Demonstration That C-Reactive Protein Decreases eNOS Expression and Bioactivity in Human Aortic Endothelial Cells

Senthil Kumar Venugopal, PhD; Sridevi Devaraj, PhD; Ivan Yuhanna, MS; Philip Shaul, MD; Ishwarlal Jialal, MD, PhD

**Background**—C-reactive protein (CRP), the prototypic marker of inflammation, has been shown to be an independent predictor of cardiovascular events. Endothelial nitric oxide synthase (eNOS) deficiency is a pivotal event in atherogenesis.

**Methods and Results**—We tested the effect of CRP on eNOS expression and bioactivity in cultured human aortic endothelial cells (HAECs). CRP decreased eNOS mRNA, protein abundance, and enzyme activity in HAECs. Furthermore, eNOS bioactivity assayed by cyclic GMP levels was significantly reduced by CRP. Preincubation of cells with CRP also significantly increased the adhesion of monocytes to HAECs.

**Conclusion**—CRP causes a direct reduction in eNOS expression and bioactivity in HAECs, further supporting its role in atherogenesis. (Circulation. 2002;106:1439-1441.)

**Key Words:** inflammation ■ C-reactive protein ■ nitric oxide synthase ■ endothelium

Inflammation seems to play a critical role in all stages of atherosclerosis, from the nascent lesion to acute coronary syndromes. CRP is a prototypic marker of inflammation and has been shown in numerous prospective studies to predict both cardiovascular events (CVE) in apparently healthy persons and a poor prognosis after acute coronary syndromes. CRP clearly is a risk marker, and much data are evolving to suggest that CRP also promotes atherogenesis. In this regard, it has been shown that CRP promotes tissue factor expression in monocytes and also induces adhesion molecule and chemokine expression in human endothelial cells (ECs). Recently, CRP was also shown to increase endothelin-1 (ET-1) release from saphenous vein ECs.

A critical enzyme present in ECs is endothelial nitric oxide synthase (eNOS). Nitric oxide derived from eNOS promotes arterial vasodilatation and inhibits smooth muscle cell proliferation, LDL oxidation, platelet adhesion and aggregation, and monocyte adhesion to endothelium. It is believed that endothelial dysfunction (decreased eNOS bioactivity) occurs very early in atherogenesis. Thus, we tested the effect of CRP on eNOS expression, enzymatic activity, and bioactivity in human aortic endothelial cells (HAECs) to ascertain if CRP impaired eNOS activity.

**Methods**

For all the experiments, HAECs (Clonetics, San Diego, Calif) were used within 5 passages. THP-1 cells, a monocytic cell line (ATCC), were used for adhesion experiments. Purity of recombinant human CRP (Calbiochem) was checked by SDS-PAGE, yielding a single band. Endotoxin was removed from CRP with Detoxigel column (Pierce Biochemicals) and found to be <0.125 endotoxin units (EU)/mL by Limulus assay (Biowhittaker).

HAECs (1x10^6 cells/mL) were used for all assays and incubated with concentrations of CRP ranging from 0 to 50 μg/mL. Cell viability as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was >95% with this dose range of CRP. Apoptosis was measured by staining cells with fluorescin isothiocyanate-labeled Annexin V (R&D Systems), followed by flow cytometry.

Cells were lysed and 30 μg of protein per well were loaded and transferred to membranes. Membranes were blocked with 5% milk and then incubated with either anti-human eNOS antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) or anti-human β-actin antibody (Sigma-Aldrich, St Louis, Mo) as a control. After washing and incubating with specific horseradish peroxidase-conjugated secondary antibodies, the membranes were developed with enhanced chemiluminescence (Amersham-Pharmacia). RNA was isolated using TRIzol (Invitrogen), and 5 μg of RNA was used first strand cDNA synthesis (Invitrogen). cDNA (100 ng) was amplified using primers (Integrated DNA Technologies) specific for eNOS (forward: 5′-GACATTT AGAGCAAAGGGCTGC-3′ and reverse: 5′-CGCGTGTGCACCTCTGG-3′) and GAPDH (forward: 5′-CCATGGCAAATTTCCATGGCA-3′ and reverse: 5′-TCTAGACGCGAGTGTCAGTCCACC-3′). eNOS was amplified for 30 cycles and GAPDH for 20 cycles. eNOS mRNA stability experiments were conducted using actinomycin D (10 μg/mL) as described previously. eNOS enzymatic activity was assessed by assaying 3H-L-arginine conversion to 3H-L-citrulline in HAEC lysates as reported previously. Bioactivity was assayed by measuring total...
cyclic GMP (cGMP) levels using the Biotrak kit (Amersham-Pharmacia). The precision of citrulline and cGMP assays was good (coefficient of variation ≤ 5%). Adhesion of THP-1 cells to EC was performed as described previously.17 All experiments were performed at least 3 times. Data are presented as mean ±SD. Paired t-tests were used to compute differences in the variables, and the level of significance was set at P<0.05.

Results

Incubation of HAECs with CRP at different time points (6, 12, and 24 hours) resulted in a decrease in eNOS protein levels at 24 hours (Figure 1A). CRP administration (5 to 50 μg/mL) resulted in a dose-dependent inhibition of eNOS mRNA levels as determined by eNOS reverse transcriptase-polymerase chain reaction using GAPDH as an internal control (Figure 1B). eNOS mRNA was maximally decreased at 12 hours. Furthermore, CRP significantly decreased eNOS mRNA stability; eNOS t1/2 without CRP 20.3±1.2 hours and with CRP (50 μg/mL) 16.6±1.0 hours, P<0.002 (n=3 experiments). Because the reduction in protein expression was at a maximum at 24 hours, further experiments were performed after a 24-hour incubation of HAECs with CRP. Boiling of CRP (100°C for 1 hour) abolished its inhibitory effect on eNOS protein. Furthermore, co-incubation of CRP with polymyxin B (25 μg/mL) did not abrogate its inhibitory effect on eNOS. No apoptosis of the cells was seen with CRP (50 μg/mL), as assessed by Annexin V staining.

Enzymatic activity of eNOS was significantly decreased at concentrations of CRP ≥10 μg/mL, as shown by decreased levels of citrulline (Figure 2). Also, eNOS bioactivity assayed as total cGMP release was decreased at CRP levels ≥5 μg/mL (Figure 2).

Furthermore, the decrease in eNOS expression and activity with CRP was associated with a significant increase in adhesion of THP-1 cells to HAECs (Table). A significant increase was observed only at concentrations of CRP ≥25 μg/mL. Flow cytometric analyses of adhesion molecules revealed a significant increase in vascular cell adhesion molecule (VCAM)-1 at a CRP concentration of 25 μg/mL (49% increase, P<0.05) and intercellular adhesion molecule (ICAM)-1 at a CRP concentration of 50 μg/mL (41% increase, P<0.05). There was no significant increase in E-selectin levels.

Discussion

CRP is a known risk marker for cardiovascular disease, and evidence continues to accumulate to support a role for CRP in atherogenesis.2-10 CRP has been seen in atherosclerotic lesions co-localizing with complement. Also, CRP promotes tissue factor expression in monocytes and induces the expression of the adhesion molecules ICAM-1, VCAM-1, and

### Table: Effect of CRP on Monocyte Adhesion to HAEC

<table>
<thead>
<tr>
<th>CRP Concentration (μg/mL)</th>
<th>Adhesion (mfi/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>66.5±7.4</td>
</tr>
<tr>
<td>CRP, 5 μg/mL</td>
<td>66.3±8.8</td>
</tr>
<tr>
<td>CRP, 10 μg/mL</td>
<td>69±8.2</td>
</tr>
<tr>
<td>CRP, 25 μg/mL</td>
<td>80.3±11.6*</td>
</tr>
<tr>
<td>CRP, 50 μg/mL</td>
<td>91.5±14.8**</td>
</tr>
</tbody>
</table>

HAECs (1×10^6 cells/mL) were incubated with different concentrations of CRP for 24 hours and then adhesion of fluorescent-labeled THP-1 cells was determined as described in Methods. Data are presented as mean±SD of 4 experiments in duplicate. mfi indicates mean fluorescence intensity. *P<0.05 compared to control. **P<0.005 compared to control.
E-selectin in human umbilical vein and coronary artery ECs.\textsuperscript{7–9} In addition, CRP has been shown to induce expression of the chemokine, monocyte chemotactic protein-1 in cultured human umbilical vein ECs\textsuperscript{10} and ET-1 from human saphenous vein ECs.\textsuperscript{11} Also, data have suggested that patients with elevated CRP levels have impaired endothelial vasoreactivity.\textsuperscript{18,19} Thus, in the present study, we tested the hypothesis that CRP could account for some of these proinflammatory effects by impairing eNOS bioactivity.

Using a variety of techniques, we convincingly show that CRP causes a decrease in eNOS expression and bioactivity, especially at the higher concentration. We show that in addition to a decrease in protein levels and mRNA (via decreasing stability of eNOS mRNA), there is a decrease in enzymatic activity. With regards to bioactivity, we show that CRP results in a decrease in cGMP and also an increase in one of the early biological events in atherosclerosis, the adhesion of human monocytes to aortic endothelium. Whereas the effect of CRP on eNOS activity was evident at 10 µg/mL, the increased adhesion of monocytes was seen at 25 µg/mL. A possible reason for this is that the inhibition of eNOS activity at 10 µg/mL was not sufficient for induction of increased adhesion. Because some of the effects that we are reporting could be induced by lipopolysaccharide (LPS), we were careful to minimize the effect of LPS in our experimental protocol. In addition to purifying our CRP through a Detoxigel column, we performed experiments in the presence of polymyxin-B. If LPS were a major factor in decreasing eNOS activity, boiling would have had no effect on inhibition of eNOS expression by the CRP preparation, whereas polymyxin-B would have prevented the reduction in eNOS abundance.

Our findings support the observation of Fichtlscherer et al.,\textsuperscript{18} who show that, in patients with angiographically documented coronary artery disease with CRP levels up to 45 µg/mL, endothelial vasoreactivity was inversely correlated with CRP ($r = -0.46$, $P=0.001$). Also, in a study of 9 healthy subjects with a body mass index of 27 ± 3.2 kg/m² and CRP levels up to 41 µg/mL, Cleland et al.\textsuperscript{19} showed that there is a relationship between CRP levels and the percentage decrease of forearm blood-flow during infusion of N-monomethyl-L-arginine, which is a substrate inhibitor of eNOS. Although both studies suggest a relationship between low-grade inflammation and impaired endothelial vasoreactivity, which implies decreased eNOS bioactivity, these studies are correlative and do not reveal causality. One cannot rule out the role of pro-inflammatory cytokines, which can attenuate eNOS bioactivity, in addition to CRP.\textsuperscript{20} In the present study, we clearly show for the first time that CRP results in a decrease of eNOS expression and bioactivity in a regulated in-vitro system using HAECs. Also, as shown previously,\textsuperscript{9} we demonstrate an increase in the expression of VCAM-1 and ICAM-1.

In conclusion, the present study presents further evidence of the critical importance of CRP in atherogenesis. Along with the capacity of CRP to induce tissue factor expression in monocytes, adhesion molecules, and MCP-1 and ET-1 in ECs, it also results in a major effect on a critical protector of the artery wall, ie, eNOS. Further studies should be directed at elucidating the molecular mechanisms via which CRP orchestrates this inhibitory effect (induction of interleukin-6, ET-1, etc.).

Acknowledgments

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References

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