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Monomeric C-reactive protein is prothrombotic and dissociates from circulating pentameric C-reactive protein on adhered activated platelets under flow

Blanca Molins[1,2], Esther Peña[1,2], Raquel de la Torre[1,2], and Lina Badimon[1,2,3]*

[1]Cardiovascular Research Center, CSIC-ICCC, Institut Investigacions Biomèdiques Sant Pau, Hospital de la Santa Creu i Sant Pau, C/ Sant Antoni Mª Claret 167, 08025 Barcelona, Spain; [2]Ciber Patofisiologia de la Obesidad y Nutrición, Institute Carlos III, Madrid, Spain; and [3]Càtedra Recerca Cardiovascular (UAB), Barcelona, Spain

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Aims We previously reported that C-reactive protein bioactivity on thrombogenesis was based on loss of its pentameric symmetry, resulting in formation of monomeric C-reactive protein. Our purpose was to provide mechanistic information on the direct effects of C-reactive protein isoforms on platelet activation and provide a C-reactive protein dissociation mechanism in circulating blood.

Methods and results C-reactive protein-induced platelet activation was evaluated by flow cytometry. Platelet aggregation, clot properties, and coagulation were also measured. Washed platelets were incubated with C-reactive protein isoforms and vasodilator-stimulated phosphoprotein (VASP) phosphorylation was analysed by western blot and immunofluorescence. C-reactive protein dissociation under flow was evaluated by confocal microscopy on the surface of adhered platelets after perfusing human blood containing pentameric C-reactive protein at different shear rates. Dissociated monomeric C-reactive protein thrombogenicity was measured in flow experiments. Platelet aggregation and flow cytometry analysis revealed that monomeric C-reactive protein significantly induced platelet aggregation, surface P-selectin and CD63 exposure, and glycoprotein IIb-IIIa activation, whereas pentameric C-reactive protein was unable to produce any effect. p38 mitogen-activated protein kinase (MAPK) and Jun N-terminal kinase (JNK) inhibitors, as well as CD36 blocking antibody partially inhibited monomeric C-reactive protein-induced platelet activation and aggregation. Additionally, monomeric C-reactive protein significantly induced VASP dephosphorylation at serine 239. We found that pentameric C-reactive protein dissociated into monomeric C-reactive protein on the surface of activated adhered platelets under flow conditions and that this generated monomeric C-reactive protein promoted further platelet recruitment.

Conclusions These data indicate that whereas serum pentameric C-reactive protein may not affect platelet activation, monomeric C-reactive protein, which dissociates from pentameric C-reactive protein on the surface of activated platelets, could contribute to atherothrombotic complications by promoting thrombosis.

Keywords C-reactive protein • Dissociation • Platelets • Thrombosis

1. Introduction

C-reactive protein is an acute-phase plasma protein that increases rapidly in response to infection, inflammation, and tissue injury.[1] It is a highly conserved protein of the pentraxin family that consists of five non-covalently bound globular subunits of 23 kDa. C-reactive protein is mainly produced in the liver, although extrahepatic synthesis has also been reported.[2,3] Small two- to five-fold increases in the baseline level of plasma C-reactive protein in asymptomatic individuals have been associated with an increased risk for cardiovascular events such as stroke and myocardial infarction.[4,5] There is emerging evidence that suggests that C-reactive protein may be a direct causative factor

* Corresponding author. Tel: +44 34 93 556 58 80; fax: +44 34 93 556 55 59, Email: lbadimon@csic-iccc.org

of coronary heart disease (CHD).[6,7] However, the causal role of C-reactive protein in atherosclerosis and its complications is controversial. Recent studies using Mendelian randomization analysis did not find a causal association between C-reactive protein levels and CHD.[8,9]

In human plasma, C-reactive protein exists as a cyclic, disc-shaped pentamer of 115 kDa (native or pentameric pentameric C-reactive protein). However, pentameric C-reactive protein can undergo dissociation, thereby acquiring distinct functionality.[10] This alternative conformation of C-reactive protein has been termed modified or monomeric C-reactive protein and has a distinct solubility (tending to be a non-soluble tissue-based protein rather than a soluble plasma-based protein), with antigenicity-expressing neoepitopes differing from pentameric C-reactive protein epitopes. Monomeric C-reactive protein is found in fibrous tissues of normal and inflammed human blood vessel intima.[11] Monomeric C-reactive protein can be produced *in vitro* from pentameric C-reactive protein by exposing pentameric C-reactive protein to urea, heat, or acidic conditions,[12] and it can also spontaneously form from pentameric C-reactive protein during storage in the absence of calcium ions. Therefore, previous results based on non-specified C-reactive protein preparations could reflect the activity of either conformation alone or the combination of both. This is important because pentameric C-reactive protein and monomeric C-reactive protein differ in their biological effects and signal through different receptors. Studies directly addressing the distinct isoforms of C-reactive protein have reported that monomeric C-reactive protein displays a proinflammatory phenotype in neutrophils and endothelial cells, whereas pentameric C-reactive protein displays anti-inflammatory activities.[13,14] We recently showed that C-reactive protein isoforms differed in their effects on thrombus growth, as monomeric C-reactive protein induced platelet adhesion and thrombus formation under flow conditions, whereas pentameric C-reactive protein produced no effect.[15] Despite the growing interest in monomeric C-reactive protein, it remains unclear how monomeric C-reactive protein is generated and whether it contributes to inflammatory processes such as atherosclerosis. C-reactive protein produced by extrahepatic cells express monomeric C-reactive protein antigenicity[16] and it has also been recently shown that cell membranes, liposomes, and static activated platelets can dissociate pentameric C-reactive protein into monomeric C-reactive protein via lysophosphatidylcholine, enhancing the relationship of C-reactive protein and inflammation. Platelets represent an important link between thrombosis, inflammation, and atherogenesis, as platelet-induced inflammatory pathways contribute to the development of atherosclerotic lesion and atherothrombosis.[17] Here, we show for the first time that dissociation of blood circulating pentameric C-reactive protein into monomeric C-reactive protein occurs on shear-induced growing thrombus and that this newly formed monomeric C-reactive protein induces further thrombus growth. Furthermore, we also show that, unlike pentameric C-reactive protein, monomeric C-reactive protein is prothrombotic and induces platelet activation and aggregation.

2. Methods

A more detailed description of this section can be found in the Supplementary material online.

2.1 C-reactive protein isoforms

High purity human pentameric C-reactive protein was purchased from Calbiochem and monomeric C-reactive protein was obtained by urea chelation from purified human C-reactive protein as previously described.[15]

2.2 Blood collection

Venous blood was drawn from non-smoking healthy volunteers with informed consent. Donors claimed not to have been taking any medication during 2 weeks prior to blood extraction. The study complied with the tenets of the Declaration of Helsinki and was reviewed and approved by the Institutional Clinical Research Committee of the Hospital de la Santa Creu i Sant Pau. In some cases, after the first extraction, donors were given a loading dose of 150 mg clopidogrel in order to test the effect of P2Y12 blockade on monomeric C-reactive protein-induced platelet activation and 12 h after clopidogrel treatment a second blood extraction was performed.

2.3 Flow cytometry

Blood was diluted 1:10 in modified Tyrode Buffer and incubated with C-reactive protein isoforms or ADP (1 μmol/L). The responses to monomeric C-reactive protein (50 μg/mL) were also studied in the presence of mitogen-activated protein kinase (MAPK) inhibitors. SB302580 (20 μM) was used to inhibit p38 MAPK, U0126 (10 μM) was used to inhibit MAPK kinase 1 and 2 (MEK 1/2), and the inhibitor SP600125 (10 μM) was used for the inhibition of Jun N-terminal kinase (JNK). The responses to monomeric C-reactive protein activation were also analysed after blocking the platelet receptors CD36 (4 μg/mL CD36-Fab antibody), FcγRIII (2.5 μg/mL of function-blocking anti-CD16 antibody, clone 3G8, Pharmingen), glycoprotein IIb-IIIa (GPIIb-IIIa) (5 μg/mL abciximab), and P2Y12 (oral treatment with clopidogrel). P-selectin and CD63 surface expression were assessed with a phycoerytrin (PE)-conjugated anti-CD62P mAb (Pharmingen) and a PE-conjugated anti-CD63 mAb (Pharmingen), respectively. GPIIb-IIIa conformational change was assessed with a FITC-conjugated PAC-1 mAb (Beckton Dickinson). Flow cytometry procedures were then performed as detailed in the Supplemental material online.

2.4 Platelet aggregation

Extent of aggregation was determined in platelet-rich plasma (PRP) with an optical aggregometer (Aggrecorder-II), as previously reported.[18] A total of 200 μL of PRP adjusted to 250 × 10^6 platelets/mL was incubated with 200 μL solution containing C-reactive protein isoforms or control buffer at indicated concentrations. The response to monomeric C-reactive protein was also studied in the presence of SB302580 (20 μM) and anti-CD36 (4 μg/mL CD36-Fab antibody).

2.5 Thrombelastographic coagulation analysis

Dynamic whole blood clot formation was performed with the rotation thromboelastometry (ROTEM®) coagulation analyzer (Pentapharm, Germany), which is based on the thrombelastograph system using the tissue factor-triggered extrinsic pathway assay (ExTEM) and the ellagic acid activated intrinsic pathway assay (InTEM). Polymerized fibrinogen/fibrin was measured using the platelet-inactivating test FIBTEM (with platelet inhibitor cytochalasin D evaluating the contribution of fibrinogen to clot formation) assay. The following variables were determined: clotting time (CT, s), clot formation time (CFT, s), clot firmness (A10, mm), and maximum clot firmness (MCF, mm).

2.6 Determination of thrombin–antithrombin complex and tissue factor-procoagulant activity

Levels of thrombin–antithrombin (TAT) were measured by enzyme immunoassay (Assay Pro) and tissue factor-procoagulant activity (TF-PCA) was measured by using a factor (F) Xa generation test.

2.7 Platelet isolation

Blood collected into acid citrate dextrose was centrifuged (15 min, 150 *g*, 20°C) to obtain PRP. PRP was removed and centrifuged (15 min 1400 *g*, 20°C) in the presence of 0.1 μg/mL of the prostaglandin E1 (PGE_1). Platelet pellets were resuspended in Hepes–Tyrode's buffer and the wash step was repeated in the presence of PGE_1. Washed platelets were then incubated (5 min, 37°C) with the different C-reactive protein isoforms (25 μg/mL) and centrifuged (15 min, 1400 *g*). When indicated, washed platelets were incubated with MAPK inhibitors prior incubation with monomeric C-reactive protein. Supernatants were removed and platelet pellets stored deep-frozen (−80°C). The pellet was lyzed with 1 mL of cold lysis buffer and then centrifuged at 10 000 *g* for 10 min at 4°C to obtain platelet lysates.

2.8 Western blot analysis

Sample extracts (25 μg protein) were resolved by 8% SDS–PAGE and electrotransferred to nitrocellulose membranes, as described previously.[19] Vasodilator-stimulated phosphoprotein (VASP) phosphorylation and VASP were detected with the phosphorylation-specific monoclonal VASP antibody 16C2 directed against the serine 239 phosphorylation site of VASP (46 kDa) (NanoTools) and a mouse monoclonal antibody that recognizes VASP (46 kDa) and VASP phosphorylated at serine 157 (50 kDa) (clone IE273 Immunoglobe). Normalization was performed against β-actin (Abcam).

2.9 Immunofluorescence

Washed platelets were fixed with 3.8% paraformaldehyde, washed with phosphate buffer saline (PBS), and immobilized on poly-L-lysine-coated coverslides. Washed platelets were then permeabilized, and stained with the appropriate antibodies, as detailed in the Supplemental material online.

2.10 Measurement of cGMP activity

Washed platelets were treated with 3-isobutyl-1-methylxanthine (IBMX) 1 mM prior monomeric C-reactive protein (25 μg/mL) stimulation and the reaction was stopped by adding an equal volume of ice-cold solution containing ethanol 90% and HCl (0.1 N) 10%. Samples were vortexed and kept in ice before centrifugation (1500 *g* for 30 min at 4°C). Supernatants were collected and evaporated before being re-suspended in assay buffer. cGMP levels were measured using commercially available cyclic GMP XP Assay Kit (Cell Signaling) following the manufacturer's instructions.

2.11 Dissociation of pentameric C-reactive protein under flow conditions on adhered platelets

Type I collagen-coated slides were prepared and placed in the flow chambers and flow experiments were performed as described in detail elsewhere,[15,20] with small modifications. Two parallel-plate perfusion chambers were placed in a series mode to analyse pentameric C-reactive protein in the proximal chamber and monomeric C-reactive protein in the distal flow-chamber. Heparinized (10 IU/mL) blood containing 5 μg/mL of pentameric C-reactive protein was perfused at constant shear rates of 250 and 1500 s^{-1}. After perfusions, collagen-coated slides were rinsed with PBS and fixed with 3.8% paraformaldehyde. Immunodetection of C-reactive protein isoforms was performed with an mAb against pentameric C-reactive protein (clone 1D6) and monomeric C-reactive protein (clone 8C10), kindly provided by Dr LA Potempa.[11] Images were recorded by fluorescence confocal microscopy (HCX PL APO 20X/0,7 IMM CORR).

Additionally, resting and effluent sheared platelets were collected and immobilized on poly-L-lysine-coated coverslides as described elsewhere[21] and immunodetection of C-reactive protein isoforms was performed as described above.

2.12 Platelet adhesion experiments under flow conditions

Type I collagen-coated slides were placed in a flow chamber and flow experiments were performed as described elsewhere.[15,20] Platelets were rendered fluorescent by the addition of mepacrine 10 μmol/L (Sigma). Blood with or without pentameric C-reactive protein (5 μg/mL) was recirculated through the flow chamber at a constant shear rate of 1500 s^{-1} for 10 min in order to dissociate pentameric C-reactive protein into monomeric C-reactive protein in the surface of adhered platelets. Immediately afterwards, flow was switched to blood without C-reactive protein that was further perfused through the chamber with the preformed thrombi for 3 min at 1500 s^{-1}. Platelet adhesion was scanned with a Leica TCS SP2 confocal laser scanning microscope and surface covered by platelets was calculated using NIH Image software (by Dr Wayne Rasband, National Institutes of Health).[21]

2.13 Statistical analysis

Results were expressed as mean ± SEM. After testing for normal distribution and equality of variances with Levene's *F*-test, Student's *t*-test or ANOVA as appropriate was used to determine statistical significance between treatments. A value of $P < 0.05$ was considered significant.

3. Results

3.1 Effect of C-reactive protein isoforms on platelet activation

Monomeric C-reactive protein increased in a dose-dependent manner the levels of platelet surface P-selectin both in the presence or absence of calcium [citrated, D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), and heparin anticoagulated blood], whereas pentameric C-reactive protein had no effect on P-selectin expression (*Figure 1A*). Treatment with 25 and 50 μg/mL of monomeric C-reactive protein significantly increased P-selectin expression ($P < 0.05$) to a similar extent as ADP (1 μM) stimulation. High doses of monomeric C-reactive protein were also able to induce a moderate increase on platelet surface CD63 in PPACK- and heparin-anticoagulated blood, but not in citrated blood ($P < 0.05$). Pentameric C-reactive protein, as expected, was unable to induce any effect on platelet CD63 expression (*Figure 1B*). Monomeric C-reactive protein also induced a significant effect on GPIIb-IIIa conformational change in a dose-dependent manner ($P < 0.05$), whereas pentameric C-reactive protein did not (*Figure 1C*). However, levels of GPIIb-IIIa activation achieved upon monomeric C-reactive protein treatment were lower than those achieved upon ADP (1 μM) stimulation.

Blockade of different platelet receptors was investigated to test their role in mediating monomeric C-reactive protein effect on platelet activation. Thus, blockade of GPIIb-IIIa ($P < 0.01$) and CD36 ($P < 0.05$) significantly inhibited monomeric C-reactive protein-induced platelet activation, suggesting a possible role for these receptors on monomeric C-reactive protein-induced platelet activation. However, the pronounced effect of GPIIb-IIIa blockade could also be related to its secondary inhibitory effect of outside-in signalling. In contrast, blockade of CD16 and P2Y12 showed no effect (*Figure 2A*).

To test the possible role of MAPK in monomeric C-reactive protein-induced platelet activation, the effect of monomeric C-reactive protein on P-selectin expression was assayed in the presence of MAPK inhibitors. Blood preincubation with the p38 MAPK inhibitor (SB203580) and JNK inhibitor (SP600125) showed a significant reduction in the monomeric C-reactive protein effect on P-selectin

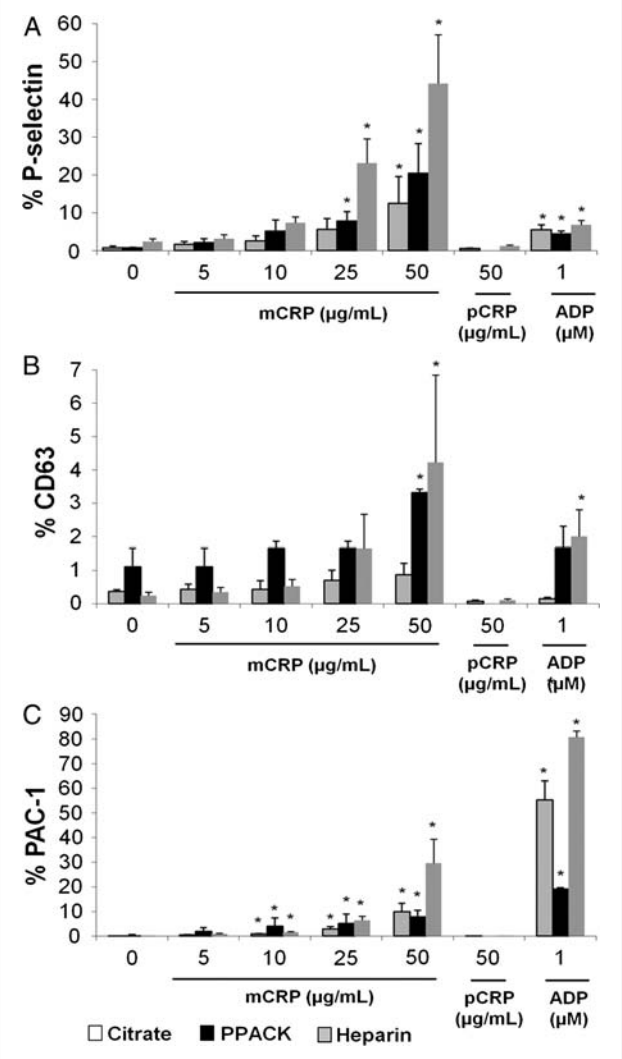

Figure 1 Effect of C-reactive protein isoforms on platelet surface P-selectin expression (*A*), CD63 expression (*B*), and PAC-1 binding (*C*). Diluted whole blood was incubated with C-reactive protein isoforms or ADP for 5 min and platelet activation was measured by flow cytometry. Results are expressed as mean values of % of CD62P-, CD63- or PAC-1-positive platelets ± SEM ($n = 8$). Statistical analysis was performed by analysis of ANOVA (*$P < 0.05$ vs. control).

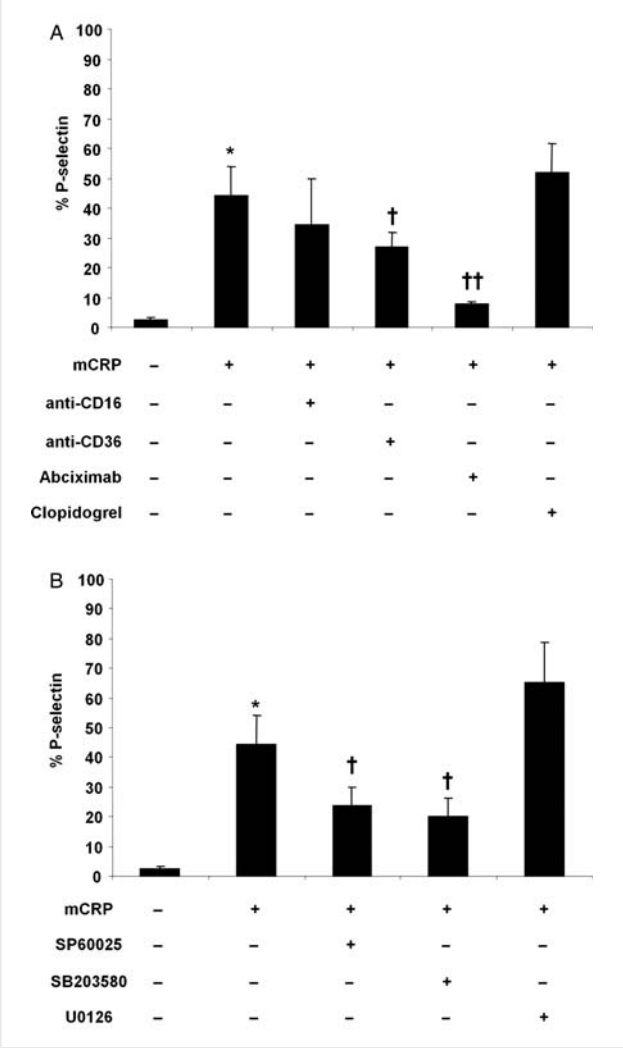

Figure 2 Flow cytometry analysis of the effect of platelet receptors blockade and MAPK inhibitors on monomeric C-reactive protein-induced platelet activation. (*A*) Effect of platelet receptors blockade on P-selectin expression of monomeric C-reactive protein-treated platelets. (*B*) Effect of MAPK inhibitors on P-selectin expression of monomeric C-reactive protein-activated platelets. Results are expressed as mean values of % of CD62P-positive platelets ± SEM ($n = 8$). Statistical analysis was performed by analysis of ANOVA (*$P < 0.05$ vs. control, †$P < 0.05$ vs monomeric C-reactive protein, ††$P < 0.001$ vs. monomeric C-reactive protein).

expression ($P < 0.05$), whereas the presence of the MEK1/2 inhibitor U0126 did not produce any effect (*Figure 2B*).

3.2 Effect of C-reactive protein isoforms on platelet aggregation and coagulation

Platelet aggregation analysis revealed that monomeric C-reactive protein induced platelet aggregation in a dose-dependent manner ($P < 0.001$), as seen in *Figure 3A*. Effects became significant at monomeric C-reactive protein concentrations >5 μg/mL. On the other hand, pentameric C-reactive protein did not produce any effect on platelet aggregation at any tested dose. Monomeric C-reactive protein-induced platelet aggregation was partially prevented when blocking the platelet receptor CD36 ($P < 0.05$) (*Figure 3B*). Similarly, the p38 MAPK inhibitor SB203580 partially inhibited monomeric C-reactive protein-induced platelet aggregation ($P < 0.05$) (*Figure 3C*).

In contrast, neither pentameric C-reactive protein nor monomeric C-reactive protein affected the clotting process since all ROTEM parameters representing the initiation (CT), propagation (CFT), and clot firmness (A10, MCF) were unaffected. Similarly, TAT levels and TF-PCA were not affected by C-reactive protein isoforms (*Table 1*).

3.3 Effect of C-reactive protein isoforms on vasodilator-stimulated phosphoprotein phosphorylation: interaction with cGMP/PKG system

Monomeric C-reactive protein induced VASP dephosphorylation at serine 239 ($P < 0.05$), as evidenced with the 16C2 antibody (*Figure 4A*), whereas pentameric C-reactive protein produced no

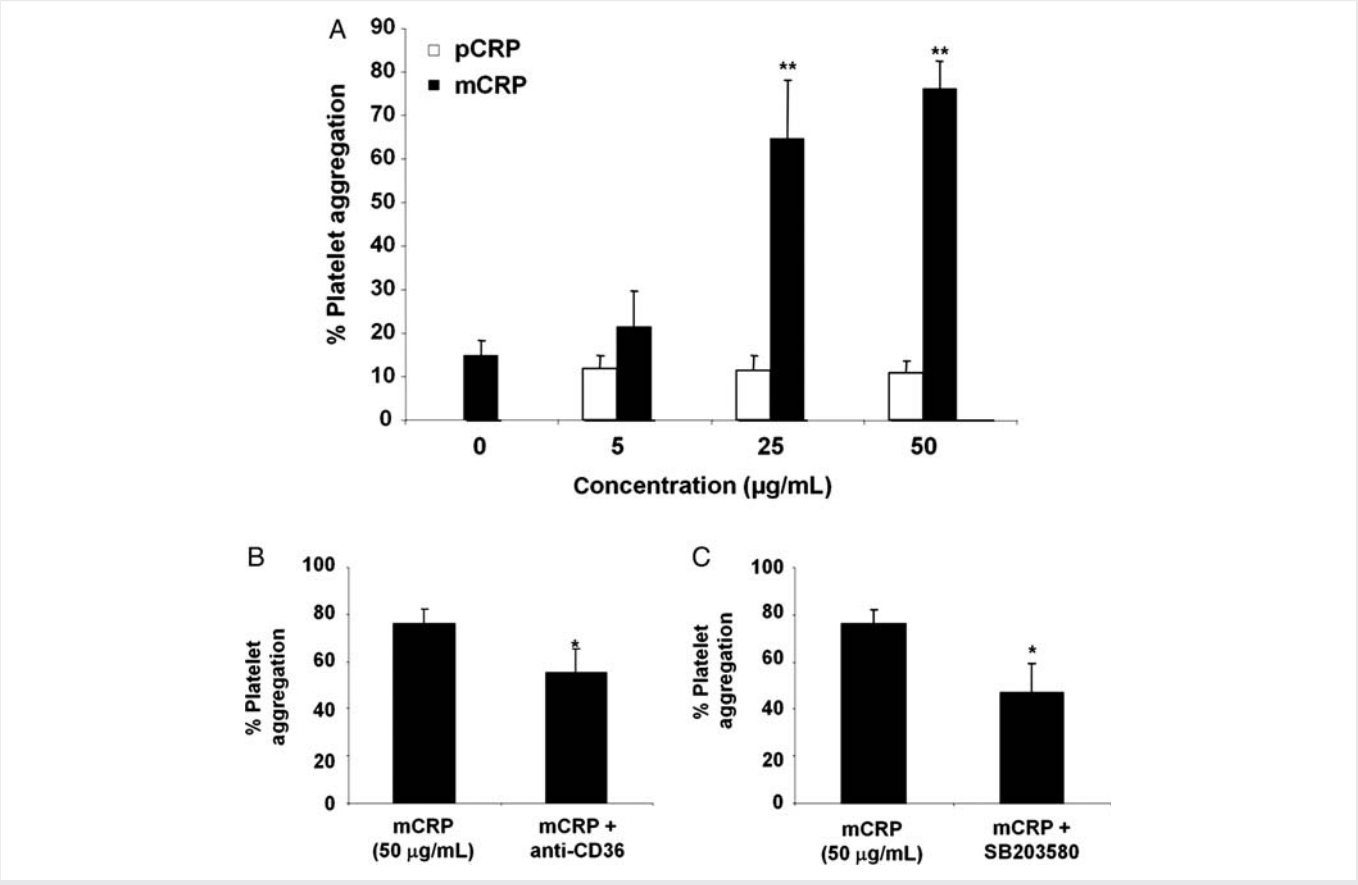

Figure 3 Effect of C-reactive protein isoforms on platelet aggregation. PRP was incubated with C-reactive protein isoforms for 5 min at 37°C and maximum platelet aggregation was measured (*A*) Aggregometry of PRP in response to C-reactive protein isoforms. (*B* and *C*) Inhibition of monomeric C-reactive protein-induced platelet aggregation in the presence of anti-CD36 (*B*) and p38 MAPK inhibitor (SB203580) (*C*). Results are expressed as mean values of maximum platelet aggregation (%) ± SEM ($n = 6$). Statistical analysis was performed by the analysis of ANOVA (**$P < 0.001$) (*A*) and by Student's *t*-test analysis (*$P < 0.05$) (*B* and *C*).

Table 1 Effect of C-reactive protein isoforms on clot formation and coagulation

	EX-Tem			In-Tem			Fib-Tem		
	CT (s)	CFT (s)	A10 (mm)	CT (s)	CFT (s)	A10 (mm)	MCF (mm)	TAT (ng/mL)	TF-PCA (mU/ml)
Control	190 ± 17	74 ± 6	56 ± 1	165 ± 7	67 ± 6	57 ± 1	16 ± 1.5	27.1 ± 1.2	0.245 ± 0.023
Monomeric C-reactive protein (25 μg/mL)	205 ± 33	64 ± 6	55 ± 2	176 ± 13	73 ± 8	54 ± 2	11 ± 1.6	28.7 ± 1.7	0.327 ± 0.043
Pentameric C-reactive protein (25 μg/mL)	177 ± 28	63 ± 4	56 ± 2	174 ± 11	63 ± 2	55 ± 1	17 ± 3	26.9 ± 0.8	0.300 ± 0.036

CT, clotting time; CFT, clot formation time; A10, clot firmness; MCF, maximum clot firmness; TAT, thrombin-antithrombin complex, TF-PCA, tissue-factor procoagulant activity.

effect. However, monomeric C-reactive protein did not affect VASP phosphorylation at S157. When using the antibody that recognizes VASP (46 kDa) and VASP phosphorylated at serine 157 (50 kDa), no significant differences were observed with either C-reactive protein or monomeric C-reactive protein (*Figure 4B*). Inhibition of p38 MAPK and JNK prior monomeric C-reactive protein treatment, although not completely, partially prevented VASP dephosphorylation at S239 ($P < 0.05$), as seen in *Figure 4C*.

Immunofluorescence analysis of VASP was also performed in platelets treated with pentameric C-reactive protein or monomeric C-reactive protein. Platelets treated with monomeric C-reactive protein showed lower VASP-phosphorylation (S239), whereas total VASP was not affected (*Figure 4D*). Untreated platelets and pentameric C-reactive protein-treated platelets showed similar levels of pVASP (green), as seen in Panel I and II (*Figure 4E*). In contrast, monomeric C-reactive protein treatment significantly reduced VASP

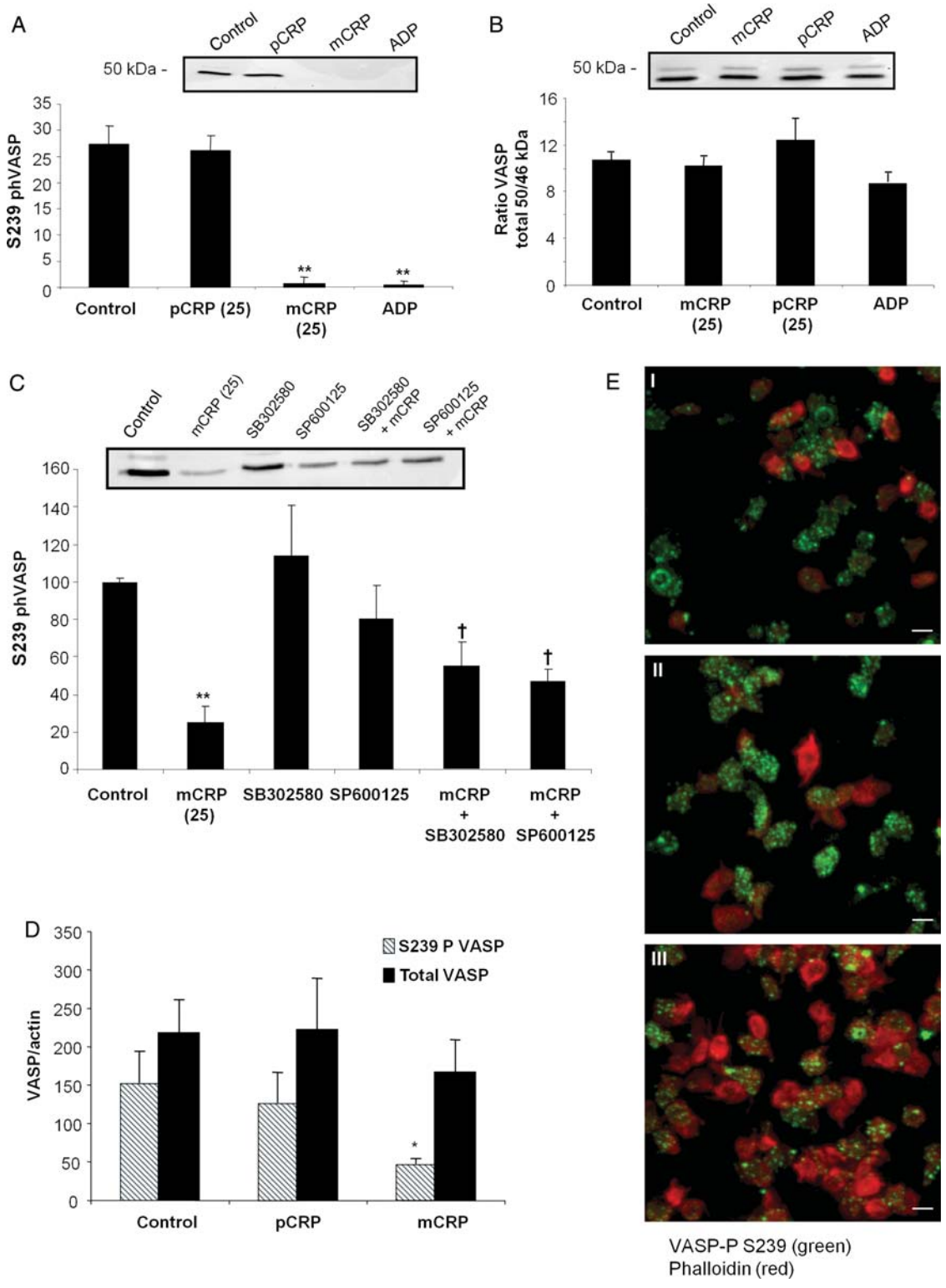

Figure 4 Effect of C-reactive protein isoforms on VASP phosphorylation. (*A*) Western blot of VASP phosphorylation at S239. (*B*) Western blot of VASP total (46 kDa) and VASP phosphorylation at S157 (50 kDa). (*C*) Western blot of VASP phosphorylation in S239 in the presence of MAPK and JNK inhibitors. Results are expressed as arbitrary units (AU) normalized by β-actin expression ± SEM (*A* and *C*) and ratio of VASP 50 kDa/46 kDa ± SEM (*B*) ($n = 6$). Statistical analysis was performed by the analysis of ANOVA (**$P < 0.001$ vs. control, *$P < 0.05$ vs. Control, $^{\dagger}P < 0.05$ vs. monomeric C-reactive protein). (*D*) Immunofluorescence analysis of VASP phosphorylation. The bars graphic shows the ratio of VASP phosphorylation vs. actin in platelets preincubated with C-reactive protein isoforms. Results are expressed as values of mean energy of VASP fluorescence intensity ± SEM divided by mean energy of actin fluorescence intensity ± SEM ($n = 5$). Statistical analysis was performed by the analysis of ANOVA (*$P < 0.05$). (*E*) Representative confocal images of VASP phosphorylated in S239 of control (I), pentameric C-reactive protein (II), and monomeric C-reactive protein (III)-treated platelets. Green: pvasodilator-stimulated phosphoprotein. Red: phalloidin (actin), $n = 5$. Scale bar: 2 μm.

phosphorylation (S239) levels, as seen in Panel III (*Figure 4E*). Furthermore, monomeric C-reactive protein-treated platelets also showed higher levels of activation as seen by shape change and number of pseudopodia. Indeed, in monomeric C-reactive protein-treated platelets pVASP seemed to start co-localizing with actin, as seen by the appearance of yellow colour.

To confirm the interaction with the cGMP/PKG system, cGMP activity was determined in washed platelets treated with monomeric C-reactive protein. In agreement with the results on VASP phosphorylation at S239, the percentage of cGMP activity was significantly reduced upon monomeric C-reactive protein (25 μg/mL) treatment (100% control vs. 56 ± 14% monomeric C-reactive protein, $P < 0.05$).

3.4 Dissociation of pentameric C-reactive protein on adhered platelets under flow

To test whether adhered or shear-activated platelets were able to dissociate pentameric C-reactive protein into its monomeric subunits, blood was perfused in the presence of pentameric C-reactive protein (5 μg/mL). Interestingly, perfusing blood either at 250 or 1500 s^{-1} for 10 min caused C-reactive protein dissociation and monomeric C-reactive protein retention on adhered platelets. Indeed, pentameric C-reactive protein was completely dissociated on the surface of activated platelets adhered to the collagen surface. As seen in *Figure 5A* and *B*, while significant amounts of monomeric C-reactive protein were detected on the surface of adhered platelets, no pentameric C-reactive protein was observed. When blood was perfused for shorter time periods (5 and 7 min) C-reactive protein was only partially dissociated as both C-reactive protein isoforms were detected on the surface of adhered platelets (data not shown).

In contrast, resting platelets *per se* did not induce C-reactive protein dissociation on their surface. As shown in *Figure 5C*, resting platelets incubated with pentameric C-reactive protein only stained positive for pentameric C-reactive protein but not for monomeric C-reactive protein. Nevertheless, this does not mean that monomeric C-reactive protein does not bind resting platelets. Indeed, monomeric C-reactive protein binds resting platelets triggering its activation.

Effluent (non-adherent) platelets that were recirculated through the flow chambers that did not adhere to the collagen surface retained pentameric C-reactive protein on their surface. As seen in *Figure 5D*, on effluent platelets that were perfused at 250 s^{-1} only pentameric C-reactive protein was detected. When platelets were perfused at a higher shear rate (1500 s^{-1}), effluent platelets showed partial dissociation of C-reactive protein since monomeric C-reactive protein was slightly detected on their surface. Nevertheless, dissociation was uncomplete as high amounts of pentameric C-reactive protein were detected on the surface of effluent platelets (*Figure 5E*). Increasing perfusion time up to 30 min did not increase C-reactive protein dissociation on the surface of effluent platelets even at high shear rate conditions (data not shown).

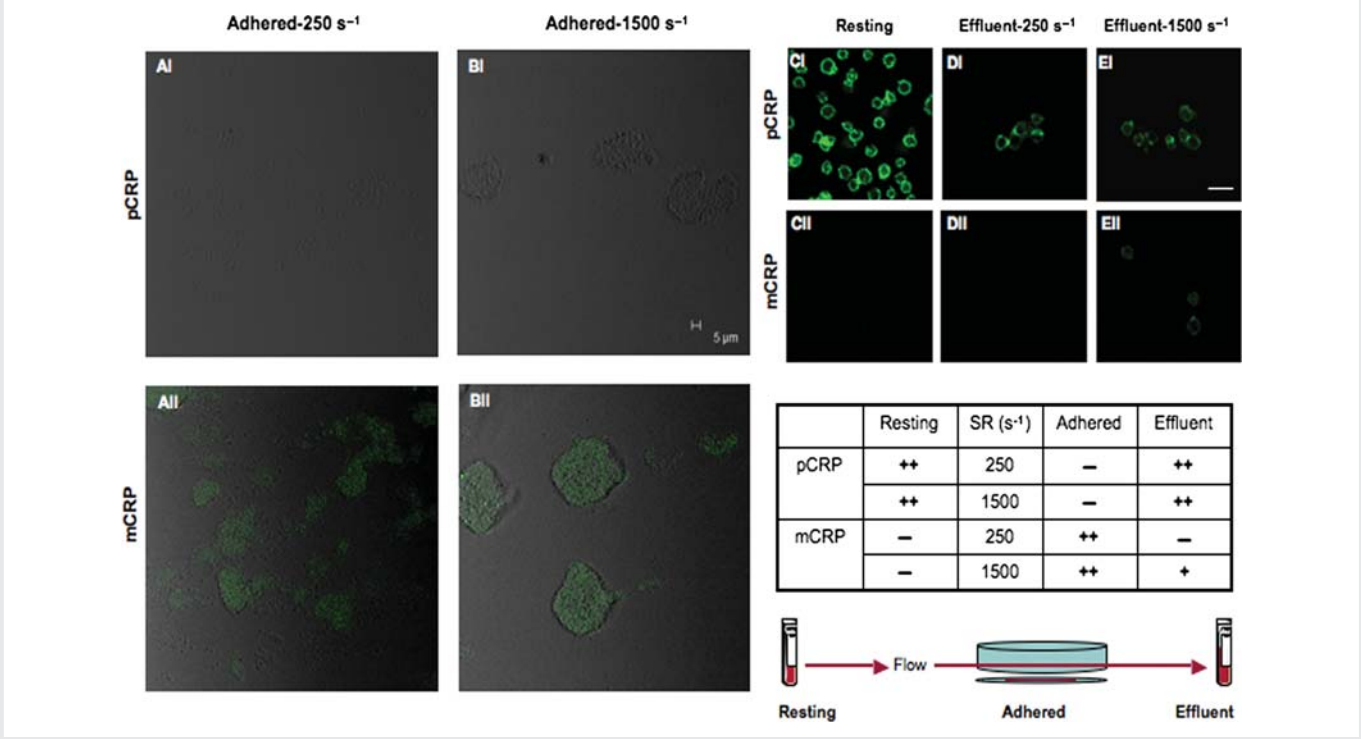

Figure 5 C-reactive protein dissociation under flow conditions. Blood containing pentameric C-reactive protein was perfused for 10 min at different shear rates over collagen-coated slides and C-reactive protein dissociation was analysed by immunofluorescence. (*A*) pentameric C-reactive protein (I) and monomeric C-reactive protein (II) on platelets adhered to collagen perfused at 250 s^{-1}. (*B*) pentameric C-reactive protein (I) and monomeric C-reactive protein (II) on platelets adhered to collagen perfused at 1500 s^{-1}. (*C*) pentameric C-reactive protein (I) and monomeric C-reactive protein (II) on resting platelets. (*D*) pentameric C-reactive protein (I) and monomeric C-reactive protein (II) on effluent platelets perfused at 250 s^{-1}. (*E*) pentameric C-reactive protein (I) and monomeric C-reactive protein (II) on effluent platelets perfused at 1500 s^{-1}. $n = 5$. Scale bar: 5 μm.

3.5 Effect of C-reactive protein dissociation on growing aggregates on further platelet deposition

To study whether platelets on which monomeric C-reactive protein was being generated were able to recruit additional platelets, a further set of perfusion experiments were performed. Blood containing pentameric C-reactive protein (5 μg/mL) was recirculated through the flow chamber containing a collagen-coated slide for 10 min to allow C-reactive protein dissociation on the growing thrombus. Afterwards, fresh untreated blood was perfused through the chamber for 3 min. As seen in *Figure 6*, monomeric C-reactive protein generated on the surface of adhered platelets showed a prothrombotic effect as it significantly increased further platelet adhesion ($P < 0.001$).

In our previous work, we showed that immobilized monomeric C-reactive protein promoted thrombus growth under flow, whereas pentameric C-reactive protein did not produce any effect on thrombus formation.[15] Therefore, the increased platelet adhesion observed after perfusing blood containing pentameric C-reactive protein is due to its dissociation into monomeric C-reactive protein which induced further platelet recruitment.

4. Discussion

Numerous epidemiological studies have shown that plasma C-reactive protein level is a powerful predictor of future cardiovascular events.[4,5] It has been shown that the lowering of C-reactive protein was related to a reduction in cardiovascular events[22] suggesting that C-reactive protein may be a direct causative factor of atherothrombosis. However, the causal role of C-reactive protein in atherothrombosis is controversial, as both proinflammatory and antiinflammatory properties have been ascribed to the molecule. Indeed, in some recent publications using transgenic murine models, no casual link between C-reactive protein and atherosclerosis was shown.[23,24] We have previously reported that C-reactive protein bioactivity on thrombus formation was based on loss of its pentameric symmetry, resulting in formation of monomeric C-reactive protein.[15] In the present study, we provide mechanistical insight on how C-reactive protein dissociates into its prothrombotic form monomeric C-reactive protein on growing platelet aggregates promoting further aggregate growth. Moreover, the opposed effects of both C-reactive protein forms on platelet activation have been mechanistically investigated in the present work.

Our study reports that C-reactive protein on its monomeric form induces platelet aggregation and activation, whereas native pentameric C-reactive protein is unable to produce any effect, even at high concentrations. However, neither pentameric C-reactive protein nor monomeric C-reactive protein seemed to affect blood coagulation, as clot properties, TAT levels and procoagulant activity were not affected by C-reactive protein treatment. In previous studies by Bisoendial *et al.*,[25] it was found that infusion of hrC-reactive protein in healthy subjects activated the coagulation system. The lack of

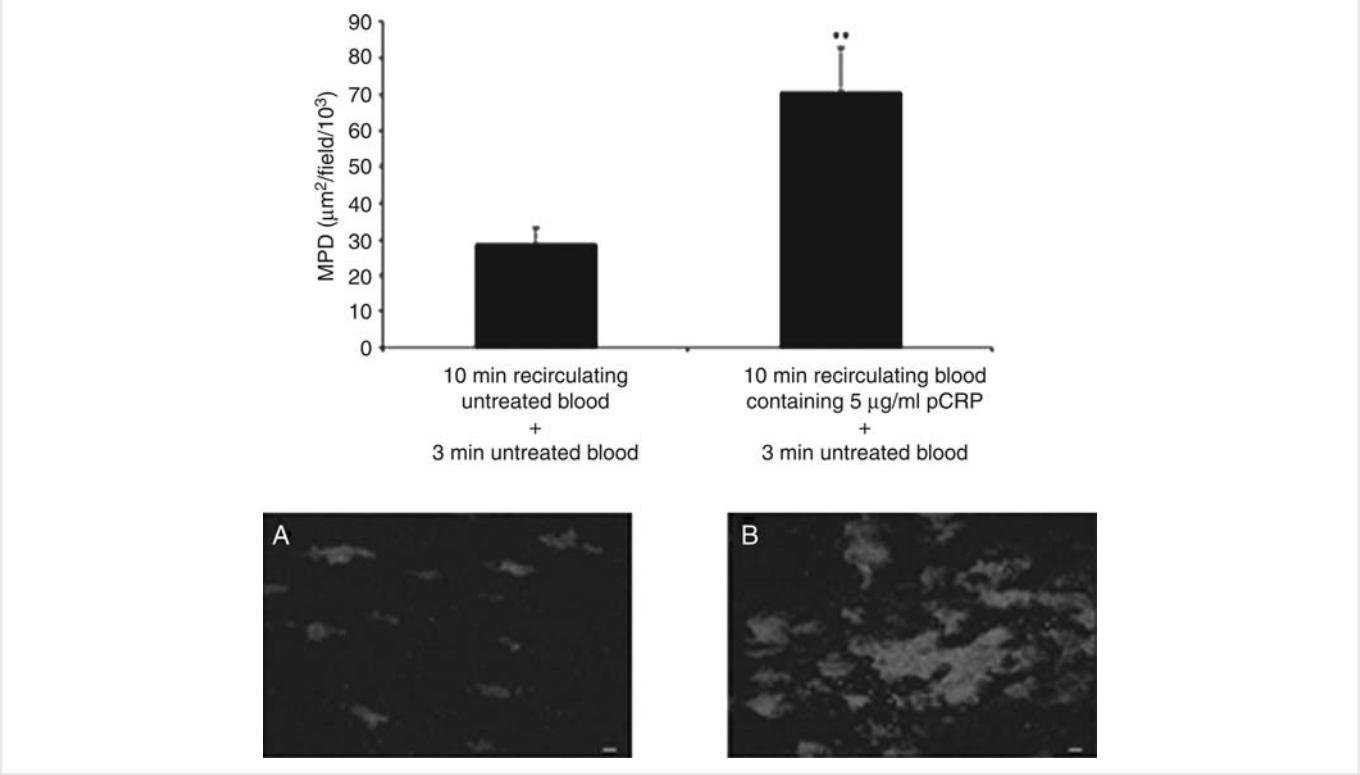

Figure 6 Effect of shear-induced C-reactive protein dissociation on mean platelet deposition (MPD). Blood containing pentameric C-reactive protein (5 μg/mL) was recirculated for 10 min through a collagen surface to induce C-reactive protein dissociation on the surface of adhered platelets and untreated blood was then perfused for 3 min at 1500 s^{-1}. Results are expressed as mean values of surface covered by platelets per analysed field (μm^2/field)/10^3 ± SEM ($n = 5$). Statistical analysis was performed by Student's *t*-test (**$P < 0.001$). Representative images of platelet adhesion under control conditions (*A*) and under shear-induced C-reactive protein dissociation conditions (*B*). Scale bar: 5 μm^2.

effect observed in our experiments could be explained by the short incubation period (15 min). In fact, in their work, Bisoendial *et al.* did not observe any effect on the activation of the coagulation system until 4 h after infusion of C-reactive protein.

Flow cytometry analysis allowed us to study the effect of C-reactive protein on the activation markers P-selectin, CD63, and on the conformational change of GPIIb-IIIa. Unlike pentameric C-reactive protein, monomeric C-reactive protein increased surface expression of P-selectin, CD63, and the active conformation of GPIIb-IIIa in a dose-dependent manner. Monomeric C-reactive protein concentrations >10 μg/mL were able to increase surface P-selectin, CD63, and PAC-1. The effect of monomeric C-reactive protein on platelet activation was higher when heparin was used as anticoagulant, thus suggesting a potential interaction between monomeric C-reactive protein and heparin that could enhance the prothrombotic properties of monomeric C-reactive protein. We also observed that monomeric C-reactive protein effect on platelet activation was mediated by p38 MAPK and JNK, since their inhibition partially prevented monomeric C-reactive protein effects. Inhibition of p38 MAPK also resulted in reduced aggregation in monomeric C-reactive protein-activated platelets. In agreement with our findings, it was shown that the proinflammatory properties of monomeric C-reactive protein in endothelial cells seem to be also mediated by a p38 MAPK-dependent mechanism and not through MEK.[14]

VASP is a 46/50 kDa protein associated with microfilaments and concentrated at sites of focal adhesion plaques. VASP is phosphorylated in response to vasodilators and platelet inhibitor drugs that increase cAMP and cGMP levels and is believed to inhibit platelet shape change by stabilizing actin filaments.[26,27] Both protein kinase (PK) A and PK G phosphorylate VASP at different sites (serine 157 and 239, respectively).[28] We observed that unlike pentameric C-reactive protein, monomeric C-reactive protein induced strong VASP dephosphorylation at S239, which correlated with platelet shape change and pVASP/actin colocalization, as observed by immunofluorescence. Because monomeric C-reactive protein induced dephosphorylation of VASP at S239 and not at S157, we concluded that the effects of monomeric C-reactive protein were produced through the PKG/cGMP machinery. Accordingly, we observed reduced levels of cGMP in monomeric C-reactive protein-treated platelets, thus confirming the interaction of monomeric C-reactive protein with the cGMP/PKG pathway. It is worth pointing that monomeric C-reactive protein reduced cGMP activity in the presence of IBMX, a non-selective phosphodiesterase (PDE) inhibitor, which indicates that the effect of monomeric C-reactive protein is not related to an increase in PDE activity but may be therefore directly interfering with the generation of cGMP.

Because commercial C-reactive protein preparations can be contaminated with sodium azide or lipopolysaccharide (LPS), we used dialysed C-reactive protein preparations to remove sodium azide and eliminate confounding factors.[29,30] In fact, non-dialysed pentameric C-reactive protein induced strong VASP phosphorylation at S239 due to the presence of sodium azide (data not shown), which is in accordance with the results of Russo *et al.*,[31] who demonstrated that sodium azide influences the responses of platelets via the cGMP/PKG/VASP pathway. Thus, previously reported inhibitory effects of pentameric C-reactive protein could be attributed to the presence of sodium azide.

The receptors that mediate monomeric C-reactive protein activities have not been fully characterized. In human neutrophils, monomeric C-reactive protein has been found to bind FcγRIII (CD16).[32] However, in endothelial cells, functional blockade of CD16 only slightly attenuates monomeric C-reactive protein-induced activation.[14] Indeed, we observed that CD16 blockade did not prevent monomeric C-reactive protein-induced platelet activation as measured by surface exposure of P-selectin, suggesting that platelets interact with monomeric C-reactive protein through other mechanisms. It has been also reported that monomeric C-reactive protein, unlike pentameric C-reactive protein or other pentraxins, had the ability to bind a variety of extracellular matrix proteins, such as collagen I and IV, fibronectin, and vitronectin, showing a propensity for non-specific binding.[33] In unpublished immunofluorescence experiments, we observed that monomeric C-reactive protein bound platelets in a non-receptor-mediated manner. Additionally, it has been recently found that monomeric C-reactive protein activates endothelial cells and monocytes through its interaction with lipid raft microdomains.[34] Monomeric C-reactive protein may promiscuously interact with platelets, triggering activation and aggregation, by interfering with the PKG/cGMP system. In fact, only blockade of the major platelet integrin GPIIb-IIIa was able to almost completely inhibit monomeric C-reactive protein-induced platelet activation. Nevertheless, the effect of GPIIb-IIIa blockade could be related to its secondary inhibitory effect of outside-in signalling rather than being the receptor of monomeric C-reactive protein itself. Besides blockade of GPIIb-IIIa, we also found that blockade of the scavenger receptor CD36 partially inhibited monomeric C-reactive protein-induced platelet activation and aggregation. CD36 recognizes multiple ligands including thrombospondin-1, collagen, oxidized low-densisty lipoproteins, and misfolded proteins.[35–38] It has been proposed that CD36 ligands generated during vascular injury, where enhanced oxidative stress is present, trigger platelet activation, and contribute to thrombus formation. Thus, it could be possible that vascular injury induces dissociation of pentameric C-reactive protein into its monomeric subunits enhancing platelet activation and thrombus formation.

Although it has not been detected in circulation, monomeric C-reactive protein is a natural constitutent of normal and inflamed human blood vessel intima. The expression of C-reactive protein mRNA in atherosclerotic tissue has been reported,[20,39] but it is unclear whether the expressed C-reactive protein is the pentameric or the monomeric isoform. The monomeric C-reactive protein concentrations used in this study may be considered similar to those achieved *in vivo* locally, at sites of injury or inflammation. In a recent study, Eisenhardt *et al.*[40] showed the presence of monomeric C-reactive protein and not pentameric C-reactive protein in human aortic and coronary atherosclerotic plaques and monomeric C-reactive protein-staining colocalized with platelets and macrophages, suggesting generation of monomeric C-reactive protein by these cells. In their work, they show that ADP-activated static platelets and apoptotic cells dissociate pentameric C-reactive protein into monomeric C-reactive protein.

Here, we show that flow-activated platelets adhered to collagen and to a lesser extent shear-activated platelets, dissociate C-reactive protein into its monomeric subunits under flow conditions, providing new insights into the *in vivo* sources and relevance of monomeric C-reactive protein. We have found that both C-reactive protein isoforms bind resting and activated platelets, but only activated platelets are able to dissociate pentameric C-reactive protein into monomeric C-reactive protein on their surface. Furthermore, we have shown for the first time that monomeric C-reactive protein generated on the

surface of adhered platelets from blood containing native C-reactive protein displays prothrombotic properties promoting further platelet recruitment. These findings could potentially explain a positive feedback mechanism in that pentameric C-reactive protein dissociation on adhered platelets could facilitate further platelet activation.

In summary, our data indicate that whereas serum pentameric C-reactive protein does not affect platelet activation, the tissue-associated form of C-reactive protein, monomeric C-reactive protein, displays a prothrombotic phenotype contributing to platelet activation. These results highlight the importance of C-reactive protein conformation on its function. Thus, it remains a future challenge to test potential therapeutic approaches aimed to block either prothrombotic properties of monomeric C-reactive protein or disscociation of pentameric C-reactive protein.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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References

1. Black S, Kushner I, Samols D. C-reactive protein. *J Biol Chem* 2004;**279**:48487–48490.
2. Jabs WJ, Theissing E, Nitschke M, Bechtel JF, Duchrow M, Mohamed S *et al*. Local generation of C-reactive protein in diseased coronary artery venous bypass grafts and normal vascular tissue. *Circulation* 2003;**108**:1428–1431.
3. Venugopal SK, Devaraj S, Jialal I. Macrophage conditioned medium induces the expression of C-reactive protein in human aortic endothelial cells: potential for paracrine/autocrine effects. *Am J Pathol* 2005;**166**:1265–1271.
4. Blake GJ, Ridker PM. C-reactive protein and other inflammatory risk markers in acute coronary syndromes. *J Am Coll Cardiol* 2003;**41**:37S–42S.
5. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med* 2002;**347**:1557–1565.
6. Jialal I, Devaraj S, Venugopal SK. C-reactive protein: risk marker or mediator in atherothrombosis? *Hypertension* 2004;**44**:6–11.
7. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest* 2003;**111**:1805–1812.
8. Elliott P, Chambers JC, Zhang W, Clarke R, Hopewell JC, Peden JF *et al*. Genetic loci associated with C-reactive protein levels and risk of coronary heart disease. *JAMA* 2009;**302**:37–48.
9. Kivimaki M, Lawlor DA, Smith GD, Kumari M, Donald A, Britton A *et al*. Does high C-reactive protein concentration increase atherosclerosis? The Whitehall II Study. *PLoS One* 2008;**3**:e3013.
10. Ji SR, Wu Y, Zhu L, Potempa LA, Sheng FL, Lu W *et al*. Cell membranes and liposomes dissociate C-reactive protein (CRP) to form a new, biologically active structural intermediate: mCRP(m). *FASEB J* 2007;**21**:284–294.
11. Diehl EE, Haines GK III, Radosevich JA, Potempa LA. Immunohistochemical localization of modified C-reactive protein antigen in normal vascular tissue. *Am J Med Sci* 2000;**319**:79–83.
12. Kresl JJ, Potempa LA, Anderson BE. Conversion of native oligomeric to a modified monomeric form of human C-reactive protein. *Int J Biochem Cell Biol* 1998;**30**:1415–1426.
13. Khreiss T, Jozsef L, Potempa LA, Filep JG. Opposing effects of C-reactive protein isoforms on shear-induced neutrophil-platelet adhesion and neutrophil aggregation in whole blood. *Circulation* 2004;**110**:2713–2720.
14. Khreiss T, Jozsef L, Potempa LA, Filep JG. Conformational rearrangement in C-reactive protein is required for proinflammatory actions on human endothelial cells. *Circulation* 2004;**109**:2016–2022.
15. Molins B, Pena E, Vilahur G, Mendieta C, Slevin M, Badimon L. C-reactive protein isoforms differ in their effects on thrombus growth. *Arterioscler Thromb Vasc Biol* 2008;**28**:2239–2246.
16. Schwedler SB, Filep JG, Galle J, Wanner C, Potempa LA. C-reactive protein: a family of proteins to regulate cardiovascular function. *Am J Kidney Dis* 2006;**47**:212–222.
17. von Hundelshausen P, Weber C. Platelets as immune cells: bridging inflammation and cardiovascular disease. *Circ Res* 2007;**100**:27–40.
18. Vilahur G, Pena E, Padro T, Badimon L. Protein disulphide isomerase-mediated LA419- NO release provides additional antithrombotic effects to the blockade of the ADP receptor. *Thromb Haemost* 2007;**97**:650–657.
19. Llorente-Cortes V, Martinez-Gonzalez J, Badimon L. Esterified cholesterol accumulation induced by aggregated LDL uptake in human vascular smooth muscle cells is reduced by HMG-CoA reductase inhibitors. *Arterioscler Thromb Vasc Biol* 1998;**18**:738–746.
20. Vilahur G, Hernandez-Vera R, Molins B, Casani L, Duran X, Padro T *et al*. Short-term myocardial ischemia induces cardiac modified C-reactive protein expression and proinflammatory gene (cyclo-oxygenase-2, monocyte chemoattractant protein-1, and tissue factor) upregulation in peripheral blood mononuclear cells. *J Thromb Haemost* 2009;**7**:485–493.
21. Molins B, Pena E, Padro T, Casani L, Mendieta C, Badimon L. Glucose-regulated protein 78 and platelet deposition: effect of rosuvastatin. *Arterioscler Thromb Vasc Biol* 2010;**30**:1246–1252.
22. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ *et al*. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med* 2008;**359**:2195–2207.
23. Tennent GA, Hutchinson WL, Kahan MC, Hirschfield GM, Gallimore JR, Lewin J *et al*. Transgenic human CRP is not pro-atherogenic, pro-atherothrombotic or pro-inflammatory in apoE−/− mice. *Atherosclerosis* 2008;**196**:248–255.
24. Teupser D, Weber O, Rao TN, Sass K, Thiery J, Fehling HJ. No reduction of atherosclerosis in C-reactive protein (CRP)-deficient mice. *J Biol Chem* 2011;**286**:6272–6279.
25. Bisoendial RJ, Kastelein JJ, Levels JH, Zwaginga JJ, van den Bogaard B, Reitsma PH *et al*. Activation of inflammation and coagulation after infusion of C-reactive protein in humans. *Circ Res* 2005;**96**:714–716.
26. Halbrugge M, Friedrich C, Eigenthaler M, Schanzenbacher P, Walter U. Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators. *J Biol Chem* 1990;**265**:3088–3093.
27. Harbeck B, Huttelmaier S, Schluter K, Jockusch BM, Illenberger S. Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin. *J Biol Chem* 2000;**275**:30817–30825.
28. Schafer A, Wiesmann F, Neubauer S, Eigenthaler M, Bauersachs J, Channon KM. Rapid regulation of platelet activation *in vivo* by nitric oxide. *Circulation* 2004;**109**:1819–1822.
29. Taylor KE, Giddings JC, van den Berg CW. C-reactive protein-induced *in vitro* endothelial cell activation is an artefact caused by azide and lipopolysaccharide. *Arterioscler Thromb Vasc Biol* 2005;**25**:1225–1230.
30. van den Berg CW, Taylor KE, Lang D. C-reactive protein-induced *in vitro* vasorelaxation is an artefact caused by the presence of sodium azide in commercial preparations. *Arterioscler Thromb Vasc Biol* 2004;**24**:e168–e171.
31. Russo I, Del Mese P, Viretto M, Doronzo G, Mattiello L, Trovati M *et al*. Sodium azide, a bacteriostatic preservative contained in commercially available laboratory reagents, influences the responses of human platelets via the cGMP/PKG/VASP pathway. *Clin Biochem* 2008;**41**:343–349.
32. Heuertz RM, Schneider GP, Potempa LA, Webster RO. Native and modified C-reactive protein bind different receptors on human neutrophils. *Int J Biochem Cell Biol* 2005;**37**:320–335.
33. Boguslawski G, McGlynn PW, Potempa LA, Filep JG, Labarrere CA. Conduct unbecoming: C-reactive protein interactions with a broad range of protein molecules. *J Heart Lung Transplant* 2007;**26**:705–713.
34. Ji SR, Ma L, Bai CJ, Shi JM, Li HY, Potempa LA *et al*. Monomeric C-reactive protein activates endothelial cells via interaction with lipid raft microdomains. *FASEB J* 2009;**23**:1806–1816.
35. Bodart V, Febbraio M, Demers A, McNicoll N, Pohankova P, Perreault A *et al*. CD36 mediates the cardiovascular action of growth hormone-releasing peptides in the heart. *Circ Res* 2002;**90**:844–849.
36. Febbraio M, Hajjar DP, Silverstein RL. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *J Clin Invest* 2001;**108**:785–791.
37. Herczenik E, Bouma B, Korporaal SJ, Strangi R, Zeng Q, Gros P *et al*. Activation of human platelets by misfolded proteins. *Arterioscler Thromb Vasc Biol* 2007;**27**:1657–1665.
38. Hoebe K, Georgel P, Rutschmann S, Du X, Mudd S, Crozat K *et al*. CD36 is a sensor of diacylglycerides. *Nature* 2005;**433**:523–527.
39. Krupinski J, Turu MM, Martinez-Gonzalez J, Carvajal A, Juan-Babot JO, Iborra E *et al*. Endogenous expression of C-reactive protein is increased in active (ulcerated noncomplicated) human carotid artery plaques. *Stroke* 2006;**37**:1200–1204.
40. Eisenhardt SU, Habersberger J, Murphy A, Chen YC, Woollard KJ, Bassler N *et al*. Dissociation of pentameric to monomeric C-reactive protein on activated platelets localizes inflammation to atherosclerotic plaques. *Circ Res* 2009;**105**:128–137.