C-Reactive Protein Isoforms Differ in Their Effects on Thrombus Growth

Blanca Molins, Esther Peña, Gemma Vilahur, Carlos Mendieta, Mark Slevin, Lina Badimon

Objective—We studied the impact of native (natCRP) and modified CRP (mCRP) isoforms on platelet adhesion and thrombus growth under arterial flow.

Methods and Results—Blood was perfused over type I collagen at a wall shear rate of 1500 s⁻¹, and platelet deposition and thrombus growth were evaluated by confocal microscopy. natCRP and mCRP were either incubated with blood before perfusion experiments or immobilized in the collagen surface and exposed to flowing blood. mCRP significantly increased platelet adhesion and thrombus growth when directly incubated with blood and when immobilized on a collagen surface (P<0.05). In contrast, natCRP did not exert any effect. Confocal immunohistochemistry revealed the presence of CRP on the surface of adhered platelets and within the thrombus and showed an upregulation of P-selectin and CD36 in effluent platelets preincubated with mCRP (P<0.05). Flow cytometry analysis of agonist-induced platelet activation demonstrated that mCRP, but not natCRP, significantly increased platelet surface P-selectin (P<0.05) without modifying CD63 and PAC-1.

Conclusions—Our data indicate that whereas serum natCRP may not affect thrombus growth, mCRP displays a prothrombotic phenotype enhancing not only platelet deposition, but also thrombus growth under arterial flow conditions. (Arterioscler Thromb Vasc Biol. 2008;28:000-0000)

Key Words: C-reactive protein ■ isoforms ■ thrombosis ■ platelets

In recent years, C-reactive protein (CRP), long associated with inflammation, has emerged as a clinical marker of future cardiovascular events among apparently healthy subjects and of worse prognosis in acute coronary patients.1–3

Thrombus formation on rupture of an atherosclerotic plaque is believed to be the responsible event for most of the coronary syndromes, in a process mainly mediated by platelet adhesion, activation and aggregation. The first response to vascular injury consists of platelet adhesion to the damaged vessel wall or to exposed tissue components, and is mediated by flow-regulated interactions that have a key influence on subsequent thrombus growth, often culminating in life-threatening complications.4,5

Long considered merely a bystander in vascular disease, new evidence indicates that CRP may be not only a marker, but also an active player in the development of cardiovascular pathology.6 The role of CRP as a modulator of inflammation and thrombosis is controversial, because both proinflammatory and antiinflammatory properties have been ascribed to the molecule.7–9 For instance, CRP inhibits neutrophil activation and adhesion,9 and blocks platelet aggregation in vitro,10,11 whereas arterial injury in CRP-transgenic mice is associated with increased thrombosis.12 Overexpression of the human CRP gene in atherosclerosis-prone mice has also shown contradictory effects on the development of atherosclerosis.13,14 To explain these apparent contradictory actions, it was proposed that distinct isoforms of CRP were formed during inflammation. The classically studied serum CRP is a pentamer composed of five noncovalently bound globular subunits arranged as a cyclic annular disk, the so-called native CRP (natCRP). natCRP can undergo subunit dissociation into individual monomeric units, as when associating with a cell-membrane.15 These subunits undergo a conformational change that significantly modifies CRP structure, solubility, and antigenicity. This form of CRP, called modified or monomeric CRP (mCRP), is found in fibrous tissues of normal and inflamed human blood vessel intima.16 Although the expression of CRP mRNA in both normal and plaque arterial tissues has been reported,17 it remains to be proven whether extrahepatic cells possess the machinery necessary to fold 5 subunits into the native pentamer. In vitro, mCRP can be produced from natCRP by exposing natCRP to heat, urea, or acidic conditions, in the absence of calcium ions.18 mCRP can also spontaneously form from natCRP during storage. Studies directly addressing the distinct isoforms of CRP have reported that mCRP displays proinflam-
matory effects on neutrophils, endothelial cells, and platelets, whereas natCRP displays antiinflammatory activities.19–21

In the present study, we investigated the relative impact of natCRP and mCRP on the dynamics of platelet adhesion and thrombus growth under defined flow conditions. Our results indicate that mCRP, but not natCRP, enhances platelet adhesion and thrombus growth.

Materials and Methods
See supplemental file (available online at http://atvb.ahajournals.org) for expanded Methods section.

CRP Isoforms Obtention
High purity human natCRP (Calbiochem) was stored in 10 mmol/L Tris, 140 mmol/L NaCl buffer (pH 8.0) containing 2 mmol/L CaCl₂ to prevent spontaneous formation of mCRP from the native pentamer. mCRP was obtained by urea chelation from purified human CRP as described by Potempa et al.18

Experimental Design
Venous blood from medication-free volunteers was withdrawn in 10 UI/mL sodium heparin. Procedures were approved by the Clinical Research Committee of our Institution.

Blood was then incubated with natCRP, mCRP, or control buffer (37°C, 10 minutes). Platelets were rendered fluorescent by the addition of mepacrine 10 µmol/L (Sigma), unless otherwise specified.

Perfusion Experiments in Flat Chamber
Glass slides were coated with type I collagen (4°C, overnight). When indicated, collagen-coated slides were incubated with 5 µg/mL of natCRP, mCRP, or blocking buffer (1% bovine serum albumin) for 3 hours at 37°C. Coated slides were placed in a parallel plate chamber. A peristaltic pump was used to perfuse blood through the chamber at a constant shear rate of 1500 s⁻¹ for 5 minutes.

Imaging of Platelet Thrombi
Platelet deposition was scanned with a Leica TCS SP2 confocal laser scanning microscope. Platelets were viewed with an APO 20X objective. Surface covered by platelets and area of individual thrombi were calculated using NIH Image software (by Dr Wayne Rasband, National Institutes of Health). Average height of platelet thrombi was calculated creating a topographical image from the spatial data set acquired and three-dimensional rotation projections were created from stack series of selected thrombi.

CRP Immunolocalization
For detection of CRP, a further set of perfusion experiments with unlabeled platelets was performed. Immunodetection of CRP on fixed slides was performed with a monoclonal anti-human CRP antibody (Sigma, clone 8) and with a monoclonal antibody (mAb) which specifically recognizes mCRP isoform (clone 8C10 kindly provided by Dr Potempa). Coverslides were incubated with Alexa Fluor 488 donkey antimouse IgG (H+L).

P-Selectin and CD36 Immunostaining
Effluent blood from perfusion experiments with blood unlabeled with mepacrine was collected and fixed with 3.8% paraformaldehyde for 30 minutes, and platelet-rich plasma was obtained by centrifugation at 200g for 17 minutes. Platelets were then isolated by centrifugation, immobilized on poly-L-lysine-coated coverslides, and incubated either with a

Figure 1. Contribution of CRP isoforms to platelet deposition, area of individual thrombi, and thrombus height. A, Mean platelet deposition (MPD). Results are expressed as mean values of surface covered by platelets per analyzed field (µm²/field)/10³ ± SEM. B, Area of individual thrombi. Results are expressed as values of surface covered by individual thrombi (µm²) ± SEM. C, Thrombus height. Results are expressed as values of height (µm) ± SEM *P<0.05; vs control.
Flow Cytometry
CRP-treated samples were diluted 1:10 in modified Tyrode Buffer and activated with collagen (5 μg/mL) or ADP (1 μmol/L). A CD41a – FITC mAb (Pharmingen) was used as an activation-independent marker of platelets for CD62P and CD63 analysis. P-selectin and CD63 were assessed with a 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). In additional experiments, the responses to CRP were studied in the presence of 2.5 μg/mL of function-blocking anti-CD16 mAb 3G8 (Pharmingen).

Statistical Analysis
Different conditions were performed at least twice in each subject and 5 subjects of each treatment were assessed. After testing for normal distribution and equality of variances with Levene F test, Student t test or ANOVA as appropriate was used to determine statistical significance between treatments. A value of P<0.05 was considered significant.

Results

Contribution of CRP Isoforms to Thrombus Formation
Preincubation of blood with mCRP increased platelet deposition in a concentration-dependent manner. Statistically significant increase was detected with concentrations higher than 1 μg/mL mCRP. In the presence of 25 μg/mL mCRP, platelet deposition was more than 3-fold higher than in untreated blood (P<0.05). On the contrary, incubation with natCRP did not produce any effect on platelet deposition at any tested concentration (Figure 1A).

To elucidate the effect of CRP on aggregate size, the area of individual thrombi formed on the collagen surface was evaluated. For this purpose we quantified the area of aggregates larger than 100 μm². Thrombus area significantly increased in samples treated with mCRP in a dose-dependent manner, with a statistically significant increase detected at concentrations higher than 1 μg/mL mCRP (P<0.05). By contrast, blood incubation with natCRP (10 μg/mL) produced inhibition on aggregate size (P<0.05) as shown in Figure 1B.

Three-dimensional topographical imaging of platelet thrombi were obtained from the spatial data set acquired by confocal microscopy. natCRP treatment did not yield any effect on thrombus height. On the contrary, 3D topographical imaging revealed that mCRP increased thrombus height in a concentration-dependent manner, with a statistically significant increase detected at concentrations higher than 1 μg/mL mCRP (P<0.05) as shown in Figure 1C.

CRP Distribution
CRP immunodetection on the platelet surface with the antibody that recognizes both CRP isoforms was more intense in perfusions ran with mCRP-treated blood than those ran when incubating blood with natCRP, as shown in Figure 2A (P<0.05). Additionally, mCRP immunodetection with anti-mCRP antibody showed lack of mCRP on the surface of control and natCRP-treated platelets, whereas mCRP was strongly immunodetected on the surface of platelets treated with mCRP (see supplemental Figure II). Because mCRP, but not natCRP, enhanced thrombus growth, CRP distribution within the thrombus volume was evaluated by 3D projections of selected thrombi (Figure 3).

CRP labeling in thrombi formed when incubating blood with natCRP appeared to be distributed as diffuse spots with a higher presence on the collagen surface (blue) than in the top edge (red) (Figure 3I: 1A through 1F). By contrast, CRP immunostaining in mCRP thrombi was found to be distributed as aggregated patches within the entire volume of the thrombus (Figure 3I: 2A through 2F). Immunodetection of mCRP revealed that mCRP was mainly localized on the thrombus growing edge (Figure 3II). See supplemental movie-file with 90° rotating animation.
P-Selectin and CD36 Immunodetection
mCRP pretreatment, but not natCRP, enhanced the expression of both P-selectin and CD36 of effluent platelets. The percentage of CD62P expression increased from 36.7 ± 4.3% in control samples, to 67.1 ± 5.0% in mCRP-treated blood (P < 0.05). Immunodetection of CD36 significantly increased up to 70% in effluent platelets incubated with mCRP compared to control and natCRP-treated platelets (P < 0.05; Figure 4).

Flow Cytometry Studies
Blood incubation solely with CRP isoforms did not induce any significant effect in the expression of the platelet activation markers P-selectin, CD63, and PAC-1. Conversely, in collagen-stimulated platelets, preincubation with mCRP significantly enhanced platelet P-selectin expression (P < 0.05), and it did not exert any effect in the expression of either CD63 nor PAC-1. Preincubation with natCRP did not produce any significant effect on the expression of CD63 and PAC-1 in either resting or collagen-stimulated platelets (Figure 5A). However, although not statistically significant, natCRP increased P-selectin expression on collagen-induced activated platelets because of the partial dissociation of natCRP into its subunits. mCRP was formed from natCRP because of the acidity of the collagen solution needed to induce platelet activation. Indeed, dot blotting of natCRP subjected to the same conditions of the flow cytometry studies confirmed the partial dissociation of natCRP into mCRP, as shown in Figure 5B. In fact, P-selectin expression on ADP-induced platelet activation was not upregulated by natCRP preincubation. On the contrary, mCRP significantly increased P-selectin expression on ADP-induced activated platelets (Figure 5C). Blockade of the FcγRIII receptor (CD16) before blood incubation with mCRP did not suppress P-selectin expression on agonist activated platelets.

Effect of Surface-Immobilized CRP Isoforms on Thrombus Formation
The effect of CRP isoforms on thrombus growth was also evaluated in perfusion experiments on surfaces coated with immobilized collagen and either natCRP or mCRP. Similarly to what we observed when CRP was directly added to blood, immobilized mCRP in the collagen surface significantly enhanced platelet deposition. Mean platelet deposition on collagen/mCRP-coated surface was 2-fold higher than in control collagen-coated surfaces (P < 0.05; Figure 6A). Immobilized mCRP also significantly increased aggregate size. Indeed, the area of individual thrombi deposited on collagen (463 ± 76 μm²) was significantly lower than that on the collagen/mCRP surface (1097 ± 278 μm²; P < 0.05; Figure 6B). Platelet deposition and aggregate size on collagen/
natCRP-coated surface did not differ significantly from platelet deposition on collagen alone. In fact, increasing concentrations of immobilized natCRP in the collagen surface did not increase platelet deposition nor aggregate size (Figure 6A and 6B).

To investigate whether the presence of circulating natCRP would affect the platelet response to immobilized mCRP we added natCRP (5 μg/mL) to the blood and measured its effects on platelet deposition and aggregate size on collagen/mCRP-coated surfaces. natCRP did not affect platelet deposition on mCRP/collagen surface, measured as mean platelet deposition and aggregate size (Figure 6A and 6B).

Three-dimensional topographical imaging of platelet thrombi revealed that immobilized mCRP significantly enhanced thrombus growth. Platelet aggregates formed on collagen/mCRP were more than 2-fold higher than those formed on collagen alone (6.17±1.07 μm on collagen versus 14.31±2.34 μm on collagen/mCRP, *P<0.05; Figure 6C).

**Figure 4.** Effect of natCRP and mCRP on CD62P and CD36 expression of effluent platelets. Photomicrographs show representative confocal images of CD62P (A) and CD36 (B) immunostained platelets; I: control; II: natCRP (5 μg/mL); III: mCRP (5 μg/mL). Scale bar is 5 μm. Expression calculated from the area of CD62P (C) or CD36 (D) positive staining normalized with the area occupied by platelets. Results are expressed as values of average %±SEM and Arbitrary Units. *P<0.05 vs control and natCRP.

**Figure 5.** Flow cytometry analysis of the effect of natCRP and mCRP on P-selectin, CD63, and PAC-1 expression of agonist-induced platelet activation. Results are expressed as % of positive platelets ±SEM *P<0.05 vs control (agonist-induced platelets without CRP preincubation). A: CD62P, CD63, and PAC-1 expression of collagen-induced platelet activation. B: Dot blotting of (a) natCRP, (b) mCRP, and (c) natCRP incubated with the collagen solution used for flow cytometry, showing the partial dissociation of natCRP into mCRP. C: CD62P expression of ADP-induced platelet activation.
Interestingly, mCRP was able to enhance thrombus growth at physiological concentrations of CRP, spanning from 1 to 25 µg/mL, which coincides with concentrations predicting cardiovascular risk. Whereas mCRP significantly enhanced platelet activation, adhesion, and thrombus growth, natCRP had no effect.

Clinically, the threshold of CRP plasma concentration associated to cardiovascular risk is more than 3 µg/mL, whereas levels higher than 10 µg/mL are usually attributed to other causes as acute infection or inflammation. Therefore used in our experiments physiological and pathophysiological concentrations of CRP, spanning from 1 to 25 µg/mL. Interestingly, mCRP was able to enhance thrombus growth at concentrations higher than 1 µg/mL, which coincides with concentrations predicting cardiovascular risk.

The postperfusion confocal analysis allowed us to study 3-dimensionally thrombus formation, measuring platelet deposition, aggregate size on a protein-coated surface, and thrombus height. Several reported effects of CRP have been shown to be calcium dependent, thus we used sodium azide or lipopolysaccharide (LPS) and when dialysed, heparin as anticoagulant instead of calcium chelators. Recent reports have questioned the validity of CRP in vitro studies. Commercial CRP preparations can be contaminated with sodium azide or lipopolysaccharide (LPS) and when dialysed, free of these factors, several of the effects of CRP are lost.

To eliminate these confounding factors we used two different purified commercial preparations and, additionally, perfusion experiments performed with control buffer had no effect on platelet adhesion and thrombus formation.

mCRP, unlike natCRP, was able to induce thrombosis by promoting platelet deposition and thrombus growth on the collagen surface. mCRP not only significantly increased platelet adhesion, but also aggregate size and thrombus height. These observations support a role for mCRP in platelet adhesion, and also on platelet to platelet interaction, which is the responsible event for thrombus growth and further vessel occlusion. Accordingly, P-selectin, which has been shown to stabilize platelet–platelet and platelet–leukocyte aggregates, was also upregulated by mCRP, as seen in effluent platelets. This increase in P-selectin might explain, partly, the mCRP enhancement of thrombus growth. Indeed, platelet surface P-selectin followed a similar pattern after collagen and ADP stimulation in flow cytometry analysis. However, mCRP was unable to induce GPIIb/IIIa activation and surface CD63 expression. These observations suggest that mCRP enhances platelet recruitment and subsequent thrombus formation by exocytosis of α-granules and platelet agonists release. In contrast to previous data, mCRP effects were not mediated through the FcγRIII receptor.

These findings raise the possibility that the increased thrombus formation after arterial injury and monocyte-platelet aggregation in human CRP-transgenic mice, could be attributed to distinct isoforms of CRP rather than natCRP itself. Moreover, the different effects of CRP isoforms found in the present study are in accordance with the opposing effects of CRP isoforms previously reported by Khreiss et al on shear-induced neutrophil-platelet aggregation. In agreement with our findings, mCRP has been shown to exert...
greater proinflammatory effects in endothelial cells and neutrophils.\textsuperscript{20,30} Paradoxically, Schwedler et al reported that natCRP promoted but mCRP reduced atherosclerosis in ApoE\textsuperscript{-/-} mice.\textsuperscript{31} These results are not necessarily contradictory with our findings because Schwedler et al studied the effect of CRP isoforms in early atherosclerosis, and designed a model of low dosing of mCRP over a long period of time, which could have heightened immune surveillance, slowing the process of atherosclerotic plaque formation. Conversely, we focused our efforts on studying the direct effect of native and modified CRP on the thrombotic complications, such as those happening on plaque rupture. Our study is, to the best of our knowledge, the first to show a causal and dual role of CRP isoforms on thrombus formation under arterial flow conditions.

How CRP mediates platelet activity still remains unclear. The presence of CRP observed on the platelet surface and within the thrombus structure after blood perfusion suggests a stable and direct interaction between platelets and CRP. When natCRP dissociates into free subunits, it yields monomeric mCRP with a loss of predominantly $\beta$-sheet secondary structure and an increase in $\alpha$-helix,\textsuperscript{30} forming insoluble aggregates, which could explain the different distribution of natCRP and mCRP within the thrombus. The observed difference in intrathrombus distribution might be attributed to a stronger interaction of mCRP with the platelet surface compared to natCRP. It is important to point out that it is not clear whether monoclonal anti–CRP-clone 8 from Sigma detects both native and modified CRP. According to the manufacturer it recognizes both isoforms. However, Schwedler et al showed that anti–CRP-clone 8 predominately recognized mCRP rather than natCRP.\textsuperscript{11} On the other hand, native CRP may likely bind to phosphatidylcholine abundantly expressed on the surface of activated platelets, and it has been shown that cell membranes dissociate natCRP to a structural intermediate (mCRP(m)), which can further detach from the membrane to form mCRP.\textsuperscript{15} Whether the staining with the anti–CRP-clone 8 antibody on the surface of adhered platelets preincubated with natCRP shows the presence of mCRP, natCRP, or the intermediate mCRP(m) is not clear. However, adhered platelets preincubated with natCRP stained positive for CRP with the anti–CRP-clone 8 and negative for mCRP with the anti-mCRP antibody (clone 8C10), suggesting the lack of presence of mCRP.

In this work we have observed that mCRP not only was able to enhance thrombosis when directly added to blood, but also when immobilized with collagen, a key component of atherosclerotic lesions. Although the presence of CRP mRNA in atherosclerotic tissue has been established,\textsuperscript{17,33} it is unclear whether the expressed CRP is the pentameric isoform or the monomeric isoform. It remains unknown whether CRP locally produced in the vessel wall\textsuperscript{14} is, indeed, mCRP that is naturally expressed in the intima.\textsuperscript{16} It is likely that collagen exposure after mechanical or spontaneous plaque rupture may also result in exposure of mCRP to blood components, leading to platelet aggregation and thrombus formation. Alternatively, inflammation may lead to formation of mCRP from natCRP within the blood stream, thus linking thrombosis and inflammation, key events in acute coronary syndromes. Interestingly, this concept supports the fact that the mere presence of CRP in plasma is not associated to platelet aggregation, but it is conceivable that mCRP, as in vascular tissue, might be one of the thrombogenic triggering factors.

In summary, our data indicate that whereas natCRP may not affect thrombus growth, mCRP displays a prothrombotic phenotype enhancing not only platelet deposition, but also thrombus growth under arterial flow conditions. Further research seems warranted to elucidate more detailed mechanisms by which CRP isoforms regulate thrombus formation, and to clarify the role of CRP in cardiovascular disease.

**Acknowledgments**

We thank Dr J. Crespo and M. Pescador for her technical help. We also thank Dr L. Potempa for kindly providing the antibodies against mCRP and natCRP.

**Sources of Funding**

This work has been possible thanks to funds provided by Ministry of Science and Education of Spain (PNS 2006/10091), Ministry of Health- Instituto Salud Carlos III (CIBEROBN- CB06/03), and Fundacion Jesus Serra. B.M. is granted with a fellowship from the Catalan Government (FI2005 DUE). G.V. is recipient of a grant from the Science and Education Spanish Ministry (JaC).

**Disclosures**

None.

**References**


8. Xia D, Samols D. Transgenic mice expressing rabbit C-reactive protein are resistant to endotoxemia. \textit{Proc Natl Acad Sci U S A}. 1997;94:2575–2580.


C-Reactive Protein Isoforms Differ in Their Effects on Thrombus Growth
Blanca Molins, Esther Peña, Gemma Vilahur, Carlos Mendieta, Mark Slevin and Lina Badimon

Arterioscler Thromb Vasc Biol. published online September 11, 2008;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2008/09/11/ATVBAHA.108.174359.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2008/09/15/ATVBAHA.108.174359.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENTAL DATA AND FIGURES

Expanded methods

Preparation, testing and storage of CRP isoforms and antibodies

Antibodies directed to natCRP (clone 1D6) and mCRP (clone 8C10) were kindly provided by our collaborator Dr Potempa. (Ying et al, 1989; Schwedler et al, 2003); and their specificity demonstrated by measurement of binding characteristics (Figure I A). High purity human natCRP (Calbiochem) was stored in 10 mmol/L Tris, 140 mmol/L NaCl buffer (pH 8.0) containing 2 mmol/L CaCl$_2$ to prevent spontaneous formation of mCRP from the native pentamer. mCRP was obtained by urea chelation from purified human CRP as described by Potempa et al. Briefly, natCRP at 1 mg/mL was chelated with 10 mmol/L ethylene diaminetetraacetic (EDTA) and incubated in 8.0 mol/L urea for 6 h at 37 °C. Urea was removed via dialysis against low ionic strength TBS (0.01 mol/L Tris-HCl and 0.05 mol/L NaCl, pH 7.3). mCRP concentration was determined by the BCA protein assay. The filtered solution was stored at 4 °C and used within 24 h.

In order to validate CRP modification, electrophoresis was performed to distinguish mCRP from natCRP, according to the method recently described by Taylor KE. Briefly, 12 % polyacrilamide SDS-containing gels were used. Samples were heated (10 min at 90 °C) for denaturing conditions. Electrophoresis was carried out at 30 mA/ 0.75 mm gel in a BioRad Power Pac 300 electrophoresis unit. Proteins were visualized using Comassie brilliant blue staining. When sample heating was omitted, natCRP ran as a broad smear with an apparent molecular weight of 40-80 kDa, while mCRP ran at a relative molecular mass of 23 kDa (Figure I B). Subjecting samples to reducing conditions and heating to 90 °C resulted in denaturation of natCRP and a relative molecular mass similar to that of mCRP was observed. natCRP was not contaminated with mCRP as no band was observed at 23 kDa. Modification of CRP by urea-chelation yielded a complete transformation of the pentameric to the monomeric CRP isoform, demonstrated by the presence of mCRP migrating as a single band of 23 kDa.
Purity of natCRP and mCRP was also shown by dot blotting where doubling dilutions of CRP preparations were bound to nitrocellulose strips and exposed to anti-nat and mCRP antibodies (1:100). Following washing and appropriate secondary antibody incubation (anti-mouse HRP conjugated; 1:100), blots were developed by ECL. Controls where primary antibodies were omitted and where CRP was replaced with BSA on the nitrocellulose membrane showed no staining (data not included). Endotoxin levels in CRP preparations were below the detection limit of the limulus assay (<0.125 EU/ml). Sodium azide was dialysed out in CRP preparations in a large volume of Tris-HCL buffer.

Dot blotting of natCRP and mCRP onto nitrocellulose (which avoids CRP transformation following mobilization onto the charged surface) followed by exposure to antibodies demonstrated the purity of the commercially obtained natCRP and complete conversion of natCRP to mCRP following urea-chelation (Figure I C).

**Experimental Design**

Blood from non-smoking healthy donors, who denied taking any antiplatelet medication for 15 days prior to blood extraction, was used for the perfusion experiments. Procedures were approved by the Clinical Research Committee of our Institution. Blood was withdrawn in 10 U/mL sodium heparin by cubital venipuncture, kept at 20 °C, and used within 2 hours of collection. Platelet count, leukocyte count, and hematocrit were all within normal ranges. Blood was then incubated at 37 °C for 10 min either with natCRP, mCRP or control buffer (which consisted on a solution of 10mM EDTA, 8M urea subjected to dialysis in a large volume of Tris-HCl, mimicking urea-chelation of natCRP). Platelets were rendered fluorescent by the addition of mepacrine 10 μmol/L (Sigma), unless otherwise specified.
Perfusion experiments in flat chamber

Glass slides were coated with 10 µg/ml of type I collagen (4ºC, overnight). Additionally, when indicated, collagen-coated slides were incubated with 5 µg/ml of either natCRP, mCRP or blocking buffer (bovine serum albumin 1 %) for 3h at 37 ºC. Immobilized CRP coating was validated by immunohistochemistry of the coated slides.

Coated slides placed in a parallel plate chamber. The flow chamber was assembled and filled with Tyrode's buffer (134 mmol/L NaCl, 0.34 mmol/l Na₂HPO₄x12H₂O, 2.9 mmol/L KCl, 12 mmol/L NaHCO₃, 1mmol/L MgCl₂x6H₂O, 20mmol/L Hepes). A peristaltic pump was used to perfuse the buffer through the chamber. After 1 min buffer preperfusion, blood was introduced into the chamber at a constant shear rate of 1500 s⁻¹ for 5 min. At the end of blood perfusion, buffer was again circulated for 1 min through the chamber under identical flow conditions. The entire system was kept at 37 ºC. After perfusions, slides were carefully removed from the system, rinsed with PBS pH 7.4 and fixed with 3.8 % paraformaldehyde for 15 min. The fixed slides were then washed with PBS and mounted on glass slides with Glycerol Mounting Medium (Dako Cytomation).

Imaging of platelet thrombi

Platelet deposition on the surface of collagen was scanned with a Leica TCS SP2 confocal laser scanning microscope. A 488 nm Ar Kr-laser was used as light source. Platelets were viewed with a HCX PL APO 20X / 0,7 IMM CORR objective. Five fields along the adhesion surface were systematically acquired for total platelet deposition and individual thrombus size analysis, discarding the entrance and exit of the flow path (field: 750 µm x 750 µm). A threshold was applied to distinguish platelets from the background, and the same value was then used for analyzing all the stacks of confocal images collected for a given experiment. Digital color images were converted into black-and-white images. The surface covered by platelets and the area of individual thrombi were calculated using NIH Image software (public domain software by Dr
The surface covered by platelets was expressed as the area covered by platelets per field analyzed (µm²/field), and the individual thrombi size was calculated by measuring the area of platelet aggregates bigger than 100 µm² and expressed in µm². Average height of platelet thrombi were calculated creating a topographic image from the spatial data set acquired (0.5 µm distance between adjacent cross-sectional images). Once the topographic image was obtained average height was calculated. Three-dimensional rotation projections were created from stack series of images of 235 µm x 235 µm x 50µm (1 µm distance) of selected thrombi. The maximum projection showing the z-value by color was created, and thereafter a 90° rotation animation was performed creating a projection every 2°. Observers for mean platelet deposition and thrombus height were blinded to treatment group.

**CRP immunolocalization**

For detection of CRP, a further set of perfusion experiments with unlabeled platelets was performed. After perfusions, collagen-coated slides were rinsed with PBS pH 7.4 and fixed with 3.8 % paraformaldehyde at room temperature and incubated in blocking buffer. Immunodetection of CRP was performed with a monoclonal anti-human CRP antibody (Sigma) which, according to the manufacturer, recognizes both native and denaturated CRP isoforms, and with a monoclonal antibody which specifically recognizes mCRP isoform. Coverslides were washed and incubated with Alexa Fluor 488 donkey anti-mouse IgG (H+L). Immunostained coverslides were washed and covered with Prolong Gold antifade reagent. Images were recorded by fluorescence confocal microscopy (HCX PL APO 63x/1.2 W Corr/0.17 CS). Controls without primary antibody showed no fluorescent labelling.
P-selectin and CD36 immunostaining

P-selectin and CD36 expression was analyzed performing perfusion experiments with human blood not labelled with mepacrine. Effluent blood was collected and fixed with 3.8 % paraformaldehyde for 30 min at room temperature. Platelet-rich plasma was obtained by centrifugation at 200 g for 17 min. Platelets were then isolated by centrifugation. Platelets were immobilized on poly-L-lysine-coated coverslides overnight, incubated with blocking buffer and afterwards incubated either with a phycoerytrin (PE) conjugated anti-CD62P monoclonal antibody (mAb) (Pharmingen) or a fluorescein isothiocyanate (FITC) conjugated anti-CD36 mAb (Pharmingen). Coverslides were washed and mounted onto slides using Prolong Gold antifade reagent. Images were recorded on a fluorescence confocal microscope (HCX PL APO 63x/1.2 W Corr/0.17 CS). Excitation was produced via the 488 laser line, and emission was measured along with interference contrast images on a separate photomultiplier for overlay.

Flow cytometry

For flow cytometry studies of P-selectin, CD63, and conformational change of GPIIb-IIIa, CRP-treated samples were diluted 1:10 in modified Tyrode Buffer. 25 µl aliquots were activated with collagen (5 µg/ml, 5 min, 37 °C) or ADP (1 µmol/L, 5 min, 37 °C). A FITC conjugated mAb to CD41a (Pharmingen) was used as an activation-independent marker of platelets for CD62P and CD63 analysis. P-selectin and CD63 surface expression were assessed with a 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). In additional experiments, the responses to CRP were studied in the presence of 2.5 µg/ml of function-blocking anti-CD16 mAb 3G8 (Pharmingen) The reaction mixture was
incubated in the dark at room temperature for 20 minutes. To assess the extent of non-specific association of proteins with platelets, blood was added to control tubes with FITC-labeled and PE-labeled non-immune immunoglobulins. The platelet population was identified based on its forward and side scatter and the association with CD41a antibody. A total of 10000 events were analyzed for percentage of positive platelets using Expo32 ADC XL 4 Color software. Fluorescence was measured with a Beckman Coulter Epics XL instrument. The fractions of the specific fluorescence-positive platelets were obtained after subtraction of non-specific fluorescence in the samples labelled with non-immune immunoglobulins. All measurements were fluorescence-compensated on a daily basis for each set of measured samples using calibration beads.
A: Binding affinity assays of anti-mCRP antibody (clone 8C10) and anti-natCRP antibody (clone 1D6). B: Analysis of natCRP (N) and urea-chelated mCRP (M) by electrophoresis. natCRP and mCRP, mixed with sample buffer were subjected to standard SDS PAGE conditions. Gels were stained with Coomassie brilliant blue. (a) Sample non-denatured; (b) denatured sample (heated and reduced). C: Dot blotting on nitrocellulose of natCRP and mCRP with (a) anti-mCRP and in (b) anti-nCRP antibodies.
Representative confocal images of CRP immunostaining (green) on adhered platelets with anti-mCRP antibody. A: control, B: natCRP (5μg/ml), C: mCRP (5μg/ml). The bars graphic shows CRP expression on adhered platelets preincubated with natCRP and mCRP. Scale bar is 20 μm.