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CRP regulates the expression and activity of tissue factor as well as tissue factor pathway inhibitor via NF-κB and ERK 1/2 MAPK pathway

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ABSTRACT

It was found that C-reactive protein (CRP) could significantly increase the expression and activity of tissue factor (TF), but decrease that of tissue factor pathway inhibitor (TFPI) in human umbilical vein endothelial cells (HUVECs) in dose- and time-dependent manners, which could be antagonized by PDTC and U0126. CRP could also increase protein expression of phosphorylated nuclear factor-kappaB (NF-κB), IκB-α and ERK1/2 in dose- and time-dependent manner. In addition, neutralizing antibody to CD32 (FcgammaR II) could significantly attenuate the expression and activity of TF and TFPI induced by CRP. These results suggest that CRP may promote coagulation by enhancing the expression and activity of TF and reducing that of TFPI by activating NF-κB and extracellular signal-regulated kinase via FcgammaR II.

1. Introduction

Increasing evidence supports the involvement of inflammation in the pathogenesis of atherosclerotic diseases [1–3]. C-reactive protein (CRP), the prototypic marker of inflammation, in addition to being a risk marker, can also act as the important predictive factor for cardiac events [4–6]. In addition, it was demonstrated that injecting highly purified CRP into humans could activate the blood coagulation system [7,8]. Hence, CRP may trigger clinical cardiac ischemic events by promoting thrombosis. Recently, increasing evidence suggested that CRP might be also an active participant in atherosclerosis [9–11].

Tissue factor (TF), a critical initiator of blood clotting, is present in atherosclerotic plaque and triggers thrombosis after plaque rupture [12,13]. Several studies demonstrated that TF played a pivotal role in the pathophysiology of acute coronary syndrome (ACS) by inducing the intracoronary thrombosis following endothelial injury [14–16]. Under physiological state, TF is hardly expressed in vascular endothelium, but when endothelial injury occurs, including endothelial dysfunction, it is sharply expressed and released into blood stream.

The integrality of vascular endothelial structure and its function are closely associated with atherogenesis, atherosclerotic development, and the final cardiac events. Over the past years, evidence has accumulated from basic to clinical studies for a close association of the degree of endothelial dysfunction and clinical cardiovascular events in patients with cardiovascular risk factors, coronary artery disease, ACS, or even heart failure. Vascular endothelium is not only the first barrier against macrophage infiltration, foam cells and atherosclerosis formation, but also the important tissue, which synthesizes and secrets coagulation and anti-coagulation factors to modulate coagulation function. However, in endothelial cells, it is unclear whether CRP can affect TF and tissue factor pathway inhibitor (TFPI) expression and activity, and how to regulate it. Therefore, the present study aimed to reveal it.

2. Materials and methods

2.1. Cell culture

Human umbilical veins endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, USA) and grown at 37 °C in 5% CO_2/95% O_2 using RPMI 1640 medium

Abbreviations: CRP, C-reactive protein; TF, tissue factor; TFPI, tissue factor pathway inhibitor; HUVECs, human umbilical vein endothelial cells; ACS, acute coronary syndrome; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; NF-κB, nuclear factor-kappaB; MAPK, mitogen-activated protein kinase

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containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Before each experiment, HUVECs were placed in medium with 1% FBS for 24 h.

2.2. Reagents

The recombined human CRP (rhCRP, Calbiochem) used in the protocols was initially dialyzed for 24 h against Dulbecco's PBS using a dialysis slide (Pierce) with a cutoff of 10 kDa to remove sodium azide, which is present as a preservative in commercial preparations of CRP and demonstrated to have vasorelaxation effect in some studies [17,18]. Endotoxin, which can affect endothelial function [19], was also removed from the commercial rhCRP by using Detoxi-Gel Columns (Pierce) and was found to be <0.05 EU/ml by the Limulus assay (Cambrex).

RPMI 1640 medium, FBS, peniciline and streptomycin were all obtained from Hyclone. PDTC, the specific inhibitor of NF-κB, U0126 [Inhibitor of ERK1/2 mitogen-activated protein (MAPK)], SB203580 (Inhibitor of p38 MAPK), and SP600125 (Inhibitor of JNK MAPK) were all obtained from Calbiochem. Antibodies of IκB-α phosphorylated at Ser32 (P-IκB-α), phosphorylated NF-κB-p65 (P-NF-κB-p65), phosphorylated ERK1/2 (P-ERK1/2), total-ERK1/2 (T-ERK1/2) and secondary antibody conjugated with horseradish peroxidase (HRP) were all purchased from Cell Signaling Technology (CST). Anti-CD16 (FcgammaR III), anti-CD32 (FcgammaR II), anti-CD64 (FcgammaR I) antibodies were purchased from Santa Cruz Biotechnology Inc.

2.3. Quantification of TF and TFPI antigen expression

HUVECs were pretreated with or without inhibitor of NF-κB (100 μmol/l PDTC), as well as MAPK inhibitors (25 μmol/l U0126, SB203580, SP600125) for 3 h, and then cultured with medium in the absence of 0–100 μg/ml purified rhCRP for 6 h or 50 μg/ml purified rhCRP for different time from 0 to 24 h. The detailed flowsheet was shown in Fig. 1. The cells were repeated freeze–thaw for three cycles and the TF extracted with a buffer saline, and then centrifuge the lysed cells to remove the cell debris. The cell lysates were prepared for the measurement of TF antigen expression according to the recommendation of kit. The ELISA kits for measurement of TF and TFPI antigen were purchased from ADI (American Diagnostic Inc.).

2.4. Determination of TF and TFPI activity

HUVECs were treated with the same methods mentioned above, and then TF and TFPI activities were determinated by chromogenic substrate with kit (ADI) according to the recommendation.

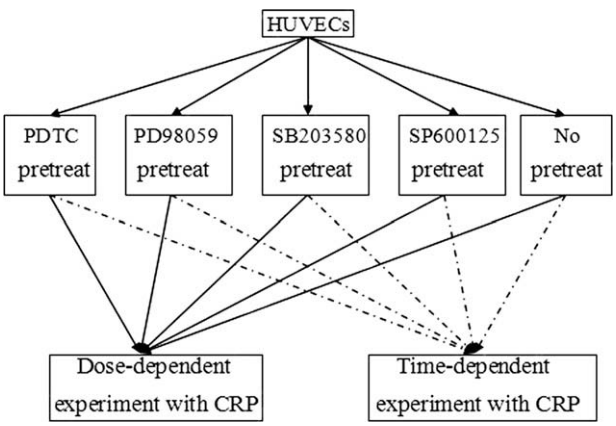

Fig. 1. Experimental flowsheet.

2.5. Measurement of mRNA expression of TF and TFPI

After the treatment for HUVECs, total RNA was isolated from the cells using Trizol according to the manufacturer's instructions (Invitrogen, USA). Reverse transcription (RT) of the RNA was performed using the ImProm-IITM Reverse Transcription System (Promega, USA). Real-time PCR was performed to determine the mRNA expression of TF and TFPI with Rotor-gene PCR System. SYBR Green PCR Master Mix was obtained from Promega (USA). Housekeeping human β-actin mRNA was also simultaneously amplified as internal control. The primers used were as follows: TF, 5′-CCT TAC CTG GAG ACA AAC CTC G-3′ (sense) and 5′-CCG TTC ATC TTC TAC GGT CAC A-3′ (antisense); TFPI, 5′-GAC TCC GCA ATC AAC CAA GGT-3′ (sense) and 5′-CTG TCT GCT GGA GTG AGA CAC C-3′ (antisense); β-actin: 5′-AGC CTC GCC TTT GCC GA-3′ (sense) and 5′-CTG GTG CCT GGG GCG-3′ (antisense).

2.6. Western blot analysis for protein expression of signaling pathway

To elucidate the mechanisms by which CRP excerts its effects on HUVECs, we tested the protein expression of NF-κB pathway (P-IκB-α, P-NF-κB-p65) and ERK1/2 MAPK pathway (P-ERK1/2, T-ERK1/2).

Total protein was extracted from cells and protein concentration was measured with Protein Extraction kit and BCA Protein Assay Kit respectively (Beyotime Institute of Biotechnology, Beijing, China). Subsequently, β-mercaptoethanol was added to a final concentration of 1%, after which each sample was denatured by boiling for 5 min, followed by heating and then subjected to 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) gel. And transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore), after blocking with 3% dry milk/0.1% Tween 20, incubated with primary antibodies in the same solution, then incubation with HRP-conjugated secondary antibodies (1:1000), proteins were visualized by ECL plus system (Beyotime Biotechnology, China), according to the instructions provided by the manufacturer. Immunoblotting signals were quantitated using an ImageMaster DVS.

2.7. Electrophoretic mobility shift assay (EMSA) for NF-κB binding activity

HUVECs were treated with the same methods mentioned above, and then nuclear protein were extracted with Nuclear Protein Extraction kit and quantified with kit mentioned above (Beyotime Institute of Biotechnology, Beijing, China) according to the recommendation.

Nuclear extracts were prepared as described above. Nuclear protein/DNA-binding reactions were performed in a volume of 20 ml containing 5 mg of nuclear extract protein, 10 mM HEPES-KOH (pH 7.9), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 2 mg poly (dI-dC) as a non-specific competitor. The probes for NF-kB was end labeled with [γ-^{32}P]ATP (DuPont, USA) by T4 polynucleotide kinase. Binding reactions were started by the addition of a [γ-^{32}P]ATP-labeled DNA probe followed by incubation at room temperature for 0 min. The oligo probe 5′-GCAGAGGGACTTTCC-GAGA-3′ containing the NF-κB binding motif was annealed to the complementary oligonucleotide and end labeled by using T4 polynucleotide kinase. Samples were electrophoresed on a native 6% polyacrylamide gel at 200 V in 0.5 TBE buffer. The gels were then dried and the bands visualized by exposure to film.

2.8. Involvement of receptor type in TF and TFPI expression and activity induced by CRP

To determine the role of Fcgamma receptors (FcgammaRs) in regulating TF and TFPI expression induced by CRP, the block of

FcgammaRs by pretreatment with specific blocking antibodies to CD16, CD32, CD64 (10 μg/ml) for 1 h prior to 6-h CRP treatment, and the expression and activity of TF as well as TFPI were measured by the above methods.

2.9. Statistics

All data were expressed as means ± S.D. Numeric values were analyzed for the presence of normal distribution. Comparisons among groups were performed by one-way ANOVA analysis. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of CRP on the levels of TF and TFPI antigen in HUVECs

HUVECs were cultured with medium in the presence of 0 μg/ml to 100 μg/ml (0, 5, 20, 50, and 100 μg/ml) rhCRP for 6 h or in the presence of 50 μg/ml rhCRP for 0–24 h (0, 2, 6, 12, and 24 h). Data in Fig. 2 showed that basic TF antigen expression was very low, and CRP could significantly increase the expression of TF antigen and decrease the expression of TFPI antigen in dose-dependent (Fig. 2A and B) and time-dependent (Fig. 2C and D) manners.

3.2. Effects of CRP on the mRNA expression of TF and TFPI in HUVECs

HUVECs were cultured and treated with the same methods mentioned above. As shown in Fig. 3, the basic mRNA expression of TF was also very low, and CRP could remarkably increase the expression of TF mRNA and decrease the expression of TFPI mRNA in dose-dependent (Fig. 3A and B) and time-dependent (Fig. 3C and D) manners.

3.3. Effects of CRP on the activities of TF and TFPI in HUVECs

After the same treatment for HUVECs mentioned above, the activities of TF and TFPI were measured according to the recommendation of kits. Results showed that the activity of TF before CRP stimulation was much lower, and CRP treatment could significantly increase the activity of TF and decrease the activity of TFPI in dose-dependent (Fig. 4A and B) and time-dependent (Fig. 4C and D) manners.

3.4. Effects of CRP on the protein expression of NF-κB and ERK1/2 MAPK pathway in HUVECs

After treatment for HUVECs, protein of cells was extracted, and Western blot was used to evaluate the protein expression of NF-κB and ERK1/2 MAPK pathway (P-NF-κB-p65, P-IκB-α, P-ERK1/2, and T-ERK1/2). As shown in Fig. 5, CRP could significantly increase the protein expression of P-NF-κB-p65 as well as P-IκB-α, in a dose-dependent manner (Fig. 5A and B), and remarkably increase the protein expression of P-ERK1/2 in a dose-dependent manner without significant influence on the protein of T-ERK1/2 (Fig. 5C).

3.5. Effects of CRP on the DNA binding activity of NF-κB in HUVECs

NF-κB transcription factor is the key expression regulator of many genes in all kinds of cells. Therefore, except for evaluation of protein expression of P-NF-κB-p65 and P-IκB-α, we also focused on the DNA binding activity of NF-κB with EMSA. Results in Fig. 6

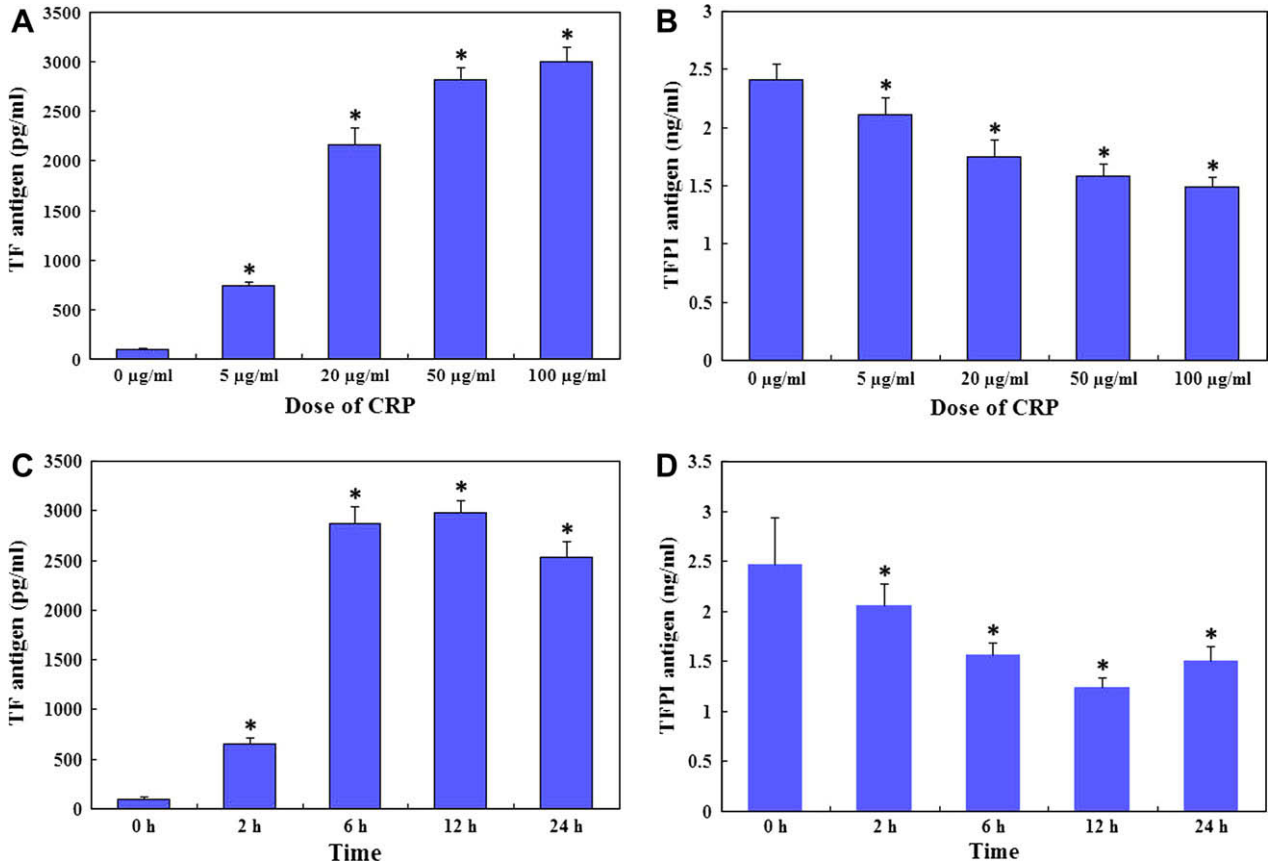

Fig. 2. Effects of CRP on the levels of TF and TFPI antigen in HUVECs. (A and B) Dose-dependent effect of CRP on the levels of TF and TFPI antigen. (C and D) Time-dependent effect of CRP on the levels of TF and TFPI antigen. * $P < 0.01$ vs. basic value.

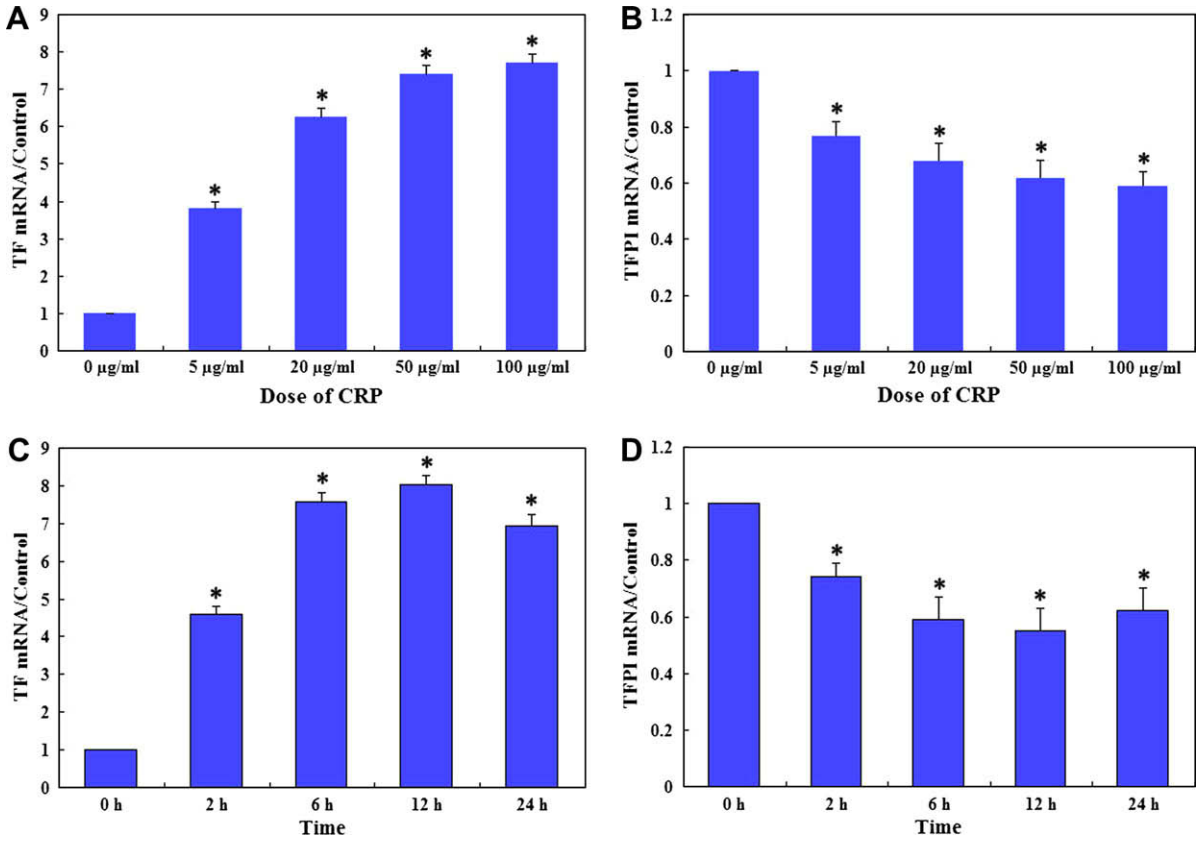

Fig. 3. Effects of CRP on the mRNA expression of TF and TFPI. (A and B) Dose-dependent effect of CRP on mRNA expression of TF and TFPI. (C and D) Time-dependent effect of CRP on mRNA expression of TF and TFPI. * $P < 0.01$ vs. basic value.

showed that CRP could significantly increase the DNA binding activity of NF-κB in dose-dependent (Fig. 6A) and time-dependent (Fig. 6B) manners.

3.6. Effects of NF-κB and MAPK pathway inhibitors on the expression and activity of TF as well as TFPI induced by CRP in HUVECs

As the important regulation pathways of signal transduction, NF-κB and MAPK pathways play pivotal role in regulating the expression of many genes. To evaluate the mechanism of CRP-induced changes of TF and TFPI expression and activity, NF-κB and MAPK pathway inhibitors (PDTC, U0126, SB203580, SP600125) were used to pretreat HUVECs for 3 h before 6-h treatment with 50 μg/ml CRP.

From the results in Fig. 7, it could be observed that PDTC and U0126 could significantly counter-regulate the effects of CRP on the expression as well as activity of TF and TFPI ($P < 0.01$), but SB203580 and SP600125 could not cause significant changes in the expression and activity of TF and TFPI ($P > 0.05$).

3.7. Effect of antibodies to Fcgamma receptors on CRP-induced TF/TFPI expression and activity

As shown in Fig. 8, after coincubation with neutralizing antibodies of CD16 (FcgammaR I), CD32 (FcgammaR II) and CD-64 (FcgammaR III), expression and activity of TF and TFPI induced by CRP were significantly attenuated by antibody to CD-32 ($P < 0.01$), but not by the antibodies to CD16 and CD-64 ($P > 0.05$).

4. Discussion

It is well accepted that inflammation plays an important role in atherogenesis, atherosclerotic development, and even cardiac events such as ACS [20–22]. CRP, as the inflammatory marker, was the important predictor of cardiovascular events and prognosis [4–6]. However, it is unclear and controversial about whether CRP directly participates in endothelial dysfunction and atherogenesis.

Normally, the levels of TF and TFPI are well balanced to prevent thrombosis or hemorrhage, but when vascular endothelium is stimulated by some factors, TF will be greatly increased and the balance between TF and TFPI will be broken to promote coagulation. Therefore, the balance between TF and TFPI also plays pivotal role in coronary events [14–16,23–25].

In the present study, purified rhCRP free of sodium azide and endotoxin, which can contaminate commercial CRP and induce artifact [26–28], was used to evaluate the effects of CRP on the expression and activity of TF and TFPI. Results demonstrated that CRP could induce the increase of TF activity and TF antigen as well as mRNA expression, and CRP could also cause the decrease of TFPI in the above parameters. In addition, CRP could enhance the DNA binding activity of NF-κB and promote the protein expression of phosphorylated NF-κB-p65 and I-κB-α. To evaluate which pathway participates in the effects of CRP on TF and TFPI expression and activity, NF-κB and MAPK pathway inhibitors were used to pretreat HUVECs before treatment with CRP. It was demonstrated that PDTC and U0126 could both significantly reduce the expression and activity of TF and TFPI induced by CRP in HUVECs, but SB203580 and SP600125 had no significant effects on the expression and activity, in addition, CRP could greatly increase the protein expression of P-ERK1/2 MAPK without significant influence on T-ERK1/2 MAPK.

However, many studies revealed that CRP could modulate gene expression via other pathways or via other receptors. For example, a study from Kuhlmann et al. found that CRP could cause a disrup-

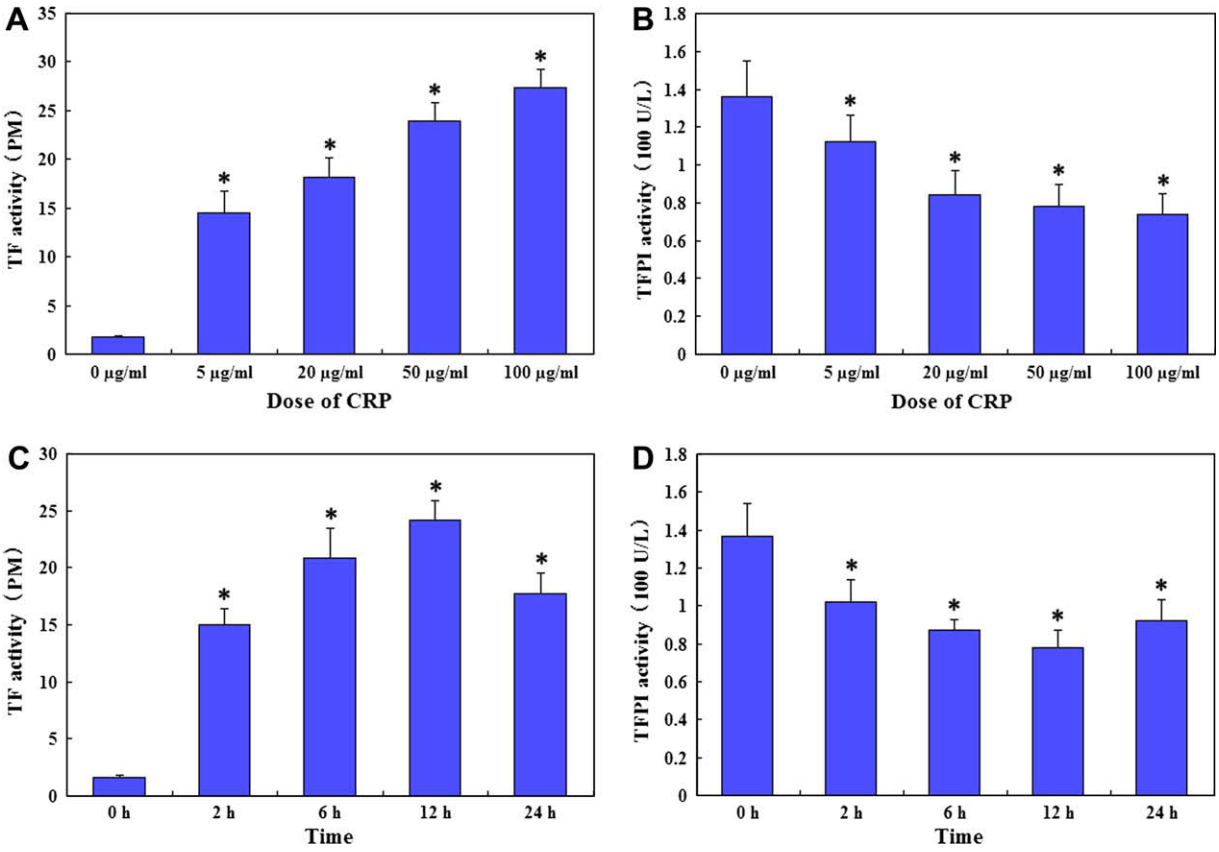

Fig. 4. Effects of CRP on the activities of TF and TFPI. (A and B) Dose-dependent effect of CRP on the activities of TF and TFPI. (C and D) Time-dependent effect of CRP on the activities of TF and TFPI. $^{*}P < 0.01$ vs. basic value.

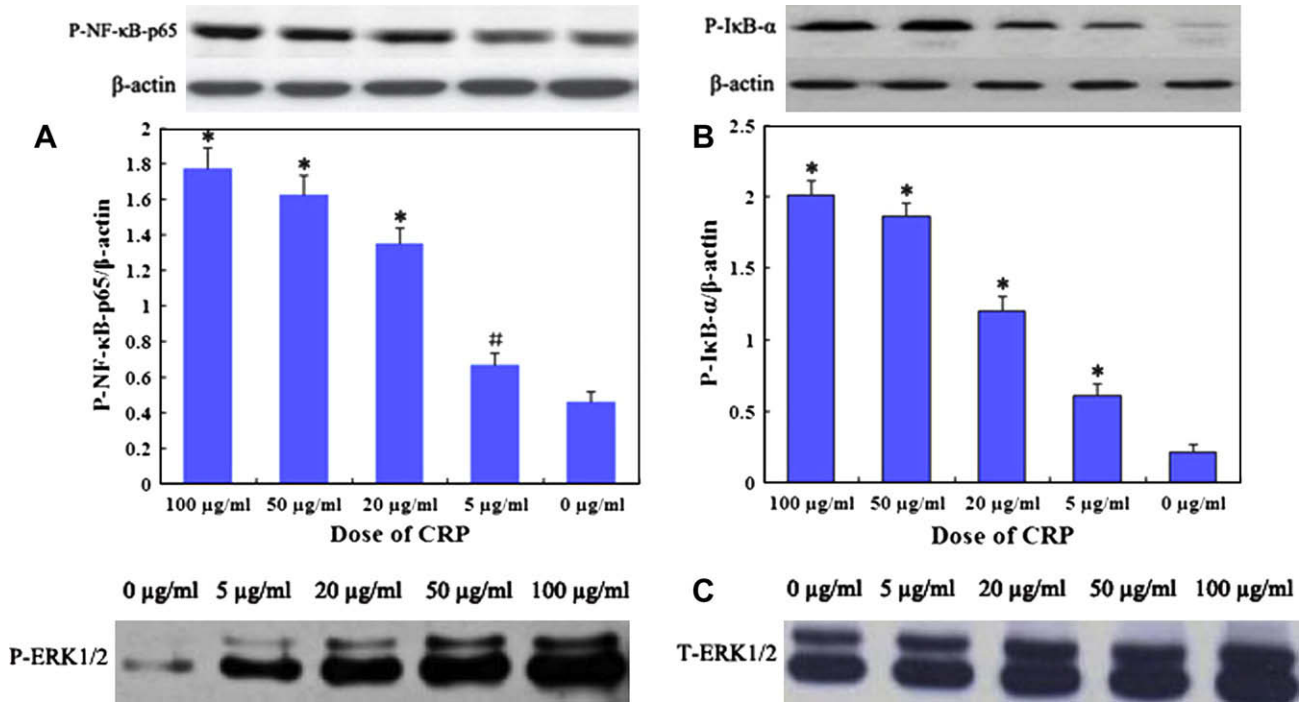

Fig. 5. Effects of CRP on the protein expression of P-NF-κB-p65 and P-IκB-α. (A) Dose-dependent effect of CRP on the protein expression of P-NF-κB-p65. (B) Dose-dependent effect of CRP on the protein expression of P-IκB-α. (C) Dose-dependent effect of CRP on the protein expression of P- and T-ERK1/2. $^{\#}P < 0.05$ and $^{*}P < 0.01$ vs. basic value.

tion of the blood–brain barrier via activation of surface Fcgamma receptors CD16/32 followed by p38-MAPK-dependent reactive oxygen species formation by the NAD(P)H-oxidase [29], which was demonstrated in bovine brain microvascular endothelial cells

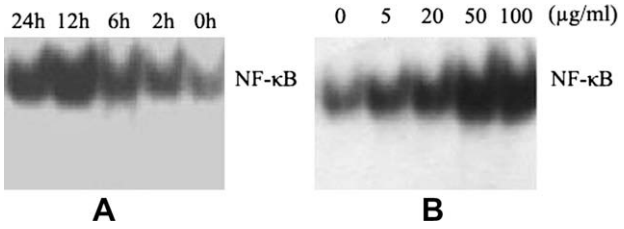

Fig. 6. Effects of CRP on the DNA binding activity of NF-κB. (A) Dose-dependent effect of CRP on the DNA binding activity of NF-κB. (B) Time-dependent effect of CRP on the DNA binding activity of NF-κB.

(BBMVEC) and EVC304. Montero et al. demonstrated that CRP could augment matrix metalloproteinase-1 (MMP-1) mRNA expression in HUVECs and aortic endothelial cells (HAECs) via p38 or ERK1/2 MAPK pathway, whereas regulate MMP-10 expression via p38 and JNK pathways [30]. In addition, Wang et al. demonstrated that CRP could significantly upregulate the expression of IL-18 via ERK MAPK pathway but not the other pathways in HUVECs [31], and Wu et al. demonstrated that CRP could increase TF and decrease TFPI expression by activating ERK1/2 but not p38 or JNK MAPK pathway in VSMCs [32]. Consequently, regulation of gene expression by CRP is complex and expression of different gene in the same cell model or same gene in different cell models may be regulated via different pathways. The results in our study suggest that CRP may promote coagulation by affecting TF and TFPI expression and activity via FcgammaR II and NF-κB as well as ERK1/2 pathway in HUVECs, and it may be independent of JNK and p38 MAPK pathways. The effects of CRP on endothelial function and atherosclerosis including the mechanisms are far from clear, and more studies, especially mechanism studies, are needed to further clarify. In one word, these findings provide new insights into the molecular mechanisms, and effects of CRP

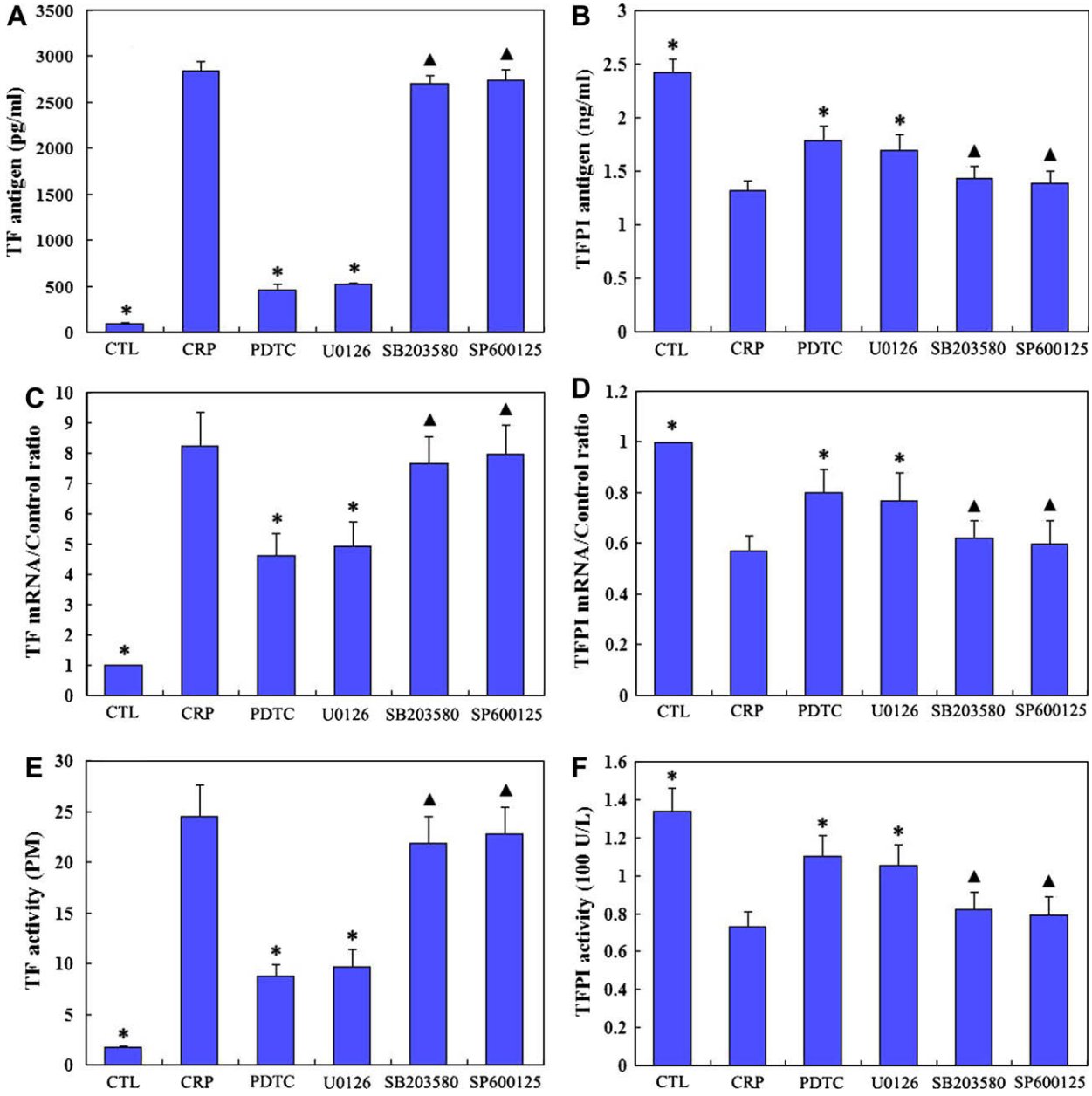

Fig. 7. Effects of NF-κB and MAPK pathway inhibitors on the expression and activity of TF as well as TFPI induced by CRP. (A and B) Effects of inhibitors on the levels of TF and TFPI antigen. (C and D) Effects of inhibitors on the expression of TF and TFPI mRNA. (E and F) Effects of inhibitors on the activity of TF and TFPI. $^{\blacktriangle}P > 0.05$ and $^{*}P < 0.01$ vs. CRP group without pretreatment of inhibitors.

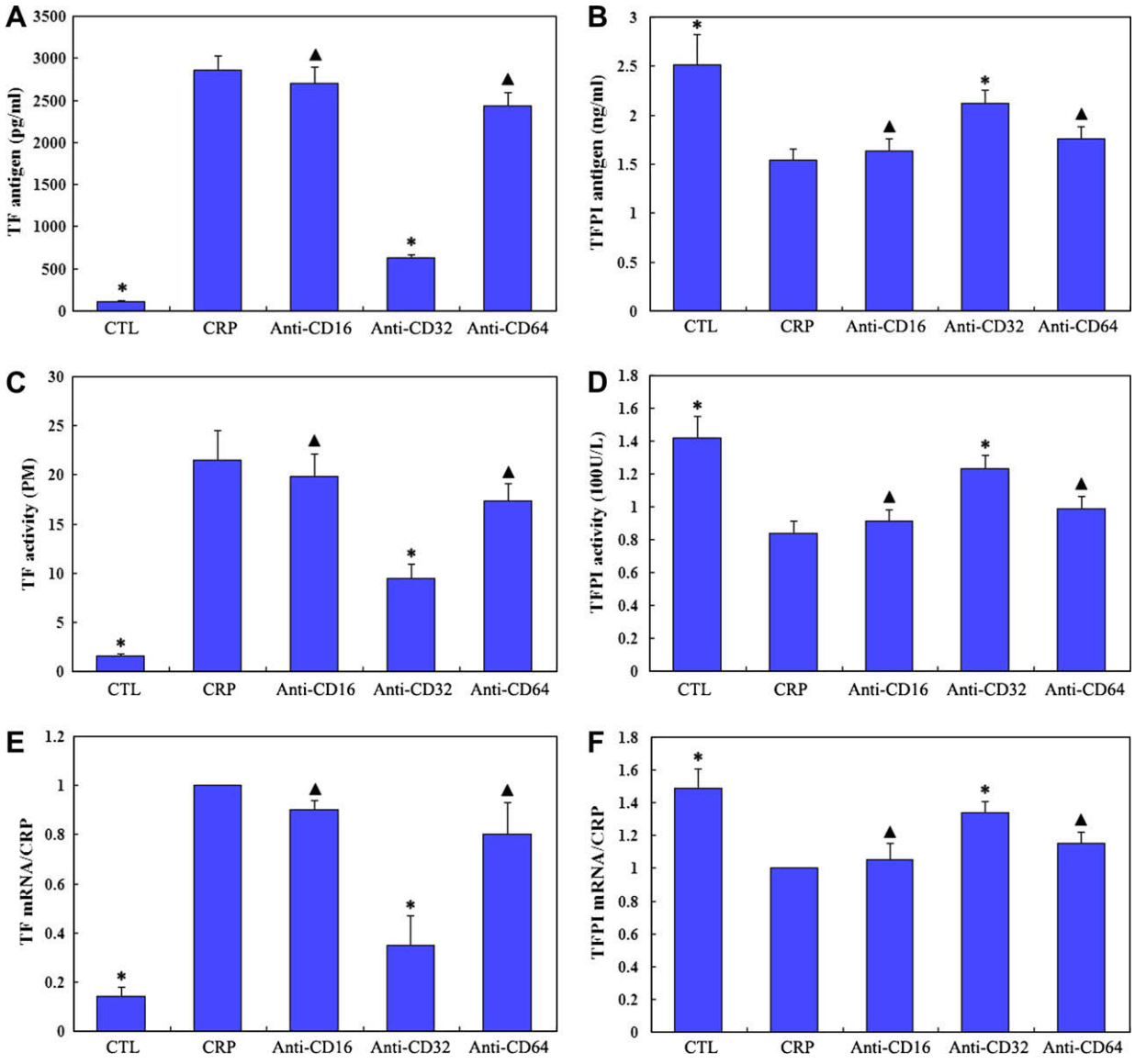

Fig. 8. Effect of antibodies to Fcgamma receptors on CRP-induced TF/TFPI expression and activity. (A and B) Effect of antibodies to Fcgamma receptors on the expression of TF and TFPI antigen. (C and D) Effect of antibodies to Fcgamma receptors on the activity of TF and TFPI. (E and F) Effect of antibodies to Fcgamma receptors on the expression of TF and TFPI mRNA. CTL = Control group. $^{\blacktriangle}P > 0.05$ and $^{*}P < 0.01$ vs. CRP group.

on the balance between TF and TFPI, and these effects may partly explain the pivotal role of CRP in increasing thrombotic events in patients with atherosclerotic diseases.

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