Background—C-reactive protein (CRP) has been suggested to actively participate in the development of atherosclerosis. In the present study, we examined the role of the potent endothelium-derived vasoactive factor endothelin-1 (ET-1) and the inflammatory cytokine interleukin-6 (IL-6) as mediators of CRP-induced proatherogenic processes.

Methods and Results—Saphenous vein endothelial cells (HSVECs) were incubated with human recombinant CRP (25 μg/mL, 24 hours) and the expression of vascular cell adhesion molecule (VCAM-1), intracellular adhesion molecule (ICAM-1), and monocyte chemoattractant chemokine-1 was determined. The effects of CRP on LDL uptake were assessed in macrophages using immunofluorescent labeling of CD32 and CD14. In each study, the effect of endothelin antagonism (bosentan) and IL-6 inhibition (monoclonal anti-IL-6 antibodies) was examined. The effects of CRP on the secretion of ET-1 and IL-6 from HSVECs were also evaluated. Incubation of HSVECs with recombinant human CRP resulted in a marked increase in ICAM-1 and VCAM-1 expression (P<0.001). Likewise, CRP caused a significant increase in monocyte chemoattractant chemokine-1 production, a key mediator of leukocyte transmigration (P<0.001). CRP caused a marked and sustained increase in native LDL uptake by macrophages (P<0.05). These proatherosclerotic effects of CRP were mediated, in part, via increased secretion of ET-1 and IL-6 (P<0.01) and were attenuated by both bosentan and IL-6 antagonism (P<0.01).

Conclusions—CRP actively promotes a proatherosclerotic and proinflammatory phenotype. These effects are mediated, in part, via the production of ET-1 and IL-6 and are attenuated by mixed ET A/B receptor antagonism and IL-6 inhibition. Bosentan may be useful in decreasing CRP-mediated vascular disease. (Circulation. 2002;105:1890-1896.)

Key Words: protein, C-reactive ■ cell adhesion molecules ■ endothelium ■ atherosclerosis ■ endothelin

Atherosclerotic cardiovascular disease represents the most common cause of death worldwide. Our knowledge of the pathogenesis and treatment of atherosclerosis has progressed exponentially, yet <50% of coronary artery disease can be ascribed to traditional risk factors, such as family history, hypercholesterolemia, smoking, diabetes, obesity, and hypertension. Attention has thus focused on identifying novel markers and mechanisms of atherosclerosis, of which C-reactive protein (CRP) has emerged as one of the most important.1–12 Indeed, the inflammatory marker CRP has been shown to predict myocardial infarction, stroke, and vascular death in a variety of settings. Elevated CRP levels are one of the strongest independent predictors of future cardiovascular events (in apparently healthy men and women) and also portend the vulnerability of an atherosclerotic plaque toward rupture.13 Although these observations have set the stage for routine CRP measurements to enter cardiovascular prediction algorithms, we are just beginning to understand the mechanisms linking elevated CRP to vascular disease. Indeed, recent studies suggest that CRP is not merely a nonspecific marker of inflammation and atherosclerosis but actively participates in lesion formation through inducing endothelial dysfunction and leukocyte activation.14–16

In the present study, we hypothesized that the proatherosclerotic and inflammatory effects of CRP may be mediated in part via increased production or action of the potent endothelium-derived vasoactive factor endothelin-1 (ET-1) and the inflammatory cytokine interleukin-6 (IL-6).

Methods

Cell Adhesion Molecule Expression

Human saphenous vein endothelial cells (HSVECs, from VEC Technologies, Inc) were grown in MCDB-131 complete medium (VEC Technologies, Inc) supplemented with 10% FBS (Gibco). HSVECs at passages 2 through 5 (n=7 per group) were incubated with 25 μg/mL of human recombinant CRP (Calbiochem) for 24 hours. The concentration of CRP used was based on previous publications.14,15 In experiments assessing the effects of ET antagonism and IL-6 inhibition, HSVECs were pretreated with 10 μmol/L bosentan (mixed ET A/B receptor antagonist, Actelion Pharmaceuticals Ltd) or 5 μg/mL anti-human IL-6 antibody (RandD Systems) for...
2 hours before being incubated with CRP as above. The concentration of the IL-6 monoclonal antibody was sufficient to neutralize >95% of cytokine activity in vitro. In a parallel series of experiments, the effects of vehicle (buffer) and anti-IL-1 monoclonal antibody (neutralizing concentrations) were tested. Anti-IL-1 served as the irrelevant antibody control. Endothelial cells were detached with nonenzymatic cell dissociation solution (Sigma) and were stained for CD54 (intracellular adhesion molecule-1 [ICAM-1]), CD106 (vascular cell adhesion molecule-1 [VCAM-1]), or CD31 (platelet-endothelial cell adhesion molecule-1 [PECAM-1]) using monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-CD54, anti-CD106, or anti-CD31 antibodies (Pharmingen), all at a 1:5 dilution. Cells were analyzed using a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. The fluorescence intensity of 10,000 cells for each sample was quantified, and unstained cells were used as controls.

Monocyte Chemoattractant Protein-1, ET-1, and IL-6 Secretion

HSVECs were cultured as described above. HSVECs at passages 2 through 5 (n = 7 per group) were incubated with 25 μg/mL human recombinant CRP (Calbiochem) for 24 hours in the presence and absence of bosentan and anti-human IL-6 antibody (R&D Systems) for 2 hours before being incubated with CRP, as described above. Culture supernatants were collected, and the secretion of monococyte chemoattractant protein-1 (MCP-1) and IL-6 was assessed by sandwich ELISA (R&D Systems). ET-1 secretion into the culture supernatant assessed with a commercial enzyme immunoassay kit was used to assess ET-1 production (American Research Products, Inc). All determinations were performed in triplicate.

Monocyte Isolation and Macrophage LDL Uptake Assay

Human monocytes were isolated from the heparinized blood of healthy volunteers, as previously described.17 Cells were adjusted with RPMI-10% FBS (Gibco) to a density of 5.0 × 10^7 cells/mL. Monocyte-derived macrophages were prepared by in vitro incubation of purified monocytes on 12-well tissue culture plates for 7 days at 37°C, 5% CO₂ in RPMI-10% FBS.

A protocol adapted from Zwaka et al was used to assess LDL uptake in macrophages. Briefly, 900 mg/L human recombinant CRP (Calbiochem) was coincubated with 1000 mg/L native LDL (Sigma) in PBS containing CaCl₂ (0.132 g/L) and MgCl₂ (0.1 g/L) at room temperature for 15 minutes. The supernatant was diluted in RPMI-10% to a final concentration of 240 mg/L CRP and 250 mg/dL LDL. The CRP/LDL coincubate was centrifuged at 15,000 rpm for 30 minutes to remove high molecular aggregates and was cooled to 4°C. Substitution with PBS instead of CRP served as a control. Macrophages were incubated for 12 hours in RPMI-2% FBS and then serum-starved for 3 hours. Cells were washed with PBS (4°C) and incubated with CRP/LDL coincubates at 4°C for 1 hour. In experiments assessing the effects of ET antagonism and IL-6 inhibition, macrophages were pretreated with 10 μmol/L bosentan (Actelion Ltd) or 5 μg/mL anti-human IL-6 antibody (R&D Systems) for 3 hours before being incubated with CRP/LDL coincubates, as described above. Macrophages were stained for CD32 and CD14 (macrophage marker) using monoclonal FITC-conjugated anti-CD32 and monoclonal PE-conjugated anti-CD14 antibodies (Pharmingen), both at a 1:5 dilution. Cells were analyzed using a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. Forward and side scatter characteristics were used to gate cell population and exclude extraneous debris. A total of 10,000 positively staining cells were analyzed.

Statistical Analysis

All data are presented as mean±SD of separate experiments. Differences between group means were determined by a one-way ANOVA followed by a Newman Keuls test for post hoc comparisons. Values of P<0.05 were considered significant.

Results

CRP Induces ET-1 and IL-6 Production

Figures 1 and 2 depict the effects of human recombinant CRP on ET-1 and IL-6 production from HSVECs. In cells stimulated with CRP, the levels of the cytokines IL-6 secreted into the medium were significantly higher at 24 hours (control 14±5 versus CRP 142±25 pg/mg, P<0.01, Figure 1). Likewise, CRP potentiated the production of ET-1 from endothelial cells (Figure 2). Because ET-1 is known to serve as a stimulus for IL-6 production, we studied the effects of CRP on IL-6 production in the presence and absence of the endothelin receptor antagonist bosentan. Bosentan did not attenuate CRP-induced IL-6 production, suggesting that this response was likely independent of ET-1 (Figure 2). Likewise, anti-IL-6 antibody did not alter the production of ET-1 in response to CRP, additionally supporting the theory that these two pathways may operate in a parallel fashion (Figure 1). Control experiments with vehicle and irrelevant monoclonal antibodies did not alter the action of CRP (not shown).

Cell Adhesion Molecule Expression

Figures 3 and 4 depict the effects of human recombinant CRP (25 μg/mL) on HSVEC ICAM-1 and VCAM-1 expression after a 24-hour incubation period in the presence of 10% FBS.
Incubation with CRP resulted in a marked increase in ICAM-1 and VCAM-1 expression ($P<0.001$). In cells preincubated with bosentan or IL-6 antibody, ICAM-1 and VCAM-1 expression was significantly attenuated ($P<0.01$). The effects of coinubcation with bosentan and IL-6 antibody were additive ($P<0.05$). Expression is related to mean fluorescence intensity from forward scatter and side scatter. Expression of PECAM-1 (CD31) was used as a marker of endothelial cell viability, and the mean fluorescence intensity was corrected for PECAM-1.

**MCP-1 Secretion in HSVECs**

Figure 5 depicts the effects of human recombinant CRP (25 μg/mL) on HSVEC secretion of MCP-1 in the presence and absence of 2 hours of pretreatment with bosentan (10 μmol/L) or an anti-human IL-6–neutralizing antibody (5 μg/mL) after a 24-hour incubation in the presence of 10% FBS. Incubation with CRP in the absence of any intervention resulted in a marked increase in the secretion of MCP-1 (from 1140±90 to 2600±210 pg/mL, $P<0.01$). When CRP was added to cells preincubated with bosentan or anti–IL-6 antibody, the increased secretion of MCP-1 was attenuated ($P<0.05$). Attenuation of CRP-induced MCP-1 production was greater during coincubation with bosentan and anti–IL-6 antibody ($P<0.01$, Figure 5), additionally supporting the theory that CRP may stimulate ET-1 and IL-6 concurrently.

**Macrophage LDL Uptake**

To investigate the role of ET-1 and IL-6 as mediators of CRP-induced LDL uptake, we conducted a flow-cytometric 2-color analysis of anti-CD32 and anti-CD14 that was previously used to demonstrate internalization and colocalization of macrophage CD32 with CRP/LDL complexes.† Figure 6 depicts the effect of human recombinant CRP (25 μg/mL) on human macrophage LDL uptake in the presence and absence of 3-hour pretreatment with bosentan (10 μmol/L) or an anti-human IL-6–neutralizing antibody (5 μg/mL). Analysis of flow cytometric data of macrophages incubated with LDL alone revealed an 89.5% positive stain for CD32 and CD14, whereas after incubation with LDL and CRP, a 61.3% positive stain for CD32 and CD14 was observed; this represents a significant uptake of LDL ($P<0.05$). When CRP/LDL was added to cells preincubated with bosentan, CRP-mediated LDL uptake observed previously was significantly attenuated ($P<0.05$). Similarly, when CRP/LDL was added to cells preincubated with anti-human IL-6 antibody, CRP-mediated LDL uptake was also significantly attenuated ($P<0.05$), suggesting that the effects of CRP on LDL uptake were mediated, in part, by the action of IL-6 and ET-1.
Discussion

Main Observations

The present study was conducted to examine the relationship between ET-1, IL-6, and the direct proinflammatory effects of CRP. To this aim, we investigated the effects of human recombinant CRP on processes of early atherosclerotic lesion development in the presence and absence of ET receptor blockade and IL-6 inhibition. The following observations were made. First, HSVECs incubated with human recombinant CRP exhibit increased secretion of ET-1 and IL-6. This seems to occur in a parallel or concurrent fashion (in contrast to ET-1–mediated stimulation of IL-6). Second, CRP augments the expression of cell adhesion molecules ICAM-1 and VCAM-1 on HSVECs; this effect is attenuated equally and independently by a mixed ETA/B receptor antagonist (bosentan) and with anti–IL-6 antibodies. Third, CRP promotes the secretion of the key leukocyte chemoattractant chemokine MCP-1 from HSVECs. This effect is also attenuated equally and independently by bosentan and IL-6 inhibition. Fourth, CRP facilitates macrophage LDL uptake, an effect inhibited by bosentan and IL-6 antibodies. These data suggest an important mechanistic relationship between ET-1, IL-6, and CRP. The ability of CRP to induce a vascular proinflammatory phenotype may be related, in part, to increased production or action of ET-1 and IL-6. By the same token, pharmacological agents such as bosentan may represent a novel approach to attenuate the vascular actions of CRP.

Inflammation Plays a Central Role in the Development of Atherosclerosis

The formation of an atherosclerotic lesion begins as a fatty streak underlining the endothelium of arteries. Recruitment of monocyte-derived macrophages and their subsequent uptake of oxidized LDL cholesterol are the major cellular events contributing to fatty-streak formation. The recruitment of monocytes to lesion-prone sites of arteries is tightly regulated by cell adhesion molecules that are expressed on the surface of endothelial cells in response to inflammatory stimuli. The selectin family of glycoproteins, P-selectin and E-selectin, are important in regulating the first step of neutrophil–endothelial cell interaction, termed leukocyte rolling. Once the leukocytes make contact with the dysfunctional endothelium, they are activated and proceed to the second step of firm adherence to the endothelium. This process is regulated by a pair of receptors, one on the surface of the neutrophil, and adhesion molecules on the endothelium (ICAM-1 and VCAM-1). The final step, ie, neutrophil migration into the subendothelial space, is likely to be stimulated in part by oxidized LDL, which can directly attract monocytes and also induce the expression of chemokines such as MCP-1 by endothelial cells. This chemoattractant chemokine is highly expressed in human atherosclerotic lesions and is believed to be the principal stimulus for monocyte recruitment into the arterial wall. Studies have demonstrated that mice lacking the leukocyte receptor for MCP-1 (CCR2) exhibit marked reductions in atherosclerotic lesion formation, providing compelling evidence that MCP-1 and its receptor play a critical role in the initiation of atherosclerosis. The development of macrophage foam cells that are present in both early and late atherosclerotic lesions. Accumulation of cholesterol in these cells is mediated primarily via uptake of LDL cholesterol via a complex series of well-regulated events. The transition from the relatively simple fatty streak to the more complex atherosclerotic lesion is characterized by the immigration of smooth muscle cells from the medial layer and into the intimal or subendothelial space. Intimal smooth muscle cells can synthesize extracellular matrix proteins that lead to the development of the fibrous cap. This phase of lesion development is influenced by interactions between monocyte/macrophages and T cells that result in a broad range of cellular and humoral responses similar to those observed in states of chronic inflammation.

It is now well accepted that inflammation plays a central role in the development of atherosclerosis and its complications. Chronic inflammation results in endothelial dysfunction and facilitates the interactions between modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall. Indeed, inflammatory processes have been implicated in each facet of atherogenesis described above. Inflammatory markers such as CRP and IL-6 not only participate in lesion formation but also alter plaque architecture in favor of rupture.

CRP: Marker and Mediator of Inflammation and Atherosclerosis

Several large-scale epidemiological studies have shown that plasma levels of high-sensitivity CRP are a strong independent predictor of endothelial dysfunction, future myocardial dysfunction, stroke, peripheral artery disease, and vascular death among individuals without known cardiovascular disease. In addition, among patients with known coronary artery disease, levels of CRP have been linked with increased vascular event rates. The latter observations are important, because recent evidence suggests that inflammation is a critical determinant of plaque stability and rupture. A direct
comparison of the magnitude of relative risk (compared with traditional risk factors) revealed that CRP is the single strongest predictor of risk, with a relative risk of 4.4 for the highest versus lowest quartile. Furthermore, in a multivariate analysis, only CRP and the ratio of total to HDL cholesterol proved to have independent predictive value once age, smoking status, obesity, hypertension, obesity, diabetes, and family history were accounted for. More recently, elevated CRP levels have been demonstrated to predict the risk of death and myocardial infarction in patients undergoing percutaneous coronary intervention (PCI) after adjustment of baseline values known to influence early events after PCI. These observations have set the stage for routine CRP assessments to enter conventional atherosclerosis prediction algorithms.

Until recently, CRP was regarded as a nonspecific marker of inflammation versus an active partaker in the process of atherogenesis. Several recent studies have now clearly demonstrated that human CRP, at concentrations known to predict increased vascular event rates, directly induces a proinflammatory and proatherosclerotic phenotype. In human umbilical vein and coronary endothelial cells, CRP has been demonstrated to increase the expression of ICAM-1, VCAM-1, and MCP-1 in a concentration-dependent fashion. CRP has been demonstrated to facilitate native LDL uptake into macrophages, an important step in foam-cell formation. CRP may also directly promote monocyte activation by stimulating the release of cytokines such as IL-1b, IL-6, and TNF-α and increasing the release of soluble IL-6 receptor. Recent evidence shows that C-reactive protein (CRP) is deposited in the arterial intima at sites of atherogenesis. Importantly, CRP deposition precedes the appearance of monocytes in early atherosclerotic lesions. In addition to the aforementioned proatherogenic actions, CRP is a well-recognized stimulator of the complement system. Complement activation plays an important role in atherogenesis, and CRP has been demonstrated to colocalize with terminal complement complexes in established coronary plaques. Lastly, data demonstrating that CRP may exaggerate lipopolysaccharide-mediated activation of endothelial cells and monocytes additionally strengthen the evidence of a direct effect of CRP on vascular inflammation.

**ET-1 and IL-6 May Mediate the Proinflammatory Actions of CRP**

The present study adds to the growing body of literature that supports a direct proinflammatory and proatherosclerotic effect of CRP. We demonstrate that CRP may evoke the production of the potent endothelium-derived vasoactive factor ET-1 and the inflammatory cytokine IL-6. Additional evidence to support a role of ET-1 and IL-6 as mediators of the actions of CRP is provided by experiments examining the effects of IL-6 inhibition and ET receptor antagonism. Both bosentan and anti–IL-6 antibodies attenuated CRP-mediated expression of adhesion molecules, MCP-1 secretion, and macrophage LDL uptake.

Although ET-1 is known to be one of the upstream activators of IL-6, we suggest that this may not be the case in HSVECs stimulated with CRP. This conclusion is based on our observation that IL-6 levels are not attenuated during concurrent ET receptor blockade. Hence, it is possible that CRP stimulates the production of IL-6 and ET-1 concurrently in a parallel fashion. Alternatively, it is possible that CRP quenches an unidentified inhibitory factor, such as nitric oxide, known to decrease IL-6 and ET-1 secretion. It is also plausible that CRP serves to activate nuclear factor-κB (NF-κB), with the resultant increase in expression of IL-6, ET-1, ICAM-1, VCAM-1, and other NF-κB–regulated systems. This however, remains to be determined.

ET-1 is one of the most potent endogenous vasoconstrictors and mediates a host of responses, including endothelial dysfunction, vasomotor contraction, leukocyte and platelet activation, and cellular proliferation. Additionally, it augments the vascular actions of other vasoactive substances, such as A-II, norepinephrine, and serotonin. In the endothelial cell, ET-1 is produced by preproET under the influence of the endothelin-converting enzyme. ET-1 exerts its biological effects via interacting with endothelin receptors (ETα and ETβ), with the former mediating the bulk of the vascular actions.

IL-6 is an important inflammatory cytokine that has been implicated in the pathogenesis and clinical course of atherosclerotic vascular disease. IL-6 is known to be secreted from several cell types, including endothelial cells, macrophages, lymphocytes, and adipocytes, and exerts its biological actions through a complex yet well-defined fashion. The IL-6 receptor complex consists of two membrane-bound glycoproteins, an 80-kD ligand-binding component (termed IL-6R), and a 130-kD signal-transducing component (termed gp130). Although IL-6 may bind the IL-6R and elicit a biological response, it also activates a soluble IL-6R (sIL-6R). The activated IL-6/sIL-6R complex serves as a potent agonist that binds the signal-transducing component of the membrane-bound receptor gp130 with high affinity. Through this mechanism, IL-6 is believed to potentiate its own biological activity and exert effects in cells that lack the IL-6R per se. Indeed the IL-6/sIL-6R complex has been demonstrated to stimulate leukocyte recruitment and promote endothelial cell inflammatory responses.

The aforementioned discussion assumes importance, because recent studies have demonstrated that CRP is a physiological regulator of sIL-6R shedding in human neutrophils and markedly increases the formation of the sIL-6R/IL-6 complex. Data from the present study suggest that CRP may also function to increase IL-6 secretion from endothelial cells. By stimulating endothelial production of IL-6 and neutrophil sIL-6R shedding, CRP may serve to markedly exaggerate the actions of IL-6 at the level of the endothelium. Because IL-6 is a potent hepatic stimulus for CRP, increased vascular production may represent a positive feedback mechanism for the continued production of CRP from the liver.

**Limitations**

Results from the present study demonstrate that the proinflammatory actions of CRP can be attenuated during mixed ETα/β receptor blockade; however, they do not allow us to comment on the relative importance of ETA versus ETB antagonism in this effect. Second, the experiments were
Conclusions

Results from the present study support a growing body of evidence suggesting that CRP exerts direct proinflammatory and proatherosclerotic effects (Figure 7). CRP directly facilitates endothelial cell adhesion molecule expression, MCP-1 production, and macrophage LDL uptake. Importantly, these effects seem to occur, in part, through an ET-1–dependent and IL-6–dependent fashion and are attenuated during pharmacological antagonism with bosentan and anti–IL-6 antibodies. This is also the first study to examine the role of CRP in saphenous vein endothelial cells and hence may have implications for saphenous vein graft atherosclerosis in patients undergoing coronary artery bypass graft surgery. Clearly, understanding the mechanisms and mediators of the proinflammatory effects of CRP may yield new therapeutic targets, such as bosentan, to predict, prevent, and treat cardiovascular disease.

Acknowledgments

This work was supported by the Heart and Stroke Foundation of Ontario (Dr Weisel, Dr Fedak, Dr Li, and Dr Mickle), Canadian Institutes for Health Research (Dr Verma, Dr Fedak, Dr Li, and Dr Mickle), and Canadian Diabetes Association (Dr Verma and Dr Weisel).

References


Endothelin Antagonism and Interleukin-6 Inhibition Attenuate the Proatherogenic Effects of C-Reactive Protein

Subodh Verma, Shu-Hong Li, Mitesh V. Badiwala, Richard D. Weisel, Paul W.M. Fedak, Ren-Ke Li, Bikramjit Dhillon and Donald A.G. Mickle

_Circulation_. 2002;105:1890-1896; originally published online April 8, 2002; doi: 10.1161/01.CIR.000015126.83143.B4
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/16/1890

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/