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 NO synthase expression and increases superoxide dismutase, does not appear to induce endothelial NO synthase expression and bioactivity in human aortic endothelial cells (HAECs). Hence, in the present report, we tested the effect of CRP on prostacyclin (PGI₂) release from HCAECs.

PGI₂ is a major product of arachidonic acid formed in macrovascular endothelium. It has potent vasodilatory and antiplatelet effects. 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. PGI₂ is generated by prostaglandin I synthase (PGIS) from the PG endoperoxide PGI₂, a rate-limiting step for PGI₂ release. Targeted deletion of PGIS resulted in hypertensive mice that exhibit renal and aortic abnormalities, including arterial sclerosis. PGI₂ receptor deficiency enhances thrombosis and atherosclerosis. Furthermore, overexpression of PGIS into rat carotid arteries significantly inhibits SMC proliferation and neointimal formation after balloon injury. PGI₂ transduces its effects through a membrane-associated receptor, IP. The response to thrombotic stimuli is reduced in mice lacking the PGI₂ receptor. 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. SDS-polyacrylamide gel electrophoresis of purified CRP revealed a single 24-kDa band. HAECs and HCAECs (1×10⁶ 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 for experiments. For F₂ isoprostane measurements, butylated hydroxytoluene (40 μmol/L) was added and samples were stored in −70°C.
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...
might contribute to atherothrombosis. Prostacyclin is a major vasorelaxant present in endothelial cells. Hence, we hypothesized that CRP could enhance endothelial dysfunction by inhibiting PGF\textsubscript{1\alpha} in addition to NO in HAECs.

PGF\textsubscript{1\alpha} is released from endothelial cells and rapidly converted to a stable metabolite, PGF-1\alpha. CRP significantly decreased PGF-1\alpha release in HAECS and HCAECs. This confirms our previous finding\textsuperscript{a} and others’ reports\textsuperscript{1,2} that CRP could induce vascular dysfunction by decreasing vasodilatory molecule release from endothelial cells. The concentrations of CRP (10 to 50 \textmu g/mL) that we have used in these experiments are seen in obesity and cardiovascular disease.\textsuperscript{3-5} Thromboxane A\textsubscript{2}, another product of arachidonic acid metabolism, is a potent platelet-aggregating agent. The ratio of TXB\textsubscript{2}/PGI\textsubscript{2} seems to be an important factor in the control of platelet aggregability in vivo.\textsuperscript{14} The increase in the TXB\textsubscript{2}/PGI\textsubscript{2} ratio with CRP supports a prothrombotic role for CRP. Also, CRP failed to affect PGIS mass. Previous reports show decreased PGF\textsubscript{1\alpha} release attributable to increased nitration of PGIS in atherosclerosis,\textsuperscript{9-11} possibly at a tyrosine present in the active site. O\textsubscript{2}\textsuperscript{-} anion is increased during inflammation and oxidative stress. When O\textsubscript{2}\textsuperscript{-} anion combines with NO, it produces ONOO\textsuperscript{-} that nitrates proteins. Tyrosine nitration of PGIS is most likely mediated by ONOO\textsuperscript{-}, which could be derived from iNOS.\textsuperscript{9-11} Our results showed that iNOS was increased by CRP in HAECs, additionally supporting a proinflammatory role for CRP. Increased O\textsubscript{2}\textsuperscript{-} anion release with CRP additionally confirms that NO produced by iNOS could combine with O\textsubscript{2}\textsuperscript{-} anions and produce ONOO\textsuperscript{-}. However, other mechanisms resulting in increased nitration cannot be excluded.\textsuperscript{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.,\textsuperscript{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 F\textsubscript{2}-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 O\textsubscript{2}\textsuperscript{-} anion release by CRP results in decreased prostacyclin release attributable to PGIS nitration. Furthermore, potent inhibitors of peroxynitrite,\textsuperscript{13} 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 cells\textsuperscript{16} and promotes tissue factor activity in monocytes,\textsuperscript{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.\textsuperscript{18} In conclusion, the present report lends additional support for a role of CRP in atherothrombosis.

Acknowledgments
This study was supported by grants from the National Institutes of Health (K24 AT 00596) and the Juvenile Diabetes Foundation.

References
C-Reactive Protein Decreases Prostacyclin Release From Human Aortic Endothelial Cells
Senthil Kumar Venugopal, Sridevi Devaraj and Ishwarlal Jialal

Circulation. 2003;108:1676-1678; originally published online September 22, 2003;
doi: 10.1161/01.CIR.0000094736.10595.A1
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/108/14/1676

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in
Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/