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Cav-1 promotes atherosclerosis by activating JNK-associated signaling

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ABSTRACT

The objective of the study is to calculate the role and underlying the molecular mechanisms of caveolin-1 (Cav-1) in atherosclerosis (AS). Cav-1 was mainly expressed in the endothelial cells of atherosclerotic lesions in both human patients and apolipoprotein E deficient ($ApoE^{-/-}$) mice. Cav-1 deficiency ($Cav\text{-}1^{-/-}$) attenuated high-fat diet (HFD)-induced atherosclerotic lesions in $ApoE^{-/-}$ mice, supported by the reduced aortic plaques. $Cav\text{-}1^{-/-}$ reduced the macrophage content and decreased the release of inflammation-related cytokines or chemokine in serum or abdominal aortas, accompanied with the inactivation of inhibitor κB kinase κ (IKKβ)/p65/IκBα signaling pathway. Also, the activity of mitogen-activated protein kinases 7/c-Jun-N-terminal kinase (MKK7/JNK) signaling was decreased by $Cav\text{-}1^{-/-}$. In addition, oxidative stress induced by HFD in $ApoE^{-/-}$ mice was alleviated by $Cav\text{-}1^{-/-}$. In response to HFD, $Cav\text{-}1^{-/-}$ markedly reduced triglyceride (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDLC) and very low-density lipoprotein-cholesterol (VLDLC) in serum of HFD-fed $ApoE^{-/-}$ mice, whereas enhanced high-density lipoprotein-cholesterol (HDLC) contents. Consistent with these findings, haematoxylin and eosin (H&E) and Oil Red O staining showed fewer lipid droplets in the liver of Cav-1-deficient mice. Further, real time-quantitative PCR (RT-qPCR) analysis indicated that $Cav\text{-}1^{-/-}$ alleviated dyslipidemia both in liver and abdominal aortas of $ApoE^{-/-}$ mice fed with HFD. Cav-1 inhibition-induced attenuation of inflammatory response, oxidative stress and dyslipidemia were confirmed in vitro using mouse vascular smooth muscle cells (VSMCs) treated with ox-LDL. Surprisingly, the processes regulated by Cav-1-knockdown could be abolished through promoting JNK activation in ox-LDL-treated VSMCs. In conclusion, Cav-1 expression could promote HFD-induced AS in a JNK-dependent manner.

1. Introduction

Atherosclerosis (AS) is a leading cause of coronary artery disease, stroke, as well as peripheral vascular diseases. Atherosclerotic lesion is recognized by the immune cells and accumulation of lipid droplets in the subendothelial space, contributing to narrowing of the arterial lumen [1]. AS is a complex process including a variety of inflammatory cells and cytokine interactions throughout different stages of its progression. It is caused by circulating low density lipoprotein (LDL), which could enter the sub-endothelial space of the blood vessel [2,3]. Once LDL is oxidized by reactive oxygen species (ROS), adhesion molecules contributes to the recruitment of monocytes and T-lymphocytes to the vessel wall, a critical factor in sustaining the inflammatory process [4]. NF-κB inhibition impairs macrophage recruitment to atherosclerotic plaques, and protects mice from atherosclerosis, demonstrating the important role of NF-κB signaling in the pathogenesis of AS [5]. In addition, lipid metabolism disorders also contribute to AS development. For instance, the abnormality of TG metabolism is a risk factor for AS since TG could be associated with atherogenic apolipoproteins [6]. Therefore, attenuating inflammatory response, oxidative stress and dyslipidemia is a key to prevent AS progression.

Caveolin-1 (Cav-1) is a marker protein for caveolae organelles and Cav-1 plays an intricate role in caveolar functioning [7]. Cav-1 has a variety of biological functions, including tumor growth and migration, lipid transport and inflammation regulation [8]. Cav-1 is an integral membrane protein, which could directly bind cholesterol [9]. Previous studies identified Cav-1 is a major plasma membrane fatty-acid binding protein in adipocytes. Cav-1 migrates from the plasma membrane to lipid droplets responding to free fatty acids [10]. Further, Cav-1 positively modulates toll like

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receptor 4 (TLR4) signaling in pulmonary endothelial cells [11]. And in murine, lipopolysaccharide (LPS)-induced inflammation was through Cav-1 induction by activating MAPKs pathway [12]. Therefore, Cav-1 is of importance in regulating inflammation and lipid metabolism, and hence could be considered as a potential target in regulating AS development.

In the study, we attempted to explore the role of Cav-1 in regulating AS pathogenesis using the Cav-1 wild type (WT) and knockout (KO) ApoE$^{-/-}$ mice. The results showed that Cav-1 was over-expressed in atherosclerotic lesions of patients compared to the normal vessel, and similar results were observed in HFD-fed ApoE$^{-/-}$ mice. Cav-1$^{-/-}$ alleviated atherosclerotic lesions in HFD-fed ApoE$^{-/-}$ mice, and reduced inflammation, oxidative stress and dyslipidemia, which was tightly associated with JNK activation.

2. Materials and methods

2.1. Patients and tissues

Human atherosclerotic plaque samples were obtained from the patients undergoing off-pump coronary artery bypass grafting surgery at the First Affiliated Hospital of Dalian Medical University (Dalian, China). All patients provided informed consent. The study was approved by the First Affiliated Hospital of Dalian Medical University and performed in line with the ethical standards.

2.2. Animals

All experimental protocols were approved by the Committee on the Ethics of Animal Experiments of the First Affiliated Hospital of Dalian Medical University. The methods were performed following the approved guidelines. Male ApoE$^{-/-}$ mice (C57/BL6 background) were purchased from the Experimental Animal Center of Dalian Medical University (Dalian, China). Cav-1$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The ApoE$^{-/-}$Cav-1$^{-/-}$ mice strain were generated through cross-breeding ApoE$^{-/-}$ and Cav-1$^{-/-}$ mice. They were backcrossed for at least 20 generations. All mice were housed in a temperature controlled facility on an artificial 12 h light-dark cycle, and they were allowed free access to water and food. All mice were divided into 3 groups (n = 6/group): 1) wild type ApoE$^{-/-}$ fed with normal chow (Con/ApoE$^{-/-}$); wild type ApoE$^{-/-}$ fed with HFD (HFD/ApoE$^{-/-}$); 3) Cav-1$^{-/-}$ApoE$^{-/-}$ group fed with HFD. Finally, the animals were killed by cervical dislocation. Blood was collected and centrifuged, and then stored at −80 °C. The aorta and liver tissues were excised from the mice, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

2.3. Cells and culture

Human aortic endothelial cells (HAECs) were purchased from ScienCell Research Laboratories (Carlsbad, USA). Primary VSMCs were isolated from mouse thoracic aortas as described previously [13]. HAECs and the derived VSMCs were cultured in ECM and DMEM (Gibco, USA), respectively, supplemented with 10% FBS (Gibco), 1% (v/v) penicillin/streptomycin, and 1% endothelial cell growth factors at 37 °C with 5% CO_2. Ox-LDL and JNK promoter of Anisomycin (ANS) were purchased from Sigma-Aldrich (USA). siRNA of Cav-1 and the negative control (NC) were synthesized by GenePharma (Shanghai, China). Lipofectamine 2000 reagent (Invitrogen CA, USA) was used for siRNA transfection.

2.4. Real time quantitative PCR (RT-qPCR) analysis

The detailed protocol for the isolation of intimal RNA from aorta and cells was the same as described previously [14]. After amplification, the threshold cycle (Ct) was analyzed and relative mRNA levels were evaluated following the $2^{-\triangle\triangle Ct}$ method. GAPDH was used as an internal control for data normalization. The sequences of the primers used are provided in Supplementary table 1.

2.5. Western blot analysis

The tissues and cells were lysed in lysis buffer and spun down at 12,000 × g for 15 min at 4 °C. The protein samples were subjected to western blotting with the indicated antibodies (Supplementary table 2) following the standard procedures [14].

2.6. Biochemical indexes measurements

Serum concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and IL-18 in ApoE$^{-/-}$ mice were determined by ELISA kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China) following the manufacturer's instructions. Measurements of malondialdehyde (MDA), superoxide dismutase (SOD), TG, TC, HDLC, LDLC and VLDLC in serum or liver were performed using commercial kits purchased from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China).

2.7. Immunohistochemical analysis

In the end, mouse aortas were removed, opened longitudinally and then stained with Oil Red O staining kit (Nanjing Jiancheng Biological Engineering Institute). The area of the atherosclerotic lesion (Oil red O area) was observed under a microscope. The aortic roots were isolated from mice, fixed with 4% paraformaldehyde, embedded in optimum cutting temperature compound (OCT), and cut into 5 μm-thick sections. The lipid deposition was analyzed using Oil Red O staining kit (Nanjing Jiancheng Biological Engineering Institute) following the manufacturer's instructions [15]. Haematoxylin and eosin (H&E) staining was performed on liver paraffin sections (5-μm thickness) after deparaffinization and rehydration to observe the distribution of lipid accumulation. For immunohistochemical staining, the slides were incubated at 4 °C overnight with primary antibodies (Supplementary table 2). And after incubation with secondary antibodies (Abcam), slides were stained with diaminobenzidine (DAB, Abcam) and counterstained with haematoxylin.

2.8. Immunofluorescent staining

IF staining was performed as previously described using antibodies listed in Supplementary table 2. The fluorescence intensity was observed with a fluorescence microscopy.

2.9. DCF-DA staining

Intracellular ROS production was calculated using a cell-permeable fluorescent probe, 2′7′-dichlorofluorescein diacetate (DCFH-DA, KeyGen BioTech) as instructed by the manufacturer.

2.10. Statistical analysis

Data are expressed as mean ± SEM. GraphPad Prism 5.0 software was used to analyze the results. For comparisons between two groups, unpaired *t*-test was performed; for comparisons among multiple groups, Oneway analysis of variance (ANOVA) test was performed. $P < 0.05$ was considered statistically significant.

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