C-Reactive Protein Inhibits Endothelium-Dependent Nitric Oxide-Mediated Dilation of Retinal Arterioles via Enhanced Superoxide Production

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PURPOSE. Elevated levels of C-reactive protein (CRP), a proinflammatory marker, are associated with systemic vascular disorders. In addition, clinical studies have implicated that elevated CRP is an independent risk factor for diabetic retinopathy and age-related macular degeneration. However, the direct effect of CRP on ocular microvascular reactivity remains unknown. The authors examined whether CRP can affect endothelium-dependent nitric oxide (NO)-mediated dilation of retinal arterioles and whether oxidative stress and distinct protein kinase signaling pathways are involved in the CRP-mediated effect.

METHODS. Porcine retinal arterioles (internal diameter, 71 ± 2 μm) were isolated and pressurized without flow for in vitro study. Diameter changes were recorded using videomicroscopic techniques. Dihydroethidium (DHE) was used to detect superoxide production.

RESULTS. Intraluminal treatment with a clinically relevant concentration of CRP (7 μg/mL, 60 minutes) significantly attenuated arteriolar dilation to endothelium-dependent NO-mediated agonists bradykinin and A23187 but not to endothelium-independent NO donor sodium nitroprusside. In the presence of superoxide scavenger TEMPOL, NAD(P)H oxidase inhibitor apocynin, p38 kinase inhibitor SB203580, simvastatin, or Rho-kinase inhibitor Y-27632, the detrimental effect of CRP on bradykinin-induced dilation was prevented. DHE staining showed that CRP produced TEMPOL-sensitive superoxide production in the arteriolar endothelium.

CONCLUSIONS. CRP inhibits endothelium-dependent NO-mediated dilation in retinal arterioles by producing superoxide from NAD(P)H oxidase, which appears to be linked with p38 kinase and RhoA/Rho-kinase activation. By impairing endothelium-dependent NO-mediated vasoactivity, CRP can potentially facilitate the development of retinal vascular diseases. In addition, statins are beneficial by preserving endothelial function, possibly through inactivation of the RhoA/Rho-kinase pathway. (Invest Ophtalmol Vis Sci. 2008;49:2053–2060) DOI: 10.1167/iovs.07-1387

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dium pentobarbital (30 mg/kg, intravenously), intubated, and venti-
lated with room air. Heparin (1000 U/kg) was administered into the
marginal ear vein to prevent clotting, and the eyes were enucleated
and immediately placed in a moist chamber on ice.

Isolation and Cannulation of Microvessels
The preparation of isolated retinal arterioles has been described in our
previous studies.25,26,27 In brief, the anterior segment and vitreous
body were removed carefully under a dissection microscope. The
eyecup was placed in a cooled dissection chamber (approximately
8°C) containing physiological salt solution (PSS; NaCl, 145.0 mM; KCl,
4.7 mM; CaCl2, 2.0 mM; MgSO4, 1.17 mM; NaH2PO4, 1.2 mM; glucose,
5.0 mM; pyruvate, 2.0 mM; EDTA, 0.02 mM; and MOPS, 3.0 mM) with
1% albumin (USB, Cleveland, OH). Second-order retinal arterioles
(40–60 μm in internal diameter in situ; 0.6–1.0 mm in length without
branches) were carefully dissected out using a Vannas spring scissors
and a pair of microdissection forceps (DuPont; Fine Science Tools,
Foster City, CA) with the aid of a stereomicroscope (model SZX12;
Olympus, Melville, NY). After careful removal of any remaining neural/
connective tissues, the arteriole was transferred for cannulation to a
poly(methacrylate) vessel chamber containing PSS-albumin solution
equilibrated with room air at ambient temperature. Both ends of the
arteriole were cannulated with glass micropipettes (tip outer di-
ameter, 50–40 μm) filled with PSS-albumin solution, and the outside of
the arteriole was securely tied to the micropipettes with 11–0 ophthalmic
suture (Alcon, Fort Worth, TX). After cannulation, the vessel and
micropipettes were transferred to the stage of an inverted microscope
(model CKX41; Olympus) coupled to a video camera (Sony DXC-190;
Labtek, Campbell, CA) and video micrometer (Cardiovascular Research
Institute; Texas A&M Health Science Center, College Station, TX) for
continuous measurement of the internal diameter. The micropipettes
were connected to independent pressure reservoirs (i.e., 30-ml glass
syringes with 10 ml PSS). By adjusting the height of the reservoirs, the
vessel was pressurized to 55 cm H2O (approximately 40 mm Hg) intraluminal pressure without flow based on pressure ranges docu-
mented in retinal arterioles in vivo22 and in the isolated, perfused
retinal microcirculation.26 Preparations with leaks were excluded from
further study.

Experimental Protocols
The human recombinant CRP (Calbiochem, San Diego, CA) used in
the following protocols was initially dialyzed for 24 hours against Dul-
becco PBS using a dialysis slide (Pierce, Rockford, IL) 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 endo-
thelial function,29 was also removed from the CRP by using detoxigel
columns (Pierce) and was found to be less than 0.06 EU/ml by the
Limulus assay (Cambrex, East Rutherford, NJ).

Cannulated arterioles were bathed in PSS at 36°C to 37°C to allow
the development of basal tone. After vessels developed a stable basal
tone (approximately 30–40 minutes), we assessed the effect of CRP on
NO-mediated vasodilation. For this purpose, the endothelium-depen-
dent NO-mediated vasodilation to bradykinin (1 × 10–12 to 1 × 10–8
M)25,30 was established before and after 30-minute intraluminal incubation
with the NO inhibitor L-NAME (10 μM).31,32 The role of stress-activated protein kinases was examined by treating the vessels with CRP combined with p38 kinase inhibitor SB203580 (0.1 μM; Calbiochem).30,31

To assess the ability of simvastatin to mitigate the effect of CRP on
NO-mediated vasodilation, vessels were treated with CRP (7 μg/ml)
combined with a clinical dose (10 nM) of simvastatin (intraluminal;
Merck Research Laboratories, West Point, PA). In addition, the role of the RhoA/Rho-kinase pathway in mediating the CRP effect was exam-
ined by treating another group of vessels with CRP in combination with
Rho-kinase inhibitor Y-27632 (0.1 μM).25

Drugs were obtained from Sigma-Aldrich (St. Louis, MO) and were
dissolved in PSS except when specifically stated otherwise. Simvastatin
was generously provided by Merck Research Laboratories and acti-
ated by alkaline hydrolysis according to the manufacturer’s instruc-
tions. Simvastatin was dissolved in ethanol, and the final concentration
of ethanol in the vessel bath was less than 0.1%.25 Vehicle control
studies indicated that this concentration of ethanol had no effect on
arteriolar function.

Detection of Superoxide
Superoxide production in isolated retinal arterioles was evaluated with the
fluorescent dye dihydroethidium (DHE).33 Isolated and pressurized retinal arterioles (70–100 μm in diameter and 1.5 mm in length) were
incubated intraluminally with PSS containing vehicle, CRP (7 μg/ml),
or CRP plus TEMPOL (1 mM) at 37°C for 60 minutes and then were
stained with DHE (4 μM) for 30 minutes. After they were washed,
arterioles were embedded in OCT compound (TissueTek; Electron
Microscopy Sciences, Hatfield, PA) for cryostat sections. Embedded
arterioles were cut into 12-μm-thick sections and placed on glass
slides. Images were taken with a fluorescence microscope (Axiovert
200; Zeiss). Fluorescence was detected with a 620/60 (590–650 nm)
bandpass 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.

Statistical Analysis
At the end of each experiment, the vessel was relaxed in an EDTA (1
mM)-calcium-free PSS to obtain its maximal diameter at 55 cm H2O
intraluminal pressure. All diameter changes in response to agonists
were normalized to this maximal vasodilation and expressed as a
percentage of maximal dilation.26 Data are reported as mean ± SEM,
and n values represent the number of vessels studied. Statistical com-
parisons of vasodilator responses were performed by two-way analysis
of variance followed by the Bonferroni multiple-range test. Changes in
resting tone by CRP or pharmacologic inhibitors were analyzed by
paired Student’s t-test. P < 0.05 was considered significant.

RESULTS
Effect of CRP on NO-Mediated Vasodilation
In this study, all vessels (n = 49) developed a similar level of
basal tone (constricted to 68% ± 2% of maximal diameter) at
36°C to 37°C bath temperature with 55 cm H2O intraluminal
pressure. Average resting and maximal diameters of the vessels
were 71 ± 2 μm and 105 ± 2 μm, respectively. Bradykinin

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The resting vascular tone was not altered by CRP (control, 68% ± 2%; 0.7 μg/mL CRP, 70% ± 3%; P = 0.60; 7 μg/mL CRP, 66% ± 3%; P = 0.43). A higher dose of CRP (35 μg/mL, n = 4) did not cause further reduction of bradykinin-induced dilation (data not shown). CRP at 7 μg/mL also significantly reduced A23187-induced vasodilation (Fig. 2B). On the other hand, the dilation of retinal arterioles to the endothelium-independent NO donor sodium nitroprusside was not affected by CRP (7 μg/mL) (Fig. 2C).

Roles of Superoxide, NAD(P)H Oxidase, Xanthine Oxidase, and p38 Kinase

To determine whether superoxide production is involved in the impairment of bradykinin-induced vasodilation, vessels were treated with CRP in the presence of a membrane-permeable superoxide scavenger TEMPOL or of specific oxidase inhibitors. In the presence of TEMPO, the inhibition of bradykinin-induced vasodilation by CRP (7 μg/mL) was prevented (Fig. 3A). This preventive effect was also found in vessels treated with NAD(P)H oxidase inhibitor apocynin (Fig. 3B). However, xanthine oxidase inhibitor allopurinol had no influence on the CRP-mediated effect (Fig. 3B). The basal tone was not significantly altered by TEMPOL (control, 64% ± 4% vs. TEMPOL, 64% ± 4%; P = 0.94), apocynin (control, 65% ± 6% vs. apocynin, 63% ± 7%; P = 0.74) or allopurinol (control, 60% ± 2% vs. allopurinol, 57% ± 5%; P = 0.67). In the presence of p38 kinase inhibitor SB203580, the inhibitory effect of CRP on vasodilation to bradykinin was also prevented (Fig. 4). The basal tone was not significantly altered by SB203580 (control, 63% ± 5% vs. SB203580, 62% ± 6%; P = 0.84). In another group of vessels, dilation to bradykinin (1 nM) was not altered after 60-minute intraluminal administration of TEMPOL (control, 54% ± 12% dilation vs. TEMPOL, 56% ± 14%; n = 3, P = 0.65) or SB203580 (control, 55% ± 11% dilation vs. SB203580, 51% ± 6%; n = 3, P = 0.61) alone.

Effect of Simvastatin and RhoA/Rho-Kinase Blockade

Coadministration of CRP and simvastatin prevented the detrimental action of CRP on bradykinin-induced vasodilation (Fig. 5A). Similarly, the inhibitory effect of CRP on the vasodilatory response to bradykinin was abolished in the presence of Rho-kinase inhibitor Y-27632 (Fig. 5B). The basal tone was not significantly altered by simvastatin (control, 59% ± 3% vs. simvastatin, 53% ± 3%; P = 0.24) or Y-27632 (control, 62% ± 2% vs. Y-27632, 59% ± 2%; P = 0.50). In addition, the vasodilatory response to bradykinin (1 nM) was not altered after 60-minute intraluminal treatment with simvastatin (control, 63% ± 10% dilation vs. simvastatin, 59% ± 12%; n = 5, P = 0.43) or Y-27632 (control, 60% ± 9% dilation vs. Y-27632, 57% ± 10%; n = 3, P = 0.23) alone.

Effect of CRP on Superoxide Production

In the absence of CRP (i.e., vehicle control), DHE fluorescence revealed sparse levels of superoxide in the vessel wall (Fig. 6). In contrast, intraluminal incubation of vessels with CRP (7 μg/mL; 60 minutes) markedly increased the superoxide level in the endothelial layer. Endothelial and smooth muscle layers were identified by setting the scanning threshold to obtain a clear background image of the vessel wall. TEMPOL markedly reduced the CRP-induced fluorescent signals for superoxide in the endothelium (Fig. 6).

DISCUSSION

The inflammatory marker CRP has recently been shown to be an independent risk for cardiovascular and peripheral arterial disease1,11 and a pathogenic factor leading to endothelial dysfunction in the cell culture model.38,39 Moreover, elevated
levels of CRP have been prospectively associated with an increased risk for hypertension\textsuperscript{40} and for type 1\textsuperscript{41} and type 2\textsuperscript{42} diabetes mellitus. Because hypertension and diabetes are major

\begin{figure}
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\includegraphics[width=\textwidth]{figure2}
\caption{Effect of CRP on retinal vascular reactivity. (A) Dilation of isolated retinal arterioles to bradykinin was examined before and after intraluminal incubation with 7 \( \mu \)g/mL CRP (\( n = 7 \)) or 0.7 \( \mu \)g/mL CRP (\( n = 5 \)) for 60 minutes. Retinal arteriolar dilation to A23187 (B, \( n = 5 \)) and sodium nitroprusside (C, \( n = 5 \)) was examined before and after intraluminal incubation with 7 \( \mu \)g/mL CRP for 60 minutes. \( *P < 0.05 \) vs. Control.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Blockade of superoxide production or NAD(P)H oxidase activation prevents CRP-induced reduction of retinal arteriolar dilation to bradykinin. (A) Dilation of retinal arterioles to bradykinin was examined before (Control, \( n = 5 \)) and after intraluminal incubation with 7 \( \mu \)g/mL CRP plus superoxide anion scavenger TEMPO (1 mM; \( n = 5 \)). (B) Dilation of retinal arterioles to bradykinin was examined before (Control, \( n = 10 \)) and after intraluminal incubation with 7 \( \mu \)g/mL CRP plus NAD(P)H oxidase inhibitor apocynin (100 \( \mu \)M; \( n = 5 \)) or xanthine oxidase inhibitor allopurinol (10 \( \mu \)M; \( n = 5 \)). \( *P < 0.05 \) vs. Control.}
\end{figure}
risk factors for retinal vascular disorders and their associations with inflammation and endothelial dysfunction have been suggested in humans with retinopathy, it is important to evaluate the direct effect of CRP on retinal microvascular function. Unfortunately, there has been no study hitherto to document the direct effect of CRP on retinal vasomotor function. The present study is the first to show that CRP significantly reduces the dilations of retinal arterioles to bradykinin and A23187 but not to sodium nitroprusside, suggesting that CRP compromises retinal endothelial function in terms of NO-mediated vasodilation.

It has been suggested that a serum CRP level lower than 1 μg/mL is considered a low cardiovascular risk for coronary artery disease. On the other hand, CRP levels between 1 and 3 μg/mL are interpreted as intermediate risks for cardiovascular events, and levels between 3 and 10 μg/mL indicate high risk. In the study by Verna et al., only CRP concentrations greater than 3 μg/mL significantly reduced NO release from cultured human umbilical vein endothelial cells. Several recent clinical studies suggest a close association between serum CRP and ocular vascular disorders related to AMD and diabetic retinopathy. It has been reported that patients with the highest quartile of CRP (greater than 6.5 μg/mL) exhibit a high risk for AMD. In addition, a more than threefold higher incidence of AMD was found in women with serum CRP levels greater than 5 μg/mL. In another clinical study, the prevalence of diabetic retinopathy was reported to be increased with higher tertiles of CRP (highest tertile range, 3–35 μg/mL). The concentrations of CRP used in the present study (0.7 and 7 μg/mL) covered the physiological and pathophysiological ranges, and only the high level of CRP exhibited inhibitory action on endothelium-dependent vasomotor function. It appears that CRP levels known to predict cardiovascular events produce adverse effects on endothelial function in the retinal microvasculature. This is consistent with the findings recently reported in coronary arteries.

In addition to the reduction of bradykinin-induced NO-mediated dilation, CRP reduced the vasodilation of retinal arterioles in response to the calcium ionophore A23187, which is known to activate eNOS by the elevation of intracellular calcium independent of receptor activation. This contention is supported by the present finding that A23187-elicited dilation of retinal arterioles was abolished by the NOS inhibitor. It has been reported that CRP (more than 10 μg/mL) decreases enzyme activity of eNOS in cultured human aortic endothelial cells. Moreover, in addition to the previously known action of CRP to attenuate eNOS expression after prolonged exposure...
dilatory function in the absence of CRP but did prevent the 
sb203580 in bovine aortic endothelial cells. in the present 
coronary artery disease.48 our findings suggest that the detri-
lated to the elevation of plasma CRP in the patients with 
stress and the reduction in NO bioavailability are closely re-
arterioles.

The mechanism responsible for the reduced NO avail-
ability by CRP remains unclear, but a plausible explanation 
could be related to increased vascular oxidative stress. Indeed, 
we found that the inhibitory effect of CRP on bradykinin-
induced dilation was prevented in the presence of a mem-
brane-permeable superoxide scavenger TEMPOL. The salutary 
effect of TEMPOL seems to be specific because this superoxide 
scavenger did not affect resting basal tone or vasodilation to 
bradykinin in the absence of CRP. Further support for super-
oxide production was revealed by DHE staining showing that 
CRP is capable of generating TEMPOL-sensitive superoxide in 
the endothelial layer of retinal arterioles. This finding is con-
sistent with recent evidence showing that CRP can increase 
the production of superoxide in cultured human aortic endo-
thelial cells9 and in porcine coronary arterioles.37 Interestingly, 
a recent clinical study reported that the increase in oxidative 
stress and the reduction in NO bioavailability are closely re-
lated to the elevation of plasma CRP in the patients with 
coronary artery disease.49 Our findings suggest that the dtri-
mental effects of CRP could possibly extend to the ocular 
circulation and may contribute in part to the development of 
retinal vascular disease.

In the vascular wall, superoxide can be generated by several 
enzymatic sources, including NAD(P)H oxidase and xanthine 
oxidase. Apocynin, a methoxy-substituted catechol isolated 
from the medicinal herb Picrorhiza kurroa,49 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.35 our study showed 
that the inhibitory effect of CRP was prevented by apocynin 
but not by allopurinol, suggesting that superoxide anions pro-
duced by NAD(P)H oxidase are responsible for the inhibitory 
action of CRP. Collectively, these findings are consistent with 
evidence indicating that NAD(P)H oxidase is the major source 
of agonist-induced superoxide production in vascular cells.50

It has been reported that the stress-activated p38 kinase is 
an important signaling molecule in response to inflammation 
and oxidative insult.51 Recently, ling et al.8 showed that p38 
kine inhibitor SB203580 partially but significantly reduced 
vascular cell adhesion molecule-1 expression induced by CRP 
in cultured endothelial cells. Kawanami et al.52 demonstrated 
that CRP-induced NF-kB activation could be inhibited by 
SB203580 in bovine aortic endothelial cells. in the present 
study, p38 kinase blockade did not influence the normal vaso-
dilatory function in the absence of CRP but did prevent the 
inhibitory effect of CRP on bradykinin-induced dilation. It ap-
pears that CRP may elicit a multiple array of functional alter-
ations in endothelial cells through p38 kinase signaling. Given 
that activation of p38 kinase has been shown to link to the 
increased superoxide production from NAD(P)H oxidase,53 it 
is likely that the observed oxidase-induced stress produced by 
CRP in the present study was mediated by p38 kinase.

A number of studies have reported a relation between 
oxidative stress and the activation of a small GTP-binding 
protein RhoA/Rho-kinase pathway. Higashi et al.34 showed that 
Rho-kinase activation leads to enhanced vascular NAD(P)H 
oxidase expression and endothelial production of superoxide. 
on the other hand, Rho-kinase blockade inhibits NAD(P)H 
oxidase activation.55 These results suggest that the RhoA/Rho-
kinase pathway may be involved in the development of oxida-
tive stress through the activation of NAD(P)H oxidase. in the 
present study, inhibition of Rho-kinase preserved NO-mediated 
dilation to bradykinin, suggesting the involvement of RhoA/ 
Rho-kinase activation in CRP-induced endothelial dysfunction 
in retinal arterioles. Our results are consistent with recent 
evidence showing that CRP can activate RhoA/Rho-kinase sig-
naling in cultured bovine aortic endothelial cells.56,57 Because 
both NAD(P)H oxidase and Rho-kinase blockade prevented the 
detrimental actions of CRP, it is possible that these events are 
linked in series with Rho-kinase activation leading to NAD(P)H 
oxidation. Alternatively, the inhibition of eNOS func-
tion might be involved because RhoA/Rho-kinase activation 
has been shown to negatively regulate eNOS activity and NO 
production in cultured human endothelial cells.58 it does not 
appear that Rho-kinase activation alters eNOS function in the 
absence of CRP because bradykinin-induced vasodilation re-
mained normal in the presence of Y-27632 alone. Future stud-
ies are required to determine the precise series of events 
leading to NAD(P)H oxidase activation and RhoA/Rho-kinase 
signaling by CRP.

Reductions in inflammation and oxidative stress or inhibi-
tion of RhoA/Rho-kinase activity by statins have been reported 
to improve endothelial function.59,60 in addition, we have 
recently demonstrated that simvastatin can elicit the dilation 
of retinal arterioles through NO production from endothelium by 
inhibiting RhoA/Rho-kinase signaling.23 therefore, it is reason-
able to consider whether statins can prevent CRP-induced 
retinal vascular dysfunction. Interestingly, Tan et al.61 reported 
that atorvastatin treatment in patients with type 2 diabetes led 
to a reduction in plasma CRP and an improvement in endothe-
lum-dependent vasodilation. However, it is unclear whether 
this beneficial effect is the result of a direct action of statin or 
the secondary effect of CRP lowering. in the present study, we 
found that a clinical dose (10 nM) of simvastatin prevented the 
inhibitory effect of CRP on endothelial function. This protec-
tive effect may be related to the reduction of oxidative stress 
because simvastatin has been shown to reduce superoxide 
formation in the rat coronary artery endothelial cells in cul-

FIGURE 6. DHE fluorescence imaging 
of superoxide in retinal arterioles. Iso-
lated and pressurized retinal arterioles 
were incubated intraluminally with ve-
lage (Control), 7 µg/mL CRP, or CRP 
+ TEMPOL (1 mM) for 60 minutes, 
followed by the addition of oxidative 
fluorescent dye DHE and imaging by 
fluorescence microscopy. Endothelial 
cells are denoted by arrowheads. Data 
shown are representative of three sep-
erate experiments.
ture. In addition, aortic and renal NAD(P)H-dependent superoxide production was reduced by simvastatin in salt-induced hypertensive rats. Therefore, it is possible that the ability of simvastatin to prevent the detrimental effect of CRP is through the inhibition of NAD(P)H oxidase-induced superoxide production the blockade of RhoA/Rho-kinase activation, as suggested in our previous studies. Although a direct action of eNOS by simvastatin cannot be excluded, our present study does not support this view because intraluminal treatment with simvastatin (10 nM) alone for 60 minutes failed to enhance bradykinin-induced vasodilation.

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 of isolated porcine retinal arterioles. The mechanism underlying the acute effect of CRP involves the activation of p38 kinase and the production of superoxide by vascular NAD(P)H oxidase. In addition, statins are beneficial by preserving endothelial function, possibly through the inactivation of the RhoA/Rho-kinase pathway and the reduction of oxidative stress. Because impaired endothelium-dependent NO-mediated dilation is a key feature of early vascular events, CRP is clearly not just only an inflammatory marker but also a mediator for the development of vascular disorders in the retinal circulation. The results obtained from the present studies may help our understanding of the pathogenesis of retinal vascular disease associated with high levels of CRP.

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