transfected with miRNA-N or miRNA-203 (the total LDH levels in cells transfected with miRNA-203 was 1.1 ± 0.2 fold of the cells transfected with miRNA-N, n = 9, $P > 0.05$). OGD up to 30 min in length did not cause an increased LDH release in cells over-expressing miRNA-203. On the other hand, OGD that was longer than 20 min significant increased the LDH release in cells expressing the control miRNA miRNA-N (Fig. 1D).

Cells over-expressing miRNA-203 also had an increased expression of phospho-Akt. However, the total Akt level was not significantly altered by miRNA-203 (Fig. 2).

4. Discussion

Isoflurane application during brain ischemia can reduce ischemic brain injury [12]. Exposure to isoflurane prior to brain ischemia also can induce tolerance to ischemia in the brain (isoflurane preconditioning-induced neuroprotection) [5,9,19]. Our results suggest that miRNA-203 is involved in isoflurane preconditioning-induced neuroprotection because isoflurane trended to increase miRNA-203 in rat cerebral cortex and significantly increased miRNA-203 expression in the neuron-like cells. Over-expression of miRNA-203 increased the tolerance of these neuron-like cells to OGD. This is the first demonstration of the involvement of miRNA in anesthetic effects on the brain. Our results also showed that over-expression of miRNA-203 increased the phospho-Akt. Activation/phosphorylation of Akt has been shown to promote cell survival and reduce ischemic brain injury [7,10]. In

addition, it has been indicated that Akt activation is involved in isoflurane-induced neuroprotection [3,10]. Thus, the increased activation/phosphorylation of Akt caused by miRNA-203 over-expression may be a mechanism for miRNA-203 to increase the tolerance of cells to OGD.

The mechanisms for miRNA-203 to increase the phosphorylation of Akt are not clear. Akt has been considered as a target gene for miRNA-203 [8,16]. Indeed, miRNA-203 over-expression decreases Akt expression in human colon cancer cells that have a mutated p53 gene. However, miRNA-203 may cause a small increase of Akt in human cancer cells that express normal p53 [8]. In our study, the total Akt expression was not changed by miRNA-203, suggesting that the increased phospho-Akt was not due to an increase in the total Akt. Thus, the increased phospho-Akt may be due to the changed expression of protein kinases or phosphatases that can phosphorylate or dephosphorylate Akt.

miRNA-203 has been shown to regulate cell differentiation and proliferation [2,18]. Recent studies have shown that miRNA-203 acts as a tumor suppressor. It can reduce cell proliferation and cause cell death [2,13,18]. Consistent with this finding, our study showed that cells over-expressing miRNA-203 had a higher LDH release under basal condition. However, these cells also had an increased tolerance to OGD. Thus, our results suggest a novel notion: cells that are not killed/injured after over-expression of miRNA-203 develop tolerance to insults, such as OGD/ischemia.

The miRNA array results from rat cerebral cortex are not fully consistent with the results from rat B35 cells. This finding is understandable because cerebral cortex contains multiple types of cells and the differentiated B35 cell cultures are relatively homogenous neuron-like cells. Anesthetic effects on miRNA expression in various types of cells may be different. Different types of neurons may respond to anesthetics differently. Also, although the differentiated B35 cells have morphological features of neurons, express neuron-specific proteins and are used as a cell model of neurons in the literature [14,17], these cells should have many features that are different from those of matured neurons *in vivo*. Nevertheless, the finding that isoflurane seems to increase the expression of miRNA-203 in the cerebral cortex under *in vivo* condition and in the B35 cells under *in vitro* condition indicate that miRNA-203 may be a target for isoflurane in the brain.

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Research highlights

- The volatile anesthetic isoflurane can alter microRNA expression in the brain cells
- The increased microRNA-203 may be involved in isoflurane preconditioning-induced neuroprotection
- Cells that tolerate a high level of microRNA-203 may develop resistance to ischemia

Fig 1. Role of miRNA-203 in isoflurane preconditioning-induced neuroprotection

A: Differentiated B35 cells were exposed to or were not exposed to 2% isoflurane for 1 h. Cells were harvested 6 h later for quantification of miRNA-203 by real-time PCR. Results are mean $\pm$ S.D. (n = 11). * P < 0.05 compared with control. **B:** Differentiated B35 cells were exposed to or were not exposed to 2% isoflurane for 1 h. They were then subjected to a 90 min oxygen-glucose deprivation (OGD) 24 h later. Lactate dehydrogenase (LDH) release assay was performed 24 h after the OGD. Results are mean $\pm$ S.D. (n = 6). * P < 0.05 compared with control. ^ P < 0.05 compared OGD only. **C:** Differentiated B35 cells were transfected with miRNA-203 duplex or Stealth RNAi negative control duplex (miRNA-N). LDH release was performed 24 h after the transfection. Results are mean $\pm$ S.D. (n = 9). * P < 0.05 compared with miRNA-N. **D:** Differentiated B35 cells were transfected with miRNA-203 duplex or the negative control duplex. They were subjected to various lengths of OGD. LDH release was performed 24 h after the OGD. Results are mean $\pm$ S.D. (n = 9). * P < 0.05 compared with the corresponding values of cells without OGD. # P < 0.05 compared the corresponding values of cells transfected with miRNA-203 duplex.

Fig 2. Effect of miRNA-203 on phospho-Akt (pAkt) and Akt expression

Differentiated B35 cells were transfected with miRNA-203 duplex or Stealth RNAi negative control duplex (miRNA-N). Cells were harvested at 24 h after the transfection for Western blotting. **A:** pAkt results. A representative image is presented on the top panel and the pooled results of pAkt after being normalized by the corresponding value of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) are presented in the bar graph. **B:** total Akt results. A representative image is presented on the top panel and the pooled results of pAkt after being normalized by the corresponding value of GAPDH are presented in the bar graph. Results are mean $\pm$ S.D. (n = 5). * P < 0.05 compared with miRNA-N.

Table 1

Expression of miRNAs in rat cerebral cortex

miRNA name	Fold change	P value
miR-350	1.58	0.0434
miR-674-3p	1.44	0.0336
miR-203	1.47	0.2478
miR-331	1.09	0.6017

The miRNAs whose expression was significantly changed after isoflurane exposure in either the cerebral cortex or rat B35 cells are listed here.

Table 2

Expression of miRNAs in rat B35 cells

miRNA name	Fold change	P value
miR-203	1.27	0.0499
miR-331	-1.32	0.0075
miR-350	1.00	0.9954
mir-674-3p	1.03	0.8700

The miRNAs whose expression was significantly changed after isoflurane exposure in either the cerebral cortex or rat B35 cells are listed here.