Conformational Rearrangement in C-Reactive Protein Is Required for Proinflammatory Actions on Human Endothelial Cells

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Background—C-reactive protein (CRP) has been suggested to actively amplify the inflammatory response underlying coronary heart diseases by directly activating endothelial cells. In this study, we investigated whether loss of the cyclic pentameric structure of CRP, resulting in formation of modified or monomeric CRP (mCRP), is a prerequisite for endothelial cell activation.

Methods and Results—We examined the impact of native CRP and mCRP on the production of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), key regulators of leukocyte recruitment, and on the expression of intercellular adhesion molecule-1 (ICAM-1), E-selectin, and vascular adhesion molecule-1 (VCAM-1) in human cultured coronary artery endothelial cells (HCAECs). Incubation with mCRP for 4 hours increased MCP-1 and IL-8 secretion and mRNA levels and expression of ICAM-1, E-selectin, and VCAM-1 protein and mRNA. Significant induction occurred at 1 to 5 μg/mL, reached a maximum at 30 μg/mL, and did not require the presence of serum. Native CRP was without detectable effects at 4 hours, whereas it enhanced cytokine release after a 24-hour incubation. An anti-FcRIII (CD16) but not an anti-FcRII (CD32) antibody produced a 14% to 32% reduction of the mCRP effects (P<0.05). mCRP but not CRP evoked phosphorylation of p38 mitogen-activated protein kinase, and inhibition of this kinase with SB 203580 reversed the effects of mCRP. Furthermore, culture of HCAECs in the presence of SB203580 markedly decreased mCRP-stimulated E-selectin and ICAM-1–dependent adhesion of neutrophils to HCAECs (P<0.001).

Conclusions—Loss of pentameric symmetry in CRP, resulting in formation of mCRP, promotes a proinflammatory HCAEC phenotype through a p38 MAPK–dependent mechanism. (Circulation. 2004;109:2016-2022.)

Key Words: proteins ▪ cell adhesion molecules ▪ signal transduction ▪ endothelium ▪ inflammation

A acute coronary artery diseases are associated with evidence of inflammation both systemically and in the arterial wall.1,2 Elevated plasma levels of C-reactive protein (CRP) are predictive of subsequent acute coronary events among apparently healthy subjects and patients with stable or unstable angina.3-5 However, the exact role and mechanisms of action of CRP as a modulator of inflammation have not been well defined, because both proinflammatory and antiinflammatory actions have been reported.6-12 Recent results suggest that CRP may directly contribute to endothelial dysfunction by inducing cytokine release and surface expression of adhesion molecules.13-15 Intriguingly, these actions were evident only after 12 to 24 hours of incubation, whereas maximum increases in adhesion molecule expression can be detected within 4 to 6 hours in response to proinflammatory cytokines or bacterial lipopolysaccharide (LPS). These observations raise the possibility that CRP may undergo structural changes to activate endothelial cells. Indeed, it has been proposed that distinct isoforms of CRP are formed during inflammation. Conformationally altered forms of CRP express several epitopes that are not present on native CRP16 and display properties distinct from those of native CRP.17-19

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Native, pentameric CRP dissociates into free subunits within a few hours after binding to plasma membrane.20 These subunits expressing several neoepitopes are referred to as modified or monomeric CRP (mCRP), mCRP antigens were detected in the wall of human normal blood vessels21 and in inflamed tissues.22

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In the present study, we investigated whether conformational rearrangement of native CRP, resulting in formation of mCRP, may be required for induction of release of interleukin-8 (IL-8) and monocyte chemotactrant protein-1 (MCP-1), key regulators of leukocyte recruitment, and expression of adhesion molecules in human coronary artery endothelial cells (HCAECs). To gain insight into the underlyling mechanisms, we also examined whether the mCRP actions on HCAECs are mediated through binding to one of the IgG receptor subtypes similarly to that reported for leukocytes and via activation of p38 mitogen-activated protein kinase (MAPK).

**Methods**

**CRP Isoforms**

High-purity (>99%) human native CRP (Calbiocrom) was stored in a NaN₃-free buffer containing CaCl₂ to prevent spontaneous formation of mCRP from the native pentamer. A recombinant form of mCRP (r₉-CRP) that cannot rearrange into a pentameric structure was engineered as described previously. Native CRP was distinguished from mCRP by binding and antigenicity differences using monoclonal antibodies described for each form of the molecule and by their secondary structure. The endotoxin levels of all CRP isoforms in the assays.

**HCAEC Stimulation**

HCAECs (passage 3, from Clonetics) were cultured in EGM-MV medium (Clonitics) supplemented with 10% FBS. Monolayers of HCAECs (passages 4 through 6) in 24-well or 96-well microplates (confluence >97%, ~28 000 cells/cm²) were incubated with native CRP or mCRP. In some experiments, HCAECs were pretreated with the MAPK kinase inhibitor PD98059 (50 μmol/L), the p38 MAPK inhibitor SB203580 (0.1 to 1 μmol/L), the phosphatidylinositol 3-kinase inhibitor wortmannin (100 nmol/L), the anti-FcyRIII (CD16) antibody 3G8, anti-FcyRIIa (CD32) antibody FL18.26, or the irrelevant antibody MOPC-21 (each at 2.5 μg/mL, Pharmingen) for 30 minutes before addition of mCRP. At the indicated times, culture supernatants were collected, and the cells were processed as described below.

**MCP-1 and IL-8 Production**

The concentrations of MCP-1 and IL-8 in culture supernatants were determined in duplicate by selective ELISAs (BD Pharmingen). Intra-assay and interassay coefficients of variation were typically <4% and <6%, respectively. There was no cross-reactivity with CRP isoforms in the assays.

**Cell Adhesion Molecule Expression**

After incubation for 4 hours, HCAECs were detached with EDTA (0.01% in PBS) from the 24-well microplates and then stained for intercellular adhesion molecule-1 (ICAM-1), E-selectin, or vascular adhesion molecule-1 (VCAM-1) using fluorescent dye–conjugated anti–ICAM-1, anti–VCAM-1 (Pharmingen) or anti–E-selectin (Serotec) antibodies as described previously. Nonspecific binding was evaluated by use of appropriately labeled mouse IgG1. Immunofluorescence (10 000 cells for each sample) was analyzed with a FACScan flow cytometer with CellQuestPro software.

**RNase Protection Assay**

For multiprobe RNase protection assays, HCAECs were lysed with 50 μL of lysis/denaturation solution (Ambion). [³²P]-labeled antisense RNA probes were generated using templates for IL-8, MCP-1, ICAM-1, E-selectin, VCAM-1, L32, and GAPDH (RiboQuant, BD Pharmingen), and the assays were performed with the Direct Protect kit (Ambion) as described previously.

**Neutrophil–Endothelial Cell Adhesion Assay**

Briefly, monolayers of HCAECs in 96-well microplates were cultured with CRP, mCRP, or LPS (1 μg/mL, a positive control) for 4 hours at 37°C and washed extensively, and 2×10⁶ human ⁵¹Cr-labeled neutrophils in 100 μL were then added. In some experiments, mCRP was added back together with neutrophils to mCRP-treated HCAECs. Some experiments were repeated using function-blocking monoclonal antibodies against E-selectin [ENA-2, 10 μg/mL, purified (ab), Monosan], L-selectin (DREG-56, 20 μg/mL), CD18 (L130, 10 μg/mL), or the irrelevant antibody MOPC-21 (20 μg/mL, all from BD Biosciences). HCAECs were incubated with neutrophils for 30 minutes at 37°C on an orbital shaker at 90 rpm. Loosely adherent or unattached cells were removed by washing, and the endothelial monolayer and the adherent neutrophils were lysed. The number of adherent neutrophils in each experiment was calculated from the radioactivity of a control sample.

**Western Blot for p38 MAPK**

Protein extracts were prepared by lysing 5×10⁶ HCAECs in 100 μL of lysis buffer. Western blot analysis of phosphorylated and total p38 MAPK was performed using the PhosphoPlus p38 MAPK antibody kit (New England Biolabs).

**Statistical Analysis**

Results are expressed 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**

mCRP Induces p38 MAPK-Dependent Expression of IL-8 and MCP-1 in HCAECs

Culture of HCAECs with mCRP for 4 hours resulted in concentration-dependent increases in IL-8 and MCP-1 release, whereas native CRP was without effect (Figure 1A). Significant induction was detected even with 1 μg/mL, which peaked at 100 μg/mL mCRP. The maximal effects of mCRP were ~50% of those of LPS 1 μg/mL (IL-8, 3.9±0.2 ng/mL; MCP-1, 11.4±0.5 ng/mL). Native CRP induced significant release of IL-8 and MCP-1 at 24-hours; however, it was a considerably less potent inducer of cytokine production than mCRP (Figure 1B). The absence of serum did not affect the responses to mCRP (Figure 1C).

Preincubation of HCAECs with SB203580 concentration-dependently decreased mCRP-induced IL-8 and MCP-1 release at 4-hours, whereas neither wortmannin nor PD98059 affected the responses to mCRP (Figure 2A). Furthermore, mCRP but not native CRP induced phosphorylation of p38 MAPK relative to unstimulated controls. Phosphorylation was rapid in onset (peak at ~30 minutes) and occurred in a concentration-dependent manner (Figure 2B).

We performed RNase protection assays on RNA extracted from HCAECs after 4 hours of incubation with mCRP. Consistent with the observations at protein levels, mCRP stimulated IL-8 and MCP-1 mRNA levels, which was suppressed by SB203580 but not by PD98059 or wortmannin (Figure 3). Native CRP did not produce detectable changes (Figure 3).
mCRP Induces Expression of Adhesion Molecules

Under our experimental conditions, 2%, 39%, and 1% of untreated HCAECs expressed E-selectin, ICAM-1, and VCAM-1, respectively (Figure 4A). Treatment with mCRP for 4 hours evoked concentration-dependent increases in the overall expression and in the percentage of HCAECs expressing these adhesion molecules, whereas native CRP was without effect (Figure 4A). As a positive control, LPS produced on average 10-, 10-, and 5-fold increases in E-selectin, ICAM-1, and VCAM-1 expression, respectively (Figure 4A). SB203580 markedly attenuated mCRP-stimulated expression of these adhesion molecules, whereas wortmannin was without effect (Figure 4B). Likewise, mCRP-induced increases in E-selectin, ICAM-1, and VCAM-1 mRNA levels that were markedly attenuated by SB203580 (Figure 3). After 24 hours of incubation, native CRP (100 μg/mL) increased ICAM-1 and VCAM-1 expression on HCAECs from 22.9±1.6 to 52.9±2.0 relative fluorescence units (RFU) and from 3.2±0.2 to 7.6±0.5 RFU, respectively (n=4, P<0.05), although it was less potent than mCRP (ICAM-1, 107.9±8.9 RFU; VCAM-1, 10.7±0.5 RFU; n=4, P<0.05 versus native CRP). Cell viability assessed by propidium iodide staining was >92% in all experiments.

mCRP Promotes Neutrophil Adhesion to HCAECs

The biological significance of adhesion molecule expression was confirmed by the significant increase of adhesion of neutrophils to HCAECs cultured with mCRP (Figure 5A). Enhanced neutrophil attachment was evident with mCRP at 1 μg/mL and reached an apparent maximum at 100 μg/mL. By contrast, native CRP even at 100 μg/mL failed to promote adherence (Figure 5A). The number of adherent neutrophils to mCRP-activated HCAECs was further enhanced when the adhesion assay was performed in the presence of mCRP (Figure 5B), indicating that mCRP activates both neutrophils and HCAECs.

Because multiple receptors are involved in neutrophil adhesion to HCAECs under nonstatic conditions and mCRP affects adhesion molecule expression on both neutrophils and HCAECs.
and endothelial cells (the present study), we assessed the contribution of L-selectin, \( \gamma \)-\( \delta \)-integrins, and E-selectin to the binding interaction by using function-blocking monoclonal antibodies. mCRP-stimulated neutrophil attachment to mCRP-activated HCAECs was blocked by antibodies against CD18 (57\%\( \pm \)4\%, \( n=6 \)), E-selectin (38\%\( \pm \)3\%), and L-selectin (14\%\( \pm \)2\%) (Figure 5C). The combination of these antibodies inhibited neutrophil adhesion by 92\%\( \pm \)3\% (Figure 5C). The number of adherent neutrophils was reduced from 2.8\( \pm \)0.2\( \times \)10\(^4 \) cells/well to 1.4\( \pm \)0.1\( \times \)10\(^4 \) cells/well when HCAECs were cultured with mCRP (30 \( \mu \)g/mL) in the absence and presence of SB203580 (\( n=6 \), \( P<0.01 \)). Neither PD98059 nor wortmannin significantly affected the neutrophil-HCAEC attachment (data not shown).

Search for mCRP Receptors on HCAECs

Because CRP binds predominantly to the low-affinity IgG Fc\( \gamma \)RIIa (CD32)\(^{23,24} \) and mCRP utilizes the low-affinity immune-complex Fc\( \gamma \)RIII (CD16)\(^{18} \) on leukocytes, we used function-blocking antibodies as competitors to assess the possible involvement of these receptors in mediating the actions of mCRP on HCAECs. Preincubation of HCAECs with the anti-CD16 antibody resulted in 14\% to 32\% attenuation of the responses to mCRP (Figure 6). Neither the anti-CD32 antibody (Figure 6) nor the irrelevant MOPC-21 antibody (data not shown) affected the responses to mCRP.

Discussion

The present results provide evidence for a novel molecular mechanism by which CRP may activate endothelial cells. This bioactivity of CRP is expressed when the pentameric structure dissociates and undergoes a conformational rearrangement, resulting in formation of mCRP.

Formation of mCRP from native CRP involves the dissociation of the CRP pentameric disk. This is accompanied by a loss of predominantly \( \beta \)-sheet secondary structure with an increase in \( \alpha \)-helix\(^{18} \) and exposure of intersubunit contact residues, in particular residues 198 to 206, the predominant neoepitope expressed on mCRP,\(^{16} \) and expression of distinct biological activities.\(^{17-19} \) For instance, native CRP inhibits whereas mCRP promotes adhesion of neutrophils to LPS-activated HCAECs.\(^{11,19} \) Furthermore, recent results suggest that aggregated (ie, structurally modified) CRP rather than native CRP may promote uptake of low-density lipoproteins by macrophages.\(^{26,27} \) To avoid the confounding effects of spon-

Figure 3. Effects of mCRP and native CRP on IL-8, MCP-1, E-selectin, ICAM-1, VCAM-1, L32, and GAPDH mRNA expression. HCAECs were cultured with CRP (100 \( \mu \)g/mL) or mCRP in presence of PD98059 (PD), SB203580 (SB), or wortmannin (Wo) for 4 hours. C indicates control (medium only). Effects of LPS (1 \( \mu \)g/mL) are shown for comparison. Shown is a representative multiprobe RNase protection assay of 4 independent experiments.

Figure 4. Induction of adhesion molecule expression by mCRP. A, Monolayers of HCAECs were cultured for 4 hours with mCRP, native CRP, or LPS (1 \( \mu \)g/mL). Adhesion molecule expression was assessed by flow cytometry and is expressed as relative fluorescence intensity (RFU) after subtracting nonspecific immunostaining. Positive cells represent percentage of HCAECs that stained positive for indicated adhesion molecule. B, HCAECs were preincubated with PD98059, SB203580, or wortmannin for 20 minutes and then challenged with mCRP (30 \( \mu \)g/mL) for 4 hours. Values are mean\( \pm \)SEM for 4 to 6 independent experiments. \( \ast P<0.05, \ast \ast P<0.01 \) vs control (cells cultured in medium alone). \( \#P<0.05, \#\#P<0.01 \) vs mCRP alone.
taneous formation of mCRP from native CRP during prolonged storage in the absence of calcium (our unpublished observations), we used CRP preparations devoid of mCRP contamination and engineered mCRP that cannot reassemble to form a pentamer.

Our study shows that mCRP, unlike native CRP, can induce cytokine release and expression of adhesion molecules on HCAECs after a 4-hour incubation period. We also examined the mechanisms of mCRP signaling in HCAECs, observing a predominant role for the p38 MAPK pathway.

At low μg/mL concentrations, mCRP induced transcription of IL-8, MCP-1, E-selectin, ICAM-1, and VCAM-1 genes within 4 hours of its addition to HCAECs. These effects were comparable in magnitude to those observed with LPS, a well-known activator of endothelial cells. Consistent with previous studies, native CRP did not evoke detectable changes at 4 hours. The CRP induction became detectable only after 6 to 12 hours of incubation, reaching maximal effects at 24 hours, coinciding with in vitro kinetics of dissociation into subunits. Although CRP clearly enhanced IL-8 and MCP-1 production at 24 hours of culture, it was a significantly less potent inducer of cytokine production than mCRP. These observations suggest that conformational rearrangement of CRP is a prerequisite for activation of HCAECs and that the amounts of mCRP generated from CRP within 4 hours are not sufficient to evoke detectable responses. Another important difference between the actions of CRP and mCRP is that the mCRP effects do not depend on, whereas the CRP effects are dependent on, an as yet unidentified serum cofactor(s). The mCRP action is based on a tissue rather than a serum environment, thus minimizing the need for serum cofactors.

The present study did not address the functional significance of mCRP-induced expression of IL-8 and MCP-1. MCP-1 and IL-8 play important roles in recruitment of monocytes into the vessel wall, and IL-8 is a key regulator of neutrophil trafficking and activation. Thus, by enhancing chemokine production, mCRP may contribute to the evolution of atherogenesis and to the widespread neutrophil activation observed in patients with unstable angina. The biological significance of adhesion molecule expression was confirmed by the significant increase of neutrophil adhesion to mCRP-activated HCAECs. These observations extend previous findings that mCRP promoted neutrophil adhesion to LPS-activated HCAECs through upregulation of CD11b/CD18 on neutrophils. Our results show that mCRP-induced expression of ICAM-1 and E-selectin also contributes to neutrophil-HCAEC attachment. Significantly higher numbers of neutrophils adhered to mCRP-activated HCAECs when the adhesion assay was performed in the presence than in the absence of mCRP, indicating that mCRP can promote...
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adhesion by activating both HCAECs and neutrophils. Leukocyte–endothelial cell interaction involves a complex interplay among adhesion molecules.31 Indeed, the anti-CD18, anti-E-selectin, and anti-L-selectin antibody alone inhibited 57%, 34%, and 14% of neutrophil attachment, respectively, whereas combination of the 3 antibodies blocked ~90% of adhesion. We also detected enhanced VCAM-1 expression after 4 hours of culture of HCAECs with mCRP, indicating that mCRP closely mimics the effect of 24 hours of incubation with CRP,13,15 although the possible role of VCAM-1 in neutrophil adhesion was not investigated.

Our results indicate that mCRP activation of HCAECs involves p38 MAPK. mCRP stimulated rapid phosphorylation of this kinase, and the specific p38 MAPK inhibitor SB203580 markedly inhibited HCAEC responses to mCRP, although the inhibition was incomplete. These results are consistent with those observed with SB203580 on thrombin-induced endothelial chemokine production and ICAM-1 expression,32,33 and suggest involvement of other intracellular signaling mechanism(s). Unlike in neutrophils,18,19 mCRP does not appear to activate the MAPK kinase and phosphatidylinositol 3-kinase pathways in HCAECs, because there was no reduction in the presence of PD98059 and wortmannin.

Little is known at present about the CRP or mCRP receptor(s) on endothelial cells. Human aortic endothelial cells may express the receptors FcγRII and FcγRII,34 which bind CRP on leukocytes.23–24 In HCAECs, an anti-FcγRII antibody failed to affect the responses to mCRP, whereas an anti-FcγRIII antibody that effectively blocked the apoptosis delaying action of mCRP in neutrophils19 produced a slight attenuation of mCRP-induced HCAEC activation. Although these observations would suggest the involvement of FcγRIII, additional studies are needed to confirm the presence of this receptor on HCAECs and to identify the major binding site(s) for mCRP on HCAECs.

Limitations of this study are that the mechanisms regulating mCRP formation in vivo are still unidentified and that mCRP levels are difficult to estimate in vivo, because unlike CRP, mCRP is expressed on cell membranes rather than in the plasma.21,22 Because the ratio of membrane-bound mCRP to mCRP in the culture medium is unknown, it is extremely complicated to measure the amount of native CRP that dissociated into free subunits in vitro. If indeed mCRP is a tissue-associated mediator, at the sites of injury it may come in contact with the endothelium and leukocytes, amplifying the proinflammatory response triggered by the initial endothelial injury.

In summary, the present results indicate that loss of pentameric symmetry in CRP, resulting in formation of mCRP, is prerequisite for the appearance of proinflammatory actions on HCAECs. Indeed, mCRP directly facilitates endothelial cell adhesion molecule expression, leukocyte adhesion, and MCP-1 and IL-8 production. Importantly, these effects are, in part, mediated by activation of the p38 MAPK pathway. These findings indicate that mCRP rather than native CRP may contribute to the development of vascular inflammation and suggest that inhibition of p38 MAPK may be a target for antiinflammatory strategies in vascular diseases.

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