C-Reactive Protein Decreases Prostacyclin Release From Human Aortic Endothelial Cells

Senthil Kumar Venugopal, PhD; Sridevi Devaraj, PhD; Ishwarlal Jialal, MD, PhD

Background—In addition to being a risk marker for cardiovascular disease, much recent data suggest that C-reactive protein (CRP) promotes atherogenesis. Decreased endothelial NO and prostacyclin (PGI2) contribute to a proatherogenic and prothrombotic state. We have shown that CRP decreases endothelial NO synthase expression and bioactivity in human aortic endothelial cells (HAECs). PGI2 is a potent vasodilator and inhibitor of platelet aggregation. Hence, the aim of this study was to examine the effect of CRP on PGI2 release from HAECs and human coronary artery endothelial cells (HCAECs).

Methods and Results—HAECs and HCAECs were incubated with human CRP (0 to 50 μg/mL for 24 hours). The release of PGF-1α, a stable product of PGI2, was also assayed in the absence and presence of a potent agonist, A23187. CRP significantly decreased PGF-1α release from HAECs under basal (48% decrease, P<0.001; n=5) and stimulated (26% decrease, P<0.01; n=5) conditions. CRP had no effect on PGI2 synthase (PGIS) mass. By increasing both superoxide and inducible NO synthase, CRP resulted in increased nitration of PGIS by peroxynitrite. The increased nitration and decreased activity of PGIS by CRP was reversed with peroxynitrite scavengers.

Conclusions—Thus, CRP decreases PGI2 release from HAECs by inactivating PGIS via nitration, additionally contributing to its atherogenicity. (Circulation. 2003;108:1676-1678.)

Key Words: nitric oxide ■ atherosclerosis ■ platelets ■ endothelium ■ inflammation

C-reactive protein (CRP), a prototypic marker of inflammation, is implicated in vascular dysfunction and in the progression of atherosclerosis. Evidence that CRP induces endothelial dysfunction includes inhibition of endothelial NO synthase (eNOS), increasing plasminogen activator inhibitor-1, endothelin-1, cell adhesion molecules, monocyte chemotactic protein-1, and monocyte-endothelial adhesion.1,2 In monocytes, CRP increases tissue factor expression, monocyte adhesion, and cytokine release.1,2 Individuals with high CRP exhibit impaired endothelial vasoreactivity.3-5 Major mediators of endothelial vasodilatation include NO and prostacyclin (PGI2). Impaired release of either could promote atherogenesis. NO can be synthesized from constitutively expressed eNOS that maintains vascular tone in the arteries, whereas inducible NO synthase (iNOS) is induced under proinflammatory states. Recently, we showed that CRP decreases eNOS expression and bioactivity in human aortic endothelial cells (HAECs).6 Hence, in the present report, we tested the effect of CRP on prostacyclin (PGI2) release from HAECs.

PGI2 is a major product of arachidonic acid formed in macrovascular endothelium. It has potent vasodilatory and antiplatelet effects.7 Its protective role in the vasculature is evident from PGI2-mediated inhibition of smooth muscle cell (SMC) proliferation and modulation of both reverse cholesterol transport from vascular cells and cellular adhesion to the vessel wall in vitro.7 PGI2 is generated by prostaglandin I synthase (PGIS) from the PG endoperoxide PGH2, a rate-limiting step for PGI2 release. Targeted deletion of PGIS resulted in hypertensive mice that exhibit renal and aortic abnormalities, including arterial sclerosis.7 PGI2 receptor deficiency enhances thrombosis and atherosclerosis.7 Furthermore, overexpression of PGIS into rat carotid arteries significantly inhibits SMC proliferation and neointimal formation after balloon injury.8 PGI2 transduces its effects through a membrane-associated receptor, IP. The response to thrombotic stimuli is reduced in mice lacking the PGI2 receptor.7 These data point to the pivotal role of PGI2 as an endogenous vasodilator and antiplatelet agent. Hence, the aim of the present study was to test if CRP modulates PGI2 release in HAECs.

Methods
Endotoxin was removed from human CRP (Sigma) using detoxigel columns (Pierce Biochemicals), and endotoxin levels were found to be <0.125 EU/mL, as described previously.6 SDS-polyacrylamide gel electrophoresis of purified CRP revealed a single 24-kDa band. HAECs and HCAECs (1×105 cells/mL, Clonetics) were maintained in EGM-2 MV and used between 2 to 6 passages. Cells were washed in serum-free media and incubated in serum-free media with CRP (0 to 50 μg/mL) for 24 hours. Supernatant was immediately stored in −70°C after experiments. For F2 isoprostane measurements, butylated hydroxytoluene (40 μmol/L) was added and samples were stored in −70°C.

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PGIS activity was quantified by assaying the stable metabolite of PGI₂. Both PGF-1α (Amersham Biosciences) and thromboxane B₂ (TXB₂; Amersham Biosciences) were quantified by ELISA. The CVs of these assays were <5%. After cell lysis and quantification of protein, 20 to 30 μg was used for Western blotting, as described previously.6 Nitration of PGIS in cell extracts was determined as described previously9 with some modifications. PGIS was immunoprecipitated from cell lysate (500 μL, 1 mg/mL) using polyclonal PGIS antibodies (Cayman Chemicals). After centrifugation, the immune complex was boiled in 2× Laemmli buffer (without DTT) and loaded on 10% SDS-PAGE gels, and Western blots were performed for nitrotyrosine using monoclonal anti-nitrotyrosine antibodies (Upstate Biotechnologies) as described previously.6 Western blots for iNOS were also performed using polyclonal antibodies (Transduction Laboratories) using β-actin as loading control. Also, Oxyblot (Serologicals Corp), a measure of protein carbonyls, was performed as per manufacturer’s instructions.

All experiments were performed at least 3 times in duplicates. Data are presented as mean±SD. The dose-response effect of CRP was analyzed by ANOVA followed by paired t tests to compute differences in the variables, and the level of significance was set at P<0.05.

**Results**

Incubation of HAECs with different doses of CRP (0 to 50 μg/mL) resulted in a dose-dependent inhibition of endogenous PGF-1α release (P<0.01 at doses ≥10 μg/mL) (Figure 1). Similar results were obtained with HCAECs (P<0.05 at doses ≥25 μg/mL). For the agonist A23187, at half maximum (2 μmol/L), PGF1-α release was also decreased significantly by CRP at concentrations ≥25 μg/mL (P<0.05). Furthermore, CRP (50 μg/mL) significantly increased the ratio of TXB2/PGF-1α (control, 0.46±0.14; CRP 50 μg/mL, 0.58±0.13; P<0.05, n=5). Trypsinized or boiled CRP did not inhibit PGF1-α release, and polymixin B did not abrogate the effects of CRP.

By Western blot, no significant changes in PGIS mass were observed between control and CRP-treated cells (Figure 2B). PGIS could either be oxidized or nitrated in HAECs by CRP, leading to PGIS inactivation, because the active site of this enzyme seems to be very susceptible to nitration.9–11 Because PGIS could be nitrated by peroxynitrite (ONOO⁻), a product of superoxide (O₂⁻) anion and NO, and we have previously shown a reduction in eNOS,6 we examined iNOS expression as a source of ONOO⁻. CRP resulted in a significant increase in iNOS protein (Figure 2A). In addition, CRP resulted in an increase in O₂⁻ anion release in HAECs using SOD-inhibitable reduction of ferricytochrome C as described previously12 (control, 0.61±0.31; CRP 50 μg/mL, 3.2±0.37 nmol/mg protein per min; n=4, P<0.01). After immunoprecipitation of PGIS, CRP increased nitration of PGIS, as evidenced by Western blots with anti-nitrotyrosine antibodies (Figure 2B). Because oxidation could also lead to decreased enzyme activity, Oxyblot was performed, and CRP failed to affect PGIS oxidation (data not shown). Potent scavengers of peroxynitrite,13 urate (1 mmol/L), and ascorbate (1 mmol/L) reversed the inhibition of PGIS by CRP (Table). Both urate and ascorbate also reduced the nitration of PGIS by CRP (data not shown).

**Discussion**

CRP is now accepted as a risk marker for atherosclerosis. However, studies performed in vitro in endothelial cells, monocytes, and SMCs suggest that CRP present in the lesion...
Effect of the Peroxynitrite Scavengers Urate and Ascorbate on CRP-Induced Inhibition of PGIS

<table>
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<tr>
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<th>PGF-1α, pg/mg protein</th>
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<tbody>
<tr>
<td>Control</td>
<td>347.1±23.6</td>
</tr>
<tr>
<td>CRP (50 µg/mL)</td>
<td>186.8±41.8</td>
</tr>
<tr>
<td>CRP (50 µg/mL) + urate (1 mmol/L)</td>
<td>369.8±79.6*</td>
</tr>
<tr>
<td>CRP (50 µg/mL) + ascorbate (1 mmol/L)</td>
<td>318.6±75.4†</td>
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*P<0.002, †P<0.02 compared with CRP alone (n=4).

might contribute to atherothrombosis. Prostacyclin is a major vasorelaxant present in endothelial cells. Hence, we hypothesized that CRP could enhance endothelial dysfunction by inhibiting PGI₂ in addition to NO in HAECS.

PGI₂ is released from endothelial cells and rapidly converted to a stable metabolite, PGF-1α. CRP significantly decreased PGF-1α release in HAECS and HCAECs. This confirms our previous finding and others’ reports that CRP could induce vascular dysfunction by decreasing vasodilatory molecule release from endothelial cells. The concentrations of CRP (10 to 50 µg/mL) that we have used in these experiments are seen in obesity and cardiovascular disease. 1-3 Thromboxane A₂, another product of arachidonic acid metabolism, is a potent platelet-aggregating agent. The ratio of TXB₂/PGI₂ seems to be an important factor in the control of platelet aggregability in vivo. 14 The increase in the TXB₂/PGI₂ ratio with CRP supports a prothrombotic role for CRP. Also, CRP failed to affect PGIS mass. Previous reports showed decreased PGI₂ release attributable to increased nitrination of PGIS in atherosclerosis, possibly at a tyrosine present in the active site. O₂⁻ anion is increased during inflammation and oxidative stress. When O₂⁻ anion combines with NO, it produces ONOO⁻ that nitrates proteins. Tyrosine nitrination of PGIS is most likely mediated by ONOO⁻, which could be derived from iNOS. Our results showed that iNOS was increased by CRP in HAECS, additionally supporting a proinflammatory role for CRP. Increased O₂⁻ anion release with CRP additionally confirms that NO produced by iNOS could combine with O₂⁻ anions and produce ONOO⁻. However, other mechanisms resulting in increased nitrination cannot be excluded. In the present study, we report that CRP increases nitrination of PGIS compared with control. This is in accord with the findings of Zou et al., who demonstrated in atherosclerotic bovine coronary arteries that there was increased PGIS nitrination and defective vasorelaxation. We also tested for the PGIS oxidation but failed to see any significant differences, which was confirmed by the F₂-isoprostane levels that were also not affected by CRP. Additional studies are needed to elucidate how CRP upregulates iNOS and induces nitrination in HAECS. In the present study, we document that increase in iNOS and O₂⁻ anion release by CRP results in decreased prostacyclin release attributable to PGIS nitrination. Furthermore, potent inhibitors of peroxynitrite, urate, and ascorbate reversed the increased nitrination and decreased activity of PGIS by CRP. Thus, given that CRP inhibits eNOS and PGIS and increases PAI-1 in aortic endothelial cells and promotes tissue factor activity in monocytes, it could be classified as a procoagulant. Support for the procoagulant activity of human CRP comes from a recent report in human CRP transgenic mice, in which the authors showed increased CRP levels and a prothrombotic phenotype after arterial injury. In conclusion, the present report lends additional support for a role of CRP in atherothrombosis.

Acknowledgments

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References

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