FEBS Letters 583 (2009) 2811–2818

FEBS Letters

journal homepage: www.FEBSLetters.org

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

YangXin Chen, JingFeng Wang *, YouJie Yao, WoLiang Yuan, MinYi Kong, YongQing Lin, DengFeng Geng, RuQiong Nie

Department of Cardiology, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, Guangzhou 510120, China

ARTICLE INFO

Article history:
Received 31 May 2009
Revised 16 July 2009
Accepted 20 July 2009
Available online 23 July 2009

Edited by Beat Imhof

Keywords:
Human umbilical vein endothelial cell
C-reactive protein
Tissue factor
Tissue factor pathway inhibitor

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

* Corresponding author. Fax: +86 20 81332623.
E-mail address: dr_wjf@hotmail.com (J. Wang).

0014-5793/$36.00
doi:10.1016/j.febslet.2009.07.037

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.

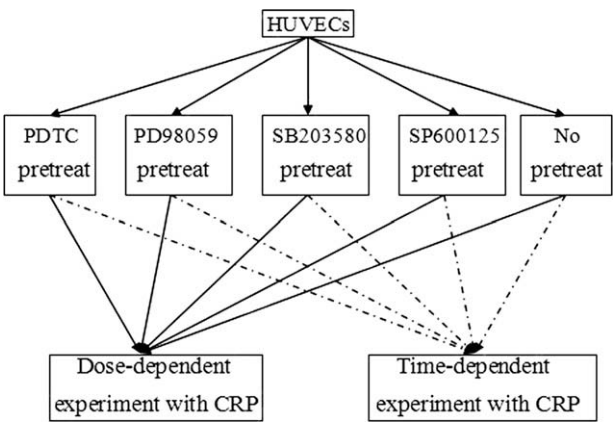

Fig. 1. Experimental flowsheet.

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