High-density lipoprotein and C-reactive protein, friend and foe in cardiovascular disease
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Reconstituted apoAI-phosphatidylcholine neutralizes atherothrombotic effects of C-reactive protein in humans

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(submitted)
Abstract

High density lipoprotein (HDL) has various anti-atherothrombotic functions and these may include inhibition of the atherothrombotic effects by C-reactive protein (CRP). We have assessed the ability of HDL to neutralize CRP-mediated activation of coagulation and inflammation. Fifteen healthy male volunteers received an infusion of highly purified recombinant human (rh)CRP. In eight of these volunteers this was preceded by an infusion of apoAI-phosphatidylcholine (apoAI-PC) three hours before. Whereas rhCRP infusion elicited a systemic inflammatory response, cytokine levels remained unaltered in subjects who received apoAI-PC prior to rhCRP infusion. Similarly, thrombin generation and activation of fibrinolysis following administration of rhCRP were abolished by pre-treatment with apoAI-PC. In volunteers receiving apoAI-PC infusion, a substantial part of the rhCRP could be found within the HDL fraction. In in vitro experiments using human endothelial cells we confirmed that apoAI-PC -preincubation with CRP completely abolished the inflammatory activity of the latter. The present findings may lend further support to the use of HDL-increasing interventions for dampening the acute inflammatory response and its consequences in acute coronary syndromes.
Introduction

Inflammation plays a major role in all phases of atherogenesis from plaque initiation to plaque rupture and atherothrombosis. In this view, C-reactive protein (CRP) has emerged as an independent predictor of cardiovascular risk in various clinical settings.\(^1\,^2\) The relation between CRP and atherothrombosis was strengthened by recent prospective studies which showed a positive linear relation between CRP change upon statin therapy and change in coronary atheroma burden\(^3\) and cardiovascular events,\(^4\) respectively, independent from changes in LDL-cholesterol. Parallel to these clinical observations, a large body of \textit{in vitro} evidence indicates that CRP exerts pro-atherogenic effects on major components of the atherosclerotic plaque such as leukocytes, endothelium and smooth muscle cells.\(^5\) Indeed, the major receptor on leukocytes by which CRP can mediate its effect has been identified as being FcyRII (CD32).\(^6\) More recently, receptors for CRP on the endothelium have also been identified when it was demonstrated that CRP exerts its biological activities in human aortic endothelial cells (HAEC) via binding and internalization through Fc receptors CD32 and CD64.\(^7\)

In addition to these receptors, various ligands of CRP have been described such as lipoproteins low density lipoprotein-cholesterol (LDL)\(^8\) and high density lipoprotein-cholesterol (HDL).\(^9,\,^{10}\) Interestingly, many of the atherothrombotic effects of CRP have been suggested to be counter regulated by HDL.\(^11,\,^{12}\) Indeed, CRP-induced atherothrombotic effects \textit{in vitro} were recently shown to be attenuated by pre-incubation with HDL.\(^13\) In the present study we investigated the capacity of HDL to inhibit the effects of an acute CRP increase in healthy human volunteers. Furthermore we wanted to evaluate what mechanisms contributed to a potential atheroprotective effect. Finally, separate experiments were performed to address the potential role of endotoxin contamination within the CRP solution.

Methods

Study Protocol

Fifteen healthy, nonsmoking men were included in this study after written informed consent was obtained. None of the volunteers had febrile illness or cardiovascular disease or were on medication. After an overnight fast, a bolus of highly purified rhCRP was given intravenously (1.25 mg per kg body weight). Blood was drawn at baseline and 1, 4, 8, and 24 hours after infusion. In eight of these volunteers this was preceded by three hours by insertion of a venous catheter, blood withdrawal and a subsequent systemic administration of apoA-I-PC disks at a dose of 80 mg/kg body weight over a period of 3 hours. Reconstituted HDL was kindly provided by CSL Limited (Sydney, Australia).

The rhCRP (BiosPacific), derived from \textit{Escherichia coli} (K12, substrain NM522), was supplied in 20 mmol/L Tris, 140 mmol/L NaCl, 2 mmol/L CaCl\(_2\), pH 7.5, and 0.05% (wt/vol) sodium azide and revealed a single 23-kDa band (>99%) after CBBR-staining (1 \textmu g; SDS-polyacrylamide
gel). Before purification, the host cell protein concentration was 85 ppm, as determined by a high-sensitive ELISA in accordance with manufacturers' instructions (Cygnus Technologies Inc). Subsequently, the rhCRP was purified using size exclusion chromatography to remove contaminants including endotoxin and sodium azide (Univalid bv). Purity as well as stability was evaluated using sequential high-performance liquid chromatography and time-of-flight mass spectrometry, showing no other protein fractions, including the monomeric variant of CRP, besides the CRP pentamer. Endotoxin levels were below 1.5 endotoxin units (EU)/mL as evaluated by Limulus assay (turbidimetric kinetic method; Bactimm bv). The rhCRP was stored in a CaCl₂-containing buffer (pH 8.5) at 0 to 4°C, and all experiments were performed within 6 weeks after rhCRP preparation.

Endotoxin (Escherichia coli lipopolysaccharide, catalog number 1235503, lot G2B274, United States Pharmacopeial Convention Inc, Rockville, USA) was administered to five healthy volunteers. Blood was withdrawn prior to infusion and at 1, 4, 8 and 24 hours infusion.

**Biochemical analysis**

Blood samples were drawn from the subjects after a 12-hour overnight fast, immediately and 3 hours after apoA-I-PC infusion. After centrifugation within 1 hour after collection, aliquots were snap-frozen in liquid nitrogen and stored at -80°C until the assays were performed. All measurements were performed at the vascular and clinical laboratory of the Academic Medical Center, University Hospital of Amsterdam. CRP concentrations were measured with high-sensitivity and immunonephelometric assays (Roche Diagnostics Corporation). IL-6 and IL-8 were assayed by cytometric bead array analysis (BD Biosciences). We measured prothrombin F1+2 (Dade-Behring) and D-dimer (Asserachrom D-dimer, Roche) using ELISAs. Total cholesterol distribution amongst the lipoproteins was measured by Fast Phase Liquid Chromatography (FPLC). In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser and an UV-975 UV/VIS detector (Jasco, Tokyo, Japan). An extra P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for in-line enzymatic reagent (Biomerieux, Marcy l’Etoile, France) addition at 0.1 ml/min. Plasma lipoprotein separations were performed using a Superose 6 HR 10/30 column (Pharmacia Biotech, Upsala Sweden) with TBS pH 7.4, as eluent at a flow rate of 0.31 ml/min. Total cholesterol was determined quantitatively using PAP 250 cholesterol enzymatic method (Biomerieux, Le Fontanille, France). Computer analyses of the chromatograms for quantitative peak integration of the lipoproteins were carried out using Borwin Chromatographic software, version 1.23 (JMBS Developments, Le Fontanil, France).

**Rat Liver Slices**

Precision-cut liver slices (10–14 mg) were prepared as described previously and stored in UW solution on ice until incubation. Slices were incubated individually at 37°C in six-well plates (Greiner) in 3.2 ml Williams’ medium E supplemented with glutamax I (GIBCO-BRL; Paisley, Scotland) and 50 mg/ml gentamicin (GIBCO-BRL) and saturated with 95% O₂-5%
CO₂. Slices were incubated for 24 h with 1, 10 and 50 ng/ml LPS and 25 and 75 µg/ml CRP for a period of five hours.

**HUVEC experiments**

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described using culture medium 199 (GIBCO-BRL, Paisley, Scotland), supplemented with 20% (v/v) foetal bovine serum, 50 µg/ml heparin (Sigma), 6.5-25 µg/ml Endothelial Cell Growth Supplement (Sigma) and 100 U/ml penicillin/streptomycin (Gibco-BRL). Upon reaching confluency, the tissue culture medium was refreshed and cells were allowed to develop their characteristic 'cobble-stone' appearance for at least 24–48 hours prior to harvesting. The cells were stimulated with CRP (25 µg/ml), with or without preincubation of apoAI-PC (10 µg/ml) 16 hours prior to CRP stimulation. The supernatant was harvested 24 hours after the CRP stimulation for IL-6 determination by an IL-6 ELISA kit (Pelikine compact human IL-6, M1916, Sanquin, the Netherlands).

**Statistical Analysis**

The results are expressed as mean ±SEM unless otherwise stated. Differences between the two groups over time were tested by 2-way analysis of variance (ANOVA) for repeated measures using SPSS for Windows (SPSS Inc., version 11.0, Chicago, Illinois, USA). Comparisons within groups were done by the Wilcoxon signed rank test. A probability (p) value of <0.05 was considered significant.

**Results**

Baseline clinical characteristics of the volunteers are summarized in Table 1. Lipid parameters, as well as systolic and diastolic blood pressures and body mass index, were not significantly different (Table 1). Hemodynamic measurements and temperature recordings were stable throughout the infusion studies and did not differ between the two groups. There was no record of any adverse effect in the volunteers.

**Plasma ApoAl and CRP levels**

Three hours after infusion of apoAI-PC levels of apoAl rose from 1.29 (1.03 to 1.55) to 2.66 g/L (2.32 to 2.94). ApoAI levels remained unaltered in volunteers that only received CRP (see fig 1A). In volunteers receiving rhCRP mono-infusion, CRP-concentrations rose from 2.5 (0.3 to 8.5) to 25.5 mg/L (21.1 to 30.4). After an initial fall, concentrations rose again to 30.7 mg/L (17.2 to 48.9) 24 hours after infusion. In volunteers that had been pretreated with apoAI-PC, rhCRP infusion resulted in a similar rise of CRP-concentrations from 1.7 (0.3 to 5.0) to 26.6 (21.4 to 32.2). A second peak however was absent and concentrations continued to fall to 12.5 (8.7 to 15.7) mg/L after 24 hours (figure 1B).
Table 1. Baseline characteristics of the study subjects

<table>
<thead>
<tr>
<th></th>
<th>rhCRP (n=7) Mean ± SD</th>
<th>ApoAI-PC &amp; rhCRP (n=8) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td>39.3 ± 16.9</td>
<td>27.6 ± 12.6</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>27.3 ± 5.2</td>
<td>22.3 ± 2.5</td>
</tr>
<tr>
<td><strong>Systolic BP, mmHg</strong></td>
<td>123 ± 8</td>
<td>131.8 ± 10.6</td>
</tr>
<tr>
<td><strong>Diastolic BP, mmHg</strong></td>
<td>79 ± 3</td>
<td>73.6 ± 5.9</td>
</tr>
<tr>
<td><strong>Glucose, mmol/L</strong></td>
<td>5.3 ± 0.4</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td><strong>hsCRP, mg/L</strong></td>
<td>1.14 ± 0.45</td>
<td>1.72 ± 0.74</td>
</tr>
<tr>
<td><strong>TC, mmol/L</strong></td>
<td>4.8 ± 1.1</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td><strong>LDL, mmol/L</strong></td>
<td>3.1 ± 1.1</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td><strong>HDL, mmol/L</strong></td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td><strong>TG, mmol/L</strong></td>
<td>1.0 ± 0.2</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; hsCRP, high-sensitivity CRP; TC, total cholesterol; LDL, low-density cholesterol; HDL, high-density cholesterol; TG, triglycerides.

Figure 1. Circulating levels of CRP and ApoAl after dual infusion of apoAI-PC and CRP compared to CRP mono-infusion. Mean (± SEM) concentrations of (A) CRP and (B) ApoAl in response to rhCRP infusion and rhCRP infusion preceeded by apoAI-PC infusion. Infusion of rhCRP led to an acute rise in CRP plasma levels followed by a secondary peak after 24 hours with the latter being prevented by apoAI-PC pretreatment. ApoAI-PC pretreatment resulted in a significant rise in ApoAl levels whereas levels remained unaltered in volunteers receiving rhCRP mono-infusion.

ApoAI-PC blunts CRP-mediated activation of inflammation and coagulation

Infusion of rhCRP elicited a systemic proinflammatory response as reflected by a monophasic rise in plasma IL-6 and IL-8 levels peaking at 4 hours after rhCRP-infusion (figure 2A and B). Such an increase in cytokine production was absent in volunteers that had been pre-treated with apoAI-PC. Mono-infusion of rhCRP elicited thrombin generation as well as activation of
Figure 2. Effects on inflammation and coagulation of dual infusion of apoAI-PC and CRP compared to CRP mono-infusion. Mean (± SEM) concentrations of (A) IL-6, (B) IL-8, (C) F1+2, and (D) D-dimers in response to rhCRP infusion and rhCRP infusion preceded by apoAI-PC infusion. Actication of inflammation and coagulation induced by rhCRP infusion was prevented by apoAI-PC pretreatment.

Fibrinolysis as indicated by elevated levels of F1+2 and D-dimer respectively (figure 2C and D). Pretreatment with apoAI-PC however prevented these CRP-mediated effects in volunteers receiving both treatments.

Atherothrombotic effects are directly mediated by CRP, not endotoxins

Several studies reporting atherogenic effects of CRP used commercial CRP which was contaminated and these effects were in fact contributable to endotoxin contamination.\(^\text{14}\)

Therefore we conducted several experiments to confirm that our findings were mediated specifically by CRP and not by contamination with lipopolysaccharide (LPS).
Rat liver slices
At different concentrations, LPS induced significant increases in production of TNF-α in rat liver slices (figure 3). Stimulation of rat liver slices with recombinant human CRP at a similar concentration reached in healthy volunteers failed to elicit any changes in TNF-α secretion.

Figure 3. Effects of stimulation of rat liver slices with CRP and LPS. Mean (± SEM) concentrations of TNF-α after stimulation of rat liver slices for a period of five hours. Stimulation with LPS at various doses (1, 10 and 50 ng/ml) resulted in significantly increased TNF-α production. In contrast, stimulation of rat with CRP liver slices failed to induce TNF-α production.

Trace amount of LPS in vivo
The same trace amount of LPS which was detectable in the recombinant CRP we used for infusion experiments (1.5 EU/ml), was administered to five healthy male volunteers. This did not result in a systemic proinflammatory state, illustrated by a stable production of IL-6 (figure 4).

Figure 4. Effects of rhCRP and LPS infusion on inflammation. Mean (± SEM) concentrations of IL-6 in response to rhCRP infusion and infusion of a trace amount LPS. Whereas infusion of rhCRP led to a significantly increased IL-6 secretion, infusion of the same trace amount of LPS which was present in rhCRP had no effect on IL-6 levels.

Aggregation of CRP and HDL
Complex formation
In order to verify whether the neutralizing effect of apoAI-PC treatment was associated with increased binding of CRP to HDL particles we analyzed lipoprotein subfractions, isolated
Figure 5. Amount of CRP detectable in the HDL fraction after dual infusion with HDL and CRP compared to CRP mono-infusion. Four hours after infusion of CRP, the amount of CRP in the HDL fraction of volunteers that had been pretreated with apoAI-PC was five-fold higher when compared to volunteers that did not receive apoAI-PC.

by FPLC. We determined concentrations of CRP within the HDL fraction of both treatment groups four hours after they had received a single bolus-infusion of CRP, when differences between the two groups were most pronounced. In volunteers that had been pre-treated with apoAI-PC, 27.8% (8.8 to 40.7) of the administered CRP was present in the HDL fraction compared to 5.5% (1.1 to 10.8) in volunteers that had only received CRP (figure 5).

HUVEC experiments
In HUVECs, stimulation with CRP for 24 hours resulted in increased IL-6 production by 25.3% compared to baseline levels (figure 6). CRP that had been heat-inactivated did not increase IL-6 production as levels remained at baseline (-4.5%). A mixture of CRP and apoAI-PC, which was prepared 1 hour before adding it to HUVECs to allow complex formation, did not change IL-6 production compared to baseline (3.5%). Furthermore, stimulation with apoAI-PC of HUVECs that had been preincubated with HDL for 16 hours resulted in reduced IL-6 production of 23.5% compared to baseline levels.

Figure 6. Effects of stimulation of HUVECs with CRP, in presence and absence of apoAI-PC. Stimulation of HUVECs with rhCRP increased IL-6 production with 25.3% compared to baseline. HUVECs stimulated with rhCRP and apoAI-PC that were added together for one hour to allow aggregation, produced a similar levels of IL-6 when compared to baseline. CRP stimulation of HUVECs which had been pretreated with apoAI-PC for 16 hours resulted in reduced IL-6 production of 23.5% compared to baseline levels.
Discussion

We show that pre-treatment with apoAI-PC effectively neutralized the atherothrombotic effects of CRP in humans (figure 2). ApoAI-PC infusion prevented a systemic proinflammatory response upon CRP challenge, as illustrated by similar levels of pro-inflammatory cytokines such as IL-6 and IL-8 before and after CRP challenge. Inhibition of the cytokine release by apoAI-PC infusion prior to the CRP challenge was accompanied by abolition of the secondary endogenous CRP peak at 24 hrs, as observed in the non-apoAI-PC treated group. In the apoAI-PC treated subjects, CRP levels decreased compliant with its reported half life of 17-19 hours. Last, thrombin generation and activation of fibrinolysis, as illustrated by elevated levels of $F_1+2$ and D-dimer, respectively, were prevented by apoAI-PC treatment.

ApoAI-PC neutralizes CRPs’ atherothrombotic effects

HDL is amongst the most powerful mediators of atheroprotection, and the strong inverse relationship between plasma HDL-cholesterol and the incidence of CVD has been established by many epidemiological surveys. Traditionally, the atheroprotective nature of HDL was thought to be confined to its role in reverse cholesterol transport (RCT), mediating transport of cholesterol from peripheral tissues, such as the arterial wall, back to the liver. Cholesterol efflux from foam cells, which is considered a critical step in RCT with respect to atherogenesis, may however only be a minor contributor to total RCT. More recently, a wide variety of anti-thrombotic and anti-inflammatory effects have been ascribed to HDL. Our findings suggest that HDL’s protective capacity may also include neutralization of the atherothrombotic effects mediated by CRP. Our findings in humans correspond with a recent study, showing the inhibitory effects of HDL on CRP-induced adhesion molecule expression in HUVECs and adherence of U937 cells to aortic endothelial cells. Main findings of this study included the observation that preincubation of HDL and its prolonged presence were obligatory for HDL’s inhibitory activity, whereas the oxidized form of its principal phospholipid was found to be the fundamental active component. The data also suggested that an interaction between HDL and endothelial cells was required for the inhibitory effect of HDL on CRP. The author speculated that this interaction may reveal ‘cryptic’ bindings sites on HDL, resulting in competitive inhibition of the interaction between CRP and ECs. In support, several groups have already shown that oxidative modification is required before CRP can bind to lipoproteins, particularly LDL particles. In addition, CRP has been suggested to bind HDL.

To analyze whether the neutralizing effect of apoAI-PC was associated with increased CRP-HDL aggregation we isolated lipoprotein subfractions using FPLC. Indeed, four hours after administration of CRP, the amount of CRP within the HDL fraction of volunteers that had received apoAI-PC treatment was five time higher when compared to volunteers that had only received CRP. In parallel, using in vitro experiments with HUVECs, we were able to confirm that pre-incubation with apoAI-PC resulted in attenuation of a CRP-mediated increase of IL-6 production, whereas CRP alone significantly stimulated endothelial IL-6 production.
note, less then 30% of CRP was found within the HDL fraction, thus alternative mechanisms may also play a role. In that respect, apoAI-PC discs may exert direct anti-inflammatory and anti-coagulant effects, which contribute to attenuation of the atherothrombotic effects of CRP.\textsuperscript{22,23}

**CRP versus endotoxin**

Numerous studies have reported on the atherothrombotic effects of CRP.\textsuperscript{5-7,13} Unfortunately, the reported effects in some of these studies, using commercial CRP, may in part be attributable to residual endotoxin in the CRP-preparate. As has recently been reviewed, however, the majority of these studies used endotoxin-purified CRP with adequate measures being taken to rule out the possibility of endotoxin-related effects.\textsuperscript{24} In these studies, control experiments showed loss of atherothrombotic effects after CRP was trypsinized, heat-inactivated, removed using antibody-coated plates or when CRP receptors were blocked with monoclonal antibodies to CD32. Limited by the fact that we study CRP-mediated effects in humans, we used different strategies to verify that the observed effects were related to CRP per se rather than contaminants. We used rhCRP, which underwent a thorough purification procedure resulting in a residual endotoxin concentration of 1.5 endotoxin units (EU)/mL. This is at least 10-fold lower than that demonstrated to elicit inflammatory and coagulation cascades.\textsuperscript{25} We confirmed the latter by infusing this low concentration of endotoxin in healthy volunteers. Indeed at this dose, no inflammatory changes could be observed (figure 4). In addition, as opposed to LPS, CRP did not stimulate production of TNF-\(\alpha\) in rat liver slices (figure 3). In line, CRP which was heat-inactivated, also lost its ability to stimulate endothelial cells to produce IL-6 (figure 6). Combined, these findings demonstrate that the atherothrombotic effects observed in healthy volunteers following infusion of CRP can indeed be attributed to CRP itself and not to LPS contamination.

**Clinical implications**

The consistent data on pro-atherogenic effects of CRP, combined with the correlation between CRP lowering and improved CV outcome in the PROVE-IT study have revolutionized the arena of cardiovascular preventive strategies. Whereas there is an ongoing debate during what stages of atherogenesis CRP antagonizing strategies may be most apt, the search for CRP-inhibiting strategies is expanding rapidly. Promising candidates include a specific small-molecule inhibitor of CRP\textsuperscript{28} and CRP antisense treatment (ISIS pharmaceuticals, USA). In the present study we show that apoAI-PC abolishes the pro-inflammatory effects of CRP. These findings provide further impetus for HDL-increasing strategies for dampening the acute inflammatory state in acute coronary syndromes.\textsuperscript{29} In addition, potential usefulness in patients with chronic inflammatory disorders such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) can be considered. It has generally been acknowledged that systemic inflammation underlies enhanced atherogenesis in these patients.\textsuperscript{30-32} Increased levels of CRP combined with decreased levels of HDL are also seen in other pro-atherogenic states such as the metabolic syndrome\textsuperscript{33} or diabetes mellitus.\textsuperscript{34} It will be a challenge
to validate whether HDL-increasing strategies have the capacity to attenuate atherogenesis in these particular patient groups, partly due to these anti-inflammatory properties. Two drugs (torcetrapib and JTT-705) that can increase HDL levels via inhibition of cholesterol ester transfer protein (CETP) are currently in the preliminary stages of clinical development and may become available in the near future. Further research will have to determine whether these novel HDL increasing strategies will have similar neutralizing effects of CRP-mediated atherothrombotic effects.

References


