

Pro-inflammatory effect of fibrinogen on vascular smooth muscle cells by regulating the expression of *PPAR α* , *PPAR γ* and *MMP-9*

SHUYUE WANG, JUNTIAN LIU, DI WU, XIAOMING PANG, JINGJING ZHAO and XIAOLU ZHANG

Department of Pharmacology, Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi 710061, P.R. China

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Abstract. Atherosclerosis is a chronic inflammatory disease in the vessel. As one of the inflammatory markers, fibrinogen has been indicated in formation and progression of atherosclerosis. However, it is completely unclear whether fibrinogen produces a pro-inflammatory effect on vascular smooth muscle cells (VSMCs). The purpose of the present study was to observe the effect of fibrinogen on the expression of peroxisome proliferator-activated receptors- α (PPAR α), PPAR γ and matrix metalloproteinase-9 (MMP-9) in VSMCs. Rat VSMCs were cultured and fibrinogen was used as a stimulant for PPAR α , PPAR γ and MMP-9 expression. mRNA expression of *PPAR α* , *PPAR γ* and *MMP-9* was identified with the reverse transcription polymerase chain reaction. Protein production of PPAR α and PPAR γ was examined by western blot analysis and the MMP-9 level in the supernatant of VSMCs was measured with the enzyme-linked immunosorbent assay. The results showed that fibrinogen downregulated mRNA and protein expression of PPAR α and PPAR γ , and upregulated mRNA and protein generation of MMP-9 in VSMCs in time- and concentration-dependent manners. The maximal inhibition of protein expression of PPAR α and PPAR γ was 71.8 and 79.9%, respectively. The maximal release of MMP-9 was 4 times over the control. The results suggest that fibrinogen exerts a pro-inflammatory effect on VSMCs through inhibiting the expression of anti-inflammatory cytokine PPAR α and PPAR γ and stimulating the production of pro-inflammatory cytokine MMP-9. The findings provide new evidence for the pro-inflammatory and pro-atherosclerotic effects of fibrinogen.

Introduction

Atherosclerosis is a chronic vascular disease that is now recognized as an inflammation of the arterial wall (1). Clinical

and experimental studies show the consistent association between various markers of inflammation and cardiovascular diseases (2,3). Previous studies indicated the important roles of pro-inflammatory cytokines in the pathogenesis of atherosclerosis, such as C-reactive protein (CRP) (4,5), interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor- α (TNF- α) (6,7).

Peroxisome proliferator-activated receptors (PPARs), as members of the nuclear receptor family of transcription factors, participate in the regulation of lipid metabolism, blood pressure, cell growth and migration, oxidative stress and inflammation (8-10). As the important anti-inflammatory cytokines, PPAR α and PPAR γ have been gaining increasing attention with regards to the study of the mechanisms involved in etiology and pathogenesis of atherosclerosis. PPAR α and PPAR γ may regulate the expression of a number of inflammatory response genes through interference with pro-inflammatory transcription factor pathways, such as activator protein-1 and nuclear factor- κ B (11). Significantly low levels of PPAR γ have been found in atherosclerotic lesions and its activation reduces monocyte recruitment by the plaque (12). This PPAR-dependent inhibition may prevent the rupture of atherosclerotic plaques and the formation of subsequent thrombosis (13).

Matrix metalloproteinases (MMPs) are specialized enzymes for the degradation of extracellular matrix. In the vessel wall, dysregulated functions of MMPs often lead to impaired endothelial barrier function, infiltration of inflammatory cells, migration and proliferation of vascular smooth muscle cells (VSMCs) and finally to the development of atherosclerosis (14). MMP-9 belongs to the gelatinase subfamily of MMPs and remodels the extracellular matrix as a part of inflammatory response, which leads to plaque destabilization and triggers atherosclerotic diseases. The plasma MMP-9 level is associated with atherosclerosis in the femoral artery (15). Therefore, MMP-9 is regarded as a promising biomarker for plaque vulnerability and cardiovascular events.

The existing evidence has confirmed that fibrinogen is a key regulator of inflammation, except for its vital roles in blood clotting. A number of studies report that a high plasma fibrinogen level is an independent and major risk marker of atherosclerosis (16,17). Additionally, a high fibrinogen level is associated with the prevalence and extent of coronary artery disease (CAD), and appears to be indicated in the pathophysiology and prognosis of CAD. This association was independent of any other atherothrombotic risk factor (18).

Correspondence to: Professor Juntian Liu, Department of Pharmacology, Xi'an Jiaotong University School of Medicine, 76 West Yanta Road, P.O. Box 58, Xi'an, Shaanxi 710061, P.R. China
E-mail: ljt@mail.xjtu.edu.cn

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Although the pleiotropic roles of fibrinogen in cardiovascular diseases have been suggested, there have been few studies demonstrating its direct pro-inflammatory effect on the vascular cells. Recently, we reported that fibrinogen, fibrin and fibrinogen degradation products (FDP) induce the CRP generation in VSMCs (19) and fibrinogen, and FDP also upregulates the expression of IL-6, TNF- α and inducible nitric oxide synthase in VSMCs (20). On the basis of our previous study, the present experiment further examined whether fibrinogen regulated the expression of PPAR α , PPAR γ and MMP-9 in VSMCs to provide more evidence for its pro-inflammatory and pro-atherosclerotic effects.

Materials and methods

Reagents. Plasminogen-depleted fibrinogen and plasmin were from Calbiochem-Merck Co. (Darmstadt, Germany). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM) was produced by Gibco BRL (Carlsbad, CA, USA). Penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rat MMP-9 enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems (Minneapolis, MN, USA). Anti- α -actin antibody was provided by ZSGB-BIO (Beijing, China). Anti-PPAR α and anti-PPAR γ antibodies were from Abcam (Cambridge, UK). PrimeScript[®] reverse transcription (RT) master mix was purchased from Takara Bio, Inc. (Shiga, Japan). Agarose gel was from Spanish Biochemicals Corp. (Pronadisa, Madrid, Spain).

Culture of rat VSMCs. Male Sprague-Dawley rats were provided by the Laboratory Animal Center of Xi'an Jiaotong University School of Medicine (Xi'an, Shaanxi, China). VSMCs were isolated from the thoracic aorta of rats and cultured using the explant method as previously described (21). In brief, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (30 mg/kg). The thoracic aorta was removed and freed of connective tissue and adherent fat. The endothelial cell layer of intima was removed mechanically and the aortic artery was cut into cubes of ~3 mm. These were subsequently placed in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C until the VSMCs exhibited a typical 'hill and valley' growth pattern. Finally, VSMCs were identified by morphological examination and showed 99% purity as estimated with the immunocytochemical staining for α -actin. The cells were passaged by brief trypsinization, and the cells between passages 3 and 8 were used for the experiments. When the cells were grown to confluence, the cells were starved for 24 h in the serum-free medium before the experiments. All the experimental procedures were performed in accordance with the international, national and institutional rules, and approved by the Institutional Animal Care Committee of Xi'an Jiaotong University.

Reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was isolated from VSMCs and reverse transcribed into complementary DNA with PrimeScript[®] RT master mix (Takara Bio, Inc.) following the manufacturer's instructions. Reaction conditions of PCR amplification were

94°C for 3 min, 35 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, and the final extension of PCR products was performed for 5 min at 72°C. Primers for rat PPAR α , PPAR γ , MMP-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) (Table I shows the sequences). GAPDH was used as an internal control. The samples were run in triplicate. PCR products were run on a 2% agarose gel containing 0.5 μ g/ml ethidium bromide and resolved by electrophoresis. Images were digitally captured and the band intensity was analyzed using Gel Pro Analyzer software, version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). The relative mRNA expression of PPAR α , PPAR γ and MMP-9 was normalized to that of GAPDH.

Western blot analysis. VSMCs were washed, lysed and homogenized in 10 mmol/l Tris-HCl (pH 7.4) containing 0.1% sodium dodecylsulfate and a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Subsequently, the protein extract was boiled in electrophoresis buffer. Total cell extract protein (25 μ g) was resolved on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skimmed dry milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with anti-PPAR α (Cat. no. ab24509) or anti-PPAR γ antibodies (Cat. no. ab27649; both from Abcam), followed by the incubation with a secondary peroxidase-conjugated antibody (Cat. no. ZB-2301; ZSGB-BIO). GAPDH was used as loading control. Signals were visualized by an enhanced chemiluminescence detection reagent. Reagents (Pierce Biotechnology, Inc., Rockford, USA) for strengthening chemiluminescence were applied to the blots and the light signals were detected by X-ray film. Optical density of the bands was scanned and quantified with Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). Data were normalized against those of the corresponding GAPDH. Results are expressed relative to the control.

ELISA. Following stimulation of the cells for the indicated time, MMP-9 concentration in the culture supernatant was measured by ELISA using the quantitative sandwich enzyme immunoassay technique according to the manufacturer's instructions.

Statistical analysis. The experiments were repeated three times and all the data are expressed as means \pm standard error of the mean. All analyses were performed by one-way analysis of variance using the SPSS 12.0 software package (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference between the groups and treatments.

Results

Fibrinogen downregulates mRNA and protein expression of PPAR α in VSMCs. Fig. 1 shows that fibrinogen significantly downregulated mRNA and protein expression of PPAR α in VSMCs in concentration- and time-dependent manners compared to control. The maximal inhibition of PPAR α was reached at 10 μ mol/l fibrinogen and the inhibitory rate was 48.9% for mRNA expression and 71.8% for protein expression.

Table I. Primers used for reverse transcription polymerase chain reaction analysis.

Gene	Primer sequence	Accession number
<i>PPARα</i>	5'-CGGGTCATACTCGCAGGAAAG-3' 5'-TGGCAGCAGTGAAGAATCG-3'	NM_013196
<i>PPARγ</i>	5'-GGAAGCCCTTTGGTGACTTTATGG-3' 5'-GCAGCAGGTTGTCTTGGATGTC-3'	NM_013124
<i>MMP-9</i>	5'-GGCACCATCATAACATCACCTA-3' 5'-GACACCAAACCTGGATGACAATG-3'	NM_031055
<i>GAPDH</i>	5'-GCCTTCTCCATGGTGGTCAA-3' 5'-GGTCGGTGTGAACGGATTTG-3'	NM_017008

PPAR α , proliferator activated receptors- α ; *MMP-9*, matrix metalloproteinase-9.

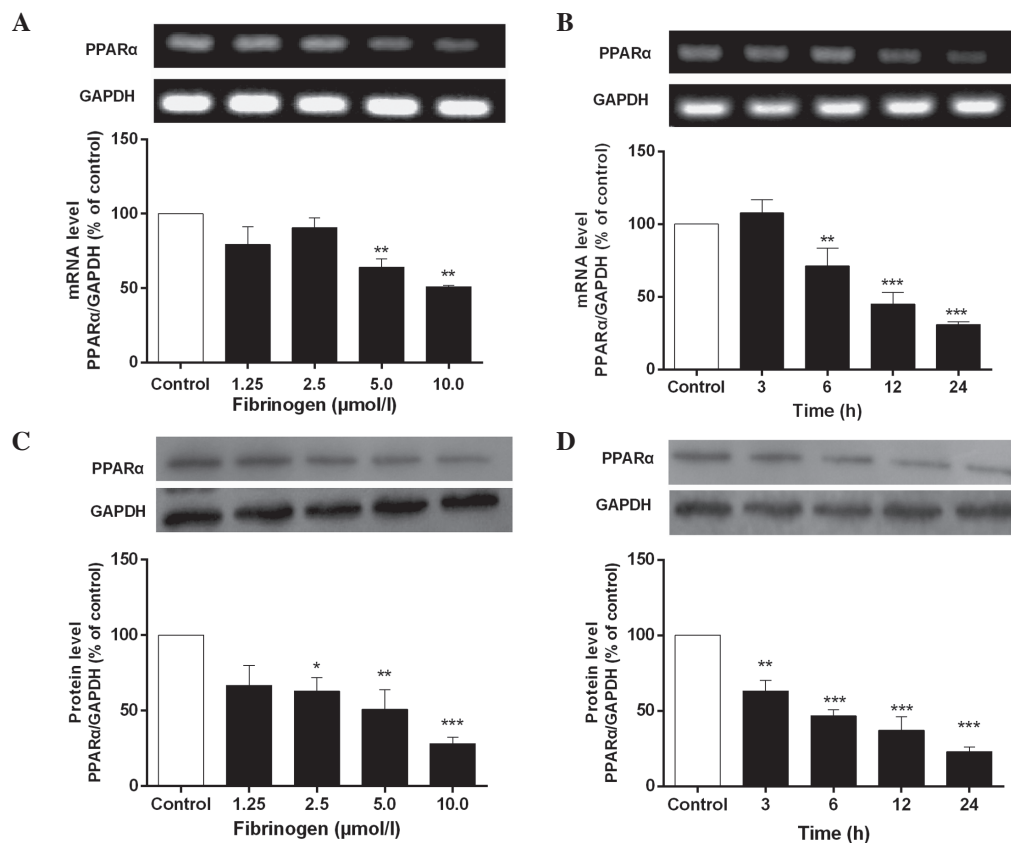


Figure 1. Fibrinogen downregulates mRNA and protein expression of proliferator activated receptors- α (PPAR α) in vascular smooth muscle cells. The cells were stimulated with the different concentrations of fibrinogen for 12 h or with fibrinogen (5 $\mu\text{mol/l}$) for the indicated time. Subsequently, mRNA and protein expression of PPAR α was determined by reverse transcription polymerase chain reaction and western blot analysis, respectively. (A and C) Concentration dependent effect of fibrinogen on mRNA and protein expression of PPAR α . (B and D) Time-dependent effect of fibrinogen on mRNA and protein expression of PPAR α . Results from three independent experiments are expressed as mean \pm standard error of the mean. *P<0.05, **P<0.01, ***P<0.001 vs. control.

Fibrinogen reduces mRNA and protein expression of PPAR γ in VSMCs. The results in Fig. 2 exhibit that fibrinogen also reduced mRNA and protein expression of PPAR γ in VSMCs in a concentration-dependent manner. mRNA and protein expression of PPAR γ was significantly reduced after stimulation of the cells with 5 $\mu\text{mol/l}$ fibrinogen for 12 h and reached a minimum at 10 $\mu\text{mol/l}$ fibrinogen. The maximal inhibition of mRNA and protein expression of PPAR γ was 63.7 and 79.9%, respectively.

Fibrinogen increases mRNA and protein expression of MMP-9 in VSMCs. As observed in Fig. 3A and B, fibrinogen at 1.25-10 $\mu\text{mol/l}$ markedly increased MMP-9 mRNA expression in VSMCs compared to the control. The results from ELISA (Fig. 3C and D) indicated that fibrinogen caused an apparent time- and concentration-dependent increase of the MMP-9 level in the culture supernatants of VSMCs. The maximal generation of MMP-9 was detected after treatment of the cells with 10 $\mu\text{mol/l}$ fibrinogen for 12 h, which was 4 times over the control.

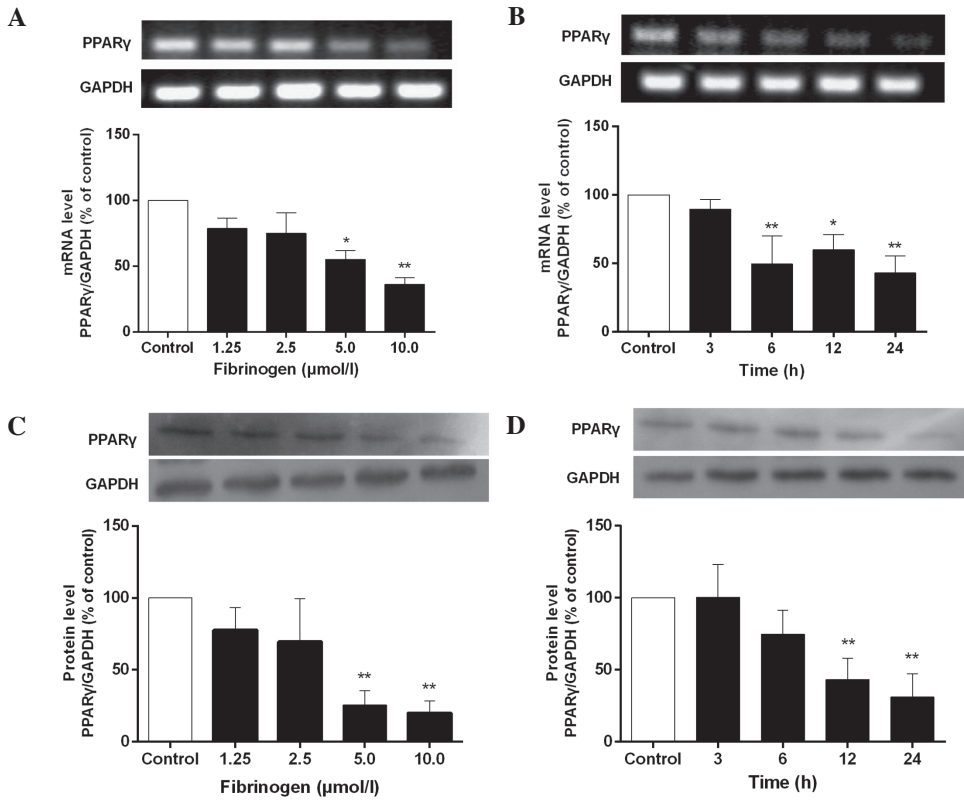


Figure 2. Fibrinogen reduces mRNA and protein expression of proliferator activated receptors- γ (PPAR γ) in vascular smooth muscle cells. The cells were stimulated with the different concentrations of fibrinogen for 12 h or with fibrinogen (5 $\mu\text{mol/l}$) for the indicated time. Subsequently, mRNA and protein expression of PPAR γ was determined by reverse transcription-polymerase chain reaction and western blot analysis, respectively. (A and C) Concentration-dependent effect of fibrinogen on mRNA and protein expression of PPAR γ . (B and D) Time-dependent effect of fibrinogen on mRNA and protein expression of PPAR γ . Results from three independent experiments are expressed as mean \pm standard error of the mean. *P<0.05, **P<0.01 vs. control.

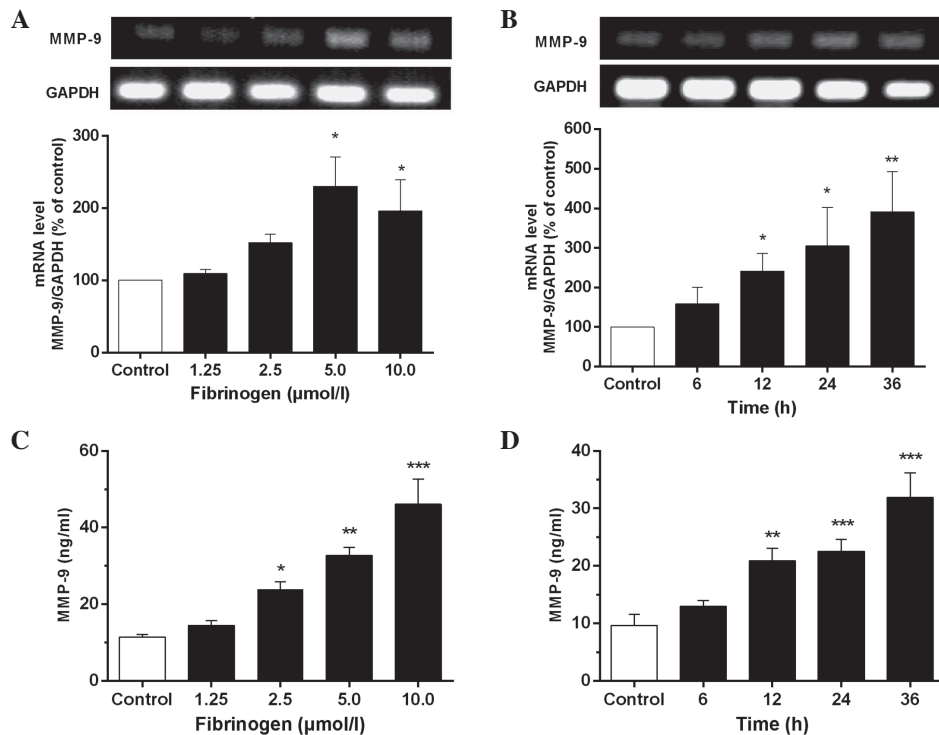


Figure 3. Fibrinogen increases mRNA and protein expression of matrix metalloproteinase-9 (MMP-9) in vascular smooth muscle cells. The cells were stimulated with the different concentrations of fibrinogen for 12 h or with fibrinogen (5 $\mu\text{mol/l}$) for the indicated time. Subsequently, MMP-9 mRNA expression was determined by reverse transcription-polymerase chain reaction and the MMP-9 concentration in the culture supernatant was measured by ELISA. (A and C) Concentration-dependent effect of fibrinogen on mRNA and protein expression of MMP-9. (B and D) Time-dependent effect of fibrinogen on mRNA and protein expression of MMP-9. Results from three independent experiments are expressed as mean \pm standard error of the mean. *P<0.05, **P<0.01, ***P<0.001 vs. control.

Discussion

An increasing body of evidence supports that fibrinogen is not only a blood coagulation factor but also an inflammatory marker (22). Fibrinogen, fibrin and FDP are components of stable and unstable atherosclerotic plaques (23). Additionally, fibrinogen has been identified as an independent risk indicator for ischemic heart disease and the severity of atherosclerosis (24). Although certain possible mechanisms regarding how fibrinogen participates in atherosclerosis have been postulated, the underlying mechanism has not yet been completely elucidated.

A number of studies report that an elevated fibrinogen level participates in the formation of atherosclerosis through activating platelet aggregation, increasing plasma viscosity, injuring endothelial cells, stimulating migration and proliferation of VSMCs (19,25). Certain studies also indicate that fibrinogen has the ability to stimulate the production of the pro-inflammatory cytokines such as monocyte chemoattractant protein-1, IL-8 and endothelin-1 in endothelial cells (26,27), and the synthesis of IL-6 and TNF- α in the peripheral blood mononuclear cells (28). Extravascular fibrinogen induces macrophage chemokine expression through Toll-like receptor 4, thereby promoting immune surveillance at sites of inflammation (29). The present study showed that fibrinogen downregulated expression of PPAR α and PPAR γ , and upregulated MMP-9 production in VSMCs at the mRNA and protein levels.

It is well-known that PPAR α and PPAR γ are involved in all the stages of atherosclerosis (30). In addition to other beneficial characteristics (31), PPAR α - and PPAR γ -mediated inhibition of atherosclerosis is also associated with their anti-inflammatory effect. *In vitro* studies demonstrate that PPAR α and PPAR γ agonists reduce the gene expression and secretion of pro-inflammatory cytokines, including TNF- α , IL-1 β and IL-6, consequently inhibiting macrophage and endothelial activation (32,33).

MMP-9 is able to efficiently degrade major components of the plaque extracellular matrix to lead to the expansion and eventual rupture of atherosclerotic plaques (34). Previous studies showed that serum MMP-9 is increased in subjects at risk of various forms of chronic inflammation and cardiovascular diseases (35). Certain studies confirm that activation of monocytes by fibrinogen increases MMP-9 secretion and MMP-9 itself enhances monocyte recruitment by the plaque (36).

In conclusion, the present results demonstrate that fibrinogen exerts a pro-inflammatory effect on VSMCs through inhibiting the expression of anti-inflammatory cytokines PPAR α and PPAR γ , and stimulating production of pro-inflammatory cytokine MMP-9, which contributes to its atherogenic effect. The findings provide new evidence for the pro-inflammatory and pro-atherosclerotic effects of fibrinogen. However, the exact molecular mechanisms for the effects remain unknown and further studies are required to characterize the mechanisms responsible for the pro-inflammatory effect of fibrinogen.

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