

C-Reactive Protein Downregulates Endothelial NO Synthase and Attenuates Reendothelialization In Vivo in Mice

Randall Schwartz,* Sherri Osborne-Lawrence,* Lisa Hahner, Linda L. Gibson, Andrew K. Gormley, Wanpen Vongpatanasin, Weifei Zhu, R. Ann Word, Divya Seetharam, Steven Black, David Samols, Chieko Mineo, Philip W. Shaul

Abstract—C-reactive protein (CRP) is an acute-phase reactant that is positively associated with cardiovascular disease risk and endothelial dysfunction. In cell culture, CRP decreases the expression of endothelial NO synthase (eNOS), which regulates diverse endothelial cell (EC) functions including migration. To determine whether CRP alters EC gene expression and phenotype in vivo, we studied CF1 transgenic mice expressing rabbit CRP (CF1-CRP) regulated by the phosphoenolpyruvate carboxykinase promoter such that levels could be altered by changing carbohydrate intake. Compared with CF1 controls with CRP of $<1 \mu\text{g/mL}$, carotid artery reendothelialization after perivascular electric injury was blunted in CF1-CRP mice, with CRP levels as low as $9 \mu\text{g/mL}$. eNOS mRNA and enzyme abundance in carotid arteries was also blunted by CRP at $9 \mu\text{g/mL}$ in vivo, and ex vivo studies of isolated arteries showed that this occurs via direct action on the endothelium. The impaired reendothelialization with CRP was mimicked by NOS antagonism in CF1 mice; conversely, in cultured ECs CRP attenuation of migration was prevented by exogenous NO. Studies of EC transfected with human eNOS 5' flanking sequence fused to luciferase indicated that CRP decreases eNOS gene transcription. Both mutagenesis and electrophoretic mobility shift assays further revealed that CRP-responsive elements reside within the first 79 bp of the eNOS promoter. Thus, CRP downregulates eNOS and attenuates reendothelialization in vivo in mice, and this action of CRP on eNOS is mediated at the level of gene transcription. (*Circ Res.* 2007;100:1452-1459.)

Key Words: C-reactive protein ■ endothelial NO synthase ■ reendothelialization

C-reactive protein (CRP) is an acute-phase reactant and a member of the pentraxin family of proteins that classically serves as an opsonin and activates complement. Its hepatic synthesis is stimulated by interleukin-6 to yield levels that can rise 500-fold within 24 to 48 hours of the initiation of an inflammatory process.¹⁻⁴ In addition to participating in immune response, CRP has received considerable attention as a risk factor for cardiovascular disease, with chronic modest elevations being associated with a greater likelihood of myocardial infarction, sudden cardiac death, stroke, and peripheral vascular disease.⁵⁻⁹ CRP is also a risk factor for the progression of subclinical vascular disease and for hypertension.^{10,11} Whether CRP plays a pathogenetic role in atherosclerosis is presently controversial.^{12,13}

In vitro studies have shown that CRP decreases the expression of endothelial NO synthase (eNOS).^{14,15} In addition to a variety of other actions important to cardiovascular health, endothelium-derived NO promotes endothelial cell (EC) growth and migration and angiogenesis, which underlie

both neovascularization and the maintenance of intimal layer integrity.¹⁶ An intact endothelial monolayer modulates hemostasis and thrombolysis and provides a nonpermeable barrier protecting vascular smooth muscle cells from circulating growth factors. Disruption of the intimal layer, either by gross denudation related to a vascular intervention or gap formation between ECs caused by disturbed shear stress, places the arterial wall at greater risk for vascular disease.¹⁷ Furthermore, whereas repeated EC removal worsens vascular lesion severity, enhanced reendothelialization attenuates lesion formation.^{18,19} CRP-induced attenuation of EC migration has been demonstrated in cell culture,¹⁵ suggesting that actions of the pentraxin on eNOS expression may result in changes in an EC phenotype of importance to vascular health. However, it is yet to be determined whether CRP modifies EC gene expression and phenotype in vivo.

The present study was designed to investigate the in vivo actions of CRP on endothelium in CRP transgenic mice. Mice expressing rabbit CRP under the regulation of the phos-

Original received December 26, 2006; revision received March 20, 2007; accepted April 10, 2007.

From the Departments of Pediatrics (R.S., S.O.-L., L.H., L.L.G., A.K.G., W.Z., D.S., C.M., P.W.S.), Internal Medicine (W.V.), and Obstetrics and Gynecology (R.A.W.), University of Texas Southwestern Medical Center, Dallas; and Department of Biochemistry (S.B., D.S.), Case Western Reserve University School of Medicine, Cleveland, Ohio.

*Both authors contributed equally to this work.

Correspondence to Philip W. Shaul, Department of Pediatrics, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390. E-mail philip.shaul@utsouthwestern.edu

© 2007 American Heart Association, Inc.

Circulation Research is available at <http://circres.ahajournals.org>

DOI: 10.1161/01.RES.0000267745.03488.47

phoenolpyruvate carboxykinase (PEPCK) promoter on a CF1 background (CF1-CRP) were used to afford predictable, direct manipulation of CRP levels in vivo via changes in carbohydrate (CHO) intake.²⁰ The use of CF1-CRP mice also avoids possible effects of contaminants or vehicle that may complicate studies with administered recombinant CRP.^{21–23} Using a perivascular electric injury model,²⁴ we tested the hypothesis that CRP attenuates reendothelialization of the carotid artery in vivo. Additional studies were performed to answer the following questions. (1) Is reendothelialization impaired at clinically-relevant levels of CRP? (2) Is the effect on reendothelialization related to eNOS downregulation? (3) Do the effects of CRP on carotid artery eNOS entail systemic mechanisms or direct action on the endothelium? (4) How does CRP downregulate eNOS expression?

Materials and Methods

Animal Model

Experiments were performed in CF1 and CF1-CRP mice that express a transgene consisting of the protein-coding region of the rabbit CRP gene linked to the promoter/regulatory region of the cytosolic form of the rat PEPCK gene.²⁰ Mice hemizygous for the transgene (CF1-CRP-low) were also studied. Additional details are in the online data supplement at <http://circres.ahajournals.org>.

Carotid Artery Reendothelialization

Carotid artery reendothelialization was studied following perivascular electric injury.²⁴ Additional details are in the online data supplement.

Quantification of eNOS mRNA

Real-time RT-PCR was performed to quantify eNOS mRNA abundance in noninjured carotid arteries of CF1 and CF1-CRP-low mice placed on the high CHO diet for 5 days.²⁰ Additional details are in the online data supplement.

Quantification of NOS Activity

NOS enzymatic activity was determined in homogenates of noninjured carotid arteries from CF1 and CF1-CRP-low mice that received the high CHO diet for 5 days by measuring 14 C-L-arginine to 14 C-L-citrulline conversion.²⁵ Further details are in the online data supplement. In additional experiments, carotid arteries from CF1 mice were incubated *ex vivo* in serum-free DMEM with human recombinant CRP (10 μ g/mL, Calbiochem) for 18 hours. Control treatment consisted of CRP vehicle (140 mmol/L NaCl, 20 mmol/L Tris-HCl, 2 mmol/L CaCl₂, 0.05% NaN₃, pH 7.5) added at the same volume.²⁶ A comparison of the characteristics of rabbit and human CRP is provided in the online data supplement.

Cell Culture

Primary bovine aortic ECs (BAECs) for use in EC migration assays were studied at passages 5 to 9.²⁶ Primary ovine ECs for use in eNOS promoter activity assays were studied at passages 4 to 6.²⁷

Endothelial Cell Migration Assay

The impact of CRP on the migration of BAECs was evaluated.²⁴ Additional details are in the online data supplement. Heat-inactivated CRP²⁶ did not alter migration (data not shown). In selected studies, cells were additionally treated with the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) (20 μ mol/L).

eNOS Promoter Activity Assay

The impact of CRP on eNOS promoter activity was evaluated in ovine ECs using a promoter-reporter plasmid containing DNA from 1.6 kbp upstream of the eNOS AUG to the transcription start site

inserted into the luciferase reporter gene plasmid pGL2 (–1624eNOS-Luc).²⁷ Progressive 5' deletion mutants were also studied, which were designated –994eNOS-Luc, –318eNOS-Luc, –279eNOS-Luc, –248eNOS-Luc, and –79eNOS-Luc. Details are in the online data supplement.

Electrophoretic Mobility Shift Assays

Following treatment of ovine ECs with vehicle or CRP, nuclear extracts were prepared and incubated with a ³²P-labeled oligonucleotide probe consisting of the 79 bp immediately upstream of the eNOS AUG.²⁷ In competition studies, nonlabeled oligonucleotide probe was added at 200-fold molar excess before the addition of the ³²P-labeled probe. Nuclear protein–DNA complexes were resolved on 5% nondenaturing polyacrylamide gels containing 1× Tris borate/EDTA buffer. Dried gels were exposed to Kodak XAR film for autoradiography.

Statistical Analysis

Data are presented as mean±SEM. Results between 2 groups were compared by Student's *t* test. ANOVA with Neuman–Keuls post hoc testing was used to assess differences between 3 or more groups. Comparisons of the degree of reendothelialization between groups were done by Mann–Whitney tests. Significance was set at *P*<0.05.

Results

Effect of CRP on Carotid Artery Reendothelialization

To first determine whether CRP affects reendothelialization, we performed perivascular electric injury experiments in 12- to 16-week-old male CF1 control versus CF1-CRP mice. Immediately following the injury procedure, the diet was switched from standard chow to a CHO-free, protein-rich chow to maximally activate transgene expression, and reendothelialization was assessed 5 days later. At the end of this regimen, CRP levels were <1 μ g/mL in CF1 controls and CF1-CRP mice had CRP levels of 160±10 μ g/mL (n=6/group). Compared with CF1 controls, reendothelialization was impaired in CF1-CRP mice (Figure 1A and 1B, respectively), as indicated by the larger area of remaining denudation that incorporated Evan's blue dye in the latter group. Cumulative studies revealed that the area of remaining denudation was 83% greater in CF1-CRP versus CF1 (Figure 1C).

To then determine whether CRP at lower levels also affects reendothelialization, additional experiments were performed in CF1 versus hemizygous CF1-CRP-low mice on high-CHO-containing diets to minimize transgene expression. This regimen yielded CRP levels of <1 μ g/mL in CF1 mice and 9±5 μ g/mL in CF1-CRP-low mice (n=6/group). Compared with CF1, reendothelialization was blunted in CF1-CRP-low mice (Figure 1D and E, respectively), and cumulative experiments indicated that the area of remaining denudation was 60% larger in CF1-CRP-low mice versus CF1 controls (Figure 1F).

Effect of CRP on eNOS Expression

It has been previously demonstrated in cultured ECs that CRP downregulates eNOS expression,^{14,15} and eNOS is critically involved in the regulation of EC migration.¹⁶ We therefore determined whether the impaired reendothelialization is associated with a change in eNOS expression. First, steady-state eNOS mRNA levels were quantified in noninjured

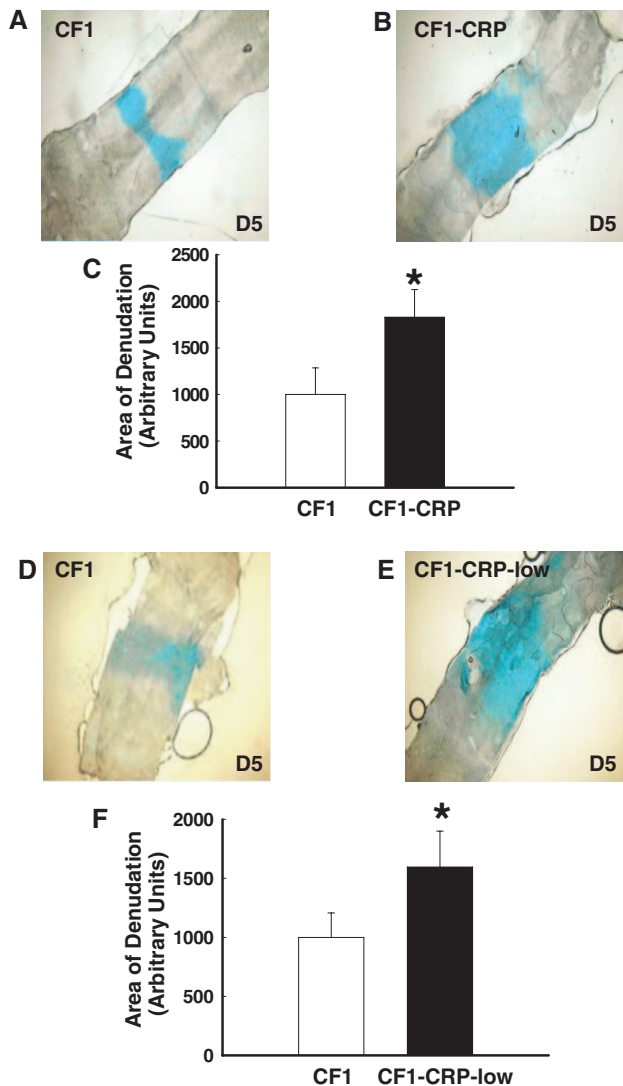


Figure 1. CRP impairs reendothelialization in mice. A and B, The intimal surface of Evan’s blue–stained arteries from a CF1 control (A) and CF1-CRP (B) mouse 5 days after injury and placement on a CHO free–diet that yields CRP levels of <1 $\mu\text{g}/\text{mL}$ and $160 \pm 10 \mu\text{g}/\text{mL}$, respectively. C, Area of denudation was quantified and is expressed in arbitrary units. The intimal surface of Evan’s blue–stained arteries from a CF1 control (D) and CF1-CRP–low (E) mouse 5 days after injury and placement on a CHO-containing diet that yields CRP levels of <1 $\mu\text{g}/\text{mL}$ and $9 \pm 5 \mu\text{g}/\text{mL}$, respectively. F, Area of denudation was quantified and is expressed in arbitrary units. Values in C and F are mean \pm SEM (n=10 to 21 mice/group). * $P < 0.05$ vs CF1.

carotid arteries from CF1 versus CF1-CRP–low mice following 5 days placement on the high-CHO-containing chow that yields CRP levels of <1 $\mu\text{g}/\text{mL}$ and 9 $\mu\text{g}/\text{mL}$, respectively. Using real-time RT-PCR, it was found that eNOS mRNA abundance was decreased by 74% by CRP (Figure 2A). In parallel, NOS enzymatic activity was decreased by 53% in the carotid arteries of CF1-CRP–low mice versus CF1 controls (Figure 2B). Thus, CRP causes a decline in both eNOS mRNA and enzyme abundance in vivo in mice.

To determine whether the reduction in eNOS in CF1-CRP–low mice is attributable to systemic actions of CRP or a direct effect of the pentraxin on the vasculature, ex vivo experi-

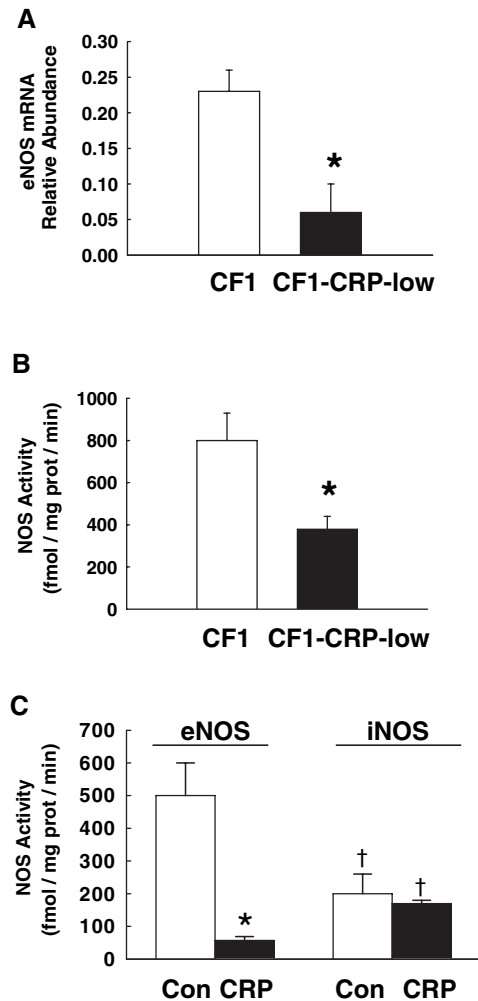


Figure 2. CRP downregulates eNOS mRNA abundance and NOS enzymatic activity in vivo in mice. A, Carotid arteries were obtained from CF1 and CF1-CRP–low mice on a CHO-containing diet that yields CRP levels of <1 $\mu\text{g}/\text{mL}$ and $9 \pm 5 \mu\text{g}/\text{mL}$, respectively, and relative abundance of eNOS mRNA was determined by real-time RT-PCR. B, Carotid arteries were obtained from CF1 and CF1-CRP–low mice under conditions outlined in A, and relative abundance of NOS enzymatic activity was determined by measurement of arginine to citrulline conversion. In A and B, values are mean \pm SEM (n=4/group); * $P < 0.05$ versus CF1. C, CRP downregulates eNOS enzymatic activity ex vivo. Carotid arteries from CF1 mice were incubated ex vivo for 18 hours under control conditions (Con) or in the presence of 10 $\mu\text{g}/\text{mL}$ human recombinant CRP. eNOS-derived and iNOS-derived calcium-dependent and calcium-independent enzymatic activity, respectively, was then determined by measurement of arginine to citrulline conversion in the absence or presence of EGTA. In C, values are mean \pm SEM (n=4/group). * $P < 0.05$ vs control, † $P < 0.05$ vs eNOS.

ments were performed with carotid arteries from CF1 mice. Arteries were incubated under control conditions or in the presence of CRP for 18 hours, and NOS enzymatic activity was evaluated in a manner that distinguished eNOS- and inducible NOS (iNOS)-derived activity based on calcium dependence. CRP had no effect on iNOS-derived NOS activity, which was less than eNOS-derived activity in control-treated arteries. However, paralleling the decline in eNOS caused by CRP in vivo, artery incubation with CRP caused a 90% decrease in eNOS-derived enzymatic activity

(Figure 2C). In contrast, heat-inactivated CRP²⁶ caused no change in eNOS-derived or iNOS-derived enzyme activity ($91 \pm 11\%$ of control activity and $101 \pm 13\%$ of control activity, respectively). Thus, CRP directly reduces arterial eNOS expression, and the changes in eNOS observed *in vivo* are not caused by CRP-induced systemic actions.

CRP, NO Deficiency, and Impaired Endothelial Cell Migration

Having demonstrated that CRP causes eNOS downregulation in association with impaired reendothelialization *in vivo*, we next determined whether the blunted reendothelialization phenotype observed in CF1-CRP mice is mimicked in the same mouse strain by NO deficiency. CF1 mice were administered drinking water or drinking water plus *N*^G-nitro-L-arginine methyl ester (L-NAME), perivascular electric injury was performed, and the area of remaining denudation was evaluated 5 days later. Compared with control mice, reendothelialization was impaired in L-NAME-treated mice (Figure 3A and 3B, respectively). Cumulative studies revealed that the area of remaining denudation was 170% larger in L-NAME-treated versus control-treated mice (Figure 3C).

To provide additional linkage among CRP, NO deficiency, and blunted EC migration, migration assays were performed with cultured BAECs. Compared with control-treated cells, migration was stimulated by vascular endothelial growth factor (VEGF) treatment (Figure 4A and 4B, respectively), and VEGF-stimulated migration was attenuated by CRP (Figure 4C). However, cotreatment with the NO donor SNAP rescued normal VEGF-stimulated migration in the presence of CRP (Figure 4D). Findings were confirmed in repeat studies, and the cumulative data are shown in Figure 4E. eNOS downregulation by CRP was also demonstrable (Fig-

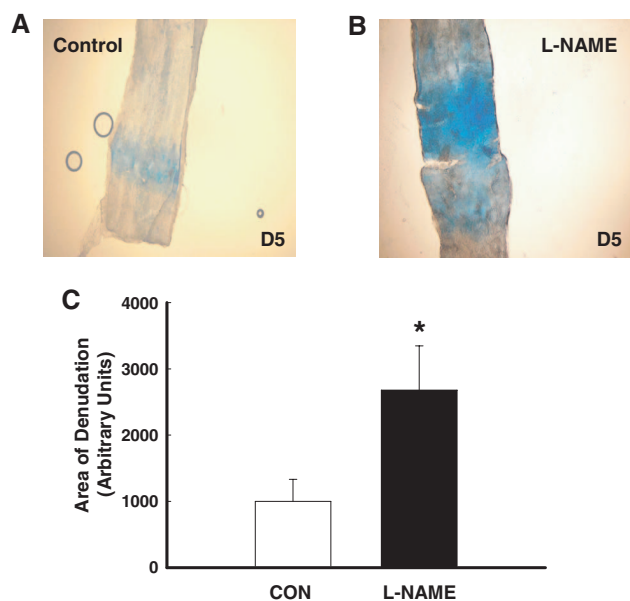


Figure 3. NOS antagonism impairs reendothelialization in mice. The intimal surface of Evan's blue-stained arteries from a CF1 control-treated (A) and CF1 L-NAME-treated (B) mouse 5 days after injury. C, Area of denudation was quantified and is expressed in arbitrary units. Values are mean \pm SEM ($n=12$ mice/group). * $P<0.05$ vs control.

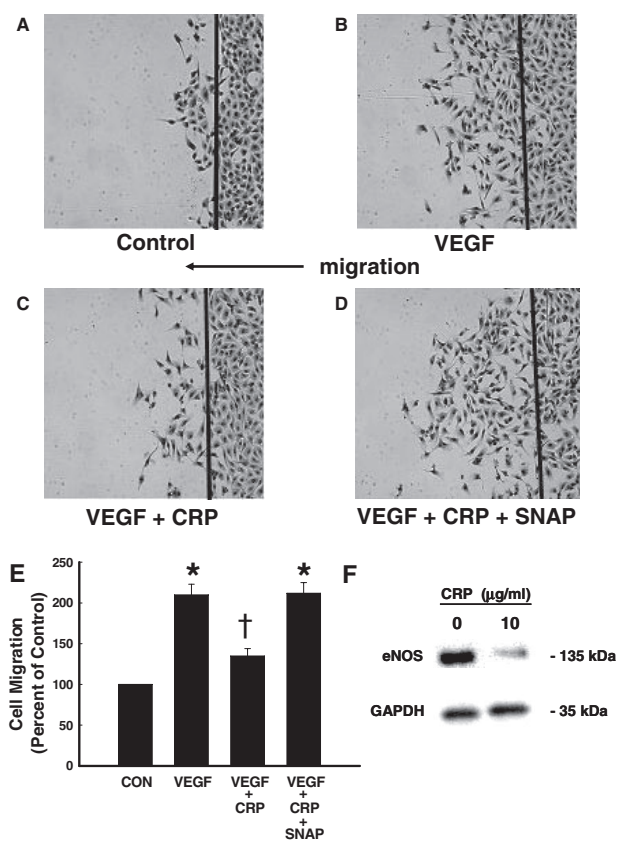


Figure 4. CRP impairs EC migration via a decline in bioavailable NO. BAECs were wounded and treated with media alone (control) (A), VEGF (50 ng/mL) (B), VEGF plus CRP (10 μ g/mL) (C), or VEGF plus CRP plus SNAP (20 μ mol/L) (D) for 24 hours, and migration was evaluated. E, Cell migration is expressed relative to control treatment; values are mean \pm SEM ($n=6$ to 8/treatment); * $P<0.05$ vs control, † $P<0.05$ vs no CRP. F, CRP downregulation of eNOS was evaluated by immunoblot analysis, with the findings shown confirmed in 3 independent experiments.

ure 4F). The collective observations made *in vivo* and in cell culture indicate that NO deficiency underlies the negative impact of CRP on EC migration.

Effect of CRP on eNOS Promoter Activity

To further understand the molecular basis of eNOS downregulation by CRP, we determined whether the pentraxin modified eNOS transcription in studies of the activity of the human eNOS promoter transfected into ovine ECs. Using the full-length promoter (-1624 eNOS-Luc), we found that treatment with CRP for 6 hours causes a decline in basal promoter activity (Figure 5A). More substantial declines in promoter activity were observed with 18 hours of CRP treatment. Polymyxin B did not alter the effect of CRP on the eNOS promoter.

The dose response to CRP was also evaluated (Figure 5B). CRP concentrations of 10 μ g/mL or more caused declines in eNOS promoter activity. In studies performed at 50 μ g/mL, recombinant and ascites-derived human CRP caused comparable $40 \pm 3\%$ and $46 \pm 2\%$ declines in eNOS promoter activity, respectively. Declines in eNOS protein expression were also apparent, particularly with CRP treatment at 10 μ g/mL or more (Figure 5B, inset). Thus, paralleling the in

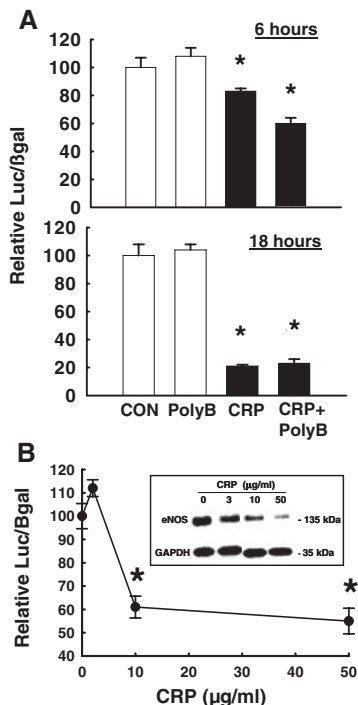


Figure 5. CRP attenuates eNOS promoter activity and protein abundance in ECs. A, eNOS promoter-reporter construct -1624eNOS-Luc was cotransfected with SV40-driven β -galactosidase plasmid (β -gal) into ovine ECs, cells were treated with control media (Con) or media containing 10 μ g/mL human recombinant CRP, with or without 25 μ g/mL polymyxin B (polyB) added, and relative activities (Luc/ β -gal) were determined in cell lysates 6 hours (top) or 18 hours (bottom) later. B, Dose response to CRP was evaluated in cells treated with 0 to 50 μ g/mL CRP for 18 hours. Parallel changes in eNOS protein abundance were demonstrated by immunoblot analysis, with the findings shown in the inset confirmed in 3 independent experiments. For A and B, values are mean \pm SEM (n=4). * P <0.05 vs no CRP.

vivo findings for CRP-induced downregulation of eNOS expression (Figure 2), eNOS gene transcription was blunted by CRP.

Promoter Elements Required for eNOS Regulation by CRP

To determine the elements in the eNOS promoter required for CRP responsiveness, the activities of progressive 5' deletion mutants were evaluated. Basal promoter activity in the absence of CRP did not change with deletion from -1624 to -994, nor with further deletion to -318. In contrast, basal promoter activity rose 5.6 ± 1.6 -fold (P <0.05) with deletion to -279 compared with full-length promoter, which is consistent with our prior findings regarding this region of the promoter in studies of eNOS regulation in airway epithelium.²⁷ In experiments comparing the CRP responsiveness of -1624eNOS-Luc, -994eNOS-Luc, -318eNOS-Luc, and -279eNOS-Luc there were equivalent 50% declines in activity in the presence of the pentraxin (Figure 6A). These findings indicate that the promoter elements responsible for CRP action reside more proximal in the eNOS 5' flanking sequence than -279.

To evaluate this further, additional 5' deletion mutants were studied. With deletion from -1624 to -248, basal

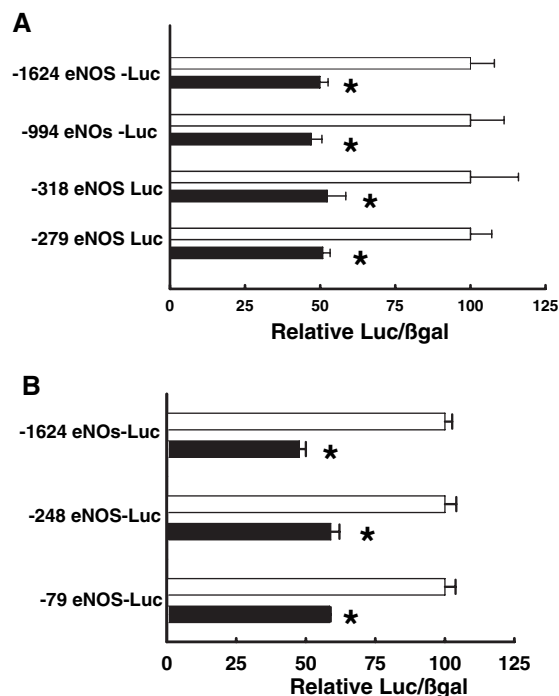


Figure 6. CRP-inhibited activity of 5' deletion mutants of the eNOS promoter. A, eNOS promoter-reporter constructs -1624eNOS-Luc, -994eNOS-Luc, -318eNOS-Luc, or -279eNOS-Luc were cotransfected with SV40-driven β -galactosidase plasmid (β -gal) into ovine ECs, cells were treated with control media (open bars) or media containing 10 μ g/mL CRP (closed bars), and relative activities (Luc/ β -gal) were determined in cell lysates 18 hours later. Results are normalized to basal activity for each promoter construct, which did not change with deletion from -1624 to -994, nor with further deletion to -318; in contrast, basal promoter activity rose 5.6 ± 1.6 -fold (P <0.05) with deletion to -279 compared with full-length promoter. B, Using the same strategy, the activities of eNOS promoter-reporter gene constructs -1624eNOS-Luc, -248eNOS-Luc, and -79eNOS-Luc were compared in the absence and presence of CRP. Results are normalized to basal activity for each construct, which did not change with deletion from -1624 to -248, nor with further deletion to -79. For A and B, values are mean \pm SEM (n=4). * P <0.05 vs no CRP.

promoter activity did not change, nor did it change with further deletion to -79. In comparisons of the impact of CRP on the activities of -1624eNOS-Luc, -248eNOS-Luc, and -79eNOS-Luc, there were equivalent 40% to 50% declines in activity in the presence of the pentraxin (Figure 6B). Thus, the CRP-responsive regulatory elements are located within 79 bp of the eNOS transcription initiation site.

The region of the eNOS promoter residing between -79 and -1 does not contain regulatory elements known to participate in eNOS gene regulation.^{28,29} We therefore performed electrophoretic mobility shift assays to provide an independent means to determine whether sequences within -79 to -1 are of relevance to CRP modulation of eNOS. Nuclear extracts from vehicle versus CRP-treated (10 μ g/mL for 24 hours) ovine ECs were incubated with a double-stranded oligonucleotide probe consisting of the 79 bp immediately upstream of the eNOS AUG. Nuclei from control cells displayed one major DNA-protein complex (Figure 7A, arrowhead). In contrast, nuclei from CRP-treated cells yielded 2 slower-migrating complexes (Figure 7A, arrows).

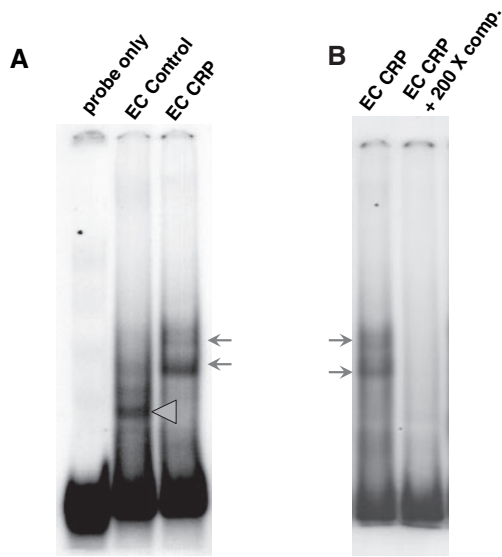


Figure 7. Electrophoretic mobility shift assays are altered by CRP exposure. A, Nuclear extracts from vehicle (control) and CRP-treated (10 $\mu\text{g}/\text{mL}$ for 24 hours) ovine ECs were incubated with labeled double-stranded oligonucleotide probe consisting of the 79 bp immediately upstream of the eNOS AUG. B, Competition reaction with unlabeled probe added at a 200-fold molar excess. Observations were confirmed in 3 independent experiments. Nuclei from control cells displayed 1 major DNA-protein complex (arrowhead). In contrast, nuclei from CRP-treated cells yielded 2 slower-migrating complexes (arrows).

The complexes were diminished by a 200-fold molar excess of unlabeled probe (Figure 7B). Thus, CRP induces nuclear protein binding to the same proximal region of the eNOS promoter that displays CRP responsiveness in promoter-reporter assays.

Discussion

Studies of cultured ECs indicate that CRP modifies the expression of important EC genes and also alters diverse EC functions.¹³ This has particularly been elucidated regarding the downregulation of eNOS expression and EC migration.^{13–15} In the present study, we demonstrated in CRP transgenic mice that the pentraxin causes impaired reendothelialization and blunted eNOS expression in the carotid artery, indicating that both endothelial gene expression and a critical ECs phenotype are altered by CRP *in vivo*.

We first demonstrated that maximal transgene activation in CF1-CRP mice induced by a CHO-free, high-protein diet, which yields CRP levels of 160 $\mu\text{g}/\text{mL}$, causes impaired reendothelialization. Minimal transgene activation was then accomplished in hemizygous CF1-CRP-low mice on a CHO-rich diet, resulting in CRP levels of 9 $\mu\text{g}/\text{mL}$, and blunted reendothelialization was still observed. This finding indicates that modest elevations in CRP that are associated with greater cardiovascular disease risk in otherwise healthy individuals in the absence of acute inflammation or infection³⁰ have adverse effects on an important EC phenotype in mice.

To determine whether the *in vivo* effect of CRP on reendothelialization of the carotid artery is related to a decline in eNOS expression, eNOS mRNA and NOS enzymatic activity were evaluated in the carotid arteries of hemizygous

CF1-CRP-low mice with CRP levels of 9 $\mu\text{g}/\text{mL}$. Both transcript abundance and enzyme activity were attenuated by CRP, consistent with previous studies in which CRP caused declines in eNOS mRNA and protein expression in cultured ECs.^{14,15} Mirroring the *in vivo* findings with the rabbit CRP transgene, *ex vivo* incubations of mouse carotid arteries with recombinant human CRP performed in the absence of serum also yielded a decline in eNOS abundance. This is not surprising because the molecular structure, binding characteristics, and functions of CRP are similar for the rabbit and human proteins (see the online data supplement). Using multiple strategies including heat inactivation of the recombinant protein, it was further revealed that the *ex vivo* observation was attributable to the actions of CRP and not contaminants or the vehicles for the CRP preparations. Although it has been a point of debate, such strategies have recently been used to exclude the involvement of contaminants or vehicle in multiple changes in gene expression and function induced by recombinant CRP in cultured ECs.¹³ From a mechanistic standpoint, the parallel *in vivo* and *ex vivo* observations further indicate that classical actions of CRP entailing the activation of complement or signaling in monocytes or other immune response cells^{1,2} do not play a role in CRP downregulation of eNOS. These cumulative results instead indicate that CRP has a direct effect on gene expression in endothelium in its native state.

To determine whether the loss in NOS activity in the carotid arteries of CF1-CRP-low mice versus CF1 controls may underlie the blunted reendothelialization observed with CRP *in vivo*, L-NAME was administered to CF1 mice and reendothelialization was evaluated. The phenotype of impaired reendothelialization seen with transgenic CRP elevation was replicated by NOS antagonism in the same mouse strain. Furthermore, in the EC migration model using BAECs in culture, blunted migration with CRP was rescued by the provision of exogenous NO. These combined observations suggest that the basis for CRP antagonism of reendothelialization lies in the downregulation of eNOS expression and the resulting decrease in bioavailable NO that is critical to the promotion of EC growth and migration.¹⁶ When considered along with our prior work demonstrating that CRP also antagonizes eNOS activation by diverse agonists,²⁶ it is apparent that the mechanisms by which CRP leads to a loss of NO are multiple. Further studies are now indicated to determine whether CRP impairs the function of endothelial progenitor cells *in vivo*, which are released from the bone marrow in an NO-dependent manner to participate in various forms of neovascularization^{31,32} and which are negatively influenced by CRP in cell culture studies.³³

In light of the well-recognized direct roles of endothelium-derived NO in the regulation of blood pressure, in the promotion of vasodilation and in the attenuation of thrombosis,³⁴ the current *in vivo* observation of eNOS downregulation by CRP may be relevant to the greater risk of hypertension and the blunted vascular responses to acetylcholine seen with elevated levels of CRP in humans,^{10,35} and also the exaggerated thrombosis reported in CRP transgenic mice.³⁶ We recently reported that the CRP transgenic mice have systolic

hypertension and supersensitivity to angiotensin II caused by a loss of vascular angiotensin receptor subtype 2 expression that is likely attributable to NO deficiency.³⁷ In contrast, despite considerable evidence for a role for inflammation in atherogenesis,³⁸ care should be taken in the extrapolation of the present findings to that disorder. Findings regarding the impact of CRP on atherosclerosis in apolipoprotein E-null mice and in hyperlipidemic rabbits have been mixed.¹² Future studies of the contribution of CRP to the pathogenesis of specific cardiovascular conditions in mice should be undertaken with the recognition that, although found to not be pertinent to the present investigation, there is uncertainty whether CRP activation of complement or signaling in immune response cells is fully operative in mice.¹²

Having demonstrated that CRP antagonism of eNOS expression occurs not only in cell culture but also in vivo, the molecular mechanisms underlying eNOS downregulation were pursued. In prior studies, CRP-induced declines in steady-state eNOS mRNA levels in cultured ECs were attributed to a 16% decrease in eNOS mRNA half-life.¹⁴ Considering that the impact of CRP on eNOS transcript abundance both in vitro¹⁴ and now in vivo exceeds the relative change reported in mRNA degradation, the effect of CRP on eNOS promoter activity was investigated. At CRP levels of 10 $\mu\text{g}/\text{mL}$ and greater, the pentraxin caused a 40% to 50% decrease in promoter activity in parallel with a decline in eNOS protein abundance, indicating that a considerable portion of the eNOS downregulation occurs at the level of gene transcription. Studies of deletion mutants of the eNOS promoter further revealed that the CRP-responsive regulatory element(s) is located within 79 bp of the 5' flanking sequence of the eNOS gene. The role of the proximal promoter in CRP action was then confirmed in electrophoretic mobility shift assays that demonstrated that the pentraxin induces nuclear protein binding to the same region. Because the region of the eNOS promoter residing between -79 and -1 does not contain sequences known to participate in eNOS gene regulation,^{28,29} studies to identify novel regulatory elements mediating CRP responsiveness are now indicated.

Pioneering experiments in cell culture have shown that multiple EC genes in addition to eNOS are likely to be modified by CRP. It has been found that CRP induces the expression of intracellular and vascular cell adhesion molecules, monocyte chemoattractant protein-1, E-selectin, the receptor for advanced glycation end products (RAGE), oxidized low-density lipoprotein receptor-1, CD40/CD40 ligand, plasminogen activator inhibitor-1, and tissue plasminogen activator, and it induces the release of endothelin-1 and interleukin-8. Furthermore, CRP impairs the expression of thrombomodulin and endothelial protein C receptor and the production of prostacyclin.¹³ Other studies performed in vitro and in vivo have demonstrated that CRP binds to and alters EC phenotype via Fc receptors, in particular CD32 (Fc receptor IIA/B) and CD64 (Fc receptor I), and that it upregulates CD32 and CD64 surface expression in cultured ECs,^{13,26} thereby potentially amplifying its own actions. Future studies of endothelial Fc receptors will provide greater understanding of the basis for CRP regulation of gene expression in endothelium in vivo.

Paralleling prior investigations of eNOS expression and action in cultured endothelium, the present studies in transgenic mice demonstrate that CRP alters EC gene expression and phenotype in vivo. These findings provide further evidence that CRP is a potential mediator of vascular dysfunction and not solely a marker of concomitant inflammation. Direct impact of CRP on EC gene transcription is also documented. Further studies in this realm will help to clarify the contribution of CRP to the pathogenesis of vascular disorders.

Sources of Funding

This work was supported by NIH grants HL75473 (to P.W.S.) and AG02467 (to D.S.), by the Donald W. Reynold's Cardiovascular Clinical Research Center (to W.V.), and by the Crystal Charity Ball Center for Pediatric Critical Care Research and the Lowe Foundation (to P.W.S.).

Disclosures

None.

References

- Black S, Kushner I, Samols D. C-reactive protein. *J Biol Chem*. 2004; 279:48487–48490.
- Du Clos TW. Function of C-reactive protein. *Ann Med*. 2000;32: 274–278.
- Du Clos TW, Mold C. The role of C-reactive protein in the resolution of bacterial infection. *Curr Opin Infect Dis*. 2001;14:289–293.
- Yeh ET, Anderson HV, Pasceri V, Willerson JT. C-reactive protein: linking inflammation to cardiovascular complications. *Circulation*. 2001; 104:974–975.
- Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, Lowe GD, Pepys MB, Gudnason V. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med*. 2004;350:1387–1397.
- Jialal I, Devaraj S, Venugopal SK. C-reactive protein: risk marker or mediator in atherothrombosis. *Hypertension*. 2004;44:6–11.
- Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest*. 2003;111:1805–1812.
- Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med*. 2002;347: 1557–1565.
- Verma S, Yeh ET. C-reactive protein and atherothrombosis—beyond a biomarker: an actual partaker of lesion formation. *Am J Physiol Regul Integr Comp Physiol*. 2003;285:R1253–R1256.
- Bautista LE, Lopez-Jaramillo P, Vera LM, Casas JP, Otero AP, Guaracao AI. Is C-reactive protein an independent risk factor for essential hypertension. *J Hypertens*. 2001;19:857–861.
- van der Meulen I, de Maat MP, Hak AE, Kiliaan AJ, Del Sol AI, Van Der Kuip DA, Nijhuis RL, Hofman A, Witteman JC. C-reactive protein predicts progression of atherosclerosis measured at various sites in the arterial tree: the Rotterdam study. *Stroke*. 2002;33:2750–2755.
- Scirica BM, Morrow DA. Is C-reactive protein an innocent bystander or proatherogenic culprit? The verdict is still out. *Circulation*. 2006;113: 2128–2134.
- Verma S, Devaraj S, Jialal I. Is C-reactive protein an innocent bystander or proatherogenic culprit? C-reactive protein promotes atherothrombosis. *Circulation*. 2006;113:2135–2150.
- Venugopal SK, Devaraj S, Yuhanna I, Shaul P, Jialal I. Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. *Circulation*. 2002;106:1439–1441.
- Verma S, Wang CH, Li SH, Dumont AS, Fedak PW, Badiwala MV, Dhillon B, Weisel RD, Li RK, Mickle DA, Stewart DJ. A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. *Circulation*. 2002;106:913–919.
- Kawasaki K, Smith RS Jr, Hsieh CM, Sun J, Chao J, Liao JK. Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis. *Mol Cell Biol*. 2003;23:5726–5737.

17. Cunningham KS, Gotlieb AI. The role of shear stress in the pathogenesis of atherosclerosis. *Lab Invest.* 2005;85:9–23.
18. Rossig L, Dimmeler S, Zeiher AM. Apoptosis in the vascular wall and atherosclerosis. *Basic Res Cardiol.* 2001;96:11–22.
19. Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M, Nickenig G. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. *Circ Res.* 2003;93:e17–e24.
20. Lin CS, Xia D, Yun JS, Wagner T, Magnuson T, Mold C, Samols D. Expression of rabbit C-reactive protein in transgenic mice. *Immunol Cell Biol.* 1995;73:521–531.
21. Pepys MB, Hawkins PN, Kahan MC, Tennent GA, Gallimore JR, Graham D, Sabin CA, Zychlinsky A, de Diego J. Proinflammatory effects of bacterial recombinant human C-reactive protein are caused by contamination with bacterial products, not by C-reactive protein itself. *Circ Res.* 2005;97:e97–e103.
22. Liu C, Wang S, Deb A, Nath KA, Katusic ZS, McConnell JP, Caplice NM. Proapoptotic, antimigratory, antiproliferative, and antiangiogenic effects of commercial C-reactive protein on various human endothelial cell types in vitro: implications of contaminating presence of sodium azide in commercial preparation. *Circ Res.* 2005;97:135–143.
23. Swafford AN Jr, Bratz IN, Knudson JD, Rogers PA, Timmerman JM, Tune JD, Dick GM. C-reactive protein does not relax vascular smooth muscle: effects mediated by sodium azide in commercially available preparations. *Am J Physiol Heart Circ Physiol.* 2005;288:H1786–H1795.
24. Seetharam D, Mineo C, Gormley AK, Gibson LL, Vongpatanasin W, Chambliss KL, Hahner LD, Cummings ML, Kitchens RL, Marcel YL, Rader DJ, Shaul PW. High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I. *Circ Res.* 2006;98:63–72.
25. Afshar S, Gibson LL, Yuhanna IS, Sherman TS, Kerecman JD, Grubb PH, Yoder BA, McCurnin DC, Shaul PW. Pulmonary NO synthase expression is attenuated in a fetal baboon model of chronic lung disease. *Am J Physiol Lung Cell Mol Physiol.* 2003;284:L749–L758.
26. Mineo C, Gormley AK, Yuhanna IS, Osborne-Lawrence S, Gibson LL, Hahner L, Shohet RV, Black S, Salmon JE, Samols D, Karp DR, Thomas GD, Shaul PW. FcγRIIB mediates C-reactive protein inhibition of endothelial NO synthase. *Circ Res.* 2005;97:1124–1131.
27. German Z, Chambliss KL, Pace MC, Arnet UA, Lowenstein CJ, Shaul PW. Molecular basis of cell-specific endothelial nitric-oxide synthase expression in airway epithelium. *J Biol Chem.* 2000;275:8183–8189.
28. Zhang R, Min W, Sessa WC. Functional analysis of the human eNOS promoter. *J Biol Chem.* 1995;270:15320–15326.
29. Karantzoulis-Fegaras F, Antoniou H, Lai S-LM, Kulkarni G, D'Abreo C, Wong GKT, Miler TL, Chan Y, Atkins J, Wang Y, Marsden PA. Characterization of the human endothelial nitric-oxide synthase promoter. *J Biol Chem.* 1999;274:3076–3093.
30. Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO III, Criqui M, Fadl YY, Fortmann SP, Hong Y, Myers GL, Rifai N, Smith SC Jr, Taubert K, Tracy RP, Vinicor F. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. *Circulation.* 2003;107:499–511.
31. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med.* 2003;9:1370–1376.
32. Duda DG, Fukumura D, Jain RK. Role of eNOS in neovascularization: NO for endothelial progenitor cells. *Trends Mol Med.* 2004;10:143–145.
33. Verma S, Kuliszewski MA, Li SH, Szmitko PE, Zucco L, Wang CH, Badiwala MV, Mickle DA, Weisel RD, Fedak PW, Stewart DJ, Kutryk MJ. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. *Circulation.* 2004;109:2058–2067.
34. Shaul PW. Regulation of endothelial nitric oxide synthase: location, location, location. *Annu Rev Physiol.* 2002;64:749–774.
35. Fichtlscherer S, Rosenberger G, Walter DH, Breuer S, Dimmeler S, Zeiher AM. Elevated C-reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease. *Circulation.* 2000;102:1000–1006.
36. Danenberg HD, Szalai AJ, Swaminathan RV, Peng L, Chen Z, Seifert P, Fay WP, Simon DI, Edelman ER. Increased thrombosis after arterial injury in human C-reactive protein-transgenic mice. *Circulation.* 2003;108:512–515.
37. Vongpatanasin W, Thomas GD, Schwartz R, Cassis LA, Osborne-Lawrence S, Hahner L, Gibson LL, Black S, Samols D, Shaul PW. C-reactive protein causes downregulation of vascular angiotensin subtype 2 receptors and systolic hypertension in mice. *Circulation.* 2007;115:1020–1028.
38. Libby P. Inflammation in atherosclerosis. *Nature.* 2002;420:868–874.

Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



C-Reactive Protein Downregulates Endothelial NO Synthase and Attenuates Reendothelialization In Vivo in Mice

Randall Schwartz, Sherri Osborne-Lawrence, Lisa Hahner, Linda L. Gibson, Andrew K. Gormley, Wanpen Vongpatanasin, Weifei Zhu, R. Ann Word, Divya Seetharam, Steven Black, David Samols, Chieko Mineo and Philip W. Shaul

Circ Res. 2007;100:1452-1459; originally published online April 19, 2007;
doi: 10.1161/01.RES.0000267745.03488.47

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circres.ahajournals.org/content/100/10/1452>

Data Supplement (unedited) at:

<http://circres.ahajournals.org/content/suppl/2007/04/19/01.RES.0000267745.03488.47.DC1>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* 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 Research* is online at:
<http://circres.ahajournals.org/subscriptions/>

Expanded Materials and Methods

Animal Model: CF1-CRP and CF1 control mice (Charles River Laboratories, Wilmington, MA) were maintained on standard chow during breeding, preweaning and postweaning periods prior to study. All experiments were performed in male mice at 12-16 weeks of age. To maximize transgene expression, the CF1-CRP mice were provided a CHO-free, high protein isocaloric diet (1). To attain CRP levels below those achieved in CF1-CRP mice, CF1-CRP were mated with CF1 mice. The resulting offspring all expressed transgene and had plasma CRP levels approximately one-half those found in CF1-CRP mice, indicating that they were hemizygous for the transgene. The hemizygotes were designated CF1-CRP-low. To minimize transgene expression in CF1-CRP-low mice, a CHO-rich isocaloric diet was administered (1). CF1 control mice received the same diet regimen during the experiments as transgenic mice. CRP levels were determined by ELISA (2,3) at the end of the protocols for carotid artery reendothelialization (see below) in additional mice that did not undergo the injury and Evan's blue dye injections; this strategy was taken to avoid both interference of phlebotomy with Evan's blue dye administration and interference in the CRP ELISA by Evan's blue dye. The care and use of all study animals was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

Carotid artery reendothelialization: Carotid artery reendothelialization was studied following perivascular electric injury in CF1, CF1-CRP and CF1-CRP-low mice as previously described (4). At 5d postinjury, animals were injected with 5% Evan's blue

dye (Sigma), arteries were harvested, and the area of denudation which incorporated the dye was quantified in a blinded manner by one investigator (DS) by image analysis using Scion Image (free software from NIH). An identical procedure was performed in additional animals on the day of perivascular electric injury, and it confirmed equal amounts of initial denudation between study groups (Supplemental Fig. 1). In prior work full reendothelialization occurred in C57BL/6 mice by 7d postinjury (4), and in preliminary studies CF1 mice also had full reendothelialization by 7d postinjury. Endothelial denudation and recovery postinjury in this model has been confirmed by immunohistochemistry for von Willebrand factor (4).

To first investigate the impact of maximal levels of CRP attainable *in vivo* with the transgene, experiments were performed in CF1 control and CF1-CRP mice placed on the CHO-free diet immediately following injury. Later studies were performed on CF1 and CF1-CRP-low mice placed in a high CHO-containing chow immediately postinjury to yield a low level of circulating CRP. In addition to testing the impact of CRP on reendothelialization, the role of endogenous NO was determined in CF1 mice by the administration of nitro-L-arginine-methyl ester (L-NAME) added to the drinking water at a dose of 100 mg/kg/d. This dose of L-NAME causes a sustained increase in blood pressure in mice to a level equivalent to the blood pressure found in mice with disruption of the eNOS gene (5). Such an elevation in BP was confirmed by radiotelemetry in additional instrumented CF1 mice (data not shown) (6). After 7d treatment with L-NAME, perivascular electric injury was performed and the mice remained on L-NAME until the area of remaining denudation was evaluated 5d postinjury.

Quantification of eNOS mRNA in mouse carotid arteries: cDNA for real-time PCR was generated from isolated RNA using a pool of oligo dT and random hexamers. Real-time PCR was performed with the ABI 7700 SDS System using the ABI protocol (<http://home.appliedbiosystems.com>). Mouse specific primers for eNOS (NM_008713) and the housekeeping gene β -2-myoglobin (β 2M, NM_009735) were designed for MGB using Primer Express 1.5a Software (ABI). They were as follows: eNOS sense 5'-CACCAGGAAGAAGACCTTTAAGGA-3' (exon 11), anti-sense 5'-CACCGTGCCCATGAGTGA-3' (exon 12) and FAM labeled probe 5'-TCTTCACTGCATTGGCT-3'; β 2M (NM_009735) sense 5'-CTATCCAGAAAACCCCTCAAATTC-3' (exon 1), anti-sense 5'-GTATGTTCCGGCTTCCCATTCTC-3' (exon 2) and FAM labeled probe: 5'-TATACTCACGCCACCCAC-3'. For quantitation, standard curves were generated for both eNOS and β 2M from mouse heart. Expression levels of eNOS were normalized to that of β 2M.

Quantification of NOS Activity in mouse carotid arteries: Immediately after harvesting, arteries were placed in ice-cold 50 mmol/L Tris buffer (pH 7.4) containing 1.0 mmol/L EDTA, 5 mmol/L mercaptoethanol, 10 μ g/mL pepstatin A, 10 μ g/mL leupeptin, 90 μ g/mL phenylmethylsulfonyl fluoride, and 1.0 μ mol/L tetrahydrobiopterin and homogenized in ground glass on ice. 50 μ L of homogenate was added to 50 μ L of the 50 mmol/L Tris buffer with final concentrations of reagents as follows: 2 mmol/L β -NADPH, 2 μ mol/L tetrahydrobiopterin, 10 μ mol/L flavin adenine dinucleotide, 10 μ mol/L flavin

mononucleotide, 0.5 $\mu\text{mol/L}$ CaCl_2 in excess of EDTA, 15 nmol/L calmodulin, 2 $\mu\text{mol/L}$ cold L-arginine, and 2.0 $\mu\text{Ci/mL}$ ^{14}C -L-arginine. After incubation at 37^o C for 60 minutes, the assay was terminated by the addition of 400 μL of 40 mmol/L HEPES buffer (pH 5.5) with 2 mmol/L EDTA and 2 mmol/L EGTA. The terminated reactions were applied to 1-mL columns of Dowex AG50WX-8 (Tris form) and eluted with 1 mL of the 40 mmol/L HEPES buffer. ^{14}C -L-citrulline was collected in scintillation vials and quantified by liquid scintillation spectroscopy. NOS activity was linear with time for up to 1h, and it was fully inhibited by 2.0 mmol/L L-NAME. In additional experiments carotid arteries from CF1 mice were incubated with recombinant human CRP ex vivo. At the amino acid level human CRP is 86% homologous to the rabbit protein which is expressed in the CF1-CRP mice, and both rabbit and human CRP bind C-polysaccharide, phosphocholine, polycations, chromatin and histones, activate complement, and protect mice from lethal pneumococcal infections (7-13). In the studies of incubated arteries the calcium-dependent and calcium-independent fractions of NOS activity were evaluated by the addition of 7.5 mmol/L EGTA. Parallel determinations on lysates of COS-7 cells expressing either eNOS or iNOS confirmed the ability to distinguish eNOS and iNOS-derived enzymatic activity by this approach. The protein content of the samples was determined by the method of Bradford (14), using bovine serum albumin as the standard.

Endothelial Cell Migration Assay: Cells were grown to near-confluence in 60-mm dishes and placed in 1% lipoprotein deficient serum (LPDS, provided by Drs. J. Goldstein and M. Brown, UT Southwestern) in Dulbecco's modified Eagle's medium

(DMEM) (Sigma) for 16h, and a defined region of cells was removed with a single-edged razor blade. Cells were treated with 0 or 50 ng/ml vascular endothelial growth factor (VEGF, Calbiochem) in the absence or presence of human recombinant CRP (10 ug/ml) in DMEM + 1% LPDS and 24 h later fixed with 3% paraformaldehyde (Sigma), permeabilized in 0.2% Triton X-100 (Bio-Rad Laboratories), stained with hematoxylin (Fisher Scientific), and viewed under an inverted microscope (Zeiss Axiovert 100M). The number of cells which had migrated past the wound edge was quantified in a minimum of 3 high power (100X) fields. Results were confirmed in a minimum of three independent experiments (4).

eNOS Promoter Activity Assay: Cells were cotransfected with -1624eNOS-Luc or deletion mutant constructs and a plasmid containing SV40-driven β -galactosidase (pSV- β -Gal: Promega Corp.) to normalize for transfection efficiency (15,16), and 24h later were placed in media and treated with either vehicle or 0 to 50 ug/ml of human recombinant CRP for 6 to 18h. The cells were lysed, extracts were centrifuged at 10,000 x g, and luciferase and β -galactosidase activity were measured (16). The results are normalized as relative luciferase light units/ β -galactosidase activity. In selected wells the cells were transfected with pGL2-Control Vector (Promega Corp.) to serve as a positive control for luciferase expression. Heat-inactivated CRP (17) did not alter eNOS promoter activity; in addition, active CRP did not change pGL2-control vector activity. Selected studies were also performed in the presence of polymyxin B to exclude effects of LPS as a possible contaminant of recombinant CRP. The actions of

human CRP preparations obtained by independent means were compared in studies of recombinant human CRP versus ascites-derived CRP (18).

References

1. Xia D, Samols D. Transgenic mice expressing rabbit C-reactive protein are resistant to endotoxemia. *Proc Natl Acad Sci U S A*. 1997;94:2575-2580.
2. Black S, Agrawal A, Samols D. The phosphocholine and the polycation-binding sites on rabbit C-reactive protein are structurally and functionally distinct. *Mol Immunol*. 2003;39:1045-1054.
3. Agrawal A, Cha-Molstad H, Samols D, Kushner I. Transactivation of C-reactive protein by IL-6 requires synergistic interaction of CCAAT/enhancer binding protein beta (C/EBP beta) and Rel p50. *J Immunol*. 2001;166:2378-2384.
4. Seetharam D, Mineo C, Gormley AK, Gibson LL, Vongpatanasin W, Chambliss KL, Hahner LD, Cummings ML, Kitchens RL, Marcel YL, Rader DJ, Shaul PW. High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I. *Circ Res*. 2006;98:63-72.
5. Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, Shesely EG, Harrison DG. Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial cell nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *Cardiovasc Res*. 1999;42:206-213.
6. Mills PA, Huetteleman DA, Brockway BP, Zwiers LM, Gelsema AJ, Schwartz RS, Kramer K. A new method for measurement of blood pressure, heart rate, and activity in the mouse by radiotelemetry. *J Appl Physiol*. 2000;88:1537-1544.

7. Lin CS, Xia D, Yun JS, Wagner T, Magnuson T, Mold C, Samols D. Expression of rabbit C-reactive protein in transgenic mice. *Immunol Cell Biol.* 1995;73:521-531.
8. Black S, Agrawal A, Samols D. The phosphocholine and the polycation-binding sites on rabbit C-reactive protein are structurally and functionally distinct. *Mol Immunol.* 2003;39:1045-1054.
9. Bach BA, Gewurz H, Osmand AP. C-reactive protein in the rabbit: isolation, characterization and binding affinity to phosphocholine. *Immunochemistry.* 1977;14:215-219.
10. Oliveira EB, Gotschlich EC, Liu TY. Comparative studies on the binding properties of human and rabbit C-reactive proteins. *J Immunol.* 1980;124:1396-1402.
11. Robey FA, Jones KD, Tanaka T, Liu TY. Binding of C-reactive protein to chromatin and nucleosome core particles. A possible physiological role of C-reactive protein. *J Biol Chem.* 1984;259:7311-7316.
12. Dougherty TJ, Gewurz H, Siegel JN. Preferential binding and aggregation of rabbit C-reactive protein with arginine-rich proteins. *Mol Immunol.* 1991;28:1113-1120.
13. Reifenberg K, Lehr HA, Baskal D, Wiese E, Schaefer SC, Black S, Samols D, Torzewski M, Lackner KJ, Husmann M, Blettner M, Bhakdi S. Role of C-reactive protein in atherogenesis: can the apolipoprotein E knockout mouse provide the answer? *Arterioscler Thromb Vasc Biol.* 2005;25:1641-1646.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-254.
15. German Z, Chambliss KL, Pace MC, Arnet UA, Lowenstein CJ, Shaul PW.

Molecular basis of cell-specific endothelial nitric-oxide synthase expression in airway epithelium. *J Biol Chem.* 2000;275:8183-8189.

16. Gibson LL, Hahner L, Osborne-Lawrence S, German Z, Wu KK, Chambliss KL, Shaul PW. Molecular basis of estrogen-induced cyclooxygenase type 1 upregulation in endothelial cells. *Circ Res.* 2005;96:518-525.

17. Mineo C, Gormley AK, Yuhanna IS, Osborne-Lawrence S, Gibson LL, Hahner L, Shohet RV, Black S, Salmon JE, Samols D, Karp DR, Thomas GD, Shaul PW. FcγRIIB mediates C-reactive protein inhibition of endothelial NO synthase. *Circ Res.* 2005;97:1124-1131.

18. Agrawal A, Simpson MJ, Black S, Carey MP, Samols D. A C-reactive protein mutant that does not bind to phosphocholine and pneumococcal C-polysaccharide. *J Immunol.* 2002;169:3217-3222.

Supplemental Figure I

Area of initial denudation is similar in control and CRP transgenic mice. The intimal surface of Evan's blue-stained arteries from an (A) CF1 control and (B) CF1-CRP mouse on the day of injury (D1). C. Area of denudation was quantified and is expressed in arbitrary units. Values in C are mean \pm SEM, n = 8 mice/group.

