C-Reactive Protein Decreases Tissue Plasminogen Activator Activity in Human Aortic Endothelial Cells
Evidence that C-Reactive Protein Is a Procoagulant

Uma Singh, Sridevi Devaraj, Ishwarlal Jialal

Objective—C-reactive protein (CRP) can promote atherothrombosis by decreasing endothelial nitric oxide synthase and prostacyclin, and by stimulating both plasminogen activator inhibitor-1 in endothelial cells and tissue factor in mononuclear cells. Plasminogen activator-1, a marker of fibrinolysis, is the primary inhibitor of tissue plasminogen activator (tPA). Thus, we tested the effect of CRP on tPA in human aortic endothelial cells.

Methods and Results—Incubation of human aortic endothelial cells with CRP (≥12.5 μg/mL) significantly decreased tPA antigen and activity. Adenyl cyclase inhibitors, an endothelin receptor antagonist, superoxide dismutase, and a nitric oxide donor failed to reverse the effect of CRP on tPA. CRP increased interleukin (IL)-1β and tumor necrosis factor (TNF)-α. Neutralization of both IL-1β and TNFα reversed the inhibition of tPA by CRP. Furthermore, in volunteers that have high CRP levels, euglobulin clot lysis time was significantly increased compared with those that have low CRP levels, providing further evidence that high CRP levels are associated with a procoagulant state.

Conclusions—CRP inhibits tPA activity via generation of proinflammatory cytokines (IL-1β and TNFα). This study provides additional novel data that CRP is a procoagulant and has implications for atherothrombosis. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: inflammation ■ endothelial cells ■ C-reactive protein ■ tissue plasminogen activator ■ procoagulant

Inflammation plays a critical role in all stages of atherosclerosis.1 C-reactive protein (CRP), a prototypic marker of inflammation, has been shown to predict cardiovascular events.2 In addition, evolving data suggest that CRP could actively participate in atherothrombosis.3–7 Evidence that CRP could promote atherothrombosis includes inhibition of endothelial nitric oxide synthase (eNOS),3,4 and prostacyclin (PGI2),5 increase of plasminogen activator inhibitor (PAI)-1,6 decrease of eNOS expression and activity,6 and stimulation of nitric oxide synthase (NOS) activity.7 Evidence that CRP could promote atherothrombosis is from human CRP transgenic (Tg) mice, in which increased CRP levels resulted in arterial thrombosis after femoral injury.8

Thrombus formation after plaque rupture is the generally accepted cause of acute coronary syndromes. Activation of the endogenous fibrinolytic system is a function of the endothelial cells of the vessel wall.9 The key enzyme in the onset of fibrinolysis is tissue plasminogen activator (tPA), which converts plasminogen to plasmin resulting in degradation of fibrin.10 Therefore, the speed and extent of local delivery of tPA during thrombus formation is important in enhancing thrombolysis.11,12 Recently, it was shown that postmyocardial infarction, an endothelial fibrinolytic response to venous occlusion, was impaired in patients with increased CRP levels.12 We previously reported that CRP induces the expression and activity of PAI-1, a marker of impaired fibrinolysis and atherothrombosis, whose action is mediated by inhibition of tPA.6 Because there is no data examining the direct effect of CRP on tPA in HAECs, we tested the effect of CRP on tPA expression and activity in HAECs.

Methods

For all the experiments, HAECs (Clonetics) were maintained in EGM-2MV and used within 3 to 5 passages. CRP (from human plasma, Sigma Chem), supplied in 20 mmol/L Tris pH 7.5, 140 mmol/L NaCl, 2 mmol/L CaCl2, and 0.1% NaN3, was purified of endotoxin by passing through a detoxigel column followed by dialysis to mimic the preparation of CRP. The cells were treated with the same volume of the resultant azide solution as used for CRP. Also, we examined the effect of LPS on tPA in HAECs at and above the concentration of LPS present in the CRP preparation to rule out a LPS-mediated effect. 2',5'-dideoxyadenosine, NOC-18 (nitric oxide donor), SQ22536 were purchased from Calbiochem. Tumor necrosis

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factor (TNF)-α-neutralizing antibody and interleukin (IL)-1 receptor antagonist were obtained from R&D systems.

**Cells Treatment**

Seventy percent to 80% confluent endothelial cells (ECs) grown in 12-well plates were washed with serum-free medium (EBM-2) and incubated for different time periods: 3, 6, 12, and 24 hours with CRP (0 to 50 μg/mL) at 37°C. Cells were treated with various inhibitors (1 hour at 37°C) before CRP exposure. Cell viability was >95% in all experiments.

**Measurement of tPA Antigen and Activity**

The secreted tPA antigen levels in the cell supernatants were measured by sandwich ELISA (Diagnostica Stago). This assay measures total circulating tPA (ie, free and complexed tPA). Also, tPA activity was assessed in culture supernatants using Chromalyze tPA reagents (BioPool International) after acification of samples to neutralize PAI-1. The inter- and intra-assay CV for these assays was <10%. tPA antigen or activity is expressed as ng or IU/mg protein, respectively.

**Western Blot for tPA**

Cells were lysed with Mammalian Protein Extraction Reagent (Pierce Biotech) and 20 μg of protein per well was loaded and transferred to membranes. Membranes were blocked with 5% milk and then incubated with either anti-human tPA rabbit antibody (1:100; Santa Cruz Biotechnology) or anti-human β-actin monoclonal antibody as control. After washing and incubating with specific horseradish peroxidase-conjugated secondary antibodies, membranes were developed with enhanced chemiluminescence.

**tPA mRNA Levels**

RNA at 3-, 6-, and 12-hour time points was isolated using Trizol (Invitrogen), and 1 μg of RNA was used for first strand cDNA synthesis (Invitrogen). cDNA (100 ng) was amplified for 35 cycles using primers (Integrated DNA Technologies) specific for tPA19 and GAPDH as reported previously. Polymerase chain reaction (PCR) products were run on 1.5% Agarose gel. Further, real-time RT-PCR was performed to confirm the results of RT-PCR. Briefly, for real-time RT-PCR, RNA was made DNA-free using Ambion’s DNA-free kit. GAPDH and tPA mRNA expression was determined using standard assay reagents (Applied Biosystems).

**Specificity of CRP-Mediated Effects**

Strategies that were used to determine whether the effect on tPA was attributed to CRP and not a contaminant included: (1) CRP was trypsinized by incubating with trypsin-coated beads (TPCK-Trypsin, Pierce Biotech), followed by centrifugation to sediment the trypsin- coated beads. Proteolytic digestion of CRP was confirmed by SDS-PAGE. (2) CRP was incubated in a boiling water bath for 1 hour. (3) CRP was removed by preabsorbing CRP on plates coated with anti-CRP IgG (Alpco Diagnostics) as reported previously to serve as negative control, and the corresponding positive control was to incubate CRP in a regular 96-well tissue culture plate without anti-CRP IgG and using the supernatant. (4) Vehicle control consisted of Buffer B. (5) Cells were pretreated with polymyxin B for 30 minutes before CRP challenge to rule out any possible LPS-mediated effect. (6) Cells were also treated with varying doses of LPS (50 pg and 1000 pg/mL) alone to determine its effect on tPA activity. (7) CRP preparation as well as 0.1% azide solution, passed separately through detoxigel column, were extensively diazylized to rule out azide mediated effect. (8) Cells were treated with mAb to CD32 (2.5 μg/mL, BD Pharmingen) for 1 hour before CRP challenge.

**Plasma Fibrinolytic Capacity**

To verify whether CRP causes decreased fibrinolytic activity in vivo as suggested by others and to confirm our in vitro findings, we measured plasma fibrinolytic capacity or euglobulin clot lysis time (ECLT), representing the balance between tPA and PAI-1 activities, by turbidimetry in the euglobulin fraction of plasma samples in 21 volunteers with high (n=10) and low CRP (n=11) levels. These volunteers had CRP levels (4.6±2.7 mg/L versus 0.35±0.30 mg/L) on 3 occasions over a 12-month period as reported previously. To a 200 μL sample, 150 μL of acetic acid (0.25%) and 1.8 mL of water was added. The sample was kept in melting ice for 30 minutes followed by centrifugation at 2000 rpm for 5 minutes. The precipitate was dissolved in 1.2 mL of Owren-Koller buffer (Diagnostica Stago) and 5 μL thrombin (100 U/mL) was added. Continuous monitoring of turbidity at 340 nm was performed for 24 hours and the time for 50% clot lysis was calculated.

**Cytokine Measurements**

tPA is known to be regulated by proinflammatory cytokines (IL-1β and TNFα). To gain mechanistic insights on the effect of CRP on tPA, we measured the secretion of these cytokines in culture. The supernatants were concentrated 4× and assayed by ELISA (R&D Systems). The intra- and inter-assay CV was <10%. Cytokine levels were expressed as pg per mg protein.

**Results**

Incubation of HAECs with CRP (0 to 50 μg/mL) for a duration of 3, 6, 12, and 24 hours resulted in a significant decrease in secreted tPA antigen at 12 hours (P<0.04 at doses ≥12.5 μg/mL; Figure 1a). Hence, all further experiments were performed after a 12-hour incubation of HAEC with CRP. In addition, tPA activity was significantly decreased (P<0.03) at all doses of CRP used (Figure 1b).

The results from various strategies used to delineate a CRP-specific effect are shown in Table 1. LPS (50 and 1000 pg/mL) alone had no effect on tPA activity. Furthermore, polymyxin B did not affect CRP-mediated tPA activity inhibition. Boiling and trypsinization abrogated this effect, indicating that the tPA inhibition is CRP-specific and not attributed to some contaminant. The treatment of cells with the same volume of azide solution as our CRP preparation did not alter tPA activity compared with control. Also, tPA activity was unaltered in cells treated with CRP that had been preabsorbed to anti-CRP IgG–coated plates but not to plates without anti-CRP IgG. Furthermore, the treatment of cells with CD32 mAb reversed the inhibitory effect of CRP (Figure 1b).

Incubation of HAECs with CRP resulted in a significant (P<0.02) decrease in tPA protein (Figure 2a). However, RT-PCR for tPA mRNA expression at either 3 hours (data not shown) or 6 hours (Figure 2b) did not reveal any change in its expression with CRP treatment. These results were confirmed by real-time RT-PCR which revealed no significant change in ΔCt value for tPA mRNA of CRP-treated cells as compared with control (ΔCt for Control: 11.84±1.21 versus CRP 25 pg/mL: 12.01±1.23). These data suggest that the inhibitory effect of CRP on tPA is posttranscriptional.

Because it has previously been shown that tPA is regulated by cAMP levels, endothelin-1, reactive oxygen species, NO levels, and proinflammatory cytokines (IL-1β and TNFα), we explored these mechanisms. Pretreatment of cells with adeny cyclase inhibitors (SQ22536 or 2′,5′-dideoxyadenosine), an ET-1 receptor blocker (bosentan), an
NO donor (NOC-18), or polyethylene glycol (PEG)-SOD had no effect on CRP-mediated tPA inhibition (Table 2). The doses of the inhibitors used were based on the published literature.19–22

CRP treatment resulted in a significant (P value for trend <0.005) increase in the secretion of IL-1 and TNF from HAEC (Table 3). Pretreatment with IL-1 receptor antagonist (IL-1 RA) and neutralizing antibody to TNF-α alone resulted in a significant (P<0.05) increase in tPA activity as compared with CRP-treated cells (Figure 3). The combination of IL-1 RA and TNF-α neutralizing antibody resulted in the greatest increase compared with CRP alone (85% of control), whereas isotope control antibody had no effect (Figure 3). The combination of IL-1 RA with soluble TNF receptor (sTNF R1- p60) reversed tPA activity to 90% of control (data not shown).

Lastly, we examined the effect of CRP on the fibrinolytic activity of plasma samples from volunteers with high CRP levels versus those with low CRP levels. The fibrinolytic activity of plasma was significantly decreased in samples with high CRP compared with samples with low CRP as evidenced by the ECLT (927±565 minutes versus 394±195 minutes, respectively; P<0.001).

**Table 1. Experiments Undertaken to Determine Whether the Inhibition of tPA by CRP Is Attributed to a Contaminant**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>tPA Activity (IU/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (No treatment)</td>
<td>5.67±0.98</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>5.02±1.89</td>
</tr>
<tr>
<td>CRP, 25 µg/ml</td>
<td>2.35±0.79*</td>
</tr>
<tr>
<td>Trypsinized CRP</td>
<td>4.65±0.93</td>
</tr>
<tr>
<td>Boiled CRP</td>
<td>4.31±0.79</td>
</tr>
<tr>
<td>LPS, 50 µg/ml</td>
<td>6.13±0.89</td>
</tr>
<tr>
<td>LPS, 1000 µg/ml</td>
<td>5.01±0.69</td>
</tr>
<tr>
<td>Polymyxin B+ CRP, 25 µg/ml</td>
<td>2.19±0.89*</td>
</tr>
<tr>
<td>Azide solution passed through column and dialyzed</td>
<td>2.19±0.89*</td>
</tr>
<tr>
<td>CRP preabsorbed to anti-IgG coated plate</td>
<td>4.99±1.02</td>
</tr>
<tr>
<td>CRP incubated with regular 96-well plate</td>
<td>3.06±1.28*</td>
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</tbody>
</table>

The values are mean±SD from 3 different experiments run in duplicates. *P<0.04 vs control.

**Table 2. Effect of CRP and Various Inhibitors on tPA Activity**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>tPA Activity (IU/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.06±0.65</td>
</tr>
<tr>
<td>CRP, 25 µg/ml</td>
<td>1.76±1.28*</td>
</tr>
<tr>
<td>CRP+Bosentan, 10 µM</td>
<td>1.31±0.89</td>
</tr>
<tr>
<td>CRP+2',5'-dideoxyadenosine, 10 µM</td>
<td>1.50±0.59</td>
</tr>
<tr>
<td>CRP+SOD2536, 100 µM</td>
<td>1.47±0.39</td>
</tr>
<tr>
<td>CRP+PEG-SOD, 125 µl/ml</td>
<td>1.21±0.60</td>
</tr>
<tr>
<td>CRP+NOC-18, 250 µl/m</td>
<td>1.46±0.42</td>
</tr>
</tbody>
</table>

The values are mean±SD from 3 different experiments run in duplicates. *P<0.028 vs control.
CRP promotes tissue factor expression and activity in mononuclear cells and induces the release of thrombomodulin in human umbilical vein ECs. In addition to augmenting PAI-1 expression and activity, CRP decreased PGI2 production and eNOS expression in vitro. Furthermore, CRP significantly increases the ratio of thromboxane B2/PGI2. Therefore, CRP increases myocardial29 and cerebral30 infarction in rats. CRP promotes tissue factor expression and activity, and CRP decreased PGI2 production5,6 CRP decreased PGI2 production5,6 and eNOS expression6,28,29 in vitro. Furthermore, CRP significantly increases the ratio of thromboxane B2/PGI2.28,29 The importance of tPA has been demonstrated with successful therapeutic outcomes after thrombolytic therapy in humans.31 There is also data suggesting that the reduced fibrinolytic activity owing to a decreased release of tPA is associated with an increased risk of thrombosis.30 Thus, impaired release of tPA from the vascular endothelium represents an important mechanism underlying the increased incidence of thrombosis. In the present study, we clearly show that CRP not only decreases tPA antigen but also tPA activity. Further, CRP treatment reduced intracellular tPA protein levels. However, CRP did not affect tPA mRNA. Recently, we reported that CRP mediates its biological effects in HAECs via FC γ receptors.32 Preincubation of HAECs with mAb to CD32 reversed CRP-mediated tPA inhibition, indicating the specificity of CRP-mediated effect via these FC γ receptors.

CRP exerted its inhibitory effect at concentrations of 12.5 to 50 μg/mL. Recently, Ridker and Cook33 have shown that CRP levels >20 μg/mL predict future cardiovascular events. CRP mRNA levels in atherosclerotic plaques are 10-fold higher than in normal arteries, suggesting that levels in the atheroma could be much higher than in serum. We showed that secreted CRP concentration increases 100-fold in HAECs exposed to macrophage conditioned medium.35 Therefore, in atherosclerotic lesions via paracrine/autocrine loops, CRP levels could be very high in certain microdomains. Furthermore, CRP levels increased from 18 to 56 μg/mL in human Tg mice after femoral wire injury resulting in a procoagulant phenotype.8 CRP infusion in humans raised the levels of CRP to 28.1 μg/mL and induced a procoagulant phenotype.36 CRP levels up to 50 μg/mL have also been reported in patients with myocardial infarction.37 Thus, the levels of CRP used in this study can clearly be attained in patients.

The mechanistic link between CRP and atherosclerosis has been the focus of much research. Recent reports suggest that CRP-induced effects are artifacts attributable to the presence of contaminants (LPS and azide) in commercial CRP.38–40 Because we have previously shown that endotoxin-purified CRP inhibits NO release via eNOS downregulation,41 we were prompted to reexamine the effect of endotoxin-purified azide-free CRP on eNOS. We show that in HAEC, this CRP also decreased both cGMP and citrulline release. Furthermore, in this article, using different strategies, we carefully rule out possible contamination by LPS or NaN3 and clearly show that tPA activity inhibition is CRP specific. Our experiments document that mAb to CD32 significantly reversed CRP-mediated tPA inhibition further supporting our hypothesis that CRP, per se, inhibits tPA. Furthermore, evidence showing the link between CRP and atherosclerosis is not limited to cell culture because the presence of CRP mRNA and protein in human atherosclerotic lesions and vascular cells has been demonstrated.34,35

Mechanistically, the endothelial tPA:Ag release is known to be upregulated by several physiological substances;41 tPA is downregulated by IL-1β as well as TNF-α,18 cAMP levels,19 endothelin-1,20 and reactive oxygen species.21 The data in this article show that an NO donor, adenylyl cyclase inhibitors, an ET-1R blocker as well as PEG-SOD do not affect CRP-mediated inhibition of tPA, hence ruling out these pathways.

CRP treatment significantly increased IL-1β and TNF-α in the present study. Previously, CRP has been shown to increase IL-1β and TNF-α in peripheral blood mononuclear cells.42 Importantly, both IL-1 and TNF inhibit tPA.18 Whereas both IL-1 RA and neutralizing antibody to TNF partially reversed the inhibition of tPA by CRP, the combi-
nation of both resulted in tPA activity that was similar to control. Similar reversal of tPA activity was obtained with the combination of IL-1 RA and soluble TNFRI. We have previously shown that endotoxin-purified CRP increases IL-8 through NF-kB, and this mechanism may be relevant to CRP-induced increase in IL-1 and TNF. Future studies will be directed to elucidate these mechanisms because it is beyond the scope of this report.

In patients admitted to the intensive care unit, CRP correlates with ECLT, an index of plasma fibrinolytic activity. Importantly, we report significantly increased ECLT and hence decreased fibrinolytic capacity in plasma from volunteers with high CRP levels. Overall, we clearly show that CRP decreases intracellular tPA and secreted tPA antigen and activity without any alteration of tPA mRNA, suggesting that CRP-mediated tPA inhibition is a posttranscriptional event. However, whether it is a translational or posttranslational event will be investigated in future studies.

Based on our findings, we postulate that CRP is a procoagulant. By inhibiting tPA activity and stimulating PAI-1 in aortic EC, CRP attenuates the fibrinolytic capacity. In the present study, we go further in making a novel observation that high CRP levels are associated with a procoagulant phenotype and impaired fibrinolysis. In this context, it is important to note that in the Physician Health Study, the greatest reduction in myocardial infarction with aspirin therapy (325 mg on alternate days) was obtained in patients with the highest CRP levels, suggesting that aspirin ameliorated the procoagulant phenotype associated with high CRP because aspirin at this dose does not decrease CRP but is a potent antiplatelet agent.

To conclude, the present study makes the novel observation that CRP inhibits tPA expression and activity via stimulation of IL-1β and TNF-α. This is additional evidence supporting the previous reports (induction of PAI-1, TF, and inhibition of PGI2 and eNOS) that CRP should be classified as a procoagulant.

Acknowledgments
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