C-Reactive Protein Inhibits Endothelium-Dependent NO-Mediated Dilation in Coronary Arterioles by Activating p38 Kinase and NAD(P)H Oxidase

Erion Qamirani, Yi Ren, Lih Kuo, Travis W. Hein

Objective—Elevated levels of C-reactive protein (CRP), a proinflammatory marker, are associated with reduced systemic endothelium-dependent NO-mediated dilation in patients with coronary artery disease; however, the direct effect of CRP on coronary microvascular reactivity remains unknown. Herein, we examined whether CRP can modulate endothelium-dependent NO-mediated dilation of coronary arterioles and whether proinflammatory signaling pathways such as stress-activated protein kinases (p38 and c-Jun N-terminal kinase [JNK]) and oxidative stress are involved in the CRP-mediated effect.

Methods and Results—Porcine coronary arterioles were isolated and pressurized without flow for in vitro study. Intraluminal treatment with a clinically relevant concentration of CRP (7 μg/mL; 1 hour) significantly attenuated the NO release and vasodilation to serotonin. Further incubation with the NO precursor L-arginine (3 mmol/L) partially restored serotonin-induced vasodilation. In the presence of superoxide scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), NAD(P)H oxidase inhibitor apocynin, or p38 kinase (an upstream activator of NAD(P)H oxidase) inhibitor SB203850, but not xanthine oxidase inhibitor allopurinol or JNK inhibitor SP600125, the detrimental effect of CRP on serotonin-induced dilation was prevented. Dihydroethidium staining showed that CRP produced SB203850- and TEMPOL-sensitive superoxide production in the arteriolar endothelium. CRP treatment of coronary arterioles significantly increased NAD(P)H oxidase activity.

Conclusions—CRP inhibits endothelium-dependent NO-mediated dilation in coronary arterioles by producing superoxide from NAD(P)H oxidase via p38 kinase activation. By impairing endothelium-dependent NO-mediated vasoreactivity, CRP could facilitate the initiation of numerous cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2005;25:995-1001.)

Key Words: C-reactive protein ■ nitric oxide ■ free radicals ■ coronary artery disease

A growing wealth of evidence supports the paradigm that inflammation plays a pivotal role in the development and progression of atherosclerosis.1 C-reactive protein (CRP) is an acute-phase inflammatory marker that has been shown in several prospective studies to be an independent risk factor for cardiovascular events such as stroke, myocardial infarction, and coronary artery disease.2-4 Recent evidence suggests that CRP may not only be a marker but also a mediator of inflammation and atherogenesis via direct effects on leukocytes and vascular cells. For example, CRP promotes monocyte chemotaxis5 and facilitates low-density lipoprotein uptake by macrophages in vitro.6 In vascular smooth muscle cells, CRP has been shown to increase angiotensin type 1 receptor number and angiotensin type 1 receptor–mediated reactive oxygen species formation,7 as well as activation of stress-activated protein kinases p38 kinase and c-Jun N-terminal kinase (JNK).8 In endothelial cells, CRP facilitated the release of plasminogen activator inhibitor-19 and endothelin-1,10 increased the expression of cell adhesion molecules,10,11 and reduced NO bioavailability.12,13

The mechanism responsible for the reduced NO bioavailability remains unclear, but a plausible explanation could be related to increased vascular oxidative stress. This idea is apparently supported by recent evidence showing that CRP can increase the production of superoxide, an NO scavenger, in cultured human aortic endothelial cells;14 however, the specific enzyme source of superoxide and the underlying signaling mechanism involved in its activation by CRP have not been examined. Interestingly, a recent clinical study has found a correlation between elevated CRP levels, increased oxidative stress, and reduced NO bioavailability in the systemic circulation of patients with coronary artery dis-
ease. However, because of the confounding influences of vascular cells with either blood-borne substances or circulating cells in vivo studies, it remains unknown whether CRP can directly influence NO-mediated function by increasing oxidative stress in the coronary circulation. Because coronary arterioles are the predominant vessels regulating blood flow in the heart, and endothelial release of NO plays an important role in vasodilation, understanding the direct impact of CRP on NO-mediated dilation of resistance vessels is an important area of investigation. In the present study, we tested the hypothesis that CRP inhibits endothelium-dependent NO-mediated dilation of coronary arterioles by increasing vascular superoxide production. By using an isolated-arteriole preparation, we examined the endothelium-dependent NO-mediated dilation of porcine coronary arterioles in the absence and presence of CRP. We also investigated whether key vascular signaling molecules in oxidative stress, such as distinct superoxide-generating enzymes and stress-activated protein kinases, are involved in the CRP-mediated effect.

Methods
Functional Assessment of Isolated Coronary Arterioles
The procedures were approved by the laboratory animal care committees at Texas A&M University and Scott & White Memorial Hospital, and have been described previously. Briefly, pigs (8 to 12 weeks old of either sex; weighing 7 to 10 kg) were anesthetized with ketamine, and ventilated with pentobarbital (20 mg/kg) and ventilated. The heart was removed and immediately placed on iced (5°C) saline. Subepicardial arteriolar branches (~1 mm in length; 40 to 100 μm in internal diameter in situ) were dissected from the surrounding cardiac tissue. Vessels were then cannulated with glass micropipettes, pressurized to 60 cm H2O intraluminal pressure, and bathed in physiological salt solution (PSS) at 37°C. The inner diameter of coronary arterioles was measured using video microscopic techniques.

The human recombinant CRP (Calbiochem) used in the following protocols was initially dialyzed for 24 hours against Dulbecco’s PBS using a dialysis slide (Pierce) with a cutoff of 10 kDa to remove sodium azide, which is present as a preservative in commercial preparations of CRP. Endotoxin, which can affect endothelial function, was also removed from the CRP by using Detoxi-Gel Columns (Pierce) and found to be <0.06 EU/mL by the Limulus assay (Cambrex). To assess the effect of CRP on NO-mediated vasodilation, dose-dependent vasodilation to an endothelium-dependent NO pathway agonist serotonin, to an endothelium-dependent hyperpolarizing factor (EDHF) pathway agonist bradykinin, or to endothelium-independent agents sodium nitroprusside and acidoxis was established before and after 60-minute intraluminal incubation of vessels with either a subclinical (0.7 μg/mL) or clinically relevant (7 μg/mL) concentration of CRP. As described previously, acidosis-induced vasodilation was studied by adding HCl (100 μmol/L) at 37°C for 60 minutes and then stained with DHE (4 μmol/L) for 30 minutes. After being washed, arterioles were embedded in OCT compound (Tissue-Tek) for cryostat sections. The embedded arterioles were cut into 12-μm thick sections and placed on glass slides. Images were taken with a fluorescence microscope (Nikon Diaphot 300) and Digital Sight Camera (model DS-5M-L1). Fluorescence was detected with a 610-nm emission filter. Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental tissues.

NAD(P)H Oxidase Activity Assay
The effect of CRP on NAD(P)H oxidase activity in isolated coronary arterioles (5 to 7 vessels per sample; ~100 μm in diameter with 1 to 2 mm in length) was determined by measuring superoxide production via lucigenin-enhanced chemiluminescence with slight modification of methods described previously. Vessels were incubated with PSS containing vehicle, CRP (7 μg/mL), CRP plus TEMPOL (1 mmol/L), or CRP plus SB203850 (0.1 μmol/L) at 37°C for 60 minutes and then stained with DHE (4 μmol/L) for 30 minutes. After being washed, arterioles were embedded in OCT compound (Tissue-Tek) for cryostat sections. The embedded arterioles were cut into 12-μm thick sections and placed on glass slides. Images were taken with a fluorescence microscope (Nikon Diaphot 300) and Digital Sight Camera (model DS-5M-L1). Fluorescence was detected with a 610-nm emission filter. Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental tissues.

RNA Isolation and RT-PCR
Subepicardial pig coronary arterioles (10 vessels per tube; 50 to 200 μm in diameter; 2 to 4 mm in length) were dissected and incubated with PSS containing vehicle or CRP (7 μg/mL) at 37°C for 60 minutes. Total RNA was then isolated from the vessels as described previously. Sets of primers specific for NAD(P)H oxidase subunit p22phox (gene accession no. M21186; sense: 5'-AGC TGT TCG GCC CCT TTA CC-3'; antisense: 5'-ACC TCG TGC ACC GGG AT -3') and GAPDH (gene accession no. 4865612M) were used as a control.
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which we have shown previously causes endothelium-dependent
modulate NO-mediated dilation, the vascular response to serotonin,
by further incubation with the NO precursor L-arginine

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\text{P} > 0.05 \text{ vs CRP}
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\text{dykinin (n = 5) and sodium nitroprusside (n = 5) was examined}
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\[
\text{after} \quad \text{intraluminal incubation with 7 \mu g/mL CRP} \quad \text{in the absence (n = 5) and presence of superoxide}
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\text{anion scavenger TEMPOL (1 mmol/L; n = 5) or NAD(P)H oxidase}
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\text{inhibitor apocynin. However, xanthine oxidase inhibitor allopurinol (10 \mu mol/L;}
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\text{n = 5). C and D, Dilation of coronary arterioles to bra-
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\text{dykinin (n = 5) and sodium nitropusside (n = 5) was examined}
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\text{before and after intraluminal incubation with 7 \mu g/mL CRP for}
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\text{60 minutes. n = number of vessels. *P < 0.05 vs control; †P < 0.05}
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\text{CRP + L-arginine.}
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U48832, sense: 5'-CCA CCC AGC GCA AGT TCC ACG GCA-3';
antisense: 5'-GGT GGT GCA GGA GGC ATT GCT GAC-3';
genes were engineered (Sigma-Genosys). Using equal amounts of total
RNA (0.1 \mu g) for each sample, RT-PCR was conducted as delineated previously. The PCR was optimized and run for 35 cycles for
two-phox genes and 30 cycles for GAPDH genes. The level of
expression of two-phox transcripts was quantified using volume
integration (Multi-Analyst software; Macintosh) and normalized to
that of GAPDH transcripts.

Data Analysis

Diameter changes in response to vasodilator agonists were normalized
to the maximum diameter changes in response to 100 \mu mol/L
sodium nitroprusside and expressed as a percentage of maximal
dilation.\textsuperscript{29} Statistical comparisons of data were performed by Stu-
dent’s \textit{t} test or by ANOVA followed by the Bonferroni multiple-
range test, as appropriate. A value of \( P < 0.05 \) was considered
significant. Data are presented as mean ± SEM.

Results

Effect of CRP on NO-Mediated Vasodilation

All isolated coronary arterioles developed a similar level of basal
tone (62 ± 1% of maximal passive diameter 95 ± 3 \mu m; range 65 to
119 \mu m) within 40 minutes. To determine whether CRP can
modulate NO-mediated dilation, the vascular response to serotonin,
which we have shown previously causes endothelium-dependent
NO-mediated vasodilation,\textsuperscript{19} was assessed before and after intralu-
minal incubation with CRP (0.7 or 7 \mu g/mL) for 60 minutes. Under
control conditions, serotonin dilated coronary arterioles in a dose-
dependent manner (Figure 1A). Subsequent incubation of vessels
with CRP did not alter basal tone (control 59 ± 2 \mu m, 62 ± 1% of
maximal diameter; 0.7 \mu g/mL CRP 61 ± 1 \mu m, 62 ± 3% of maximal
diameter; 7 \mu g/mL CRP 60 ± 2 \mu m, 63 ± 1% of maximal
diameter). However, the vasodilation to serotonin was significantly
attenuated after incubation with 7 \mu g/mL CRP but not 0.7 \mu g/mL
CRP (Figure 1A). Further incubation of the CRP (7 \mu g/mL)-treated
vessels with the NO precursor L-arginine (30 minutes) partially
restored serotonin-induced dilation (Figure 1B), thus suggesting the
inhibitory action of a clinically relevant concentration of CRP on the
NO signaling pathway. CRP (7 \mu g/mL) alone did not alter vasodi-
lation to endothelium-dependent EDHF pathway agonist bradyki-
nin (Figure 1C) or to endothelium-independent agonists sodium
nitropusside (Figure 1D) and acidosis (pH 7.1; control 51 ± 1% of
maximal dilation; CRP 54 ± 3% of maximal dilation; n = 4).

Roles of Superoxide, NAD(P)H Oxidase, and
Xanthine Oxidase in CRP-Induced
Vascular Dysfunction

To determine whether superoxide production is involved in the
impairment of serotonin-induced vasodilation, vessels were treated with CRP in the presence of either a membrane-
permeable superoxide scavenger TEMPOL or specific oxi-
dase inhibitors. In the presence of TEMPOL, the impairment of serotonin-induced NO-mediated vasodilation by CRP (7
\mu g/mL) was prevented (Figure 2A). This preventive effect
was also found in the vessels treated with NAD(P)H oxidase
inhibitor apocynin. However, xanthine oxidase inhibitor al-
llopurinol had no influence on the CRP-mediated effect (Figure 2B).

Roles of p38 Kinase and JNK in CRP-Induced
Vascular Dysfunction

To determine whether p38 kinase and JNK contribute to the
CRP-mediated inhibition of serotonin-induced vasodilation, vessels were treated with CRP in the presence of specific
kinase inhibitors. In the presence of p38 inhibitor SB203850 but
not JNK inhibitor SP600125, the detrimental effect of CRP on the vasodilatory response to serotonin was prevented (Figure 3).

Effect of CRP on Serotonin-Induced
NO Production

To support the functional study, coronary arteriolar produc-
tion of NO in response to serotonin was determined before
and after incubation with CRP (7 μg/mL; 60 minutes). Basal NO production under resting conditions was ≈50 nmol nitrite/g protein (Figure 4). Serotonin (0.1 μmol/L) stimulated a nearly 3-fold increase in NO production from control vessels. However, the serotonin-stimulated increase in NO production was almost completely abolished after incubation with CRP. In the presence of TEMPOL (1 mmol/L), CRP had no inhibitory effect on serotonin-induced NO production (Figure 4).

Effect of CRP on Vascular Superoxide Production

The ability of CRP to induce superoxide production in isolated coronary arterioles was determined by histochemical staining for superoxide. In the absence of CRP (ie, vehicle control), DHE fluorescence revealed sparse levels of superoxide in the vessel wall (Figure 5). In contrast, intraluminal incubation of vessels with CRP (7 μg/mL; 60 minutes) markedly increased superoxide in the endothelial layer. The endothelial and smooth muscle layers were identified by setting the scanning threshold to obtain a clear background image of the blood vessel. SB203850 and TEMPOL markedly reduced the CRP-induced fluorescent signals for superoxide in the endothelium (Figure 5).

Effect of CRP on NAD(P)H Oxidase Activity

The influence of CRP on vascular NAD(P)H oxidase activity was evaluated with a lucigenin chemiluminescence superoxide detection assay and presented in Figure 6. There was no superoxide production in the buffer containing lucigenin and the substrates NADPH and NADH (data not shown). In the absence of NADPH and NADH, the basal level of superoxide production from homogenates of control and CRP-treated (7 μg/mL, 60 minutes) vessels was relatively low (0.12±0.08 RLU·min⁻¹·μg⁻¹). In the presence of NADPH and NADH, the superoxide production in control vessel homogenates was slightly but significantly increased (1.30±0.46 RLU·min⁻¹·μg⁻¹). In contrast, CRP treatment caused a nearly 20-fold increase in the NADPH/NADH-driven superoxide production (Figure 6A). This CRP effect was prevented by apocynin (Figure 6A) but not by allopurinol (data not shown).

Effect of CRP on p22phox Expression

To determine whether CRP treatment (7 μg/mL; 60 minutes) affects NAD(P)H oxidase expression, p22phox (NAD(P)H oxidase subunit) mRNA levels were evaluated by RT-PCR. The mRNA expression of p22phox did not differ significantly
between vessels treated with PSS or CRP for 60 minutes, as demonstrated by the normalization of p22phox transcripts with GAPDH transcripts (Figure 6B).

**Discussion**

The present study implicates that CRP can directly modulate coronary arteriolar function by influencing NO bioavailability. This was demonstrated by the attenuation of endothelium-dependent NO-mediated dilation to serotonin after a short-term intraluminal incubation of vessels with a clinically relevant concentration of CRP (7 μg/mL). The mechanism behind the observed inhibitory effect of CRP involves activation of p38 kinase and generation of superoxide, a direct NO scavenger, by NAD(P)H oxidase. It appears that CRP did not exert a general impairment of endothelial or smooth muscle vasodilatory function because vasodilations to endothelium-dependent EDHF pathway agonist bradykinin or endothelium-independent pharmacological agent sodium nitroprusside and physiological stimulus acidosis were not affected.

Accumulating evidence suggests that CRP is not only a risk marker for cardiovascular disease but also could be a mediator of vascular inflammatory and atherogenic events, such as endothelial dysfunction and impaired vascular reactivity. For example, CRP has been shown to reduce agonist-stimulated NO release from cultured venous endothelial cells. Furthermore, recent clinical evidence demonstrates an inverse correlation between CRP levels and endothelial vascular reactivity in the coronary circulation of patients with coronary artery disease. Maintaining NO bioavailability in the vasculature is crucial because NO is a potent vasodilator and an antithrombogenic agent. Diminished endothelium-dependent NO-mediated vasodilation and enhanced platelet/endothelium interaction are the earliest detectable vascular changes before atherosclerotic plaque development. Because serotonin is released by activated platelets, inhibiting serotonin-stimulated endothelial release of NO by CRP can potentially induce vasospasm and also favor the promotion of thrombosis for the development of atherosclerosis and other inflammatory vascular diseases. Although previous studies have established the effect of CRP on stimulated NO release from cultured endothelial cells, the direct effect of CRP on NO-dependent vasoreactivity is not known. In the present study, we demonstrate for the first time that CRP attenuates endothelium-dependent NO-mediated dilation by diminishing NO bioavailability in isolated coronary arterioles. Partial restoration of the serotonin-induced dilation after administration of L-arginine (NO precursor) confirmed the CRP-mediated reduction in NO bioavailability. Importantly, restoration of the dilation to serotonin did not occur in CRP-treated vessels after administration of PSS (data not shown). We provided further evidence for the reduction in NO bioavailability by demonstrating that CRP reduces stimulated NO release from isolated coronary arterioles. It is worth noting that the 2 concentrations of CRP used in our study (0.7 μg/mL and 7 μg/mL) were clinically relevant. Patients without inflammation generally have serum CRP levels <1 μg/mL, which is interpreted as low cardiovascular risk. On the other hand, CRP levels between 1 and 3 μg/mL are interpreted as an intermediate risk for cardiovascular events, and levels between 3 and 10 μg/mL indicate high risk. In the study by Verma et al, only CRP concentrations >3 μg/mL significantly reduced NO release from cultured human umbilical vein endothelial cells. Our study is consistent with these findings because only the higher concentration of CRP (7 μg/mL) had an effect on endothelium-dependent NO-mediated vasodilation, suggesting that CRP levels known to predict adverse cardiovascular events can also impair coronary microvascular endothelial function.

Elevated levels of reactive oxygen species, which mediate oxidative stress, have been implicated in contributing to the development of endothelial dysfunction in patients with coronary artery disease. Interestingly, coinfusion of the reactive oxygen species scavenger vitamin C improved the acetylcarnitine-stimulated forearm blood flow responses in patients with elevated CRP and coronary artery disease. Although it has been shown that stimulation of cultured endothelial and smooth muscle cells with CRP can increase the production of reactive oxygen species, it remains unknown whether this protein can sufficiently increase oxidative stress and influence NO production for vasodilation in the intact microvessels. We found that the effects of CRP on serotonin-induced dilation and NO release were prevented in the presence of the membrane-permeable superoxide scavenger TEMPOL. The effect of TEMPOL seems to be specific because this superoxide scavenger did not affect...
resting basal tone or vasodilation to sodium nitroprusside, as shown in our previous study. Further support for superoxide production was revealed by DHE staining showing that CRP is capable of generating TEMPO-sensitive superoxide in the endothelial layer of the microvascular wall. Although our findings indicate that acute exposure to CRP can impair NO bioavailability via increased oxidative stress, additional mechanisms, such as reduced expression of endothelial NO synthase, may be accountable for the reduced NO bioavailability by chronic exposure of CRP under clinical settings.12,13

Superoxide can be generated by several enzymatic sources in vascular cells, including NAD(P)H oxidase and xanthine oxidase. The activation of vascular NAD(P)H oxidase is dependent on the assembly of its membrane-bound (gp91phox and p22phox) and cytoplasmic (p40phox, p47phox, p67phox, and the small GTPase rac) subunits.35 Apocynin, a methoxy-substituted catechol isolated from the medicinal herb Picrohriza kurroa,37 inhibits NAD(P)H oxidase activation by interfering with the assembly of the enzyme subunits. On the other hand, allopurinol, a pseudosubstrate for xanthine oxidase, competitively inhibits the enzyme by binding to its active site.26 Our study shows that CRP-induced impairment of NO-mediated vasodilation was prevented by apocynin but not by allopurinol, suggesting that superoxide anions produced by NAD(P)H oxidase are responsible for the detrimental effect of CRP. This conclusion was supported by data obtained with lucigenin-enhanced chemiluminescence detection of superoxide. Numerous studies have used this technique to measure NAD(P)H oxidase activity in vascular cells and tissue.30,31,36–40 Because vascular NAD(P)H oxidase can use NADPH41 and NADH42 as electron donors for superoxide production, we supplied both substrates to increase NAD(P)H oxidase activity in arteriolar homogenates. A relatively low basal level of NADPH/NADH-driven superoxide production was detected in control vessel homogenates. However, treatment with CRP caused a significant increase in vascular NAD(P)H oxidase activity, an effect that was prevented specifically by apocynin. Collectively, these findings are consistent with the evidence indicating that NAD(P)H oxidase is the major source of agonist-induced superoxide production in vascular cells.36

The possible mechanisms leading to the CRP-induced increase in superoxide production by NAD(P)H oxidase include alteration of enzyme expression or activation. It does not appear that upregulation of NAD(P)H oxidase was a contributing factor because the gene expression of p22 phox, an essential and abundant oxidase subunit in coronary microvascular endothelial cells,40 was not altered by CRP treatment. On the other hand, the acute regulation of NAD(P)H oxidase by CRP may be dependent on various activation pathways involving protein kinases. Numerous studies have shown that the stress-activated kinases p38 and JNK are important signaling molecules in inflammation and oxidative stress.43,44 The intracellular substrates for these protein kinases include transcription factors, as well as cytosolic proteins.45 Interestingly, results from cultured lung endothelial cell studies suggest that p38 kinase can contribute to the regulation of NAD(P)H oxidase by activating cytosolic subunits p47phox and p67phox.45 In addition, recent evidence has shown that CRP can increase the activity of p38 and JNK in cultured vascular smooth muscle cells.8 Based on these observations, we examined whether blockade of p38 or JNK signaling could preserve endothelium-dependent NO-mediated vasodilation. Our results show that inhibition of p38 but not JNK prevented the CRP-induced impairment of serotonin-induced dilation. Furthermore, p38 blockade attenuated the vascular production of superoxide in the presence of CRP. Together, our findings suggest that the p38 signaling could be involved in the CRP-induced activation of vascular NAD(P)H oxidase.

In conclusion, we have demonstrated for the first time that CRP, at a concentration known to predict vascular disease, directly inhibits the endothelium-dependent NO-mediated dilation and attenuates the stimulated release of NO in isolated coronary arterioles. The mechanism of the observed acute CRP effects involves the activation of p38 kinase and production of superoxide by vascular NAD(P)H oxidase. Because impaired endothelium-dependent NO-mediated dilation is a key feature of early atherogenesis, CRP is clearly not just a marker, but can be a mediator of cardiovascular disease.

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C-Reactive Protein Inhibits Endothelium-Dependent NO-Mediated Dilation in Coronary Arterioles by Activating p38 Kinase and NAD(P)H Oxidase

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