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,

Abbreviations used: AAPH, 2,2'-azobis-(2-amidinopropane) hydrochloride; ApoB, apolipoprotein B; CEase, cholesterol esterase; CRP, C-reactive protein; IL, interleukin; LDL, low-density lipoprotein(s); lyso-PC, lysophosphatidylcholine; MCP-1, monocyte chemoattractant protein-1; OHpβCD, hydroxypropyl-β-cyclodextrin; PC, phosphatidylcholine; PLA₂, phospholipase A₂; SM, sphingomyelin; SMase, sphingomyelinase; Try/CEase-LDL, LDL modified with a combination of trypsin and cholesterol esterase.

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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 ($\rho = 1.019\text{--}1.050$ 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 written consent, and the procedure met the legal requirements of the local ethical committee. Apolipoprotein B-100 (ApoB-100) of LDL was ^3H -labelled by the Bolton–Hunter procedure [26] with *N*-succinimidyl [2,3- ^3H]propionate (Amersham Biosciences, Uppsala, Sweden) to yield [^3H]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 Na ^{125}I (Amersham Biosciences), using IODO-GEN® Pre-coated Tubes (Pierce, Rockford, IL, U.S.A.), essentially as described previously [27]. Briefly, Na ^{125}I 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 (Amersham Biosciences), equilibrated, and eluted with 10 mM Tris/2 mM CaCl $_2$ /150 mM NaCl, pH 7.4. Protein-bound and free ^{125}I were determined by a standard trichloroacetic acid precipitation procedure. The specific activity of ^{125}I -CRP was 2900–4800 c.p.m./ng of protein, and the amount of free ^{125}I was $\approx 6\%$. The reactions were allowed to proceed at room temperature. The native conformation of the iodinated CRP preparations were confirmed by demonstrating unchanged Ca $^{2+}$ -dependent binding to phosphocholine.

Binding of ^{125}I -CRP to LDL modified in solution

LDL (1–2 mg/ml of protein) was lipolysed with 100 munits/ml sphingomyelinase (SMase) from *Bacillus cereus* (Sigma) in PBS at +37 °C for 18 h or with 60 munits/ml (0.05 $\mu\text{g}/\text{ml}$) phospholipase A $_2$ (PLA $_2$) from bee venom (Sigma) at +37 °C for 18 h in the presence of 2 % (w/v) fatty-acid-free BSA (Sigma) in PLA $_2$ buffer (10 mM Hepes/2 mM CaCl $_2$ /2 mM MgCl $_2$ /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 $\mu\text{g}/\text{ml}$ trypsin from bovine pancreas

(Sigma) in PBS at +37 °C for 2 h and then with 80 $\mu\text{g}/\text{ml}$ CEase from *Candida cylindracea* (Boehringer Mannheim, Mannheim, Germany) in PBS at +37 °C for 2 h. Before CEase treatment, trypsin was inhibited with 64 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor (Sigma) in PBS. LDL (0.5–1 mg/ml protein) was oxidized by incubation with 5 μM CuSO $_4$ 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-amidino-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 $\times$ 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 $\mu\text{g}/\text{ml}$) in buffer B (10 mM Tris/2 mM CaCl $_2$ /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 $\mu\text{g}/\text{ml}$) of CRP were added in 50 μl of buffer B in the presence of 3 % 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 Ca $^{2+}$ 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 $\mu\text{g}/\text{ml}$ native [^3H]LDL and [^3H]LDL modified with SMase, PLA $_2$, Try/CEase, copper oxidation or vortex-mixing revealed that 14–19 ng of LDL was bound to the wells.

Binding of ^{125}I -CRP to LDL modified on microtitre wells

Microtitre wells (Labsystems) were coated with native LDL (50 $\mu\text{g}/\text{ml}$) 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 $\mu\text{g}/\text{ml}$) PLA $_2$ in 50 μl of PLA $_2$ buffer in the presence of 2 % (w/v) fatty-acid-free BSA. Coated LDL was also modified with 10 $\mu\text{g}/\text{ml}$ trypsin in 50 μl PBS for 1 h at +37 °C, after which the trypsin was inhibited with 160 $\mu\text{g}/\text{ml}$ trypsin inhibitor in 50 μl of PBS and LDL was further modified with 20 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$) 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 Ca $^{2+}$ 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 [^3H]LDL revealed that the modifications of

LDL particles with SMase, PLA₂ 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/acetic acid/water (80:18.5:1.2:0.3, by vol.) solvent, and phospholipids were separated in chloroform/methanol/acetic 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, Muttens, 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]LDL 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 (33 000 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

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

LDL was modified in solution by PLA₂, 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, PLA₂ 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.

	Composition [µg/mg (%)]			
	LDL	SMase-LDL	PLA ₂ -LDL	Try/CEase-LDL
(a) Modification of LDL in solution				
NC	530 ± 45 (12)	481 ± 46 (12)	488 ± 47 (14)	1542 ± 151 (50)
TG	173 ± 41 (4)	149 ± 45 (4)	144 ± 33 (4)	0 (0)
CE	1903 ± 292 (42)	1941 ± 352 (48)	1957 ± 277 (56)	0 (0)
PC	1456 ± 44 (32)	1446 ± 72 (36)	0 (0)	574 ± 14 (19)
Lyso-PC	84 ± 43 (2)	0 (0)	558 ± 30 (16)	673 ± 104 (22)
SM	342 ± 26 (8)	0 (0)	322 ± 41 (9)	313 ± 21 (10)
(b) Modification of LDL immobilized on microtitre wells				
NC	494 ± 69 (16)	442 ± 87 (17)	452 ± 64 (17)	752 ± 145 (36)
CE	2069 ± 115 (67)	1771 ± 80 (69)	1876 ± 70 (68)	932 ± 219 (45)
PC	390 ± 104 (13)	352 ± 84 (14)	110 ± 12 (4)	292 ± 57 (14)
SM	155 ± 9 (5)	9 ± 9 (0.4)	166 ± 19 (6)	95 ± 22 (5)

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 PLA₂ 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 PLA₂, 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-(human CRP) antibodies. Consistent with the above results with iodinated CRP, we found that binding of native CRP to LDL was significantly increased after treatment of LDL with Try/CEase (results not shown). In the second type of binding experiment, we studied binding of modified [³H]LDL to CRP immobilized on microtitre wells. By analogy to the results shown above, native LDL did not bind to CRP, and LDL modified by SMase or by Try/CEase bound most strongly to CRP (not shown).

In the third type of binding experiment with modified LDL, we studied the binding of ¹²⁵I-CRP to LDL that had been modified after immobilization to microtitre wells. In this system also, CRP bound to LDL modified by Try/CEase and to LDL modified by

Figure 2 Binding of CRP to microtitre wells coated with native LDL and LDL modified after immobilization

Microtitre wells were coated with native LDL, and non-specific binding to the wells was blocked by incubating them with buffer B containing 3% BSA. The wells were then incubated with PLA_2 , SMase or trypsin/CEase for 1 h at $+37^\circ\text{C}$, as described in the Materials and methods section. After washing, the wells were incubated for 2 h at room temperature with the indicated concentrations of ^{125}I -CRP in 50 μl of buffer B, washed, and detached, and the bound ^{125}I -CRP was quantified. The data points indicate the specifically bound ^{125}I -CRP, calculated by subtracting the amounts of ^{125}I -CRP bound to the control wells (coated only with BSA) from the amounts of ^{125}I -CRP bound to the LDL-coated wells. The data points are means $\pm$ S.E.M. for three independent experiments. * $P < 0.05$ against native LDL.

SMase (Figure 2). Similar to modification of LDL in the fluid phase, modification after LDL immobilization by SMase effectively hydrolysed sphingomyelin, and Try/CEase effectively hydrolysed cholesteryl esters and generated non-esterified cholesterol in LDL (Table 1b). The binding to LDL modified by PLA_2 was weak and did not differ from binding to native LDL. The differences in the CRP/LDL ratios are likely to be due to the fact that, when the particles are modified after immobilization, they remain as single particles, whereas modification in the fluid phase leads to aggregation and/or fusion of the particles [34,35]. As shown in Table 1(b), SMase, PLA_2 and Try/CEase had effectively modified LDL lipids also when LDL particles were immobilized on microtitre wells.

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

Figure 3 Susceptibility of cholesterol to cholesterol oxidase in native and modified LDL

LDL was modified in solution by SMase or Try/CEase. Portions (200 μg) of native and modified LDL were oxidized with 100 units/ml cholesterol oxidase from *Streptomyces* sp. in 1 ml of PBS at $+37^\circ\text{C}$ for different periods of time. Lipids were extracted and separated in 100% dichloromethane by HPTLC, and cholestenone was quantified by densitometry. The data points represent means $\pm$ S.E.M. for three independent experiments. * $P < 0.05$ against native LDL.

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.

in LDL. To further study the role of non-esterified cholesterol in binding of CRP to modified LDL, we studied how modification of non-esterified cholesterol in Try/CEase-LDL by cholesterol

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.

Composition [μg/mg (%)]			
	LDL	+ Cyclodextrin	+ Cholesterol–cyclodextrin complexes
(a) Native LDL			
NC	622 ± 43 (18)	154 ± 77 (6)	1882 ± 245 (43)
CE	1838 ± 197 (53)	1908 ± 232 (69)	1914 ± 327 (43)
PC	821 ± 58 (24)	563 ± 46 (20)	484 ± 27 (11)
SM	192 ± 8 (6)	123 ± 11 (4)	133 ± 2 (3)
(b) Try/CEase-LDL			
	Try/CEase-LDL	+ Cyclodextrin	+ Cholesterol–cyclodextrin complexes
NC	956 ± 42 (38)	204 ± 26 (14)	1211 ± 138 (52)
CE	800 ± 199 (32)	700 ± 171 (50)	658 ± 110 (28)
PC	597 ± 24 (24)	404 ± 28 (29)	360 ± 11 (15)
SM	166 ± 6 (7)	105 ± 4 (7)	120 ± 4 (5)

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

Figure 5 Binding of CRP to 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 by Try/CEase (B) as described in the Materials and methods section. The wells were then incubated with 15 mM cyclodextrin or cholesterol–cyclodextrin complexes for 1 h at +37 °C, washed, and further incubated with 20 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. The values are means ± S.E.M. for three independent experiments.

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.

the particles paralleled conversion of cholesterol into cholestenone, confirming the importance of the 3β-hydroxy group of cholesterol in CRP binding.

To elucidate whether CRP can bind directly to cholesterol, we studied the binding of CRP to microtitre wells coated with non-esterified cholesterol. We found that CRP bound to non-esterified cholesterol in a Ca²⁺-dependent manner (Figure 7A). Binding of radioiodinated CRP to cholesterol was efficiently competed for by unlabelled CRP, confirming that the binding is not caused by changes in CRP by radioiodination (results not shown). In contrast with cholesterol, no binding of CRP to immobilized

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 phosphocholine (Figure 7B). Finally, the binding of CRP to non-esterified cholesterol was inhibited by phosphocholine (Figure 7C), suggesting that cholesterol bound to the phosphocholine 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 phosphocholine. 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 phosphocholine [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 phosphocholine [39]. Surfaces of plasma lipoprotein particles like the surfaces of eukaryotic cells contain large amounts of phosphocholine 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 A_2 , 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 phospho-

lipase A_2 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 A_2 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 A_2 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 phosphocholine groups of the superficially located PC molecules and binding of CRP to the exposed phosphocholine. The importance of proper exposure of the phosphocholine 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 phosphocholine 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 SM in 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 phosphocholine were similar, and phosphocholine 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 phosphocholine binding site in CRP, and we speculate that this interaction could involve the 3β -OH group of cholesterol and the Ca^{2+} ions bound to CRP. This hypothesis is strongly supported by our findings that both oxidation of the 3β -OH group of cholesterol and chelation of Ca^{2+} -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 γ 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 induced in the arterial intima by, for example, extracellular lipid droplets, vesicles and cholesterol crystals.

The Wihuri Research Institute is maintained by the Jenny and Antti Wihuri Foundation. This work was supported by grants from the Academy of Finland, the Juselius Foundation and the Federation of Finnish Insurance Companies.

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Received 28 March 2002/25 June 2002; accepted 9 July 2002

Published as BJ Immediate Publication 9 July 2002, DOI 10.1042/BJ20020492