Original Paper

Fibrinopeptide A Induces Expression of C-Reactive Protein via the ROS-ERK1/2/P38-NF- κ B Signal Pathway in Vascular Smooth Muscle Cells

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Key Words

Fibrinopeptide A • CRP • Vascular smooth muscle cells • Inflammation • Atherosclerosis

Abstract

Background/Aims: Atherosclerosis is a chronic inflammatory disease in the artery walls. Fibrinopeptide A (FPA) is a biomarker of the activation of coagulation system, and a high concentration of FPA in blood occurs in patients with ischemic heart disease etc. However, there exist few studies on the pathological effects of FPA in cardiovascular system. Therefore, the present study examined the effect of FPA on CRP expression in VSMCs and the molecular mechanisms. **Methods:** mRNA and protein expression was identified by quantitative real-time PCR and Western blot, respectively. Reactive oxygen species (ROS) and the immunofluorescence staining were observed by a fluorescence microscope. Plasma FPA and CRP level was determined by ELISA. **Results:** FPA induced the expressions of CRP, IL-1 β and IL-6 in VSMCs, and anti-IL-1 β and anti-IL-6 neutralizing antibodies partially reduced FPA-induced CRP expression in VSMCs. The subchronic administration of FPA to rats increased FPA level in plasma and CRP expression in the aortic artery walls. The further studies showed that FPA promoted superoxide anion generation in VSMCs. Antioxidant NAC antagonized FPA-stimulated superoxide anion generation and inhibited FPA-induced CRP expression in VSMCs. FPA activated ERK1/2 and p38 phosphorylation, and PD98059 and SB203580 reduced FPA-induced CRP expression. Moreover, NAC inhibited the activation of ERK1/2 and p38. In addition, FPA enhanced NF- κ B level in the nuclei of VSMCs, and PDTC reduced FPA-induced expression of CRP. **Conclusions:** FPA induces CRP expression in VSMCs via ROS-ERK1/2/p38-NF- κ B signal pathway. This finding for the first time provides an experimental evidence for pro-inflammatory effect of FPA.

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Introduction

Atherosclerosis is a disease associated with chronic inflammation in the artery wall and inflammation is involved in the different phases of the disease from the plaque formation to the plaque rupture [1, 2]. Data from the clinical and experimental researches show that inflammatory markers like cytokines, acute-phase response proteins and cellular adhesion molecules are able to predict the event of atherosclerosis [3]. As an acute-phase response protein, C-reactive protein (CRP) is considered as an independent risk factor of atherosclerosis. CRP is not only a predictor of atherosclerosis but also plays a pivotal role in the progression of atherosclerosis [4]. CRP regulates the activities and expressions of many factors participating in atherogenesis [5], such as evoking interleukin-6 and endothelin-1 releases [6], inducing the expressions of adhesion molecules and monocyte chemoattractant protein-1 (MCP-1) [7]; promoting the uptake of low-density lipoprotein by macrophage and stimulating the migration and proliferation of vascular smooth muscle cells (VSMCs) [8].

VSMCs are involved in the whole progress of atherosclerosis, especially in the plaque formation [9-12]. Although the circulating CRP is mainly from hepatocytes and non-hepatocytes including neurons, renal epithelium and respiratory tract, a growing evidence has identified that VSMCs also produce CRP [13, 14]. Moreover, the locally produced CRP in the vessel wall may play a direct and key role in the progress of atherosclerosis and cardiovascular complications.

The earlier studies showed that fibrinogen and its degradation products are involved in the development of atherosclerosis by mediating inflammatory responses and participating in thrombosis formation [15-19]. Fibrinopeptide A (FPA) is a peptide of 16 amino acids which occurs as a natural consequence of hemostasis from proteolysis of fibrinogen's A α chain [20]. The peptide exists in blood of normal healthy individuals at the concentration of 0.5-2.0 nM, and is 2-10 time higher in blood of the patients with thrombotic disease, pulmonary embolism and acute ischemic cardiovascular disease [21-26]. Surprisingly, so far there have been few studies on the biological activity of FPA. The limited reports show that FPA causes an irreversible damage to endothelial cells [27], stimulates the secretions of IL-8, MCP-1, macrophage migration inhibitory factor and serpin E1 in dermal microvascular endothelial cells [28]; promotes an infiltration of leukocytes and the production of the pro-inflammatory cytokine MCP-1 in the inflammation site after the injection into BALB/C mice [29]. In the present research, we examined the effect of FPA on CRP expression in VSMCs and probed whether reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF- κ B) signal pathway played a role in FPA-induced CRP expression.

Materials and Methods

Reagents

Dulbecco's High Glucose Modified Eagles Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). FPA, PD98059, SB203580, SP600125, N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTTC), thenoyltrifluoroacetone (TTFA) and diphenyleneiodonium (DPI) were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal CRP antibody and mouse α -smooth muscle actin antibody were provided by Abcam (Cambridge, MA, USA). Rabbit monoclonal β -actin antibody was ordered from Zhuangzhi Biotech (Xi'an, China). NF- κ B activation and nuclear translocation kits, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA), ERK1/2 and phospho-ERK1/2 antibodies were obtained from Beyotime (Jiangsu, China). Phospho-p38 and p38 antibody were from Cell Signaling Technology (Danvers, MA, USA). Relative second antibodies were provided by CW Biotech (Beijing, China). Rabbit monoclonal lamin B1 antibody was from Abnova (Taipei, Taiwan, China). Anti-rat IL1 β and anti-rat IL-6 neutralizing antibodies were ordered from R&D Systems (MN, USA). ELISA kits for detecting IL-1 β and IL-6, and for assay of FPA were from West Tang (Shanghai, China) and MLBIO Biotech (Shanghai, China) respectively.

Culture of rat VSMCs

The primary VSMCs separated from the thoracic aorta of male Sprague Dawley (SD) rats were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a 5% CO₂ incubator as previously described [30, 31]. The cells showed the typical “hill and valley” morphology and 99% purity as identified by the immunofluorescence staining with α -smooth muscle-actin antibody (showed in Fig 1). The cells between passages 3 and 8 were used in the present study. The cells were incubated in the serum-free medium for an additional 12 h before the experiments. All experimental procedures were strictly performed according to the international, national and institutional rulers, and approved by the Institutional Animal Care Committee of Xi'an Jiaotong University.

Assessment of the viability of VSMCs by the MTT methods.

VSMCs were incubated with 10⁻⁴-1.0 μ M FPA for 12 h or with 10⁻² μ M FPA for 0-24 h. Then, 20 μ l MTT (5 mg/ml, Invitrogen, Carlsbad, CA, USA) was added to each well. After the incubation at 37°C for 4 h, the culture medium was carefully removed and the formed formazan crystal was dissolved in 150 μ l DMSO (Sigma-Aldrich, St. Louis, MO, USA) with vigorous shaking for 10 min. Finally, the optical density was determined at 490 nm with micro-plate reader (Bio-Rad, Hercules, CA, USA).

Quantitative real-time PCR

Total RNA was extracted from VSMCs using RNAfast100 purification kit (Xianfeng Biotech, Shaanxi, China). Complementary DNA (cDNA) was synthesized from total RNA (2 μ g) by Revert Aid™ First Strand cDNA synthesis kit (TaKaRa, Tokyo, Japan) in accordance with the method provided by the manufacturer. Amplification of cDNA was accomplished by primer pairs specific for rat (showed in Table 1). Quantitative real-time PCR was performed using Agilent MX3000 Real-Time PCR System (Santa Clara, CA, USA). The reaction products were measured by detecting the binding of SYBR Green I to DNA using SYBR Green PCR Master Mix (Roche, Basel, Switzerland). Optimization of the amplification reaction was assured by a dissociation curve analysis. All samples were run in triplicate, and then analyzed using the 2^{- $\Delta\Delta$ CT} methods. The level of mRNA was expressed as relative to internal control GAPDH.

Western blot

VSMCs were washed 3 times with the ice-cold PBS after the treatments. The whole cell lysates were acquired using the lysis buffer supplemented with protease inhibitor cocktail and phosphatase inhibitors (Roche, Basel, Switzerland). Concentration of the protein was measured by BCA protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amount of the protein (40 μ g) was loaded, separated by 10% SDS-PAGE, and blotted onto PVDF membrane (0.45 μ m, GE Healthcare, Buckinghamshire, UK). The membranes were incubated with anti-CRP (1:800 dilution), anti- β -actin (1:2000 dilu-

Fig. 1. Characterization of the primary cultured VSMCs derived from the thoracic aorta of male Sprague-Dawley rats. The cells were stained with α -smooth muscle actin antibody and labeled with the cy3 conjugated antibody (left), with DAPI (middle) and the merged field of red and purple color (right). The cells were observed at magnification of 200 $\times$ under a fluorescence microscope.

Table 1. Primers used for quantitative real-time PCR

Gene	Primer sequence	Accession number	Product size (bp)
CRP	5'-TTGGTGGGAGACATTGGAGA-3'	NM_017096.3	99
	5'-AACATTGGGGCTGAATACCCTAC-3'		
IL-6	5'-GATTGTATGAACAGCGATGATGC-3'	NM_012589.2	145
	5'-AGAAACGGAACCTCAGAAGACC-3'		
IL-1 β	5'-CCCAACTGGTACATCAGCACCTCTC-3'	NM_031512.2	76
	5'-CTATGTCCCGACCATTTGCTG-3'		
GAPDH	5'-TGGAGTCTACTGGCGTCTT-3'	XM_017593963.1	138
	5'-TGTCATATTTCTCGTGGTTCA-3'		

tion), anti-p38 (1:1000 dilution) or anti-phospho-p38 (1:500 dilution), anti-ERK1/2 (1:1000 dilution) or anti-phospho-ERK1/2 (1:800 dilution), anti NF- κ B p65 (1:800 dilution) or anti-lamin B (1:2000 dilution) antibodies at 4°C overnight. After washed three times, the membranes were incubated with the relevant second antibodies (1:3000 dilution) for 1.5 h and then the immune complexes were enhanced by chemiluminescence.

Enzyme-linked immunosorbent assay (ELISA)

VSMCs were cultured in 96-well plate and stimulated with 10^{-3} - $1.0 \mu\text{M}$ FPA for 12 h or with $10^{-2} \mu\text{M}$ FPA for the indicated time. Then, the supernatant was collected for assaying levels of IL-1 β and IL-6 by ELISA kit specific for rat IL-1 β and IL-6.

Neutralizing experiment

VSMCs were pretreated with anti-IL-1 β and anti-IL-6 neutralizing antibodies (5 $\mu\text{g}/\text{ml}$) alone or in combination for 1 h and then stimulated with FPA ($10^{-2} \mu\text{M}$) for additional 12 h. Finally, CRP protein expression was detected by western blot.

Determination of CRP expression in VSMCs and in the vessel wall with the immunofluorescence staining

In the *in vitro* experiment, VSMCs were incubated with the primary mouse anti-smooth muscle-actin antibody and with the secondary cy3 conjugated antibody for the identification of VSMCs after the cells were fixed and blocked. Next, the cells were treated with the primary rabbit anti-CRP antibody and with the secondary Alexa fluor 488 conjugated antibody for the staining of CRP. Subsequently, the cells were stained in the 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) solution. Each step above was followed by washing three times. Finally, the cells on the coverslips were preserved in anti-fade mounting medium and CRP expression was observed under fluorescent microscope (Olympus Tokyo, Japan).

In the *in vivo* experiment, the aortic artery was immediately isolated after blood samples were collected. Frozen sections (5 μm) were incubated with rabbit anti-CRP antibody (1:50 dilution) and mouse anti-smooth muscle actin antibody (1:100 dilution) at 4°C overnight. After washed three times, the sections were incubated with goat anti-rabbit IgG (Alexa Fluor 488 conjugated) and goat anti-mouse IgG (Cy3 conjugated) antibodies for 30 min at 37°C followed by the DAPI (10 mg/ml) staining for 3 min at 37°C. Finally, the sections were observed under fluorescence microscope (Olympus, Tokyo, Japan).

Measurement of superoxide anion generation in VSMCs

VSMCs were exposed to FPA ($10^{-2} \mu\text{M}$) for 3 h after the pretreatment with NAC ($10^4 \mu\text{M}$), TTFA (10 μM), DPI (10 μM) for 1 h. Then, the cells were loaded with $\text{H}_2\text{DCF-DA}$ (10 μM) for 1 h and washed with PBS three times. Fluorescence images were acquired at the excitation wavelength of 488 nm and the emission wavelength of 525 nm with fluorescence microscope (Olympus, Tokyo, Japan). Fluorescence intensity was measured and analyzed from the fluorescence images with the Image-pro plus software (Version X; Media Cybernetics, Silver Springs, MD, USA). The relative fluorescence intensity was taken as the average of values from six repeated experiments.

Assay of NF- κ B activation in VSMCs by the immunofluorescence staining

VSMCs were incubated with the primary rabbit anti-NF- κ B p65 antibody at 4°C overnight, and then with the secondary Cy3-labeled antibody for 3 h at 37°C. The cells were stained in DAPI (10 mg/ml) for 3 min at 37°C. Finally, the cells on the coverslips were preserved in anti-fade mounting medium, and the activated NF- κ B subunit p65 was observed under fluorescent microscope (Olympus, Tokyo, Japan).

Animal model

To observe whether FPA induces CRP generation in vascular smooth muscle tissue *in vivo*, a model of rat with the high level of blood FPA was established. Male SD rats (n = 16, weight: 180-220 g) were randomly assigned to the model and control groups [32, 33]. The rats in the model group were subcutaneously delivered with 17.0 $\mu\text{g}/\text{kg}/\text{h}$ FPA (dissolved in normal saline) for 14 days through the implanted osmotic mini-pumps (Model 2004, ALZET, Cupertino, CA, USA). Rats in control group received normal saline in the same way as the model group. At the end of the experiment, rats were sacrificed under the deep anesthesia with chloral hydrate. Blood samples were collected via the abdominal aorta for measuring plasma FPA and

CRP levels with ELISA. Then, the thoracic aorta of rats was removed for detecting expressions of CRP, p38, p-p38, ERK1/2 and p-ERK1/2 by western blot and the immunofluorescence staining. All rats were provided by the Experimental Animal Center of Xi'an Jiaotong University School of Medicine. Rats had free access to food and water, and were maintained in a constant environment with a conventional 12 h/12 h light/dark cycle.

Statistical analysis

Data were shown as means $\pm$ SEM. Statistical significance between two groups was tested by Student's t test. Statistical significance between multiple groups was assessed by one-way ANOVA followed by Tukey's test. All statistical analyses were performed by the GraphPad Prism (Version 6; La Jolla, CA, USA). $p < 0.05$ was considered to be statistically significant.

Results

Effect of FPA on the viability of VSMCs

Fig. 2A showed that FPA from 10^{-4} μ M to 1.0 μ M did not produce a significant effect on the viability of VSMCs. Similarly, incubation of VSMCs with 10^{-2} μ M FPA for 6 to 24 h did not affect the viability of VSMCs (Fig. 2B).

FPA stimulates CRP expression in VSMCs

The results in Fig. 3 displayed that stimulation of VSMCs with FPA at 10^{-2} to 1.0 μ M for 12 h increased mRNA and protein expression of CRP in VSMCs in a concentration-dependent manner (Fig. 3A and 3B). Incubation of VSMCs with 10^{-2} μ M FPA for 6 to 24 h up-regulated mRNA and protein expression of CRP in VSMCs in a time-dependent way. Compared with control, mRNA and protein expression of CRP was obviously increased at 6 h and 12 h respectively (Fig. 3C and 3D). The immunofluorescence staining showed that there was a weakly positive staining of CRP in control VSMCs. However, treatment of the cells with 10^{-2} μ M FPA for 12 h up-regulated CRP expression in VSMCs in comparison with control (Fig. 4).

Fig. 2. Effect of FPA on the viability of VSMCs. The cells were incubated with the different concentrations of FPA for 12 h or with 10^{-2} μ M FPA for the indicated time. Then, the cell viability was assayed by the MTT method. Results eight independent experiments were expressed as mean $\pm$ SEM.

Fig. 3. FPA induces mRNA and protein expression of CRP in VSMCs. The cells were treated with the different concentrations of FPA for 12 h or with 10^{-2} μ M FPA for the indicated time. Then, mRNA and protein expression of CRP was identified by quantitative real-time PCR and western blot, respectively. (A) Concentration-dependent increase of CRP mRNA expression, (B) Concentration-dependent increase of CRP protein expression, (C) Time-dependent increase of CRP mRNA expression, (D) Time-dependent increase of CRP protein expression. Results from three independent experiments were expressed as mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ vs. control.

Fig. 4. The immunofluorescence staining of CRP expression in VSMCs (200×). The cells were incubated with 10^{-2} μ M FPA for 12 h. Then, the fluorescence was observed under fluorescence microscope. The right panels were the merged field of purple color (for cell nucleus) and green (for CRP).

Fig. 5. FPA increases mRNA and protein expressions of IL-1 β and IL-6 in VSMCs. The cells were treated with 10^{-3} – 1.0 μ M FPA for 12 h or with 10^{-2} μ M FPA for the indicated times. Then, mRNA and protein expressions of IL-1 β and IL-6 were identified by quantitative real-time PCR and ELISA, respectively. (A) Concentration-dependent increase of IL-1 β mRNA expression, (B) Time-dependent increase of IL-1 β mRNA expression, (C) Concentration-dependent increase of IL-6 mRNA expression, (D) Time-dependent increase of IL-6 mRNA expression, (E) Concentration-dependent increase of IL-1 β protein expression, (F) Time-dependent increase of IL-1 β protein expression, (G) Concentration-dependent increase of IL-6 protein expression, (H) Time-dependent increase of IL-6 protein expression. Results from six independent experiments for ELISA and three independent experiments for quantitative real-time PCR were expressed as mean $\pm$ SEM. * p <0.05 and ** p <0.01 vs. control.

FPA increases IL-1 β and IL-6 expressions in VSMCs

In order to further ensure the pro-inflammatory effect of FPA on VSMCs, we observed effect of FPA on the expressions of IL-1 β and IL-6 in VSMCs. The results showed that FPA increased both IL-1 β and IL-6 mRNA expressions in VSMCs and IL-1 β and IL-6 levels in the supernatant of the cultured VSMCs in concentration- and time-depended fashions (Fig. 5).

Effect of anti-IL-1 β and anti-IL-6 neutralizing antibodies on FPA-induced CRP expression in VSMCs

It is known that IL-1 β and IL-6 stimulate CRP expression in VSMCs [34], and FPA-induced CRP expression in VSMCs is possibly attributed to the release of IL-1 β and IL-6. So, we conducted the neutralizing experiment to identify whether FPA directly induced CRP expression in VSMCs. As shown in Fig. 6, anti-IL-1 β and anti-IL-6 neutralizing antibodies alone or in combination did not alter FPA-induced expression of CRP in VSMCs. The result suggests that FPA directly induces CRP expression in VSMCs.

ROS are involved in FPA-induced CRP expression in VSMCs

As seen from Fig. 7A, the production of superoxide anion was significantly increased after the incubation of VSMCs with FPA. However, pre-incubation of the cells with TTFA (complex II inhibitor), DPI (NADPH oxidase inhibitor) and antioxidant NAC reduced FPA-induced production of superoxide anion in VSMCs. The further study indicated that NAC nearly abolished FPA-induced CRP expression in VSMCs (Fig. 7B), suggesting that ROS are involved in FPA-induced CRP expression.

FPA induces CRP expression in VSMCs via ERK1/2/p38 pathway

Fig. 8A and 8B exhibited that pretreatment of VSMCs with PD98059 (ERK1/2 inhibitor) and SB203580 (p38 inhibitor) weakened FPA-induced expression of CRP. Although JNK inhibitor SP600125 mildly reduced FPA-induced CRP expression, there was no significant statistical difference in CRP expression compared with FPA alone (Fig. 8C), indicating that FPA induces CRP expression in VSMCs via ERK1/2 and p38 pathway.

Fig. 6. Effect of anti-IL-1 β and anti-IL-6 neutralizing antibodies on FPA-induced CRP expression in VSMCs. The cells were pretreated with anti-IL-1 β or/and anti-IL-6 neutralizing antibodies for 1 h before the stimulation with FPA for 12 h. Then, CRP protein expression was detected by western blot. Results from three independent experiments were expressed as mean $\pm$ SEM. ** p <0.01 vs. control.

Fig. 7. Involvement of ROS in FPA-induced CRP expression in VSMCs. (A) The cells were exposed to FPA (10⁻² μM) for 3 h after the pretreatment with TTFA (10 μM), DPI (10 μM), NAC (10⁴ μM). Then, ROS was observed under fluorescence microscope (200 $\times$): (a) Control, (b) FPA alone, (c) FPA + TTFA, (d) FPA + DPI, (e) FPA + NAC, (f) Ros up: a positive control. The relative fluorescence intensity was quantified from the fluorescence images. (B) The cells were pretreated with NAC (10⁴ μM) for 1 h before the incubation with 10⁻² μM FPA for 12 h and then, CRP protein expression was detected by western blot. Data from three independent experiments were expressed as mean $\pm$ SEM. ** p <0.01 vs. control, ## p <0.01 vs. FPA alone.

Fig. 8. FPA induces CRP expression in VSMCs via MAPK pathway. The cells were pretreated with SB203580, SP600125 or PD98059 for 1 h before the incubation with 10^{-2} μ M FPA for 12 h. Then, CRP protein expression was assayed by western blot. Results from three independent experiments were expressed as mean $\pm$ SEM. ** p <0.01 vs. control, # p <0.05 vs. FPA alone.

FPA induces CRP expression in VSMCs through the ROS-ERK1/2/p38 signal pathway

In order to clarify the relationship between ROS and ERK1/2/p38 pathways in FPA-induced CRP expression, VSMCs were pretreated with antioxidant NAC, ERK1/2 inhibitor PD98059 or p38 inhibitor SB203580 before stimulated with FPA at 10^{-2} μ M. Then, the phosphorylation of ERK1/2 and p38 was detected. The results showed that FPA activated the phosphorylation of ERK1/2 and p38 in VSMCs, while NAC, PD98059 and SB203580 decreased the expressions of the phosphorylated ERK1/2 and p38. These implicate that FPA induces CRP expression through the ROS-ERK1/2/p38 signal pathway (Fig. 9).

Fig. 9. FPA induces CRP expression in VSMCs through the ROS-ERK1/2/p38 signal pathway. The cells were pretreated with NAC, PD98059 or SB203580 for 1 h before the incubation with FPA at 10^{-2} μ M for 1.5 h. Then, the phosphorylation of ERK1/2 and p38 was detected by western blot. Data from three independent experiments were expressed as mean $\pm$ SEM. ** p <0.01 vs. control, ## p <0.01 vs. FPA alone.

NF- κ B mediates FPA-induced CRP expression in VSMCs

Western blot analysis showed that FPA enhanced NF- κ B level in the nuclei of VSMCs (Fig. 10A). The immunofluorescence staining revealed that fluorescence for the cytosolic NF- κ B was stronger in FPA-treated cells than control cells (Fig. 10B), suggesting that more NF- κ B is activated in the cytoplasm of VSMCs. The further experiment manifested that pretreatment of VSMCs with NF- κ B inhibitor PDTC reduced FPA-induced expression of CRP (Fig. 10C), verifying NF- κ B-mediated CRP expression induced by FPA in VSMCs.

FPA stimulates CRP expression in vascular smooth muscle of rats

The results from the *in vivo* experiment showed that the subchronic administration of FPA to rats for 14 days obviously increased FPA (Fig. 11A) and CRP concentrations

Fig. 10. NF-κB mediates FPA-induced CRP expression in VSMCs. (A) The cells were treated with $10^{-2} \mu\text{M}$ FPA for 12 h, and NF-κB level in the nuclei of VSMCs was detected by western blot. (B) The cells were treated with $10^{-2} \mu\text{M}$ FPA for 12 h and then, the activated NF-κB subunit p65 were examined by the Cy3-labeled immunofluorescence staining, and the nuclei were stained by DAPI (200×): (Upper) the activated NF-κB subunit p65 (red), (middle) the cellular nuclei (blue), (below) the merged images. (C) The cells were pretreated with PDDC for 1.5 h before the incubation with $10^{-2} \mu\text{M}$ FPA for 12 h, and then CRP protein expression was determined by western blot. Results from three independent experiments were expressed as mean $\pm$ SEM. ** $p < 0.01$ vs. control, ## $p < 0.01$ vs. FPA alone.

Fig. 11. FPA stimulates CRP expression in the aortic artery walls of rats in vivo. After the administration of FPA ($17 \mu\text{g/kg/h}$) to rats for 14 days, plasma FPA and CRP levels were detected by ELISA, and CRP expression in the aortic artery walls of rats was examined with the immunofluorescence staining and western blot. The phosphorylation of ERK1/2 and p38 in the aortic artery walls was detected by western blot. (A) Plasma FPA, (B) Plasma CRP, (C) CRP expression in the aortic artery walls, (D) Expressions of ERK1/2, p-ERK1/2, p38 and p-p38 in the aortic artery walls, (E) The immunofluorescence staining of CRP in the vessel wall (200×): The right panels were the merged field of red (for α-actin) and green (for CRP), and purple color represented the cell nucleus. Results were expressed as mean $\pm$ SEM ($n = 6$). ** $p < 0.01$ vs. model.

in plasma (Fig.11B) and CRP expression in the aortic artery walls of rats (Fig. 11C). The immunohistochemical staining exhibited that a weak staining of CRP existed in the aortic artery walls of control rats. However, CRP expression was potently increased in the aortic artery walls of FPA-treated rats (Fig. 11E). The images of CRP and α-actin coexisting

indicated that VSMCs were a possible origin of CRP (Fig. 11E, merged panels). In addition, FPA enhanced levels of the phosphorylated ERK1/2 and p38 in the aortic artery walls of rats (Fig. 11D).

Discussion

Atherosclerosis is a common cardiovascular disease and the main pathological basis of the ischemic cardiocerebrovascular diseases. Although the pathogenesis of atherosclerosis is not well defined, more and more evidence supports that atherosclerosis is a chronic inflammatory disease in the artery walls. The inflammation plays an important role in the formation and progression of atherosclerosis. Among the multiple inflammatory factors, CRP is regarded as a representative molecule. The clinical observations find that a high level of FPA in blood means the activation of coagulation system [35] and occurs in the patients with venous thrombosis, pulmonary embolism and ischemic heart disease as a biomarker. However, few reports on the pathological effects of FPA in the cardiovascular system are indexed in literature. Therefore, the present study examined the pro-inflammatory effect of FPA on VSMCs and the molecular mechanisms. The results showed that FPA stimulated mRNA and protein expressions of CRP, IL-1 β and IL-6 in VSMCs *in vitro*, and anti-IL-1 β or anti-IL-6 neutralizing antibody alone or in combination did not change FPA-induced protein expression of CRP in VSMCs. The animal experiment confirmed that the subchronic administration of FPA to rats increased FPA and CRP levels in plasma and CRP expression in the aortic artery walls. These results suggest that FPA is not simply a biomarker of the coagulation activation, but also produces a pro-inflammatory effect on VSMCs by stimulating CRP, IL-1 β and IL-6 expressions.

ROS are both the important second messenger and the direct participant of oxidative stress [36]. Low concentration of ROS is permanently produced in cells, which plays a role as signal molecules. Researches show that ROS participate in the production of many inflammatory cytokines by the different signal pathways such as MAPK and NF- κ B [37, 38]. In the study, we found that FPA stimulated the production of superoxide anion in VSMCs, and the NADPH oxidase inhibitor DPI, mitochondrial respiratory complex II inhibitor TTFA and antioxidant NAC all inhibited FPA-induced the generation of superoxide anion in VSMCs, suggesting that the NADPH oxidase pathway and mitochondria-derived signal pathway are involved in FPA-elicited ROS generation. The abnormal generation of ROS plays important role in CRP expression, and this has been proved in many kinds of cell lines or tissues such as VSMCs [13], endothelial cells [39], liver cells [40] and macrophages [41]. The present study exhibited that antioxidant NAC abolished CRP expression induced by FPA, demonstrating that ROS mediate FPA-induced CRP expression in VSMCs.

MAPK is activated in the inflammatory process of atherosclerosis. ROS are able to regulate MAPK pathway involved in inflammatory process [42, 43]. The current experiment showed that FPA increased the expressions of phosphorylated ERK1/2 and p38 *in vivo* and *in vitro*, and ERK1/2 inhibitor PD98059 and p38 inhibitor SB203580 reduced FPA-induced CRP expression *in vitro*, implicating that the activated ERK1/2 and p38 participate in FPA-induced CRP production. However, JNK inhibitor SP600125 did not significantly affect FPA-induced CRP expression. In order to understand the roles of ROS in the inflammatory process, we detected the effect of the ROS scavenger NAC on the activation of ERK1/2 and p38. The results show that antioxidant NAC reduced the expressions of the phosphorylated ERK1/2 and p38. Collectively, these confirm that ROS are the upstream molecules of ERK1/2/p38 and regulate ERK1/2/p38 activation, and FPA induces CRP expression via the ROS-ERK1/2/p38 signal pathway. But, we do not rule out other signal pathways mediating FPA-induced CRP expression, since ERK1/2 and p38 inhibitors partially inhibited FPA-induced CRP expression.

The existed evidence certifies that MAPK may activate NF- κ B [44]. NF- κ B activation regulates the generation of mediators resulting in the thrombotic potential of human

atherosclerotic plaques, such as tissue factor, matrix metalloproteinases and inflammatory cytokines [45]. The present experiment observed that FPA activated NF- κ B and enhanced NF- κ B level in the nuclei of VSMCs, and the selective NF- κ B inhibitor PDTC reduced FPA-induced expression of CRP. These confirm that NF- κ B participates in FPA-induced CRP expression in VSMCs.

In conclusion, the present study demonstrates that FPA induces CRP expression in VSMCs via the ROS-ERK1/2/p38-NF- κ B signal pathway. This finding for the first time provides an evidence for pro-inflammatory effect of FPA on VSMCs.

Disclosure Statement

No conflict of interests exists.

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