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ORIGINAL RESEARCH

Intermedin$_{1-53}$ Improves Atherosclerosis by Reducing Local Endothelial Damage via AMPK Signaling Pathway in Obese apoE-Deficient Mice

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Background: Atherosclerotic cardiovascular diseases (CVD) are commonly found in obesity. Endothelial inflammation accompanied by oxidative stress is a crucial risk factor and a key initiating step for the pathogenesis of atherosclerosis (AS). In the present study, the role and mechanism of intermedin (IMD), a potent active peptide, in endothelial damage in AS in obese apolipoprotein E-deficient (apoE$^{-/-}$) mice were investigated.

Methods and Results: In vivo, IMD$_{1-53}$ was infused via Alzet mini-osmotic pump in apoE$^{-/-}$ mice with high-fat diet (HFD) for 4 weeks. In vitro, palmitic acid (PA) and oxidized low density lipoprotein (Ox-LDL) were used to stimulate human umbilical vein endothelial cells (HUVECs) for exploring the potential mechanism of IMD$_{1-53}$ action on endothelial damage. We found that IMD$_{1-53}$ application remarkably improved plasma lipid profiles, hepatic lipid accumulation and its cholesterol levels, and vascular lipid accumulation and lesion sizes. Moreover, IMD$_{1-53}$ markedly increased eNOS expression and decreased the levels of vascular inflammatory factors and ROS. In vitro, the combination of PA and Ox-LDL caused more severe inflammatory and oxidative damages and lower expression of eNOS, which were significantly inhibited by IMD$_{1-53}$. IMD$_{1-53}$ notably induced AMPK phosphorylation, and the inhibition of AMPK activation markedly reversed the anti-inflammatory and antioxidant effects of IMD$_{1-53}$ on PA and Ox-LDL-treated HUVECs.

Conclusion: IMD$_{1-53}$ improves AS partially by reducing endothelial inflammatory and oxidative damage via AMPK signaling pathway and decreasing vascular lipid accumulation involving the improvement of lipid profiles in blood and in liver in a state of obesity.

Keywords: intermedin, atherosclerosis, endothelium, inflammation, oxidative stress

Introduction

Cardiovascular diseases (CVD) have become one of the main causes of mortality worldwide, and atherosclerosis (AS) is a crucial underlying pathology of cardiovascular events such as stroke and myocardial infarction in obese people.[1,2] It is well known that metabolic dysregulation and a chronic inflammatory state exist in obesity. Proinflammatory factors, adipokines and fatty acids are involved in the regulation of inflammatory responses, creating an inflammatory condition and promoting the initiation and progression of AS.[3] Moreover, hyperlipidemia causes endothelial inflammatory and oxidative damage that is a well-defined risk factor of AS,[4] especially apoB-containing low-density lipoproteins (LDL), which can drive the development of AS even in the absence of other risk factors.[5] As a long chain saturated fatty acid, palmitic acid (PA) is a major component of free fatty acids in plasma that also promotes atherosclerotic progression and endothelial damage and dysfunction in people with obesity.[6,7] Clinical trials for lipid-lowering drugs such as statins which can reduce atherosclerotic damage, but due to statins resistance or intolerance in some patients, as well as side

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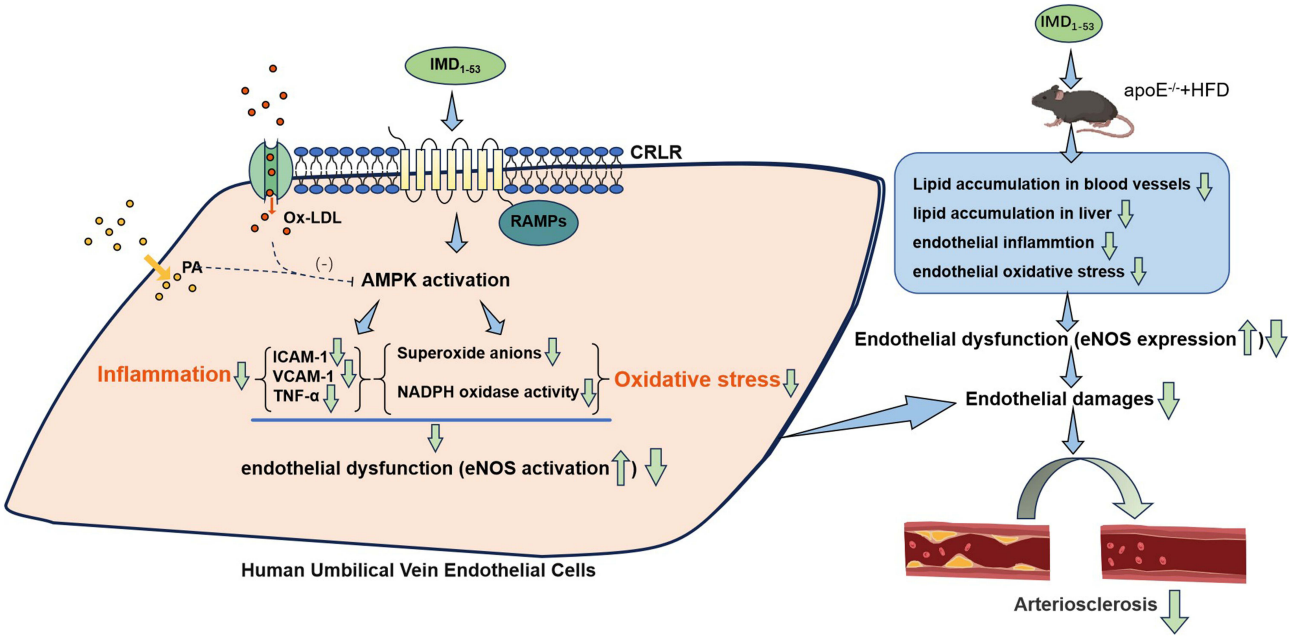
Graphical Abstract



effects in long-term use, the application of statins are limited.[8] Furthermore, statins treatment alone decreased the risk of main vascular diseases by only about 40%-50%,[9] suggesting that residual cardiovascular risks remain. Therefore, developing effective non-statin lipid-lowering therapies with fewer side effects are required for many patients who cannot be treated with statins alone. Meanwhile, in addition to lipid-lowering therapies, novel therapeutic approaches are needed to be identified.

As an independent atherosclerotic risk factor, obesity involves the enhanced systemic inflammation in obese individuals.[10] AS is a lipid-driven, chronic inflammatory disease involving endothelial cells (ECs), smooth muscle cells and macrophages.[11] Lipid accumulation and blood saturated fatty acids cause inflammatory and oxidative damages that are confirmed to be a key mechanism of AS, so it also provides a complementary therapeutic approach, namely lipid-lowering, anti-inflammatory and antioxidant therapies.[12–14] At the innermost layer of aorta, endothelial cells serve as gatekeepers of aortic homeostasis by mediating vascular tone, leukocyte adhesion/extravasation, vascular permeability and hemostasis. Inflammatory or oxidative damages of ECs and endothelial dysfunction are crucial events in the initial step of AS, so during the initiation and progression of AS, endothelial cells are constantly subjected to inflammatory and oxidative damages.[14,15] Endothelial dysfunction is an important risk factor for the progression of CVD, and ECs play multiple important roles throughout the whole process of AS, so protecting endothelial cells from inflammation and oxidative stress is pivotal for the therapeutic strategies.[14–16]

As an endogenous active peptide, intermedin (IMD) belongs to the calcitonin/calcitonin gene-related peptide (CGRP) family and involves cardiovascular homeostasis. It can exert multiple protective roles in some cardiovascular diseases.[17] After proteolytic cleavage, IMD_{1-53} is produced from the prepro-peptide of IMD at Arg94-His95.[18] IMD can exert various protective effects on endothelial cells under different harmful stimuli.[19–22] For instance, IMD in vitro protects human umbilical vein endothelial cells (HUVECs) against amiodarone-induced damage or ischemia reperfusion injury,[19,20] and it in vivo can relieve sepsis-caused inflammation in the endothelial barrier,[21] and protect endothelial cell monolayers against thrombin-induced barrier failure.[22] Previous studies have reported that IMD1-53 in $apoE^{-/-}$ mice could reduce atherosclerotic lesions by attenuating the macrophage foam-cell formation[23,24] and modifying serum lipid profiles.[25] Moreover, IMD1-53 can also attenuate atherosclerotic plaque vulnerability by alleviating endoplasmic

reticulum stress regulated macrophage apoptosis, and subsequent NLRP3-caused inflammation.[26] However, this study mainly explored the direct roles of IMD in endothelial inflammatory and oxidative damages and function, which could further complement the mechanism research of IMD in inhibiting AS. After all, obesity and endothelial dysfunction are important risk factors for the development of AS. Moreover, our previous studies have not demonstrated the direct role and mechanism of IMD in endothelial cells in the environment of high lipid metabolism.

As is well known, blood saturated fatty acids cause inflammatory and oxidative damages, which is a key factor of AS. Therefore, we want to focus on the role and mechanism of IMD under the condition of the combination of PA and Ox-LDL in endothelial cells. Mechanistically, we will clarify the exact signaling pathway of IMD, namely PKA, Akt or AMPK signaling, by which IMD exerts protective effects on endothelial cells treated with PA and Ox-LDL.

It is worth studying and elucidating the effect on endothelium and the related mechanism of IMD in AS in a state of obesity. So in this study, we aim to demonstrate the role and the related mechanism of IMD1-53 in endothelial damage in AS in apoE$^{-/-}$ mice with high-fat diet (HFD) feeding, and in vitro by using Ox-LDL together with PA in endothelial cells to simulate AS in obesity.

Materials and Methods

Materials

Human IMD_{1-53} was synthesized in Phoenix Pharmaceuticals Corp (Burlingame, CA, USA). Alzet Mini-osmotic Pumps (model 2004) were purchased from DURECT Corp (Cupertino, CA, USA). PA was purchased from (Kunchuang, Xian, China), Ox-LDL was from (Yeasen, Shanghai, China), and Compound C (AMPK activation inhibitor) was purchased from (Aladdin, Shanghai, China). Fetal bovine serum, streptomycin/penicillin, 0.25% trypsin-EDTA, DMEM and trypsin were bought from Thermo Fisher Scientific (Pudong New District, Shanghai, China). Primary antibodies for Mac-3, intercellular cell adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein 1 (MCP-1) were obtained from Abcam PLC (Cambridge, UK); and NOX2, NOX4 and GAPDH were purchased from Proteintech (SANYING, Wuhan, China); and CRLR, RAMP2, RAMP3, vascular cell adhesion molecule-1 (VCAM-1), TNF-α, T-Akt, P-Akt, Akt, P-Akt (ser473), AMPK, P-AMPKα (Thr172), PKA, P-PKA (Thr198), P-eNOS and eNOS were bought from Affinity Biosciences (Pottstown, PA, USA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa C, CA, USA). The oil red O was bought from Sigma Aldrich (St. Louis, MO, USA). Other reagents and chemicals reached the analytical standards.

Animals and Treatment

Twenty-week-old male apoE$^{-/-}$ mice were obtained by Beijing HFK Bioscience Co., LTD, and they were randomly divided into three groups (n = 10 per group): (1) apoE$^{-/-}$ group: mice with normal diet for 4 weeks; (2) apoE$^{-/-}$ plus high-fat diet (HFD) group (apoE$^{-/-}$+HFD group): mice with a HFD (fat provided 60% kilocalories) for 4 weeks; (3) apoE$^{-/-}$ plus HFD plus IMD_{1-53} group (apoE$^{-/-}$+HFD+IMD_{1-53} group). At the same time as the HFD treatment, IMD_{1-53} (300 ng/kg/h) was dissolved in sterile saline and subcutaneously applied to the mice for 4 weeks by using Alzet mini-osmotic pumps (model 2004, Cupertino, CA, USA). All mice were fed at the Animal Center of Peking University Health Science Center (Beijing, China). The Animal Care Committee of Peking University Health Science Center (Beijing, China) approved the animal experimental protocols and care, and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) were complied in this study.

Lipid Assay

At the end of experiment, the plasma from blood samples was collected by centrifugation. Separation of plasma lipoproteins including high density lipoprotein (HDL), very low density lipoprotein (VLDL) and low density lipoprotein (LDL) was performed using fast performance liquid chromatography (FPLC) as previously described.[27] Plasma triglycerides (TG) and total cholesterol (TC) were tested by using colorimetric methods with kits from Zhong Sheng Biotechnology (Beijing, China).

Oil Red O Staining

Lipid accumulation in aorta, arterial roots or liver was shown by Oil-red O staining. Aortas from the heart to the iliac arteries were collected for en face analysis and opened longitudinally; kept the aortas for 2 hours in the working solution including Oil-red O following 70% ethanol for 5 min and washed with Deionized water. For liver or arterial roots samples analysis, the optimal cutting temperature (OCT) compound was used to embed the liver tissues and arterial roots with heart for obtaining frozen sections. 10 μm cross sections were prepared, and Oil red O was used for staining. As previously described,[22] vascular lipid accumulation was assessed and quantified.

Hematoxylin/Eosin (HE) Staining

4% paraformaldehyde was used to fix the liver tissues for 24 hours, and the liver tissues were embedded in paraffin. As previously described,[24] 7-μm cross sections were cut for HE staining for morphological analysis.

Immunohistochemistry Staining

7-μm paraffin sections of arterial roots with the heart were used for Immunohistochemical staining. After rehydrated and antigen-retrieved, the sections underwent the incubation process with primary antibodies for Mac-3 (1:100 dilution), MCP-1 (1:100 dilution) and ICAM-1 (1:100 dilution) at 4°C overnight, then underwent another incubation process with HRP-conjugated secondary antibodies for 1 hour. They were stained with chromogen diaminobenzidine (Zhongshan Golden Bridge Biotechnology, Beijing, China). A Leica fluorescence microscopy (Leica Imaging Systems, Cambridge, UK) was used to acquire the images.

Cell Culture and Treatment

HUVECs were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Briefly, Endothelial Cells Medium (ECM, cat. #1001, Sciencell, USA) containing 100 U/mL penicillin and 100 μM/mL streptomycin and 10% fetal bovine serum (FBS) was used to culture HUVECs in an incubator with 5% CO_2 at 37°C. 100 nM $IMD_{1\text{-}53}$ was applied to cells for 30 min, then the cells were treated with PA combined with Ox-LDL for 24 h. For inhibition of signaling, Compound C (CC, 10 μM), namely AMPK activation inhibitor, was applied before $IMD_{1\text{-}53}$ administration.

Measurements of ROS Level and NADPH Oxidase Activity

In HUVECs, the ROS level and the activity determination of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase were examined with the enhanced lucigenin-derived chemiluminescence method. To trigger the photon emission, both 100 μM NADPH and dark-adapted 5 μM lucigenin were added into the protein suspension, and the background chemiluminescence was examined using a luminometer (Turner, CA, USA). The data were obtained according to the average of ten measurements in 10 minutes and presented as the mean of light unit (MLU)/min/mg protein.

DHE Staining

DHE staining was used to detect reactive oxygen species (ROS) generation in HUVECs and arterial roots. 10 μM DHE in PBS was used to incubate HUVECs in six-well plates and paraffin-embedded tissue sections at 37°C in a dark environment for 30 min. Then, they were washed with PBS three times and observed by using a fluorescence microscopy (DP70, Olympus Optical, Tokyo, Japan).

Western Blotting

According to the manufacturer's protocol, total proteins were extracted from cells or aorta with RIPA buffer (Epizyme, Shanghai, China) and quantified with a BCA protein assay kit (Beyotime, Shanghai, China). After electrophoresis and electrotransfer, the membranes were blocked with 5% non-fat milk for 3 hours at room temperature and incubated with CRLR (1:1000), RAMP1/2/3 (1:1000), ICAM-1 (1:2000), VCAM-1 (1:2000), NOX2 (1:2000), NOX4 (1:1000), eNOS (1:1000), TNF-α (1:1000), Akt (1:2000), p-Akt (Ser473, 1:2000), AMPK (1:2000), P-AMPKα (Thr172, 1:2000), PKA

(1:2000), P-PKA (Thr198, (1:2000), P-eNOS (Ser1177, 1:2000) and GAPDH (1:3000) antibodies at 4°C overnight and then incubated with the corresponding HRP-conjugated secondary antibodies. Protein band intensities were normalized with nonphosphorylated total-PKA, total-Akt, total-AMPK or GAPDH levels. Odyssey Imaging System (LI-COR Biosciences, Lincoln, Nebraska) was used to quantify the signals, and the protein expression level was assessed by Image J software.

Statistical Analysis

The GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, CA, USA) was used for Statistical analysis. All data are expressed as mean ± SEM. The unpaired Student's *t*-test was used to identify significant differences between the two groups. Differences among the different animal groups were evaluated using one-way ANOVA followed by the Newman-Keuls test for more than 2 groups. Statistical significance was accepted at $p < 0.05$.

Results

IMD_{1-53} Alleviated Vascular Lipid Accumulation in $apoE^{-/-}$ Mice Fed with HFD

Intimal lipoprotein accumulation is the initial process in AS and drives the development of AS.[13,14,28,29] To explore the effect of IMD_{1-53} on lipid accumulation in vascular wall, twenty-week-old male $apoE^{-/-}$ mice by feeding a HFD for 4 weeks were used to induce AS. Firstly, body weight (BW) was markedly increased in the $apoE^{-/-}$+HFD group when compared to the $apoE^{-/-}$ group. However, there was no significant alteration in BW after IMD_{1-53} application (Figure 1A), which is consistent with previous study.[22] After isolating aortas and opening longitudinally, we found much more white fatty streaks or fibrous plaque in the intima of $apoE^{-/-}$ mice with HFD, which were obviously

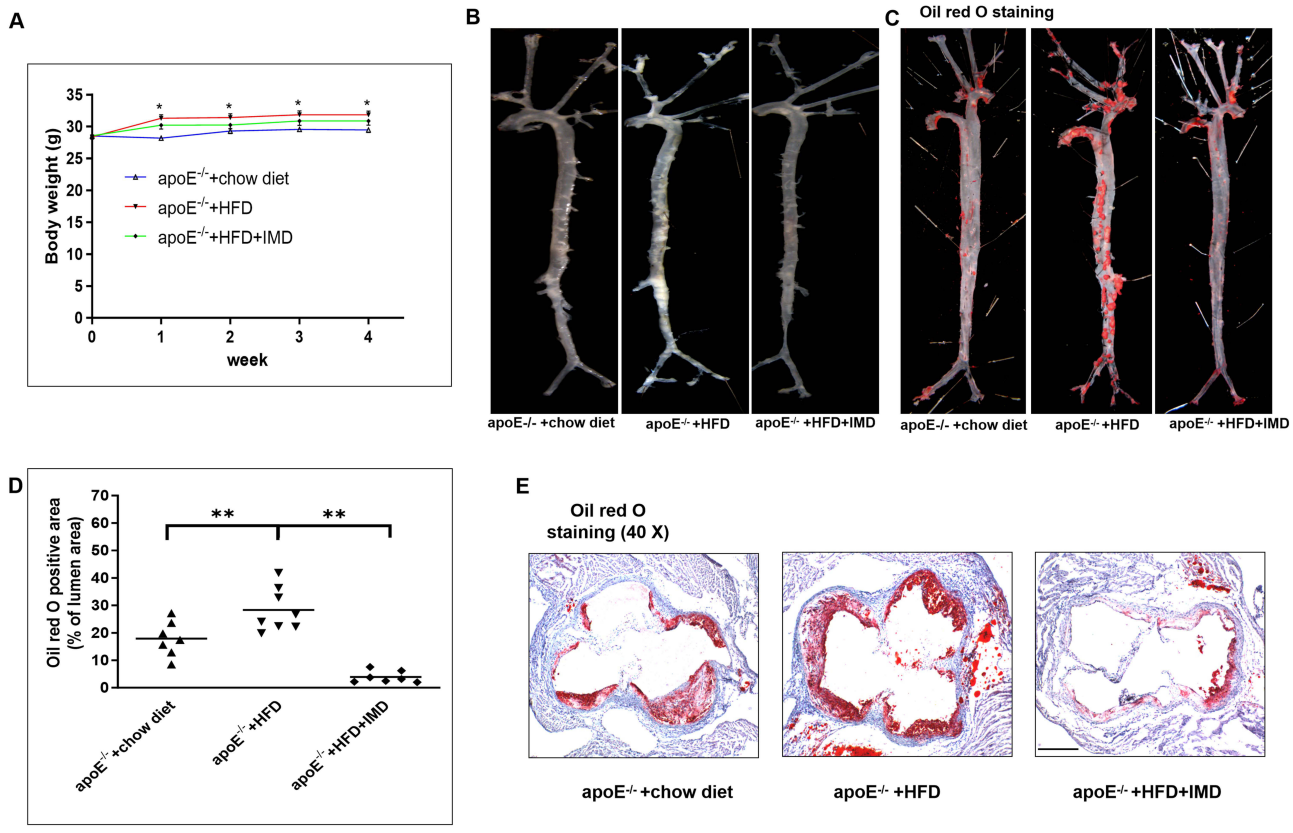


Figure 1 The effects of IMD_{1-53} on vascular lipid accumulation in $apoE^{-/-}$ mice. Twenty-week-old male $apoE^{-/-}$ mice were fed a normal diet or a high-fat diet (HFD) without IMD_{1-53} or with IMD_{1-53} for 4 weeks. (**A**) Body weight of three groups, *p<0.05 vs $apoE^{-/-}$ group; (**B**) Representative view of en face-prepared aortas without staining; (**C**) Representative image of en face-prepared aortas with Oil Red O staining; (**D**) Quantification of Oil Red O positive area in aortic roots; (**E**) Representative image of Oil Red O staining in aortic roots. Scale bar = 125 μm. **p<0.01. Data are mean±SEM. n=7-10. $ApoE^{-/-}$ mice, namely apolipoprotein E-deficient mice.

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improved by IMD_{1-53} application (Figure 1B). Oil Red O staining further confirmed a marked reduction of vascular lipid accumulation in IMD_{1-53}-treated obese $apoE^{-/-}$ mice (Figure 1C). Moreover, IMD_{1-53} treatment resulted in an 84% reduction of lipid content in the aortic root (Figure 1D and E). Collectively, these results demonstrated that IMD_{1-53} decreased vascular lipid accumulation and atherosclerotic lesion size in obese $apoE^{-/-}$ mice.

IMD_{1-53} Improved Lipid Profiles in Plasma in $apoE^{-/-}$ Mice with HFD Feeding

Hyperlipidemia is a well-known risk factor of AS, and could induce endothelial dysfunction, which makes intimal retention of lipoprotein easily.[29,30] Therefore, to explore the mechanism of IMD_{1-53} alleviating vascular lipid accumulation, we evaluated the circulatory lipid concentrations at different time points after HFD feeding and IMD_{1-53} treatment. As shown in Figure 2A, we could visually observe the notable improvement of lipids in plasma after IMD_{1-53} treatment. Indeed, IMD_{1-53} significantly improved the plasma lipid profiles at different time points including lowering the levels of TG, TC, LDL-C and VLDL-C compared with $apoE^{-/-}$ mice with HFD feeding (Figure 2B–F). Meanwhile, IMD_{1-53} markedly increased HDL-C levels (Figure 2G). These results suggest that IMD_{1-53} can reduce vascular lipid accumulation partly via improving lipid profiles in blood.

IMD_{1-53} Ameliorated Liver Lipid Accumulation in $apoE^{-/-}$ Mice with HFD Feeding

As well known, the liver is the main organ for cholesterol metabolism. Surprisingly, a significantly increased visceral adipose accompanied by fatty liver was visually observed in $apoE^{-/-}$ mice with HFD feeding, which was significantly reversed by IMD_{1-53} application (Figure 3A). Moreover, IMD_{1-53} treatment also markedly decreased the liver weight to BW ratio, hepatic TG and TC levels (Figure 3B–D). HE and Oil Red O staining also showed that $apoE^{-/-}$ mice with HFD feeding had a higher degree of steatosis, which was effectively relieved by IMD_{1-53} treatment (Figure 3E and F). These data indicate that IMD_{1-53} could ameliorate liver lipid accumulation in $apoE^{-/-}$ mice.

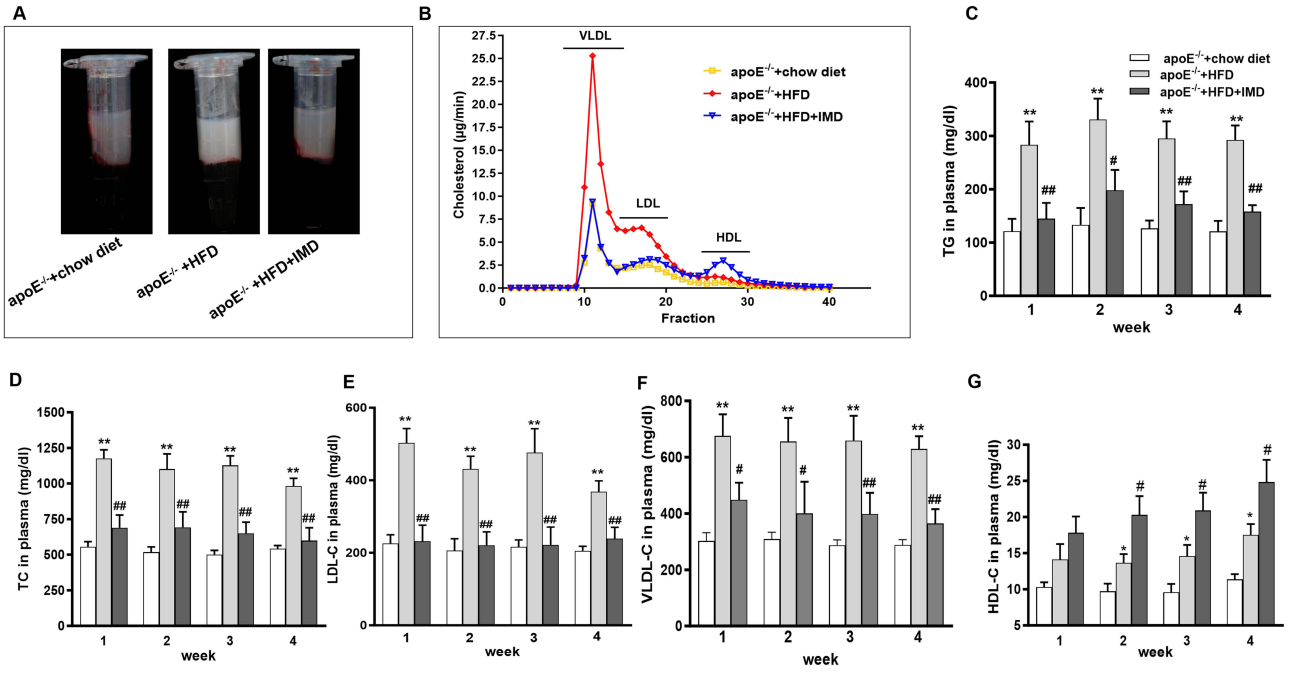

Figure 2 The effects of IMD_{1-53} on lipid profiles in plasma in $apoE^{-/-}$ mice. Twenty-week-old male $apoE^{-/-}$ mice were fed a normal diet or a HFD without IMD_{1-53} or with IMD_{1-53} for 4 weeks. (**A**) Representative view of plasma after centrifugation; (**B**) FPLC profiles of plasma lipoproteins from different groups; (**C** and **D**) Plasma triglyceride (TG) and total cholesterol (TC) levels were measured; (**E-G**) Lipoprotein fractions (LDL-C, VLDL-C and HDL-C) were isolated and the cholesterol levels of each fraction were measured. *p<0.05 and **p<0.01 vs $apoE^{-/-}$ group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs $apoE^{-/-}$+HFD group. Data are mean±SEM. n=9-10.

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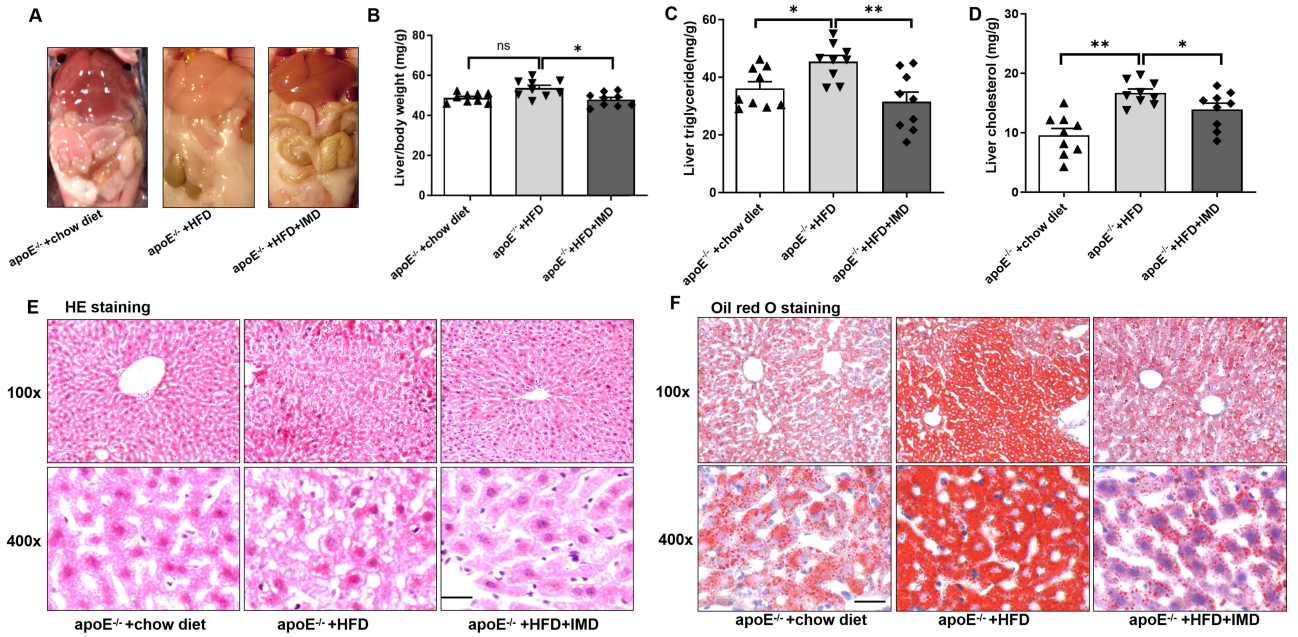

Figure 3 The effects of IMD_{1-53} on liver lipid accumulation in $apoE^{-/-}$ mice. Twenty-week-old male $apoE^{-/-}$ mice were fed a normal diet or a HFD without IMD_{1-53} or with IMD_{1-53} for 4 weeks. (**A**) Representative view of visceral adipose after dissection; (**B**) Liver weight to body weight ratio of mice from each group; (**C** and **D**) liver triglyceride and cholesterol levels were measured; (**E**) Representative image of HE-stained liver steatosis, white vacuole in hepatocytes showed the location of lipid droplets; (**F**) Representative image of Oil Red O-stained liver steatosis, lipid droplets of hepatocytes were stained into salmon pink. Scale bar = 50 μm. *p<0.05 and **p<0.01. ns, no significance. Data are mean± SEM. n=9-10.

IMD_{1-53} Improved Endothelial Dysfunction by Inhibiting Inflammatory and Oxidative Damage in $apoE^{-/-}$ Mice with HFD Feeding

Vascular endothelium is an important barrier that is essential for the integrity and function of blood vessels. Hyperlipidemia can induce endothelial dysfunction which upregulates inflammation-related proteins or chemokines such as ICAM-1, MCP-1, and Mac-3.[30] Indeed, our immunostaining results revealed that ICAM1, MCP-1 and Mac-3 were notably increased in $apoE^{-/-}$ mice with HFD feeding, and they were significantly reduced with IMD_{1-53} treatment (Figure 4A–C). Hyperlipidemia can also induce oxidative stress and decrease the expression of eNOS (being responsible for NO production which relaxes blood vessels and has antithrombotic and anti-inflammatory effects) in endothelium.[31,32] In this study, we found that aortic superoxide anion levels were remarkably increased (Figure 4D and E), but eNOS expression was dramatically decreased in $apoE^{-/-}$ mice with HFD feeding, which were also markedly reversed by IMD_{1-53} treatment (Figure 4F). These results indicate that IMD_{1-53} improves endothelial dysfunction partially by reducing inflammatory response and oxidative damage and increasing eNOS expression.

IMD_{1-53} Inhibited Inflammatory and Oxidative Damage and Increased eNOS Activation via AMPK Signaling Pathway in HUVECs Stimulated by PA and Ox-LDL

PA and Ox-LDL-caused endothelial dysfunction is mainly involved in inflammation and oxidative stress.[6,28] In the present study, PA was adopted to mimic the high-saturated fatty acids state of obesity, and Ox-LDL simulated dyslipidemia in AS. To verify whether IMD_{1-53} could suppress PA and Ox-LDL-evoked inflammatory and oxidative damage in HUVECs, we examined the protein levels of inflammation-related cytokines including VCAM-1, ICAM-1 and TNF-α, and oxidative stress-related indicators including the protein levels of NOX2 and NOX4 which are the essential catalytic subunits of NADPH oxidase, superoxide anion levels and NADPH oxidase activity. We found that they were further significantly increased in HUVECs-incubated by PA and Ox-LDL compared with PA treatment alone (Figure 5A–H), and which were effectively inhibited by the application of IMD_{1-53} (Figure 6A–H), suggesting that IMD_{1-53} has anti-inflammatory and antioxidant capacities in endothelium in AS state. The activation of PKA, Akt or AMPK signaling

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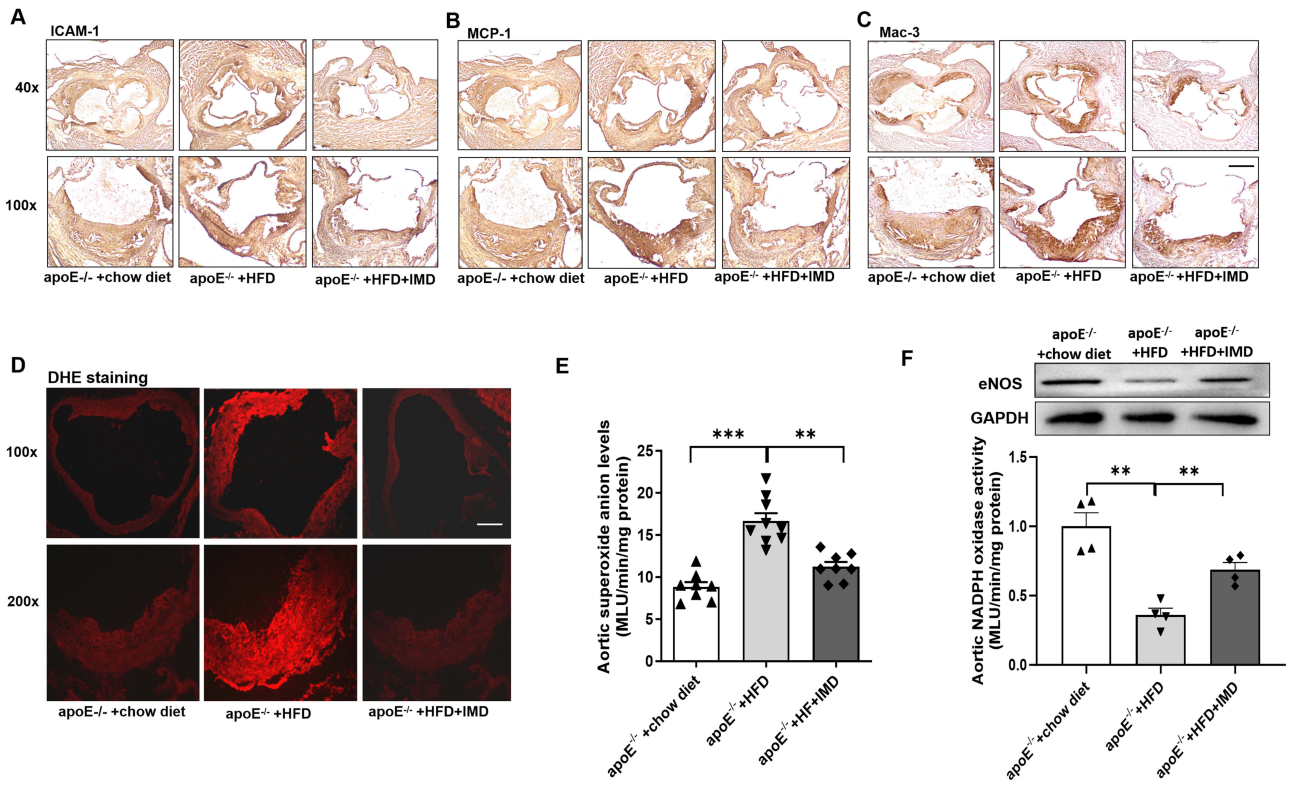

Figure 4 The effects of IMD_{1-53} on the atherosclerotic plaque inflammation, oxidative stress and endothelial eNOS protein expression in $apoE^{-/-}$ mice. Twenty-week-old male $apoE^{-/-}$ mice were fed a normal diet or a HFD without IMD_{1-53} or with IMD_{1-53} for 4 weeks. Representative immunohistochemical staining images of ICAM-1 (**A**), MCP-1 (**B**) and Mac-3 (**C**); DHE staining of intracellular ROS and superoxide anion levels measured by the enhanced lucigenin-derived chemiluminescence method (**D** and **E**); The levels of vascular eNOS protein expression determined by Western blotting method (**F**). ICAM-1, inter-cellular adhesion molecule 1; MCP-1, monocyte chemotactic protein 1; Mac-3, macrophage specific antigen. Scale bar = 50 μm.**p<0.01 and ***p<0.001 vs indicated group. Data are mean±SEM. n=4-10.

pathways involves IMD's effects on inflammation and oxidative stress in previous studies.[33–35] So the phosphorylation of PKA, Akt and AMPK was determined by Western blotting method in HUVECs stimulated by PA and Ox-LDL, respectively. As shown in Figure 7, IMD_{1-53} administration notably enhanced AMPK phosphorylation, rather than PKA and Akt phosphorylation (Figure 7A–C). Moreover, AMPK activation inhibitor significantly inhibited AMPK phosphorylation caused by IMD_{1-53} (Figure 7D), indicating that AMPK activation in endothelial cells may be associated with the protective mechanism of IMD_{1-53} on endothelial dysfunction. Indeed, the pretreatment of CC (an inhibitor of AMPK activation) effectively blocked the roles of IMD_{1-53} in inflammatory and oxidative damage in HUVECs stimulated by PA and Ox-LDL (Figure 8A–H). In addition, eNOS activation was determined by examining its phosphorylation level. The significant reduction of eNOS phosphorylation in HUVECs stimulated by PA and Ox-LDL was improved by IMD_{1-53} application, which was also inhibited by the inhibition of AMPK activation (Figure 8I). These results suggest that IMD_{1-53} via AMPK pathway can effectively inhibit PA and Ox-LDL-induced inflammatory and oxidative damage in HUVECs, and it may perform a protective role in endothelial dysfunction in AS in a state of obesity.

Discussion

Endothelial damage is associated with inflammation and oxidative stress, which promotes the progression of AS and predicts the future of cardiovascular events.[14,36,37] We explored the role and the related mechanism of IMD_{1-53}, a cardiovascular active peptide, in the endothelial damage of AS. ApoE-deficient dyslipidemic mice fed with an HFD were used in vivo study, and HUVECs-stimulated by PA and Ox-LDL were used in vitro research. The results showed that the coexistence of dyslipidemia and obesity indeed remarkably augmented AS involving the deterioration of lipid profiles in blood, increased vascular and hepatic lipid accumulation and hepatic cholesterol levels, and aggravated endothelial inflammation and oxidative stress, which effectively inhibited by IMD_{1-53} administration. In this study, PA

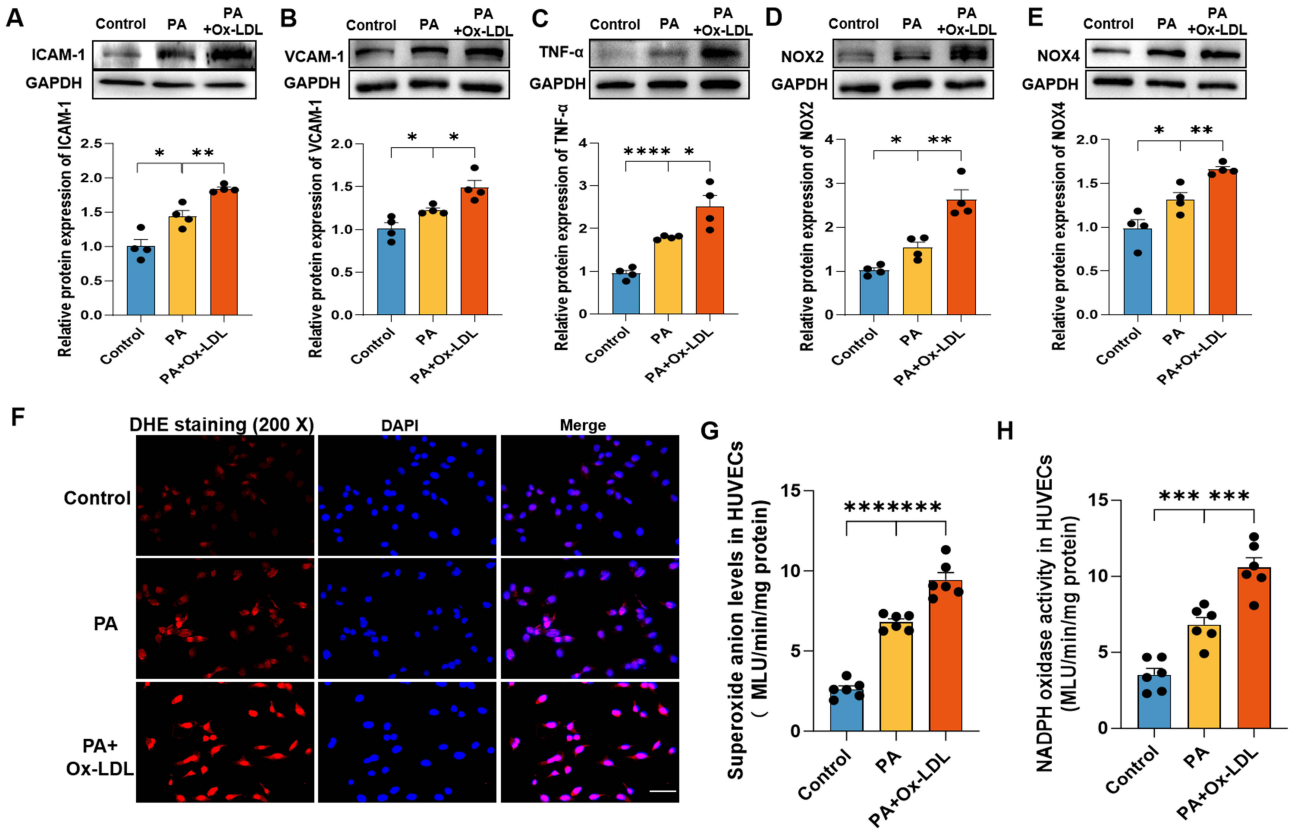

Figure 5 The effects of PA and Ox-LDL combination on inflammation and oxidative stress in HUVECs. The protein expression levels of inflammation-related factors ICAM-1, VCAM-1 and TNF-α in HUVECs stimulated by PA (200 μM) and Ox-LDL (100μg/mL) for 24 h. They were determined by Western blotting method to assess inflammation (**A-C**). Oxidative stress was evaluated by the levels of protein expression of NOX2 and NOX4, two essential catalytic subunits NADPH oxidase (NOX) (**D** and **E**), intracellular ROS levels detected by DHE staining (**F**), superoxide anion levels (**G**) and NADPH oxidase activity (**H**). Scale bar = 100 μm. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs indicated group. Data are mean±SEM. n=4-6.

Abbreviations: PA, palmitic acid; Ox-LDL, oxidized low density lipoprotein; ICAM-1, inter-cellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

and Ox-LDL further enhanced inflammatory and oxidative responses in HUVECs that were also significantly suppressed by IMD_{1-53} application. Moreover, IMD_{1-53} increased vascular eNOS expression and promoted AMPK activation, and AMPK inhibitor significantly reversed the roles of IMD_{1-53} in endothelial cells in vitro.

IMD, a novel active peptide of CGRP family, has important physiological and pathophysiological effects on the cardiovascular system.[18,38] For instance, IMD exerts vasodilator effects to lower blood pressure, enhances myocardial contractility and increases the perfusion of heart.[38] IMD application also shows protective effects in some CVD models, including ischemia-reperfusion injury,[39,40] vascular calcification,[41,42] abdominal aortic aneurysm,[43] cardiac hypertrophy[44] and cardiac fibrosis.[33] Previous studies have found that IMD alleviated atherosclerotic lesions by suppressing the macrophage foam-cell formation in $apoE^{-/-}$ mice,[19,20] increasing cholesterol efflux[45] and modifying serum lipid profiles.[21] Previous studies found that IMD_{1-53} could attenuate atherosclerotic plaque vulnerability via the inhibition of apoptosis triggered by endoplasmic reticulum stress, and subsequent NLRP3-caused inflammation in macrophages.[22] Endothelium suffers from inflammatory and oxidative damages that serves interrelated roles in the progression of AS and other vascular diseases.[12,14,37] However, the previous studies have not explored the exact role and the related mechanism of IMD_{1-53} in the endothelium, especially in AS under obesity condition. In this study, we not only discovered that IMD_{1-53} effectively inhibited endothelial inflammation and oxidative stress, but also clarified the possible mechanism that AMPK signaling pathway by which IMD_{1-53} plays a protective role in endothelial cells.

ApoE deficiency exacerbates AS. However, the coexistence of dyslipidemia and obesity synergistically exacerbates the severity of AS. It has been reported that obesity induced by HFD can intensify AS.[22] The synergistic roles of

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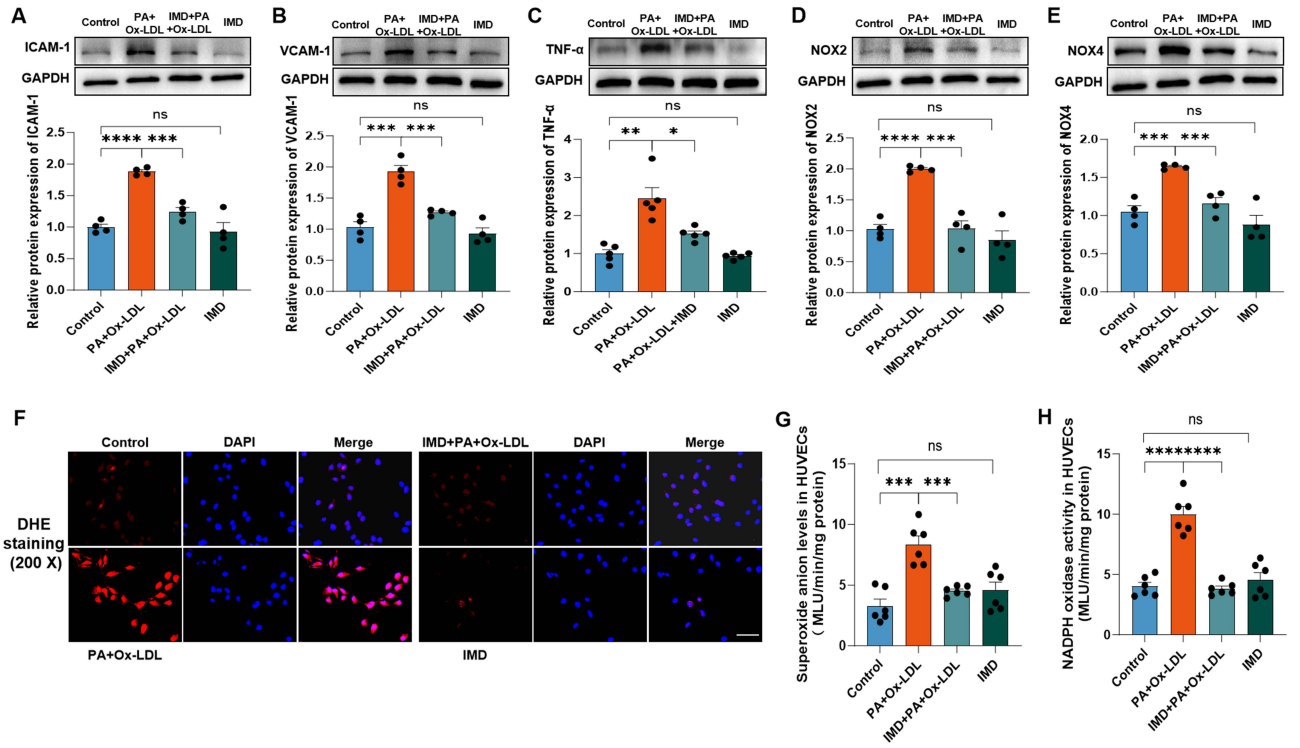

Figure 6 The effects of IMD_{1-53} (100 nmol/L) on inflammation and oxidative stress in HUVECs stimulated by PA and ox-LDL. The protein expression levels of inflammation-related factors ICAM-1, VCAM-1 and TNF-α were determined to assess inflammation (**A**–**C**). The levels of protein expression of NOX2 and NOX4 (**D** and **E**), intracellular ROS levels (**F**), superoxide anion levels (**G**) and NADPH oxidase activity (**H**) were detected to evaluate oxidative stress. Scale bar = 100 μm. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs indicated group. ns, no significance. Data are mean±SEM. n=4-6.

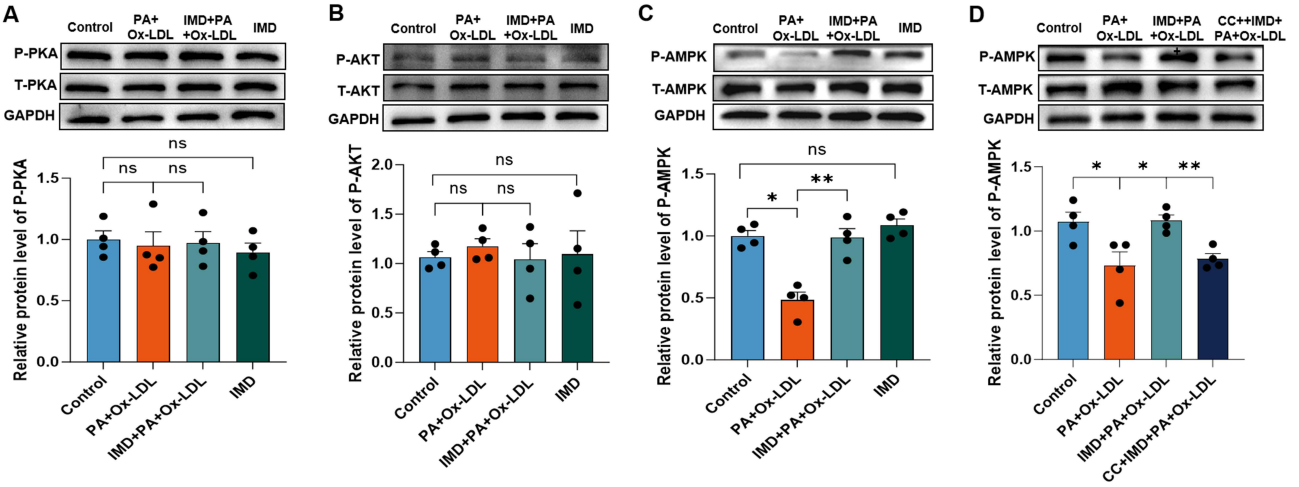

Figure 7 The effects of IMD (100 nmol/L) on the activation of the PKA, Akt or AMPK in HUVECs stimulated by PA and ox-LDL, and the effect of AMPK activation inhibitor CC on the IMD_{1-53} response to AMPK activation. The protein expression levels of phosphorylation of PKA, Akt and AMPK were determined by Western blotting method to assess the effects of IMD_{1-53} on the activation of PKA, Akt or AMPK signaling pathway (**A**–**C**). For IMD_{1-53} treatment, the cells were treated with 100 nM IMD_{1-53} for 30 min, then treated with PA combined with Ox-LDL for 24 h. For inhibition of signaling, 10 μM AMPK activation inhibitor Compound C (CC, 10 μmol/L) was added into the medium for 30 min before IMD_{1-53} application (**D**). Scale bar = 100 μm. *p<0.05 and **p<0.01 vs indicated group. ns, no significance. Data are mean±SEM. n=4-6.

dyslipidemia and obesity in endothelial damage are closely associated with a mass of metabolic factors, such as low high-density lipoprotein cholesterol levels, high triglyceride and glucose levels, and so on. In this study, apoE deficient mice with HFD diet resulted in a more severe lipid disorder in blood and more pronounced endothelial inflammatory response, oxidative stress and dysfunction correlated with decreased expression of eNOS, increased inflammatory factors VCAM-

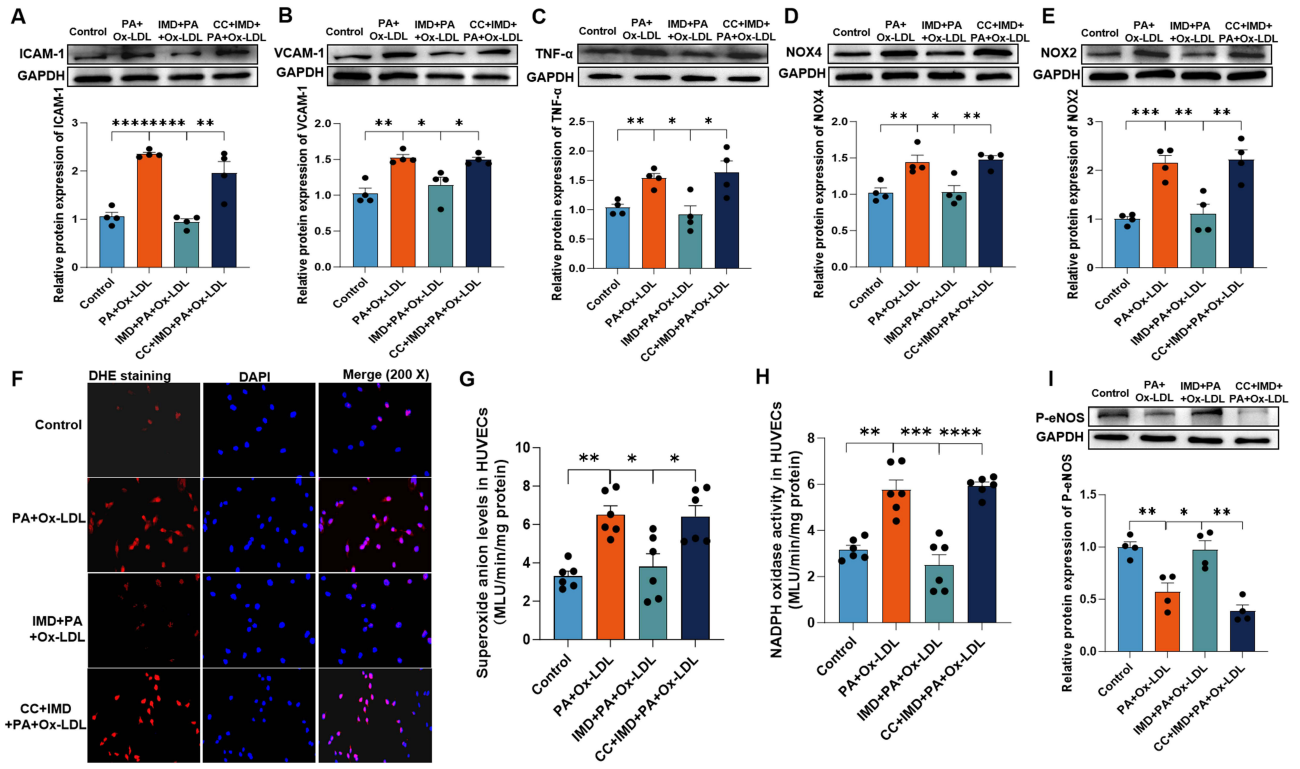

Figure 8 The effects of AMPK activation inhibitor CC on the IMD_{1-53} responses to PA and OX-LDL-caused inflammation, oxidative stress and the decrease in eNOS activation in HUVECs. The protein expression levels of inflammation-related factors ICAM-1, VCAM-1 and TNF-α were determined to assess inflammation (**A**–**C**), the levels of protein expression of NOX2 and NOX4 (**D** and **E**), intracellular ROS levels (**F**), superoxide anion levels (**G**) and NADPH oxidase activity (**H**) were detected to evaluate oxidative stress, and endothelial nitric oxide synthase (eNOS) activation was determined by examining its phosphorylation protein level (**I**). For inhibition of signaling, 10 μM AMPK activation inhibitor Compound C (CC, 10 μmol/L) was added into the medium for 30 min before IMD_{1-53} application. Scale bar = 100 μm. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs indicated group. Data are mean±SEM. n=4-6.

1, MCP and Mac-3, and elevated ROS levels. However, IMD_{1-53} administration not only significantly attenuated vascular and hepatic lipid accumulation, but also improved plasma lipid profiles. More importantly, IMD_{1-53} application effectively relieved endothelial inflammatory and oxidative damages, and increased eNOS expression. Therefore, our results suggest that IMD_{1-53} exerting a protective effect on endothelial damage may partially involve the suppression of endothelial inflammation and oxidative stress, and the improvement of lipid profiles in blood and lipid metabolism in liver. Previous studies indicate that metabolic dysfunction-associated steatotic liver disease is closely related to AS and cardiovascular disease.[46,47] However, the precise effects and molecular mechanisms of IMD_{1-53} in improving lipid metabolism require further exploration.

As a saturated fatty acid, PA involves the inflammatory process in CVD.[36] Under obesity condition, the increased PA can trigger a pro-inflammatory response in endothelium by activating immune cells such as macrophages.[48] Ox-LDL is widely confirmed to involve inflammation and oxidative stress in endothelial cells.[28] In this study, the results indicated that PA and Ox-LDL promoted marked endothelial damage which manifested by an obvious decrease in eNOS activation and notable increases in proinflammatory related factors and superoxide anion levels in HUVECs as well as the increased activity and protein expression (essential catalytic subunits NOX2 and NOX4) of NADPH oxidase. However, IMD_{1-53} application promoted the improvement of endothelial damage, including increasing eNOS activation and diminishing inflammatory factors levels, superoxide production and NADPH oxidase activity. So these data indicate IMD_{1-53} is a potent peptide in improving endothelial dysfunction in AS in obesity state.

AMP-activated protein kinase (AMPK) is a crucial enzyme in cellular metabolism and energy balance. It has been shown to exert protective roles in CVD involving its anti-inflammatory and antioxidant properties.[49,50] The activation of the AMPK pathway can inhibit lipid accumulation and improve intracellular lipid metabolism.[51] Moreover, AMPK activation can perform multiple anti-atherosclerotic roles to promote vascular health.[50] A number of cholesterol-lowering

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drugs activate AMPK that facilitate their beneficial roles in the cardiovascular system.[51,52] The downstream signals are activated by the interaction of IMD with its receptor, and the main signals identified include cAMP/PKA, PI3K/Akt and AMPK.[18,38] It was reported that IMD can activate the phosphorylation of AMPK to exert its protective roles in some studies.[35] In the present study, PA and Ox-LDL treatment caused the reduced AMPK activation in HUVECs, which was associated with decreased levels of AMPK phosphorylation, whereas $IMD_{1\text{-}53}$ significantly upregulated AMPK phosphorylation but not Akt or PKA phosphorylation. Moreover, the inhibition of AMPK activation effectively prevented the anti-inflammatory and antioxidant roles of $IMD_{1\text{-}53}$, which may account for the finding that $IMD_{1\text{-}53}$ activating AMPK signaling pathway could improve endothelial damage via the suppression of inflammation and oxidative stress.

An intact endothelium is a major factor for the conservation of vascular homeostasis, and endothelial nitric oxide synthase (eNOS)-derived NO can relax vessels and exert antithrombotic and anti-inflammatory roles in the vasculature.[53,54] The reduced NO bioavailability will lead to endothelial dysfunction involving the progression of hypertension and AS.[54] AMPK activation can directly lead to eNOS phosphorylation which increases NO bioavailability in endothelial cells. However, AMPK inhibition reduces eNOS phosphorylation, leading to the impaired NO generation. In this study, $IMD_{1\text{-}53}$ increased eNOS activation in PA and Ox-LDL-treated HUVECs, which was reversed by the inhibition of AMPK activation, suggesting that $IMD_{1\text{-}53}$ increased eNOS activity to protect endothelial function against inflammatory and oxidative damages in AS in obese status.

Conclusions

In conclusion, obesity and dyslipidemia-associated risk factors, palmitic acid and Ox-LDL synergistically exacerbate endothelial inflammation, oxidative stress and dysfunction. IMD1-53 is a potent anti-atherosclerotic peptide by improving lipid profiles in blood and in liver and activating AMPK pathway to inhibit inflammatory and oxidative damages for relieving endothelial dysfunction. Thus, the ability of $IMD_{1\text{-}53}$ for improving lipid profiles and endothelial dysfunction makes it a promising lead compound for the prevention and treatment of AS in people with obesity.

Data Sharing Statement

The data of this study will be available from the authors according to reasonable requests.

Consent for Publication

This study is comprised of animal data without any human data.

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Disclosure

There are no competing interests.

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