Original Article
Overexpression of PER2 inhibits the progression of atherosclerosis via the Akt-eNOS signaling in apolipoprotein e-null mice

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Abstract: Atherosclerosis is an inflammatory disease. The circadian clock gene has a connection with inflammation, including vascular inflammation. However, whether circadian clock gene has a regulatory effect on atherosclerosis is rarely studied. Our aim is to determine whether *Period 2* (*Per2*), a main clock gene, exerts a therapeutical effect on atherosclerosis in Apolipoprotein E-null (ApoE$^{-/-}$) mice. Atherosclerotic mice were divided into three groups, including the Control group (mice received an intravenous injection of phosphate buffered saline at 13 weeks old), the Ad-GFP group (mice received an intravenous infection of adenovirus vector carrying GFP at 13 weeks old) and the Ad-PER2 group (mice received an intravenous infection of adenovirus vector carrying PER2 at 13 weeks old). PER2 overexpression decreased lesion areas of aorta and aortic root, levels of inflammatory factors and adhesion molecules, and the serum levels of total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C). Moreover, PER2 exerted an inhibitory effect on atherosclerosis by activating the protein kinase B-endothelial nitric oxide synthase (Akt-eNOS) signaling pathway in ApoE$^{-/-}$ mice. Taken together, our findings suggest that PER2 has an inhibitory effect on atherosclerosis in ApoE$^{-/-}$ mice via activation of the Akt-eNOS pathway.

Keywords: Atherosclerosis, PER2, Akt-eNOS pathway, inflammation, adhesion molecules

Introduction

Atherosclerosis is considered as a complex inflammatory disease which could cause serious cardiovascular diseases to human health [1]. The accumulation of lipid fibrous tissue and inflammatory cells in arteries is the main characteristic of atherosclerosis [2]. Monocytes in the endothelial dysfunction adhere to the artery wall and move to the tunica intima where they differentiate to macrophages and then engulf modified LDL to form early atherosclerosis lesions [3]. Inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) contribute to the inflammatory response of multiple chronic inflammatory disorders [4]. Inflammatory cytokines expressed on vascular and extravascular tissues elicit the development of coronary lesion [5]. Cellular adhesion molecules such as cellular adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and selectins (P, E and L) mediate early phase of atherosclerosis [6]. For instance, VCAM-1, originated from cytokine-stimulated endothelial cells, products accumulation of inflammatory cells by binding monocytes and T lymphocytes in the initiation of atherosclerotic plaque [7, 8].

Circadian genes such as *CLOCK, BMAL1, NPAS2, Cryptochrome* genes (*Cry1/2*), and *Period* genes (*Per1/2/3*) are the main mediators of circadian clock to achieve biological synchronization [9]. Circadian disruption is responsible for inflammatory immune responses and vascular disease [10, 11]. *Per2*, a clock gene, plays an important role in maintenance of normal cardiovascular functions, mutating which could lead to premature vascular senescence that correlates with impaired endothelial function and angiogenesis [12], and impair nitric

oxide (NO) release and vascular relaxation [13]. Additionally, *Per2* plays a crucial role in control of cardiac metabolism and myocardial ischemia [14, 15]. Although recent evidence has implicated *Per2* gene in cardiovascular dysfunctions and cardiovascular events physiologically and pathologically, no investigation directly implicate the association of *Per2* gene and atherosclerosis.

Akt, a serine-threonine kinase, has been thought as a key factor in protection of cardiomyocytes against apoptosis through eNOS phosphorylation induced by activation of Akt [16, 17]. eNOS the main producer of NO, has various originations such as endothelium, the heart and blood vessels, cardiac myocytes and blood platelets. The phosphorylation of eNOS is stimulated by its upstream activator Akt at site Ser^{1177}, allowed by endothelial NO generation, which leads to cell proliferation, migration, and angiogenesis [18]. Endothelial NO plays a crucial role in vascular protection, because it prevents vasoconstriction, thrombus and atheroma formation via enhancement of vascular relaxation and inhibition of platelet aggregation, monocytes adhesion and vascular smooth muscle cell (VSMC) proliferation [19, 20]. Recently, several studies have reported that the Akt/eNOS pathway could mediate the amelioration of atherosclerosis [21].

In the present study, the effect of PER2 overexpression on the prevention of atherosclerosis was studied in ApoE$^{-/-}$ mice. In addition, the underlying mechanism responsible for the inhibition of atherosclerosis was investigated.

Materials and methods

Animals and experimental design

Male apolipoprotein E-deficient mice (ApoE$^{-/-}$, 5 weeks old, weighing 20-25 g) were purchased from Beijing Biocytogen Co., Ltd (Beijing, China) and raised as the standard protocols. All the mice were housed under normal specific pathogen-free (SPF) conditions. Room temperature of the housing facility was maintained at 25 ± 1°C with a relative humidity of 60 ± 10% and a 12-h light/dark cycle. To establish atherosclerotic mouse models, mice were fed on high-cholesterol diet (20% fat, 20% sugar and 1.25% cholesterol) with free access to water in the whole experimental process. Wild-type mice (5

weeks old, weighing 20-25 g) also purchased from Biocytogen were fed normal rodent chow with free access to water (defined as the normal group). All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee of the First People's Hospital of Shangqiu.

At 13 weeks old, atherosclerotic mice were randomly divided into three groups (8 in each group). Mice received an injection of phosphate buffered saline were defined as the control group. Mice were injected with adenovirus vector carrying GFP were defined as the Ad-GFP group. Mice were injected with adenovirus vector carrying PER2 were defined as the Ad-PER2 group. Mice were injected with PBS, PER2-expressing adenovirus vector or GFP-adenovirus vector (1 × 10^8 pfu in 100 µl of PBS; Biowit, Shenzhen, China) by tail vein injection for 4 weeks. All the mice were euthanized with sodium pentobarbital at 17 weeks old. The mRNA and protein levels of PER2 in aorta and inflammatory cytokines as well as adhesion molecules in aortic endothelial cells were determined by using qRT-PCR and western blot analysis, respectively.

To confirm the regulatory effect of the Akt/eNOS signaling on atherosclerosis, ApoE$^{-/-}$ mice were divided into three groups (8 in each group). At 9 weeks old, L-NAME, an eNOS inhibitor, was dissolved in water and added directly to the drinking water of the animals for 8 weeks. The L-NAME concentration in each group is 0, 0.1 and 0.2 g/L. All the mice were euthanized with sodium pentobarbital to collect aortas and aortic roots. The protein levels of Akt, 3-phosphoinositide-dependent kinase-1 (PDK1), and eNOS in aorta were detected by using western blot analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

After the mice were humanely sacrificed, total RNA was isolated from the mouse aorta and aortic endothelial cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA of 5 µg was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA). The cDNAs were used as templates for qRT-PCR to measure the gene expression. The qRT-PCR 10 µl system contained 5 µl SsoFast™ EvaGreen®

Supermix (Bio-Rad), 1 μl of cDNA and 2 μl of each of the forward and reverse primers (1 μM). The PCR amplification was performed with initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 20 s, and melt curve from 65 to 95°C. β-actin was used as internal reference gene for normalizing gene expression. The data obtained were calculated by $2^{-\Delta\Delta CT}$.

Tissue preparation and analysis

The en-face preparation was prepared as follows: mice were euthanized and the perfusion of aortic tree was performed using PBS through the left ventricle. Subsequently, the aorta was separated from the aortic arch to approximately the iliac bifurcation. After overnight fixation of the isolated aortas in 4% paraformaldehyde, the adventitia was removed from the surrounding material and the aortas were cut longitudinally and secured on a silicone-coated dish with black paraffin under a dissecting microscope.

The aortic section was soak in 60% isopropanol (Aladdin, shanghai, China) for 25 s and then stained with oil red (Sigma, St. Louis, MO, USA) for 8 min. Next, soaking the slide in 60% isopropanol for 10 s again to remove redundant dye. To determine the plaque size in the aortic root, The cryosections of the aortic root were soaked in 60% isopropanol for 30 s and then in oil red O for 20 min. Sections were rinsed 3 times and counterstained with hematoxylin (Amresco, Shanghai, China). The images were captured and analyzed using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

Western blot analysis

After mice tissues of the aorta were homogenized and aortic endothelial cells were lysed, the protein concentration were determined using the BCA protein quantification kit (Pierce Chemical Co., Rockford, IL, USA). Fifty μg proteins was fractionated by 12% SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane (Amersham, Little Chalfont, UK) followed by incubation with TBS containing 0.1% Tween-20 and 5% skimmed milk in Tris-buffered saline for 1 h at room temperature to block non-specific binding. After blocking, the filters were incubated with primary antibody at 4°C overnight. The primary antibodies are as followed: anti-TNF-α (Abcam, Cambridge, UK), anti-IL-1β (Abcam), anti-IL-6 (Abcam), anti-VCAM-1 (Abcam), anti-ICAM-1 (Abcam), anti-E-selectin (Abcam), anti-Akt (Cell Signaling Technology, Danvers, MA, USA), anti-Akt (phospho Ser473, Cell Signaling Technology), anti-3-Phosphoinositide-dependent Kinase-1 (PDK1) (Abcam), anti-eNOS (phospho Ser1177, Abcam) and anti-GAPDH (Abcam). After washing and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam) for 1 h, membranes were visualized with Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Serum sample analysis

Under anesthesia, blood samples were obtained from mouse orbital venous plexus using capillary glass tubes followed by the centrifugation at 3000 *g* for 5 min. Levels of total cholesterol (TC), triglyceride (TG), free fatty acids (FFA), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using ELISA kit (Dirui, Changchun, China), according to the manufacturer's instructions.

Statistical analysis

Data were expressed as the mean ± SD. Comparisons among different groups were undertaken using the using the ANOVA. The criteria of statistical significance were a *P* value less than 0.05. All statistical analyses were done with SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA).

Results

PER2 was down-regulated in atherosclerotic mice

Previous studies showed that Clock protein mutant increases atherosclerosis in ApoE^{-/-} mice and main clock genes, including *Per2*, were significantly attenuated in human plaque-derived vascular smooth muscle cells (VSMCs) compared with normal human carotid VSMCs [22, 23]. To understand the expression of PER2 in atherosclerotic mice, mRNA and protein levels of PER2 in aorta were analyzed. The results showed that the level of PER2 in control and
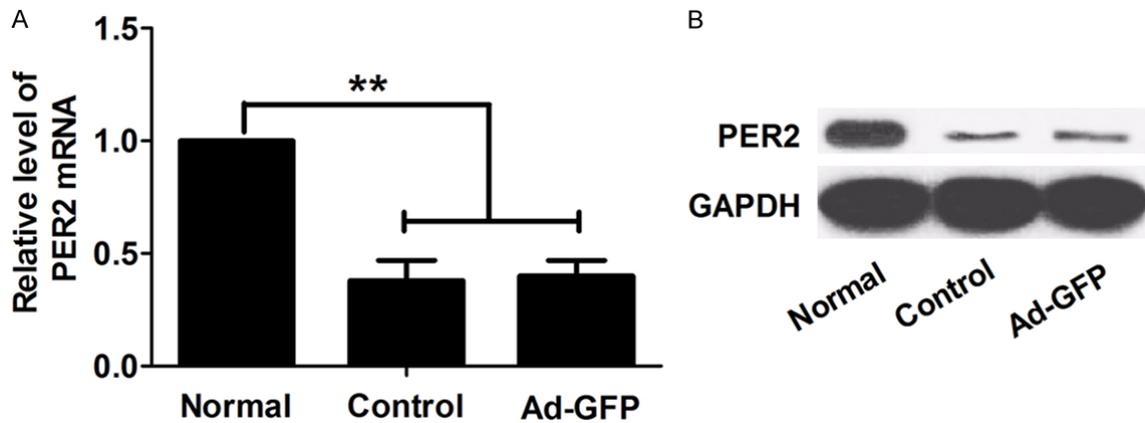
Figure 1. Expression of PER2 in atherosclerotic mice. The mRNA level (A) and protein level (B) of PER2 in aorta of mice were determined using qRT-PCR and western blot, respectively. $^{**}P < 0.01$. $n = 8$.
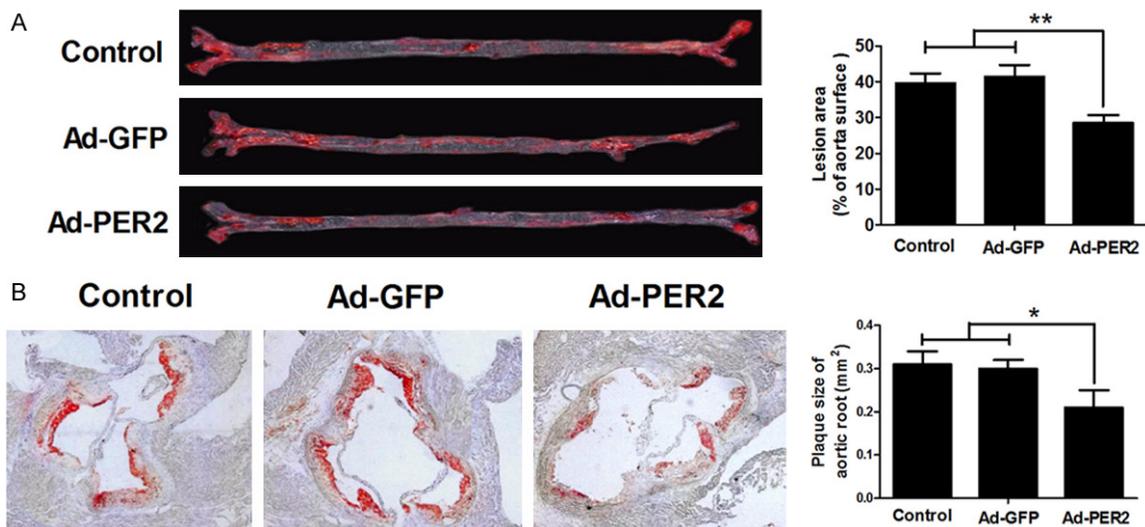
Figure 2. Overexpression of PER2 relieved atherosclerosis. A. Quantitative analysis of atherosclerotic lesion area of aorta showed that PER2 overexpression relieved atherosclerosis. B. Overexpression of PER2 decreased the plaque size of aortic root. $^{*}P < 0.05$, $^{**}P < 0.01$. $n = 8$.

Ad-GFP groups were significantly decreased compared to the normal group (**Figure 1A** and **1B**). Thus, we hypothesized that overexpression of PER2 might contribute to prevention of atherosclerosis.

Overexpression of PER2 attenuated atherosclerosis

We examined PER2 mRNA and protein levels in aorta after mice were injected with PER2-expressing adenovirus vector. The results showed that PER2 mRNA was markedly increased in the Ad-PER2 group compared with the control or Ad-GFP group (Data not shown).

To exam the role of PER2 on atherosclerosis, lesion areas of aorta and aortic root were analyzed. The results showed that the Ad-PER2 group had a remarkable smaller areas compared with the Ad-GFP or control group (**Figure 2A** and **2B**), which suggested that overexpression of PER2 significantly attenuated atherosclerosis.

Overexpression of PER2 altered lipid profiles

To further explore the effect of PER2 overexpression on atherosclerosis, body weight and serum levels of LDL-C, HDL-C, total cholesterol, triglycerides, and FFA were measured. As
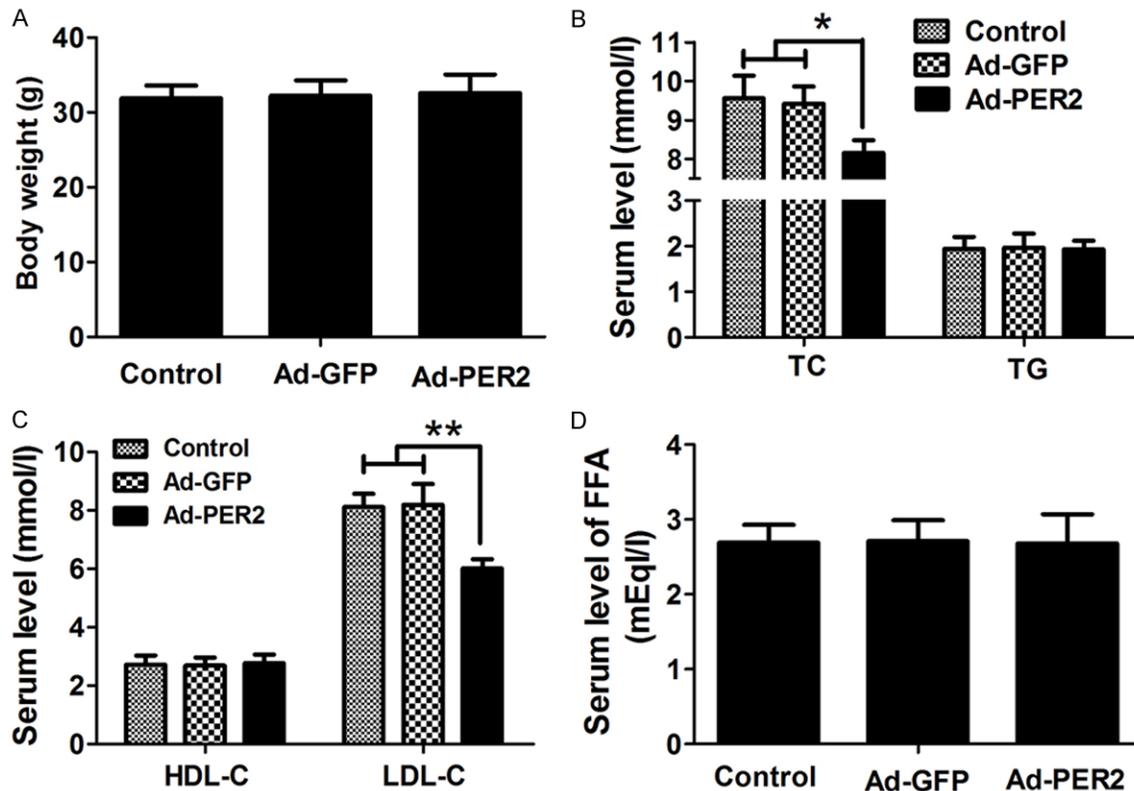
Figure 3. Effects of overexpression of PER2 on body weight and metabolic parameters in ApoE$^{-/-}$ mice. TC: total cholesterol; TG: triglyceride; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; FFA: free fatty acids. $^{*}P < 0.05$, $^{**}P < 0.01$. $n = 8$.

shown in **Figure 3A-D**, overexpression of PER2 significantly decreased the serum levels of total cholesterol and LDL-C, compared with the Ad-GFP or control group. Nevertheless, body weight and the serum levels of HDL-C, triglycerides, and FFA were almost unchanged in the Ad-PER2 group compared with those in the Ad-GFP or control group.

Overexpression of PER2 decreased the levels of inflammatory factors and adhesion molecules in ApoE$^{-/-}$ mice

To examine whether the overexpression of PER2 has a negative effect on inflammatory cytokines expression, mRNA levels of TNF-α, IL-6 and IL-1β in the aortic endothelial cells were determined by qRT-PCR (**Figure 4A-C**), and protein levels were determined by western blot (**Figure 4D**). Overexpression of PER2 markedly decreased levels of TNF-α, IL-6 and IL-1β in the Ad-PER2 group compared with the Ad-GFP or control group. To evaluate the effect of PER2 overexpression on the adhesion molecules in

aortic tissues, qRT-PCR and western blot analysis were performed. The mRNA levels of these adhesion molecules were markedly decreased in the Ad-PER2 groups, compared with the Ad-GFP or control group (**Figure 4E-G**). Western blot analysis showed that the levels of VCAM-1, ICAM-1 and E-selectin proteins had a similar trend in these groups (**Figure 4H**).

The Akt-eNOS signaling was activated by overexpression of PER2

It was reported that the Akt/eNOS signaling pathway are involved in atherosclerotic inflammatory reaction [21] and circadian clock enhances NO production by regulating phosphorylation of eNOS to protect endothelial function from injury [24]. To examine whether the Akt-eNOS signaling is activated by overexpression of PER2, total (t)-Akt, phosphorylated (p)-Akt, PDK1, t-eNOS and p-eNOS were measured by using western blot analysis in aortic tissues. The results showed that the expression levels of p-Akt, PDK1 and p-eNOS in the
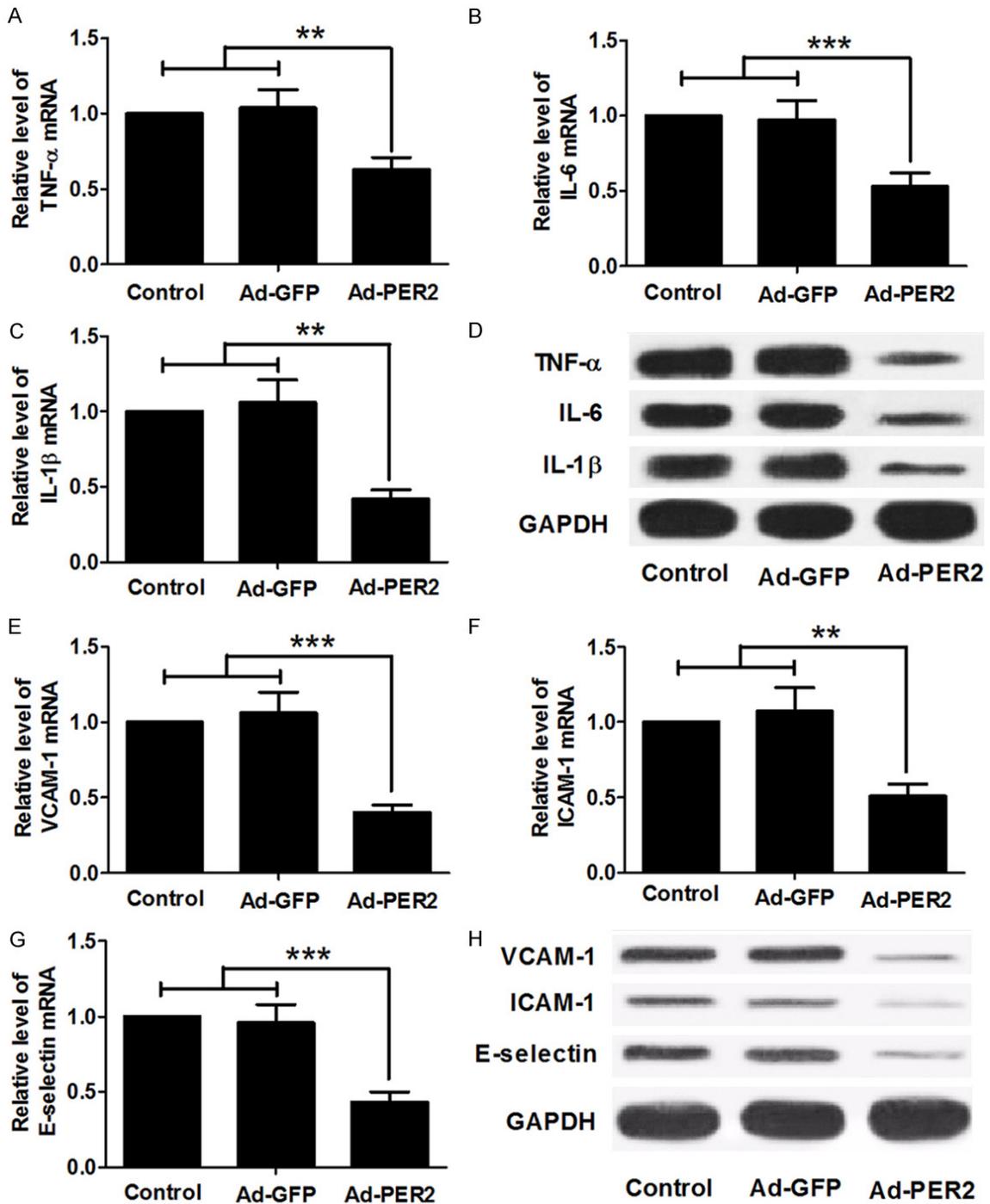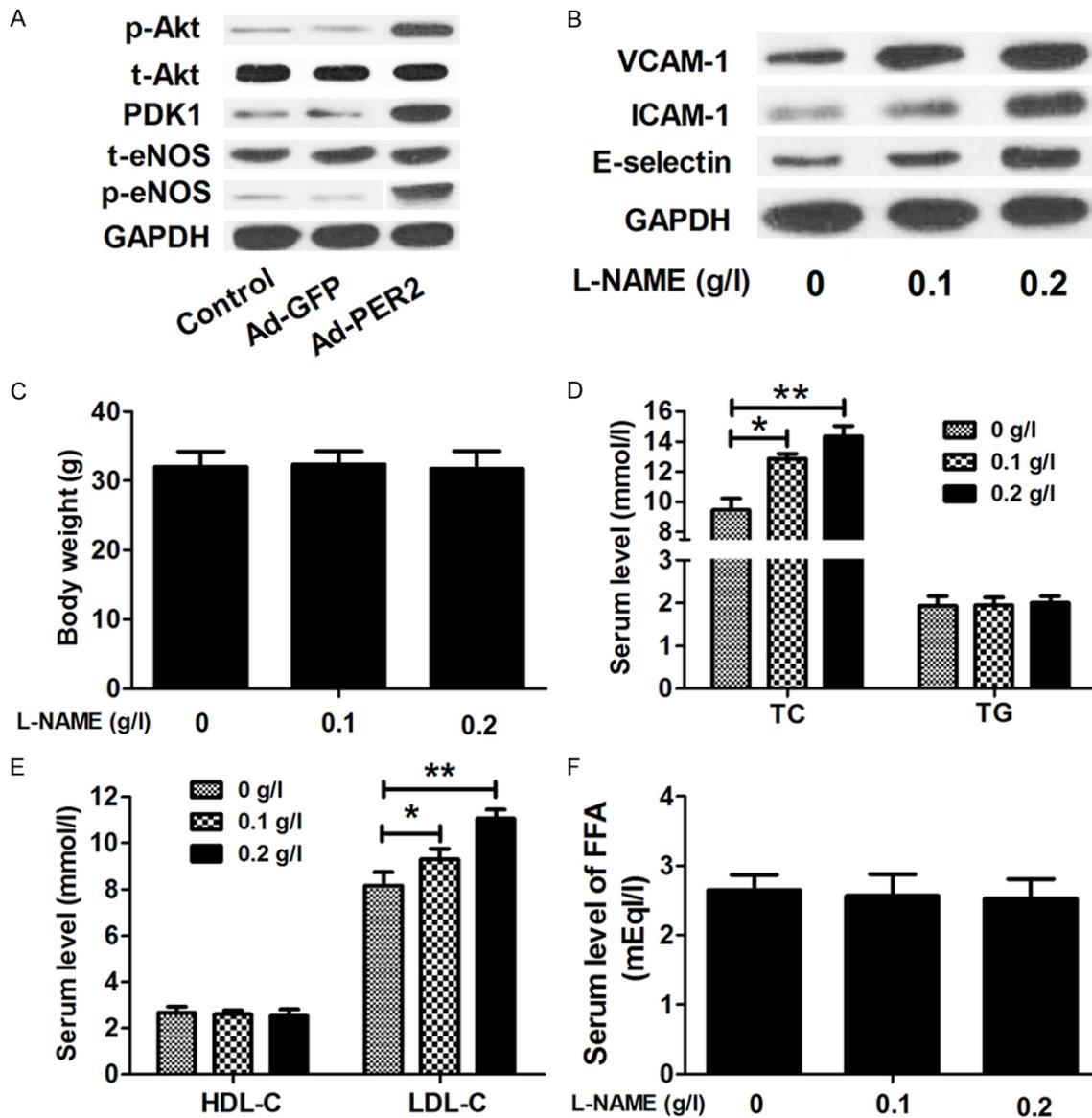
Figure 4. Overexpression of PER2 reduced levels of inflammatory factor and adhesion molecules in ApoE$^{-/-}$ mice. The mRNA (A-C) and protein levels (D) of TNF-α, IL-6 and IL-1β in atherosclerotic mice were detected by qRT-PCR and western blot analysis. Overexpression of PER2 decreased the levels of TNF-α, IL-6 and IL-1β. The mRNA (E-G) and protein levels (H) of VCAM-1, ICAM-1 and E-selectin in atherosclerotic mice were tested by qRT-PCR and western blot analysis. Overexpression of PER2 obviously decreased the levels of VCAM-1, ICAM-1 and E-selectin. $^{**}P < 0.01$, $^{***}P < 0.001$. $n = 8$.

Ad-PER2 group were significantly increased compared with the Ad-GFP or control group. The levels of t-Akt and t-eNOS were unchanged in these groups (**Figure 5A**). These findings indicated that overexpression of PER2 activated the Akt/eNOS signaling pathway in ApoE$^{-/-}$ mice.

Figure 5. Overexpression of PER2 activated the Akt-eNOS signaling pathway. A. The expression levels of p-Akt, PDK1, and p-eNOS were increased by overexpression of PER2 in atherosclerotic mice. The expression levels of t-Akt and t-eNOS were unchanged. B. The levels of VCAM-1, ICAM-1 and E-selectin were increased in atherosclerotic mice treated with eNOS inhibitor L-NAME. C. Body weight of mice was not affected by L-NAME. D-F. Blocking the Akt-eNOS signaling pathway by L-NAME increased serum levels of TC and LDL-C in a concentration-dependent manner. Serum levels of TG, HDL-C, and FFA were not affected by L-NAME. $^*P < 0.05$, $^{**}P < 0.01$. $n = 8$.

To further identify the effect of the Akt/eNOS signaling on atherosclerosis, we detected the levels of VCAM-1, ICAM-1 and E-selectin in endothelial cells in ApoE$^{-/-}$ mice treated with L-NAME, an eNOS inhibitor. As shown in **Figure 5B**, L-NAME significant increased the levels of VCAM-1, ICAM-1 and E-selectin in a concentration-dependent manner. Additionally, we evaluated the body weight and lipid profiles in ApoE$^{-/-}$ mice treated with L-NAME to further understand the effect of the Akt/eNOS signal-

ing. We found that serum levels of total cholesterol and LDL-C were increased in mice treated L-NAME in a concentration-dependent manner, while body weight and the serum levels of HDL-C, triglycerides, and FFA were unchanged among these groups (**Figure 5C-F**).

Discussion

In the current study, the findings suggest that PER2 is beneficial to control atherosclerosis as

a regulator in the inflammatory process. Moreover, by over-expressing PER2 in ApoE$^{-/-}$ mice, we provide evidence that PER2 may contribute to relieve atherosclerosis via the Akt-eNOS signaling pathway. Our findings might shed lights on development of effective treatments for cardiovascular disease therapy.

Dysregulated lipid metabolism is of special interest in atherosclerosis. For example, high serum concentration of TC and LDL-C are always involved in atherosclerosis formation. In this study, as expected, atherosclerotic process elicited aberrant lipid profiles with high serum levels of TC and LDA-C, while those metabolic parameters were alleviated markedly by overexpression of PER2. These findings suggested that PER2 is capable to attenuated atherosclerosis by interacting with the lipid. Atherosclerosis is known as an inflammatory disease to cause cardiovascular disorder, therefore, in the development of atherosclerosis, multiple of pro-inflammatory factors and cell adhesion molecules are involved to facilities this progression [25]. For example, inflammatory mediators including TNF-α, IL-6 and IL-1β are measured as the characteristic of cardiovascular disease [5, 26]. In this study, the levels of these inflammatory mediators reduced markedly by overexpression of PER2, which indicates that PER2 possesses anti-inflammatory effects and could impede atherosclerosis formation.

The formation of atherosclerosis involves migration of blood leukocytes and medial smooth muscle cells into the arterial intima to form lesions [3]. Adhesion molecules such as VCAM-1, ICAM-1 and E-selectin expressed on vascular endothelium and circulating leukocytes may be one of the contributions to lesion initiation in the earliest stages of atherogenesis, because they could recruit blood monocytes and lymphocytes to the intima in which these cells mature into macrophages and then turn into foam cells to develop and progress into the atherosclerosis progressively [6, 26]. In the present study, the data demonstrated that overexpression of PER2 has significantly inhibitory effect to the expression of these genes. Cardiovascular system owns typical circadian rhythmicity, because pressure and heart rates have distinct circadian pattern physiologically and pathophysiologically [27]. As an important *Period* gene, *Period 2* gene possesses diverse

biological functions such as the regulation of bone formation [28], tumor suppression [29], and periodic pattern maintain [30] and regulation of vascular endothelial function [31]. In line with these findings, our results indicated that PER2 suppressed atherosclerosis and stabilized plaques in ApoE$^{-/-}$ mice.

Through regulation of vascular tone and relaxation and maintenance of vascular integrity, endothelium-derived relaxing factor nitric oxide, a gaseous transmitter, plays a vital role in endothelium events, dysfunction of which involved in the pathogenesis of various vascular disease including atherosclerosis, hypertension and aging [32, 33]. eNOS, the mainly generator of NO, catalyzes conversion of L-arginine to L-citrulline and NO [34]. Akt, a serine-threonine kinase, regulates several biological processes in protection of cardiomyocytes against apoptosis through eNOS phosphorylation [35]. Various studies have demonstrated that the circadian clock regulates phosphorylation of eNOS which enhance NO production to protect endothelial function from injury and maintain circadian rhythm [24]. In parallel with these studies, a similar trend occurred in this study, the p-Akt, PDK1 and p-eNOS increased significantly by overexpression of PER2, which indicated that the Akt-eNOS signaling was activated by PER2, even though the complicated mechanism of this finding is still being explored. In addition, the eNOS inhibitor L-NAME increased VCAM-1, ICAM-1, Selectins-E, TC and LDL-C. These results suggest that the ApoE$^{-/-}$ mice require the Akt/eNOS signal pathway to eliminate the atherosclerosis.

In conclusion, PER2 has a significantly inhibitory effect on atherosclerosis via the Akt-eNOS pathway. Our data also demonstrated that manipulation of PER2 attenuates the inflammatory progression by inhibiting the inflammatory factors and adhesion molecules. Furthermore, overexpression of PER2 suppressed lesion area significantly and attenuated atherosclerosis by altering aberrant lipid profiles. These results indicate that PER2 is a promising target for atherosclerosis treatment.

Acknowledgements

Disclosure of conflict of interest

None.

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