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C-Reactive Protein-Bound Enzymatically Modified Low-Density Lipoprotein Does Not Transform Macrophages into Foam Cells

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Atherosclerosis is a disease of the arterial wall that leads to heart attack and stroke. Atherosclerosis begins when low-density lipoprotein (LDL)3 is deposited in artery walls. The trapped LDL has increased susceptibility to modifications, such as oxidation and enzymatic proteolysis. Modified LDL is engulfed by macrophages to form foam cells that contribute to the development of atherosclerosis (1–3). It is, therefore, important to capture and inactivate LDL to prevent formation of macrophage foam cells.

C-reactive protein (CRP) is a pentameric protein composed of five identical 23,028 Da-subunits (4, 5). CRP binds to phosphocholine (PC)-containing substances, such as pneumococcal C-polysaccharide (PnC) and damaged cells of the myocardial infarcts (6–9). CRP also binds to non-PCh substances, such as phosphoethanolamine (PEt), dAMP, galactose-containing substances, and cholesterol (10–15). Calcium ions are required for these binding reactions of CRP. There are five PCh-binding sites in CRP, one located on each subunit. The PCh-contacting amino acids in the PCh-binding site of CRP are Phe66 and Glu81. Phe66 interacts with the methyl groups of choline while Glu81 interacts with the nitrogen atom of choline. The phosphate group of PCh coordinates directly with the CRP-bound Ca2+ (4, 5).

CRP localizes with LDL and macrophages in the atherosclerotic lesions (16–19). In vitro, native pentameric CRP binds to modified forms of LDL, such as oxidized LDL and enzymatically modified LDL (E-LDL), in a Ca2+-dependent and reversible manner (19–26). Aggregated forms of native pentameric CRP have been shown to bind to native LDL as well (27–29). The participation of the PCh-binding site of CRP in binding to modified forms of LDL has been demonstrated by the inhibition of binding of CRP to LDL by PCh. Because CRP binds to modified LDL, the possibility that CRP may play a role in the uptake of modified LDL by macrophages has been investigated (29–32). However, the effect of CRP on the accumulation of lipid droplets made up of cholesteryl esters in LDL-treated macrophages, which is a hallmark of macrophage foam cells, has not been reported.

In this study, we further explored CRP-E-LDL interactions and investigated the formation of macrophage foam cells by unbound and CRP-bound E-LDL.

Materials and Methods

CRP mutants

We performed site-directed mutagenesis and constructed two mutant forms of CRP, F66A and F66A/E81A, by substituting Phe66 and Glu81 with Ala at the PCh-binding site of CRP. The method of construction, expression, and some biochemical characterization of the CRP mutants have been described earlier (14, 33).
Preparation of CRP
Native CRP was purified from pleural fluid as described earlier (33, 34). Purified CRP was stored in TBS at −80 °C. After thawing, however, the stored CRP was not used as such in the experiments. Instead, CRP was centrifuged and subjected to gel filtration on a Superose 12 column (10/300 GL; GE Healthcare) using the Biologic Duo Flow protein purification system (Bio-Rad) to remove fragmented, denatured, monomeric, and aggregated CRP from the purified, stored, native pentameric CRP preparations. The column was eluted with TBS, and the fractions containing pentameric CRP were collected, diluted in appropriate Ca2+-containing buffer as described below, and then used in the assays the same day. After the purification step, we labeled our CRP as freshly purified CRP. The F66A and F66A/E81A CRP mutants were purified from CHO cell culture supernatants by immunoaffinity chromatography as described previously (33).

Preparation of E-LDL
E-LDL was prepared as described previously, with some modifications (19, 20). First, native LDL was isolated from normal human plasma donated by healthy laboratory volunteers according to a published method (35). The concentration of protein in the LDL preparations was measured using a protein assay kit (Bio-Rad); Native LDL was dialyzed against 5 mM Vero-nal buffer containing 0.1% NaCl (pH 7.3). Native LDL (5 mg/ml) was treated with plasmid (0.1 U/ml; Roche) and EDTA (0.5 mM) and incubated for 24 h at 37 °C with shaking. Cholesterol esterase (40 μg/ml; Roche) was added next and incubated for 24 h at 37 °C with shaking. Then, once again, plasmid and cholesterol esterase were added together and incubated overnight at 37 °C with shaking. Finally, the mixture was centrifuged at 10,000 rpm for 5 min and after protein estimation, the supernatant was aliquoted and stored at 4 °C. This preparation of E-LDL was used in the solid-phase E-LDL-binding assays.

Solid-phase E-LDL-binding assay
Microtiter wells were coated with 10 μg of E-LDL in TBS overnight at 4 °C. Wells were then blocked with TBS containing 0.5% gelatin. CRP, diluted in TBS-Ca (TBS containing 0.1% gelatin, 0.02% Tween 20, and 2 mM CaCl2), was added in duplicate wells. After incubation for 3 h at 37 °C, the wells were washed with TBS-Ca. Affinity-purified polyclonal rabbit anti-CRP Abs (1 μg/ml) were used to detect bound CRP (34). HRP-conjugated goat anti-rabbit IgG (Pierce) was used as the secondary Ab. Color was developed, and the absorbance was read at 405 nm in a microtiter plate reader (Molecular Devices).

To determine the effects of PCh (Sigma-Aldrich), dAMP (Sigma-Aldrich; D6250), and PEt (Sigma-Aldrich; P0503) on CRP-E-LDL interaction, the E-LDL-binding assays were performed as mentioned above, except that CRP was added to E-LDL-coated wells in the presence of PCh, dAMP, or PEt. For all statistical comparisons, results were compared by two-tailed unpaired t test.

PnC-binding assay
Binding activity of CRP for PCh-containing ligands other than E-LDL was determined by using PnC (Statens Serum Institut) as described previously (33). Microtiter wells were coated with PnC (2 μg/ml) and used for the treatment of macrophages. For all the experiments as follows. The E-LDL or CRP-E-LDL complexes were collected from several rounds of gel filtration, pooled, and concentrated to 1 mg/ml for a second round of CRP-treatment and gel filtration. Finally, the CRP-E-LDL complex was cleaned again by passing it through the gel filtration column, concentrated to 200 μg/ml, and used for the treatment of macrophages. For some experiments, the CRP-E-LDL complexes were treated with 10 mM EDTA for 15 min at room temperature to dissociate CRP from E-LDL. Before using the CRP-E-LDL complexes for the treatment of cells, an aliquot of the complex was subjected to SDS-PAGE (10%) and Western blot using polyclonal anti-CRP (Sigma-Aldrich) and polyclonal anti-apolipoprotein B (ApoB) (Calbiochem) Abs to confirm the presence of CRP and E-LDL in the complex.

Preparation of CRP-E-LDL complex
Purified CRP (200 μg) was mixed with E-LDL (200 μg) in TBS containing 2 mM CaCl2, in a final volume of 2.5 ml. CRP was used in excess to saturate all E-LDL molecules with CRP. After 30 min at room temperature, the mixture was concentrated to 440 μl and subjected to gel filtration on a Superose 12 column to remove unbound CRP and eluted with TBS containing 2 mM CaCl2. The fractions in the first peak appearing after the void volume and containing the CRP-E-LDL complexes were collected from several rounds of gel filtration, pooled, and concentrated to 1 mg/ml for a second round of CRP-treatment and gel filtration. Finally, the CRP-E-LDL complex was cleaned again by passing it through the gel filtration column, concentrated to 200 μg/ml, and used for the treatment of macrophages. For some experiments, the CRP-E-LDL complexes were treated with 10 mM EDTA for 15 min at room temperature to dissociate CRP from E-LDL. Before using the CRP-E-LDL complexes for the treatment of cells, an aliquot of the complex was subjected to SDS-PAGE (10%) and Western blot using polyclonal anti-CRP (Sigma-Aldrich) and polyclonal anti-apolipoprotein B (ApoB) (Calbiochem) Abs to confirm the presence of CRP and E-LDL in the complex.

Determination of macrophage foam cell formation
Monocytes were isolated using Ficoll-gradient centrifugation from EDTA-containing blood donated by healthy laboratory volunteers. Monocytes were transformed into macrophages in chambered coverglasses (Lab-Tek; Nunc) by culturing for 6 days at 37 °C with 5% CO2. The cells were cultured for 3 days in RPMI 1640 containing t-Glu, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES and then for 3 days in serum-free medium. The resulting serum-starved macrophages were used in the experiments as follows. The E-LDL or CRP-E-LDL complexes at various concentrations (based on the total protein concentration) were added to the cells and incubated at 37 °C. The CRP-E-LDL complexes, used in the experiment shown in Fig. 5, were prepared by gel filtration. The CRP-E-LDL complexes prepared by sucrose density gradient ultracentrifugation can also be used to treat macrophages. The cells were then washed with PBS and fixed for 15 min in 4% paraformaldehyde in PBS. Cells were stained with Nile red (Sigma-Aldrich) for 5 min, followed by washing with PBS. Foam cell images were captured with a Nikon Diaphot 200 fluorescence microscope. Pictures were taken using a ×40 objective lens and ×8 ocular lens at 480 nm excitation and 510 nm emission settings. Final images were prepared using Adobe Photoshop. In the cytoplasm, the accumulation of lipid droplets formed of cholesterol esters derived from the processing of E-LDL is the hallmark of foam cell formation (3, 36). Nile red, at 480 nm excitation and 510 nm emission, stains these lipid droplets bright yellow (37).

Results

The E-LDL-binding site of CRP
To define the binding site on CRP for E-LDL, we used two mutant forms of CRP, F66A and F66A/E81A. Both F66A and F66A/E81A CRP mutants bound to E-LDL in a dose-dependent manner, as well as did the native CRP (Fig. 1A). The relative binding avidities of F66A (0.84 ± 0.11) and F66A/E81A CRP (1.68 ± 0.41) for E-LDL were not significantly different from that of native CRP (Fig. 1B). These results indicated that Phe66 and Glu81 at the PCh-binding site of CRP in E-LDL are not significantly different from that of native CRP (Fig. 1A). In the binding site on CRP for E-LDL interaction. In the presence of 10 mM PCh, the binding of all CRP species to E-LDL was inhibited at all concentrations of CRP (Fig. 1C), suggesting the involvement of the PCh-binding site of CRP in
binding to E-LDL. The next assay was designed to determine the IC₅₀ values of PCh for the inhibition of binding of CRP to E-LDL (Fig. 1D). The IC₅₀ of PCh for both F66A (0.48 ± 0.13 mM) and F66A/E81A CRP (1.28 ± 0.16 mM) were significantly lower than that of native CRP (2.19 ± 0.20 mM). These data indicated that the CRP mutants, that do not bind to immobilized PCh-containing substances, such as PnC, PCh-BSA, and PCh-Sepharose (14, 33), retain some binding avidity for fluid-phase free PCh.

PEt enhanced the binding of CRP to immobilized E-LDL

The unexpected finding that blocking of the PCh-binding site with PCh inhibited, but the mutation of the PCh-contacting amino acids did not inhibit, CRP-E-LDL interaction prompted us to investigate the effects of additional blockers, dAMP and PEt, of the PCh-binding site of CRP on CRP-E-LDL interaction. In the presence of 10 mM dAMP, the binding of CRP to E-LDL was inhibited at all concentrations of CRP (Fig. 2A), confirming the requirement of an empty PCh-binding site in CRP for binding to E-LDL.

PEt (Fig. 2B), which lacks a choline group, was evaluated next (Fig. 2C). PCh and dAMP inhibited the binding of CRP to E-LDL but, surprisingly, PEt did not do so. Instead, PEt, in a dose-dependent manner, enhanced the binding of CRP to E-LDL. In the presence of PEt, the amount of CRP required for 50% of maximal binding of CRP to E-LDL was 93.5 ± 0.8% less compared with that required in the absence of PEt (Fig. 2D). As a control, we performed PnC-binding assay, and, as expected, PEt inhibited CRP-PnC interaction (Fig. 2E). To reassess the enhanced reactivity of PEt-complexed CRP selectively toward E-LDL, we compared the binding of CRP to PnC and E-LDL in the presence and absence of PEt in a single assay (Fig. 2F). Again, PEt inhibited CRP-PnC interaction but enhanced the potency of CRP to interact with E-LDL. Thus, upon complexing with PEt, CRP was converted into a potent E-LDL-binding protein and became unable to bind to the noncholesterol PCh-containing ligand PnC.

PEt enhanced the binding of CRP to fluid-phase E-LDL

We next performed sucrose density gradient ultracentrifugation to investigate the interaction between CRP and purified E-LDL in the
fluid-phase (Fig. 3). When E-LDL alone was centrifuged, the majority of E-LDL remained at the top of the gradient (gradient A). When CRP alone was centrifuged, the majority of CRP remained in fractions 5 and 6 at the bottom of the gradient (gradient B). When mixed together, CRP was bound to E-LDL (compare the gradient in C with A and B). The complexes appeared in fractions 3 and 4 of the gradient (gradient in C), although the complexes were not visible as bands. The formation of two types of complexes could be due to the heterogeneity of E-LDL molecules. PEt was not required for the binding of CRP to fluid-phase E-LDL. In the presence of PEt (gradient in D), however, protein-containing white bands (indicated by red arrow) were observed in both fractions 3 and 4. The amount of both complexes was increased in the presence of PEt. Coomassie blue staining showed the presence of CRP in fractions 3 and 4 from reactions C and D (lanes 4–7). Because E-LDL was not visible in the photograph of the stained gel, we performed Western blotting using anti-ApoB Abs. Western blotting showed the presence of E-LDL in these fractions (lanes 11–14). Thus, in the presence of PEt, binding of CRP to fluid-phase E-LDL was enhanced.

CRP-bound E-LDL did not transform macrophages into foam cells

E-LDL, also a constituent of atherosclerotic lesions, causes formation of macrophage foam cells in vitro (36). We investigated whether CRP-bound E-LDL behaved differently from unbound E-LDL in forming foam cells. We prepared a CRP-E-LDL complex that we processed by gel filtration to obtain a clear homogeneous solution of the complex, before treating macrophages with the complex (Peak I, Fig. 4A). The fractions in peak II contained unbound CRP (data not shown). The appearance of free unbound excess CRP in peak II indicated that the E-LDL in peak I was saturated with CRP and that the peak I contained only CRP-E-LDL complex, devoid of both free E-LDL and CRP. Examination of the proteins in peak I by Western blotting (Fig. 4B) revealed the presence of both CRP (lanes 2–4) and E-LDL (lanes 5–7) in the complex.

We treated human macrophages with E-LDL and with CRP-E-LDL complexes and evaluated formation of foam cells using Nile red staining (Fig. 5A). There was no foam cell formation in untreated cells (A1). Treatment of macrophages with E-LDL resulted in the formation of foam cells (A2 and A3). The ability of E-LDL to convert macrophages into foam cells was abolished when the cells were treated with CRP-E-LDL complexes for 4 h (A4, A5, and A6). These results also confirmed that the CRP-E-LDL complexes were either devoid of free E-LDL or contained only minimal amount of free E-LDL not sufficient to cause foam cell formation. We did not perform any quantitative assay on CRP-E-LDL complex-treated cells because no yellow staining was observed reflecting total absence of lipid droplets. To further assess that CRP-bound E-LDL was unable to induce the formation of foam.
cells, we treated CRP-E-LDL complex with EDTA to dissociate CRP from E-LDL. When the macrophages were treated with E-LDL and CRP in the presence of EDTA, the formation of foam cells was observed again (A7 and A8). Identical results were obtained when the cells were treated with CRP-E-LDL complexes for 36 h (Fig. 5B). Identical results were obtained when the cells were treated with CRP-PEt-E-LDL complexes (Fig. 5C), indicating that the presence of PEt in the CRP-E-LDL complex did not influence the ability of CRP to prevent foam cell formation.

Discussion
We investigated the binding of CRP to E-LDL and the effects of CRP on E-LDL-induced formation of macrophage foam cells. The storage of CRP causes spontaneous dissociation of pentameric CRP into monomeric CRP, and the monomeric CRP also binds to E-LDL (24, 38); therefore, in all experiments, we used freshly purified CRP to avoid contamination with monomeric CRP. Thus, the results reported in this paper apply to native pentameric CRP only. Our major findings were as follows. 1. The PCh-binding site of CRP participated in binding to E-LDL, but the PCh-contacting amino acids Phe66 and Glu81 in the PCh-binding site of CRP were not critical for binding of CRP to E-LDL. 2. Blocking of the PCh-binding site of CRP with PEt, but not with PCh and dAMP, dramatically increased the binding of CRP to both solid-phase and fluid-phase E-LDL. 3. The PEt-mediated increase in the binding of CRP to E-LDL was selective for E-LDL, because PEt inhibited the binding of CRP to PnC, suggesting that PEt-complexed CRP may be unable to bind to other PCh-containing noncholesterol substances. 4. The ability of E-LDL to transform macrophages into foam cells was abolished when the cells were treated with CRP-E-LDL complexes, indicating that CRP-bound E-LDL was inactive in causing the formation of foam cells. This function of CRP required that E-LDL must be bound to CRP. The mere presence of CRP with E-LDL was not sufficient to prevent foam cell formation. 5. The presence of PEt in the CRP-E-LDL complex did not impair the ability of CRP to prevent foam cell formation.

The findings that PCh inhibited CRP-E-LDL interaction, but that the mutation of the PCh-contacting amino acids in CRP did not abolish CRP-E-LDL interaction, suggested that the manipulation of the PCh-binding site of CRP using small-molecule compounds might selectively abolish CRP’s PCh-binding activity without affecting its E-LDL-binding activity. In this study, we show one such compound, PEt, which inhibits CRP-PnC interaction but enhances CRP-E-LDL interaction. The enhancement of the binding of CRP to E-LDL by PEt was not obvious. However, based on the data obtained from the solid-phase E-LDL-binding assays and CRP-binding sucrose flotation experiments, we provide a possible mechanism of action of PEt on CRP in enhancing the E-LDL-binding function of CRP. CRP, when alone, may be binding to only a certain population of E-LDL molecules in the E-LDL preparations. PEt, probably by inducing a structural alteration or aggregation of CRP, may confer CRP the ability to bind to those E-LDL molecules also to which CRP cannot bind alone.

A PCh-based compound was recently reported to inhibit the deposition of CRP at the myocardial infarcts by blocking the PCh-binding site of CRP (9). However, such a compound may lessen the beneficial effects of CRP on foam cell formation, because PCh inhibits CRP-E-LDL interaction. In contrast, a PEt-based compound would inhibit the deposition of CRP at the myocardial infarcts but would not lessen the beneficial effects of CRP. It would also allow CRP to capture and inactivate E-LDL.

The moieties on the LDL molecule that have been reported to interact with CRP include ApoB, cholesterol, and PCh (19–28). Our data obtained from the CRP mutants indicate that PCh groups in E-LDL are not necessary for binding to CRP and, therefore, modification of native LDL to expose the PCh groups is not required for binding to CRP. Our results support the interpretation that PEt-complexed CRP binds E-LDL through cholesterol (15, 21). This interpretation is also supported by our finding that PEt inhibits binding of CRP to PnC, which does not contain cholesterol.

CRP has been shown to exert proinflammatory and proatherogenic activities in many experiments using cultured vascular cells (39, 40). The results obtained from cell culture experiments using purified CRP remain a subject of concern because of the presence of sodium azide and endotoxins in CRP and also because of the presence of modified forms of CRP, such as aggregated CRP, in preparations of native pentameric CRP (41–44). Indeed, it has been confirmed that some, if not all, of the in vitro proinflammatory functions of CRP were not due to azide and endotoxins (45–47). It will be interesting to investigate the activities of PEt-complexed CRP in experiments using cultured mammalian cells.

Because CRP binds to modified LDL, the possibility that CRP may play a role in the uptake of modified LDL by macrophages has been investigated previously (29–32). These studies, using Abs to CRP and ApoB, or labeled LDL, revealed that CRP did not
prevent uptake of modified LDL by macrophages. When measured indirectly by CD32 internalization, CRP was found to increase LDL uptake; however, the involvement of CD32 was questioned (29, 32). These studies did not reveal the function of CRP in preventing formation of foam cells, probably because Nile red staining was not used to visualize cholesteryl esters. In addition, between our and previously reported experiments, there are differences in the preparation and quality of CRP-LDL complexes used for the treatment of macrophages. We filtered the mixture of CRP, Ca\textsuperscript{2+} and E-LDL, which was slightly turbid, through a gel filtration column to produce a clear and homogeneous solution of the CRP-E-LDL complex appropriate for the treatment of macrophages. While doing so, we routinely noticed deposition of unidentified materials on top of the gel in the column, indicating that the mixture of CRP and E-LDL in the presence of Ca\textsuperscript{2+} contained some insoluble aggregates of E-LDL. In previous studies, however, the macrophages were treated with a mixture of CRP and modified LDL without filtration. For reproducibility of our results, it is necessary that E-LDL be filtered either before or after mixing with CRP by gel filtration and that the CRP-E-LDL complex be devoid of any free, insoluble E-LDL. Indeed, when we treated the macrophages with the mixture of CRP and E-LDL in the presence of Ca\textsuperscript{2+} without removing uncomplexed E-LDL, the foam cells were formed (data not shown).

Although we have not determined the effects of CRP on the formation of macrophage foam cells by other forms of modified LDL, such as oxidized LDL, the current findings raise the possibility that if CRP is present in a sufficient amount in the arterial wall and if each LDL molecule retained in the arterial wall becomes CRP-bound, CRP may be capable of preventing foam cell formation in vivo. The mechanism of action of CRP in the prevention of foam cell formation, however, is not clear. There are two possible mechanisms. Either the CRP-bound E-LDL was not taken up by macrophages or the CRP-bound E-LDL complexes were taken up but not processed to form cholesteryl esters.

In addition to the beneficial function of CRP on E-LDL-mediated formation of foam cells that we report here, it has been shown earlier that complement activation by E-LDL halts before detrimental terminal sequence.

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