

Pravastatin inhibits fibrinogen- and FDP-induced inflammatory response via reducing the production of IL-6, TNF- α and iNOS in vascular smooth muscle cells

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Abstract. Atherosclerosis is a chronic inflammatory response of the arterial wall to pro-atherosclerotic factors. As an inflammatory marker, fibrinogen directly participates in the pathogenesis of atherosclerosis. Our previous study demonstrated that fibrinogen and fibrin degradation products (FDP) produce a pro-inflammatory effect on vascular smooth muscle cells (VSMCs) through inducing the production of interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α) and inducible nitric oxide synthase (iNOS). In the present study, the effects of pravastatin on fibrinogen- and FDP-induced expression of IL-6, TNF- α and iNOS were observed in VSMCs. The results showed that pravastatin dose-dependently inhibited fibrinogen- and FDP-stimulated expression of IL-6, TNF- α and iNOS in VSMCs at the mRNA and protein level. The maximal inhibition of protein expression of IL-6, TNF- α and iNOS was 46.9, 42.7 and 49.2% in fibrinogen-stimulated VSMCs, and 50.2, 49.8 and 53.6% in FDP-stimulated VSMCs, respectively. This suggests that pravastatin has the ability to relieve vascular inflammation via inhibiting the generation of IL-6, TNF- α and iNOS. The results of the present study may aid in further explaining the beneficial effects of pravastatin on atherosclerosis and related cardiovascular diseases. In addition, they suggest that application of pravastatin may be beneficial for prevention of atherosclerosis formation in hyperfibrinogenemia.

Introduction

Inflammation is important in the development and progression of atherosclerosis (1,2). The inflammatory processes are characterized by increased circulating levels of pro-inflammatory cytokines, including interleukin (IL)-1 β , IL-6, tumor necrosis

factor- α (TNF- α) and inducible nitric oxide synthase (iNOS), soluble adhesion molecules, and cytokine-responsive acute phase protein such as C-reactive protein (CRP) (3,4).

Fibrinogen, the acute phase reactant in humans, is derived predominantly from hepatocytes in response to IL-6 and is then secreted into the systemic circulation (5). Recent studies have reported that in addition to its predictive role in determining cardiovascular risk, fibrinogen also exerts a direct pro-inflammatory role on the different vascular cells implicated in atherogenesis (6,7). Fibrinogen stimulates the production of inflammatory cytokines (IL-6, TNF- α , IL-1 β , IL-8 and endothelin-1) in endothelial cells (7,8), macrophages (9), monocytes (10) and neutrophils (11). In addition, fibrinogen participates in the formation of atherosclerosis lesions through promoting platelet aggregation (12), increasing plasma viscosity (13), injuring endothelial cells (14,15), and stimulating migration and proliferation of vascular smooth muscle cells (VSMCs) (16,17). Furthermore, fibrinogen is degraded by plasmin to a series of fibrin degradation products (FDPs), which share similar bioactivities to fibrinogen and also are associated with the progression of atherosclerosis (17,18). Our previous studies showed that fibrinogen and FDP upregulate the expression of CRP, IL-6, TNF- α and iNOS in VSMCs (19,20).

3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) effectively lower the serum cholesterol level and reduce cardiovascular events and mortality in patients with or without coronary artery disease (21). The beneficial effects of statins have also been characterized by their non-lipid related mechanisms, such as preventing inflammation, improving endothelial function, inhibiting activation of VSMCs, stabilizing atherosclerotic plaques, and preventing thrombosis formation (21,22). Since chronic inflammation in the vessel wall is important in atherogenesis, and hyperfibrinogenemia is associated with cardiovascular disorders, including atherosclerosis, the aim of this study was to observe whether pravastatin regulated fibrinogen- and FDP-induced production of IL-6, TNF- α and iNOS, and to highlight its anti-inflammatory effect and anti-atherosclerotic action.

Materials and methods

Reagents. Plasminogen-depleted fibrinogen and plasmin were obtained from Calbiochem (Merck, Darmstadt, Germany).

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Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were produced by Gibco-BRL (Carlsbad, CA, USA). Pravastatin and thrombin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat IL-6 and TNF- α ELISA kits were obtained from Alpha Diagnostics International (San Antonio, TX, USA). Polyclonal rabbit anti-rat iNOS antibody (cat. no. bs-0162R) was provided by Beijing Biosynthesis Biotechnology (Beijing, China). TRIzol reagent kits were provided by Invitrogen Life Technologies (Carlsbad, CA, USA). The Revert AidTM First Strand cDNA Synthesis kit was obtained from Fermentas (St. Leon-Rot, Germany). Agarose gels were from Spanish Biochemicals Corp. (Madrid, Spain).

Culture of rat VSMCs. Male Sprague-Dawley rats (n=12; age, 2-3 months; weight, 100-150 g) were obtained from the Laboratory Animal Center of Xi'an Jiaotong University School of Medicine (Xi'an, China). VSMCs were prepared from the thoracic aorta of using the explant method as described previously (23). Briefly, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (30 mg/kg; Sigma-Aldrich). The thoracic aorta was removed and freed of connective tissue and adherent fat. The endothelial cell layer of the intima was removed mechanically, and the aortic artery was cut into sections of ~3 mm. These were then placed in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C until VSMCs exhibited a typical 'hill and valley' growth pattern. Finally, VSMCs were identified with the morphological examination, and showed 99% purity as estimated with the immunocytochemical staining for α -actin. The cells were observed using an optical microscope (Eclipse E600; Nikon Corporation, Tokyo, Japan) and the positive ratio of immunocytochemical staining for α -actin was analyzed with Image-Pro Plus 5.0 software (Media Cybernetics Inc., Rockville, MD, USA). The cells were passaged by brief trypsinization (MP Biomedicals, Santa Ana, CA, USA), and the cells between passages 3 and 8 were used for the experiments. When the cells were grown to confluence, they were starved for 24 h in serum-free medium prior to the experiments. All 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 (Xi'an, China).

Experimental protocols. VSMCs were cultured in 6-well plates at a density of 5x10⁶ cells/well. When cells had been starved for 24 h in the serum-free medium, the cells were divided into the following groups: Control, cells treated with fibrinogen or FDP, and cells treated with fibrinogen or FDP with pravastatin (10, 30 or 100 μ mol/l). Following pretreatment with pravastatin for 30 min, the cells were stimulated with 5 μ mol/l fibrinogen for 9 h, or 1 ml FDP diluted in 30 ml physiological saline (1/30 FDP) for 3 h, as previously described by Naito *et al* (17). Then, the supernatant was collected. IL-6 and TNF- α levels were determined using an ELISA, and iNOS protein expression was detected with immunofluorescence. mRNA expression of IL-6, TNF- α and iNOS were determined by reverse transcription-polymerase chain reaction (RT-PCR).

Preparation of FDP. About 6.3 units of human thrombin were mixed with 3 ml fibrinogen (50 μ mol/l), and incubated overnight at 37°C. The formed clots were homogenized for 10 sec, and centrifuged at 600 x g for 10 min. Then, the supernatant was removed, and the clots were washed three times with physiological saline. Digestion of the clots was conducted in Tris-buffered saline containing physiological levels of calcium (10 mmol/l Tris, 100 mmol/l NaCl and 2.5 mmol/l CaCl₂, pH 7). The clotted fibrin was suspended in 5 ml buffer, and 2 units of human plasmin were added to form FDP. Subsequently, the samples were placed on a rotator in a 37°C incubator for 48 h, and sterilized by passing the samples through a 0.22- μ m pore sterilization filter (17). Finally, the samples containing FDP were diluted with physiological saline. FDP concentrations were expressed as the diluted proportion.

ELISA. VSMCs were cultured in 6-well plates at a density of 5x10⁶ cells/well. The cells were stimulated with fibrinogen (5 μ mol/l) or FDP (1/30) for the indicated time after pretreatment for 30 min with pravastatin at 10, 30 or 100 μ mol/l. Then, the supernatant was collected for assay of IL-6 and TNF- α by ELISA kits specific for rat IL-6 and TNF- α according to the manufacturer's instructions.

RT-PCR. Total RNA was extracted and complementary DNA (cDNA) was synthesized from 1 μ g of total RNA with random hexamer primers using Revert AidTM First Strand cDNA Synthesis kit according to the manufacturer's instructions. PCR amplification was performed through 35 cycles at 94°C for 30 sec, 54°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 IL-6, TNF- α , iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed with Beacon designer 4.0 (Palo Alto, CA, USA) (Table I). GAPDH was used as an internal control. The samples were run in triplicate. Equal volumes of the reaction mixture from each sample were loaded on a 2% TAE agarose gel (Spanish Biochemicals Corp., Madrid, Spain) containing ethidium bromide, and resolved by electrophoresis. Images were digitally captured using a gel analysis system (JS-680D; Shanghai Peiqing Science & Technology Co., Ltd., Shanghai, China), and band intensity was analyzed using Gel Pro Analyzer software, Version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). The relative amount of each mRNA was normalized to the housekeeping gene (GADPH) mRNA.

Immunofluorescent analysis of iNOS. VSMCs were plated on cover slips in 6-well plates until 90% confluency. Then, the treated cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 15 min. The cell membrane was permeabilized with 0.2% Triton X-100-PBS (Amresco, LLC, Solon, OH, USA) for 20 min, and the non-specific binding sites were blocked with 10% goat serum. Following pretreatment with rabbit anti-rat iNOS antibody (1:200 diluted in PBS) overnight at 4°C, the cells were washed three times with phosphate-buffered saline, and incubated with the secondary antibodies conjugated to fluorescein isothiocyanate for 15 min at room temperature. Finally, the immunolabeled cells were observed under a fluorescent microscope (BX-51 Olympus, Tokyo, Japan), and fluorescent intensity of iNOS was

Table I. Primer sequence used for RT-PCR analysis.

Gene	Primer sequence	Accession number
IL-6	5'-TCCAGCCAGTTGCCTTCTTG-3' 5'-AGCCACTCCTTCTGTGACTC-3'	NM_012589
TNF- α	5'-CCAGAACTCCAGGCGGTGTC-3' 5'-GGCTACGGGCTTGTCACTCG-3'	NM_012675
iNOS	5'-GGAAGAGACGCACAGGCAGAG-3' 5'-GCAGGCACACGCAATGATGG-3'	NM_012611
GAPDH	5'-GCCTTCTCCATGGTGGTGAA-3' 5'-GGTCGGTGTGAACGGATTTG-3'	NM_017008

IL, interleukin; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase.

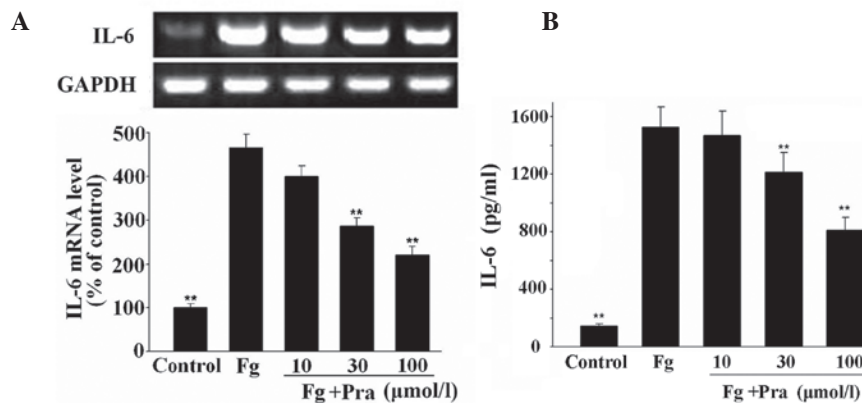


Figure 1. Effect of Pra on Fg-induced expression of IL-6 in VSMCs. VSMCs were stimulated with 5 μ mol/l Fg for 9 h after pretreatment for 30 min with different concentrations of Pra. Then, (A) mRNA and (B) protein expression of IL-6 were determined by reverse transcription-quantitative polymerase chain reaction and ELISA, respectively. Values from three independent experiments are expressed as the mean \pm standard deviation. ** P <0.01 vs. Fg. Pra, pravastatin; IL, interleukin; VSMCs, Vascular smooth muscle cells; Fg, fibrinogen.

detected and analyzed with Image-Pro Plus software (Media Cybernetics., Inc., Rockville, MD, USA). Data were expressed as relative to the control.

Statistical analysis. Data are expressed as the mean \pm standard deviation. The experiments were repeated three times, and the differences between means were examined for statistical significance (P <0.05) by one-way analysis of variance followed by Fisher's exact test. SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Pravastatin inhibits fibrinogen- and FDP-induced IL-6 expression in VSMCs. As shown in Figs. 1 and 2, mRNA and protein expression of IL-6 was low in the unstimulated VSMCs. However, mRNA and protein expression of IL-6 was significantly increased following stimulation of VSMCs with fibrinogen or FDP (P <0.01 vs. control). However, pravastatin dose-dependently suppressed fibrinogen- and FDP-stimulated IL-6 expression in VSMCs in protein and mRNA levels (P <0.01 vs. fibrinogen or FDP alone). The inhibitory rates in protein levels were 3.7, 20.7 and 46.9% in fibrinogen-stimulated

VSMCs, and were 13.1, 27.9 and 50.2% in FDP-stimulated VSMCs with the increase of pravastatin concentration.

Pravastatin attenuates fibrinogen- and FDP-induced TNF- α expression in VSMCs. As observed in Figs. 3 and 4, fibrinogen and FDP produced a significant increase in the mRNA and protein expression of TNF- α in VSMCs (P <0.01 vs. control), while pretreatment of the cells with pravastatin inhibited fibrinogen- and FDP-induced mRNA and protein expression of TNF- α in a concentration-dependent manner (P <0.01 vs. fibrinogen or FDP alone). The maximal inhibition of fibrinogen- and FDP-induced TNF- α protein expression was 42.7 and 49.8%, respectively.

Pravastatin reduces fibrinogen- and FDP-stimulated iNOS expression in VSMCs. The results shown in Figs. 5 and 6 indicate that mRNA and protein expression of iNOS in VSMCs was significantly enhanced after exposure of the cells to the same concentration of fibrinogen and FDP (P <0.05 vs. control). However, pretreatment of the cells with pravastatin antagonized the stimulatory effect of fibrinogen and FDP on mRNA and protein expression of iNOS in a dose-dependent manner (P <0.05 or P <0.01 vs. fibrinogen or FDP alone). The

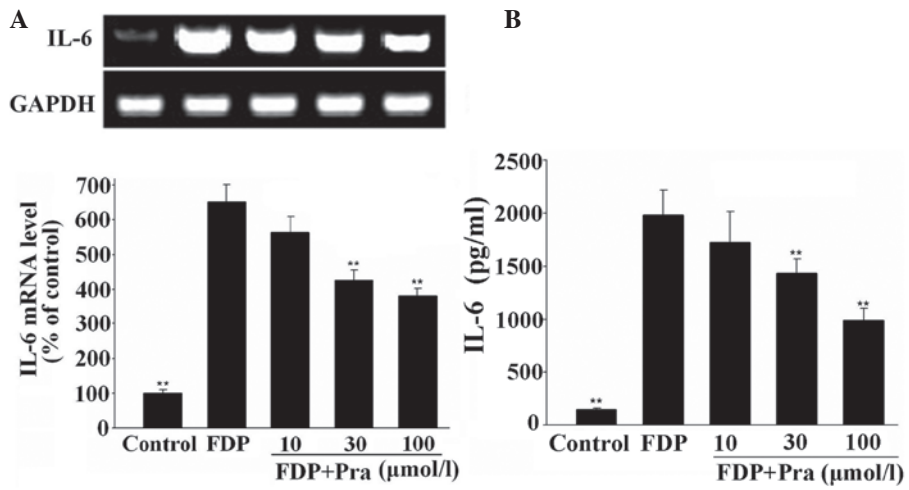


Figure 2. Effect of Pra on FDP-induced expression of IL-6 in VSMCs. VSMCs were stimulated with 1/30 FDP for 3 h after pretreatment for 30 min with different concentrations of Pra. Then, (A) mRNA (B) protein expression of IL-6 were determined by reverse transcription-polymerase chain reaction and ELISA, respectively. Values from three independent experiments are expressed as the mean ± standard deviation. **P<0.01 vs. FDP. Pra, pravastatin; FDP, fibrin degradation products; IL, interleukin; VSMCs, vascular smooth muscle cells.

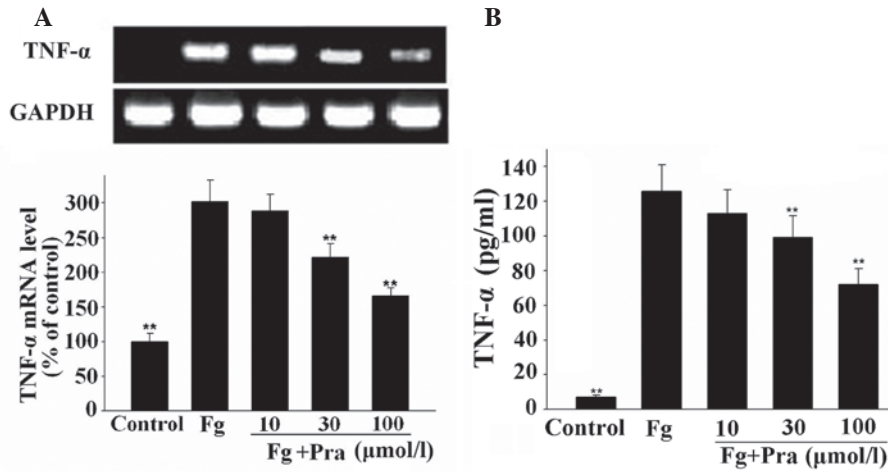


Figure 3. Effect of Pra on Fg-stimulated expression of TNF-α in VSMCs. VSMCs were stimulated with 5 μmol/l Fg for 9 h after pretreatment for 30 min with the different concentrations of Pra. Then, (A) mRNA and (B) protein expression of TNF-α were determined by reverse transcription-polymerase chain reaction and ELISA, respectively. Values from three independent experiments are expressed as the mean ± standard deviation. **P<0.01 vs. Fg. Pra, pravastatin; Fg, fibrinogen; TNF-α, tumor necrosis factor-α; VSMCs, vascular smooth muscle cells.

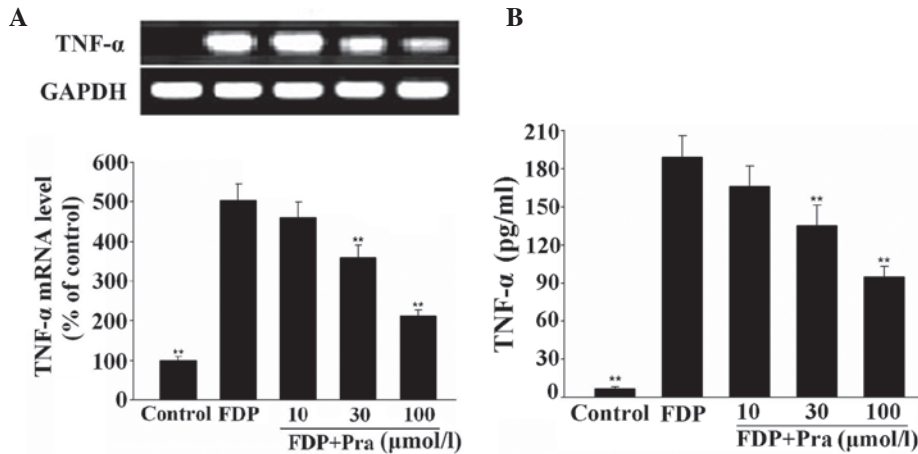


Figure 4. Effect of Pra on FDP-stimulated expression of TNF-α in VSMCs. VSMCs were stimulated with 1/30 FDP for 3 h after pretreatment for 30 min with different concentrations of Pra. Then, (A) mRNA and (B) protein expression of TNF-α were determined by reverse transcription-polymerase chain reaction and ELISA, respectively. Values from three independent experiments are expressed as the mean ± standard deviation. **P<0.01 vs. FDP. Pra, pravastatin; FDP, fibrin degradation products; TNF-α, tumor necrosis factor-α; VSMCs, vascular smooth muscle cells.

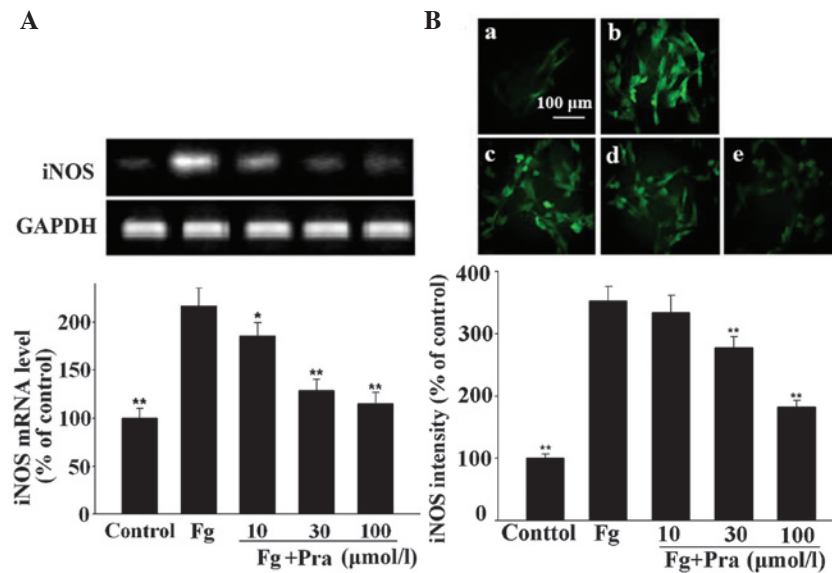


Figure 5. Effect of Pra on Fg-induced expression of iNOS in VSMCs. VSMCs were stimulated with 5 $\mu\text{mol/l}$ Fg for 9 h after pretreatment for 30 min with different concentrations of Pra. Then, mRNA and protein expression of iNOS was determined by reverse transcription-polymerase chain reaction and immunofluorescence, respectively. (A) mRNA expression of iNOS. Protein expression of iNOS in (Ba) control, (Bb) Fg alone, (Bc) Fg+10 $\mu\text{mol/l}$ Pra, (Bd) Fg+30 $\mu\text{mol/l}$ Pra and (Be) Fg+100 $\mu\text{mol/l}$ Pra. Values from three independent experiments are expressed as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. Fg. Scale bar, 100 μm . Pra, pravastatin; Fg, fibrinogen; iNOS, inducible nitric oxide synthase; VSMCs, vascular smooth muscle cells.

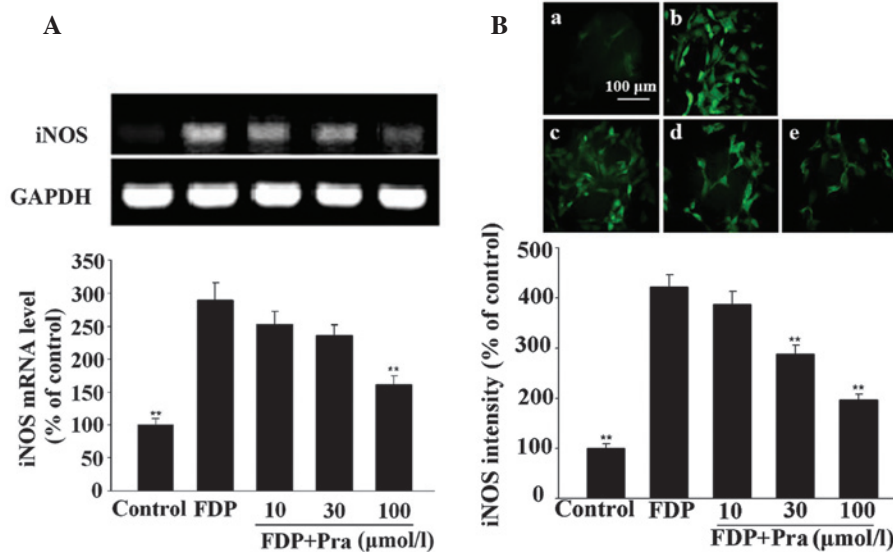


Figure 6. Effect of Pra on FDP-induced expression of iNOS in VSMCs. VSMCs were stimulated with 1/30 FDP for 3 h after pretreatment for 30 min with the different concentrations of Pra. Then, mRNA and protein expression of iNOS was determined by reverse transcription-polymerase chain reaction and immunofluorescence, respectively. (A) iNOS mRNA expression. iNOS protein expression in (Ba) control, (Bb) FDP alone, (Bc) FDP+10 $\mu\text{mol/l}$ Pra, (Bd) FDP+30 $\mu\text{mol/l}$ Pra and (Be) FDP+100 $\mu\text{mol/l}$ Pra. Values from three independent experiments are expressed as the means \pm standard deviation. ** $P < 0.01$ vs. FDP. Scale bar, 100 μm . Pra, pravastatin; FDP, fibrin degradation products; iNOS, inducible nitric oxide synthase; VSMCs, vascular smooth muscle cells.

maximal inhibition reached 49.2% for fibrinogen-induced iNOS protein expression and 53.6% for FDP-induced iNOS protein expression.

Discussion

Atherosclerosis is considered to be a chronic inflammatory disease that leads to a number of cardiovascular diseases (CVDs). Statins have been demonstrated to decrease

cardiovascular events through their pleiotropic properties, including anti-inflammatory action (24). However, the precise mechanisms by which statins inhibit inflammatory responses remain unclear.

VSMCs are the major players implicated in atherogenesis. Migration and proliferation of VSMCs are the critical events in the initiation and progression of atherosclerosis as well as in the inflammatory processes (6,2). IL-6 and TNF- α are the important pro-inflammatory cytokines involved in

atherogenesis, which stimulate the release of other inflammatory mediators, increase expression of adhesion molecules in endothelial cells, promote release of various chemokines to recruit monocytes to the injury site and enhance their adhesiveness to endothelium (25,26). A clinical study suggests that high serum levels of IL-6 and TNF- α is associated with a worse prognosis in patients after acute myocardial infarction (27).

It is known that iNOS exists in human atherosclerotic lesions as several factors present in atherosclerotic plaques, such as TNF- α , IL-1 and γ -interferon, are able to induce iNOS expression in VSMCs. High iNOS activity produces large quantities of NO, which may lead to cellular damage, inflammation, apoptosis and peroxynitrite formation. Furthermore, peroxynitrite may cause oxidative damage to endothelial cells and VSMCs, and therefore is important in the pathology of atherosclerosis (28-30).

Although the role of fibrinogen in the clotting cascade has been well defined, its role in the inflammatory response is less well understood. It has long been demonstrated that fibrinogen and its fragments are incorporated into atherosclerotic plaques as the plaques develop (31). Fibrinogen is also converted into a fibrin matrix at the site of inflammation, and fibrin(ogen) deposition may directly participate in the inflammatory response by providing a scaffold for inflammatory cell adhesion and migration, and for subsequent remodeling of the tissue with extracellular matrix (32). Our previous studies found that fibrinogen and FDP are able to produce a pro-inflammatory effect on VSMCs via stimulating the generation of CRP, IL-6, TNF- α and iNOS, which suggests a novel mechanism involving fibrinogen in atherogenesis (19,20).

In view of the pro-inflammatory effect of fibrinogen on VSMCs, which is involved in its pro-atherosclerotic process, the inhibitory effect of pravastatin on fibrinogen- and FDP-stimulated generation of inflammatory cytokines, such as IL-6, TNF- α and iNOS, was observed in VSMCs. The results showed that pravastatin at the concentrations used in the present study significantly inhibited fibrinogen- and FDP-stimulated expression of IL-6, TNF- α and iNOS in VSMCs at the mRNA and protein levels, suggesting that pravastatin is able to exert an anti-inflammatory effect via decreasing fibrinogen- and FDP-induced expression of IL-6, TNF- α and iNOS in VSMCs.

Our previous study demonstrated that lovastatin reduces nuclear factor- κ B activation induced by CRP in human vascular endothelial cells (33), and Han *et al* in turn demonstrated that simvastatin inhibits angiotensin II-induced CRP expression in human aortic endothelial cells through interfering with AT₁-reactive oxygen species-mitogen activated protein kinase (MAPK) signaling pathway (unpublished data). The MAPK signaling pathway mediates the inflammatory response induced by inflammatory cytokines in the atherogenesis-related cells (34-37). Thus, it was inferred that pravastatin, analogous to lovastatin and simvastatin, reduces fibrinogen- and FDP-stimulated generation of IL-6, TNF- α and iNOS in VSMCs possibly by inhibiting the MAPK signaling pathway. Further studies are required to characterize the mechanisms responsible for the inhibitory effect of pravastatin.

In conclusion, the present results demonstrate that pravastatin is able to inhibit fibrinogen- and FDP-stimulated

generation of IL-6, TNF- α and iNOS in VSMCs thus relieving the inflammatory response in the vessel wall involved in atherogenesis. This may aid in further explaining the beneficial effects of pravastatin on atherosclerosis and CVDs. It also suggests that application of pravastatin may be beneficial for the prevention of atherosclerosis in hyperfibrinogenemia.

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