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LncRNA-CARl in a rat model of myocardial infarction

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Abstract. – OBJECTIVE: To investigate how long non-coding ribonucleic acid-cardiac apoptosis-related (lncRNA-CARL) regulates apoptosis of primary endothelial cells. The specific role of lncRNA-CARL in the occurrence and development of myocardial infarction (MI) and atherosclerosis is also explored.

MATERIALS AND METHODS: A rat model of arteriosclerosis was prepared by extracting myocardial endothelial cells of rats. After the overexpression or inhibition of lncRNA-CARL, cell counting kit-8 (CCK-8) assay was used to detect cell activity. LncRNA-CARL plasmids were constructed and injected into the carotid artery in rats, and hematoxylin and eosin staining (HE) was used to observe the neointima of the carotid artery in rats. The activity of apoptosis protein Caspase-3 in endothelial cells was detected by Caspase-3 activity assay kit. Expressions of prohibitin-2 (PHB2), B-cell lymphoma-2 (Bcl-2) and Bcl-2-Associated X (Bax) protein were gauged by Western blot.

RESULTS: The over-expression of lncRNA-CARL in primary endothelial cells in rats could increase cell viability. LncRNA-CARL also down-regulated the expressions of PHB2 and Bax, reduced the activity of Caspase-3 and increased the expression of anti-apoptotic protein Bcl-2. LncRNA-CARL inhibition could significantly increase Caspase-3 activity and Bax expression, whereas decrease Bcl-2 expression ($p<0.05$). Local silencing of lncRNA-CARL in rats resulted in decreased intravascular intima-media thickness ratio and Bcl-2 expression, as well as increased activity of Caspase-3 and Bax expression ($p<0.05$).

CONCLUSIONS: Long non-coding ribonucleic acid-cardiac apoptosis-related lncRNA (lncRNA-CARL) regulates cell apoptosis and participates in the occurrence and development of MI.

Key Words:
lncRNA-CARL, Myocardial infarction, Apoptosis, Bcl-2, Bax.

Introduction

Myocardial infarction (MI) refers to acute lumen occlusion caused by rupture of plaque, hemorrhage, the formation of thrombosis or coronary spasm on the basis of coronary atherosclerosis1. Interruption or sharp decrease of coronary arteriovenous blood flow leads to acute ischemia of cardiac myocytes dominated by coronary arterial veins. Sustained acute ischemia results in ischemic necrosis of the cardiac myocytes[2]. Acute MI (AMI) is mainly characterized by sudden and rapid onset and late diagnosis, which has a poor treatment effects at present. Therefore, effective prevention and early treatment of AMI are particularly important. The main treatment methods of MI include thrombolytic therapy, emergency percutaneous coronary intervention operation (PCI), coronary artery bypass graft (CABG) surgery and so on[3]. In China, the incidence rate of AMI is 45/100,000 to 55/100,000, accounting for 25% of the total number of deaths per year in China[4]. Coronary heart disease caused by AMI has become one of the main causes of disability and mortality in urban and rural residents[5]. Long non-coding ribonucleic acid (lncRNA) is a class of ncRNA with over 200 base pair (bp) in length. It has highly conserved sequence elements, specific spatial secondary structure and complex sub-cellular location, which can be located in the nucleus or cytoplasm[6]. LncRNA is widely involved in the regulation of a large number of physiological activities and the pathological processes of diseases[7]. The abnormal expression of lncRNA is closely correlated with lots of diseases (such as metabolic diseases, neurodegenerative diseases, autoimmune diseases and tumors)[8,9]. Recent studies have suggested that lncRNAs have a high degree of tissue specificity in myocardial tissues. Most cardiac specific lncRNAs have unique regulatory and functional characteristics for the remodeling and regeneration of cardiac myocytes and cardiac function. Cardiac specific lncRNAs can be served as target molecules and biomarkers associated with the development of heart diseases10. Other studies have found that some of the-

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se lncRNAs can be detected in body fluids. It is reported that lncRNAs have a close relationship with the occurrence of cardiovascular disease, which participate in many important regulatory processes such as genomic imprinting, X chromosome silencing, chromatin modification, transcriptional activation, transcriptional interference and intranuclear trafficking[11,12]. The research on the function and mechanism of lncRNA is in the initial stage, among which studies on lncRNA and cardiovascular diseases are relatively few. However, previous investigations[13] have shown that lncRNA is closely correlated with the occurrence and development of atherosclerosis and other cardiovascular diseases. In the process of aging, lncRNA-cardiac apoptosis-related lncRNA (lncRNA-CARL) can isolate heterogeneous nuclear ribonucleoprotein A1 (hnRPA1)[14] and stabilize p16INK expression, indicating its correlationship with aging. Scholars[15,16] have shown that lncRNA-CARL can inhibit the mitosis and apoptosis of mitochondria in cardiac myocytes by combining with MI-related micro ribonucleic acid-539 (miR-539) and down-regulating PHB2. The primary purpose of this study was to evaluate the expression and significance of lncRNA-CARL in MI.

Materials and Methods

Experimental Materials

A total of 20 male specific pathogen free (SPF) Sprague-Dawley (SD) rats (180-200 g) and male clean C57BL/6J mice (18-20 g) were bought from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). This study was approved by the Animal Ethics Committee of Jining Medical University Animal Center. Ltd. 293FT, human embryonic kidney cells were purchased from the Cell Bank of Chinese Academy of Sciences (Beijing, China). The recombinant shuttle plasmids and packaging plasmid spRev, pVSV-G, pGag/Pol were constructed by Shanghai Shenggong Industrial Bio Engineering Co., Ltd. (Shanghai, China). Polyclonal antibodies of Bcl-2, Bax, β-ACTIN, PHB2 were obtained from CST (Danvers, MA, USA); Anti-alkaline phosphatase labeled Goat anti-rabbit IgG from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China); Cell counting kit-8 (CCK-8) was obtained from Dojindo (Kumamoto, Japan); Caspase 3 activity assay kit was obtained from Shanghai Beyotime Biological Engineering Co. Ltd. (Shanghai, China); LncRNA-CARL plasmid and Real-Time-PCR matched primers were constructed from Invitrogen (Carlsbad, CA, USA); Real-Time PCR kit was obtained from TaKaRa (Otsu, Shiga, Japan).

Experimental Study of Endothelial Cell Atherosclerosis Model in Rats

The primary endothelial cells of rats were prepared in the SPF animal laboratory, with the temperature of (22±2)°C. After one week of adaptive feeding, the primary myocardial endothelial cells were extracted from the rats. 10% chloral hydrate (300 mg/kg) was injected into the intraperitoneal of rats for anesthesia. After being anaesthetized, the heart and aorta were removed rapidly, the left ventricular myocardial tissue was retained. Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) with 20% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) was used for cell culture in a 5% CO2 incubator at 37°C. After cells were adhered to the dish bottom wall, High Modified Dulbecco's Modified Eagle Medium (HDMEM) containing high-fat serum (cholesterol 50 mg/dL) was replaced. Atherosclerotic model was then prepared. Logarithmic growth phase cells were used for all experiments.

Construction of Cell Lentivirus Vector and Cell Transfection

LncRNA-CARL plasmid, packaging plasmid and liposome Turofect were co-transfected into 293FT cells. Virus supernatant after transfection for 48 h and 72 h was collected, centrifuged and concentrated. The corresponding lentiviruses were re-suspended in serum-free DMEM and then stored at -80°C. The rat primary endothelial cells were seeded on 24 well plates. When the cell fusion rate reached 50%, lentivirus fluid was transfected. Meanwhile, polybrene reagent was added to enhance the infection efficiency, and then incubatedat 37°C with 5% CO2 incubator. After 24 h, the medium was replaced to the new full medium and continued to culture until the cells fusion rate reached over 90%. The experiment was divided into negative control group, lncRNA-CARL overexpression group and lncRNA-CARL inhibition group.

Detection of Cell Activity by CCK-8

Cells in the logarithmic growth phase after transfection were inoculated into 96 well plates (100 μL / hole) incubated at 37°C in 5% CO2 for 24 h. Cells in each group were given doxorubicin

and paclitaxel combined with 24 h culture medium. 10 μL cell counting kit-8 (CCK-8) solution were added into each group, then incubated in 5% CO2 at 37°C for 24 h. Optical density (OD) value was measured at 450 nm and the cell inhibition curve was drawn. Inhibitory rate of cells proliferation was calculated according to the formula: inhibitory rate = (1-ODdrug/ODcontrol) × 100%. The experiment was repeated for at least 3 times.

Models of Atherosclerotic Disease in Mice

Establishment of a Locally lncRNA-CARL Silent Mouse Carotid Artery Injury Atherosclerosis Model

Mouse carotid artery injury is a classical animal model of atherosclerosis. The specific procedures were as follows: mice received intraperitoneal injection of 10% chloral hydrate (350 mg/kg) for anesthesia. After routine skin disinfection, subcutaneous tissue and muscle were cut to expose and separate the left carotid artery. A rough guide wire inserted tube was placed for 2-3 times of continuous pump for arterial intimal injury. LncRNA-CARL was silenced by administration of 30 μL recombinant virus in the lesioned artery intima and incubated for 30 min at room temperature. After suturing the wound, atherosclerosis was induced by 60% high fat diet after the mice were awakened.

After Intraperitoneal Injection of HE Staining in Mice

The carotid artery specimens were dehydrated and transparent, and 10 continuous sections (5 μm/sheets) were sectioned with paraffin embedding. Hematoxylin was stained and xylene was transparent. The neointima of carotid artery was observed by microscope.

Detection of Endothelial Cells in Mice

Detection of Caspase-3 activity by Caspase-3 Activity Detection Kit

The cell suspension was prepared from the endothelial cells of the myocardium. After cells were washed twice with phosphate-buffered saline (PBS), cells were treated with lysis buffer and centrifuged at 10000 rpm at 0°C for 5 min. 50 μL reaction buffer diluted with buffer (reaction buffer 10 mM DTT) and 5 μL mM LEHD-pNA 4 substrate were added in each well of 96-well plates, and incubated at 37°C for 1 h. The absorbance at 405 nm was detected by a microplate reader. The Caspase-3 active unit (U) = (absorbance of each sample - absorbance of the blank group)/the slope of the standard curve. Each group had 3 replicates.

Western Blotting Detection of PHB2, Bcl-2, Bax Protein Expression Level

The cell suspension was prepared from the endothelial cells of the myocardium. After cells were washed twice with phosphate-buffered saline (PBS), they were treated with lysis buffer and centrifuged at 10000 rpm at 0°C for 10 min. The concentration of protein extracted was measured via bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). 50 μg protein were subjected to 8% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane and sealed with gelatin for 2 h. Membranes were incubated with rabbit anti-human PHB2, Bcl-2, Bax protein-specific polyclonal antibodies (diluted at 1:500, Cell Signaling Technology, Danvers, MA, USA) and mouse anti-human β-actin monoclonal antibody (diluted at 1:1000, Zhongshan Golden Bridge, Beijing, China) at 4°C overnight. Then, membranes were incubated with specific horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000) for 1 h, and washed with Tris-Buffered Saline with Tween-20 (TBST) for 3 times. The protein bands were developed using enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 software (Chicago, IL, USA) was used for statistical analysis. The results were expressed by (`x+s), t-test was used for the intergroup differences. $p<0.05$ suggested that the difference was statistically significant.

Results

Detection of Cell Activity via CCK-8 Assay

The cell viability was detected by CCK-8 assay. Compared with that of the control group, the cell activity in lncRNA-CARL overexpression group increased, and the cell apoptosis decreased. The cell activity of lncRNA-CARL inhibition group decreased compared with that of control group, and the difference was statistically significant ($p<0.05$, Figure 1).

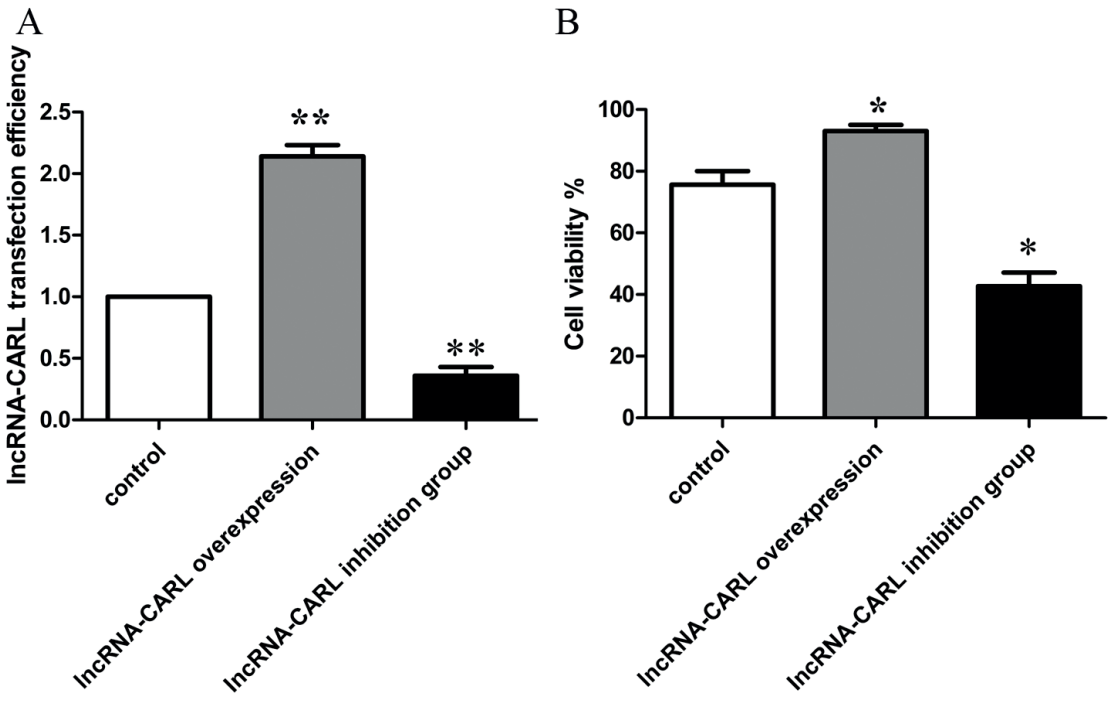

Figure 1. Transfection efficiency detection and cell viability detection by CCK-8 method. ***A,*** Transfection efficiency. ***B,*** Cell viability detection by CCK-8; ($^{*}p<0.05$ control with transfected vector virus group; $^{**}p<0.01$ control with transfected vector virus group).

Hematoxylin and Eosin Stain Results

Hematoxylin and eosin (HE) staining showed that compared with the control group, the in vivo/medium thickness ratio of mice was significantly decreased after lncRNA-CARL silence. The results indicated that lncRNA-CARL silence can significantly reduce the neointimal growth of injured arteries (Figure 2).

Detection Caspase-3 Activity

Caspase-3 activity assay showed that compared with the control group, the activity of Caspase-3 decreased and the cell apoptosis decreased in the lncRNA-CARL overexpression group. The activity of Caspase-3 increased and the cell apoptosis was promoted in lncRNA-CARL inhibition group ($p<0.05$) (Figure 3A). After silencing of lncRNA-CARL in vivo, the Caspase-3 activity of mouse vascular endothelial cells increased, and the difference was statistically significant ($p<0.05$). The results indicated that lncRNA-CARL silencing can increase the occurrence of apoptosis, as shown in Figure 3B.

Detection of Protein Levels of PHB2, Bcl-2 and Bax via Western Blot

Western blot showed decreased pro-apoptotic protein Bax and increased anti-apoptosis protein Bcl-2 in primary endothelial cells with lncRNA-CARL overexpression. The opposite results were found in lncRNA-CARL inhibition group, and the difference was statistically significant ($p<0.05$) (Figure 4). After silencing of lncRNA-CARL in vivo, the level of anti-apoptotic protein Bcl-2 decreased significantly, and the level of pro-apoptotic protein Bax increased significantly. The difference was statistically significant ($p<0.05$) (Figure 5).

Discussion

In recent years, researches[17,18] have shown that lncRNA plays an important biological function in the development, pluripotency, cell growth and apoptosis of stem cells. However, the role of lncRNAs in cardiovascular diseases is not quite clear yet[19]. LncRNA-CARL can isolate heterogeneous nuclear ribonucleoprotein A1 (hnRPA1), thus stabilizing p16INK expression and inhibiting cell apoptosis[20,21]. In the early myocardial ischemia stage of AMI, the main form of cardiac myocyte death is cell apoptosis presenting in the whole pathophysiological process of myocardial injury[22]. Studies have shown that the expression level of ln-

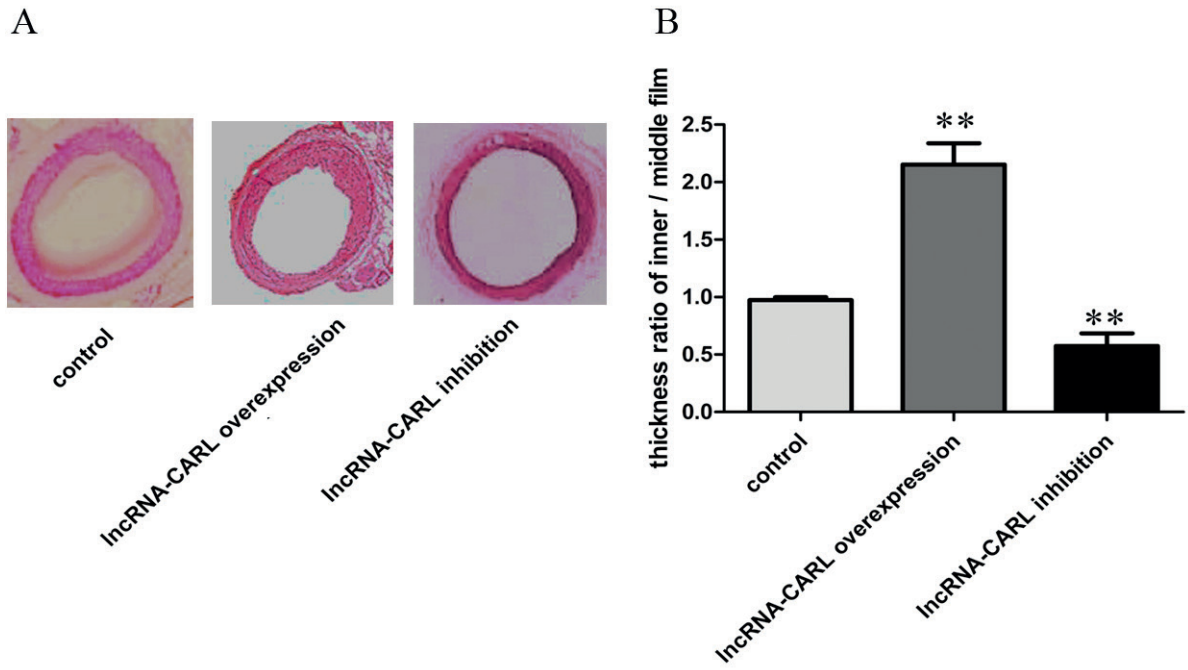

Figure 2. Hematoxylin and eosin (HE) stain detection (x200). ***A,*** Carotid intima HE staining in control group. ***B,*** Thickness ratio of inner / middle membrane. ($^{*}p<0.05$ control with transfected vector virus group; $^{**}p<0.01$ control with transfected vector virus group).

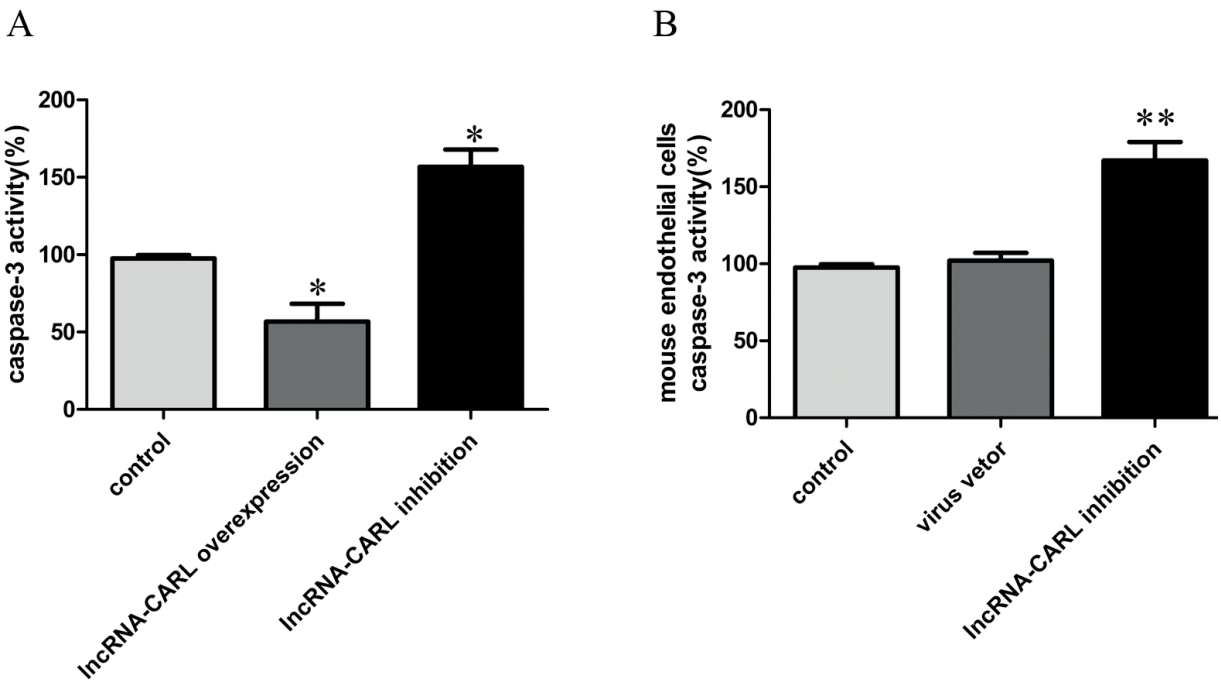

Figure 3. Detection results of Caspase-3 activity. ***A,*** Detection Caspase-3 activity of primary endothelial cells. ***B,*** Detection Caspase-3 activity of mouse endothelial cells. ($^{*}p<0.05$ control with transfected vector virus group; $^{**}p<0.01$ control with ransfected vector virus group).

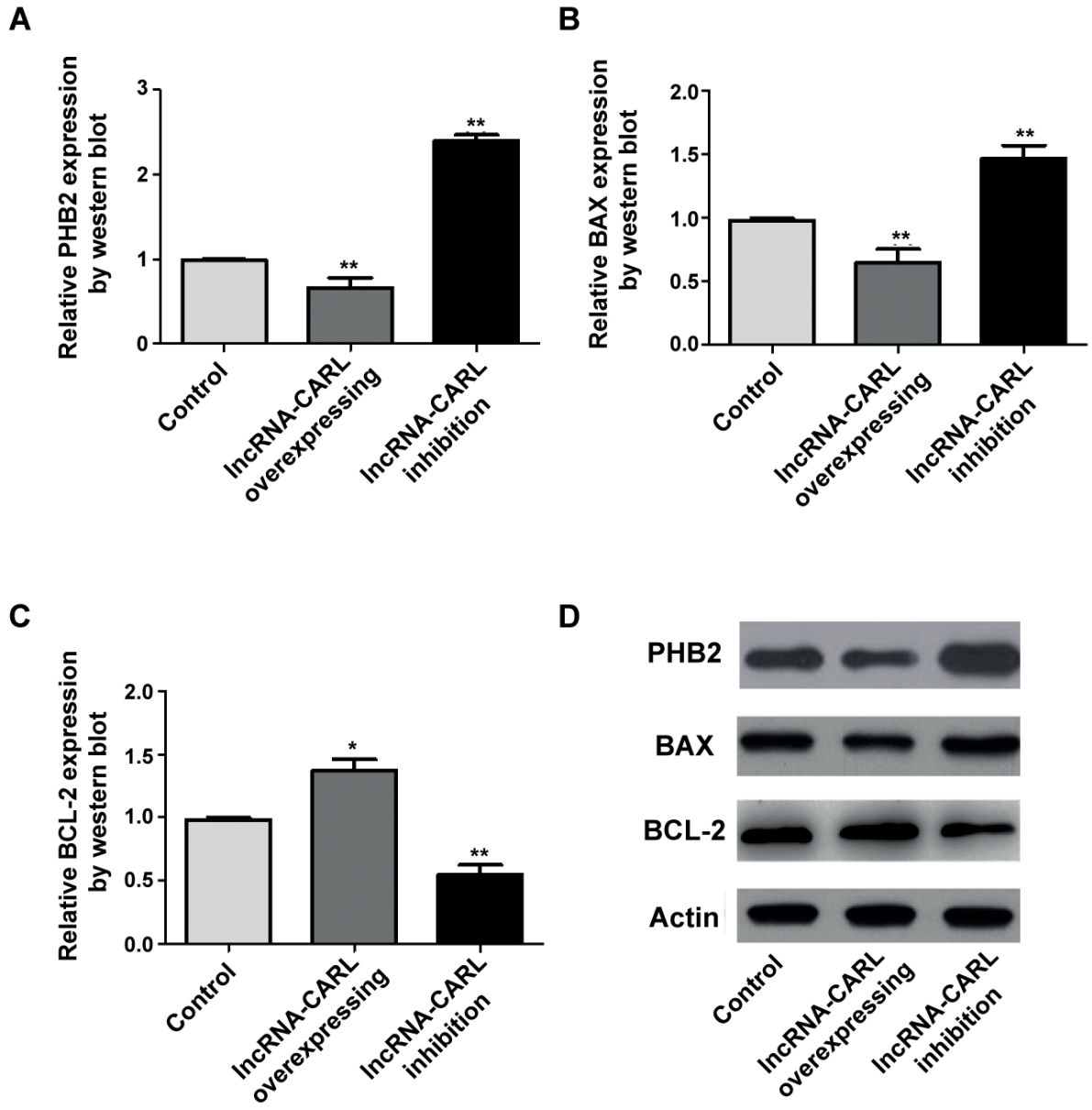

Figure 4. Protein expression detection in primary endothelial cells. ***A-B,*** and ***C,*** Detection of protein expression in the primary endothelial cells. D, Detection of expression of protein in the primary endothelial cells. (*p<0.05 control with transfected vector virus group; **p<0.01 control with transfected vector virus group).

cRNA-CARL in apolipoprotein E (ApoE)-/- atherosclerotic plaque tissues in rats was significantly decreased. It suggested that lncRNA-CARL may be correlated with the occurrence and development of AS23. However, whether lncRNA-CARL participates in the process of mediating the occurrence and development of MI is unclear.

The biological function of lncRNA-CARL was observed after primary culturing of endothelial cells in rats and hyperlipidemic arteriosclerosis model. Compared with those in the control group, the cell survival rate was significantly decreased and the activity of Caspase-3 was significantly increased in lncRNA-CARL over-expression group. The cell survival rate in lncRNA-CARL inhibition group was significantly increased, and the activity of Caspase-3 was significantly decreased. The data indicated that over-expression of lncRNA-CARL can significantly promote the occurrence of apoptosis and affect the cell pro-

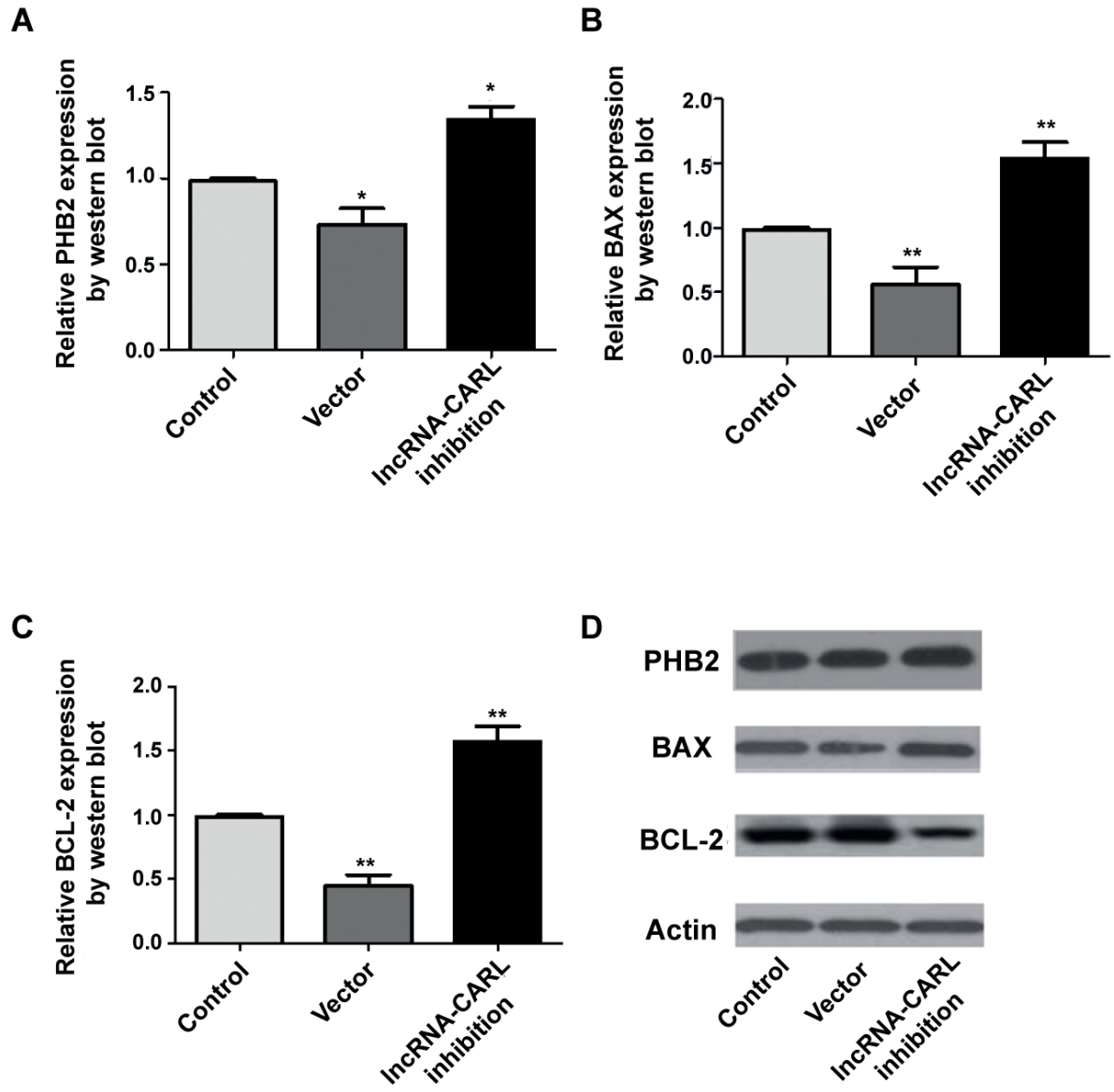

Figure 5. Protein expression detection in mouse model cells. ***A-B***, and ***C,*** Detection of protein expression in mouse model cells. ***D,*** Detection of expression of protein in mouse model cells. ($^{*}p<0.05$ control with transfected vector virus group; $^{**}p<0.01$ control with transfected vector virus group).

liferation process. At the same time, the results showed that the level of pro-apoptotic protein Bax was increased significantly and the level of anti-apoptotic protein Bcl-2 was decreased in lncRNA-CARL over-expression group. The opposite results were found in lncRNA-CARL inhibition group, indicating that lncRNA-CARL silencing inhibited apoptosis by regulating apoptosis-associated proteins.

In order to detect the effects of lncRNA-CARL on the endothelial neointima and endothelial cell apoptosis in atherosclerosis in vivo, the rat model of atherosclerosis and the rat model of lncRNA-CARL local silencing atherosclerosis were established24. The results showed that intravascular intima-media thickness ratio was decreased after local silencing of lncRNA-CARL in vivo. Our results indicated that lncRNA-CARL silencing can inhibit the neointimal

growth of injured artery, reduce the activity of apoptotic protein Caspase-3, upregulate Bcl-2 expression and downregulate Bax expression. The inhibition of cell apoptosis after silencing of lncRNA-CARL suggested that lncRNA-CARL can effectively inhibit the development of atherosclerosis. This demonstrated that lncRNA-CARL can affect the proliferation and apoptosis of endothelial cells in atherosclerotic MI *in vitro*. LncRNA-CARL was further confirmed that it can affect atherosclerosis by regulating cell proliferation and apoptosis. However, how lncRNA-CARL participates in the regulation of cell proliferation and apoptosis still need to be further discussed.

Conclusions

We showed that LncRNA-CARL regulated cell proliferation and apoptosis of myocardial endothelial cells, thus inhibiting the occurrence and development of MI. Understanding the mechanism of lncRNA in regulating apoptosis and proliferation can provide a strong basis for elucidating the pathophysiological role and regulatory mechanism of lncRNA in cardiovascular diseases. AMI has the characteristic of sudden onset. Early prevention, diagnosis and treatment can effectively control its incidence rate[25].

Conflict of Interest

The Authors declare that they have no conflict of interest.

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