Opposing Effects of C-Reactive Protein Isoforms on Shear-Induced Neutrophil-Platelet Adhesion and Neutrophil Aggregation in Whole Blood

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Background—Plasma C-reactive protein (CRP) level is a powerful predictor of cardiovascular events. However, it is not known whether CRP could affect neutrophil-platelet adhesion and neutrophil aggregation, key events in acute coronary syndromes. Emerging in vitro evidence suggests that some bioactivities of CRP are expressed on loss of the pentameric symmetry, resulting in formation of modified or monomeric CRP (mCRP).

Methods and Results—We studied the impact of human native CRP and bioengineered mCRP that cannot rearrange into the pentameric structure on the kinetics of neutrophil-platelet adhesion and neutrophil aggregation in whole blood subjected to shear (\(\sim 100 \text{ s}^{-1}\)) using real-time flow cytometry. Shear resulted in upregulation of platelet P-selectin expression, leading to platelet capture of neutrophils and subsequent neutrophil aggregation, which was dependent on P-selectin, L-selectin, and CD18. Native CRP at clinically relevant concentrations markedly attenuated these changes. The residual amount of neutrophil adhesion was blocked with anti-CD18 or anti-CD11b antibody. By contrast, mCRP concentration-dependently enhanced shear-induced platelet P-selectin expression and increased the rate and extent of formation of both neutrophil-platelet and neutrophil-neutrophil aggregates. Complete abrogation of platelet-neutrophil adhesion and neutrophil aggregation required both anti–P-selectin and anti-CD18 antibodies but not anti–L-selectin antibody. The CRP action was markedly inhibited by an anti–CD32 antibody, whereas the mCRP effects were significantly attenuated by an anti–CD16 antibody.

Conclusions—These results indicate that native CRP inhibits platelet activation and prevents platelet capture of neutrophils, whereas mCRP displays potent prothrombotic activities under low levels of shear. Thus, mCRP rather than native CRP may precipitate acute coronary syndromes. (*Circulation* 2004;110:2713-2720.)

Key Words: neutrophils ■ platelets ■ cell adhesion molecules ■ inflammation ■ heart disease
In the present study, we investigated the impact of native CRP and mCRP on the dynamics and molecular constituents that support neutrophil-platelet and neutrophil-neutrophil aggregates in human whole blood using real-time flow cytometry. Our results indicate that native CRP inhibits, whereas mCRP accelerates, hydrodynamic shear-induced platelet-neutrophil adhesion and subsequently neutrophil aggregation.

**Methods**

**CRP Isoforms**

High-purity (>99%) human native CRP (Calbiotech) was stored in NaN3-free 20 mmol/L Tris, 150 mmol/L NaCl buffer (pH 7.5) containing 2 mmol/L CaCl2 to prevent spontaneous formation of mCRP from the native pentamer. A recombinant form of mCRP (r-mCRP; purity, >97%) that cannot rearrange into a pentameric structure was engineered and characterized as described.24 Native CRP was distinguished from mCRP by binding and antigenicity differences24 and by their secondary structure.25 The endotoxin level of all peptide solutions was below the detection limit (0.125 endotoxin units/mL, corresponding to 0.01 ng/mL lipopolysaccharide [LPS]) that cannot rearrange into a pentameric Limulus assay (Sigma).

**Detection of Neutrophil-Platelet Conjugates by Flow Cytometry**

Venous blood (10 mL, anticoagulated with sodium heparin, 50 U/mL) was obtained from healthy, nonsmoking volunteers (male and female, 23 to 55 years old; leukocyte count, 3800 to 8800/μL) who had denied taking any medication for at least 2 weeks. The Clinical Research Committee approved the experimental protocols. Blood was kept at 20°C and medication for at least 2 weeks. The Clinical Research Committee approved the experimental protocols. Blood was kept at 20°C and used within 2 hours of collection. Two-hour storage of blood resulted in 17±6% increases in neutrophil CD11b/CD18 expression without increasing platelet P-selectin expression (7.9±1.0 versus 8.4±0.7 fluorescence units) detected immediately after blood collection, n=4. To circumvent the possible confounding effects of this slight neutrophil activation, the order of measurements within each experiment was randomized. Blood obtained from the same persons (at different times) was used to study both CRP and mCRP.

Formation of neutrophil aggregates and neutrophil-platelet conjugates was monitored in real time in accordance with the technique of Konstantopoulos et al28 with modifications. In brief, blood (100 μL) was diluted 1:6 with HEPES buffer. Platelets were labeled with an anti–CD61-FITC mAb followed by staining of leukocytes with LDS-751. A, Control (unsheared) blood sample at 0 minutes showing single neutrophils with and without platelets. An LDS-751 fluorescence threshold (dashed line) was set to exclude unbound erythrocytes and single platelets from display. Sample was then subjected to hydrodynamic shear (≈100 s−1) at 37°C for 14 minutes. Vertical line (FITC-fluorescence threshold) separates single neutrophils (left) from neutrophil platelets (right). Horizontal line separates single neutrophils from neutrophil aggregates. B, Representative histogram of neutrophil aggregation after 14 minutes of shear. LDS-751 mean fluorescence values for neutrophil doublets, triplets, and quartets and larger aggregates are integral multiples of singlet value. RFU, relative fluorescence units.

![Figure 1](http://circ.ahajournals.org/)

**Figure 1.** Real-time flow cytometry analysis of neutrophil-platelet and neutrophil-neutrophil aggregates. Whole blood was diluted 1:6 with HEPES buffer. Platelets were labeled with an anti–CD61-FITC mAb followed by staining of leukocytes with LDS-751. A, Control (unsheared) blood sample at 0 minutes showing single neutrophils with and without platelets. An LDS-751 fluorescence threshold (dashed line) was set to exclude unbound erythrocytes and single platelets from display. Sample was then subjected to hydrodynamic shear (≈100 s−1) at 37°C for 14 minutes. Vertical line (FITC-fluorescence threshold) separates single neutrophils (left) from neutrophil platelets (right). Horizontal line separates single neutrophils from neutrophil aggregates. B, Representative histogram of neutrophil aggregation after 14 minutes of shear. LDS-751 mean fluorescence values for neutrophil doublets, triplets, and quartets and larger aggregates are integral multiples of singlet value. RFU, relative fluorescence units.
quantified by gating this population on the basis of LDS-751 fluorescence. Neutrophil conjugates defined as particles that expressed LDS-751 fluorescence levels greater than those of single neutrophils with or without bound platelets (Figure 1A) were quantified on the basis of the LDS-751 staining, in which mean fluorescence intensity was an integral multiple of the singlet neutrophil fluorescence value (Figure 1B). The ratio of neutrophils in aggregates to the total number of neutrophils was calculated by use of the formula %aggregation=(2D+3T+4Q)/S + 2D+3T+4Q, where S represents singlet neutrophils and the neutrophil aggregate sizes are given as D (doublets), T (triplets), and Q' (quartets and larger unresolved neutrophil aggregates).

Platelet P-Selectin Expression
For detection of P-selectin expression, shear was initiated in diluted blood samples in which leukocytes were not stained. At the indicated times, 5-μL aliquots were removed and incubated with R-phycocyanin-conjugated anti-P-selectin mAb CRC81 (Caltag Laboratories) for 3 minutes at 37°C. Samples were then diluted 1:1000 with HEPES buffer and analyzed by flow cytometry. Ten thousand events were acquired per sample.

Statistical Analysis
Results are presented as mean±SEM. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test) followed by Dunn’s multiple-contrast hypothesis test to identify differences between various treatments or by the Mann-Whitney U test for unpaired observations. Values of P<0.05 were considered significant for all tests.

Results
Native CRP Attenuates, Whereas mCRP Accelerates, Hydrodynamic Shear-Induced Formation of Neutrophil-Platelet Aggregates
Consistent with previous reports, 28-31 29±3% of neutrophils were adhered to platelets in diluted whole blood before shear was applied, whereas neutrophil homotypic adhesion was <2% (Figure 2). Platelet adhesion to neutrophils and neutrophil aggregation increased rapidly on application of shear, reaching a plateau at ~10 minutes (Figure 2). At 14 minutes, 82±4% of neutrophils had platelets adherent to their surface, and 54±3% of neutrophils were in aggregates of ≥2 cells (Figure 2, B and C). No changes were detected in the absence of shear (Figure 2, B and C).

Preincubation of blood samples for 5 minutes with native CRP produced decreases in neutrophil-platelet adhesion and neutrophil aggregation at all time points under shear without affecting the kinetics of adherence (Figure 2, A–C). The CRP inhibition was concentration-dependent; statistically significant inhibition was detected even with 1 to 5 μg/mL, which peaked at 25 μg/mL (Figure 2, D and E). CRP effectively inhibited the adhesive events even when it was added on initiation of shear, although the degree of inhibition was somewhat lower than that detected after preincubation with CRP (data not shown). Heat inactivation of CRP (30 minutes at 100°C) resulted in a complete loss of its activity (data not shown). Because preincubation of unshaved blood with mCRP for 10 minutes already produced detectable increases in cell adherence, mCRP was added to blood on application of shear. mCRP (25 μg/mL) enhanced the rate and extent of formation of neutrophil-platelet and neutrophil-neutrophil conjugates (Figure 2, B and C). Platelet adhesion to neutrophils reached a plateau at ~5 minutes (Figure 2B). At 14 minutes of shear mixing, the number of neutrophils that had platelets adherent to their surface (95±1%) or that were in aggregates of ≥2 cells (78±2%) were significantly (P<0.01) higher than those subjected to shear only. The mCRP actions were concentration-dependent; statistically significant potentiation was detected with 1 μg/mL, which peaked at 25 μg/mL (Figure 2, D and E). Addition of LPS (Escherichia coli) increased neutrophil-platelet and neutrophil aggregates (E) as assed after 5 minutes of shear (when mCRP effect reached plateau) and 14 minutes of shear, respectively. Values are mean±SEM for 4 to 8 experiments using blood from different donors. *P<0.05; **P<0.01 vs shear alone.

Figure 2. Kinetics of neutrophil-platelet and neutrophil aggregate formation and its modulation by CRP isoforms. Platelets were labeled with CD61-FITC, followed by staining of leukocytes with LDS-751. Diluted blood samples were subjected to hydrodynamic shear (~100 s⁻¹). At indicated time points, samples were injected directly into a FACScan flow cytometer. A, Representative scatterplots of real-time detection of neutrophil-platelet adhesion kinetics in absence or presence of native CRP or mCRP (25 μg/mL). B and C, Time course of neutrophil-platelet adhesion (B) and neutrophil aggregates (C) in presence of CRP or mCRP (25 μg/mL). D and E, Concentration-dependent actions of CRP and mCRP on neutrophil-platelet (D) and neutrophil aggregates (E) as assessed after 5 minutes of shear (when mCRP effect reached plateau) and 14 minutes of shear, respectively. Values are mean±SEM for 4 to 8 experiments using blood from different donors. *P<0.05; **P<0.01 vs shear alone.
Opposing Effects of Native CRP and mCRP on Hemodynamic Shear-Induced Expression of Platelet P-Selectin

Both platelet P-selectin expression and the number of platelet bound to neutrophils increased with time of shear, as evidenced by the 1.7-fold increase in P-selectin fluorescence and the 4-fold increase in platelet CD61-FITC fluorescence of single neutrophils that were bound to platelets, respectively, after 14 minutes of shear (Figure 3). Preincubation of blood samples with native CRP concentration-dependently attenuated these events, indicating inhibition of platelet activation and adherence (Figure 3). Statistically significant inhibition was detected with 5 μg/mL CRP. In the presence of 25 μg/mL CRP, P-selectin expression after 14 minutes of shear did not differ from that of unsheared (time 0) platelets (Figure 3B). By contrast, mCRP concentration-dependently increased the rate and extent of CD62P expression and augmented CD61-FITC fluorescence intensity associated with neutrophil-bound platelets (Figure 3, B and D). A statistically significant increase was detected with 5 μg/mL mCRP, which peaked at 25 μg/mL.

Dependence of Homotypic and Heterotypic Neutrophil Adhesion on Selectins and β2-Integrins

To assess the contribution of multiple adhesion molecules to neutrophil-platelet and neutrophil-neutrophil adhesion in sheared suspensions,28–30 we used function-blocking mAbs. Anti-P-selectin mAb decreased the rate of adhesive interactions, resulting in ~30% inhibition of neutrophil-platelet adhesion and ~55% inhibition of neutrophil-neutrophil conjugates after 14 minutes of shear (Figure 4). Blocking of either CD18 or L-selectin alone did not significantly decrease either neutrophil-platelet or neutrophil-neutrophil adhesion (Figure 4). However, a combination of anti–P-selectin and anti–L-selectin mAbs resulted in 64% inhibition of neutrophil-platelet adhesion after 14 minutes of shear (Figure 4). Simultaneous blockade of P-selectin and CD18 almost completely inhibited neutrophil-platelet adhesion and reduced neutrophil aggregation by ~73% (Figure 4).

In CRP-treated blood samples, P-selectin blockade produced only slight decreases, whereas simultaneous P-selectin and CD-18 blockade resulted in significant decreases in cell-cell interactions compared with those of native CRP alone (Figure 4). L-selectin blockade was without effect. A combination of native CRP with anti–P-selectin and anti–CD18 mAbs yielded almost complete blockade of neutrophil-platelet adhesion and inhibited neutrophil homotypic adhesion by ~75% after 14 minutes of shear (Figure 4).

The mCRP-accelerated neutrophil-platelet adhesion and neutrophil aggregation were significantly reduced at all time points under shear in the presence of anti–P-selectin mAb, whereas neither anti–L-selectin nor anti–CD-18 mAb alone produced significant inhibition (Figure 4). A combination of anti–P-selectin mAb and anti–L-selectin mAb did not result in significantly greater inhibition than that observed with anti–P-selectin mAb alone (Figure 4). A combination of anti–P-selectin and anti–CD18 mAbs inhibited neutrophil-platelet adhesion by ~94% and neutrophil aggregation by ~81% after 14 minutes of shear (Figure 4).

Blocking Mac-1 with anti-CD11b mAb alone did not yield significant inhibition (Figure 5). However, a combination of anti–P-selectin and anti–Mac-1 mAbs was as effective as anti–P-selectin plus anti–CD18 mAbs in blocking platelet-neutrophil and neutrophil-neutrophil adhesion (Figure 5).

Involvement of CD16 and CD32 in Native CRP and mCRP Signaling

We used function-blocking mAbs to CD16 (FcγRIII) and CD32 (FcγRIIa) as competitors to determine the IgG receptor...
subtype mediating the actions of mCRP and CRP, respectively.\textsuperscript{25,32,33} None of these antibodies by themselves significantly affected the parameters studied (Figure 6). The anti-CD32 mAb effectively, although never completely, prevented the inhibitory action of CRP on shear-induced neutrophil-platelet adhesion, neutrophil aggregation, CD61-FITC fluorescence of single neutrophils, and platelet P-selectin expression, whereas the anti-CD16 mAb was without detectable effects (Figure 6). Conversely, the anti-CD16 mAb, but not the anti-CD32 mAb, significantly, although never completely, attenuated the effects of mCRP (Figure 6). The irrelevant antibody MOPC-21 had no detectable effects on the responses to either CRP or mCRP (data not shown).

**Discussion**

Numerous epidemiological studies have shown that plasma CRP level is a powerful predictor of future cardiovascular diseases.\textsuperscript{1–5} However, the pathophysiological importance of CRP is far from being fully understood. The present study provides evidence for a dual role of CRP isoforms in the regulation of shear-induced platelet adherence to neutrophils and neutrophil aggregation, critical events underlying ACS. We report that native CRP suppresses, whereas mCRP significantly enhances, the dynamics and extent of these adhesive interactions.

We used a flow cytometry assay to monitor real-time transition of platelets from the “resting” to activated state and to study the dynamics of platelet adherence to neutrophils and formation of neutrophil aggregates,\textsuperscript{28,30} as may occur in the circulating blood of patients with ACS. Apparently more neutrophils are adhered to platelets in diluted heparinized blood (as reported by previous\textsuperscript{28–31} studies and the present studies) than in blood collected into EDTA and formaldehyde.\textsuperscript{34} These discordant results might be attributed to differences in blood collection and handling rather than to a significant platelet activation, because we detected low levels of platelet P-selectin expression in unsheared samples. Shear mixing of diluted blood samples evokes negligible platelet aggregation,\textsuperscript{28} thereby eliminating the possibility of passive neutrophil entrapment in platelet aggregates. Low levels of hydrodynamic shear induce platelet activation, as evidenced by enhanced P-selectin expression. Likely mechanisms for platelet activation may involve hemolysis and release of ADP,\textsuperscript{28,35} contact with foreign surfaces and altered O\textsubscript{2} levels may function as additional signals. Activated platelets then bind to neutrophils, and this heterotypic cell interaction is a prerequisite for neutrophil aggregation.\textsuperscript{28} Our results show that native CRP concentration-dependently attenuated upregulation of P-selectin and consequently platelet adherence to neutrophils and neutrophil aggregation. The inhibition was detectable at CRP levels of 1 to 5 μg/mL, which are useful for predicting cardiovascular risk.\textsuperscript{2,3} Native CRP failed to affect the percentage of neutrophils that were bound to platelets, although it reduced neutrophil-associated platelet CD61-FITC fluorescence, indicating decreases in the number of platelets attached to single neutrophils. Flow cytometry did not allow determination of the number of platelets bound per neutrophil and assessment of whether platelets might have attached to neutrophils as single cells or small aggregates. Electron microscopy studies showed that most of the neutrophils were adherent to at least 2 platelets and that platelets either formed bridges between neutrophils or attached to a periphery away from the neutrophil-neutrophil contact region.\textsuperscript{28} These observations support the notion that critical
numbers of bound platelets (>2) may be necessary to induce neutrophil activation.\textsuperscript{28} The CRP inhibition of P-selectin expression is consistent with its inhibitory action on platelet reactivity in response to various agonists, including ADP.\textsuperscript{20–22} These findings raise the possibility that increased thrombus formation after arterial injury in human CRP-transgenic mice\textsuperscript{23} or left ventricular thrombus formation in patients with myocardial infarction\textsuperscript{16} could be attributed to distinct isoforms of CRP, perhaps directly derived from CRP, rather than to native CRP itself.

Native, pentameric CRP dissociates into free subunits after binding to plasma membranes\textsuperscript{37} or in a denaturing or oxidative environment,\textsuperscript{24} yielding mCRP. This is accompanied by a loss of predominantly \(\beta\)-sheet secondary structure, with an increase in \(\alpha\)-helix\textsuperscript{25} and expression of neoepitopes and distinct biological activities.\textsuperscript{24–27} Our study revealed that mCRP, unlike native CRP, induced P-selectin expression and enhanced the dynamics and extent of neutrophil-platelet adhesion and neutrophil aggregation during shear. Oxidized or heat-aggregated (ie, structurally modified) CRP has previously been reported to induce human platelet activation and aggregation in platelet-rich plasma in vitro.\textsuperscript{22,38} Of note, endotoxin levels in our CRP preparations were lower than the median plasma endotoxin value in a random population of men and women.\textsuperscript{39} However, neither LPS (at a concentration 2-fold higher than might be present in our protein preparations) nor heat-inactivated CRP evoked detectable effects, indicating that CRP or mCRP signaling was responsible for the observed effects.

Platelet-neutrophil adhesion is modeled as a multiple-step process.\textsuperscript{29,40} Platelet P-selectin binding to PSGL-1 on neutrophils mediates initial tethering, followed by their firm adhesion dependent on CD11b/CD18 (Mac-1) binding to GPIb\textsuperscript{41} and/or junctional adhesion molecule-3.\textsuperscript{42} In agreement with previous reports,\textsuperscript{28,40} we also found that P-selectin is crucial for platelet capture of neutrophils, and only combined P-selectin and CD18 or Mac-1 blockade resulted in almost complete abrogation of neutrophil-platelet adhesion under shear. L-selectin is also involved in this interaction, because a combination of anti-L-selectin and anti-P-selectin mAbs was more effective at blocking platelet adhesion than anti-P-selectin antibody alone. In the presence of native CRP, anti-P-selectin mAb did not produce a significant additive inhibition, indicating that prevention of P-selectin expression or interference with P-selectin binding by native CRP was critical for inhibition of subsequent cell-cell adhesion. In the

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**Figure 5.** Dependence of platelet-neutrophil and neutrophil-neutrophil adhesion on Mac-1 (CD11b). Diluted blood was incubated for 10 minutes with a panel of blocking mAbs, as indicated. Samples were preincubated with native CRP (25 \(\mu\)g/mL) for 5 minutes. mCRP (25 \(\mu\)g/mL) was added on initiation of shear. Adhesion kinetics was monitored for neutrophil-platelet conjugates (A) and neutrophil aggregation (B). Values are mean±SEM for 5 experiments using blood from different donors. *\(P<0.05\), **\(P<0.01\) vs shear (no antibodies). Comparisons were made separately for each group (ie, shear only or mCRP-treated samples).

**Figure 6.** Involvement of CD32 in CRP signaling and CD16 in mCRP signaling. Diluted blood was incubated for 10 minutes with function-blocking anti-CD16 or anti-CD32 mAb, as indicated. Samples were preincubated with native CRP (25 \(\mu\)g/mL) for 5 minutes. mCRP (25 \(\mu\)g/mL) was added on initiation of shear. Adhesion kinetics was monitored for neutrophil-platelet conjugates (A), neutrophil aggregation (B), mean platelet CD61-FITC fluorescence intensity of single neutrophils with attached platelets (C), and platelet P-selectin (CD62P) expression after 14 minutes of shear (D). Values are mean±SEM for 4 to 5 experiments using blood from different donors. *\(P<0.05\), **\(P<0.01\).
presence of mCRP, antibody blocking also revealed the involvement of P-selectin and Mac-1 in adhesive interactions. Anti-L-selectin mAb produced little or no further inhibition of native CRP or mCRP responses, consistent with CRP- and mCRP-induced L-selectin shedding from neutrophils.\textsuperscript{16,26} Unlike native CRP, mCRP can directly upregulate neutrophil CD11b/CD18 expression.\textsuperscript{26} This action might have contributed to the enhanced formation of neutrophil aggregates, because CD18 blockade resulted in significantly higher decreases in the number of neutrophil aggregates in blood subjected to shear in the presence than the absence of mCRP. It should be noted that adhesion molecules that mediate neutrophil adhesion to immobilized platelets under higher shear levels may differ from those observed in the present study.

Our results point toward the involvement of different Fcγ receptors in native CRP and mCRP signaling. Thus, the actions of native CRP appear to be mediated predominantly through the low-affinity IgG FcγRIIa (CD32), whereas the actions of mCRP are mediated predominantly via the low-affinity immune complex binding IgG receptor FcγRIIIB (CD16). These observations are consistent with previous studies identifying CD32 as the primary binding site for human CRP and mCRP responses, consistent with CRP- and mCRP-induced L-selectin shedding from neutrophils.\textsuperscript{16,26} Anti-L-selectin mAb produced little or no further inhibition of mCRP-induced L-selectin shedding from neutrophils.\textsuperscript{16,26} This action might have contributed to the enhanced formation of neutrophil aggregates, because CD18 blockade resulted in significantly higher decreases in the number of neutrophil aggregates in blood subjected to shear in the presence than the absence of mCRP. It should be noted that adhesion molecules that mediate neutrophil adhesion to immobilized platelets under higher shear levels may differ from those observed in the present study.

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Disclosure

Dr. Potempa is an inventor on more than 50 patents that involve modified forms of C-reactive protein. He was employed by NextEra Therapeutics, Inc., of Barrington, Ill, and is currently employed by Immtech International, Inc, of Vernon Hills, Ill. NextEra Therapeutics and Immtech International are biotechnology companies that (in aggregate) are the primary owners of intellectual property developed on the modified C-reactive protein from Immtech (until 1999) and from NextEra (from 1998 to 2002). He no longer has a relationship with NextEra Therapeutics.
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