Loss of Pentameric Symmetry in C-Reactive Protein Induces Interleukin-8 Secretion Through Peroxynitrite Signaling in Human Neutrophils

Tarek Khreiss,* Levente József,* Lawrence A. Potempa, János G. Filep

Abstract—Plasma levels of C-reactive protein (CRP), nitrotyrosine, and interleukin-8 (IL-8) are known predictors of acute cardiovascular events. Peroxynitrite (ONOO⁻) may function as an intracellular signal for the production of IL-8; however, it is not known whether CRP regulates these events. Emerging evidence suggests that some bioactivities of CRP are expressed only when the pentameric structure of CRP is lost, resulting in formation of monomeric or modified CRP (mCRP). We studied the impact of human native CRP and bioengineered mCRP that cannot rearrange into the pentameric structure on ONOO⁻ formation and ONOO⁻-mediated IL-8 gene expression in human leukocytes. Incubation of human whole blood or isolated neutrophils with mCRP (0.1 to 100 μg/mL) for 4 hours increased IL-8 gene expression and secretion that was blocked ≈70% by the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). In neutrophils, mCRP simultaneously increased superoxide production and endothelial nitric oxide synthase-mediated NO formation, leading to enhanced ONOO⁻ formation, and consequently activation of nuclear factor-κB and activator protein-1. Native CRP had no detectable effect at 4 hours, whereas it enhanced IL-8 release after a 24-hour incubation that was blocked by L-NAME. An anti-CD16 antibody, but not an anti-CD32 antibody, produced 60% to 70% reductions in mCRP-stimulated NO formation and IL-8 release (both P<0.05). These results suggest that loss of the pentameric symmetry in CRP, resulting in formation of mCRP, leads to IL-8 release from human neutrophils via peroxynitrite-mediated activation of nuclear factor-κB and activator protein-1. (Circ Res. 2005;97:690-697.)

Key Words: C-reactive protein ■ leukocytes ■ interleukins ■ signal transduction ■ inflammation

Epidemiological and clinical studies have shown strong and consistent relationships between circulating markers of inflammation and risk prediction of future coronary artery disease (CAD). Among these markers, elevated plasma levels of C-reactive protein (CRP) are predictive for subsequent acute coronary events among apparently healthy men and women and patients with stable or unstable angina.1,2 The C-X-C chemokine interleukin-8 (IL-8) is a sensitive marker of unstable CAD.3 Increases in IL-8 levels may coincide with increased CRP levels,4 though IL-8 does not directly stimulate CRP gene expression.5,6 Increased plasma levels of nitrotyrosine, a “molecular fingerprint” for nitric oxide-derived oxidants,7 are also associated with the presence of CAD.8 Statin therapy produced reductions in nitrotyrosine concentrations similar to those in total cholesterol and CRP.8 It is uncertain, however, whether changes in these inflammatory markers occur independently or whether they are interrelated.

Widespread neutrophil activation has also been detected in patients with acute CAD, though the existence of a correlation between neutrophil activation and plasma CRP remains controversial.9,10 Neutrophil infiltration into plaques was found to be actively associated with acute coronary events.11 Recent results suggest that peroxynitrite (ONOO⁻), formed in a reaction of superoxide with NO,7 functions as an intracellular messenger to mediate IL-8 gene expression in lipopolysaccharide (LPS) or cytokine-stimulated human leukocytes through activation of the transcription factors nuclear factor (NF)-κB and activator protein-1 (AP-1).12–15 CRP induces cytokine release from endothelial cells.16–18 Recent results suggest that conformational rearrangement in native CRP, leading to the formation of monomeric or modified CRP (mCRP), is required for activation of endothelial cells.19 CRP and mCRP bind to the distinct receptors FcγRIIIa (CD32) and FcγRIII (CD16), respectively, on human neutrophils20–22 and exert opposing actions. For instance, native CRP inhibits
neutrophil activation, adherence, and trafficking.\textsuperscript{5,23–25} whereas mCRP promotes neutrophil adhesion to endothelial cells\textsuperscript{19} and suppresses neutrophil apoptosis.\textsuperscript{26}

In the present study, we investigated the impact of native pentameric CRP and mCRP on IL-8 production in human whole blood and isolated neutrophils. To gain insight into the underlying molecular mechanisms in neutrophils, we characterized the immunoglobulin (Ig) G receptor subtype (ie, the CRP receptor) involved and examined whether induction of IL-8 gene and protein expression is mediated through stimulation of ONOO\(^{-}\)-dependent activation of NF-\(\kappa\)B and AP-1.

### Materials and Methods

**CRP Isoforms**

High purity (>99\%) human native CRP (Calbiochem) was stored in Na\(_2\)F-free 20 mM/L Tris, 150 mM/L NaCl buffer (pH 7.5) containing 2 mM/L CaCl\(_2\) to prevent spontaneous formation of mCRP from the native pentamer. A recombinant form of mCRP, (rCRP, purity >97\%) that cannot rearrange into a pentameric structure was engineered, characterized, and compared with mCRP prepared from native CRP by urea elution as described.\textsuperscript{26} Native CRP was distinguished from mCRP by binding and antigenicity differences\textsuperscript{27} and by their secondary structure.\textsuperscript{26} The endotoxin of all protein solutions was below the detection limit (0.125 EU/mL).

**Cell Stimulation**

Venous blood (anticoagulated with sodium heparin, 50 U/mL) was obtained from 24 healthy volunteers who had denied taking any medication for at least 2 weeks. The Clinical Research Committee approved the experimental protocols. Neutrophil granulocytes (purity >95\%, viability >97\%) were isolated as described.\textsuperscript{23} Whole blood aliquots or isolated neutrophils (5 \(\times\) 10\(^6\) cells/mL) in microcentrifuge tubes were placed on a rotator and challenged with native CRP or mCRP with or without the NO synthase inhibitor N\(_o\)-nitro-L-arginine methyl ester (L-NAME) (1 mM/L) at 37\(\degree\)C in 5\% CO\(_2\) atmosphere. In additional experiments, the responses to CRP and mCRP were studied in the presence of the specific NF-\(\kappa\)B inhibitor pyrrolidine dithiocarbamate (PDTC, 100 \(\mu\)mol/L), function-blocking antibodies against CD32 mAb FLI-8.26 (isotype: IgG1), or the irrelevant class-matched anti-CD16 monoclonal antibody (mAb) 3G8 (isotype: IgG1), anti-CD14, anti-CD15, and anti-CD16 mAbs (Coulter). The endotoxin of all protein solutions was below the detection limit (0.125 endotoxin units/mL, corresponding to \(\approx\)0.01 ng/mL LPS) of the Limulus assay (Sigma).

**Measurements of IL-8 mRNA Expression and Secretion**

Levels of IL-8 in plasma or culture medium were determined by a selective enzyme immunoassay (OptEIA, BD Biosciences). Intracellular and interassay coefficients of variation were typically <4\% and <6\%, respectively. IL-8 and GAPDH mRNA expression was assayed in RNase protection assays with the Direct Protect kit (Ambion) as described previously.\textsuperscript{12}

**Nitric Oxide and Superoxide Formation**

Intracellular formation of NO was monitored by flow cytometry after incorporation of diaminofluorescein diacetate (5 \(\mu\)mol/L, Clontech) into neutrophils.\textsuperscript{19} Superoxide production was determined as superoxide dismutase-inhibitable reduction of ferricytochrome c.\textsuperscript{15}

**Expression of NO Synthase Isoforms**

Total RNA was isolated from 1\(\times\)10\(^7\) neutrophils using Trizol reagent (Invitrogen). cDNA was prepared with superscript reverse transcriptase (Invitrogen). The following primers were used for subsequent PCR analysis: human inducible NOS (iNOS), sense 5\'-TCTTCTGGCCACCTTTGTGAG-3\' and antisense 5\'-GTTGTCATCAGCTGTGAC-3\'; human endothelial NOS (eNOS), sense 5\'-GTGATGGCGAAGCGAGTGAAG-3\' and antisense 5\'-GGAGCCCGAACACACAGA3C-3'; human neuronal NOS, sense 5\'-CTTCTGGCAACACGCCTTATTTG-3\' and antisense 5\'-TGGAGACTGATCACTGAGCGCTTG-3'; \(\beta\)-actin, sense 5\'-ATGGCAATCCTGGCTTGAC-3' and antisense 5\'-AGCATTTGCGGTGCACGATGG-3'. The corresponding 412-base pair, 421-base pair, 458-base pair, and 500-base pair fragments were amplified enzymatically by 40, 35, 40, and 25 repeated cycles, respectively, and subjected to electrophoresis on 1.2\% agarose containing ethidium bromide.

**Detection of Peroxynitrite**

NO-dependent fluorescence of rhodamine, an oxidation product of dihydrorhodamine 123 (DHR 123),\textsuperscript{29} and nitrotyrosine formation were measured as markers of ONOO\(^{-}\) formation. DHR 123 (20 \(\mu\)mol/L, Molecular Probes) was added to some samples during the last 60 minutes of incubation in the presence or absence of L-NAME (1 mM/L), and fluorescence was analyzed by a flow cytometer (FACScan, Becton Dickinson).\textsuperscript{12} The NO synthase blocker-inhibitable proportion of DHR oxidation can be attributed to ONOO\(^{-}\) because ONOO\(^{-}\) readily oxidizes DHR 123, whereas NO does not.\textsuperscript{29} DHR 123 oxidation by mCRP was also determined in neutrophils pretreated with the calmodulin inhibitor W7 (20 \(\mu\)mol/L), the broad PKC inhibitor GF109203X (200 \(\mu\)mol/L), Clontech) as described.\textsuperscript{26}

**Calcium Mobilization Assay**

Intracellular Ca\(^{2+}\) concentration was monitored in Fura-2/AM (1 \(\mu\)mol/L)-loaded neutrophils in a Perkin-Elmer spectrophotometer (excitation: 340 nm, emission: 510 nm) as previously described.\textsuperscript{10}

**Western Blot Analysis**

Protein extracts were prepared by lysing 2\(\times\)10\(^7\) neutrophils in 100 \(\mu\)L of lysis buffer, and immunoblot analysis of phosphorylated and total Akt was performed using the Phospho Plus Akt antibody kit (New England Biolabs) as described.\textsuperscript{26}

**Neutrophil Viability**

Neutrophil viability was assessed by flow cytometry immediately after staining with propidium iodide (0.5 \(\mu\)g/mL).

**Statistical Analysis**

Results are expressed as mean \(\pm\) SEM. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test) followed by Dunn’s multiple contrast hypothesis tests to identify differences
between various treatments. Repeated measures were analyzed by the Friedman test followed by the Wilcoxon-Wilcon test. Values of \( P < 0.05 \) were considered significant.

**Results**

**Effects of CRP and mCRP on IL-8 Production and IL-8 mRNA Expression**

Incubation of human whole blood with mCRP for 4 hours resulted in concentration-dependent IL-8 release, whereas native CRP was without effect (Figure 1). Significant induction was detected with 5 \( \mu \)g/mL, which peaked at 100 \( \mu \)g/mL mCRP (Figure 1B). Native CRP started to increase IL-8 release only after 8 hours of incubation; however, it was a considerably less potent inducer of IL-8 production than mCRP at any time points studied (Figure 1A). Both mCRP- and CRP-induced IL-8 release were markedly, though never completely, inhibited by L-NAME (Figure 1A). Likewise, mCRP, but not CRP, induced concentration-dependent IL-8 release from isolated neutrophils after 4 hours of incubation (Figure 1C). On a molar basis, bioengineered mCRP and mCRP prepared from native CRP evoked similar IL-8 release (Figure 1D). Recombinant mCRP-induced (50 \( \mu \)g/mL) IL-8 release (5.6±0.7 ng/mL) was unaffected by the formyl-peptide receptor antagonist N-t-Boc-Phe-Leu-Phe-Leu-Phe (50 \( \mu \)mol/L) (5.4±0.5 ng/mL, n=6, \( P > 0.1 \)). Therefore, because of the enhanced solubility, bioengineered mCRP was used in subsequent experiments. Heat inactivation of native CRP (60 minutes at 100°C) resulted in a complete loss of its activity (data not shown). Also, LPS (Escherichia coli serotype O111:B4) at 0.02 ng/mL, a concentration 2-fold higher than the maximum level of LPS contamination in our protein solutions, did not induce detectable IL-8 release (0.24±0.03 ng/mL versus 0.23±0.03 ng/mL in unstimulated neutrophils, n=4, \( P > 0.1 \)).

We performed RNase protection assays on RNA extracted from leukocytes after 4 hours of incubation with mCRP. Consistent with the observations at the protein level, mCRP evoked concentration-dependent increases in IL-8 mRNA expression, which were suppressed by L-NAME (Figure 2). Native CRP at 100 \( \mu \)g/mL did not produce detectable changes (Figure 2).

**mCRP Induces NO, Superoxide, and Peroxynitrite Formation in Neutrophils**

Incubation of neutrophils for 4 hours with mCRP led to simultaneous increases in \( \text{O}_2^- \) and NO production. Significant increases were detected with 5 \( \mu \)g/mL and peaked at 100 \( \mu \)g/mL mCRP (Figure 3). The increases in \( \text{O}_2^- \) and NO...
mCRP: 29/H11006/H11006/H11001 4; mCRP: 50/H11006 4; L-NAME 3; control: 22 (0.5 mmol/L) to L-NAME-treated neutrophils restored DHR 123 cytochrome c in the culture medium. ONOO− measured as superoxide dismutase-inhibitable reduction of ferricytochrome c in the culture medium. ONOO− formation was assessed as L-NAME-inhibitable oxidation of DHR 123 to rhodamine and is expressed as RFU. Values are mean±SEM (n=4 to 7). *P<0.05; **P<0.01; ***P<0.001 vs unstimulated (control).

Figure 3. mCRP induces formation of superoxide, NO, and ONOO−. Neutrophils (5×106 cells/mL) were challenged with mCRP at 37°C for 4 hours. Intracellular formation of NO was monitored using diaminoflorescein, and is expressed as relative fluorescence units (RFU). Diaminoflorescein fluorescence was reduced 98% by L-NAME (1 mmol/L). Superoxide formation was measured as superoxide dismutase-inhibitable reduction of ferricytochrome c in the culture medium. ONOO− formation was assessed as L-NAME-inhibitable oxidation of DHR 123 to rhodamine and is expressed as RFU. Values are mean±SEM (n=4 to 7). *P<0.05; **P<0.01; ***P<0.001 vs unstimulated (control).

production coincided with increases in NO-dependent DHR123 oxidation, indicating enhanced ONOO− formation (Figure 3). Addition of the NO donor spermine NONOate (0.5 mmol/L) to L-NAME-treated neutrophils restored DHR 123 oxidation (rhodamine fluorescence in relative fluorescence units, control: 22±4; mCRP: 50±4; L-NAME+mCRP: 29±3; spermine NONOate+L-NAME+mCRP: 48±4; n=4; P>0.1 compared with mCRP). To confirm ONOO− formation, we measured cellular nitrotyrosine content. Neutrophils exposed to mCRP for 4 hours contained significantly higher amounts of nitrotyrosine than unchallenged cells, and the increases in nitrotyrosine correlated with those in DHR 123 oxidation (Figure 3).

To identify the NOS isof orm(s) responsible for enhanced NO production, we performed reverse transcription polymerase chain reaction on cDNA prepared from neutrophils. These assays resulted in the amplification of eNOS mRNA, but not neuronal NOS and iNOS, in freshly isolated cells and in response to mCRP at 2 and 4 hours after addition of the protein (Figure 4). mCRP did not affect eNOS mRNA expression (Figure 4).

To characterize the proximal signaling events associated with mCRP-induced ONOO− formation, we monitored calcium mobilization. mCRP evoked a rapid increase in intracellular Ca2+ similar to that observed with fMLP (Figure 5A), and transiently enhanced (peak at around 2 minutes) phos-
whether the mCRP actions described above were also mediated via CD16. The anti-CD16 mAb partially prevented mCRP-induced NO formation assessed at 4 hours, and IL-8 release assessed at 4 and 24 hours after addition of mCRP, whereas the anti-CD32 or the irrelevant MOPC-21 antibody had no detectable effects (Figure 8). Increasing the concentration of the anti-CD16 mAb did not result in further inhibition (data not shown). At 24-hours culture, native CRP-induced IL-8 release was also attenuated by the anti-CD16 mAb, but not by the anti-CD32 mAb or MOPC-21 (Figure 8B).

Discussion

The present results provide evidence for a novel mechanism by which CRP may affect the inflammatory process by stimulating ONOO⁻ formation and signaling in human neutrophils. These actions are expressed when the pentameric structure undergoes a conformational rearrangement, however, leading to formation of mCRP. Our results also suggest a potential link between CRP, IL-8, nitrotyrosine, and neutrophil activation, as it may occur in the blood of patients with acute CAD.
Our study using human whole blood and isolated neutrophils demonstrates that mCRP stimulates a rapid (within 4 hours) and potent synthesis of IL-8. This is a primary response to mCRP that requires de novo protein synthesis and transcription of the IL-8 gene. Comparison of plasma and culture medium IL-8 levels suggests that ~70% of IL-8 release was of neutrophil origin in blood. Native CRP did not evoke detectable changes at 4 hours. This was unexpected, for native CRP induced rapid (within minutes) shedding of L-selectin from the surface of neutrophils, and cannot be explained by the slight differences in the viability of mCRP or CRP-treated neutrophils. Human blood contains all the yet unidentified serum cofactors that were required for CRP activation of endothelial cells. The CRP stimulation became detectable only after 8 hours of incubation, coinciding with in vitro kinetics of dissociation into subunits. Although CRP clearly enhanced IL-8 production at 8 to 24 hours of incubation, it was a considerably less potent inducer of IL-8 release than mCRP. These observations suggest that conformational rearrangement of CRP is required to induce IL-8 production in neutrophils, and that the amounts of mCRP generated from CRP within 4 hours are not sufficient to evoke detectable increases in IL-8. The time course of neutrophil activation by native CRP appears to be similar to that of CRP activation of endothelial cells. Neither LPS (at a concentration 2-fold higher than might be present in our protein preparations) nor heat-inactivated CRP evoked detectable IL-8 release, indicating that CRP and mCRP signaling was responsible for the observed effects.

Blockade of NO synthesis with L-NAME inhibited to a similar degree mCRP-induced IL-8 release and nuclear accumulation of NF-κB and AP-1 in neutrophils, coinciding with suppression of IL-8 mRNA expression. Interestingly, L-NAME also attenuated native CRP-induced IL-8 release, and the degree of inhibition was comparable to that detected with mCRP. These results pointed to the involvement of NO in mediating these responses. Previous studies have shown that ONOO− rather than NO by itself mediates IL-8 release from human neutrophils in response to LPS or cytokines. Accordingly, we found that mCRP simultaneously enhanced superoxide and NO formation, coinciding with increases in NO-dependent oxidation of DHR123 and nitration of protein tyrosine residues. A significant portion of rhodamine fluorescence in mCRP-stimulated neutrophils can be attributed to ONOO−, because it depends on NO-related species (it can be inhibited by L-NAME), whereas NO per se does not oxidize DHR 123. Further, the NO donor spermine NONOate restored DHR 123 oxidation in L-NAME–treated neutrophils, indicating that L-NAME does not inhibit NADPH oxidase activation. Although nitrotyrosine is often considered as a distinct “molecular fingerprint” of ONOO−
formations, peroxidase-dependent tyrosine nitration has also been described. Interestingly, in human neutrophils, ONOO− appears to be the predominant mechanism for tyrosine nitration.

Reverse-transcriptase polymerase chain reaction amplified eNOS, but not iNOS and neuronal NOS-specific products, in unchallenged neutrophils and in neutrophils treated with mCRP for up to 4 hours. These results point toward increased eNOS activity as the source for enhanced NO production in response to mCRP. Previous studies on constitutive NOS expression in human neutrophils yielded contradictory results, as both the absence and presence of eNOS and neuronal NOS have been reported. Contaminating cells or differences in the NOS assays used might account for this apparent discrepancy. Unstimulated human neutrophils do not express iNOS, whereas iNOS-positive neutrophils have been detected in tissue exudates and after more than 16 hours incubation with cytokines. Whether mCRP could induce iNOS expression after prolonged incubation periods remains to be investigated. There is compelling evidence that eNOS-derived NO contributes to ONOO− formation in amounts sufficient to activate signaling mechanisms and even to induce cell damage.

Ca2+ mobilization and activation of calmodulin and PI3-kinase appears to be required for mCRP-induced ONOO− formation. Ca2+ transients control calmodulin-mediated eNOS activation and the activity of PLCγ1 and PLCγ2, which through formation of diacylglycerol and activation of PI3-kinase lead to activation of NADPH oxidase. The inhibitory actions of W7, wortmannin, and DPI on DHR 123 oxidation were not additive with those of L-NAME, suggesting that these compounds inhibited the same reaction, ie, the ONOO−-dependent oxidation through suppression of either NO or O2− formation. PKC appears to play a minor role in mCRP signaling, for GF109203X inhibited only a small portion of DHR 123 oxidation by mCRP.

The IL-8 gene contains cis-regulatory elements for NF-κB, AP-1, and NF-IL-6. Of these transcription factors, NF-κB plays a key role in the induced expression of IL-8. Accordingly, we found that mCRP stimulates nuclear accumulation of NF-κB and AP-1, and inhibition of NF-κB activation with PDTC decreased IL-8 production by 66%. The mechanism of action of PDTC has not been fully defined, but likely involves inhibition of formation of oxidants that would result in activation of Iκα-κB kinase and/or enhancing phosphorylation of IκB. Thus, NF-κB activation could be attributed to decomposition products of ONOO− rather than the parent molecule itself.

Our results indicate that mCRP-induced IL-8 gene expression and release are predominantly mediated through the low affinity immune complex binding IgG receptor CD16, for the function-blocking anti-CD16 mAb 3G8, but not anti-CD32 mAb, markedly, though never completely, inhibited NO formation and IL-8 production in neutrophils. Furthermore, only the anti-CD16 mAb attenuated native CRP-induced IL-8 release at 24 hours incubation. These observations imply that during 24-hours culture, conformational rearrangement might have occurred in native CRP, yielding mCRP, because native CRP does not bind to CD16. We cannot exclude the possibility that mCRP may bind different sites from IgG on CD16, for blockade of the epitope defined by the mAb 3G8 reduced ≈80% of mCRP binding at 0°C. Alternatively, mCRP might interact with other as yet unidentified cell surface molecules. These may include direct binding to positively charged residues on proteins and direct interaction with the lipid membrane (Potempa et al, unpublished observations, 2005). The slight inhibition of mCRP activation of endothelial cells by anti-CD16 mAb lends support to the existence of mCRP receptor(s) other than CD16.

The mechanisms that induce conformational rearrangement in native CRP in vivo are still unknown. Native, pentameric CRP dissociates into free subunits after binding to plasma membranes or in denaturing or oxidative environment, yielding mCRP. The percentage of CRP that might have dissociated into subunits during a 24-hour incubation period, however, remains to be determined. Unlike CRP, mCRP appears to be predominantly membrane-bound, therefore making its detection rather difficult. Because the commercial CRP antibody (clone 8) mainly recognizes mCRP, tissue immunostaining attributed to native CRP in previous studies might have been due to mCRP. Neutrophils may come into contact with mCRP expressed in the arterial wall at sites of endothelial injury or perhaps on other leukocytes, thereby aggravating the inflammatory response and contributing to plaque destabilization. Stimulated neutrophils release ONOO−, though the ratio of intracellularly retained and released ONOO− cannot be deduced. Increased ONOO− release is consistent with enhanced nitration of plasma proteins and may contribute to tissue damage. Finally, our results do not exclude contribution of other cells to elevated plasma levels of nitrotyrosine and IL-8. Indeed, mononuclear leukocytes and endothelial cells also release IL-8 in response to native CRP or mCRP, thereby increasing plasma cytokine levels in patients with CAD.

In summary, the present results indicate that loss of pentameric symmetry in CRP, resulting in formation of mCRP, stimulates IL-8 production by human neutrophils through ONOO−-mediated activation of NF-κB and AP-1. These observations point toward neutrophils as a major source of nitrosative stress and IL-8 and may provide a potential link between CRP, neutrophil activation, plasma nitrotyrosine, and IL-8, all implicated in predicting future acute CAD.

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