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Original article

Pioglitazone up-regulates long non-coding RNA MEG3 to protect endothelial progenitor cells via increasing HDAC7 expression in metabolic syndrome

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ABSTRACT

Long non-coding RNA (lncRNA) maternally expressed 3 (MEG3) is expressed in endothelial cells and involved in angiogenesis and vascular function. It was proposed that MEG3 participates in the process of endothelial progenitor cells (EPCs) functions in metabolic syndrome (MetS). In this study, the circulating EPCs number and function were decreased in MetS subjects. The MEG3 expression was expressed at a lower level and microRNA-140-5p (miR-140-5p) was expressed at a higher level in circulating EPCs of subjects with MetS. Pioglitazone reversed the alterations of EPCs function and the expression levels of MEG3 and miR-140-5p in EPCs. In bone marrow-derived EPCs exposed to palmitate, down-regulation of miR-140-5p canceled the increase of MEG3 expression level induced by Pioglitazone. Overexpression of MEG3 resulted in the down-regulation of miR-140-5p. The luciferase reporter assay and RIP assay showed that MEG3 targeted miR-140-5p. In addition, the HDAC7 expression levels were regulated by miR-140-5p and MEG3. These findings demonstrated that Pioglitazone up-regulated MEG3 expression to protect EPCs via decreasing miR-140-5p expression and increasing HDAC7 expression in MetS, which may be a novel therapeutic target for preventing and treating MetS.

1. Introduction

The metabolic syndrome (MetS) comprises a cluster of metabolic disorders, with insulin resistance (IR) and adiposity as central features [1]. The incidence of MetS is rapidly increasing with the alteration of diets. Approximately 35% of United States adults have the MetS and this appears to be a very common syndrome globally. MetS have has a 2 folds increase in cardiovascular outcomes and a 1.5 fold increase in all-cause mortality [2]. Therefore, the prevention and treatment are significant for reducing the incidence of cardiovascular diseases (CVD).

Endothelial progenitor cells (EPCs), a sub-type of progenitor cells, can be isolated from bone marrow, umbilical vessels, and peripheral blood of adults. EPCs have the capacity to circulate, proliferate, and differentiate into mature endothelial cells [3]. In recent years, the emerging experiments demonstrated that the MetS subjects without diabetes or CVD have decreased EPC number and impaired functionality as compared with control subjects, which indicates that EPCs defect is an independent risk factor for MetS and involved in the promoting the development of CVD [4].

Pioglitazone is a prescription drug of the class thiazolidinedione (TZD) with hypoglycemic action to be used for the treatment of type 2 diabetes mellitus (T2DM) [5]. Treatment with Pioglitazone as monotherapy or combination therapy led to sustained, positive effects on important components of metabolic syndrome in patients with type 2 diabetes, independent of effects on blood glucose control [6]. It has been shown that Pioglitazone increases the numbers and improves the functional capacity of EPCs [7,8] and PI3K/Akt signal pathway may be mediated in this process [9]. However, the molecular mechanisms of Pioglitazone on EPCs regulation is little known.

Long non-coding RNA (lncRNA) with more than 200 nucleotides are non-protein coding transcripts, regulates gene expression at the level of chromatin modification, transcription and post-transcriptional processing [10]. Another primary class of ncRNA

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is microRNA (miRNA), assembles into RNA-induced silencing complex (RISC) and activates the complex to target genes to regulate the gene expression [11]. The recent studies have demonstrated that lncRNA may regulate miRNAs expression, subsequently modulating the target genes of miRNAs in post-transcriptional level [12]. Maternally expressed 3 (MEG3) is expressed in endothelial cells of arterial [13], and MEG3 knock-out increases the expression of VEGF signaling pathway genes in the brain [14]. The cross-sectional studies discloses that circulating miR-140-5p is increased in T2DM patients and in morbidly obese patients [15,16]. Histone deacetylase 7 (HDAC7) has been reported to influence endothelial cell migration and modulate angiogenesis. Accordingly, we proposed that MEG3 participates in the protective role of EPCs via regulation of miRNA and its target gene expression in metabolic syndrome with Pioglitazone treatment.

2. Materials and methods

2.1. Study participants

All subjects were recruited from Henan Provincial People's Hospital and completed written informed consent. This study was approved by the Ethics Committee of Henan Provincial People's Hospital. Sixty subjects with MetS and 30 healthy controls were investigated. 30 of MetS subjects were treated with Pioglitazone (30 mg qd for 4 weeks) and the remaining 30 MetS subjects without any treatment. MetS was defined using the criteria of the National Cholesterol Education Program Adult Treatment Panel-III10. Control subjects needed to have $\leq$2 features of MetS and not be on blood pressure (BP) medications. None of the subjects had diabetes, cardiovascular disease or were on medications known to affect EPC biology. For all subjects, the other exclusion criteria were described in previous study [17]. After physical examination, the fasting venous blood was collected and serum was obtained. Serum lipids, glucose, liver function indexes and renal function indexes were measured by automated biochemical analyzer in Clinical Laboratory.

2.2. Detection of circulating EPC number

1 ml of fresh heparinized whole blood was used to assess the circulating EPCs number by flow cytometry. In brief, 100 μL peripheral blood was incubated with monoclonal antibodies against human KDR followed by Allophycocyanin(APC)-conjugated secondary antibody, with the FITC-conjugated monoclonal antibodies against human CD34, and/or with PE-conjugated monoclonal antibody against human CD133. After incubation, samples were lysed and then washed with PBS. 2% paraformaldehyde was used to fix cells before analysis. Each analysis included 100,000 events. EPCs were reported as a percentage of total events.

2.3. EPC isolation

The Peripheral blood specimens were obtained from subjects with MetS or without MetS. The total mononuclear cells (MNCs) were isolated by density gradient centrifugation with Histopaque-1077 (Sigma, USA), and incubated with endothelial growth medium at 6-well plates for 4 h. Then the medium was replaced and non-adherent cells were removed. Collected the early and late EPCs and defined with flow cytometry. The early and late EPCs were characterized as previous described [18].

Bone marrow derived EPCs were isolated from mice. The C57BL/6J mice aged eight weeks were sacrificed. Femurs, tibias, and ilia were surgically dissected, and the adhering tissues were completely removed. The bone marrow cells were harvested and resuspended into EGM-2 medium with a 25-gauge needle, and then seeded on 1% gelatine coated petri dishes at 37 °C for 3 days. After removing non-adherent cells the fresh EGM-2 medium was supplemented in adherent cells. Repeated this procedure every 2 days until day 14 and then the Bone marrow derived EPCs were identified by morphology.

2.4. EPCs adhesion

Harvested the EPCs on day 7 and washed with PBS. 0.5 mmol/L EDTA in PBS was used to detach ECPs. After centrifugation and re-suspension in medium containing 5% FBS, the EPCs were plated in a fibronectin-coated 6-well plate and cultured for 30 min and then washed with PBS for three times. The adhesion cells were counted and the relative adhesion was calculated.

2.5. EPCs migration

Before the migration assay, EPCs were placed in M199 medium with 1% fetal bovine serum (FBS) on day 13 for 16 h to induce serum starvation. A modified Boyden chamber technique was used to evaluate EPC migratory function in MetS subjects with or without treatment. In brief, EPCs were trypsinized and resuspended in M199 medium with 1% FBS to the concentration of 2.0×10^4 cells/well. 100 μL EPCs suspension was plated to the transwell gelatin-coated insert (Coring, USA) with 8.0-μm proes. The lower chamber was added with 500 μL M199 medium with 1% FBS containing 100 ng/ml human VEGF and cultured for 20 h. Fixed and stained the migrated cells on the lower surface of the membrane with haematoxylin and eoxsin. The migrated cells were calculated with the average of numbers in 5 representative fields per well.

2.6. EPCs tube formation

The tube formation of EPCs was assessed by the In Vitro Angiogenesis Assay Kit (Chemicon, USA). After unfreezing overnight at 4 °C, the ECMatrix gel solution was mixed with ECMatrix diluent buffer and placed in a 96-well plated for 1 h at 37 °C. The harvested late EPCs (1×10^4 cells) were placed onto a matrix with EGM-2 MV medium and incubated at 37 °C for 16 h. To observe the tube formation using inverted light microscope ($100\times$) in five representative fields. The relative tube formation of EPCs was calculated.

2.7. Real-time PCR

Trizol reagent (TaKaRa, China) was utilized to isolate total RNA from circulating EPCs of subjects or bone marrow-derived EPCs. The RNA concentration and quality were evaluated using spectrophotometer and denatured gel electrophoresis, respectively. 1 μg of total RNA was used to reverse transcribe to cDNA by ImProm-II™ Reverse Transcription System (Promega, USA). The expression levels of MEG3 and miR-140-5p were quantified with Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) using Applied Biosystems 7900 Fast Real-Time PCR System (Applied Biosystems, USA). MEG3 and miR-140-5p expression were normalized with GAPDH and U6, respectively. HDAC7 mRNA expression was normalized with β-Actin. The specific primers were as follows: MEG3 forward primer, 5′-GAGTGTTTCCCTCCC-CAAGG-3′, reverse primer, 5′-GCGTGCCTTTGGTGATTCAG-3′; GAPDH forward primer, 5′-GGGAGCCCAAAAGGGTCAT-3′, reverse primer, 5′-GAGTCCTTCCACGATACCAA-3′; miR-140-5p forward