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Detection and analysis of apoptosis- and autophagy-related miRNAs of mouse vascular endothelial cells in chronic intermittent hypoxia model

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ABSTRACT

Endothelial dysfunction is the main pathogenic mechanism of cardiovascular complications induced by obstructive sleep apnea/hyponea syndrome (OSAHS). Chronic intermittent hypoxia (CIH) is the primary factor of OSAHS-associated endothelial dysfunction. The hypoxia inducible factor (HIF) pathway regulates the expression of downstream target genes and mediates cell apoptosis caused by CIH-induced endothelial injury. miRNAs play extensive and important negative regulatory roles in this process at the post-transcriptional level. However, the regulatory mechanism of miRNAs in CIH tissue models remains unclear. The present study established a mouse aortic endothelial cell model of CIH in an attempt to screen out specific miRNAs by using miRNA chip analysis. It was found that 14 miRNAs were differentially expressed. Of them, 6 were significantly different and verified by quantitative real-time PCR (Q-PCR), of which four were up-regulated and two were down-regulated markedly. To gain an unbiased global perspective on subsequent regulation by altered miRNAs, we established signaling networks by GO to predict the target genes of the 6 miRNAs. It was found that the 6 identified miRNAs were apoptosis- or autophagy-related target genes. Down-regulation of miR-193 inhibits CIH induced endothelial injury and apoptosis- or autophagy-related protein expression. In conclusion, our results showed that CIH could induce differential expression of miRNAs, and alteration in the miRNA expression pattern was associated with the expression of apoptosis- or autophagy-related genes.

1. Introduction

Obstructive sleep apnea/hyponea syndrome (OSAHS) is a sleep-related hypoxia-reoxygenation (H/R) syndrome, clinically characterized by complete or incomplete obstruction of the upper airway, resulting in snoring accompanied with apnea and/or hypoventilation [1]. Chronic intermittent hypoxia (CIH) is an important pathophysiologic mechanism of sleep apnea, and the underlying basis for cardiovascular and other organ injuries in OSAHS patients [2]. It is currently recognized that endothelial cell injury plays a crucial role in OSAHS-related cardiovascular diseases. Research on vascular endothelial cell injury using CIH animal models is vulnerable to be affected by multiple factors in the body, while in vitro culture of endothelial cells is relatively simple and less likely to be affected by exogenous factors and therefore can be used to establish a CIH model of endothelial cell injury [3]. However, the mechanism underlying CIH-induced endothelial cell injury has not been well known. Related studies have demonstrated that miRNAs participate in the modulation of endothelial and vascular functions by regulating the expression of genes closely related to the endothelial cell function [4,5]. miRNAs are a group of newly discovered endogenous non-coding single-strand RNAs with about 22 nucleotides in length, and regulate gene expression at the transcriptional or post-transcriptional level via complementary base pairing [6,7]. In recent years, many associations between disease mechanisms and specific miRNAs have been identified by using large-scale microarray profiling and genetic approaches [8]. Some studies have also provided an overview about the role of miRNAs in the development of CIH-induced cardiomyocyte injury [9]. However, the association between miRNAs and CIH-induced endothelial cell injury remains elusive.

In the present study, we established a C57 mouse model of primarily cultured endothelial cell injury under the condition of CIH to detect changes in the expression of miRNAs in endothelial cells by using the miRNA microarray technique, in an attempt to screen out significantly expressed miRNAs by quantitative real-time PCR (qRT-PCR). The result

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of Gene Oncology (GO) analysis showed that miRNAs played an important role in CIH by regulating the expression of apoptosis- and autophagy-related genes.

2. Materials and methods

2.1. Reagents

Materials used in this study were Dulbecco's modified Eagle's medium (DMEM), dimethylsulfoxide (DMSO), newborn calf serum (NCS), streptomycin, penicillin, trypsin-EDTA (GIBCO), CCK-8 cell counting kit and Annexin V-FITC apoptosis detection kit (Sigma). Antibodies used in this study were anti-capase3 polyclonal antibody (CST), anti-caspase9 polyclonal antibody (Santa Cruz), anti-bcl-2 polyclonal antibody (Santa Cruz), anti-PARP polyclonal antibody (Santa Cruz), anti-LC3 polyclonal antibody (Santa Cruz), and anti-beta-Actin antibody (CST).

2.2. Instruments

The instruments used in this study included a constant temperature and humidity incubator (Thermo), a biological phase contrast microscope (Olympus), a high-speed refrigerated centrifuge (Eppendorf), a cryogenic refrigerator (Samsung), a microplate reader (Thermo), and a flow cytometer (Thermo).

2.3. Methods

2.3.1. Culture and grouping of mouse aortic endothelial cells

Male C57 mice weighing 20 g were sacrificed with 30 mg/kg pentobarbital sodium. The aorta pectoralis was removed from each mouse immediately and rinsed with PBS to remove blood to improve the visibility of the tissue. Then the aortic endothelial tissue was cut into pieces (< 0.5 cm), washed with 0.01 mol/L PBS containing 100 μg/mL streptomycin and 100 IU/mL penicillin, digested by addition of 2.5 g/L pepsin and 2 g/L type II collagenase, seeded to a 6-well plate at 1×10^8 cells/L, and incubated in a 95% O_2, 5% CO_2, 37 °C and saturated humidity incubator in DMEM containing 20% FBS, 100 μg/mL streptomycin and 100 IU/mL penicillin. The medium was replaced every 2–3 days. Cells growing to 80% confluence were passaged at a ratio of 1:3, and 4–6 passage cells were used for subsequent experiments. For phenotypic analysis, fluorescein isothiocyanate (FITC-F) or phycoerythrin (PE) was used. The expression of the following markers was investigated: CD105, CD90, CD34, KDR, CD133 and CD14 (Sigma, St. Louis, MO). Isotype-identical antibodies served as controls (PharMingen). For analysis of CD105, CD90, CD34, KDR, CD133 and CD14, the cells were further incubated with a biotinylated anti-rat IgG (H1L) antibody made in horse (Vector Laboratories) and with FITC-conjugated streptavidin (Caltag, South San Francisco, CA). After treatment, the cells were fixed in 1% paraformaldehyde. Quantitative analyses were performed using a FACSCalibur flow cytometer and FlowJo software (Flowjo, Ashland, OR, USA). Vascular endothelial cells were divided into two groups: normal control and CIH.

In order to further identified the characteristic of the isolated aortic endothelial cells. In vitro neovascularization assays were performed in human fibrin matrices. In brief, aortic endothelial cells were seeded onto matrigel (BD Bioscience)-coated plate in EBM medium and incubated at 37 °C for 12 h, tubular structures of EPCs in the matrigel were analyzed by phase-contrast microscopy.

2.3.2. In vitro establishment of the mouse aortic endothelial cell model of CIH

Routinely cultured mouse aortic endothelial cells were treated in serum-free medium for 12 h till synchronization, and then cultured in serum-free medium to prepare intermittent and persistent hypoxic injury models. Serum-free culture under normal oxygen conditions was used as the control. In the CIH group, cells were maintained at 37 °C at 5% CO_2 in a chamber (Oxycycler model A42, Biospherix) in which O_2 levels were alternated between 21% for 5 min and 1% for 10 min, for a total of 64 cycles (18 h). Cells in the control group were maintained under normoxic condition. After hypoxic treatment, the medium was washed off, and the mouse aortic endothelial cells were returned to the maintenance medium (NCS-DMEM) in the normal incubator for 60-min recovery.

2.3.3. Cytoactive analysis

Cells from control or CIH groups were suspended, counted and adjusted to a concentration of 5–10 × 10^4/ml. Each well was added with 100 μl well mixed cell suspension at a density of 5000–10,000 cells/well. The marginal wells were filled with sterile PBS. The seeded plate was incubated in the 5% CO_2 and 37 °C incubator for 16–48 h until single-layer cells spread all over the bottom of the 96-well plate, and then observed under the inverted microscope for change in cell morphology. After addition of 10 μl MTT solution (5 mg/ml, 0.5% MTT) to each well, cells were cultured for additional 4 h, and after removing the supernatant, washed with PBS for 2–3 times. Optical density (OD) was read at 490 nm on the microplate reader.

2.3.4. Western blot analysis

The expression of apoptosis-related proteins was determined by Western blot analysis as described by Zhu et al. (Zhu et al., 2011). Briefly, the extracted protein was mixed with the buffer solution, boiled, electrophoresed by 10% SDS-PAGE, and transferred to the membrane using the semi-dry transfer method. The primary antibodies, including caspase 3 (1: 500), caspase 9 (1: 400), LC3 (1: 200), and GAPDH (1:300), were diluted. The membrane was then incubated at 4 °C overnight with different primary antibodies. Mouse anti rabbit horseradish peroxidase (HRP) conjugated secondary antibodies (Sigma, US) were diluted at concentrations of 1:8000, 1:4000, 1:4000, 1:3000. The membrane was then incubated with the secondary antibodies at room temperature for 1 h, laminated, exposed and analyzed. Images were collected on the Alpha Imager HP Fluorescent and Visible Light Gel Imaging System and analyzed using IPP 6.2 image analysis software.

2.3.5. Detection of vascular endothelial cell apoptosis by flow cytometry

The Falcon tube was numbered according to the serial number of the specimen. Cells were washed twice with cool PBS, and prepared with 1 × Binding Buffer to a 1 × 10^6 cells/ml suspension. The Falcon tube was added with 100 μl cell suspension, Annexin V and nuclear acid dye, mixed gently, placed at room temperature (20–25 °C) away from light for 15 min, and then tested with Annexin V-Biotin reagent. After washing cells with 1 × Binding Buffer once and removing the supernatant, 0.5 μg v-FITC reagent dissolved in 100 μl 1 × Binding Buffer was added to the test tube, mixed gently, added with 5 μl PI, and placed at room temperature (20–25 °C) away from light for 15 min. After adding 400 μl 1 × Binding Buffer to each tube, flow cytometry was performed within 1 h.

2.3.6. Detection of miRNA chip

Mouse aortic endothelial cells were lyzed by addition of 1 ml RNA extraction reagent TRIzol (Invitrogen) to extract RNA. RNA OD was measured at 260 nm/280 nm using the NanoDrop™ fluorospectrometer. miRNAs were isolated from 50–100 μg total RNA by using the miRNA isolation kit (Invitrogen). Denaturing agarose gel electrophoresis was performed using 1% formaldehyde electrophoresis reagent. miRNAs were labeled with Hy3 or Hy5 fluorescence using the miRCURY™ Array Power Labeling Kit to obtain the fluorescent probe that can be hybridized with the chip. The labeled probe was hybridized with the miRCURY™ chip under the standard condition using the MAUI hybridization system. The fluorescence intensity of the chip was scanned with the Agilent chip scanner and analyzed using Agilent feature