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Expression of miRNA-155 in carotid atherosclerotic plaques of apolipoprotein E knockout ($ApoE^{-/-}$) mice and the interventional effect of rapamycin

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ABSTRACT

Carotid atherosclerosis (AS) is an inflammatory process and is the primary pathogenesis of cerebrovascular disease. Many factors are responsible for development of atherosclerosis such as inflammation and autophagy. It is reported that microRNAs (miRNAs) could regulate the development of atherosclerosis through targeting autophagy-related genes. Many studies have demonstrated that miRNA-155 could regulate autophagy in macrophages or tumor cells. However, the role of miRNA-155 on autophagy in carotid plaques is not yet known. In this study, we explore the expression of miRNA-155 and autophagy-related proteins in carotid plaques of $ApoE^{-/-}$ mice and the interventional effect of rapamycin. We compared the expression of miRNA-155 and autophagy-related proteins between the control, model and rapamycin groups using qRT-PCR and western blot. Compared to the control group, we found the miRNA-155 and LC3-II expression was up-regulated ($P < 0.05$), expression ratio of phosphorylated mammalian target of rapamycin to total mammalian target of rapamycin (p-mTOR/mTOR) was down-regulated in model group ($P < 0.05$), but atherosclerotic lesions were still aggravated. These results following rapamycin group indicated that miRNA-155 and LC3-II expression was significantly up-regulated ($P < 0.05$), the expression ratio of p-mTOR/mTOR was significantly down-regulated ($P < 0.05$), and atherosclerotic lesions were reduced. Our results showed in the early stages of atherosclerotic plaques development, effective autophagy could attenuate atherosclerosis in $ApoE^{-/-}$ mice. Furthermore, our results also demonstrated that rapamycin might promote the activation of the autophagy by enhancing the expression of miRNA-155, which delays the development of atherosclerotic plaques.

1. Introduction

Carotid atherosclerosis (AS) is a chronic inflammatory disease characterized by the formation of atherosclerotic plaques, which primarily occurs in medium-to-large-sized arteries [1]. The unstable atherosclerotic plaques were believed to be the main pathogenesis of cerebrovascular disease [2]. Recently, growing evidences notes that autophagy is up-regulated in response to stimulation of oxidized low density-lipoprotein (ox-LDL), inflammation and metabolic stress conditions in atherosclerotic plaques [3].

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase, which is the key to regulate autophagy via the PI3K/Akt/mTOR pathway [4]. mTOR is suppressed in response to mTOR inhibitors or inadequate nutrients, significantly up-regulating level of autophagy [5]. Rapamycin is an mTOR inhibitor, so it has been selected for inducing autophagy and modulating atherosclerotic plaques stability [6].

MicroRNAs (miRNAs) are small non-coding RNAs that bind to 3′ untranslated regions (UTRs) of mRNA, regulating gene expression and repressing protein translation in many pathological and physiological conditions [7,8]. Existing studies showed that overexpression of miRNA-155 increased autophage activity possibly by down-regulating mTOR signaling in the process of cell metabolism [9]. Our study aims to investigate the expression of miRNA-155 and autophagy-related proteins in carotid plaques of $ApoE^{-/-}$ mice and to explore the interventional effect of rapamycin.

2. Materials and methods

2.1. Reagents

Oral rapamycin (purity 98%) was purchased from Shanghai Zhong Kang Weiye Biotechnology Company, quantitative Real-Time PCR (qRT-PCR) Reverse Transcription kit was obtained from TaKaRa (No.

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420a); mRNA primers were designed and purchased along with TRIzol from Sangon Biological Engineering (Shanghai) Company. RIPA cracking liquid was bought from Dalian Takara Company, antibodies for mTOR (7C10), LC3B (D11), p-mTOR (D9C2) were from Cell Signaling Technology (Danvers, MA, USA) CST.

2.2. Instruments

Low speed centrifuge (heal force), reverse transcription apparatus (Day Biochemical Science and Technology Beijing Company), qRT-PCR (FTC-3000, Funglyn Biotech), protein electrophoresis apparatus (Bio-Rad Company, USA), TS-92 universal table was from sea outlets Kirin medical instrument factory, and ultra-low temperature freezer (MDF-38) were purchased from SANYO.

2.3. Animal models

All experiment procedures followed ARRIVE guidelines [10]. Thirty-six 6-week-old male ApoE$^{-/-}$ mice (in C57BL/6J background) and a western-type diet (0.25% cholesterol and 15% fat) were obtained from Beijing Hua Fu Kang Biotechnology Company. All the mice were raised under standard conditions of room temperature, dark-light cycles in the center of Qingdao University experiment.

Diet and water were provided optionally for 2 weeks before surgery. All mice aged 8 weeks or more were randomly divided into 3 groups ($n = 12$ mice/group) for treatment: control group (the carotid wound was closed in one layer with interrupted silk sutures, 0.5 mg/kg/d; PBS), model group (silastic collars were placed bilaterally around the common carotid arteries, 0.5 mg/kg/d; PBS) [11] and the rapamycin group (silastic collars were placed bilaterally around the common carotid arteries, 0.5 mg/kg/d; rapamycin) [12]. All the mice were fed with a Western-type diet (0.25% cholesterol and 15% fat) and free access to water for 8 weeks. After treatment, all the mice were sacrificed by exsanguination [11].

2.4. Detection of serum plasma lipid levels

Eight weeks after treatment, all the animals were sacrificed by femoral artery exsanguination. Blood samples were centrifuged to collect serum and measure the levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-c) by routine biochemical methods.

2.5. Histological observation and transmission electron microscopy (TEM)

After animals were sacrificed, the carotid arteries were immediately extracted and respectively fixed with 4% paraformaldehyde for 12 h and embedded in paraffin. The carotid arteries were continuously cut into serial sections at the distal and proximal to carotid collar placement and randomly selected five pictures to observe external elastic membrane and plaque areas. Fresh frozen carotid artery samples were fixed with 3% glutaraldehyde, after a series of dehydration in a gradient ethanol and embedding was performed in Araldite. The samples were placed on copper wire for observation on TEM.

2.6. QRT-PCR

Total RNA (include miRNAs) from the right common carotid arteries (weighing 20 mg/group) were isolated using TRIzol reagent, total RNA was reverse-transcribed into cDNA, and cDNAs samples were taken for qRT-PCR. The PCR primers for miRNA-155 were TGC GGT TAA TGC TAA TCG TGA TAG G, the PCR primers for mTOR were 5′-GCC CTC ACC TCA CAA GAG AT-3′ (forward) and 5′-GCT CTC TCA CCC AGC AGA AC-3′ (reverse); the PCR primers for LC3-II were 5′-CCT TCT TCC TGC TGG TGA AC-3′ (forward) and 5′-TTT CCT GGG AGG CGT AGA C-3′ (reverse), those for GAPDH (U6) were 5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′ (forward) and 5′-AAA TGA GCC CCA GCC TTC ATG-3′ (reverse). The PCR reaction conditions as follows: the miRNA-155: degeneration (95 °C for 10 min), denature (95 °C for 15 s), annealing and extension (60 °C for 1 min) followed by 40–45 cycles; LC3-II, mTOR: degeneration (95 °C for 30 s), denature (95 °C for 5 s), annealing (60 °C for 20 s), extension (72 °C for 30 s) followed by 40 cycles. Three independent experiments were performed at the same experimental conditions. All the values that we acquired were calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$) and treated for statistical analysis with GAPDH as internal control.

2.7. Western blot analysis

The carotid arteries were frozen in liquid nitrogen and treated with RIPA lysis buffer. Total protein extracted was quantified using the BSA protein assay kit. Protein samples (20 μg/group) was separated on a 10% SDS-PAGE and electrophoretically transferred to a polyvinylidenedifluoride (PVDF) membrane (Millipore). Subsequently, following procedures: the membranes were subject to shaking and blocking with 5% non-fat dry milk in PBS or 4% BSA for 2 h at room temperature, followed by incubation in primary antibody and blocked at 4 °C overnight, and then anti-rabbit or anti-mouse horseradish peroxidase (HRP)-labeled secondary antibodies for 2 h at room temperature. We detected the bands, using enhanced chemiluminescence (ECL) detection kits. The relative quantity of protein was measured by ImageJ software.

2.8. Statistical analysis

All results were presented as the mean ± standard error of the mean (SEM), and at least three sets of independents experiments were performed. Differences between groups were analyzed by one-way ANOVA, followed by Bonferoni's multiple comparison test to determine difference between groups. Statistical analysis was performed by Statistical Package for the Social Sciences (SPSS) v19 (SPSS, Chicago, IL, USA), $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Detection of plasma lipid levels in ApoE$^{-/-}$ mice

The levels of TC, TG and LDL-c are increased in model group compared to the control group($P < 0.05$);while the level of TC, TG and LDL-c in rapamycin group are decreased compared to the model group($P < 0.05$) (Table 1).

3.2. Histological observation of unstable carotid atherosclerotic plaques

From the histological observation of carotid artery, we found that the intima of the control group arrowed integrally and regularly, with no obvious lipid laden foam cells deposited. While in the model group, because of the accumulation of damaged factors in development of AS, the intima of the model group were obviously proliferated and disarranged, and there appeared a large number of lipid laden foam cells in the intima. In the rapamycin group, when mice were fed with rapamycin at the early stage of AS, the intima was thinner than the model group, and it

Table 1
Plasma lipid levels of TC, TG and LDL-c in ApoE$^{-/-}$ mice (mmol/L, $\bar{x} \pm s$).

	TC (mmol/L)	TG (mmol/L)	LDL-c (mmol/L)
Control group ($n = 12$)	10.05 ± 4.41	1.51 ± 0.45	1.22 ± 0.90
Model group ($n = 12$)	26.64 ± 0.58*	3.78 ± 1.24*	16.96 ± 4.12*
Rapamycin group ($n = 12$)	18.43 ± 1.41#	2.77 ± 0.97#	11.45 ± 3.39#

* Compared to the control group, $P < 0.05$.
\# Compared to the model group, $P < 0.05$.