Original Paper

Endothelial GPR124 Exaggerates the Pathogenesis of Atherosclerosis by Activating Inflammation

Dong-Mei Gong^{a,b} Yan-Li Zhang^b Dan-Yang Chen^b Ling-Juan Hong^b
Feng Han^b Qi-Bing Liu^c Jian-Jun Jiang^d Ying-Mei Lu^a

^aSchool of Medicine, Zhejiang University City College, Hangzhou, Zhejiang, ^bInstitute of Pharmacology and Toxicology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, ^cSchool of Pharmacy, Hainan Medical College, Haikou, ^dDepartment of Cardiovascular Diseases, Taizhou Hospital, Wenzhou Medical University, Taizhou Zhejiang, China

Key Words

GPR124 • Endothelial • Atherosclerosis • Nitrosative stress • Inflammasome

Abstract

Background/Aims: Endothelial cell dysfunction is the principal pathological process underlying atherosclerotic cardiovascular disease. G protein-coupled receptor 124 (GPR124), an orphan receptor in the adhesion GPCR subfamily, promotes angiogenesis in the brain. In the present study, we explored the role of endothelial GPR124 in the development and progression of atherosclerosis in adult mice. **Methods:** Using tetracycline-inducible transgenic systems, we generated mice expressing GPR124 specifically under control of the *Tie-2* promoter. The animal model of atherosclerosis was constructed by intravenously injecting AAV-PCSK9^{DY} into tetracycline-regulated mice and feeding the mice a high-fat diet for 16 consecutive weeks. Biochemical analysis and immunohistochemistry methods were used to address the role and mechanism of GPR124 in the pathological process of atherosclerosis. **Results:** Higher TC (total cholesterol) and LDL-C (low density lipoprotein cholesterol) levels in serum and greater lipid deposition in the aortic sinus were found in atherosclerotic mice with GPR124 overexpression, coincident with the elevated proliferation of smooth muscle cells. We observed an elevation of ONOO⁻ in the aortic sinus in this model by using immunofluorescence, and the experiments showed that the specific overexpression of GPR124 in the endothelium induced the up-regulation of CD68, NLRP3 and caspase-1 levels in the aortic sinus. **Conclusion:** The above results indicate that manipulating GPR124 in the endothelium may contribute to delayed pathological progression of atherosclerosis.

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D.-M. Gong and Y.-L. Zhang contributed equally to this work.

Jian-Jun Jiang
and Ying-Mei Lu

Department of Cardiovascular Diseases, Taizhou Hospital, Wenzhou Medical College, Taizhou Zhejiang, School of Medicine, Zhejiang University City College, Hangzhou, Zhejiang (China); E-Mail jiangjj@enzemed.com, lufx@zju.edu.cn

Introduction

Atherosclerosis is recognized as a chronic inflammatory disease leading to arterial plaque formation and vessel narrowing in vascular beds [1, 2]. The endothelial cell dysfunction is present in the early stages of atherosclerotic cardiovascular disease [3]. Accumulated evidence from experimental and clinical data have illuminated a role for endothelial inflammation that contributes to atherogenesis, characterized by the aberrant expression of proinflammatory cytokines [4-6]. However, the molecular basis for the mechanisms that incite inflammation in endothelial cells is poorly understood. New insights into the mechanisms of endothelial inflammation may lead to novel therapeutic strategies with the potential to improve prognosis.

The orphan G protein-coupled receptor (GPCR) GPR124/tumor endothelial marker 5 is highly expressed in the endothelium of the brain [7]. GPR124 exhibits neuronal and non-neuronal expression profiles [8, 9]. GPR124 expression during capillary morphogenesis is induced by the small GTPase Rac and mediates the contact inhibition of proliferation in endothelial cells during angiogenesis [10]. GPR124 has a long Ser/Thr-rich N terminus containing a leucine-rich repeat (LRR), an immunoglobulin (Ig) domain, and a hormone-binding domain (HBD), indicating that some of these receptors participate in the immune or reproductive systems [11]. The expression pattern of LRR and Ig domain-containing protein is expressed in a wide variety of tissues in the early mouse embryo [12]. Several proteins carry LRR and Ig domains as Trk receptors [12].

The developmental functions of GPR124 have been widely studied, and complete or endothelial-specific GPR124 deletion results in blood-brain barrier (BBB) leakage and embryonic lethality from brain-specific angiogenesis arrest [7]. GPR124 functions cell autonomously in the endothelium to regulate sprouting, migration, and the developmental expression of the Glucose transporter 1 (Glut1) [8]. Gpr124 was identified as a ligand-specific coactivator of canonical Wnt signaling [13]. Nevertheless, the role of GPR124 in cardiovascular diseases of adult animals remains largely unknown. Therefore, these findings raise an interesting question of whether endothelial GPR124 plays a critical role in determining the effects of risk factors on endothelial function during the pathological process of cardiovascular diseases in adult animals.

Accumulating evidence indicates that the molecular and cellular mechanisms associated with inflammasome signaling are potentially important during pathological disease processes [14-16]. Importantly, NLRP3 inflammasomes are required for atherogenesis and are activated by cholesterol crystals is an early event of inflammation [17]. Moreover, aberrant elevation of NLRP3 observed in the aortas of patients with coronary atherosclerosis, suggesting a clinical association between aortic expression levels of NLRP3 and the severity of coronary artery disease [18].

In the present study, a combination of tetracycline-regulated systems and biochemical strategies was used to investigate the role of GPR124 in endothelial function during the pathological process of cardiovascular diseases. This study is the first to reveal that endothelial GPR124 plays a critical role in the modulation of the endothelial inflammatory process and provides possibilities for new therapeutic regimes to treat atherosclerosis pathologies.

Materials and Methods

Inducible overexpression of endothelial GPR124 in vivo

To generate a conditional transgenic mouse model based on a tetracycline-regulated system, the expression of the reverse tetracycline transactivator (rtTA) was driven by the mouse endothelial-specific receptor tyrosine kinase (Tie2) promoter (pRP.ExSi-Tie2-RTTA). The co-injection of rtTA and Gpr124 transgenes (pRP.ExSi-TRE3G-Gpr124) led to compound hemizygous animals that exhibited unregulated Gpr124 gene expression. Doxycycline (Dox) sucrose solution (2 mg/mL) was administered to turn on GPR124 expression.

Plasmid and adeno-associated virus (AAV)

The human mutant liver targeting pSB-D374Y-PCSK9 plasmid was a gift from Juan A. Bernal (Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain) [19]. The shuttle vector pAAV-PCSK9^{DY} was generated by direct cloning of the Psil-BglII fragment from pSB-D374Y-PCSK9 and packaging into capsids from AAV-9 (provided by Hanbio, Shanghai, China). The specification and efficiency of knocking down PCSK9 were examined by western blot analysis of HEK293T cells co-transfected with AAV-PCSK9^{DY} and AAV-empty construct. AAV-PCSK9^{DY} (1×10^{12} V.G./ml) and AAV-empty (1×10^{12} V.G./ml) of the AAV2/9 serotype were packaged by Hanbio Biotechnology.

Generation of the mouse model of atherosclerosis

The atherosclerotic mouse model was constructed by intravenously injecting tetracycline-regulated mice with AAV-PCSK9^{DY}, which drives PCSK9^{DY} gene expression in hepatocytes under a liver-specific promoter [19], and the animals were fed a high-fat diet for 16 consecutive weeks. Mice weighing 25–27 g were housed under climate-controlled conditions with a 12-h light/dark cycle and provided access to standard food and water. The animals were acclimated to their environment for at least 1 week prior to the initiation of the experimental protocols. All experimental protocols and animal handling procedures were approved by the Committee for Animal Experiments at Zhejiang University in China.

Measurement of LDL-C and TC in serum

Peripheral blood, collected from mice prior to decapitation, was used to determine serum concentrations of LDL-C and TC. After a 10-min centrifugation at 3000 rpm at 4 °C, 300 µl of lipid extracts from the bottom layer was transferred into a new brown auto-sampler vial. The serum levels of TC and LDL-C were quantified using enzymatic kits from BioSino Bio-Technology & Science Inc. (Beijing, China).

Immunohistochemistry assessment of atherosclerotic lesion formation

Serial cryosections (6-mm) were cut distally from the aortic root and stained with Oil Red O (Cat# O0625-25G, Sigma-Aldrich, St. Louis, MO, USA). For immunohistochemistry staining, aortic sections were incubated with primary antibodies α-SMA (1:300, Cat# ab7817, Abcam), CD68 (1:300, Cat# MCA1957, Bio-Rad), nitrotyrosine (1:2000, Cat# 05-233, Millipore), NLRP3 (1:25, Cat# MAB 7578, R&D Systems), or caspase-1 (1:50, Cat# sc-56036, Santa Cruz Biotechnology) overnight at 4°C. The secondary fluorescent antibodies included Alexa Fluor 488-conjugated anti-rabbit IgG, Alexa Fluor 594-conjugated anti-mouse IgG, Alexa Fluor 594-conjugated anti-goat IgG (Invitrogen) (1:300). After DAPI counterstaining, the slices were rinsed in 3% BSA-PBS and mounted onto glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired using a Zeiss LSM 510 confocal microscope [20].

Evaluation of peroxynitrite formation

The aortic sections were also incubated with the peroxynitrite-sensitive probe NP3 (1:2000 dilution of a 20 mM stock) [21] for 30 min at 37°C. After staining with NP3, the sections were incubating with propidium iodide (PI) (1:500) (BD Bioscience). Slices were mounted with VectaShield (Vector Labs) on glass slides. Images were acquired using a Zeiss LSM 510 confocal microscope [20].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). Unless otherwise noted, significant differences were determined by either unpaired two-tailed Student's t-test or one-way analysis of variance (ANOVA). The results are presented as the mean ± S.E.M., and P < 0.05 indicated statistical significance.

Results

TC and LDL-C protein levels were increased in atherosclerotic mice with GPR124 overexpression

Tetracycline-inducible transgenic systems [reverse tetracycline transactivator (rtTA) or “Tet-On”] allow for the reversible temporal regulation of transgene expression [22, 23].

The atherosclerotic animal model was constructed by intravenously injecting tetracycline-regulated mice with AAV-PCSK9^{DY}, which drives PCSK9^{DY} gene expression in hepatocytes under a liver-specific promoter (Fig. 1A and B), and feeding these animals with a high-fat diet for 16 consecutive weeks (Fig. 1C). The pathological atherosclerotic-related indicators serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) were tested (Fig. 1D and E). The data showed increased serum levels of TC and LDL-C in AAV-PCSK9^{DY}-injected mice compared to C57BL/6J (WT) mice, GPR124 (-Dox) mice and GPR124 overexpression mice (+Dox) (Fig. 1D and E). Furthermore, significantly higher serum levels of TC and LDL-C were observed in the atherosclerotic model upon doxycycline administration (+Dox, AAV-PCSK9^{DY}) compared to control (-Dox, AAV-PCSK9^{DY}) (Fig. 1D and E). Therefore, inducible endothelial-specific GPR124 overexpression exaggerates the elevation of TC and LDL-C levels in an atherosclerotic model.

Fig. 1. TC and LDL-C protein levels are increased in atherosclerotic mice with GPR124 overexpression. The atherosclerotic animal model was constructed by intravenously injecting AAV-PCSK9^{DY} into tetracycline-regulated mice, and feeding these mice a high-fat diet for 16 consecutive weeks. A: Generation of endothelium-specific GPR124-overexpressing mice. Representative genotyping results for the identification of GPR124-overexpressing mice by PCR. B: The quantitative analysis of the relative mRNA level of GPR124 (mean $\pm$ SEM), * P <0.05, unpaired Student's t test. C: Diagrammatic representation of the inducible strategy in atherosclerotic mice. D: Changes in serum concentrations of LDL-C in the indicated groups. E: Changes in serum concentrations of TC in the indicated groups. Error bars represent the mean $\pm$ S.E.M (n=5). ## P <0.01 and * P <0.05, one-way ANOVA.

Atherosclerotic model with GPR124 overexpression exaggerated lipid deposition in the aortic sinus

Atherosclerosis mostly affects large and medium elastic arteries [24]. In the present study, we assessed atherosclerotic lesions in mouse models using precision sectioning of the aortic sinus combined with immunohistochemical analysis. The results confirmed previous observations that the aortic sinus is a susceptible region following chronic high-fat Western-type diet feeding. As shown in Fig. 2, lipid deposition in the aortic sinus caused vessel wall thickening in atherosclerotic mice following doxycycline administration (+Dox, AAV-PCSK9^{DY}) (Fig. 2C) compared to C57BL/6J mice (Fig. 2A) or mice with no doxycycline administration (-Dox, AAV-PCSK9^{DY}) (Fig. 2B). The quantification results showed that the lesion area was significantly increased in atherosclerotic mice with doxycycline administration (+Dox, AAV-PCSK9^{DY}) (Fig. 2D).

GPR124 overexpression in the endothelium induced the abundant proliferation of smooth muscle cells in the aortic sinus

To ascertain the proliferation of smooth muscle cells in the atherosclerotic plaque, alpha smooth muscle actin (α -SMA) was detected by immunofluorescence in mice fed a high-fat diet for 16 consecutive weeks. The expression of α -SMA is regulated by hormones and cell

Fig. 2. Increased atherosclerotic lesions in atherosclerotic mice with GPR124 overexpression. A: Representative image of aortic tissue stained with oil red O in C57BL/6J mice. B: Representative image of aortic tissue stained with oil red O in mice (-Dox, AAV-PCSK9^{DY}). C: Representative image of aortic tissue stained with oil red O in mice (+Dox, AAV-PCSK9^{DY}); Scale bar=50 μ m. D: The quantitative analysis in atherosclerotic lesions in the indicated groups (mean $\pm$ SEM, n=5). The atherosclerotic lesion areas were significantly increased in mice with doxycycline administration (+Dox, AAV-PCSK9^{DY}). ***P<0.001; unpaired Student's t test.

Fig. 3. GPR124 overexpression in endothelium induces the abundant proliferation of smooth muscle cells in the aortic sinus. A: Representative immunostaining data showing the expression of α -SMA (red) in the aortic sinus; DAPI counterstaining (blue) indicates nuclear localization. Scale bar=50 μ m. B: Visualization of gray scale intensities of α -SMA for each group in (A) by using 3D mesh plots.

proliferation and is altered during the pathological process of atherosclerosis [25]. As shown in Fig. 3, α -SMA protein levels were significantly increased in the aortic sinus of atherosclerotic mice upon doxycycline administration compared with that of atherosclerotic mice with no doxycycline administration.

Atherosclerotic mice with endothelial GPR124 overexpression showed exaggerated nitro-stress in the aortic sinus

To clarify the physiological and pathological relevance of the present observations, further investigations were needed. Peroxynitrite (ONOO⁻) is a reactive nitrogen species that induces cellular stress and inflammation [26-28]. Our previous studies implicated ONOO⁻ in

Fig. 4. Atherosclerotic model with endothelial GPR124 overexpression exaggerates nitrosative stress in aortic sinus. A: The immunohistochemical expression of nitrotyrosine (red) was examined in the aortic sinus of WT and AAV-PCSK9^{Dy} (-DOX or +DOX) mice. DAPI counterstaining (blue) indicates nuclear localization. Scale bar=50 μm. B: Visualization of nitrotyrosine gray scale intensities for each group in (A) by using 3D mesh plots. C: The immunohistochemical expression of NP3 (blue) was examined in the aortic sinus of WT and AAV-PCSK9^{Dy} (-DOX or +DOX) mice. PI counterstaining (red) indicates nuclear localization. Scale bar=50 μm. D: Quantification of data in (C).

Fig. 5. Atherosclerotic model with endothelial GPR124 overexpression shows macrophage activation. A: The immunohistochemical expression of CD68 (red) was examined in the aortic sinus of WT and AAV-PCSK9^{Dy} (-DOX or +DOX) mice. DAPI counterstaining (blue) indicates nuclear localization. Scale bar=50 μm. B: Visualization of CD68 gray scale intensities for each group in (A) by using 3D mesh plots.

various redox-related diseases, and its nitrosative damage has been implicated in vascular diseases [29-31]. Here, to explore whether endothelial GPR124 overexpression exaggerates nitrosative stress in atherosclerotic damage, we detected ONOO⁻ formation by using a nitrotyrosine-specific antibody (Fig. 4A and 4B) and the fluorescent probe NP3 (Fig. 4C and 4D). As shown in Figures 4A-D, ONOO⁻ formation in lesion areas was increased in atherosclerotic mice with doxycycline administration compared with that in atherosclerotic mice with no doxycycline administration. These results indicate that the atherosclerotic model with endothelial GPR124 overexpression exaggerates nitrosative stress in the aortic sinus.

Effects of the endothelial overexpression of GPR124 on NLRP3 inflammasome signaling in atherosclerotic model mice

Atherosclerosis is a chronic inflammatory disease. Lipid deposition induces a series of inflammatory events, leading to damage of vascular endothelial cells. To explore whether GPR124 is involved in inflammasome signaling in the pathogenesis of atherosclerosis, the activation of macrophages was assessed by CD68 staining (Fig. 5), and the expression of NLRP3 (Fig. 6) and caspase-1 (Fig. 7) was examined in the aortic sinus. The images and fluorescence intensity showed that the expression of CD68 (Fig. 5), NLRP3 (Fig. 6) and caspase-1 (Fig. 7) was increased in the aortic sinus of atherosclerotic mice. These results showed that the expression of CD68, NLRP3 and caspase-1 was higher in atherosclerotic mice with GPR124 overexpression (+Dox, AAV-PCSK9^{DY}).

Discussion

In the present study, both endothelial-specific gene overexpression and fluorescent immunohistochemical strategies were used to investigate the role of GPR124 in the pathological process of atherogenesis in adult mice. To the best of our knowledge, this is the first study to show that endothelial GPR124 plays a critical role in the

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Fig. 6. Effects of the endothelial overexpression of GPR124 on NLRP3 inflammasome signaling in an atherosclerotic mouse model. A: The immunohistochemical expression of NLRP3 (red) was examined in the aortic sinus of WT and AAV-PCSK9^{DY} (-DOX or +DOX) mice. DAPI counterstaining (blue) indicates nuclear localization. Scale bar=50 μm. B: Visualization of NLRP3 gray scale intensities for each group in (A) by using 3D mesh plots.

Fig. 7. GPR124 overexpressed in endothelium increases caspase-1 expression. A: The immunohistochemical expression of caspase-1 (red) was examined in the aortic sinus of WT and AAV-PCSK9^{DY} (-DOX or +DOX) mice. DAPI counterstaining (blue) indicates nuclear localization. Scale bar=50 μm. B: Visualization of caspase-1 gray scale intensities for each group in (A) by using 3D mesh plots.

modulation of endothelial inflammatory processes, which exaggerate the pathogenesis of atherosclerosis by activating nitrosative stress and NLRP3 inflammasome signaling (Fig. 8).

These results demonstrate that inducible endothelium-specific expression of GPR124 illuminates the functions of atherogenesis, which are poorly understood. Uncontrolled transgenic expression of a given gene typically leads to embryonic lethality that precludes further studies in adults [32]. For instance, a *Gpr124* conditional knockout in the endothelia of adult mice resulted in BBB leakage in mouse models of both ischemic injury and glioblastoma [33]. GPR124 overexpression produced hyperproliferative vascular malformations in brain [34]. Here, we propose that the GPR124 receptor is an important initiator of the endothelial inflammatory response and a potential drug target for atherosclerosis. In a cardiovascular disease context, Calderón-Zamora L and colleagues reported that GPR124 receptors might be involved in the development of hypertension in spontaneously hypertensive rats [9].

Nitrosative stress originates from peroxynitrite formed by the reaction of superoxide with nitric oxide [28, 35, 36]. The pathogenic role of peroxynitrite in atherosclerosis has been evidenced by the increased expression of iNOS and 3-nitrotyrosine within human atherosclerotic lesions [37]. Here, increases in nitrotyrosine were observed in the aortic sinus of atherosclerosis mice, and these increases were further exaggerated in endothelial-specific GPR124-overexpressing mice. In fact, nitrotyrosine binding to β -VLDL apoproteins was observed in hypercholesterolemia rabbits [38]. GPR124 not only increases serum cholesterol and lipid deposition in the aortic sinus but also aggravates smooth muscle cell proliferation. Apolipoprotein B-100, the main LDL protein, has found to have tyrosine nitration and cysteine oxidation caused by peroxynitrite, leading to protein unfolding and contributing to atherosclerotic plaque deposition [39, 40]. Thus, we predicted that endothelial-specific GPR124 overexpression will increase peroxynitrite formation and affect several downstream lipids and proteins that are targets for protein tyrosine nitration.

Here, a markedly increased number of CD68-positive macrophages were observed in GPR124-overexpressing mice. The macrophage content of the atherosclerotic vessel depends on the extent of the inflammatory reaction [41, 42]. Notably, several recent studies suggested that nitrosative stress is a key trigger of inflammasome activation [14, 43]. In the present study, an elevation in NLRP3 in the aortic sinus of atherosclerosis mice was observed, and a further dramatic increase was evidenced in atherosclerotic mice with GPR124 overexpression. Although the mechanism by which endothelial-specific GPR124 overexpression exaggerates the inflammatory response must be further clarified in future studies, the activation of the NLRP3 inflammasome may facilitate the retention of macrophages in the arterial wall and ultimately induce the formation of foam cells [44, 45]. Accordingly, arglabin, an NLRP3 inflammasome inhibitor, exhibits effective anti-inflammatory and antiatherogenic activities in ApoE2Ki mice fed a high-fat Western-type diet [46]. These reports can explain the aggravated inflammatory response observed here and support efforts to prevent this response by inhibiting inflammasome signaling.

Fig. 8. Diagrammatic representation of endothelial GPR124 exaggerates the pathogenesis of atherosclerosis by activating nitrosative stress and NLRP3 inflammasome signaling in atherosclerotic mice.

Conclusion

Taken together, the present results indicate that inducible endothelial-specific GPR124 overexpression exaggerates atherosclerosis pathogenesis by activating nitrosative stress and NLRP3 inflammasome signaling *in vivo*. The functional tropism of GPR124 suggests this receptor as a potential therapeutic target for atherosclerosis pathologies.

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Disclosure Statement

Nothing to report.

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