patient-oriented research

Effects of CRP infusion on endothelial function and coagulation in normocholesterolemic and hypercholesterolemic subjects

Radjesh J. Bisoendial,[*] John J. P. Kastelein,[*] Stephan L. M. Peters,[†] Johannes H. M. Levels,[*] Rakesh Birjmohun,[*] Joris I. Rotmans,[*] Daniel Hartman,[§] Joost C. M. Meijers,[*] Marcel Levi,[*] and Erik S. G. Stroes[1,*]

Department of Vascular Medicine* and Department of Pharmacology and Pharmacotherapy,[†] Academic Medical Center, Amsterdam, The Netherlands; and deCODE Genetics,[§] Reykjavik, Iceland

Abstract C-reactive protein (CRP) has been suggested to exert direct adverse effects on the vasculature in experimental setups, including endothelial dysfunction and proinflammatory changes. Here, we assessed the consequences of 1.25 mg/kg highly purified recombinant human CRP, administered as an intravenous bolus, in six patients with familial hypercholesterolemia (FH) and six normocholesterolemic subjects. Endothelium-dependent and -independent vasoreactivity to serotonin and nitroprusside, respectively, were assessed using venous occlusion plethysmography before and after CRP infusion. For biochemical analyses, blood was drawn at different time points. At baseline, FH patients showed blunted endothelium-dependent vasodilation (maximum, 89.2 ± 30.0% vs. 117.7 ± 13.1% in normolipidemic subjects; $P = 0.037$). Procoagulant activity was also higher in FH patients, illustrated by increased prothrombin fragment 1+2 (F_{1+2}) levels ($P = 0.030$) and plasminogen activator inhibitor type-1 (PAI-1) activity ($P = 0.016$). Upon CRP challenge, endothelium-dependent vasodilator capacity further deteriorated in FH patients ($P = 0.029$), whereas no change in vascular reactivity was observed in normolipidemic subjects. Additionally, coagulation activation was augmented in FH patients compared with normolipidemic subjects ($P = 0.009$ for F_{1+2} levels; $P = 0.018$ and $P = 0.003$ for PAI-1 antigen and activity, respectively). No difference in inflammatory responses was observed between groups. In hypercholesterolemic patients, CRP aggravates endothelial dysfunction and also evokes augmented procoagulant responses. These findings suggest that particularly in hypercholesterolemia, CRP-lowering strategies should be considered in addition to LDL reduction.—Bisoendial, R. J., J. J. P. Kastelein, S. L. M. Peters, J. H. M. Levels, R. Birjmohun, J. I. Rotmans, D. Hartman, J. C. M. Meijers, M. Levi, and E. S. G. Stroes. **Effects of CRP infusion on endothelial function and coagulation in normocholesterolemic and hypercholesterolemic subjects.** ***J. Lipid Res.*** **2007.** 48: **952–960.**

Supplementary key words inflammation • atherothrombosis • C-reactive protein

Low density lipoprotein-cholesterol (LDL-C) is a principal risk factor for atherogenesis. More recently, the impact of classical risk factors on atherogenesis has been acknowledged to increase in a proinflammatory environment. Thus, direct interactions have been reported between LDL-C and inflammatory activation. Oxidative modification of LDL-C may evoke a strong inflammatory response (1), whereas inflammatory activation causes redox dysregulation and upregulation of lectin-like oxidized low density lipoprotein (OxLDL) receptor-1 (LOX-1) (2), promoting LDL uptake within the arterial wall.

C-reactive protein (CRP), in its role as inflammatory marker, has emerged as a relevant predictor of future cardiovascular events (3, 4). CRP constitutes a member of the innate host defense against pathogens, and among its activities are regulation of the complement system (5), binding to OxLDL (6) as well as facilitating its clearance, and mediating neutrophil responses and phagocytosis (7). More recently, experimental studies have revealed that CRP itself may also exert proatherogenic actions, particularly at the level of the arterial wall and inflammatory cells (8, 9). Whereas opposing observations in human CRP-transgenic mice have ignited debate on the in vivo relevance of these findings, it has also become clear that the CRP-transgenic mouse model is not suitable to provide solid answers in this particular case (10, 11). As a consequence, human studies addressing the concept of CRP adversely affecting the vessel wall are warranted. In this respect, we recently found that CRP infusion elicits the activation of inflammatory and coagulation pathways in healthy volunteers (12).

Manuscript received 24 October 2006 and in revised form 26 December 2006.

Published, JLR Papers in Press, January 26, 2007.
DOI 10.1194/jlr.P600014-JLR200

[1] To whom correspondence should be addressed.
e-mail: e.s.stroes@amc.uva.nl

To date, we have not addressed whether or not CRP may impair endothelium-derived vasomotor function, as indicated by several in vitro studies (13). We also considered that CRP infusion would have a greater impact on endothelial vasoreactivity in humans with hypercholesterolemia. The basis for this hypothesis are several observations in different animal species of hypercholesterolemia showing exaggerated responses, including endothelial dysfunction, to experimental endotoxemia (14, 15). Thus, to further explore the potential proatherogenic function of CRP in humans, we evaluated the impact of CRP infusion on vascular reactivity, inflammation, and coagulation in subjects with increased LDL-C levels [familial hypercholesterolemia (FH)] as well as in normolipidemic subjects.

METHODS

Study design

Twelve healthy, nonsmoking, male individuals, including six FH heterozygotes [age, 36.3 ± 11.5 years (mean ± SD)] recruited from Dutch kindreds with known LDL receptor mutations and six normolipidemic subjects (35.8 ± 10.9 years), were enrolled in the study. The FH subjects were carriers of the N543H/2393del9 combo-mutation with on-treatment LDL-C levels of 3.6 ± 1.8 mmol/l. Written informed consent was obtained from all participants. The study protocol was approved by the Institutional Review Board of the Academic Medical Center in Amsterdam. The FH patients had no history of overt cardiovascular disease, and none of them was receiving any drugs besides statins. Before inclusion, statin use in the FH cohort had been stable for many years. Lipid-lowering medication and antioxidant supplementation was discontinued for 6 weeks. All subjects were free from febrile illness at least 4 weeks before the study. They refrained from alcohol- and caffeine-containing drinks for >24 h before the study. During the statin washout period, FH patients were instructed by the department's dietician to maintain their lipid-lowering diet, as recommended by the American Heart Association/National Cholesterol Education Program-1 (step 1 diet). Based on results from the dose-escalation study, as described below, an intravenous bolus of 1.25 mg/kg CRP was administered after an overnight fast. Endothelium-dependent and -independent vascular reactivity was assessed under standardized conditions before and 6 h after CRP infusion. Blood was drawn at baseline and at 1, 4, and 8 h after infusion.

Dose-escalation study

To establish an adequate CRP concentration for examining the acute effects of CRP under both normocholesterolemic and hypercholesterolemic conditions, a dose-escalation infusion was performed in healthy volunteers different from those participating in the main study. Thus, 21 men were randomly assigned to one of three dose groups: 7 in the 0.25 mg/kg group (52.1 ± 5.8 years), 7 in the 0.5 mg/kg group (52.0 ± 5.1 years), and 7 in the 1.25 mg/kg group (35.9 ± 17.2 years).

Recombinant human CRP

Escherichia coli-derived recombinant human (rh) CRP (BiosPacific) was supplied in 20 mM Tris, 140 mM NaCl, 2 mM $CaCl_2$, pH 7.5, and 0.05% (w/v) sodium azide and revealed a single 23 kDa band (>99%) after Coomassie Brilliant Blue R-250 staining (1 μg; SDS-polyacrylamide gel). The host cell protein concentration was 85 ppm before purification, as determined by a highly sensitive ELISA in accordance with the manufacturers' instructions (Cygnus Technologies, Inc.). Subsequently, the rhCRP was purified using size-exclusion chromatography to remove contaminants, including endotoxin and sodium azide (Univalid BV, Leiden, The Netherlands). High purity was demonstrated by high-pressure size-exclusion chromatography HPLC and reverse-phase HPLC as well as time-of-flight mass spectrometry, showing no other protein fractions than rhCRP. As evaluated by the Limulus assay (turbidimetric kinetic method; ACC, Inc., East Falmouth, MA), endotoxin levels in the end product were <1.5 endotoxin units/ml at 0.91 mg/ml rhCRP. Cell culture experiments using human umbilical vein endothelial cells revealed no toxicity of the end product with regard to cell viability and cell growth. Single-dose toxicity studies in mice (n = 6) reaching CRP levels greater than four times that obtained in humans demonstrated no direct effects of the end product on temperature, blood pressure, or heart rate. The end product was stored under sterile conditions in a $CaCl_2$-containing buffer (pH 8.5) at 0–4°C, and all experiments were performed within 4 weeks after preparation. We repeatedly evaluated the rhCRP content by HPLC analysis during the course of the protocol. Pentameric 125 kDa protein was the sole protein present; no aggregate formation and/or monomeric protein could be observed during the study period. Thus, HPLC analysis clearly showed one peak corresponding to the CRP pentamer, even after 4 and 8 weeks. The latter finding underscores the stability of our product. Thus, at the time of presentation, there was only the CRP pentamer.

Vascular function

Assessment of vascular function was performed using venous occlusion plethysmography (EC4; Hokanson, Inc.) (16). The nondominant brachial artery was cannulated using a 20 gauge polyethylene catheter under local anesthesia. At least 45 min after cannulation, forearm blood flow (FBF), expressed in ml/min/100 ml of forearm tissue volume (FAV), was measured simultaneously in both arms. During each measurement, blood pressure cuffs around both upper arms were inflated (40 mmHg) using a rapid cuff inflator. Synchronously, bilateral wrist cuffs were inflated to greater than systolic blood pressure to exclude the hand circulation (200 mmHg). Intra-arterial blood pressure and heart rate were monitored continuously. Next, FBF responses to cumulative doses of serotonin (5-HT; Sigma Chemical) (0.6, 1.8, and 6 ng/100 ml FAV/min) and nitroprusside (SNP; Spruyt Hillen, Utrecht, The Netherlands) (6, 60, 180, and 600 ng/100 ml FAV/min) were measured. All agents were administered intra-arterially for 6 and 4 min at each dose, respectively, using a constant-rate infusion pump. Six measurements during the last 2 min (steady state) were averaged to determine mean FBF. The two different infusion blocks were initiated after a 15 min rest period or until FBF had returned to baseline. Six hours after intravenous infusion of CRP, the two infusion blocks were repeated. Additionally, B-mode ultrasound measurement of carotid artery intima-media thickness was performed using an Acuson 128XP/10v equipped with a 7.0 MHz linear array transducer, as described previously (17).

Biochemical analyses

Blood samples were processed immediately by centrifugation at 1,700 *g* for 15 min and stored at −80°C until analysis. Total cholesterol, high density lipoprotein-cholesterol (HDL-C), and triglycerides were determined enzymatically (Boehringer Mannheim, Mannheim, Germany), and the interassay and intra-assay coefficients of variation (CV) were <2% for all three. LDL-C was calculated using the Friedewald equation. Apolipoprotein B-100 (apoB-100) and apoA-I were assayed by rate nephelometry (CV <

5% for both). CRP was measured by a high-sensitivity method (Roche Diagnostic Corp., Basel, Switzerland) and (in case CRP > 10 mg/ml) by immunonephelometry (P800 analyzer; Roche Diagnostic Corp.) (CV < 4% for both). Interleukin-8 (IL-8) was assayed by Cytometric Bead Array Analysis (BD Biosciences), whereas ELISAs were used to measure fragment 1+2 (F_{1+2}; Dade-Behring, Marburg, Germany), plasminogen activator inhibitor type-1 (PAI-1) antigen (Monozyme, Charlottelund, Denmark), soluble E-selectin, and monocyte chemoattractant protein-1 (both R&D Systems, Abingdon, UK) (CV < 10% for all assays). PAI-1 activity (CV < 6%) was determined on an automated coagulation analyzer (Behring Coagulation System) with reagents and protocols from the manufacturer (Dade Behring).

Statistical analysis

Unless stated otherwise, data are expressed as means ± SD. Descriptive statistics between both groups were compared by means of Student's *t*-test or the Mann-Whitney test (nonparametric), as appropriate. Statistical analysis was performed for the FBF responses and clinical parameters between both groups using two-way ANOVA for repeated measures (SPSS for Windows 12.0; SPSS, Inc.). For comparisons within groups, the Wilcoxon signed rank test or the Mann-Whitney *U* test was used, as appropriate. $P < 0.05$ was considered significant.

RESULTS

Baseline clinical characteristics

Plasma levels of total cholesterol, LDL-C, and apoB-100 (all $P < 0.010$) were significantly higher in FH patients (**Table 1**). Concomitantly, HDL-C ($P = 0.045$) and apoA-I ($P = 0.004$) levels were lower in the FH cohort. Compared with normolipidemic subjects, higher body mass index values were found in the FH patients ($P = 0.025$), whereas blood pressure, heart rate, and FBF were not different. There was a trend toward higher intima-media thickness values in FH patients compared with normolipidemic subjects, whereas the latter did not reach statistical significance because of the small sample size.

Dose-escalation study

Upon CRP infusion, plasma CRP levels increased, peaking after 1 h, from 2.5 ± 4.0 to 7.9 ± 4.0, from 2.4 ± 3.3 to 13.1 ± 2.8, and from 1.3 ± 0.9 to 28.1 ± 5.9 mg/l in the 0.25, 0.5, and 1.25 mg/kg group, respectively (**Fig. 1**). Subsequently, F_{1+2} levels increased from 0.8 ± 0.3 to 1.9 ± 0.8 nmol/l ($P = 0.018$) in the 1.25 mg/kg group and from 0.8 ± 0.2 to 1.1 ± 0.3 nmol/l ($P = 0.028$) in the 0.5 mg/kg group, peaking after 4 h. Noticeably, a dose-dependent activation of thrombin generation was observed at 4 h ($P < 0.0001$). Also, CRP infusion resulted in a dose-dependent increase in plasma IL-6 levels (overall $P = 0.018$), reaching statistical significance only in the highest dose group at 4 h (from 33.8 ± 29.8 to 72.4 ± 40.9 pg/ml; $P = 0.018$). Augmented thrombin generation and inflammatory responses were absent in the lowest dose group.

CRP levels before and after CRP infusion

Based on the results from the dose-escalation study, 1.25 mg/kg CRP was administered in the FH patients and normolipidemic subjects (**Fig. 2**). During the infusion studies, none of the subjects experienced adverse effects. Body temperature and hemodynamics remained constant throughout the experiment. At baseline, CRP levels were 1.8 ± 1.7 mg/l in FH patients and 2.8 ± 3.0 mg/l in normolipidemic subjects ($P = 0.340$). After CRP infusion, CRP levels increased to 30.0 ± 3.5 and 26.2 ± 2.9 mg/l in FH patients and normolipidemic subjects, respectively.

Vascular function

Intra-arterial infusion of the endothelium-dependent vasodilator 5-HT increased FBF in a dose-dependent manner in both groups (**Fig. 3**). At baseline, maximal 5-HT-induced vasodilation was lower in FH patients (89.2 ± 30.0%; $P = 0.037$) compared with normolipidemic subjects. Six hours after CRP infusion, the blunted endothelial vasodilator response to 5-HT decreased even further in the FH patients (maximum, 42.3 ± 25.7%; $P = 0.029$). In normolipidemic subjects, maximal FBF responses to 5-HT were similar before (117.7 ± 13.1%) and after (110.3 ± 22.0%) rhCRP infusion. The change in 5-HT-induced vasodilation upon CRP infusion was significantly more pronounced in FH patients than in controls ($P = 0.014$). Maximal SNP-mediated vasodilation at baseline was

TABLE 1. Demographic and baseline parameters of the study cohort

Parameters	Familial Hypercholesterolemia Patients (n = 6)	Mean (SD) Median (95% CI)	Normolipidemic Subjects (n = 6)	Mean (SD) Median (95% CI)	*P*
Age (years)	36.3 (11.5)	41.5 (24.2–48.4)	35.8 (10.9)	31.5 (24.4–47.3)	0.870
Body mass index (kg/m^2)	28.3 (4.1)	27.5 (24.1–32.6)	23.2 (2.5)	22.2 (20.5–25.8)	0.025
Systolic blood pressure (mmHg)	130 (4)	129 (125–134)	131 (8)	133 (123–139)	0.470
Diastolic blood pressure (mmHg)	83 (6)	82 (76–90)	80 (4)	82 (76–84)	0.430
Basal forearm blood flow (ml/100 ml forearm tissue volume/min)	2.8 (0.9)	2.7 (1.8–3.7)	2.7 (2.4)	2.0 (0.2–5.1)	0.210
Total cholesterol (mmol/l)	6.4 (0.9)	6.4 (5.5–7.3)	4.0 (0.8)	3.9 (3.1–4.8)	0.004
LDL-cholesterol (mmol/l)	4.6 (1.1)	4.6 (3.4–5.7)	2.2 (0.6)	2.2 (1.6–2.9)	0.004
HDL-cholesterol (mmol/l)	1.0 (0.2)	1.0 (0.8–1.2)	1.2 (0.2)	1.2 (1.0–1.4)	0.045
Triglycerides (mmol/l)	1.7 (1.4)	1.2 (0.2–3.2)	1.1 (0.9)	0.8 (0.2–2.1)	0.340
Apolipoprotein A-I (g/l)	0.8 (0.1)	0.8 (0.7–0.9)	1.1 (0.1)	1.1 (1.0–1.1)	0.004
Apolipoprotein B-100 (g/l)	1.2 (0.3)	1.1 (0.9–1.4)	0.7 (0.2)	0.7 (0.5–0.9)	0.006
High-sensitivity C-reactive protein (mg/l)	1.8 (1.7)	1.5 (0–3.6)	2.8 (3.0)	2.3 (−0.3–6.0)	0.340
Intima-media thickness ($\times 10^{-1}$ mm)	8.3 (1.5)	7.9 (6.6–9.9)	6.9 (0.7)	6.9 (6.2–7.6)	0.109

CI, confidence interval.

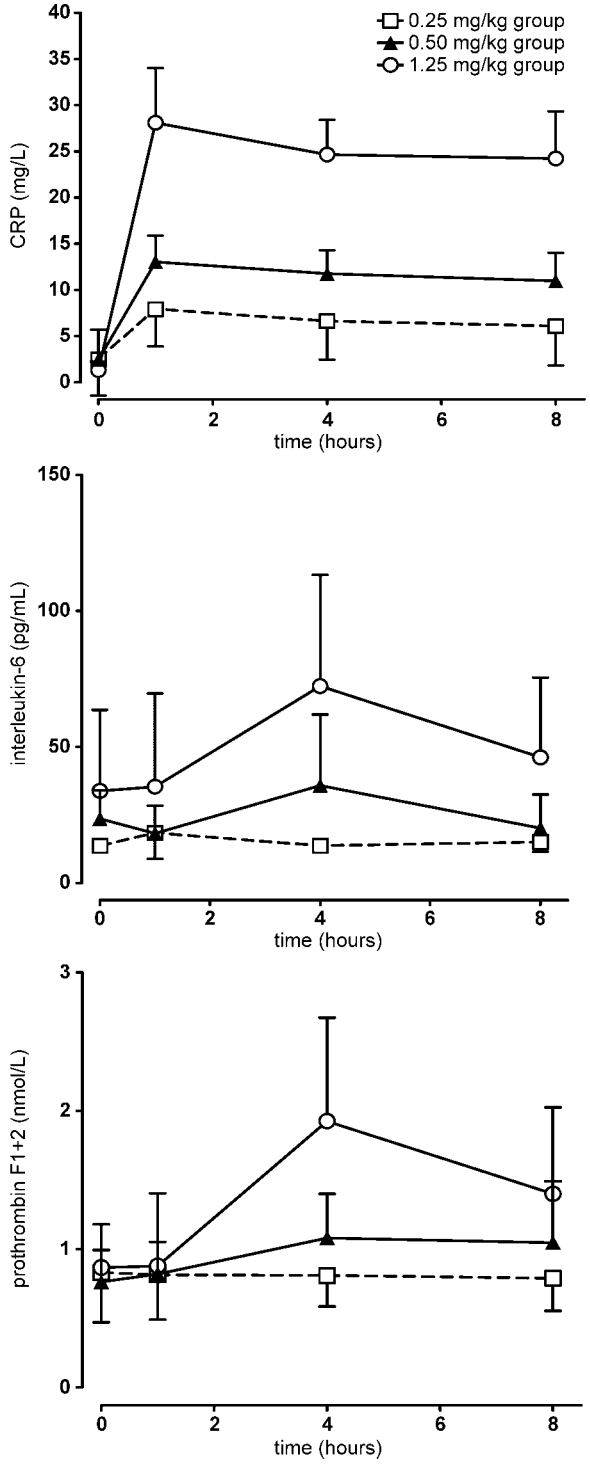

Fig. 1. Dose-escalation study with levels of C-reactive protein (CRP), interleukin (IL)-6, and fragment 1+2 (F_{1+2}) before and after CRP infusion. Values shown are concentrations (means ± SD) of CRP, IL-6, and F_{1+2} in response to recombinant human (rh) CRP infusion. The rhCRP infusion was associated with an acute and dose-dependent increase in CRP levels, peaking after 1 h. Concentrations of ≥10 mg/l (dose of 0.5 mg/kg body weight) were required to activate both the inflammatory (IL-6) and coagulation (F_{1+2}) pathways. Notably, moderate CRP increases in the physiological range were not associated with activation of either of these pathways.

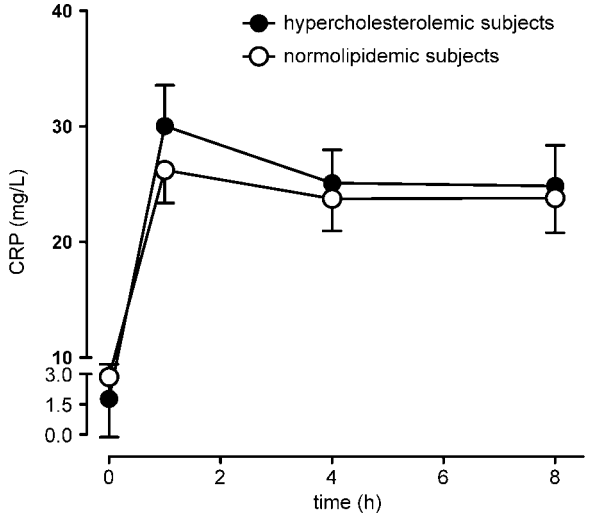

Fig. 2. CRP levels before and after CRP infusion. At baseline, CRP levels were 1.8 ± 1.7 mg/l in familial hypercholesterolemia (FH) patients and 2.8 ± 3.0 mg/l in normolipidemic subjects (P = 0.340). After CRP infusion, CRP levels increased maximally to 30.0 ± 3.5 and 26.2 ± 2.9 mg/l in FH patients and normolipidemic subjects, respectively. Values shown are means ± SD.

not significantly different between FH patients and normolipidemic subjects and remained unaffected upon CRP infusion within both groups [FH patients, 284.0 ± 83.3% (before) and 277.7 ± 61.7% (after); P = 0.110; normolipidemic subjects, 334.6 ± 146.3% (before) and 341.8 ± 94.7% (after); P = 0.550].

Coagulation and fibrinolysis

At baseline, F_{1+2} levels were increased in FH patients compared with normolipidemic subjects (0.8 ± 0.2 vs. 0.6 ± 0.1 nmol/l; P = 0.030) (**Fig. 4**). Additionally, PAI-1 activity was higher in FH patients than in normolipidemic subjects (5.8 ± 1.0 vs. 3.7 ± 1.4 U/ml; P = 0.016). No significant differences regarding baseline values were observed for PAI-1 antigen.

Four hours after CRP infusion, levels of F_{1+2} increased to a greater extent in FH patients compared with normolipidemic subjects (3.1 ± 0.7 vs. 2.0 ± 1.1 nmol/l; P = 0.009). In a similar manner, PAI-1 antigen (185.8 ± 109.3 vs. 54.2 ± 25.2 ng/ml; P = 0.018) and PAI-1 activity (11.0 ± 3.6 vs. 4.7 ± 1.5 U/ml; P = 0.003) increased to a greater extent in FH patients compared with normolipidemic subjects, both peaking at 4 h.

Inflammation

CRP infusion was associated with a transient increase in plasma levels of MCP-1 (for both groups, $P < 0.01$ vs. baseline) and IL-8 (for both groups, $P \leq 0.05$ vs. baseline), all peaking after 4 h (**Fig. 5**). However, no differences in response were seen between groups. Moreover, CRP infusion was followed by a gradual increase in plasma concentration of soluble E-selectin (for both groups, $P < 0.05$ vs. baseline), indicating endothelial cell activation, again not significantly different between groups. During the 8 h

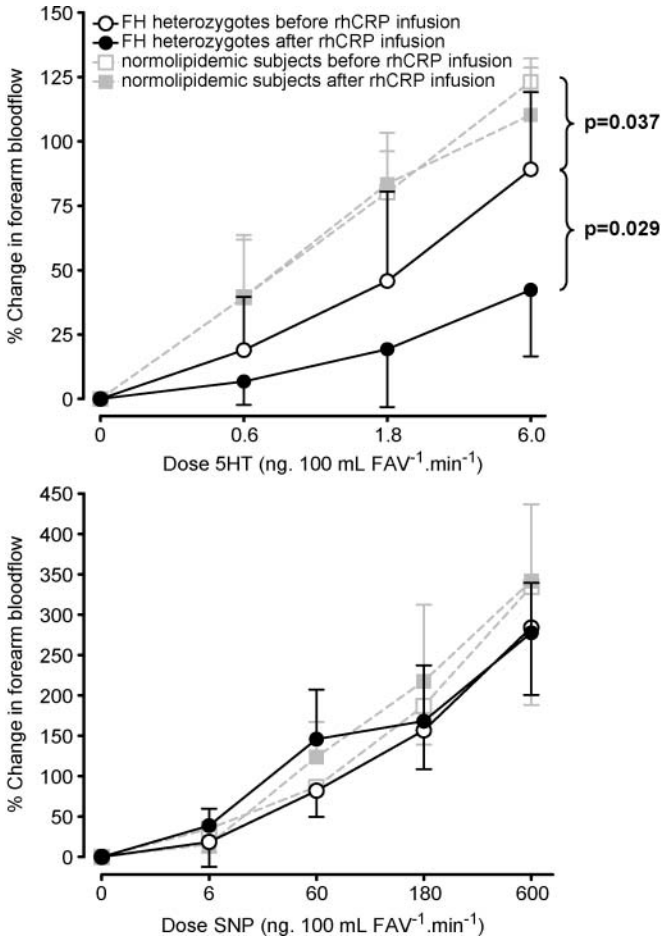

Fig. 3. Endothelial vasodilator capacity before and after CRP infusion. Forearm blood flow (FBF) dose-response curves to the endothelium-dependent vasodilator 5-HT before and after rhCRP infusion are shown for FH patients (before, open black circles; after, closed black circles) and normolipidemic subjects (before, open gray squares; after, closed gray squares). At baseline, maximal 5-HT-induced vasodilation was lower in FH patients ($P = 0.037$) compared with normolipidemic subjects. After rhCRP infusion, the FBF response to 5-HT in the FH patients deteriorated significantly ($P = 0.029$), whereas no additional effects were seen in normolipidemic individuals. The change in 5-HT-induced vasodilation upon CRP infusion was significantly more pronounced in FH patients than in controls ($P = 0.014$). SNP-mediated vasodilation was not affected by CRP infusion in either group. FAV, forearm tissue volume. Values shown are means ± SD.

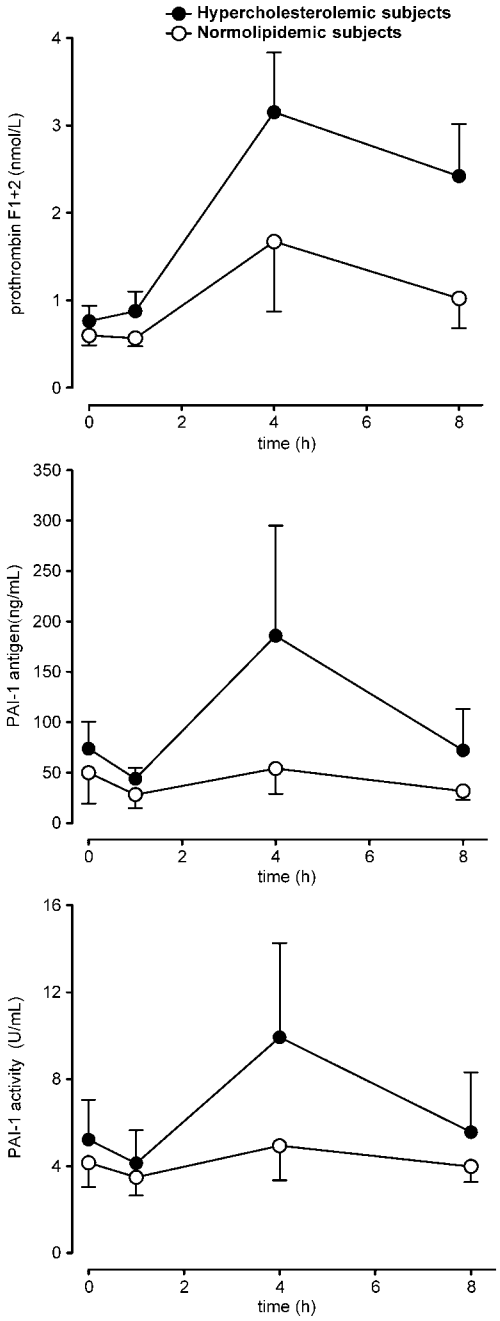

Fig. 4. Effects of rhCRP infusion on coagulation and fibrinolysis. Clinical responses of F_{1+2}, plasminogen activator inhibitor type-1 (PAI-1) antigen, and PAI-1 activity to rhCRP infusion in FH patients (closed circles) and normolipidemic subjects (open circles) are shown. At baseline, F_{1+2} levels ($P = 0.030$) and PAI-1 activity ($P = 0.016$) were higher in FH patients than in normolipidemic subjects. Moreover, levels of F_{1+2} increased to a greater extent in FH patients than in normolipidemic subjects (from 0.8 ± 0.2 to 3.1 ± 0.7 nmol/l vs. from 0.6 ± 0.1 to 2.0 ± 1.1 nmol/l; $P = 0.009$). Also, CRP-mediated increases in PAI-1 antigen levels (from 73.8 ± 26.9 to 185.8 ± 109.3 ng/ml vs. from 50.0 ± 30.5 to 54.2 ± 25.2 ng/ml; $P = 0{\cdot}018$) as well as PAI-1 activity (from 5.8 ± 1.0 to 11.0 ± 3.6 U/ml vs. from 3.7 ± 1.4 to 4.7 ± 1.5 U/ml; $P = 0.003$) were significantly greater in FH patients than in normolipidemic individuals. Values shown are means ± SD.

observation, no changes occurred in individual plasma lipid concentrations (data not shown).

DISCUSSION

A single bolus infusion of 1.25 mg/kg CRP elicited a progressive decline of endothelium-dependent vasodilation in hypercholesterolemic individuals. In contrast, CRP had no effect on vascular reactivity in normolipidemic subjects. Concomitantly, CRP-mediated procoagulant responses were augmented in FH patients, whereas inflam-

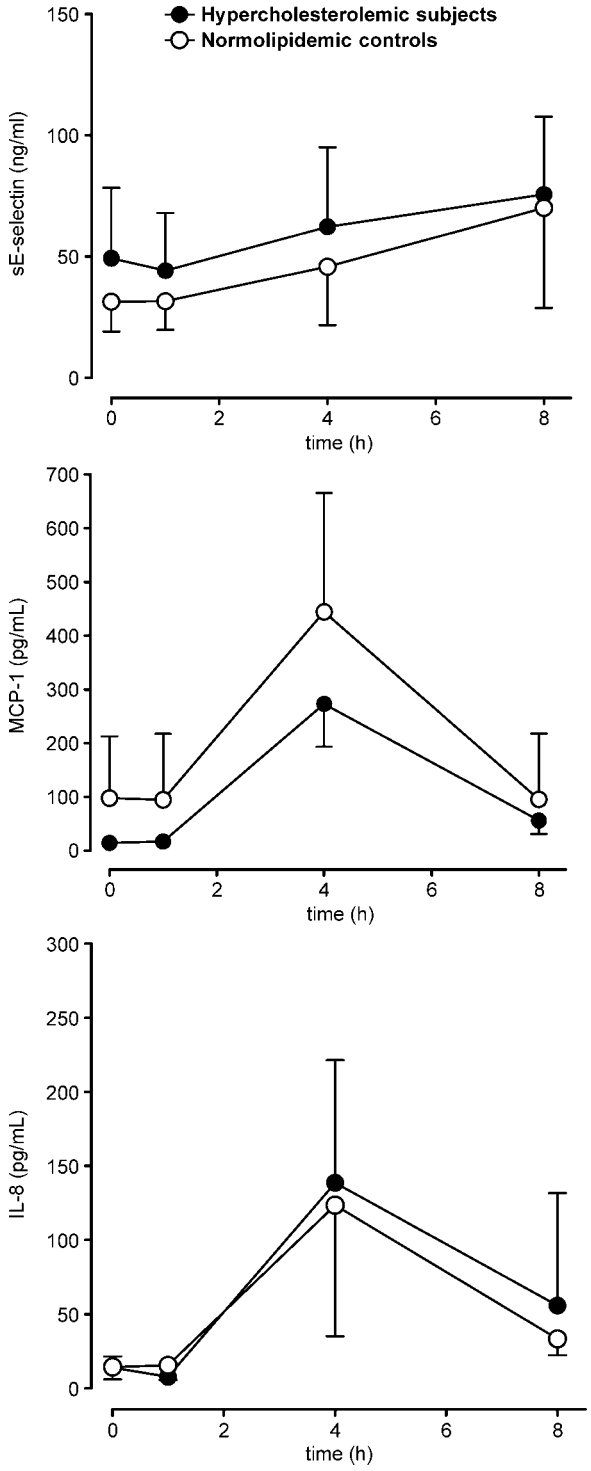

Fig. 5. Effects of rhCRP infusion on inflammation Clinical responses of soluble E-selectin (sE-selectin), MCP-1, and IL-8 to rhCRP infusion in FH patients (closed circles) and normolipidemic subjects (open circles) are shown. CRP elicited endothelial cell activation and proinflammatory responses in both FH patients and normolipidemic subjects; however, there were no differences in response between these groups. Values shown are means ± SD.

matory responses were not different between both groups. These data underscore direct adverse effects of CRP in humans, which appear to be augmented in the presence of increased LDL-C levels.

Dose-escalation study

The dose-escalation study confirmed dose-dependent effects of CRP on inflammation and coagulation. Activation of these pathways became apparent at CRP concentrations of >10 mg/l, at least in an acute setting. In this respect, CRP levels of >3 mg/l have already been associated with increased cardiovascular events in large observational studies (18). Because our dose-escalation study only evaluated a single bolus of rhCRP infusion, resulting in acute, short-term CRP increase, the present study cannot address what the impact of moderate CRP increase during prolonged periods would be on inflammatory and coagulation activation.

CRP and vasoreactivity

The endothelium is considered the first-line defense against atherogenic insults. In agreement with this, endothelial dysfunction has been shown to predict future cardiovascular events (19). As a consequence, the impact of CRP on the endothelium, and in particular endothelial nitric oxide synthase (eNOS), has been studied on numerous occasions. In cultured endothelial cells, CRP decreased eNOS mRNA and protein as well as nitric oxide (bio)activity within 24 h (20, 21). Direct vascular effects of CRP involved the attenuation of serotonin-induced vasodilation after 1 h of exposure to 7 mg/l CRP in porcine vessels (22), whereas the sensitivity to acetylcholine increased after a 4 h exposure to 200 mg/l CRP in aortas of Sprague-Dawley rats (23). In this study, we show that CRP up to 26 mg/l had no effect on endothelial vasoreactivity in normocholesterolemic men after 6 h. Whereas discrepancies in dose and exposure time as well as species differences should be taken into account, the present findings imply that a moderate CRP increase is unlikely to induce endothelial dysfunction in otherwise healthy individuals. In fact, these data are in accordance with the findings of Clapp et al. (23), who showed normalization of endothelial dysfunction several hours after typhoid vaccination in spite of increased CRP levels.

FH is characterized by the early loss of nitric oxide activity, coinciding with accelerated atherogenesis (16). FH patients in our study showed mildly increased LDL-C levels compared with normolipidemic subjects. Baseline endothelial vasodilator response to serotonin was clearly impaired. Underlying mechanisms include OxLDL-mediated displacement and uncoupling of eNOS, producing superoxide anions rather than nitric oxide (24). In contrast to normolipidemic subjects, CRP infusion caused marked deterioration of endothelium-dependent vasodilation in FH patients. Apparently, the protective capacity characteristic of the "healthy" endothelium fails in FH patients (16). Several mechanisms may be involved here. First, CRP's antagonism of eNOS has been attributed to a de-

creased stability of eNOS mRNA (21) as well as an attenuated eNOS phosphorylation at serine 1,179 (25). These mechanisms are mediated by CRP-dependent activation of the FcγRIIB receptor. Second, CRP may reduce prostacyclin secretion and stimulate superoxide anion release from NAD(P)H oxidase via p38 kinase activation (22, 26). Last, CRP was reported to dose- and time-dependently induce LOX-1, which is crucial for OxLDL's detrimental effects on endothelial function in human endothelium (27).

CRP and coagulation

Stimulation of thrombin generation and PAI-1 by CRP in FH patients was more pronounced than in normolipidemic subjects. CRP has been reported to promote tissue factor expression on mononuclear cells and, in addition, endothelial PAI-1 expression and activity (28, 29). Given the prominent role of the endothelium for counteracting procoagulant pathways, the augmented responses in FH patients can be linked to decreased nitric oxide activity, deteriorating upon CRP infusion. Increased LDL-C and reduced nitric oxide activity, both features of FH, are associated with a higher state of coagulation activation. In turn, interventions aiming at lipid lowering and stimulation of eNOS activity favorably affect "blood thrombogenicity" (30, 31).

Because hepatic LDL receptor-related proteins have been implicated in mediating the cellular degradation of thrombin (32) and several fibrinolytic compounds, such as the tissue plasminogen activator-PAI-1 complex (33), the higher concentration of both factors in FH patients may additionally be explained by impaired blood clearance associated with reduced expression of hepatic LDL receptor-related proteins. Collectively, these data indicate that CRP-induced activation of procoagulant pathways may be linked to decreased nitric oxide activity and may increase the risk for arterial thrombotic events, particularly under hypercholesterolemic conditions.

CRP and inflammation

Among its proinflammatory effects, CRP has been shown to promote monocyte-endothelium interaction by stimulating the release of chemoattractant IL-8 and antagonizing eNOS activity (21, 34). Furthermore, CRP can induce an inflammatory phenotype of venous endothelium, including upregulation of adhesion molecules and MCP-1, which in part has been attributed to the release of endothelin-1 and IL-6 (35). In addition, CRP elicits an inflammatory phenotype in artery endothelial cells (9, 36) and phagocytes (8). For that matter, binding and internalization of CRP by Fcγ receptors, activation of the nuclear factor κB and p38 mitogen-activated protein kinase signaling pathways, and upregulation of the CD40/CD40 ligand signaling pathway have been implicated in the proinflammatory effects of CRP on cellular functions (7, 36–38). In agreement with these in vitro data, we observed an inflammatory response upon infusing CRP in human subjects that overall did not significantly differ between both groups. During the 8 h observation, we also did not notice any change in individual plasma lipid concentrations in either group. Hence, the differences between FH patients and normolipidemic subjects with regard to CRP-mediated endothelial dysfunction and activation of coagulation cannot be attributed to changes in proinflammatory mediators.

Study limitations

The mean LDL-C levels in this FH cohort were only modestly increased. The latter pertains to the fact that all FH patients were carriers of the N543H/2393del9 combomutation, which is characterized by modest increases of LDL-C (39). That the potentiation of CRP's effects already occurred at these modestly increased LDL-C levels underscores the potential relevance of these observations for non-FH patients with increased LDL levels.

Recombinant CRP carries the risk of contaminants within the CRP-containing solution. In this study, several observations argue against a major role for contaminants. First, we were unable to detect tissue necrosis factor release upon rhCRP infusion (12), which is a hallmark of endotoxin exposure. Second, infusion of *E. coli* endotoxin at a dose that equaled the dose coinfused during CRP challenge was not accompanied by inflammatory activation in healthy volunteers, thus excluding a causal role of trace amount endotoxin within the CRP solution in mediating the inflammatory responses observed after infusion of rhCRP (40). Finally, the absence of an effect on vascular function in normolipidemic subjects, combined with a significant effect of CRP in hypercholesterolemic patients, suggests a selective, rather than contaminant-driven, response. Considering a specific CRP-mediated response, its effects on endothelial cells have been demonstrated to be receptor-mediated (25, 41).

The number of subjects studied here is limited, and confounding roles of low HDL-C and higher body mass index in the responses of FH patients cannot be excluded. Even with these limitations, CRP's direct adverse effects in humans appear to be aggravated in the presence of hypercholesterolemia.

Clinical implications

CRP has been increasingly recognized to play an active role throughout various stages of atherosclerosis, although its precise role has not been fully elucidated. This concept has gained further support from data obtained in the REVERSAL and PROVE-IT trials, indicating that lower CRP levels after statin therapy are associated with better cardiovascular outcome independent of LDL-C reduction (42, 43). More recently, a small molecule inhibitor of CRP was also shown to limit infarct size in rats undergoing experimental myocardial infarction with concomitant CRP infusion (44). These findings lend support to CRP-lowering strategies predominantly during the final stages of the disease. Interestingly, we now show that also at early stages, CRP, in conjunction with LDL, is associated with the induction of a proatherogenic state, including the induction of endothelial dysfunction, inflammation, and

coagulation activation. With emerging CRP-inhibiting strategies, such as small molecules or antisense strategies (http://www.isispharm.com), it will be a challenge to evaluate the impact of long-term CRP lowering on cardiovascular outcome.

This work was supported in part by a grant from Pfizer. J.J.P.K. is an established investigator of the Netherlands Heart Foundation (2000D039). The sponsors of this study had no role in study design, data collection, data analysis, data interpretation, or writing of this report.

REFERENCES

1. Liu, Y., L. M. Hulten, and O. Wiklund. 1997. Macrophages isolated from human atherosclerotic plaques produce IL-8, and oxysterols may have a regulatory function for IL-8 production. *Arterioscler. Thromb. Vasc. Biol.* **17:** 317–323.
2. Kume, N., T. Murase, H. Moriwaki, T. Aoyama, T. Sawamura, T. Masaki, and T. Kita. 1998. Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. *Circ. Res.* **83:** 322–327.
3. Danesh, J., J. G. Wheeler, G. M. Hirschfield, S. Eda, G. Eiriksdottir, A. Rumley, G. D. Lowe, M. B. Pepys, and V. Gudnason. 2004. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N. Engl. J. Med.* **350:** 1387–1397.
4. Ridker, P. M., N. Rifai, L. Rose, J. E. Buring, and N. R. Cook. 2002. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N. Engl. J. Med.* **347:** 1557–1565.
5. Volanakis, J. E. 1982. Complement activation by C-reactive protein complexes. *Ann. N. Y. Acad. Sci.* **389:** 235–250.
6. Chang, M. K., C. J. Binder, M. Torzewski, and J. L. Witztum. 2002. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: phosphorylcholine of oxidized phospholipids. *Proc. Natl. Acad. Sci. USA.* **99:** 13043–13048.
7. Bharadwaj, D., M. P. Stein, M. Volzer, C. Mold, and T. W. Du Clos. 1999. The major receptor for C-reactive protein on leukocytes is fcgamma receptor II. *J. Exp. Med.* **190:** 585–590.
8. Ballou, S. P., and G. Lozanski. 1992. Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein. *Cytokine.* **4:** 361–368.
9. Pasceri, V., J. T. Willerson, and E. T. Yeh. 2000. Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation.* **102:** 2165–2168.
10. Paul, A., K. W. Ko, L. Li, V. Yechoor, M. A. McCrory, A. J. Szalai, and L. Chan. 2004. C-reactive protein accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *Circulation.* **109:** 647–655.
11. Trion, A., M. P. de Maat, J. W. Jukema, A. van der Laarse, M. C. Maas, E. H. Offerman, L. M. Havekes, A. J. Szalai, H. M. Princen, and J. J. Emeis. 2005. No effect of C-reactive protein on early atherosclerosis development in apolipoprotein E*3-leiden/human C-reactive protein transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1635–1640.
12. Bisoendial, R. J., J. J. Kastelein, J. H. Levels, J. J. Zwaginga, B. van den Bogaard, P. H. Reitsma, J. C. Meijers, D. Hartman, M. Levi, and E. S. Stroes. 2005. Activation of inflammation and coagulation after infusion of C-reactive protein in humans. *Circ. Res.* **96:** 714–716.
13. Jialal, I., S. Devaraj, and S. K. Venugopal. 2004. C-reactive protein: risk marker or mediator in atherothrombosis? *Hypertension.* **44:** 6–11.
14. Brito, B. E., E. L. Romano, and C. Grunfeld. 1995. Increased lipopolysaccharide-induced tumour necrosis factor levels and death in hypercholesterolaemic rabbits. *Clin. Exp. Immunol.* **101:** 357–361.
15. Cerwinka, W. H., M. H. Cheema, and D. N. Granger. 2001. Hypercholesterolemia alters endotoxin-induced endothelial cell adhesion molecule expression. *Shock.* **16:** 44–50.
16. Stroes, E., J. Kastelein, F. Cosentino, W. Erkelens, R. Wever, H. Koomans, T. Luscher, and T. Rabelink. 1997. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J. Clin. Invest.* **99:** 41–46.
17. de Groot, E., J. W. Jukema, A. D. Montauban van Swijndregt, A. H. Zwinderman, R. G. Ackerstaff, A. F. van der Steen, N. Bom, K. I. Lie, and A. V. Bruschke. 1998. B-mode ultrasound assessment of pravastatin treatment effect on carotid and femoral artery walls and its correlations with coronary arteriographic findings: a report of the Regression Growth Evaluation Statin Study (REGRESS). *J. Am. Coll. Cardiol.* **31:** 1561–1567.
18. Ridker, P. M., and N. Cook. 2004. Clinical usefulness of very high and very low levels of C-reactive protein across the full range of Framingham risk scores. *Circulation.* **109:** 1955–1959.
19. Schachinger, V., M. B. Britten, and A. M. Zeiher. 2000. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation.* **101:** 1899–1906.
20. Verma, S., C. H. Wang, S. H. Li, A. S. Dumont, P. W. Fedak, M. V. Badiwala, B. Dhillon, R. D. Weisel, R. K. Li, D. A. Mickle, et al. 2002. A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. *Circulation.* **106:** 913–919.
21. Venugopal, S. K., S. Devaraj, I. Yuhanna, P. Shaul, and I. Jialal. 2002. Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. *Circulation.* **106:** 1439–1441.
22. Qamirani, E., Y. Ren, L. Kuo, and T. W. Hein. 2005. C-reactive protein inhibits endothelium-dependent NO-mediated dilation in coronary arterioles by activating p38 kinase and NAD(P)H oxidase. *Arterioscler. Thromb. Vasc. Biol.* **25:** 995–1001.
23. Clapp, B. R., G. M. Hirschfield, C. Storry, J. R. Gallimore, R. P. Stidwill, M. Singer, J. E. Deanfield, R. J. MacAllister, M. B. Pepys, P. Vallance, et al. 2005. Inflammation and endothelial function: direct vascular effects of human C-reactive protein on nitric oxide bioavailability. *Circulation.* **111:** 1530–1536.
24. Vasquez-Vivar, J., B. Kalyanaraman, P. Martasek, N. Hogg, B. S. S. Masters, H. Karoui, P. Tordo, and K. A. Pritchard. 1998. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc. Natl. Acad. Sci. USA.* **95:** 9220–9225.
25. Mineo, C., A. K. Gormley, I. S. Yuhanna, S. Osborne-Lawrence, L. L. Gibson, L. Hahner, R. V. Shohet, S. Black, J. E. Salmon, D. Samols, et al. 2005. FcgammaRIIB mediates C-reactive protein inhibition of endothelial NO synthase. *Circ. Res.* **97:** 1124–1131.
26. Venugopal, S. K., S. Devaraj, and I. Jialal. 2003. C-reactive protein decreases prostacyclin release from human aortic endothelial cells. *Circulation.* **108:** 1676–1678.
27. Li, L., N. Roumeliotis, T. Sawamura, and G. Renier. 2004. C-reactive protein enhances LOX-1 expression in human aortic endothelial cells: relevance of LOX-1 to C-reactive protein-induced endothelial dysfunction. *Circ. Res.* **95:** 877–883.
28. Devaraj, S., D. Y. Xu, and I. Jialal. 2003. C-reactive protein increases plasminogen activator inhibitor-1 expression and activity in human aortic endothelial cells: implications for the metabolic syndrome and atherothrombosis. *Circulation.* **107:** 398–404.
29. Cermak, J., N. S. Key, R. R. Bach, J. Balla, H. S. Jacob, and G. M. Vercellotti. 1993. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. *Blood.* **82:** 513–520.
30. Baetta, R., M. Camera, C. Comparato, C. Altana, M. D. Ezekowitz, and E. Tremoli. 2002. Fluvastatin reduces tissue factor expression and macrophage accumulation in carotid lesions of cholesterol-fed rabbits in the absence of lipid lowering. *Arterioscler. Thromb. Vasc. Biol.* **22:** 692–698.
31. Corseaux, D., T. Le Tourneau, I. Six, M. D. Ezekowitz, E. P. Mc Fadden, T. Meurice, P. Asseman, C. Bauters, and B. Jude. 1998. Enhanced monocyte tissue factor response after experimental balloon angioplasty in hypercholesterolemic rabbit: inhibition with dietary L-arginine. *Circulation.* **98:** 1776–1782.
32. Stefansson, S., D. A. Lawrence, and W. S. Argraves. 1996. Plasminogen activator inhibitor-1 and vitronectin promote the cellular clearance of thrombin by low density lipoprotein receptor-related proteins 1 and 2. *J. Biol. Chem.* **271:** 8215–8220.
33. Bu, G., E. A. Maksymovitch, and A. L. Schwartz. 1993. Receptor-mediated endocytosis of tissue-type plasminogen activator by low density lipoprotein receptor-related protein on human hepatoma HepG2 cells. *J. Biol. Chem.* **268:** 13002–13009.
34. Devaraj, S., P. R. Kumaresan, and I. Jialal. 2004. Effect of C-reactive protein on chemokine expression in human aortic endothelial cells. *J. Mol. Cell. Cardiol.* **36:** 405–410.
35. Verma, S., S. H. Li, M. V. Badiwala, R. D. Weisel, P. W. Fedak, R. K. Li, B. Dhillon, and D. A. Mickle. 2002. Endothelin antagonism and

interleukin-6 inhibition attenuate the proatherogenic effects of C-reactive protein. *Circulation.* **105:** 1890–1896.
36. Devaraj, S., B. Davis, S. I. Simon, and I. Jialal. 2006. CRP promotes monocyte-endothelial cell adhesion via Fcgamma receptors in human aortic endothelial cells under static and shear flow conditions. *Am. J. Physiol. Heart Circ. Physiol.* **291:** H1170–H1176.
37. Lim, M. Y., H. Wang, A. M. Kapoun, M. O'Connell, G. O'Young, H. A. Brauer, G. R. Luedtke, S. Chakravarty, S. Dugar, G. S. Schreiner, et al. 2004. p38 inhibition attenuates the pro-inflammatory response to C-reactive protein by human peripheral blood mononuclear cells. *J. Mol. Cell Cardiol.* **37:** 1111–1114.
38. Lin, R., J. Liu, N. Peng, G. Yang, W. Gan, and W. Wang. 2005. Lovastatin reduces nuclear factor kappaB activation induced by C-reactive protein in human vascular endothelial cells. *Biol. Pharm. Bull.* **28:** 1630–1634.
39. Koeijvoets, K. C., A. Wiegman, J. Rodenburg, J. C. Defesche, J. J. Kastelein, and E. J. Sijbrands. 2005. Effect of low-density lipoprotein receptor mutation on lipoproteins and cardiovascular disease risk: a parent-offspring study. *Atherosclerosis.* **180:** 93–99.
40. Bisoendial, R., R. Birjmohun, T. Keller, S. van Leuven, H. Levels, M. Levi, J. Kastelein, and E. Stroes. 2005. In vivo effects of C-reactive protein (CRP)-infusion into humans. *Circ. Res.* **97:** e115–e116.
41. Devaraj, S., T. W. Du Clos, and I. Jialal. 2005. Binding and internalization of C-reactive protein by Fcgamma receptors on human aortic endothelial cells mediates biological effects. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1359–1363.
42. Nissen, S. E., E. M. Tuzcu, P. Schoenhagen, T. Crowe, W. J. Sasiela, J. Tsai, J. Orazem, R. D. Magorien, C. O'Shaughnessy, and P. Ganz. 2005. Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. *N. Engl. J. Med.* **352:** 29–38.
43. Ridker, P. M., C. P. Cannon, D. Morrow, N. Rifai, L. M. Rose, C. H. McCabe, M. A. Pfeffer, and E. Braunwald. 2005. C-reactive protein levels and outcomes after statin therapy. *N. Engl. J. Med.* **352:** 20–28.
44. Pepys, M. B., G. M. Hirschfield, G. A. Tennent, J. R. Gallimore, M. C. Kahan, V. Bellotti, P. N. Hawkins, R. M. Myers, M. D. Smith, A. Polara, et al. 2006. Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature.* **440:** 1217–1221.