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 (eNOS) 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
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. Nitration of PGIS in cell extracts was determined as described previously 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 X 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. 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. 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, 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 previously (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, 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...
CRP-Induced Inhibition of PGIS

Effect of the Peroxynitrite Scavengers Urate and Ascorbate on CRP-Induced Inhibition of PGIS

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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 PG12 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 finding9 and others' reports1,2 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.3–5 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 show decreased PG1 release attributable to increased nitration of PGIS in atherosclerosis,9–11 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 which nitrates proteins. Tyrosine nitration of PGIS is most likely mediated by ONOO which could be derived from iNOS.9–11 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 nitration cannot be excluded.15 In the present study, we report that CRP increases nitration of PGIS compared with control. This is in accord with the findings of Zou et al,9 who demonstrated in atherosclerotic bovine coronary arteries that there was increased PGIS nitration 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 nitration 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 nitration. Furthermore, potent inhibitors of peroxynitrite, urate, and ascorbate reversed the increased nitration and decreased activity of PGIS by CRP. Thus, given that CRP inhibits eNOS and PGIS and increases PAI-1 in aortic endothelial cells16 and promotes tissue factor activity in monocytes,17 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.18 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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