# Inflammation and Endothelial Function: Direct Vascular Effects of Human C-Reactive Protein on Nitric Oxide Bioavailability

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# Inflammation and Endothelial Function

## Direct Vascular Effects of Human C-Reactive Protein on Nitric Oxide Bioavailability

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***Background***—Circulating concentrations of the sensitive inflammatory marker C-reactive protein (CRP) predict future cardiovascular events, and CRP is elevated during sepsis and inflammation, when vascular reactivity may be modulated. We therefore investigated the direct effect of CRP on vascular reactivity.

***Methods and Results***—The effects of isolated, pure human CRP on vasoreactivity and protein expression were studied in vascular rings and cells in vitro, and effects on blood pressure were studied in rats in vivo. The temporal relationship between changes in CRP concentration and brachial flow-mediated dilation was also studied in humans after vaccination with *Salmonella typhi* capsular polysaccharide, a model of inflammatory endothelial dysfunction. In contrast to some previous reports, highly purified and well-characterized human CRP specifically induced hyporeactivity to phenylephrine in rings of human internal mammary artery and rat aorta that was mediated through physiological antagonism by nitric oxide (NO). CRP did not alter endothelial NO synthase protein expression but increased protein expression of GTP cyclohydrolase-1, the rate-limiting enzyme in the synthesis of tetrahydrobiopterin, the NO synthase cofactor. In the vaccine model of inflammatory endothelial dysfunction in humans, increased CRP concentration coincided with the resolution rather than the development of endothelial dysfunction, consistent with the vitro findings; however, administration of human CRP to rats had no effect on blood pressure.

***Conclusions***—Pure human CRP has specific, direct effects on vascular function in vitro via increased NO production; however, further clarification of the effect, if any, of CRP on vascular reactivity in humans in vivo will require clinical studies using specific inhibitors of CRP. (***Circulation*. 2005;111:1530-1536.**)

**Key Words:** endothelium ■ inflammation ■ nitric oxide

C-reactive protein (CRP) is an exquisitely sensitive systemic marker of inflammation and tissue damage,[1] the concentration of which is associated with future development of atherothrombotic events both in patients with known cardiovascular disease[2–4] and in healthy individuals in the general population, albeit less strongly in the latter than previously suggested.[5] CRP is present in most atherosclerotic lesions,[6,7] and via its binding to lipids and lipoproteins and its capacity to activate the classic complement pathway, it has the potential to contribute to atherogenesis.[1] Recently, it has been suggested that CRP also affects nitric oxide (NO) pathways,[8,9] and this would be of interest in relation to the association between endothelial dysfunction and atherosclerosis. However, it would be remarkable if CRP, the plasma concentration of which can cover a dynamic range of up to 10 000-fold (0.05 to 500 mg/L) with a half-time of ≈24 hours,[10,11] was actually an important regulator of tone of either resistance or conduit vessels in vivo. We have therefore investigated the effect of highly purified and very-well-characterized human CRP on arterial tone and observed hyporeactivity to phenylephrine (PE) mediated by increased rather than reduced NO production.

## Methods

### Human CRP

Malignant effusion fluids removed for therapeutic purposes were collected from patients without active infections. Source material tested negative for hepatitis B and C, anti-HIV-1, and HIV-2. CRP was purified by sequential DEAE anion exchange and ligand-elution calcium-dependent affinity chromatography on phosphoethanolamine-carboxyhexyl Sepharose.[12,13] After calcium chelation and concentration,

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the CRP was buffer exchanged into 10 mmol/L Tris, 140 mmol/L NaCl, 2 mmol/L $CaCl_2$, pH 8.0 (Tris-saline-calcium buffer) that contained no preservatives or bacteriostatic agents. The CRP was >99% pure by overloaded silver-stained 8%- to 18%-reduced SDS-PAGE, was all in its native pentameric form by analytical size-exclusion chromatography, and was fully functional by calcium-dependent binding to immobilized phosphoethanolamine. CRP concentration in the final preparation was established by absorbance at 280 nm, corrected for light scattering at 320 nm and using the specific extinction coefficient for pure CRP (1.75 for 1 mg/mL) established by interferometric measurement of refractive index.[14] The Gram-negative bacterial lipopolysaccharide (LPS) content was 100 pg/mg of CRP, measured by the kinetic chromogenic *Limulus* amebocyte lysate assay (BioWhittaker Europe S.P.R.L.). Plasma CRP in the in vivo study of experimental inflammation was determined with a high-sensitivity, automated, microparticle-enhanced latex turbidimetric immunoassay[15] (COBAS MIRA; Roche Diagnostics GmbH). The lower limit of detection was 0.2 mg/L, with an interassay coefficient of variation of 4.2% at 4 mg/L and 6.3% at 1 mg/L.

## In Vitro Studies

### *Organ Bath*

Rings of thoracic aorta from male Sprague-Dawley rats and of human internal mammary artery from bypass operation were incubated in 0.5 mL of serum-free Dulbecco's modified Eagle medium (GibcoBRL Life Technologies) containing human CRP (2 to 200 mg/L) or Tris-saline-calcium buffer in 95% $O_2$/5% $CO_2$ at 37°C for 4 hours and then mounted in organ baths containing Krebs solution. Changes in isometric tension were measured with a force-displacement transducer (FT03) connected through a MacLab to a computer. Endothelium was considered intact if acetylcholine (ACh; $10^{-6}$ mol/L) produced >50% reversal of contraction to $10^{-7}$ mol/L PE. When endothelium was removed by gentle rubbing, rings exhibited <1% relaxation to ACh. Concentration-response curves to $10^{-9}$ to $10^{-5}$ mol/L PE were constructed, and rings were then submaximally precontracted with PE (to 80% of maximal contraction to 48 mmol/L KCl) before relaxation with $10^{-9}$ to $10^{-5}$ mol/L ACh or sodium nitroprusside. In some experiments, vessels were preincubated for 30 minutes with either $3\times10^{-4}$ mol/L nitro-L-arginine methyl ester (L-NAME) or $10^{-5}$ mol/L 1400W {*N*-[3-(aminomethyl)benzyl]acetamidine, AG Scientific Inc} to block NO synthesis; $10^{-5}$ mol/L actinomycin D for 4 hours to block protein synthesis; $10^{-4}$ mol/L methoxyacetylserotonin (MAS) for 30 minutes before the addition of CRP/vehicle to block tetrahydrobiopterin ($BH_4$) synthesis; 10 μg/mL polymyxin to bind LPS; or $8.69\times10^{-3}$ mol/L RMM, a novel low-molecular-weight inhibitor of CRP binding.[16]

### *Endothelial Cell Culture*

Eighty-percent–confluent passage-5 human coronary aortic endothelial cells (HCAECs; Promocell, Heidelberg, Germany) grown in the manufacturer's growth medium supplemented with 2% vol/vol fetal calf serum, 0.05 mg/mL amphotericin B, and 50 μg/mL gentamicin were incubated for 24 hours with pure human CRP (final concentration 50 mg/L) or an equal volume of Tris-saline-calcium buffer. Cells were lysed with 0.01 mol/L sodium phosphate buffer containing 1% vol/vol Nonidet, 1% wt/vol sodium deoxycholate, 0.1% vol/vol SDS, and EDTA-free complete protease inhibitor (Roche), disrupted by sonication, and collected by centrifugation.

### *Western Blotting*

Equal quantities of protein solution were mixed with loading buffer, heated to 99°C for 10 minutes, separated by SDS-PAGE, and electrotransferred to nitrocellulose membranes (Hybond-P). These were blocked with 5% vol/vol milk solution for 3 hours before overnight incubation with rabbit anti-human endothelial NO synthase (eNOS; c-20) or a GTP cyclohydrolase-1 (GTPCH-1) anti-peptide antibody followed by a horseradish peroxidase–linked secondary antibody (Santa Cruz Biotechnology). Membranes were developed with the ECL plus system (Amersham). Coomassie blue staining of gels confirmed equal protein transfer. Scion image 4.02 software was used for semiquantitative analysis of Western blots.

## Human Studies

All human studies were approved by the institutional research ethics committees, and all participants gave informed consent.

### *Experimental Inflammation*

Twelve healthy, nonsmoking male volunteers (mean age [SD] 28 [6] years) received *Salmonella typhi* capsular polysaccharide vaccine 0.025 mg (Typhim Vi, Pasteur Merieux MSD) by gluteus intramuscular injection at 8 AM on day 2 of a 4-day study.[17] Venous blood was taken before and 4, 8, 32, and 56 hours after injection to provide plasma in lithium heparin and citrate. CRP was assayed as described above, and interleukin (IL)-6 and IL-1 receptor antagonist (IL-1Ra) were assayed by ELISA (R & D Systems).[17] NO-mediated, endothelium-dependent vasodilation was assessed by high-resolution ultrasound of the brachial artery during reactive hyperemia before and 8, 32, and 56 hours after vaccination.[18] Vessel diameter was measured with edge-detection software (Brachial Tools) and expressed as the percentage change from baseline. Endothelium-independent dilation was assessed after 25 μg of sublingual nitroglycerin. Four subjects were identically reassessed in the absence of an inflammatory stimulus.

### *Cohort Study*

Plasma samples from 579 healthy, middle-aged, male Northwick Park Heart Study II participants[19] were used to study correlations between plasma neopterin concentration (as an index of pterin pathway activation) and CRP, measured on a BN Prospec (Dade Behring). Interassay and intra-assay coefficients of variation were <4% and <2%, respectively, with a CRP detection limit of 0.20 mg/L. Neopterin was measured by ELISA (Brahms) with a lower limit of detection of 2 nmol/L and intra-assay and interassay coefficients of variation of 3.5% and 6.5%, respectively.

## Rat In Vivo Study

Male Sprague-Dawley rats (mean [SD] weight 310.8 g [11.3]) with free access to food and water were anesthetized by intraperitoneal injection of isoflurane 60 mg/kg. A jugular line was sited for infusions, and a carotid line was sited for continuous monitoring of blood gas and pressure (P23XL transducer, Viggo-Spectramed) on a precalibrated PowerLab system (AD Instruments). A tracheostomy tube was sited, but the animals continued to breathe spontaneously. After stabilization, 3 consistent hemodynamic measurements at 5-minute intervals were obtained before injection with CRP 20 mg/kg (n=6) or control buffer (n=7) in a volume of 400 μL. Mean arterial blood pressure was recorded before CRP or buffer and every 15 minutes thereafter for 240 minutes. Blood for human CRP measurement was collected 5 minutes after injection.

## Statistical Analysis

Data are presented as mean and SEM unless otherwise stated or as geometric mean and approximate SD for CRP. Concentration-response curves were compared by 2-way ANOVA. Plasma concentrations of analytes and endothelial function measures were analyzed by paired *t* test with correction for multiple comparison, linear regression, or ANOVA. Significance was taken as $P\leq0.05$.

# Results

## CRP Causes PE Hyporeactivity in Vascular Rings by an Endothelium-Dependent Mechanism

Human CRP (200 mg/L) caused hyporeactivity to PE in rat aortic rings similar to that observed with *S typhi* LPS (50 ng/mL; Figure 1A; $P<0.0001$ for both CRP and LPS compared with control). Similar effects were seen at concentrations of CRP observed in health (2 mg/L) or mild inflammation (20 mg/L), with no indication of a substantial dose-response relationship over this time course (Figure 1B). This effect was also seen in the physiological, homologous system of human internal mammary artery rings (Figure 1C;

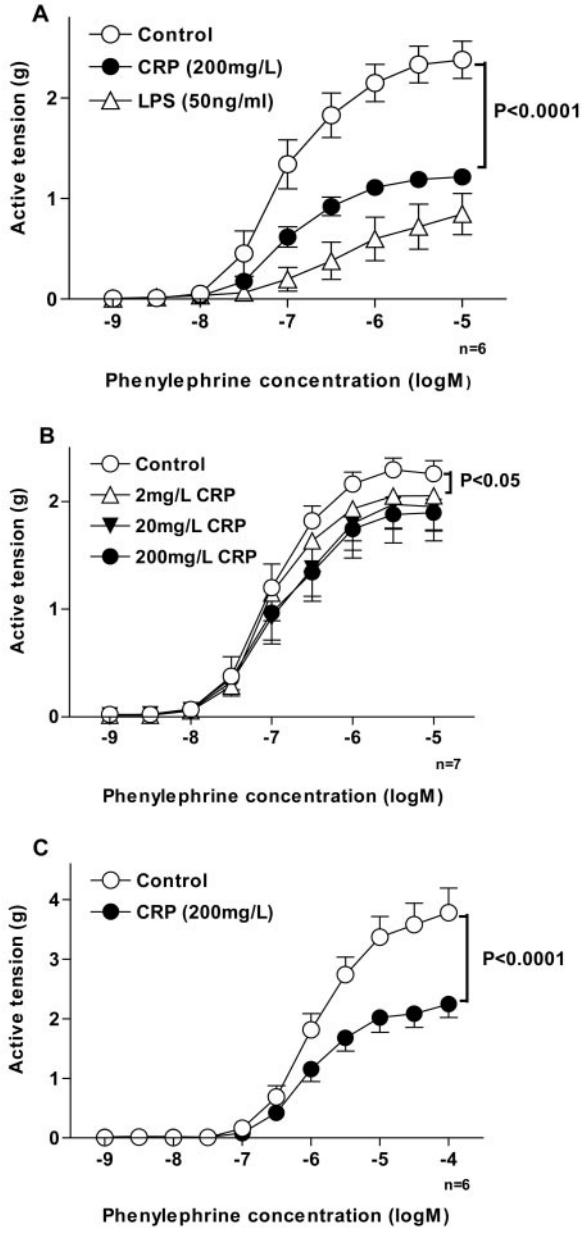


**Figure 1.** Effect of CRP on PE concentration response of arterial rings in vitro. A, Rat aorta after exposure for 4 hours to CRP or LPS, compared with control (n=6; $P<0.0001$ for both CRP and LPS vs control). B, Rat aorta after exposure for 4 hours to different concentrations of CRP (n=7; $P<0.05$ for all CRP concentrations vs control). C, Human internal mammary artery after exposure for 4 hours to CRP, compared with control (n=6; $P<0.0001$ for CRP vs control).

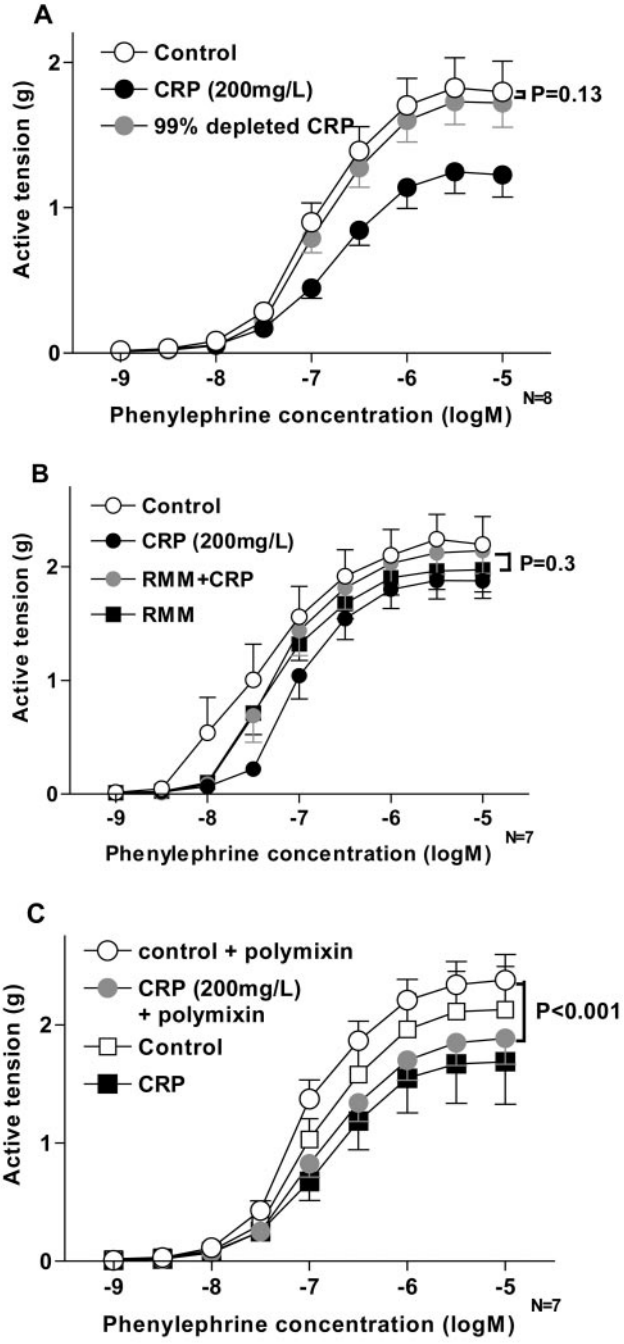


**Figure 2.** Specificity of CRP effect. A, Preabsorption of CRP preparation with Sepharose-phosphoethanolamine (n=8; $P=0.13$ for depleted CRP solution vs control). B, Inhibition by presence of RMM throughout incubation with CRP (n=7; $P=0.3$ for RMM with CRP vs RMM alone). C, No effect of addition of 10 $\mu$g/mL polymyxin to CRP (n=7; $P<0.001$ for CRP in presence of polymyxin vs polymyxin alone).

$P<0.0001$), which shows that it is not simply a heterologous, cross-species phenomenon. The effects were specific to CRP because they were abolished when the CRP was preincubated with Sepharose-phosphoethanolamine, to which all the CRP binds avidly so that no CRP was detectable in the fluid phase (Figure 2A). The action of CRP was also blocked by RMM (Figure 2B). This novel low-molecular-weight compound is bound by CRP with a $K_d$ of $\approx$500 nmol/L and potently inhibits specific calcium-dependent binding of CRP to its known ligands, so the vascular effect observed here requires ligand binding by CRP. The effects of the CRP preparation were not explained by the presence of LPS, because the stock CRP solution and its buffer, which had no effect, both contained <0.5 ng/mL LPS. Furthermore, preincubation of the CRP with polymyxin to bind LPS did not prevent PE hyporeactivity (Figure 2C). Endothelial denudation before incubation with CRP (Figure 3A) or non–isoform-selective NO synthase inhibition with L-NAME (Figure 3B) after incubation with CRP prevented the effect of CRP, which

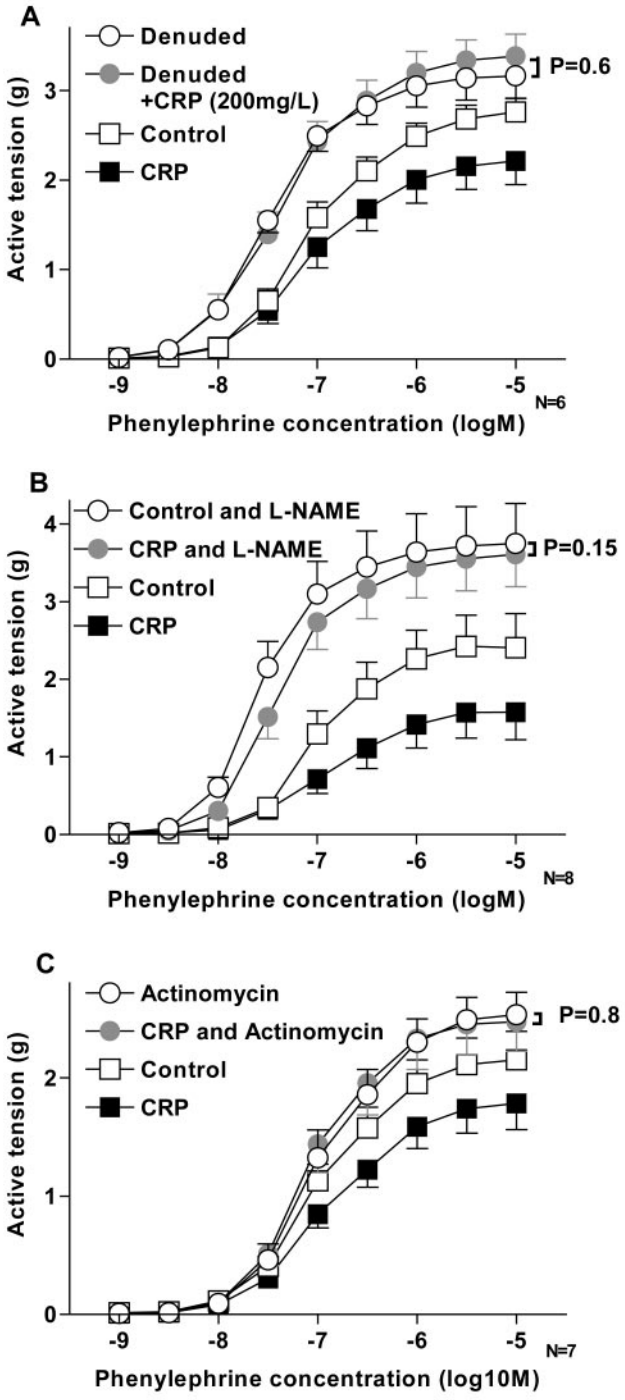


**Figure 3.** Effects of endothelial denudation, NO, and actinomycin D on CRP effect in rat aorta. A, Endothelial denudation abolishes CRP-induced hyporeactivity to PE (n=6; $P=0.6$ for CRP vs control). B, L-NAME abolishes CRP-induced hyporeactivity to PE (n=8; $P=0.15$ for CRP in presence of L-NAME vs L-NAME alone). C, Actinomycin D prevents CRP-induced hyporeactivity to PE (n=7; $P=0.8$ for CRP with actinomycin D vs actinomycin D alone).

suggests that PE hyporeactivity is mediated by endothelium-derived NO. Actinomycin D blocked CRP-induced hyporeactivity to PE (Figure 3C), which indicates that new protein synthesis is required.

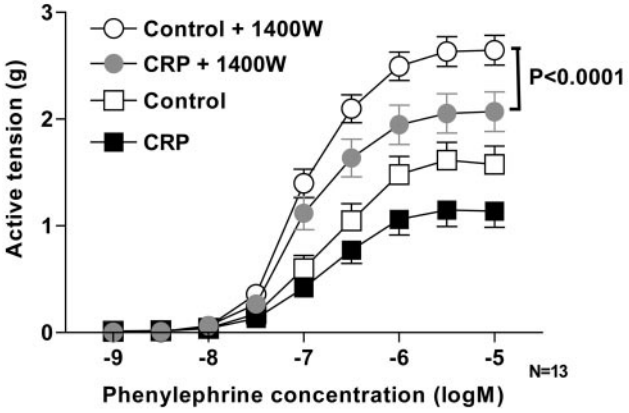


**Figure 4.** Effect of selective iNOS inhibition in rat aorta. Selective iNOS inhibition with 1400W does not alter CRP-induced hyporeactivity to PE (n=13; $P<0.0001$ for CRP plus 1400W vs 1400W alone).

## CRP Augments NO Availability Independently of Inducible NO Synthase Induction

To exclude a contribution from inducible NO synthase (iNOS)-derived NO, studies were performed with the iNOS-specific inhibitor 1400W. Although 1400W increased PE constriction in control vessels, which indicates a degree of iNOS induction during the incubation period, it did not affect the PE hyporeactivity induced by CRP, which suggests that this effect is independent of iNOS (Figure 4). In a separate series of experiments, 1400W markedly attenuated the hyporeactivity induced by LPS, which is considered to be mediated by iNOS-derived NO (n=4; $P<0.0001$; data not shown).

## CRP Augments NO Availability by Increasing Synthesis of $BH_4$

In rat aortic rings incubated with CRP and precontracted with PE, there was a small but significant increase in the sensitivity to the endothelium-dependent vasodilator ACh (Figure 5A; $P<0.0001$), with no change in the response to the endothelium-independent vasodilator sodium nitroprusside (Figure 5B), consistent with increased NO availability. Despite increased NO availability and the requirement for new protein synthesis, CRP did not alter eNOS expression in HCAECs (Figure 6A); however, NO availability can be increased through elevations in $BH_4$, in the absence of transcriptional induction of eNOS.[20,21] In HCAECs in culture, CRP upregulated expression of GTPCH1, the rate-limiting enzyme in $BH_4$ synthesis (Figure 6B). Moreover, MAS, an inhibitor of sepiapterin reductase, the terminal enzyme in $BH_4$ biosynthesis, prevented CRP-mediated hyporeactivity to PE (Figure 6C).

## Elevations in CRP Coincide With Recovery of Endothelial Function After Experimental Inflammation

In healthy volunteers, vaccination with *S typhi* capsular polysaccharide[17] caused a significant reduction in NO-dependent flow-mediated dilation of the brachial artery (7.7±0.8% before vaccination versus 6.0±0.9% 8 hours after vaccination, $P=0.05$; Figure 7A). At the time of maximal

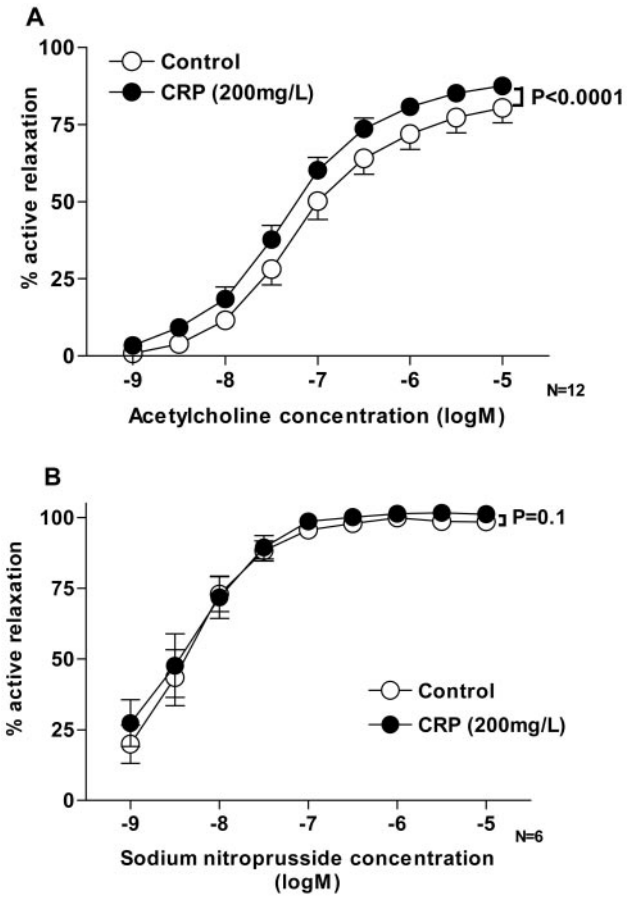


**Figure 5.** Effect of CRP on vasodilator responses in endothelium-intact rat aorta. A, Enhanced response to endothelium-dependent vasodilation induced by ACh (n=12; $P<0.0001$ for CRP vs control). B, No alteration of endothelium-independent vasodilation induced by sodium nitroprusside in rat aortic rings (n=6; $P=0.3$ for CRP vs control).

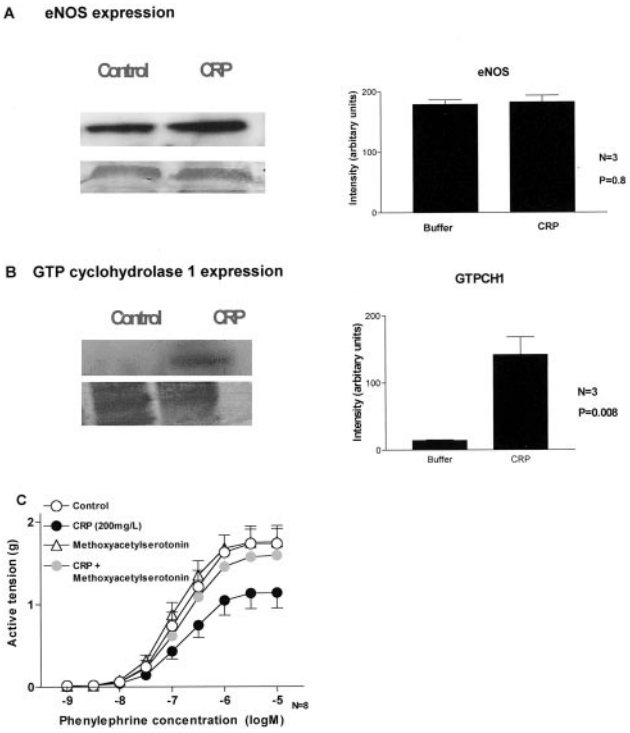


**Figure 6.** Mechanism of CRP effect on NO synthesis. eNOS and GTPCH-1 expression in HCAECs. A, No change in eNOS expression. Western blot for eNOS (above) and Coomassie stain for total protein (below) after incubation of cells for 24 hours with 50 mg/L CRP or control medium. B, Increased expression of GTPCH-1. Western blot for GTPCH-1 (above) and Coomassie stain for total protein (below) after incubation of cells for 24 hours with 50 mg/L CRP or control medium. C, Specific inhibition of $BH_4$ synthesis in rat aorta by MAS blocks CRP effect (n=8; $P<0.0001$ for CRP alone vs CRP plus MAS).

endothelial dysfunction, 8 hours after vaccination, plasma CRP concentration had not changed significantly (0.89 [0.88] mg/L at baseline versus 1.06 [0.92] mg/L at 8 hours). However, by 32 hours after vaccination, coincident with recovery of endothelial function, CRP had risen 3-fold to 2.7 (1.8) mg/L ($P<0.01$ compared with baseline; Figure 7A). In contrast, IL-6 and IL-1Ra were both elevated as early as 4 hours after vaccination, before the development of maximal endothelial dysfunction, and had returned to baseline by 32 hours (IL-6 0.87±0.08 pg/mL at baseline and 5.8±1.6 pg/mL at 4 hours, and IL-1Ra 265±49 pg/mL at baseline and 363±101 pg/mL at 4 hours, $P<0.05$ for both comparisons; Figure 7B). Baseline vessel diameters, response to nitroglycerin, and other parameters were unaltered during the study. Neither CRP nor flow-mediated dilation varied in time-control studies in 4 subjects who were not vaccinated ($P=0.6$ and 0.5, respectively).

## Correlation of CRP With Neopterin

In 579 healthy middle-aged men evaluated in the absence of clinically apparent infection or inflammation, plasma CRP correlated with plasma neopterin, albeit weakly ($r=0.11$, $P=0.007$; Figure 8).

## Effect of Human CRP on Blood Pressure in Rats

Immediately after injection of human CRP, rats had plasma concentrations of 303 to 342 mg/L, which corresponded to a major human acute-phase response. However, there was no consistent difference in blood pressure compared with control animals, mean (SD) initial and final blood pressures being 95.8 (3.4) and 87.8 (8.8) mm Hg compared with 95.7 (7.2) and 87.5 (8.3) mm Hg in controls (Figure 9).

# Discussion

Functionally active, highly purified human CRP directly increased rather than decreased NO bioavailability in blood vessels in vitro, in contrast to previous reports[8,22] that used commercially sourced CRP preparations. This action was seen in both human and rat vessels over a range of concentrations of CRP, although without a significant concentration-response relationship within the time studied. This phenomenon was robust and specific, being abolished by affinity removal of CRP from solution before addition to the vessel preparation. The effect was also blocked by a specific inhibitor of calcium-dependent ligand binding by CRP and was not attributable to LPS contamination. Few recent reports on actions of CRP in vitro[8,9,23–25] describe characterization of the source, purity, assay, or functional integrity of the preparation used, the use of specificity controls, or the

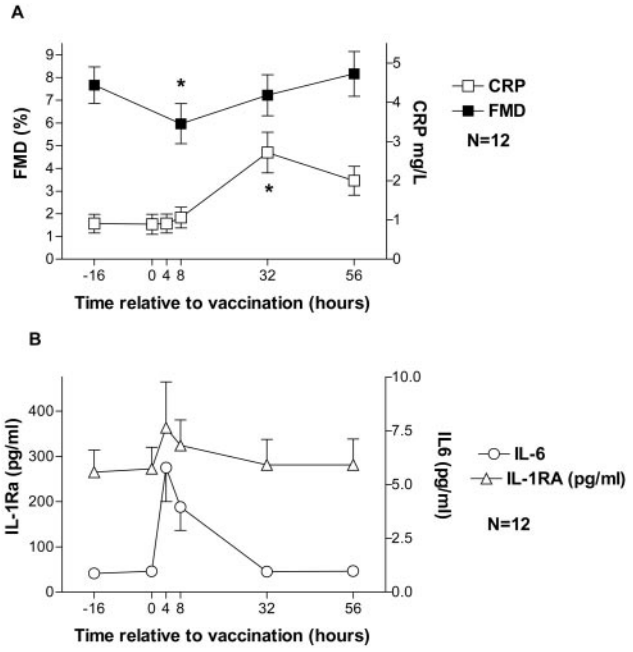


**Figure 7.** Time course of change in flow-mediated dilatation (FMD) of brachial artery after *S typhi* vaccination of healthy volunteers. A, FMD (■) and plasma CRP concentration (□; n=12; $P \leq 0.05$ by 1-way ANOVA for both CRP and FMD). B, Plasma concentration of IL-6 (○) and IL-1Ra (△) in same experiment (n=12; $P < 0.05$ by 1-way ANOVA for both IL-6 and IL-1Ra).

removal of preservatives[26] such as sodium azide with known vasoactive effects[27,28] that are present in commercial CRP preparations.

In contrast to a previous report of relaxation of human artery by recombinant human CRP in vitro,[26] an effect that may be due to the presence of sodium azide in the buffer,[29] the effect observed here was prevented by endothelial denudation or by inhibitors of NO synthase and was consistent with activation of eNOS rather than induction of iNOS. Although it required new protein synthesis, the action of CRP was independent of increased eNOS expression. Instead, CRP induced the expression of GTPCH-1, the rate-limiting enzyme in the synthesis of $BH_4$. Increases in the concentration

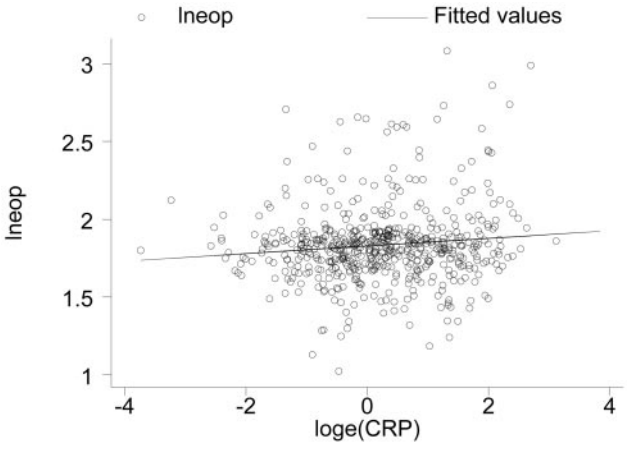


**Figure 8.** Correlation between plasma neopterin and CRP (both log transformed) among 579 men from Northwick Park Heart Study II.

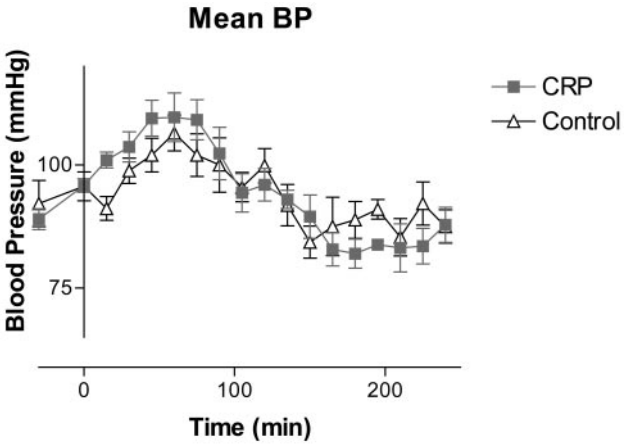


**Figure 9.** Mean blood pressure (BP) before and after injection of male Sprague-Dawley rats with human CRP 20 mg/kg (n=6) or control buffer (n=7).

of $BH_4$ mediated by inflammatory cytokines,[20] or estrogens,[21] can lead to increases in NO synthesis from eNOS, independent of altered enzyme expression. The correlation of CRP and neopterin, a byproduct of GTPCH-1 activity, in a large cohort of healthy individuals is consistent with a possible link between CRP and GTPCH-1 activation in vivo but obviously does not prove it. Whether CRP is capable of altering NO synthase activity by other mechanisms (eg, phosphorylation) requires further study.

It has been suggested, on the basis of previous limited in vivo association studies, that increased circulating CRP may cause endothelial dysfunction.[30,31] We examined this in a model of inflammatory endothelial dysfunction in humans in which, after intramuscular administration of *S typhi* capsular polysaccharide vaccine, impaired flow-mediated dilation of the brachial artery at 8 hours is preceded by increases in plasma IL-6 and IL-1Ra concentration. However, with a suitably precise and high-sensitivity assay, there was no significant increase in CRP concentration before the development of endothelial dysfunction. CRP increased significantly only after the nadir of the flow-mediated dilation response, and its rise coincided with recovery of endothelial function. Because flow-mediated dilation is almost exclusively dependent on the release of endothelial NO,[32] this observation is consistent with our in vitro findings of increased NO generation from the endothelium of isolated vascular rings, although it does not prove a causal relationship. Direct vascular effects could thus conceivably be a function of CRP relevant to cardiovascular disease and to changes in vascular reactivity seen during episodes of systemic inflammation. Although the enormous dynamic range of CRP in vivo argues strongly against CRP having a major role in modulation of vascular tone, it is consistent with the poor concentration-response relationship observed here in vitro and with the absence of an acute effect of human CRP on blood pressure in rats in vivo. Elucidation of these questions, and other aspects of the possible role of CRP in cardiovascular disease, will require the introduction of selective drugs or other therapeutic interventions that specifically block the actions of CRP in vivo.

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