Binding of C-reactive protein to modified low-density-lipoprotein particles: identification of cholesterol as a novel ligand for C-reactive protein

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C-reactive protein (CRP), an acute-phase reactant, is present in atherosclerotic human arterial intima in association with lipids. In the present work we studied interactions between CRP and LDL on microtitre wells, where either CRP or LDL was immobilized. LDL was modified by vortex-mixing, oxidation, or by lipolysis with phospholipase A₂ or with sphingomyelinase or a combination of trypsin and cholesterol esterase. We found that CRP bound only to LDL modified by trypsin/cholesterol esterase or by sphingomyelinase and that this binding was Ca²⁺-dependent. In these two forms of modified LDL, non-esterified cholesterol was susceptible to cholesterol oxidase, indicating exposure of non-esterified cholesterol on particle surfaces and suggesting a role for non-esterified cholesterol in mediating CRP binding. Consistent with this hypothesis were the following findings: (i) increasing the amount of non-esterified cholesterol in LDL with cyclodextrin increased, and decreasing its amount decreased, the binding of CRP to LDL; (ii) modification of non-esterified cholesterol in LDL by cholesterol oxidase decreased the binding of CRP to LDL; and (iii) CRP bound to purified non-esterified cholesterol. The binding was Ca²⁺-dependent and could be competed out with phosphocholine. Taken together, these findings suggest that CRP can bind to modified lipoproteins, notably to the non-esterified cholesterol on their surface. These interactions may be related to the suggested role of CRP in the local inflammation present in atherosclerotic plaques.

Key words: atherosclerosis, cholesterol, inflammation.

INTRODUCTION

There is compelling evidence that inflammation plays an important role in atherosclerosis [1]. A major cause of chronic inflammation in the atherosclerotic arterial wall is the presence of extracellularly located low-density-lipoprotein (LDL) particles, notably their modified lipids. Even minimally oxidized LDL contains bioactive phospholipids capable of activating endothelial cells to induce secretion of monocyte chemoattractant protein-1 (MCP-1) [2]. Oxidized LDL and LDL lipolysed with phospholipase A₂ contain lysophosphatidylcholine (lyso-PC), which, at low concentrations, has various proinflammatory effects on arterial cells and, at high concentrations, is cytotoxic [3]. LDL modified by a protease and cholesterol esterase (CEase) has been shown to be a potent inducer of adhesion and transmigration of monocytes and T cells through the endothelium [4]. It also stimulates the secretion of MCP-1 and interleukin-6 (IL-6) by macrophages and promotes smooth-muscle-cell proliferation and foam-cell formation [5].

Of the above-listed proinflammatory mediators, IL-6 is of particular interest, since it triggers the synthesis of acute-phase reactants, such as the C-reactive protein (CRP), in the liver. CRP is an 120 kDa acute-phase protein with a suggested role in the recognition and clearance of damaged cells, modified self-structures and microbial products. CRP has the structure of a disc consisting of five symmetrically assembled 21 kDa protomers [6]. The ‘recognition face’ of CRP contains two Ca²⁺ ions and binds to the phosphocholine moieties of pneumococcal polysaccharides [7] and of membrane phospholipids [8], to apoptotic cells [9], fibronectin [10] and histone–chromatin complexes [11] and to small nuclear ribonuclear particles [12] in a Ca²⁺-dependent manner and to polycations in a Ca²⁺-independent manner [13]. The ‘effector face’ of CRP, in turn, binds sub-component C1q of the complement system and Fcγ receptors [14,15]. Thus CRP can opsonize its ligands either directly (via Fcγ receptors) or indirectly via activation of the classical complement pathway, generating ligands for complement receptors [16]. Importantly, CRP-induced complement activation usually proceeds only to the C3 level, since CRP also binds Factor H, a complement inhibitor that accelerates the decay of the C3 and C5 convertases [9,17].

Human atherosclerotic [18,19] lesions have been shown to contain CRP. In atherosclerotic lesions, the amount of CRP was found to correlate with the degree of atherosclerotic involvement [20]. It is noteworthy that, in areas rich in extracellular lipids, CRP was found to co-localize with the terminal complement complex in the atherosclerotic lesions [21]. Although rabbit and rat CRP have been shown to interact with lipoproteins in vitro and in blood plasma in vivo, human CRP is not associated with lipoproteins in blood plasma [22]. Since human lipoproteins,
notably the LDL particles, are considered to be in native form in the circulation, but in modified form in atherosclerotic lesions, it is possible that, in atherosclerotic lesions, the CRP binds to the modified LDL. Interestingly, Bhakdi et al. [23] recently showed that CRP binds to LDL modified by a combination of trypsin and CEase (Try/CEase) in vitro and suggested that CRP binds to phosphatidylcholine (PC) molecules in the modified LDL. Here, we studied binding of CRP to LDL modified in a variety of ways (oxidative, lipolytic, and proteolytic agents and enzymes) potentially relevant to atherosclerosis [24] to find the critical components in LDL for CRP binding. We found that modifications that increased the amount of non-esterified cholesterol on the surfaces of LDL-particles triggered CRP binding to these particles. Most importantly, CRP bound directly to non-esterified cholesterol. These findings reveal a novel ligand for CRP, namely cholesterol, and provide a plausible explanation for the retention of CRP in human atherosclerotic lesions.

**MATERIALS AND METHODS**

**Isolation and labelling of LDL**

Human LDL (ρ = 1.019–1.050 g/ml) was isolated from plasma of healthy volunteers by sequential ultracentrifugation [25] and dialysed extensively against buffer A (150 mM NaCl/1 mM EDTA, pH 7.4). The volunteers gave their informed consent, and the procedure met the legal requirements of the local ethical committee. Apolipoprotein B-100 (Apo-B-100) of LDL was ²H-labelled by the Bolton–Hunter procedure [26] with N-succinimidyld-[²H]propionate (Amershams Biosciences, Upsala, Sweden) to yield [²H]LDL. The amounts of LDL are expressed in terms of protein. Protein concentrations were determined by the procedure of Lowry with BSA (Sigma, St. Louis, MO, U.S.A.) as standard.

**Labelling of CRP**

Human CRP (100 µg; Sigma) was labelled with 0.5 mCi of Na¹²⁵I (Amershams Biosciences), using IODO-GEN® Pre-coated Tubes (Pierce, Rockford, IL, U.S.A.), essentially as described previously [27]. Briefly, Na¹²⁵I was added to the protein solution after activation in the IODO-GEN®tube. NaI (0.1 mM) was used as a carrier iodide and 0.2 % (w/v) tyrosine as an iodination scavenger. The excess of free iodide was separated from the iodinated protein by gel filtration on a PD-10 column (Amershams Biosciences), equilibrated, and eluted with 10 mM Tris/2 mM CaCl₂/150 mM NaCl, pH 7.4. Protein-bound and free¹²⁵I were determined by a standard trichloroacetic acid precipitation procedure. The specific activity of ¹²⁵I-CRP was 2900–4800 c.p.m./ng of protein, and the amount of free¹²⁵I was ≥ 6 %. The reactions were allowed to proceed at room temperature. The native conformation of the iodinated CRP preparations were confirmed by demonstrating unchanged Ca²⁺-dependent binding to phospholipids.

**Binding of ¹²⁵I-CRP to LDL modified in solution**

LDL (1–2 mg/ml of protein) was lipolysed with 100 munits/ml phospholipase A₂ (PLA₂) from bee venom (Sigma) at PBS at +37 °C for 18 h or with 60 munits/ml (0.05 µg/ml) phospholipase A₁ (PLA₁) from Bacillus cereus (Sigma) in PBS at +37 °C for 18 h or with 60 munits/ml (0.05 µg/ml) phospholipase A₂ (PLA₂) from bee venom (Sigma) at +37 °C for 18 h in the presence of 2 % (w/v) fatty-acid-free BSA (Sigma) in PLA₂ buffer (10 mM Hepes/2 mM CaCl₂/2 mM MgCl₂/140 mM NaCl, pH 7.4). Cholesterol esters of LDL were hydrolysed with CEase. For CEase treatment, LDL (1–2 mg/ml protein) was first treated with 40 µg/ml trypsin from bovine pancreas (Sigma) in PBS at +37 °C for 2 h and then with 80 µg/ml CEase from Candida cylindracea (Boehringer Mannheim, Mannheim, Germany) in PBS at +37 °C for 2 h. Before CEase treatment, trypsin was inhibited with 64 µg/ml soybean trypsin inhibitor (Sigma) in PBS. LDL (0.5–1 mg/ml protein) was oxidized by incubation with 5 µM CuSO₄ in PBS at +37 °C for 2 h or 18 h. The reactions were terminated by addition of 1 mM EDTA. The degree of LDL oxidation was monitored by electrophoretic mobility on agarose gel (Paragon Lipo; Beckman Coulter, Fullerton, CA, U.S.A.) relative to native LDL and was found to be 1.5-fold in 2 h-oxidized and 4-fold in 18 h-oxidized LDL. LDL was also oxidized with 25 mM 2,2-azobis-(2-aminooxy)-propane hydrochloride (AAPH; Polysciences, Warrington, PA, U.S.A.) in buffer A for 18 h at +37 °C [28] or with 0.8 mM hypochlorite (YA-Kemia, Helsinki, Finland) in buffer A for 15 min on ice [29]. LDL (1 mg/ml protein) was vortex-mixed in PBS at room temperature for 60 s at full speed.

After each modification LDL was re-isolated by gel filtration on a Bio-Gel A-0.5 m column (1 cm × 50 cm; Bio-Rad, Hercules, CA, U.S.A.) at +4 °C in 5 mM Tris/1 mM EDTA/150 mM NaCl, pH 7.4. Microtitre wells (Labsystems, Helsinki, Finland) were coated with native or modified LDL (50 µg/ml) in buffer B (10 mM Tris/2 mM CaCl₂/150 mM NaCl, pH 7.2) at +4 °C for 18 h. Non-specific binding sites of the wells were blocked by incubation with 3 % (w/v) BSA in buffer B for 1 h at room temperature. Unlabelled and radioiodinated CRP were mixed to yield a specific radioactivity of 33000 c.p.m./µg, and different concentrations (1–30 µg/ml) of CRP were added in 50 µl of buffer B in the presence of 3 % (w/v) BSA and incubated for 2 h at room temperature. The wells were washed three times with 250 µl of buffer B in the presence of 3 %, BSA, detached, and their radioactivities were determined by liquid-scintillation counting. To study the effect of Ca²⁺ on the binding, the experiment was also done in buffer C (10 mM Tris/4 mM EDTA/150 mM NaCl, pH 7.2) in the presence of 3 % BSA. BSA-coated wells served as controls for non-specific binding. Experiments with microtitre wells coated with 50 µg/ml native [²H]LDL and [²H]LDL modified with SMase, PLA₂, Try/CEase, copper oxidation or vortex-mixing revealed that 14–19 ng of LDL was bound to the wells.

**Binding of ¹²⁵I-CRP to LDL modified on microtitre wells**

Microtitre wells (Labsystems) were coated with native LDL (50 µg/ml) in 50 µl of buffer B at +4 °C for 18 h. Non-specific binding sites of the wells were blocked by incubation with 3 % BSA in buffer B for 1 h at room temperature. The coated LDL was modified on wells with 2 munits/ml SMase in 50 µl of PBS or 1200 munits/ml (1 µg/ml) PLA₂, in 50 µl of PLA₂ buffer in the presence of 2 % (w/v) fatty-acid-free BSA. Coated LDL was also modified with 10 µg/ml trypsin in 50 µl PBS for 1 h at +37 °C, after which the trypsin was inhibited with 160 µg/ml trypsin inhibitor in 50 µl of PBS and LDL was further modified with 20 µg/ml CEase in 50 µl of PBS for 1 h at +37 °C. The wells were washed twice with buffer B and different concentrations (1–30 µg/ml) of radioiodinated CRP mixed with unlabelled CRP to obtain a specific radioactivity of 33000 c.p.m./µg were incubated in 50 µl of buffer B in the presence of 3 % BSA for 2 h at room temperature. The wells were washed and their radioactivities were determined as described above. To study the effect of Ca²⁺ ions on the binding, the experiment was also done in buffer C in the presence of 3 % BSA. BSA-coated wells served as controls for non-specific binding. Experiments with microtitre wells coated with [²H]LDL revealed that the modifications of
LDL particles with SMase, PLAn or Try/CEase did not release LDL from the wells.

**Analysis of lipid composition of modified LDL**

Lipids from LDL samples modified in solution were extracted by the procedure of Bligh and Dyer [29a]. Lipids from LDL samples modified on microtitre wells were extracted with hexane/propan-2-ol (3:2, v/v), dried, and again extracted by the Bligh and Dyer [29a] method as a further purification step. Neutral lipids were separated by high-performance TLC on HPTLC Silica Gel 60 (Merck, Darmstadt, Germany) in hexane/ether/acetate acid/water (80:18.5:1:2:0.3, by vol.) solvent, and phospholipids were separated in chloroform/methanol/acetate acid/water (55:33:8.7:3:8, by vol.) solvent. Finally, the lipids were visualized by dipping the plates into CuSO₄ solution, heating at 180 °C, and quantified with a Camag TLC Scanner 3 (Camag, Muttenz, Switzerland) with purified lipids from Sigma as standards.

**Oxidation of cholesterol by cholesterol oxidase**

LDL was modified in solution by SMase and by Try/CEase, as described above. Native and modified LDL (200 µg) were oxidized with 100 munits/ml cholesterol oxidase from *Streptomyces* sp. (Calbiochem, San Diego, CA, U.S.A.) in 1 ml of PBS at 37 °C for various periods of time, as described previously [30]. After oxidation, lipids were extracted from LDL and the amount of 4-cholesten-3-one (cholestenone) produced from cholesterol was determined from the lipid extracts by TLC. The samples were separated on HPTLC Silica Gel 60 in 100%, dichloromethane [31]. Purified cholesterol and cholestenone (Sigma) were used as standards.

**Modulation of the amount of non-esterified cholesterol in LDL by cyclodextrin**

Native LDL was coated on microtitre wells and the non-specific binding sites of the wells were blocked by incubation with 3% BSA in buffer B for 1 h at room temperature. The coated LDL was modified with Try/CEase as described above. The amount of non-esterified cholesterol in LDL was decreased by incubation with 20 mM hydroxypropyl-β-cyclodextrin (OHpβCD; Trappsol; Cyclodextrin Technologies Development Inc., Gainesville, FL, U.S.A.) in PBS at +37 °C for 1 h. The wells were washed, and the binding of radioiodinated CRP was studied as described above. It was also confirmed by the use of [3H]cholesterol that OHpβCD treatments did not release coated LDL particles from the microtitre wells.

The amounts of non-esterified cholesterol in native LDL and in Try/CEase-modified LDL (Try/CEase-LDL) were modulated by incubating LDL with OHpβCD or OHpβCD-cholesterol complexes. OHpβCD–cholesterol complexes were prepared by a slight modification of the method of Yancey et al. [32] at an 18:1 molar ratio of OHpβCD to non-esterified cholesterol (Sigma). Non-esterified cholesterol was dried under N2, and OHpβCD in PBS was added to the dried lipid. The mixture was incubated at +37 °C for 48 h in a shaker until the solution of the OHpβCD–cholesterol complexes became clear. LDL was coated on microtitre wells and, in some experiments, LDL was modified with Try/CEase after immobilization, as described above. LDL was incubated with 15 mM OHpβCD or OHpβCD-cholesterol complexes in PBS at +37 °C for 1 h. The wells were washed twice with buffer B and the binding of radioiodinated CRP (20 µg/ml) was analysed as described above. The change in the lipid content of LDL after modification by OHpβCD was studied as described above.

**Binding of 125I-CRP to Try/CEase-LDL oxidized with cholesterol oxidase**

Microtitre wells were coated with native LDL in 50 µl of buffer B at +4 °C for 18 h. Non-specific binding sites of the wells were blocked by incubation with 3% BSA in buffer B for 1 h at room temperature. Native LDL was modified on wells with Try/CEase as described above. The wells were washed twice with buffer B and incubated with various concentrations of cholesterol oxidase from *Streptomyces* sp. in 100 µl of PBS for 1 h at +37 °C. The wells were washed twice and incubated with 30 µg/ml radioiodinated CRP (33000 c.p.m./µg) in 50 µl of buffer B in the presence of 3% BSA for 2 h at room temperature. The wells were washed three times with 250 µl of buffer B in the presence of 3% BSA, detached, and their radioactivities were determined by liquid-scintillation counting. BSA-coated wells served as controls for non-specific binding. The lipid composition of Try/CEase-LDL oxidized by cholesterol oxidase was determined by TLC as described above.

**Binding of 125I-CRP to non-esterified cholesterol and phosphocholine**

Microtitre wells were coated with non-esterified cholesterol (100 µg/ml) in 50 µl of PBS containing 2% (v/v) ethanol and with phosphocholine coupled to albumin (phosphocholine–BSA; Biosearch Technologies Inc., Novato, CA, U.S.A.) (10 µg/ml) in 50 µl of PBS at +4 °C for 18 h. Experiments with [14C]cholesterol showed immobilization of ≥ 1.5 µg of non-esterified cholesterol to the wells. Non-specific binding sites of the wells were blocked by incubation with 3% BSA in buffer B for 1 h at room temperature and the binding of (1–30 µg/ml) radioiodinated CRP in buffer B was studied as described above. To study the effect of Ca2+ on the binding, the experiment was also performed in buffer C in the presence of 3% BSA. BSA-coated wells served as controls for non-specific binding.

To study the competing effect of phosphocholine on the binding of CRP to non-esterified cholesterol, microtitre wells were coated with 100 µg/ml of non-esterified cholesterol. The non-specific binding sites of the wells were blocked and the wells were incubated with 30 µg/ml radioiodinated CRP together with various concentrations (0–90 µg/ml) of phosphocholine (Sigma) in 50 µl of buffer B in the presence of 3% BSA for 2 h at room temperature. The wells were washed and the bound radioactivities were determined. Experiments with [14C]cholesterol showed that phosphocholine did not solubilize coated cholesterol from the microtitre wells.

**Statistical analyses**

The data points in all Figures are means ± S.E.M. for three independent experiments, and significance was determined with Student’s unpaired *t* test. *P < 0.05* was considered statistically significant.

**RESULTS**

To study the interactions between CRP and modified LDL under physiological ionic conditions, binding experiments of three different types were performed in microtitre wells. First, we studied the binding of 125I-CRP to LDL which was modified in solution and then immobilized to the microtitre wells. 125I-CRP
with the indicated concentrations of 125I-CRP in the presence (native or modified LDL, and non-specific binding to the wells was blocked by incubating them with buffer B or C containing 3% BSA. The wells were then incubated for 2 h at room temperature to activation of the endogenous PLA
cholesterol in LDL, but also generated lyso-PC, possibly due to activation of the endogenous PLA
binding was strongest to LDL modified by SMase or by Try/CEase (Figure 1A). As shown in Table 1, SMase-treatment had hydrolysed all the sphingomyelin (SM) in the particles. Moreover, Try/CEase treatments effectively hydrolysed cholesteryl esters, generating a 4-fold increase in non-esterified cholesterol in LDL, but also generated lyso-PC, possibly due to activation of the endogenous PLAs of LDL. In addition to treatment of LDL by a combination of trypsin and CEase, we studied whether treatment of LDL with trypsin or CEase alone would increase binding of CRP to the particles. We found that trypsin alone had no effect on CRP binding. Moreover, CEase alone only marginally increased binding of CRP to LDL (not shown), a finding consistent with the findings that this enzyme is unable to effectively hydrolyse LDL lipids when apoB-100 is intact [33]. We found that the binding of 125I-CRP to LDL was consistently increased to some extent after treatment with PLAs, which had hydrolysed virtually all PC in LDL (Table 1), but that binding to LDL modified by copper oxidation or vortex-mixing was as low as binding to native LDL. In addition to oxidation of LDL with copper, we studied LDL oxidation with AAPH and hypochlorite, but found no significant increase in CRP binding to the modified LDL particles (results not shown). As shown in Figure 1B, 125I-CRP failed to bind to any of the modified LDL in the presence of EDTA, i.e. binding to modified LDL was completely Ca2+-dependent. To ensure that the increased binding of CRP to modified LDL is not an artifact due to iodination of CRP, control binding experiments were conducted with native CRP. In this assay, CRP was detected by polyclonal rabbit anti-

Figure 1 Binding of CRP to native and modified LDL coated on microtitre wells

LDL was modified in solution by PLAs, SMase, copper oxidation (‘ox-LDL’), trypsin/CEase or by vortex-mixing as described in the Materials and methods section. Microtitre wells were coated with native or modified LDL, and non-specific binding to the wells was blocked by incubating them with buffer B or C containing 3% BSA. The wells were then incubated for 2 h at room temperature with the indicated concentrations of 125I-CRP in the presence (A; + Ca2+) or absence (B; + EDTA) of Ca2+. After washing, the wells were detached and the bound 125I-CRP was quantified. The data points indicate the specifically bound 125I-CRP obtained by subtracting the amounts of 125I-CRP bound to the control wells (coated only with BSA) from the amounts of 125I-CRP bound to the LDL-coated wells. The data points are means ± S.E.M. for three independent experiments. *P < 0.05 against native LDL.

Table 1 Analysis of the lipid composition of LDL modified in solution (A) and on microtitre wells (B)

LDL was modified in solution (a) and after immobilization to microtitre wells (b) by SMase, PLAs or by Try/CEase as described in the Materials and methods section. Lipids were extracted and analysed by TLC as described in the Materials and methods section. The results are expressed as µg of lipid/mg of LDL protein and in parenthesis as percentages of different lipid classes in LDL. The data points are means ± S.E.M. for three independent experiments. Further abbreviations: NC, non-esterified cholesterol; TG, triacylglycerols; CE, cholesteryl esters.

<table>
<thead>
<tr>
<th>Composition [µg/mg (%)]</th>
<th>LDL</th>
<th>SMase-LDL</th>
<th>PLAs-LDL</th>
<th>Try/CEase-LDL</th>
</tr>
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<tbody>
<tr>
<td>(a) Modification of LDL in solution</td>
<td></td>
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</tr>
<tr>
<td>NC</td>
<td>530 ± 45 (12)</td>
<td>481 ± 46 (12)</td>
<td>488 ± 47 (14)</td>
<td>1542 ± 151 (50)</td>
</tr>
<tr>
<td>TG</td>
<td>173 ± 41 (4)</td>
<td>149 ± 45 (4)</td>
<td>144 ± 33 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CE</td>
<td>1903 ± 292 (43)</td>
<td>1941 ± 352 (46)</td>
<td>1967 ± 277 (56)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PC</td>
<td>1456 ± 44 (32)</td>
<td>1446 ± 12 (36)</td>
<td>0 (0)</td>
<td>574 ± 14 (19)</td>
</tr>
<tr>
<td>Lyso-PC</td>
<td>84 ± 43 (2)</td>
<td>0 (0)</td>
<td>558 ± 30 (16)</td>
<td>673 ± 104 (22)</td>
</tr>
<tr>
<td>SM</td>
<td>342 ± 26 (8)</td>
<td>0 (0)</td>
<td>322 ± 41 (0)</td>
<td>313 ± 21 (10)</td>
</tr>
<tr>
<td>(b) Modification of LDL immobilized on microtitre wells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>509 ± 49 (16)</td>
<td>442 ± 67 (17)</td>
<td>452 ± 64 (17)</td>
<td>752 ± 145 (36)</td>
</tr>
<tr>
<td>CE</td>
<td>2099 ± 115 (67)</td>
<td>1771 ± 80 (69)</td>
<td>1870 ± 70 (68)</td>
<td>932 ± 219 (45)</td>
</tr>
<tr>
<td>PC</td>
<td>390 ± 104 (13)</td>
<td>325 ± 64 (14)</td>
<td>110 ± 12 (4)</td>
<td>292 ± 57 (14)</td>
</tr>
<tr>
<td>SM</td>
<td>155 ± 9 (5)</td>
<td>9 ± 0.4 (0)</td>
<td>166 ± 19 (6)</td>
<td>95 ± 22 (5)</td>
</tr>
</tbody>
</table>
As shown in Table 1(b), SMase, PLA₂ phase leads to aggregation and they remain as single particles, whereas modification in the fluid fact that, when the particles are modified after immobilization, the differences in the CRP concentrations of 125I-CRP in 50 µl of buffer B, washed, and detached, and the bound 125I-CRP was quantified. The data points indicate the specifically bound 125I-CRP, calculated by subtracting the amounts of 125I-CRP bound to the control wells (coated only with BSA) from the amounts of 125I-CRP bound to the LDL-coated wells. The data points are means ± S.E.M. for three independent experiments. *P < 0.05 against native LDL.

Next we set out to elucidate the mechanism of the increased CRP binding to LDL modified by a combination of trypsin and CEase or SMase. Non-esterified cholesterol is known to bind to SM and to reside in sphingomyelin-containing domains on the surface of LDL [36]. This raised the possibility that the binding of CRP to the modified particles involved non-esterified cholesterol. We first studied whether the surface exposure of non-esterified cholesterol was increased in the modified LDL. This was performed by studying the susceptibility of non-esterified cholesterol to cholesterol oxidase, i.e., conversion of non-esterified cholesterol to cholestenone. As shown in Figure 3, both the rate of cholesterol oxidation and the maximal amount of cholestenone formed were greatly increased in LDL modified by Try/CEase. Moreover, oxidation of non-esterified cholesterol was significantly more rapid in SMase-modified LDL than in native LDL. Thus exposure of non-esterified cholesterol was increased both in Try/CEase-modified and in SMase-modified LDL. To study the role of non-esterified cholesterol on particle surfaces in CRP binding, we first decreased its amount by incubating modified LDL with cyclodextrin, which is known to remove non-esterified cholesterol from membranes [37].

Cyclodextrin treatment of Try/CEase-LDL (Figure 4) efficiently decreased the binding of CRP to these particles, which is compatible with the hypothesis that non-esterified cholesterol is important for the binding of CRP to LDL.

Next, we incorporated non-esterified cholesterol into native LDL and Try/CEase-LDL with the aid of cholesterol-cyclodextrin complexes, and studied the binding of CRP to these particles. When non-esterified cholesterol was increased in the particles, binding of CRP to both native LDL and Try/CEase-LDL was significantly enhanced (Figure 5). Indeed, the very low basal binding of CRP to the native LDL particles was increased by about 20-fold, and the high basal binding of CRP to the Try/CEase-LDL was increased by about 3-fold. By contrast, in the absence of cholesterol, cyclodextrin did not affect the binding of CRP to native LDL, but effectively blocked its binding to Try/CEase-LDL, resulting in almost complete inhibition of CRP binding to this species of LDL.

Analysis of the lipid composition revealed that treatment with cyclodextrin decreased the content of non-esterified cholesterol in LDL by 67% and in Try/CEase-LDL by 63%, with no major effect on phospholipid content of native or Try/CEase-LDL (Table 2). The treatment of LDL with cholesterol–cyclodextrin complexes increased the content of non-esterified cholesterol in LDL by 2.4-fold and in Try/CEase-LDL by 1.4-fold. Interestingly, treatment with cholesterol–cyclodextrin complexes removed 54% of PC and 50% of SM from native LDL and 38% of PC and 29% of SM from Try/CEase-LDL, suggesting that cholesterol–cyclodextrin complexes partly exchanged cholesterol for phospholipids.

The above data revealed the strong dependence of the binding of CRP to LDL on the content of non-esterified cholesterol.
Figure 4  Binding of CRP to microtitre wells coated with native LDL which was subsequently modified with trypsin/CEase

Microtitre wells were coated with LDL and the immobilized LDL was modified by Try/CEase as described in the Materials and methods section. The wells were then incubated in the absence or presence of 20 mM cyclodextrin for 1 h at +37 °C, washed, and further incubated with the indicated concentrations of 125I-CRP for 2 h at room temperature. After incubation, the wells were washed and detached, and the bound 125I-CRP was quantified. The data points indicate the specifically bound 125I-CRP obtained by subtracting the amounts of 125I-CRP bound to the control wells (coated only with BSA) from the amounts of 125I-CRP bound to the LDL-coated wells. The data points represent means ± S.E.M. for three independent experiments. *P < 0.05 against native LDL.

oxidase affects binding of CRP to the particles. As shown in Figure 6(A), increasing concentrations of cholesterol oxidase, an enzyme converting the 3β-hydroxy group of cholesterol to a ketone group, progressively reduced the binding of CRP to Try/CEase-LDL. Moreover, lipid composition analysis of Try/CEase-LDL oxidized by increasing concentrations of cholesterol oxidase (Figure 6B) showed that the decreased CRP binding to

Table 2  Analysis of the lipid composition of native LDL (a) and Try/CEase-LDL (b) modified with cyclodextrin or with cholesterol–cyclodextrin complexes

Microtitre wells were coated with native LDL (a) and the immobilized LDL was modified on wells with Try/CEase (b) as described in the Materials and methods section. LDL and Try/CEase-LDL were then incubated with 15 mM cyclodextrin or cholesterol–cyclodextrin complexes, the wells were washed, and lipids were extracted and analysed by TLC as described in the Materials and methods section. The results are expressed as μg of lipid/mg of LDL protein and in parentheses as percentages of different lipid classes in LDL. The data points are means ± S.E.M. for three independent experiments. For further abbreviations, see the legend to Table 1.

<table>
<thead>
<tr>
<th>Composition [μg/mg (%)]</th>
<th>LDL</th>
<th>+ Cyclodextrin</th>
<th>+ Cholesterol–cyclodextrin complexes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(a) Native LDL</td>
<td>Try/CEase-LDL</td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>622 ± 43 (18)</td>
<td>595 ± 42 (38)</td>
<td>980 ± 199 (32)</td>
</tr>
<tr>
<td>CE</td>
<td>1838 ± 197 (53)</td>
<td>800 ± 199 (32)</td>
<td>597 ± 24 (24)</td>
</tr>
<tr>
<td>PC</td>
<td>821 ± 58 (24)</td>
<td>206 ± 26 (14)</td>
<td>404 ± 28 (29)</td>
</tr>
<tr>
<td>SM</td>
<td>192 ± 8 (6)</td>
<td>123 ± 11 (4)</td>
<td>105 ± 4 (7)</td>
</tr>
<tr>
<td></td>
<td>(b) Try/CEase-LDL</td>
<td></td>
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</tr>
<tr>
<td>NC</td>
<td>956 ± 42 (38)</td>
<td>595 ± 42 (38)</td>
<td>956 ± 42 (38)</td>
</tr>
<tr>
<td>CE</td>
<td>800 ± 199 (32)</td>
<td>206 ± 26 (14)</td>
<td>800 ± 199 (32)</td>
</tr>
<tr>
<td>PC</td>
<td>597 ± 24 (24)</td>
<td>404 ± 28 (29)</td>
<td>597 ± 24 (24)</td>
</tr>
<tr>
<td>SM</td>
<td>166 ± 6 (7)</td>
<td>105 ± 4 (7)</td>
<td>166 ± 6 (7)</td>
</tr>
</tbody>
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C-reactive protein binds to modified low-density lipoproteins

Figure 6  Binding of CRP to Try/CEase-LDL oxidized by cholesterol oxidase

Microtitre wells were coated with native LDL and the immobilized LDL was modified by Try/CEase as described in the Materials and methods section. The wells were then incubated with the indicated concentrations of cholesterol oxidase from Streptomyces sp. in 100 μl of PBS for 1 h at +37 °C. (A) Binding of CRP to the wells was studied by incubating the wells with 30 μg/ml [125I]-CRP for 2 h at room temperature. After incubation, the wells were washed and detached, and the bound [125I]-CRP was quantified. The data points indicate the specifically bound [125I]-CRP obtained by subtracting the amounts of [125I]-CRP bound to the control wells (coated only with BSA) from the amounts of [125I]-CRP bound to the LDL-coated wells. (B) Lipids were extracted from the wells and separated in 100% dichloromethane by HPTLC, and cholesterol and cholestenone were quantified by densitometry. The data points are means ± S.E.M. for three independent experiments. *P < 0.05 against the sample without cholesterol oxidase.

Figure 7  Binding of CRP to non-esterified cholesterol and phosphocholine coated on microtitre wells

Microtitre wells were coated with non-esterified cholesterol (A) or phosphocholine coupled to albumin (B), and non-specific binding to the wells was blocked by incubating the wells with buffer B or C containing 3% BSA. The wells were then incubated for 2 h at room temperature with the indicated concentrations of [125I]-CRP in the presence or absence of Ca²⁺. After incubation, the wells were washed and detached, and the bound [125I]-CRP was quantified. (C) Microtitre wells were coated with non-esterified cholesterol and non-specific binding to the wells was blocked by incubating the wells with buffer B containing 3% BSA. The wells were then incubated with 30 μg/ml of [125I]-CRP together with the indicated concentrations of phosphocholine for 2 h at room temperature. After incubation, the wells were washed and detached, and the bound [125I]-CRP was quantified. The data points indicate the specifically bound [125I]-CRP obtained by subtracting the amounts of [125I]-CRP bound to the control wells (coated only with BSA) from the amounts of [125I]-CRP bound to the wells coated with non-esterified cholesterol or phosphocholine. The data points are means ± S.E.M. from three independent experiments. *P < 0.05 against the sample with EDTA (A and B) or against the sample without phosphocholine (C).
cholestenone could be detected (results not shown), further confirming the specificity of binding of CRP to cholesterol. The affinity of CRP for cholesterol was found to be similar to that for phosphatidylcholine (Figure 7B). Finally, the binding of CRP to non-esterified cholesterol was inhibited by phosphatidylcholine (Figure 7C), suggesting that cholesterol bound to the phosphatidylcholine binding site on CRP.

**DISCUSSION**

CRP is an important indicator of inflammation and is probably involved in restoring homeostasis after tissue injury. The reports on CRP deposition in human atherosclerotic lesions prompted us to study the interactions of CRP with native and modified lipoproteins. The present results showed that, although little or no CRP bound to native LDL, significant amounts of CRP bound to LDL modified by SMase or by trypsin/CEase. Moreover, modulation of the amount of non-esterified cholesterol in the modified LDL particles by cyclodextrin affected their ability to bind CRP. Finally, we demonstrated that CRP can bind directly to non-esterified cholesterol. The results suggest a role for CRP in modulating the clearance of and the immune response elicited by modified lipoproteins.

The classical, i.e. the first discovered and most studied, ligand of CRP is phosphatidylcholine. CRP was initially discovered because of its ability to precipitate pneumococci carrying C-polysaccharide [38]. Subsequently, it was discovered that this property of CRP was due to its binding to pneumococcal phosphatidylcholine [7], known to be associated with the cell-wall teichoic acid via N-acetylgalactosamine. In addition to *Pneumococcus*, other Gram-positive bacteria, the Gram-negative bacterium *Haemophilus influenzae* and eukaryotic cells also contain phosphatidylcholine [39]. Surfaces of plasma lipoprotein particles like the surfaces of eukaryotic cells contain large amounts of phosphatidylcholine esterified with diacylglycerol and with a sphingosine backbone to form the two major membrane phospholipids: PC and SM respectively. Although human CRP can interact with PC vesicles containing lyso-PC [8], human CRP does not interact with native lipoproteins or cell membranes [9,22]. The reason for the lack of binding is not entirely clear, but could be the high level of organization of the normal, undisturbed physiological membranes. However, we found (results not shown) that, after prolonged storage or derivatization into monomeric/aggregated form with urea/EDTA [40], CRP was able to bind freshly isolated native LDL, a finding consistent with the recently observed ability of aggregated CRP to bind to native LDL [41]. The relevance of this finding may be related to the observations suggesting that CRP undergoes denaturation at sites of inflammation [42]. In a recent study, CRP was shown to mediate uptake of native LDL by macrophages [43]. We found no difference in the uptake or degradation of native LDL by human monocyte-derived macrophages, whether CRP was absent or present in the incubation medium (results not shown). This finding is consistent with little or no binding of native, i.e. non-denatured, CRP to native LDL.

When cells die, the highly organized structure of cell membranes is disturbed. During apoptosis, cessation of membrane ‘flip-flop’ leads to exposure of phosphatidylserine on the plasma membrane [44]. This makes the membrane susceptible to secretory group IIa phospholipase A₂, with ensuing generation of lyso-PC on the cell membrane [45]. CRP was recently shown to bind to apoptotic cells and facilitate their clearance [9]. This is consistent with the previous finding that CRP binds to membranes containing lyso-PC [8]. Since the secretory type of phospholipase A₂ is present extracellularly in human atherosclerotic lesions [46] and has been shown to modify LDL in vitro [47], we also studied its effect on LDL binding to CRP. Treatment of LDL with phospholipase A₂ was found to increase the binding of CRP to LDL, but the effect was modest. This may partly be explained by the findings that phospholipase A₂ treatment decreases LDL size and makes the particles more rigid, most likely because of penetration of the lyso-PC molecules toward the particle core [36]. We found that enrichment of LDL with exogenous lyso-PC without concomitant loss of PC effectively increased the binding of CRP to LDL (results not shown). It is likely that the lyso-PC enrichment resulted in loosening of the LDL surface and disruption of the surface lipid organization, with ensuing exposure of the phospholipid groups of the superficially located PC molecules and binding of CRP to the exposed phospholipid. The importance of proper exposure of the phospholipid groups in membranes was recently highlighted in experimental studies in which binding of CRP to phospholipid monolayers was found to be increased when a fraction of the phospholipid had a spacer exposing the phospholipid groups from the membrane [48].

Most interesting were the present findings that CRP effectively bound to LDL modified by either SMase or trypsin/CEase, since similar modifications of LDL are expected to occur in the arterial intima in vivo. The apparent liberation of non-esterified cholesterol by CEase treatment and the suggestion that also SMase treatment can liberate non-esterified cholesterol that is tethered by SMin native LDL [36] led to the hypothesis that non-esterified cholesterol can influence the binding of CRP to LDL. This hypothesis gained support from the findings that modulation of the level of non-esterified cholesterol with cyclodextrin and cholesterol–cyclodextrin complexes caused parallel changes in the binding of CRP to LDL. The effect of cholesterol on CRP binding may be due to (i) cholesterol-induced alteration in the organization of the LDL surface allowing binding of CRP to surface phospholipids or (ii) binding of CRP directly to cholesterol. Although we were unable to rule out the possibility that cholesterol enhanced the binding of CRP to phospholipids, we were able to show direct binding of CRP to purified cholesterol. Interestingly, the kinetics of the binding of CRP to immobilized cholesterol and to phosphatidylcholine were similar, and phosphatidylcholine efficiently inhibited the binding of CRP to cholesterol. Although additional studies are needed for further characterization of the interaction between cholesterol and CRP, it is reasonable to assume that cholesterol binds to the phosphatidylcholine binding site in CRP, and we speculate that this interaction could involve the 3/-OH group of cholesterol and the Ca²⁺ ions bound to CRP. This hypothesis is strongly supported by our findings that both oxidation of the 3/-OH group of cholesterol and chelation of Ca²⁺-ions by EDTA blocked the binding of CRP to modified LDL. Cholesterol, when present in excessive amounts in membranes, can undergo lateral phase separation into ordered immiscible domains. Cholesterol domains have been shown to be present in model membranes enriched in cholesterol, in membranes of arterial smooth-muscle cells isolated from aortas of cholesterol-fed rabbits, and in macrophages and fibroblasts enriched in cholesterol in the presence of an acyl-CoA:cholesterol acyltransferase inhibitor [49,50]. It is likely that more or less similar cholesterol domains are formed also on the surface of LDL when LDL is enriched in cholesterol by cyclodextrin, or by CEase treatments, or when SM has been degraded. As CRP is unable to bind to native LDL despite the presence of separate cholesterol molecules on its surface, it is possible that CRP binds to clustered cholesterol molecules, forming domains in modified LDL.
The potential biological significance of the interaction between CRP and cholesterol in the arterial intima relies on the fact that substantial amounts of non-esterified cholesterol are often deposited in human atherosclerotic lesions in lipoproteins, in non-esterified cholesterol-rich vesicles and in cholesterol crystals. The pathophysiological consequences of CRP binding to these structures remain to be shown. It is possible that such binding of CRP has a role in the clearance of non-esterified cholesterol from the intima, since macrophages can take up CRP-opsonized particles directly via Fc receptors [15] and, perhaps more importantly, via complement receptors, due to CRP-induced deposition of iC3b on target structures [9]. Although CRP may have a detrimental pro-inflammatory role in advanced lesions containing necrotic cells, such as in acute myocardial infarction [51], CRP deposition may be beneficial in early lesions because of its ability to aid in the clearance of modified self-structures in a non-inflammatory fashion [9]. Furthermore, CRP could limit activation of the terminal complement pathway, which can be inflammatory fashion [9]. Furthermore, CRP could limit activation of the terminal complement pathway, which can be inflammatory fashion [9]. Furthermore, CRP could limit activation of the terminal complement pathway, which can be inflammatory fashion [9]. Furthermore, CRP could limit activation of the terminal complement pathway, which can be inflammatory fashion [9]. Furthermore, CRP could limit activation of the terminal complement pathway, which can be inflammatory fashion [9]. Furthermore, CRP could limit activation of the terminal complement pathway, which can be inflammatory fashion [9]. 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