Selective Activation of Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Mediates C-Reactive Protein–Evoked Endothelial Vasodilator Dysfunction in Coronary Arterioles

Travis W. Hein,* Erion Qamirani,* Yi Ren, Xin Xu, Naris Thengchaisri, Lih Kuo

Rationale: Studies in cultured endothelium implicate that lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) or Fcγ receptor II (CD32) contributes to the proatherogenic effects of C-reactive protein (CRP). However, the identity of the receptors linking to deleterious actions of CRP in vasomotor regulation remains unknown.

Objective: We tested the hypothesis that LOX-1 contributes to adverse effects of CRP on endothelium-dependent vasomotor function in resistance arteries.

Methods and Results: Porcine coronary arterioles were isolated for vasoreactivity study, dihydroethidium fluorescence staining of superoxide, immunohistochemical localization of receptors, immunoprecipitation of receptor/CRP interaction, and protein blot. Intraluminal treatment of pressurized arterioles with a pathophysiological level of CRP (7 µg/mL; 60 minutes) attenuated endothelium-dependent nitric oxide–mediated and prostacyclin-mediated dilations to serotonin and arachidonic acid, respectively. LOX-1 and CD32 were detected in the endothelium of arterioles. Blockade of LOX-1 with either pharmacological antagonist k-carrageenan or anti–LOX-1 antibody prevented the detrimental effect of CRP on vasodilator function, whereas anti-CD32 antibody treatment was ineffective. Denudation of endothelium and blockade of LOX-1 but not CD32 prevented CRP-induced elevation of superoxide in the vessel wall. CRP was coinmunoprecipitated with LOX-1 and CD32 from CRP-treated arterioles. Similarly, LOX-1 and CD32 blockade prevented CRP-induced arteriolar expression of plasminogen activator inhibitor-1, a thrombogenic protein.

Conclusions: CRP elicits endothelium-dependent oxidative stress and compromises nitric oxide–mediated and prostacyclin-mediated vasomotor function via LOX-1 activation. In contrast, both LOX-1 and CD32 mediate plasminogen activator inhibitor-1 upregulation in arterioles by CRP. Thus, activation of LOX-1 and CD32 may contribute to vasomotor dysfunction and proatherogenic actions of CRP, respectively. (Circ Res. 2014;114:92-100.)

Key Words: nitric oxide ■ oxidative stress ■ vasodilation

Inflammation-related cardiovascular disturbance is regarded as a pivotal event in the development of atherosclerosis. C-reactive protein (CRP), a biomarker of inflammation and cardiovascular deterioration, is becoming an emerging risk factor for cardiovascular disease. The landmark Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) study recently showed that statin therapy significantly reduced the incidence of major cardiovascular events, in apparently healthy individuals with elevated CRP (≥2 mg/mL). Despite the lack of direct evidence for a causal link of CRP, accumulating findings suggest that CRP is actively involved in promoting adverse cardiovascular outcomes because of its proatherogenic effects on vascular cells. In cell culture models, CRP, at relatively high pharmacological concentrations (25–100 µg/mL), has been shown to elevate reactive oxygen species generation from vascular cells and impair nitric oxide (NO) synthase (NOS) activity and NO production, as well as prostacyclin (PGI2) release, from endothelial cells. Extending the studies from a homogeneous cell type in culture, we recently demonstrated in intact coronary arterioles that CRP, at a clinically relevant concentration (7 µg/mL), impairs endothelium-dependent NO-mediated and PGI2-mediated dilations by reducing NO bioavailability and inhibiting PGI synthase activation, respectively. The detrimental effect of CRP on vasodilator function depended on endothelial production of reduced form of nicotinamide dinucleotide phosphate (NAD[P]H) oxidase–derived superoxide, and impair nitric oxide synthase (NOS) activity and NO production, and nitric oxide (NO) synthase (NOS) activity and NO production, respectively.
and subsequent formation of peroxynitrite rather than inactivation of NOS or cyclooxygenase. Several clinical studies have also demonstrated a strong correlation between diminished vascular reactivity and increased coronary microvascular resistance in association with high levels of CRP (3–10 μg/mL). In addition, local production of CRP within the coronary circulation has been associated with impaired coronary endothelium–dependent vasodilation in patients with acute coronary syndrome. Taken together, experimental and clinical findings support the notion of CRP as a mediator of vascular dysfunction in the microcirculation, the primary site for blood pressure and flow control.

Despite the overwhelming evidence from both animal and human studies assigning a pathophysiologic role to CRP, the identity of the receptor in intact blood vessels mediating deleterious actions of CRP has not been determined. Previous studies in cultured aortic endothelial cells have provided disparate results, implicating that CRP activates Fcγ receptor II (CD32) or lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in THP-1 cells. Because LOX-1, a scavenger receptor, can bind to several pathogenic ligands and cause endothelial dysfunction similar to that induced by CRP, we propose that CRP inhibits endothelium-dependent vasodilation via generation of oxidative stress by activating LOX-1 in intact arterioles. To test this hypothesis without the confounding influences from hemodynamic and humoral factors associated with in vivo preparations, porcine coronary arterioles were isolated and pressurized for vasomotor assessment and biochemical/immunohistochemical studies.

**Methods**

Please see the Online Data Supplement for an expanded Methods section.

**Functional Assessment of Isolated Coronary Arterioles**

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996). All animal procedures were approved by the Scott & White Institutional Animal Care and Use Committee and have been described previously. Pigs were anesthetized with 2% to 4% isoflurane, and the heart was quickly excised. Individual subepicardial arterioles (40–100 μm in internal diameter in situ) were dissected from the surrounding cardiac tissue for in vitro study. Vessels were cannulated and pressurized to 60 cm-H₂O intraluminal pressure without flow. The human recombinant CRP and high purity serum CRP (Calbiochem) used in the following protocols were prepared as reported previously.

After coronary arterioles developed stable baseline tone, the vasodilator function was assessed by challenging the vessels with an endothelium-dependent NO-mediated agonist serotonin before and after extraluminal incubation of vessels with NO inhibitor Nω-nitro-l-arginine methyl ester (10 μmol/L) or 60-minute intraluminal incubation with a clinically relevant concentration of CRP (7 μg/mL). To confirm the activity of recombinant CRP, human serum–derived native CRP (Calbiochem) was used. To verify the specific action of CRP, an anti-CRP monoclonal antibody was used (clone CRP-8; Sigma; 35 μg/mL). The roles of LOX-1 and CD32 in mediating the CRP effect were examined in a separate group of vessels by determining dilation to serotonin before and after coadministration of CRP with an anti–LOX-1 antibody (5 μg/mL; Abcam) or an anti-CD32 antibody (5 μg/mL; Santa Cruz Biotechnology). k-Carrageenan (125 μg/mL), a LOX-1 antagonist, and catalase (1000 U/mL) the hydrogen peroxide scavenger, were administered to determine the role of LOX-1 and hydrogen peroxide, respectively, in the observed CRP effect. In another cohort of vessels, vasodilation to arachidonic acid (10 μmol/L), an endothelium-dependent PGI₂-mediated agonist, was examined before and after incubation with PGI₂ synthase inhibitor trans-2-phenyl cyclopropylamine (TPC; 100 μmol/L) or after 60-minute intraluminal incubation with recombinant CRP (7 μg/mL) in the absence or presence of an anti–LOX-1 antibody (5 μg/mL), TPC plus an anti–LOX-1 antibody, or an anti-CD32 antibody (5 μg/mL). In preliminary studies, dilations of coronary arterioles to serotonin and arachidonic acid were repeatable without any sign of tachyphylaxis (data not shown).

Diameter changes in response to vasodilator agonists were normalized to the maximal diameter change in response to 100 μmol/L sodium nitroprusside in EDTA (1 mmol/L)-Ca²⁺–free physiologic saline solution and expressed as % maximal dilation. Data are presented as mean±SEM. Statistical comparisons of vasomotor responses under different treatments were performed with 1- or 2-way ANOVA followed by the Bonferroni multiple range test, as appropriate. Differences in resting diameter before and after pharmacological interventions were compared with paired Student’s t-test. A value of P<0.05 was considered significant.

**Detection of Superoxide**

Real-time detection of superoxide production from isolated porcine coronary arterioles in the presence or absence of endothelium was conducted using dihydroethidium (DHE) with slight modification from a previous study. Images were obtained before and after extraluminal incubation of vessels with NOS inhibitor N G-nitro-L-arginine methyl ester (10 μmol/L), TPC, or catalase (1000 U/mL). The hypoxanthine-xanthine oxidase system was used to distinguish between superoxide and xanthine oxidase-generated oxidative metabolites. Diameters were measured before and after extraluminal incubation of vessels with NOS inhibitor N G-nitro-L-arginine methyl ester (10 μmol/L) or catalase (1000 U/mL). Treatment effects were examined in a separate group of vessels by determining NOS activity and hydrogen peroxide scavengers, were administered to determine the role of LOX-1 and hydrogen peroxide, respectively, in the observed CRP effect. In another cohort of vessels, vasodilation to arachidonic acid (10 μmol/L), an endothelium-dependent PGI₂-mediated agonist, was examined before and after incubation with PGI₂ synthase inhibitor trans-2-phenyl cyclopropylamine (TPC; 100 μmol/L) or after 60-minute intraluminal incubation with recombinant CRP (7 μg/mL) in the absence or presence of an anti–LOX-1 antibody (5 μg/mL), TPC plus an anti–LOX-1 antibody, or an anti-CD32 antibody (5 μg/mL). In preliminary studies, dilations of coronary arterioles to serotonin and arachidonic acid were repeatable without any sign of tachyphylaxis (data not shown).

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**Immunohistochemical Detection of LOX-1 and CD32**

Coronary arterioles were prepared for immunohistochemical analysis as described previously. Sections (10-μm thick) were immunolabeled with anti–LOX-1 polyclonal antibody (1:150 dilution), anti-CD32 monoclonal antibody (1:100 dilution), anti–endothelial NOS (eNOS) monoclonal antibody (1:100 dilution), anti-CRP monoclonal antibody (1:100 dilution, co-staining with anti–LOX-1), or anti-CRP polyclonal antibody (1:100 dilution, co-staining with anti-CD32). The eNOS staining was used as a marker for endothelial layer localization. Images were observed using fluorescence microscopy as described previously.

**Cell Culture**

Human coronary artery endothelial cells (HCAEC) were purchased from Genlantis. Cells at passage 3 or 4 were pretreated with anti-CD32 antibody (5 μg/mL), anti–LOX-1 antibody (5 μg/mL), or vehicle control (physiological saline solution) for 30 minutes, and then recombinant CRP (7 μg/mL) was added to the cells for 12 hours. After the 12-hour incubation, cells were sonicated, and the protein

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concentration in the supernatant was measured. The cell lysates were used for Western blot analysis as described previously.10

Small Interfering RNA Transfection
HCAEC were transfected with 20 nmol/L control small interfering RNA (siRNA; scrambled nontargeting sequence; sc-37007; Santa Cruz Biotechnology) or 20 nmol/L LOX-1 siRNA (sc-40185; Santa Cruz Biotechnology) using Lipofectamine 2000 transfection reagent (Invitrogen) or were treated without siRNA–Lipofectamine. The cell lysates were used for Western blot analysis to verify the efficacy of protein knockdown by siRNA.

Immunoprecipitation and Western Blot Analysis
To determine whether CRP binds to LOX-1 and CD32 in intact vessels, isolated coronary arterioles were incubated with vehicle (physiological saline solution) or recombinant CRP (7 µg/mL) for 60 minutes. Protein isolated from each vessel sample was used for immunoprecipitation and Western blot analysis as described previously.10

Because activation of CD32 by CRP has been shown in cultured cells to alter the expression of thrombogenic proteins such as plasminogen activator inhibitor-1 (PAI-1),35 we assessed whether CRP influences PAI-1 protein expression in intact blood vessels. In this regard, coronary arterioles were isolated and incubated with vehicle, recombinant CRP (7 µg/mL), CRP plus anti–LOX-1 antibody (5 µg/mL), or CRP plus anti-CD32 antibody (5 µg/mL) for 12 hours. The possible roles of both LOX-1 and CD32 activation in CRP-induced endothelial PAI-1 expression were also assessed in cultured HCAEC as described above. Protein isolated from each vessel and cell sample was used for Western blot analysis with an anti–PAI-1 monoclonal antibody (1:500; R&D Systems). Statistical comparisons of data were performed with 1-way ANOVA followed by the Bonferroni multiple range test. A value of \( P < 0.05 \) was considered significant. Data are presented as mean±SEM.

Results
Role of LOX-1 in CRP-Induced Endothelial Dysfunction
All isolated coronary arterioles developed a similar level of basal tone, \( \approx 61 \pm 1 \% \) of maximal passive diameter (87 µm; range, 57–118 µm). Serotonin dilated coronary arterioles in a concentration-dependent manner (Figure 1), and this dilation was significantly reduced by \( \text{N}^2\text{-nitro-L-arginine methyl ester} \) (Figure 2A). In another set of experiments, administration of recombinant CRP to the lumen of the vessel for 60 minutes did not alter basal diameter (control, 54±5 µm; CRP, 55±5 µm) but significantly attenuated the dilation to serotonin (Figure 1) in a comparable manner as that produced by \( \text{N}^2\text{-nitro-L-arginine methyl ester} \) (Figure 2A). In contrast, CRP did not affect endothelium-independent vasodilation to an NO donor sodium nitroprusside (Online Figure 1). The converse effect of recombinant CRP was neutralized in the presence of an anti-CRP monoclonal antibody (Figure 2A). In the presence of LOX-1 antagonist \( \kappa\)-carrageenan19 or anti–LOX-1 antibody, the adverse effect of CRP on serotonin-induced vasodilation was also eliminated (Figure 2B). However, blocking CD32 with an anti-CD32 antibody had no influence on the inhibitory action of CRP (Figure 2B). Arachidonic acid (10 µmol/L) elicited a significant dilation of coronary arterioles, and this dilation was nearly abolished by PGI2 synthase inhibitor TPC

Figure 1. Effect of C-reactive protein (CRP) on serotonin-induced dilation of coronary arterioles. Representative tracing showing concentration-dependent dilation of isolated and pressurized porcine coronary arterioles to serotonin before and after recombinant CRP (rCRP) treatment. The maximal diameter was established in response to sodium nitroprusside (SNP; 10–4 mol/L) at the end of the experiment.

Figure 2. C-reactive protein (CRP) inhibits nitric oxide (NO)–mediated dilation by activating lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). A, Coronary arteriolar dilation to serotonin was examined before and after extraluminal incubation with NO synthase inhibitor \( \text{N}^2\text{-nitro-L-arginine methyl ester} \) (L-NAME; n=5) or intraluminal incubation with 7 µg/mL CRP in the absence (n=7) and presence of anti-CRP antibody (35 µg/mL; n=4). B, Vasodilation to serotonin was examined before and after incubation with 7 µg/mL CRP in the absence (n=7) and presence of LOX-1 antagonist \( \kappa\)-carrageenan (125 µg/mL; n=5), anti–LOX-1 antibody (5 µg/mL; n=10), or anti-CD32 antibody (5 µg/mL; n=4). Diameter changes to serotonin were normalized to the maximal dilation in response to sodium nitroprusside (10–4 mol/L). *\( P < 0.05 \) vs control. Ab indicates antibody; and n, number of vessels (1 per animal).
Role of LOX-1 in CRP-Induced Superoxide Production

Because we previously showed that elevated superoxide production contributes to CRP-induced impairment of serotonin-induced vasodilation, we determined whether LOX-1 activation influences superoxide levels in live, pressurized coronary arterioles. In the absence of CRP (ie, vehicle control), real-time DHE fluorescence revealed sparse levels of superoxide in the arteriolar wall (Figure 4). However, intraluminal incubation of vessels with CRP (7 μg/mL; 60 minutes) markedly increased 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL)-sensitive superoxide levels about 3-fold, predominantly in the endothelial cells with longitudinal alignment of nuclear staining and sparse transverse nuclear staining of smooth muscle cells. In the presence of an anti-LOX-1 antibody but not an anti-CD32 antibody, the elevated superoxide level in the endothelium and smooth muscle was significantly reduced (Figure 4). In the absence of endothelium, both longitudinal and transverse DHE signals were negligible with (Figure 4) or without (data not shown) CRP treatment. In these endothelium-denuded vessels, the vasodilation to serotonin (0.1 μmol/L) was abolished (control: 63±4% dilation versus denudation: 3±1% dilation; n=4), and the endothelium-independent vasodilation to sodium nitroprusside (10 μmol/L) was unaltered (control: 85±6% dilation versus denudation: 80±9% dilation; n=4). In the presence of an effective concentration of catalase, a scavenger of hydrogen peroxide, the inhibitory effect of CRP on vasodilation to serotonin remained (Online Figure II).

Cellular Localization of LOX-1 and CD32 in Coronary Arterioles

Immunostaining results indicated that LOX-1 staining was primarily localized to the endothelial layer and was closely associated with eNOS signals (Figure 5A). After treating the vessels with CRP, this peptide was detected in the location highly expressed with LOX-1 (Figure 5B). CD32 was also expressed primarily in the endothelial layer of coronary arterioles and overlapped with eNOS and CRP (Figure 5C and 5D). Support for the binding of CRP with LOX-1 and CD32 was provided by Western blot data showing detection of CRP in immunoprecipitated LOX-1 or CD32 protein from coronary arterioles pretreated with CRP only (Figure 6).

Roles of LOX-1 and CD32 in CRP-Induced Increase in PAI-1 Expression

The positive coimmunoprecipitation of CRP with both LOX-1 and CD32 suggests a close interaction of these receptors with CRP. Because CRP has been shown to promote PAI-1 expression via CD32 activation in cultured endothelial cells, we examined the roles of LOX-1 and CD32 in regulating the expression of PAI-1 in intact coronary arterioles. After 12-hour incubation of coronary arterioles with CRP, PAI-1
protein expression increased ≈3-fold (Figure 7). The CRP-induced increase in PAI-1 expression was abolished by either an anti–LOX-1 or an anti-CD32 antibody (Figure 7). There was no greater inhibitory effect after combined treatment with both antibodies (n=2, data not shown). However, the ability of CRP to increase PAI-1 expression in cultured HCAEC was inhibited by an anti-CD32 antibody but was unaffected by an anti–LOX-1 antibody (Figure 8). Notably, the specificity of the anti–LOX-1 and anti-CD32 antibodies for the cognate proteins was confirmed via immunoblots of control nontreated HCAEC (Online Figure IIIA). In addition, the LOX-1 antibody detected the knockdown of LOX-1 protein expression by ≈75% in HCAEC after LOX-1 siRNA, but not scrambled siRNA, treatment. Both LOX-1 and scrambled siRNA had no effect on the actin protein expression (Online Figure IIIB). These data support the specificity and potency of the LOX-1 antibody used in the present study.

Discussion

Recent clinical and experimental findings support CRP as a mediator of inflammation and atherogenesis. One of the
earliest manifestations of atherosclerosis is impairment of endothelial cell function and vascular reactivity. In the current study, we showed that acute exposure of coronary arterioles to CRP, at a concentration known to predict cardiovascular risk, elevated vascular superoxide and led to impaired endothelial function for NO- and PGI$_2$-mediated vasodilations through the activation of endothelial LOX-1. By stimulating LOX-1, CRP could contribute to oxidative stress-induced endothelial dysfunction, which is commonly associated with inflammatory cardiovascular diseases. We have also observed a similar inhibitory effect on the endothelium-dependent vasodilation by CRP derived from human serum (Online Figure IV) or by the native form of CRP from ascitic/pleural fluid, further supporting the detrimental actions of CRP with clinical relevance.

Controversial evidence has been reported for the putative CRP receptor in the vascular cells. Earlier reports in cultured human and bovine aortic endothelial cells have indicated that CRP activates CD32, whereas recent studies in the same type of cell lines have shown that CRP can also bind to LOX-1. Although CRP has been shown to increase endothelial expression of both CD32 and LOX-1, the receptors responsible for the alteration of vasomotor function of intact arterioles by CRP remain unknown. In the current study, we demonstrate for the first time in the intact coronary microvasculature that CRP inhibits endothelium-dependent dilation and promotes superoxide production by activating LOX-1 rather than CD32 because pharmacological blockade of LOX-1 with k-carrageenan or exposure of the vessels to an anti-LOX-1 antibody, but not to an anti-CD32 antibody, protected the vessels from CRP insult.

LOX-1 exhibits broad ligand specificity by binding to polyanionic compounds, apoptotic cells, and activated platelets, in addition to oxidized low-density lipoprotein. After ligand binding, LOX-1 could mediate a wide array of proatherogenic events, including activation of endothelial NAD(P)H oxidase for superoxide production and reduction of endothelial NO bioavailability through superoxide scavenging. Interestingly, our previous studies showed that the adverse effect of CRP on endothelial NO-mediated dilation and NO production in response to serotonin is also sensitive to the pharmacological blockade of NAD(P)H oxidase. However, it is unclear whether there is a direct link of CRP to LOX-1 activation and the subsequent oxidative stress. In the present study, we demonstrated that CRP markedly increased superoxide levels in small coronary arterioles, which was prevented by the blockade of LOX-1 but not CD32. This CRP-evoked LOX-1-dependent oxidative stress was prevented by endothelial removal (Figure 4), suggesting the crucial role of the endothelium in LOX-1 activation and superoxide production. Furthermore, CRP also promotes the generation of peroxynitrite, a product of superoxide and NO interaction, and consequently compromises PGI$_2$ release and PGI$_2$-mediated dilation in response to arachidonic acid. The adverse effect seems to be mediated by the LOX-1 activation because blockade of this receptor effectively preserved vasodilation to arachidonic acid in a manner sensitive to a PGI$_2$ synthase inhibitor (Figure 3). Taking into consideration the broad binding capabilities of LOX-1 and the similar properties between CRP and LOX-1 in eliciting endothelial dysfunction, it is likely that CRP interacts with LOX-1 to exert its adverse effect on eNOS- and cyclooxygenase-related signaling pathways for vasomotor regulation via oxidative insult (Figure 4).

LOX-1 is highly expressed in both endothelial cells and smooth muscle cells in large conduit arteries associated with atherosclerotic lesions. However, its expression in normal small resistance vessels has not been documented. In the current study, distinct from previous reports on large conduit vessels, we demonstrated for the first time that LOX-1 is present in the coronary arteriolar wall, primarily in the endothelial layer (Figure 5). This may explain, in part, the evidence that coronary resistance arterioles display little smooth muscle remodeling and anatomic lesions in contrast to their upstream atherosclerotic conduit arteries. CRP was detected in the region heavily expressed with LOX-1 after administration of this peptide. Overlapping of LOX-1 with eNOS expression and coimmunoprecipitation of CRP with LOX-1 support the notion that endothelial LOX-1 activation contributed to the rapid adverse effect of CRP on eNOS-mediated vascular regulation. Although we have also shown that CD32 is expressed in the endothelial region of coronary arterioles (Figure 5) and coimmunoprecipitates with CRP (Figure 6), the vasomotor results indicate that CRP/CD32 binding does not contribute to vasodilator dysfunction of arterioles. Our findings suggest that the binding of CRP to LOX-1 is critical for initiating the impairment of endothelium-dependent vasodilation in the intact coronary arterioles. It is noted that although LOX-1 was found primarily in the endothelial layer, superoxide levels were elevated in both the endothelium and smooth muscle after CRP exposure. Because CRP induced negligible DHE signals in the absence of endothelial cells (Figure 4), this
result supports the idea that increased superoxide production by CRP–LOX-1 interaction is initiated in the endothelium as the primary source. It has been shown that hydrogen peroxide, a downstream product from superoxide dismutase, can impair vasomotor function.\textsuperscript{29,46} Inhibition of hydrogen peroxide formation by cell-permeable polyethylene-glycolated catalase has been reported to prevent hydrogen peroxide signaling in the vasculature.\textsuperscript{57} To determine whether hydrogen peroxide contributed to the observed vascular dysfunction in our study, the vessels were pretreated with this modified catalase, and the effect of CRP was subsequently investigated. However, this modified catalase caused loss of basal tone of coronary arterioles, and the vasomotor activity could not be further studied. Nevertheless, using an effective concentration of unmodified catalase,\textsuperscript{29,30} we found that the adverse effect of CRP remained (Online Figure II), suggesting nominal contribution of hydrogen peroxide in mediating the CRP effect in our preparation.

Under physiological conditions, the endothelial cell release of NO and PGI\textsubscript{2} plays an important role not only in regulating vascular tone, but also in maintaining an antithrombogenic layer. Both NO and PGI\textsubscript{2} can increase local tissue perfusion and maintain microvascular homeostasis by eliciting vasodilation and inhibiting platelet and inflammatory cell adherence to the vessel wall. The endothelium can also influence vessel patency by producing fibrinolytic system factors including tissue plasminogen activator and its inhibitor PAI-1.\textsuperscript{48} PAI-1 inhibits tissue plasminogen activator, which is the primary serine protease that converts plasminogen to plasmin resulting in degradation of platelet-fibrin thrombi. Because elevated plasma levels of PAI-1 are associated with coronary artery disease,\textsuperscript{49,50} CRP may have a direct effect on the fibrinolytic system as suggested by the evidence of downregulation of tissue plasminogen activator\textsuperscript{51} and upregulation of PAI-1 by CRP stimulation in cultured endothelial cells.\textsuperscript{35,52–56} These molecular findings may explain the observed enhancement of thrombosis in CRP transgenic mice with arterial injury\textsuperscript{57} and the promotion of inflammation and coagulation in parallel with PAI-1 elevation in response to intravenous infusion of CRP in healthy human subjects.\textsuperscript{58} However, the receptors contributing to the CRP effect remain to be determined. Furthermore, although CRP has been implicated to regulate PAI-1 levels via selective activation of endothelial LOX-1,\textsuperscript{15,18,27,68} our disparate results in arterioles versus HCAEC. However, it has been shown that only native CRP binds to CD32 and LOX-1.\textsuperscript{15,18,27,68} In the present study, the pentameric CRP was used as verified by native gel electrophoresis under nonreducing conditions\textsuperscript{56} (Online Figure V). Our findings provide new evidence that CRP selectively activates LOX-1 in the microvasculature to exert an adverse effect on endothelium-dependent vasomotor function but upregulates prothrombogenic protein PAI-1 via combined LOX-1/CD32 activation.

In summary, the present study demonstrates the novel finding that porcine coronary arterioles display functional LOX-1 and CD32 and that CRP elicits vasodilator dysfunction via selective activation of endothelial LOX-1. Furthermore, CRP can increase the arteriolar expression of PAI-1 after activation of these receptors. It seems that activation of both LOX-1 and CD32 by CRP in arterioles are necessary for vasodilation and promote a thrombotic milieu by reducing protective endothelial factors (NO and PGI\textsubscript{2}) and upregulating prothrombogenic factor PAI-1. The abundant expression of LOX-1 and CD32 in the endothelium of coronary resistance vessels may participate in the dysregulation of coronary flow and myocardial ischemia in patients with cardiac syndrome X, corresponding to elevated CRP levels and impaired endothelium-dependent vasodilator function\textsuperscript{69} (see Editorial).\textsuperscript{70}

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Disclosures
None.

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Supplemental Methods

Functional Assessment of Isolated Coronary Arterioles
The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal procedures were approved by the Scott & White Institutional Animal Care and Use Committee, and have been previously described. Briefly, pigs (8-12 weeks old of either sex; 7-12 kg) were anesthetized with 2-4% isoflurane and ventilated. The heart was removed and immediately placed on iced (5°C) saline. Individual subepicardial arterioles (~1 mm in length; 40-100 µm in internal diameter in situ) were dissected from the surrounding cardiac tissue. Vessels were then cannulated with glass micropipettes, pressurized to 60 cmH₂O intraluminal pressure and bathed in physiological salt solution (PSS) at 37°C. The pressurized vessel developed resting basal tone within 40 minutes of equilibration period. The inner diameter of coronary arterioles was measured using video microscopic techniques and recorded with a PowerLab data acquisition system (ADInstruments). 1

The human recombinant C-reactive protein (CRP, Calbiochem) and high purity, serum CRP (Calbiochem) used in the following protocols were dialyzed for 24 hours against Dulbecco’s phosphate buffered saline 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 coronary arteriolar endothelial function, 2 was also removed from the CRP by using Detoxi-Gel Columns (Pierce) and was found to be at the level [<0.06 EU/mL or 6 pg/mL by Limulus assay (Cambrex)] insufficient to affect vasomotor function. 2,3 The purity of recombinant CRP was assessed under reducing conditions by SDS-PAGE (4-15%, Bio-Rad) and detected via immunoblot with an anti-CRP antibody (1:100 dilution, Santa Cruz Biotechnology), which showed a single band at about 27 kDa. Native gel electrophoresis of recombinant CRP (25 µg) under non-reducing conditions was performed as described previously, 4 followed by Coomassie Blue staining, to confirm that native pentameric CRP (120 kDa) was used in the following studies. The vasodilator function of coronary arterioles was assessed by challenging the vessels with an endothelium-dependent nitric oxide (NO)-mediated agonist serotonin before and after 40-minute extraluminal incubation of vessels with NO synthase (NOS) inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME; 10 µmol/L) or 60-minute intraluminal incubation with a clinically relevant concentration of CRP (7 µg/mL). 6 To confirm the activity of recombinant CRP, human serum-derived natural CRP (Calbiochem) was used. To verify the specific action of CRP, an anti-CRP monoclonal antibody 7 was employed (clone CRP-8, Sigma; 35 µg/mL). The roles of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) and CD32 in mediating the CRP effect were examined in a separate group of vessels.
by determining dilation to serotonin before and after co-administration of CRP with anti-LOX-1 antibody (5 µg/mL; Abcam)\textsuperscript{9} or anti-CD32 antibody (5 µg/mL; Santa Cruz Biotechnology)\textsuperscript{10} for 60 minutes. κ-Carrageenan (125 µg/mL), a LOX-1 antagonist,\textsuperscript{8,11} and catalase (1000 U/mL),\textsuperscript{12} the hydrogen peroxide scavenger, were administered to determine the role of LOX-1 and hydrogen peroxide, respectively, in the observed CRP effect. In another cohort of vessels, vasodilation to arachidonic acid (10 µmol/L),\textsuperscript{14} an endothelium-dependent PGI\textsubscript{2}-mediated agonist, was examined before and after 40-minute incubation with PGI\textsubscript{2}-synthase inhibitor trans-2-phenyl cyclopropylamine (TPC, 100 µmol/L)\textsuperscript{14} or following 60-minute intraluminal incubation with recombinant CRP (7 µg/mL) in the absence or presence of an anti-LOX-1 antibody (5 µg/mL), TPC plus an anti-LOX-1 antibody, or an anti-CD32 antibody (5 µg/mL).

At the end of each experiment, diameter changes in response to vasodilator agents were normalized to the maximal diameter changes in response to 100 µmol/L sodium nitroprusside and expressed as a percentage of maximal dilation.\textsuperscript{15} We have previously shown that this concentration of sodium nitroprusside produced maximal dilation of isolated vessels because their diameters were not further increased by a calcium free solution containing EDTA (1 mmol/L).\textsuperscript{16} Data are presented as mean ± SEM and one or two vessels were used from each heart. Statistical comparisons of vasomotor responses under different treatments were performed with one- or two-way ANOVA followed by the Bonferroni multiple-range test, as appropriate. Differences in resting diameter before and after pharmacological interventions were compared with paired Student’s t-test. A value of P < 0.05 was considered significant.

Detection of Superoxide
Real-time detection of superoxide production from isolated porcine coronary arterioles in the presence or absence of endothelium was conducted using dihydroethidium (DHE) with slight modification from a previous study.\textsuperscript{16} Isolated and pressurized coronary arterioles were incubated intraluminally with PSS containing vehicle, CRP (7 µg/mL), CRP plus TEMPOL (1 mmol/L), CRP plus anti-LOX-1 antibody (5 µg/mL, Abcam), or CRP plus anti-CD32 antibody (5 µg/mL, Santa Cruz Biotechnology) at 37°C for 60 minutes. In separate experiments, the role of the endothelium in the CRP-induced superoxide production was evaluated in coronary arterioles following air bolus injection to remove endothelial cells as performed previously.\textsuperscript{17} The denuded vessels that exhibited normal basal tone, showed no vasodilation to endothelium-dependent vasodilator serotonin (0.1 µmol/L), and showed unaltered response to endothelium-independent vasodilator sodium nitroprusside (SNP, 10 µmol/L) were then incubated intraluminally with PSS containing vehicle or CRP for 60 minutes. To assess fluorescence intensity, DHE (4 µmol/L) was added in each light-protected vessel chamber for 5 minutes. The DHE was then washed out and the DHE signal was detected (800 ms exposure rate) with a 620/60 nm emission filter on a fluorescence microscope (Axiovert 200, Zeiss). The resulting images were quantitatively analyzed for DHE fluorescence intensity using ImageJ software (National Institutes of Health) with the following procedure. The average fluorescence intensity was determined for the DHE-treated control vessels from each experimental group and then all individual control and CRP-treated vessels from the same group were normalized to the corresponding average value. Statistical comparisons of data were performed with one-way analysis of variance followed by the Bonferroni multiple-range test. A value of P < 0.05 was considered significant. Data are presented as mean ± SEM.

Immunohistochemical Detection of LOX-1 and CD32
Isolated coronary arterioles (40-100 µm in diameter and 1.5 mm in length) were embedded in Tissue Tek O.C.T. compound for cryostat sections. Some vessels were pressurized and incubated intraluminally with CRP (7 µg/mL) at 37°C for 60 minutes as described above for
vasomotor study before embedding. The embedded arterioles were cut into sections 10-µm-thick and placed on glass slides. The tissue slides were incubated with blocking diluent (5% normal rabbit serum and 1% BSA in PBS) at room temperature for 1 hour and then with anti-LOX-1 polyclonal antibody (1:150 dilution, Abcam), anti-CD32 monoclonal antibody (1:100 dilution, Santa Cruz Biotechnology), anti-endothelial NOS (eNOS) polyclonal antibody (1:100 dilution, Santa Cruz Biotechnology), anti-CRP monoclonal antibody (1:100 dilution, Sigma; co-staining with anti-LOX-1), or anti-CRP polyclonal antibody (1:100 dilution, Santa Cruz Biotechnology; co-staining with anti-CD32) at 4°C overnight. The eNOS staining was utilized as a marker for endothelial layer localization. The slides were then rinsed and incubated with the secondary antibody, FITC-conjugated anti-IgG (1:150 dilution, Jackson ImmunoResearch Laboratories) or Cy3-conjugated anti-IgG (1:150 dilution, Jackson ImmunoResearch Laboratories) at room temperature for 1 hour. Subsequently, the slides were sealed by a coverslip with mounting media (Sigma). Control tissues were exposed for the same duration to non-immune goat serum (1:150 dilution, Sigma) and secondary antibody and were used to establish background staining. Images were taken with a fluorescence microscope (Nikon Diaphot 300) and Digital Sight Camera (model DS-5M-L1). Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for both control and experimental tissues. Merged images were created using NIH ImageJ software.

Cell Culture
Human coronary artery endothelial cells (HCAEC) were purchased from Genlantis were cultured in T-75 flasks with 15 mL of MesoEndo Cell Growth Medium (Cell Applications). Cells were maintained at 37°C in 5% CO₂ atmosphere. The medium was changed every 48 hours and the cells were subcultured onto 60-mm tissue culture dishes when the cells reached 80% confluence. Cells were cultured to 90% confluence and then the medium was replaced with the starvation medium (Cell Applications) for 24 hours. Cells at passage 3 or 4 were pretreated with anti-CD32 antibody (5 µg/mL, Santa Cruz Biotechnology), anti-LOX-1 antibody (5 µg/mL, Abcam) or vehicle control (PSS) for 30 minutes and then recombinant CRP (7 µg/mL) was added to the cells at 37°C for 12 hours. After the 12-hour incubation, cells were sonicated on ice and the protein concentration in the supernatant was measured by BCA protein assay kit (Pierce). The cell lysates were utilized for Western blot analysis as described below.

siRNA Transfection
HCAEC were transfected with 20 nmol/L control siRNA (sc-37007, Santa Cruz Biotechnology) or 20 nmol/L LOX-1 siRNA (sc-40185, Santa Cruz Biotechnology) using Lipofectamine™ 2000 (Invitrogen) transfection reagent or were treated without siRNA-Lipofectamine™ at 37°C. A higher concentration of 100 nmol/L LOX-1 siRNA did not cause further knockdown of LOX-1 in pilot studies, so studies were completed with 20 nmol/L LOX-1 siRNA. After 16 hours, the siRNA-Lipofectamine™ complexes and medium were removed and fresh MesoEndo Cell Growth Medium (Cell Applications) was added to the cells at 37°C for 48 hours. Protein was then isolated from cells as described above and utilized for Western blot analysis with the LOX-1 antibody (Abcam) to verify efficacy of protein knockdown by siRNA.

Immunoprecipitation and Western Blot Analysis
To determine whether CRP binds LOX-1 and CD32 in intact vessels, isolated coronary arterioles (40 to 100 µm in diameter and 1.5 mm in length; 6-7 vessels per sample) were incubated with PSS containing vehicle or recombinant CRP (7 µg/mL) at 37°C for 60 minutes. All samples were then washed and lysis buffer was added. After sonication on ice, the protein
concentration in the supernatant was measured by BCA protein assay kit (Pierce). Equal amounts of protein (10-20 µg) from each sample were incubated with an anti-CD32 antibody (1:100 dilution, Santa Cruz Biotechnology) or an anti-LOX-1 antibody (1:50 dilution, Abcam). Immune complexes were precipitated with 20 µl Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). For Western blot analysis, total immune complexes or protein samples from vessel lysate (20 µL) were separated by Tris-Glycine SDS-PAGE (4-15%, Bio-Rad), transferred onto nitrocellulose membrane, and then incubated with an anti-CRP antibody (1:1000 dilution, Santa Cruz Biotechnology). After incubation with appropriate secondary antibody, membranes were developed by enhanced chemiluminescence (Pierce). Membranes containing the total vessel lysate samples were stripped and re-probed with anti-actin antibody (1:1000, Santa Cruz Biotechnology) to confirm equal loading.

Because activation of CD32 by CRP has been shown in cultured cells to alter the expression of thrombogenic proteins such as plasminogen activator inhibitor-1 (PAI-1), we assessed whether CRP influences PAI-1 protein expression in intact blood vessels. In this regard, coronary arterioles were isolated and pretreated with PSS containing vehicle or antibodies (anti-LOX-1 or anti-CD32) for 30 minutes, and then incubated with vehicle, recombinant CRP (7 µg/mL), CRP plus anti-LOX-1 antibody (5 µg/mL), or CRP plus anti-CD32 antibody (5 µg/mL) at 37°C for 12 hours. The samples (4-5 vessels per sample) were sonicated on ice and the protein concentration in the supernatant was measured by BCA protein assay kit (Pierce). The possible role of both LOX-1 and CD32 activation in CRP-induced endothelial PAI-1 expression was also assessed in cultured HCAEC as described above. Equal amounts of protein (2 µg for vessel lysate and 5 µg for cell lysate) were diluted in sample buffer and separated by Tris-Glycine SDS-PAGE (4-15% Tris-HCl Ready Gels, Bio-Rad). The proteins were transferred onto a nitrocellulose membrane, blocked for 2 hours in Tris-buffered saline containing 3% non-fat dry milk and Tween-20 at room temperature, and then incubated with anti-PAI-1 monoclonal antibody (1:500; R&D Systems) overnight at 4°C. After washing, the membranes were incubated with appropriate horseradish peroxidase secondary antibody (1:1000; Santa Cruz Biotechnology) for 2 hours at room temperature, followed by detection of immunoreactive proteins using enhanced chemiluminescence (Thermo Scientific). Membranes were then stripped and re-probed with an anti-actin antibody (1:1000, Santa Cruz Biotechnology) to confirm equal protein loading. Densitometric analysis of immunoblots was performed with NIH ImageJ software. Statistical comparisons of Western blot data were performed with one-way analysis of variance followed by the Bonferroni multiple-range test. A value of P < 0.05 was considered significant. Data are presented as mean ± SEM.

Supplemental References


Online Figure I. CRP does not alter endothelium-independent dilation to sodium nitroprusside

Coronary arteriolar dilation to nitric oxide donor sodium nitroprusside was examined before and after intraluminal incubation with 7 µg/mL recombinant CRP (n = 3). n = number of vessels (one per animal).
Online Figure II. Unmodified catalase does not alter CRP-induced impairment of endothelium-dependent nitric oxide-mediated vasodilation

Coronary arteriolar dilation to serotonin was examined before and after intraluminal incubation with 7 μg/mL recombinant CRP (n = 3) or CRP plus 1000 U/mL catalase (n = 3). n = number of vessels (one per animal). *P < 0.05 vs. Control (analysis of variance followed by the Bonferroni multiple-range test).
Online Figure III. Protein expression of LOX-1 and CD32 in HCAEC

(A) Immunoblots show detection of LOX-1 and CD32 expression in cultured HCAEC. (B) HCAEC were treated with LOX-1 siRNA or nontargeting scrambled siRNA in the presence of Lipofectamine™ 2000 transfection reagent, or without siRNA-Lipofectamine (Control). Immunoblots show the specific knockdown of LOX-1 protein by LOX-1 siRNA without altering expression of actin. LOX-1 expression for each sample was normalized with corresponding actin level. Data represent three independent experiments with each sample in duplicate. *P < 0.05 vs. Control or Scrambled.
Online Figure IV. Serum-derived CRP inhibits endothelium-dependent nitric oxide-mediated vasodilation

Coronary arteriolar dilation to serotonin was examined before and after intraluminal incubation with 7 µg/mL human serum-derived CRP (n = 3). n = number of vessels (one per animal). *P < 0.05 vs. Control (analysis of variance followed by the Bonferroni multiple-range test).
Online Figure V. Coomassie Blue staining of native pentameric CRP

Native gel electrophoresis under non-reducing conditions followed by Coomassie Blue staining shows detection of native pentameric form of recombinant CRP (25 µg) at about 120 kDa.