A Self-Fulfilling Prophecy
C-Reactive Protein Attenuates Nitric Oxide Production and Inhibits Angiogenesis

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Background—Given the central importance of nitric oxide (NO) in the development and clinical course of cardiovascular diseases, we sought to determine whether the powerful predictive value of C-reactive protein (CRP) might be explained through an effect on NO production.

Methods and Results—Endothelial cells (ECs) were incubated with recombinant CRP (0 to 100 μg/mL, 24 hours), and NO and cyclic guanosine monophosphate (cGMP) production was assessed. The effects of CRP on endothelial NO synthase (eNOS) protein, mRNA expression, and mRNA stability were also examined. In a separate study, the effects of CRP (25 μg/mL) on EC cell survival, apoptosis, and in vitro angiogenesis were evaluated. Incubation of ECs with CRP resulted in a significant inhibition of basal and stimulated NO release, with concomitant reductions in cGMP production. CRP caused a marked downregulation of eNOS mRNA and protein expression. Actinomycin D studies suggested that eNOS downregulation was related to decreased mRNA stability. In conjunction with a decrease in NO production, CRP inhibited both basal and vascular endothelial growth factor–stimulated angiogenesis as assessed by EC migration and capillary-like tube formation. CRP did not induce EC survival but did, however, promote apoptosis in a NO-dependent fashion.

Conclusions—CRP, at concentrations known to predict adverse vascular events, directly quenches the production of the NO, in part, through posttranscriptional effect on eNOS mRNA stability. Diminished NO bioactivity, in turn, inhibits angiogenesis, an important compensatory mechanism in chronic ischemia. Through decreasing NO synthesis, CRP may facilitate the development of diverse cardiovascular diseases. Risk reduction strategies designed to lower plasma CRP may be effective by improving NO bioavailability. (Circulation. 2002;106:913-919.)

Key Words: angiogenesis ■ nitric oxide ■ interleukins ■ protein, C-reactive ■ cells

The inflammatory marker C-reactive protein (CRP) has been shown to predict myocardial infarction, stroke, and vascular death in a variety of settings. CRP levels are one of the strongest independent predictors of future cardiovascular events in apparently healthy men and women and also portend the vulnerability of an atherosclerotic plaque to rupture. Although these remarkable observations have set the stage for routine CRP measurements to enter cardiovascular prediction algorithms, one question remains poorly defined: What is the mechanism through which CRP predicts, with such accuracy, the development of diverse vascular insults? Here we show that one of the missing links between CRP and cardiovascular disease may be decreased production and action of the multifactorial vasoactive peptide, nitric oxide (NO).

Methods

Cell Culture
Human saphenous vein and umbilical vein endothelial cells (ECs) were grown in MCDB-131 complete medium supplemented with 10% fetal bovine serum. ECs were plated into 6-well plates and grown to confluence before treatment. ECs between passages 2 and 5 were used for the studies outlined below. Human recombinant CRP (Calbiochem) was used in all studies described. Given the concern surrounding the potential contamination of CRP with endotoxin, a number of control experiments were performed. A single 23-kDa band ascertained purity. After purification of CRP via a commercial...
Detoxigel column, endotoxin levels were evaluated by Limulus assay. In other control experiments, we studied the effect of a recombinant NH2-terminal bactericidal permeability-increasing protein (rBPI), and observed no effect of this endotoxin inhibitor on the CRP-mediated upregulation of adhesion molecules (not shown).

With the use of similar methods, other investigators have reported proinflammatory actions of purified, endotoxin-free CRP.14

Assessment of NO Release and cGMP Production

ECs (n=10 wells per group) were incubated with human recombinant CRP (1, 3, 5, 25, 50, and 100 µg/mL, 24 hours) and NO production was determined spectrophotometrically by measuring its final stable equimolar degradation products, nitrite and nitrate. Total nitrite was quantified after the reduction of all nitrates with nitrate reductase (Boehringer Mannheim). After the conversion of nitrate to nitrite, total nitrite was determined spectrophotometrically at 540 µm by employing the Griess reaction. The measurement of nitrite was performed in a total of 5.0 mL of Tris-buffered saline (Tris 25 mmol/L, NaCl 138 mmol/L, MgCl2 0.49 mmol/L, CaCl2 0.68 mmol/L, glucose 3.0 mmol/L, and pH 7.4) to avoid phosphate interference with the assay procedure. Extracellular fluid was collected and concentrated by freeze-drying and reconstituted in glass-distilled water. Total nitrate concentration was calculated from a standard curve constructed over the linear range of the assay and expressed as µmol/L per milligram protein. Because we have previously demonstrated the ability of CRP to augment endothelin-1 (ET-1) and interleukin-6 (IL-6) production,15 we evaluated the effects of CRP on NO production in the presence and absence of bosentan (ET, receptor antagonist, 10 µmol/L, Actelion Pharmaceuticals Inc) and antihuman IL-6 antibody (5 µg/mL, RandD Systems) for 2 hours before being incubated with CRP as above. The concentration of the IL-6 monoclonal antibody was sufficient to neutralize >95% of cytokine activity in vitro. In a separate experiment, the effects of CRP (25 µg/mL, 24 hours) on bradykinin-stimulated NO release were evaluated. In these experiments, ECs incubated with CRP were exposed to 10–8 mol/L bradykinin or vehicle for 30 minutes. The supernatant was then harvested for NO measurement. To evaluate the effects of CRP on the bioactivity of NO, we assessed intracellular cyclic guanosine monophosphate (cGMP) concentrations in ECs incubated with and without CRP. cGMP was determined by a commercial enzyme immunoassay (Amersham). After treatment, the confluent cell culture plates were subjected to ice-cold ethanol (65%). The cells were scraped and then centrifuged at 2000 rpm for 15 minutes at 4°C. The supernatant was transferred to fresh tubes and freeze-dried. The extracts were dissolved in 100 µL of manufacturer’s assay buffer before analysis. The extracted intracellular cGMP was assayed by the enzyme-immunoassay kit and expressed as fmol/mg protein. The cross-reactivity of the assay <0.000008 for adenosine-3’, 5’-cyclic monophosphate (cAMP) and <0.000004 for GMP, where the reactivity was 100 for cGMP.

Western Blot Analysis

The effects of CRP on endothelial nitric oxide synthase (eNOS) protein expression were determined by Western blotting with the use of an anti-eNOS monoclonal antibody. Briefly, EC lysates were fractionated through a 4% stacking and 10% running SDS-PAGE gel of an anti-eNOS monoclonal antibody. Briefly, EC lysates were fractionated through a 4% stacking and 10% running SDS-PAGE gel and the fractionated proteins were transferred to PVDF membranes. Blots were blocked for 1 hour at room temperature with blocking buffer (5% nonfat milk in 10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20, Anti-eNOS monoclonal IgG (Transduction Laboratories, Lexington, Ky), at a dilution of 1:2500, was reacted with the blots overnight at 4°C. After washing (2X for 15 minutes in 1X TTBS), the blots were incubated with the secondary antibody (horseradish peroxidase–conjugated goat anti-mouse immunoglobulin antibody (BioRad, Hercules, Calif) at 1:3000 dilution for 1 hour at room temperature. Visualization was performed with enhanced chemiluminescence. Densitometric analysis of Western blots was performed with the use of PDI Imageware System. Western blotting of the structural protein α-tubulin was also performed to confirm equal loading.

Assessment of Cell Injury and Apoptosis

The effects of CRP on endothelial nitric oxide synthase (eNOS) production in the presence and absence of CRP (25 µg/mL, 24 hours) on basal NO production in human ECs incubated in 20% FBS EC basal medium-2 (Clonetics) in the presence of CRP were evaluated in vitro with the use of densitometry and ImageMaster software. The eNOS mRNA signal was normalized for the corresponding ribosomal 28S band. The effects of CRP (25 µg/mL) on mRNA stability were also evaluated. For this purpose, confluent ECs were incubated with CRP in media containing 2.5 µg/mL actinomycin D. Culture media was removed at 0, 8, and 24 hours after actinomycin D treatment and RNA extracted for Northern blot analysis.

Cell Injury and Apoptosis

For the assessment of cellular injury, cells were stained with trypan blue. Injured cells were unable to exclude the dye and were subsequently stained blue. For detection of apoptosis, ECs were washed with PBS and fixed in 4% formaldehyde after which they were stained with 4′,6-diamidino-phenylindole (DAPI; 0.2 µg/mL in 10 mmol/L Tris/HCl, 10 mmol/L EDTA and 100 mmol/L NaCl) for 30 minutes. Cells were washed with PBS and DAPI-positive cells were evaluated by fluorescence microscopy.

In Vitro Angiogenesis: Scratch Wound Assay and Capillary-Like Tube Formation

A wounding migration assay was performed to ascertain the effects of CRP on angiogenesis. EC migration was determined with the use of similar methods, other investigators have reported the effects of CRP on angiogenesis. EC migration was determined with the use of Matrigel tubes formed to confirm equal loading.

Northern Blot Analysis and mRNA Stability

Total cellular RNA was isolated by lysis of cells in guanidinium isothiocyanate followed by phenol extraction. Northern blot analysis was conducted by subjecting 10 µg of RNA sample to electrophoresis in 1% agarose gel containing formaldehyde; then the RNA was transferred to a nylon membrane and UV-cross-linked. The eNOS cDNA probes were labeled with [32P]dCTP by a random primer kit (Amersham), and hybridized with the membranes in the presence of 50% formaldehyde and 10% dextran sulfate at 42 °C (12 hours). The hybridized blots were washed and exposed to an x-ray film (Amersham) for 48 hours, and autoradiographic results quantified with the use of densitometry and ImageMaster software. The eNOS mRNA signal was normalized for the corresponding ribosomal 28S band. The effects of CRP (25 µg/mL) on mRNA stability were also evaluated. For this purpose, confluent ECs were incubated with CRP in media containing 2.5 µg/mL actinomycin D. Culture media was removed at 0, 8, and 24 hours after actinomycin D treatment and RNA extracted for Northern blot analysis.

Results

CRP Attenuates NO Release and cGMP Production

Figure 1A depicts the effects of human recombinant CRP (100 µg/mL, 24 hours) on basal NO production in human ECs incubated in 20% FBS EC basal medium-2 (Clonetics) basic medium with the presence or absence of CRP (25 µg/mL) and interventions as mentioned above at 37°C for 24 hours under a 5% CO2 atmosphere. The morphological changes were observed and photographed with the use of a phase contrast microscope. Each well was photographed 4 times at random. The length of the tube was measured at 40× magnification with Scion image (Scion Corp, NIH, Beta 4.0.2), and expressed as mm/mm².

Statistical Analysis

All values are presented as mean±SEM. Comparisons between multiple treatment groups were done with the use of a 1-way ANOVA followed by a Newman-Keul’s test. Unpaired data were analyzed by a 2-tailed Student’s t test. Differences were considered significant at P<0.05.

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saphenous vein ECs. Basal NO release was markedly attenuated by CRP, with maximal inhibition observed at \(5 \mu g/mL\) \((P<0.001)\). Increases in CRP concentrations beyond \(5 \mu g/mL\) did not further attenuate NO release, demonstrating a potent NO quenching effect of low concentrations of CRP.

In addition to inhibiting basal NO production, CRP (25 \(\mu g/mL\)) inhibited bradykinin-stimulated NO production (Figure 1B) in human umbilical vein ECs \((P<0.01)\). The majority of the vascular actions of NO are mediated via the second messenger cGMP. Therefore, measuring cGMP levels is a surrogate marker of bioactivity of eNOS-derived NO. Figure 1C depicts the effects of human recombinant CRP on cGMP production. cGMP levels, determined by enzyme immunoassay, were markedly lower in cells treated with CRP \((P<0.001)\). We have previously demonstrated that CRP increases EC production of ET-1 and IL-6. To examine the contribution of ET-1 and IL-6 toward CRP’s effects on NO production, we studied the effects of coinoculation with bosentan and anti-IL-6 antibodies. The ability of CRP (25 \(\mu g/mL\)) to attenuate basal NO production was unaffected during either ET receptor blockade or IL-6 inhibition (Figure 2).

CPR Attenuates eNOS Protein, mRNA Expression, and Stability

To examine whether the effects of CRP on NO release were secondary to decreased eNOS, we studied eNOS protein expression by Western blotting in saphenous vein ECs incubated with and without CRP (25 \(\mu g/mL\), 24 hours). In the presence of CRP, basal eNOS protein expression was markedly downregulated \((P<0.001)\) (Figure 3, E and F). A similar effect was noted at concentrations of 5 \(\mu g/mL\) (not shown). Northern blot analysis revealed that CRP caused a significant decrease in mRNA levels (Figure 3, A and B). eNOS mRNA half-life was assessed in actinomycin D–treated ECs incubated with CRP (25 \(\mu g/mL\), 24 hours). CRP caused a significant reduction in eNOS mRNA stability (half-life \(\sim14\) hours in CRP-treated cells versus \(>24\) hours in control ECs) (Figure 3, C and D).

Effects of CRP on Cell Injury and Apoptosis

Figure 4 depicts the effects of CRP on cell injury and apoptosis. Cell injury, assessed by trypan blue exclusion, was similar in CRP-treated versus untreated cells (Figure 4A). Evaluation of apoptosis by DAPI staining revealed greater proportion of apoptotic cells in the CRP-treated versus untreated group (Figure 4B). This effect appeared to be NO dependent because it was reversed during coincubation with sodium nitroprusside.

CPR Inhibits Angiogenesis In Vitro

Because CRP decreased the release of NO, we hypothesized that a key NO-mediated event, ie, angiogenesis, would also be attenuated under both basal and VEGF-stimulated conditions. Angiogenesis was evaluated in vitro by capillary-like

**Figure 1.** A, CRP inhibits basal NO production. Human recombinant CRP causes a marked, sustained, and concentration-dependent decrease in nitric oxide production in human saphenous vein ECs. ECs were incubated with CRP for a period of 24 hours and NO production assessed spectrophotometrically by measuring its final stable equimolar degradation products, nitrite and nitrate. C indicates control. **\(P<0.01,\) significantly different from other groups. \(^*P<0.01,\) different from other groups. B, Bradykinin (BK)–stimulated NO production is attenuated by CRP. Human umbilical vein ECs were incubated with CRP (25 \(\mu g/mL\)) and then exposed to 10 \(^{–6}\) mol/L bradykinin or vehicle for 30 minutes. **\(P<0.01;\) \(^*P<0.001,\) different from all groups. C, CRP decreases cGMP production, the second messenger of NO. ECs were incubated with CRP (25 \(\mu g/mL\), 24 hours) and intracellular cGMP production assessed by a commercial enzyme immunoassay kit and normalized for protein content. \(^*P<0.01,\) all data are expressed as mean±SEM. \(n=8\) to 10 per group.

**Figure 2.** Effects of CRP (25 \(\mu g/mL\), 24 hours) on basal NO production in human umbilical vein ECs in the presence or absence of anti–IL-6 monoclonal antibodies (5 \(\mu g/mL\)) or bosentan (10 \(\mu g/mL\)). Interventions were added 2 hours before the addition of CRP. The effects of CRP to blunt NO production are not attenuated during ET receptor blockade or IL-6 inhibition. **\(P<0.01,\) different from all groups.
tube formation and EC migration assays, respectively. The effects of CRP and VEGF on capillary-like tube formation are depicted in Figure 5. CRP markedly reduced tube length under both basal and VEGF-stimulated conditions (P<0.01). Further evidence for an antiangiogenic effect of CRP was derived from the wounding migration assay. CRP inhibited both basal and VEGF-stimulated EC migration (P<0.01, Figure 6). Taken together, these data suggest that the ability of CRP to directly quench NO production is associated with diminished angiogenesis, a key compensatory mechanism to chronic ischemia.

Discussion
NO is the key endothelium-derived relaxing factor that plays a pivotal role in the maintenance of vascular tone and reactivity. In addition to being the main determinant of basal vessel tone, NO opposes the actions of potent endothelium-derived contracting factors, such as angiotensin II and ET-1, and promotes angiogenesis, an important compensatory mechanism to long-standing ischemia. Decreased production and/or action of NO are central to the pathogenesis of atherosclerotic vascular disease via promoting vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, oxidation, thrombosis, impaired coagulation, and vascular inflammation. Indeed, decreased NO production has been implicated in the pathogenesis and clinical course of all known cardiovascular diseases and is associated with future risk of adverse cardiovascular events. Declining levels of NO adversely affect plaque architecture, facilitating rupture and inciting acute coronary syndromes and myocardial infarction. In the endothelium, NO is synthesized from L-arginine under the influence of the eNOS and exerts its effects via production of cGMP.

Given the central importance of NO in the development and manifestations of cardiovascular diseases, we sought to determine whether the powerful predictive value of CRP might be explained through a direct effect on decreasing NO production. We found, that human recombinant CRP, at concentrations known to predict adverse cardiovascular events, caused a marked, sustained, and dose-dependent decrease in NO production in venous ECs. To confirm the functional significance of these data, we assessed the effects of human recombinant CRP on the production of cGMP, suggesting that reduction in NO release was associated with decreased second messenger bioactivity. To determine the mechanism, we examined the effects of CRP on the expression of eNOS, the rate-limiting enzyme in the production of NO. We show that CRP caused a marked downregulation of eNOS protein and transcript expression, in part via an effect of CRP to attenuate eNOS mRNA stability. mRNA levels represent the net balance between gene transcription and production. Figure 4. A, Effects of CRP (5 and 25 μg/mL, 24 hours) on human umbilical vein EC cell survival, assessed by trypan blue exclusion. At the concentrations studied, CRP did not promote cell death. B, Effects of CRP on apoptosis, assessed by DAPI staining. CRP (25 μg/mL, 18 hours) caused an increase in DAPI-positive apoptotic cells. This effect was restored by the exogenous NO donor, sodium nitroprusside (SNP). *P<0.01, different from control and CRP+SNP.
mRNA degradation, the latter being dependent on nucleotide sequence motifs, usually located in the 3′ untranslated region of the gene. The ability of CRP to attenuate eNOS mRNA half-life is analogous to the effects of cytokines (tumor necrosis factor-α), lipopolysaccharides, hypoxia, and oxidized LDL. In this regard, it is important to note that CRP has been shown to augment the EC production of IL-6, an important inflammatory cytokine. It is plausible, therefore, that the effects of CRP on eNOS may be secondary to an increase in cytokine production. However, in our studies, preincubation with an anti-IL-6 antibody did not prevent CRP-mediated decreases in NO release (Figure 2). Whether CRP increases the production of tumor necrosis factor-α, a potent destabilizer of eNOS mRNA, is an important question that remains to be determined. The effects of CRP on eNOS mRNA expression could also be due to a diminished transcription rate. Because nuclear run-on assays were not performed, this mechanism cannot be excluded.

A growing body of evidence supports the notion that NO is a key modulator of angiogenesis. Angiogenesis is a key compensatory mechanism in response to chronic ischemia and has emerged as an important therapeutic target for ischemic heart disease. NO donors promote EC proliferation and migration, whereas NOS inhibitors block VEGF-induced EC proliferation and capillary-like tube formation. VEGF-induced angiogenesis is critically dependent on NO release. Our data suggest that the ability of CRP to attenuate NO production is associated with a marked reduction in vitro angiogenesis; both wound cell migration and capillary-like tube formation were inhibited by CRP, at concentrations known to predict adverse cardiovascular outcomes.
In addition to promoting angiogenesis, NO has been suggested to augment cell survival and inhibit apoptosis induced by a variety of stimuli. Although a number of NO-mediated antiapoptotic effects have been described, one important mechanism involves nitrosylation of caspases. In states of decreased NO bioavailability, ECs are unable to produce enough NO required for S-nitrosylation and inactivation of caspases, in turn facilitating apoptotic cell death. Because CRP reduced the production of NO, we hypothesized that EC apoptosis would be augmented in CRP-treated cells. Indeed, results from the present study suggest that CRP promotes apoptosis, and that this occurs in a NO-dependent fashion because it is inhibited by the exogenous NO donor sodium nitroprusside.

An Integrated Perspective
The mechanistic basis of the CRP-atherosclerosis connection may lie in the ability of CRP to directly modulate the production of endothelium-derived vasoactive factors. To the best of our knowledge, this is the first evidence that CRP can profoundly downregulate the production of NO, the central “controller” of cardiovascular homeostasis. This effect appears to occur in part via an effect of CRP on eNOS mRNA stability. In a synchronous fashion, CRP augments the production of the potent endothelium-derived constrictor, ET-1, and a key inflammatory cytokine, IL-6 (Figure 7). These events in turn facilitate the expression of early atherosclerotic processes, including adhesion molecule expression, chemokine production, and macrophage LDL-uptake, and uncover a prothrombotic and proinflammatory phenotype. Through decreasing NO production, CRP may also facilitate EC apoptosis and attenuate important compensatory mechanisms to ischemia-like angiogenesis. Thus, CRP may not just be a marker of atherosclerosis and coronary events, but also a
mediator of this disease because it contributes to the substrate underlying lesion formation, plaque rupture, and coronary thrombosis. In the same context, strategies designed to lower plasma CRP may be effective in reducing risk of cardiovascular events by virtue of directly improving NO bioavailability and endothelial function. In this way, the ability of CRP to attenuate NO release is nothing short of a self-fulfilling prophecy.

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References


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