Lectin-like Oxidized LDL Receptor 1 Is Involved in CRP-Mediated Complement Activation

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BACKGROUND: C-reactive protein (CRP) is purported to be a risk factor that acts independently of LDL cholesterol in predicting all-cause mortality in patients with ischemic heart disease. Lectin-like oxidized LDL receptor 1 (LOX-1) impairs endothelial function and exacerbates myocardial injury. We recently demonstrated that CRP increased vascular permeability through direct binding to LOX-1. Here we examined, using a hypertensive rat model, whether LOX-1 is involved in CRP-induced complement activation.

METHODS AND RESULTS: In the cultured LOX-1–expressing cell line hLOX-1-CHO, CRP increased complement activation, but did not do so in native CHO cells. Depleting C1q from serum abolished CRP-induced complement activation. Incubation of CRP with serum on immobilized recombinant LOX-1 similarly showed that CRP activated C1q-requiring classical complement pathway in a LOX-1–dependent manner. Interestingly, the interaction between CRP and LOX-1 was dependent on Ca2+/H+ ion and competed with phosphocholine, suggesting that LOX-1 bound to the B-face of CRP with a phosphocholine-binding domain. This was in contrast to Fcγ receptors, to which CRP bound in A-face with complement-binding domain. In vivo, intradermal injection of CRP to hypertensive SHRSP rats induced complement activation detected by C3d deposition and leukocyte infiltration around the injected area. Anti–LOX-1 antibody reduced the extent of complement activation and leukocyte infiltration.

CONCLUSIONS: LOX-1 appears to be involved in CRP-induced complement activation, and thus may serve to locate the site of CRP-induced complement activation and inflammation.

C-reactive protein (CRP) is an acute-phase reactant protein predominantly produced in liver. The biological roles of CRP in the immune system have been well established. CRP binds to a variety of ligands such as phosphocholine residues, modified LDL, and damaged cells; activates complement; and opsonizes biological particles (1, 2). The plasma concentration of CRP increases in response to tissue damage, infection, and trauma; hence it is used as a clinical marker of systemic inflammation (3).

Inflammation has been proposed to play a critical role in the multiple stages of coronary heart disease, including atherogenesis, thrombogenesis, and myocardial damage after ischemia (4, 5). Since the demonstration of an association of CRP concentrations with subsequent cardiovascular outcomes, CRP has also received extensive attention as a clinical marker of cardiovascular disease (6). Lines of laboratory evidence have shown that CRP concentrations correlate with degree of atherosclerotic lesion size and that CRP deposits in atherosclerotic lesions along with complement proteins (7, 8). The Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) has demonstrated that statins reduced the incidence of cardiovascular events in people without hyperlipidemia but with high CRP concentrations, highlighting the possibility of CRP as a therapeutic as well as prognostic marker (9).

Despite accumulating evidence of the association of CRP with cardiovascular diseases, the causative role of CRP has been controversial (10, 11). For instance, results of atherosclerosis experiments in apolipoprotein E–knockout mice overexpressing human CRP were conflicting; whereas 1 study found a positive relationship between CRP concentrations and atherosclerotic lesion size (12), 2 other reports did not (13, 14). In contrast to the contradicting results on the role of CRP in atherosclerosis, the role of CRP in myocardial ischemic damage seems to be consistent. The plasma CRP concentration increases following myocardial ischemia, and pharmacological evidence...
supports that CRP activates complement system and aggravates myocardial injury after ischemia-reperfusion in several species, including humans (15–18).

Recently, we showed that CRP bound to CHO cells (hLOX-1-CHO) expressing human lectin-like oxidized LDL receptor 1 (LOX-1), and that CRP bound to recombinant human LOX-1 in a cell-free system (19, 20). We also showed that CRP bound to LOX-1 and increased vascular permeability in hypertensive rats (19). These data indicate that LOX-1, along with Fcγ receptors, works as a receptor for CRP. LOX-1 was originally identified as a receptor for atherogenic oxidized LDL in vascular endothelial cells (21). LOX-1 contributes to multiple stages of cardiovascular disease such as endothelial dysfunction, atherogenesis, myocardial ischemia-reperfusion injury, and restenosis after balloon injury (22–28). In this study, we examined whether LOX-1 promotes CRP-induced complement activation by interacting with CRP to develop inflammatory pathogenic response.

Materials and Methods

CRP
We purchased human CRP purified from pleural fluid (AG723) from Millipore. Sodium azide in the solution was extensively removed by dialyzing 3 times against a 3000-fold volume of PBS (8 mmol/L Na₂HPO₄ · 2 H₂O, 1.5 mmol/L KH₂PO₄, 137 mmol/L NaCl, 3 mmol/L KCl). Gram-negative bacterial endotoxin was undetectable by limulus amoeocyte lysate (Cape Cod), which can detect concentrations as low as 30 U/L.

COMPLEMENT ACTIVATION ASSAY ON CULTURED CELLS
The CHO cell line hLOX-1-CHO, which expresses human LOX-1 in a tetracycline-inducible manner, was maintained with Ham’s F12, 10% fetal bovine serum, and 1% antibiotics-antimycotics (Invitrogen) as described (19). We induced expression of human LOX-1 in hLOX-1-CHO seeded in 96-well plates (#655090, Greiner) by incubating overnight at 4 °C for 8 h. After washing the cells with PBS, we added 10% human complement serum (Sigma) or C1q-depleted serum (Sigma) at 37 °C for 1 h with or without CRP, boiled CRP (95 °C, 5 min), or CRP plus polymyxin B (5 mg/L, MP Biomedicals) in 10 mmol/L HEPES, 150 mmol/L NaCl, and 2 mmol/L CaCl₂ (pH 7.0). We detected C3d deposition with rabbit anti-human C3d antibody (3.5 mg/L, Dako) in combination with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:2000, GE Healthcare). We determined peroxidase activity with the 3,3′,5,5′-tetramethylbenzidine (TMB) Peroxidase EIA Substrate Kit (Bio-Rad).

CRP-LOX-1 INTERACTION ASSAY BY ELISA
Recombinant human LOX-1 (0.2 μg) or heat-inactivated BSA (0.2 μg, Sigma) was immobilized to each well of a 96-well plate (Maxisorp, Thermo Fisher Scientific) by incubating in PBS overnight at 4 °C. After 2 washes with PBS, the plates were blocked with 300 μL of 20% ImmunoBlock (DS Pharma)/PBS at 4 °C for 8 h. After an additional 2 washes with PBS, the plates were incubated with 1% human complement serum (Sigma) or 3% C1q-depleted serum (Sigma) at 37 °C for 1 h with or without CRP, boiled CRP (95 °C, 5 min), or CRP plus polymyxin B (5 mg/L, MP Biomedicals) in 10 mmol/L HEPES, 150 mmol/L NaCl, and 2 mmol/L CaCl₂ (pH 7.0). We detected C3d deposition with rabbit anti-human C3d antibody (3.5 mg/L, Dako) in combination with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:2000, GE Healthcare). We determined peroxidase activity with the 3,3′,5,5′-tetramethylbenzidine (TMB) Peroxidase EIA Substrate Kit (Bio-Rad).
(1:20 000, Pierce) in 1% BSA/PBS. After 5 washes with PBS, we determined the peroxidase activity with the TMB Peroxidase EIA Substrate kit.

**ANIMALS**

This study was approved by the institutional ethics review board of National Cerebral and Cardiovascular Center (#10034). We used male Wistar Kyoto rats (WKY/Izm, Disease Model Cooperative Research Association) and stroke-prone spontaneously hypertensive rats (SHRSP/Izm, Disease Model Cooperative Research Association), age 8 weeks, for the experiments. The rat experiments were conducted in accordance with our previous report (19). CRP was intradermally injected with anti–LOX-1 antibody (TS20, 3 μg) (29) or nonimmune mouse IgG (3 μg, Sigma) as described (19). Four hours after the injection, the rats were killed by anesthetics, and their skin was isolated and snap frozen for immunohistochemical analyses.

**IMMUNOHISTOCHEMISTRY**

For C3d staining, 7-μm-thick fresh frozen sections were fixed with acetone for 15 min, washed with PBS 3 times, and treated with 3% H₂O₂/PBS for 1 h at room temperature. For myeloperoxidase (MPO) staining, 3-μm-thick paraffin sections were deparaffinized and treated with 3% H₂O₂/PBS for 1 h at room temperature. After washing with PBS 3 times, the sections were incubated with 20% (vol/vol) BlockAce (DS Pharma)/PBS supplemented with 5% donkey serum (Millipore) or 3% goat serum (Vector Laboratories), followed by incubation with a primary antibody at room temperature for 1 h. The primary antibodies used were 1 mg/L rabbit anti–human MPO antibody (Thermo Fisher Scientific) and 1 mg/L rabbit anti–human C3d antibody. As negative controls, we used rabbit IgG (Dako). The sections were then washed with PBS and incubated with a biotinylated anti–mouse/rabbit IgG antibody (Dako) for 1 h at room temperature. We visualized antibody binding with streptavidin/HRP (Dako) and Envision kit/HRP [3,3′-diaminobenzidine (DAB)] (Dako). The sections were then counterstained with Mayer’s hematoxylin (Wako Pure Chemical Industries), and subjected to microscopic observation.

**STATISTICS**

All data represent mean (SE). Statistical analyses were performed by 1-way ANOVA followed by Dunnett test with a software Prism version 5.0 (GraphPad). A P value <0.05 was considered to be statistically significant.

**Results**

**CRP-INDUCED COMPLEMENT ACTIVATION IN LOX-1-EXPRESSING CHO CELLS**

We first investigated CRP-induced complement activation in LOX-1–expressing CHO cells. To ensure the specificity of the activation, we used hLOX-1-CHO, which expressed human LOX-1 driven by tetracycline-inducible promoter (19). We assessed complement activation by detecting C3d, which is a degradation product of C3b generated by complement activation. In cultured cells, C3d deposition was increased in hLOX-1-CHO, which expressed human LOX-1 driven by tetracycline-inducible promoter (19). We assessed complement activation by detecting C3d, which is a degradation product of C3b generated by complement activation. In cultured cells, C3d deposition was increased in hLOX-1-CHO compared with control CHO cells. CRP (10 mg/L) enhanced the complement activation in hLOX-1-CHO. (A), Complement activation detected by C3d deposition on the cells. More intense activation of complement system was observed in hLOX-1-CHO compared with control CHO cells. CRP (10 mg/L) enhanced the complement activation in hLOX-1-CHO. (B), Complement activation in C1q-depleted serum on hLOX-1-CHO. Human serum used in (A) was replaced with C1q-depleted human serum. Depletion of C1q completely suppressed the complement activation reaction depending on LOX-1 and CRP. N.S., not significant. Significant differences between the indicated groups, **P < 0.01, ***P < 0.001.
LOX-1 still increased C3d deposition on hLOX-1-CHO cells in the absence of CRP, which was probably due to the interaction of LOX-1 with unknown cell-derived materials, possibly dead cells. Background C3d deposition on control CHO was also diminished by C1q depletion.

**ACTIVATION OF CLASSICAL COMPLEMENT PATHWAY BY CRP VIA LOX-1 IN CELL-FREE SYSTEM**

We further investigated CRP-induced complement activation in a cell-free system by use of recombinant human LOX-1 immobilized on an ELISA plate. Complement activation was detected only when CRP was applied to the recombinant LOX-1-coated wells; it was undetectable in BSA-coated wells or in the absence of CRP (Fig. 2A). Replacement of CRP with boiled CRP abolished complement activation. Supplementation of polymyxin B, which neutralizes lipopolysaccharide to the reaction, did not affect complement activation. These data suggest that the complement activation observed therein should be an effect of protein in CRP solution, but not caused by lipopolysaccharide. Without C1q, complement activation was not observed even in the presence of CRP (Fig. 2B). The complement activation was clearly observed in the LOX-1–coated well only when C1q was supplemented. As expected, CRP-independent C3d deposition in the presence of LOX-1 was not observed, indicating that C3d deposition on hLOX-1-CHO cells in the absence of CRP should be due to the reaction against cell-derived materials. These results suggest that LOX-1–CRP interaction activated the C1q-dependent classical pathway of complement system.

**INHIBITION OF CRP BINDING TO LOX-1 BY PHOSPHOCHOLINE**

It is known that CRP binds phosphocholine in the presence of Ca^{2+} to induce complement activation (1). The complement activation domain is located in the A-face of CRP, whereas the phosphocholine binding domain is on the other side in B-face. Therefore, knowledge of how the binding of CRP occurs in relation to Ca^{2+} and phosphocholine would give us an idea about the mode of CRP action. CRP bound to LOX-1 depending on the concentration of CaCl_{2} (0–2 mmol/L) (Fig. 3A). The binding was completely abolished by EDTA. We also observed competitive binding between CRP and phosphocholine to LOX-1. In the presence of CaCl_{2}, CRP–LOX-1 binding was inhibited by phosphocholine in a dose-dependent manner (0–0.1 mmol/L) (Fig. 3B). These results suggest that LOX-1 might interact with the B-face of CRP, which bound phosphocholine in a Ca^{2+}-dependent manner.

![Fig. 2. CRP-induced complement activation in a cell-free system.](image-url)
We previously reported that intradermal CRP injection induced vascular hyperpermeability around injection sites in SHRSP hypertensive rats. Because this phenomenon occurred in a LOX-1–dependent manner, we used the same specimen to analyze CRP-induced complement activation in vivo. Immuno-histochemistry of dermal tissues of SHRSP rats injected with CRP showed significantly stronger signals of C3d, especially around blood vessels, compared with BSA-injected rats (Fig. 4). From the morphology of the blood vessels, veins rather than arteries showed stronger signals of C3d. In tissues where anti–LOX-1 antibody was simultaneously injected with CRP, C3d generation was less pronounced compared with IgG plus CRP–injected rats.

**SUPPRESSION OF NEUTROPHIL-INFILTRATION BY ANTI–LOX-1 ANTIBODY**

We further observed CRP-induced leukocyte infiltration. Immunohistochemistry with anti-MPO antibody similarly showed that CRP induced leukocyte infiltration, and simultaneous injection of anti–LOX-1 antibody suppressed leukocyte infiltration (Fig. 5).

**Discussion**

This study demonstrated that LOX-1 enhanced CRP-induced activation of classical complement pathway in vitro by reconstitution experiments. This study also demonstrated the involvement of LOX-1 in CRP-induced complement activation and infiltration of polymorphonuclear cells in vivo, as evidenced by significant suppression by anti–LOX-1 antibody.

**CRP-INDUCED COMPLEMENT ACTIVATION IN DISEASE MODELS**

CRP is alleged to be a cardiovascular risk marker; however, the causal role of CRP in cardiovascular disease has been controversial. Several rat studies using human CRP did suggest that CRP promotes cardiovascular injury. For instance, exogenously administered human CRP increased infarct size in coronary-ligated rats via a complement-dependent mechanism, and a chemical inhibitor of CRP efficiently suppressed human CRP effects on rat myocardial infarction (16, 17).

However, the mechanism by which CRP induces complement activation in vivo has not been fully understood. We have shown interaction of CRP with LOX-1 in our 2 previous studies (19, 20) and the present study. We have also shown that human CRP increased vascular permeability in rats (19). Therefore, in this study we examined the possible involvement of LOX-1 in CRP-induced complement activation. We used a hypertension rat model, SHRSP, in which LOX-1 is highly expressed (19, 30). As expected, CRP induced complement activation in vitro and in vivo, and the activation largely depended on the function of LOX-1.

We also observed LOX-1–dependent leukocyte infiltration into tissues. This might be due to the function of LOX-1 as an adhesion molecule (31) and also to its ability to serve as a CRP-tethering site that generates chemotactic factors, including C3a and C5a, through complement activation. Taking into account that it was a CRP-triggered response, the latter case is more plausible.
In vitro analyses showed that immobilized LOX-1 could be the site for complement activation, recruiting CRP and C1q. LOX-1 might be tethering CRP on the surface of cells and providing the sites for assembling components of complement system. Because phosphocholine effectively competed with CRP for the binding to LOX-1 in this study, it is presumable that LOX-1 bound to the B-face of CRP, which chelated phosphocholine. The other face of CRP, the A-face, is known to activate the C1q-dependent classical complement pathway by binding C1q (Fig. 6) (32, 33).

In contrast to LOX-1, Fcγ receptors, well-known receptors of CRP, bind to the A-face of CRP. The crystal structure of a Fcγ receptor–CRP complex has been solved to demonstrate their binding manner (34). The B-face of CRP recognizes phosphocholine epitope of bacteria to opsonize them. The A-face of CRP then binds to Fcγ receptors for ingesting the CRP-opsonized bacteria (35). With accumulated evidence (11, 36), the roles of Fcγ receptors in CRP-induced signal transduction in endothelial cells are thought to be established.

However, the CRP-induced complement activation observed in disease models could not be directly explained by the function of Fcγ receptors. Fcγ re-
Receptors would conceal the complement activation domain of CRP by binding to its A-face. Although CRP activates the complement system, if circulating CRP should freely activate complement system wherever it travels, systemic inflammation would occur throughout the body, which would be disastrous. It is natural to presume, therefore, that some control mechanism works so that the complement activation should occur only within limited locations. It is known that CRP also has a function to suppress the later phase of the complement system by activating the complement inhibitor, factor H (37, 38), while, in a rabbit myocardial reperfusion injury model, CRP promotes membrane attack complex formation in cardiac tissue (39). LOX-1 might be of importance in such localized and limited activation of complement system induced by CRP, by binding to the other face of CRP, the B-face.

From the data of competitive inhibition by phosphocholine against CRP binding to LOX-1, there are 2 possibilities for the manner of CRP binding. One is that phosphocholine bound to the B-face of CRP mediates the binding to LOX-1 with its negative charge, since ligand binding surface of LOX-1 is known to be composed of positively charged amino acids that bind negatively charged molecules. Therefore, CRP-bound phosphocholine might compete with the excess amount of free phosphocholine. The second possibility is that phosphocholine might work as a molecular switch to regulate receptor selectivity of CRP by blocking CRP-LOX-1 binding. In this case, if the B-face is not occupied by phosphocholine, it is available for LOX-1 to activate complement system. If the B-face is occupied by phosphocholine or phosphocholine-like ligand, then the A-face is used for the binding to Fcγ receptors. Perhaps membrane-bound (i.e., LOX-1–bound) CRP can direct complement activation, but phosphocholine-bound CRP is needed for engagement of Fcγ receptors. Interestingly, CRP induces LOX-1 expression via Fcγ receptors in endothelial cells to increase endothelial uptake of oxidized LDL and adhesiveness of monocytes (40), indicating that there is cross-talk between Fcγ receptors and LOX-1. Therefore, it is likely that some CRP-induced phenomena mediated by Fcγ receptors might be caused by LOX-1 located downstream of Fcγ receptors’ signal transduction.

LIMITATION OF THE STUDY
In the present study, we did not use primary cultured vascular cells, but rather a LOX-1–expressing CHO cell line as a representative of cells stimulated by LOX-1–inducible signals, to demonstrate the potential role of LOX-1 in CRP-mediated complement activation. In addition, we did not analyze aortic tissue after systemic administration of CRP. We instead analyzed local response following intradermal injection of CRP, partly because atherosclerosis in the aorta does not progress even after a year of loading high-fat diet in the stroke-prone spontaneously hypertensive rats used in this study. These differences in study design limit the interpretation of the results of our study regarding the mechanisms of atherothrombosis, but still allow us to suggest the potential role of LOX-1 in CRP-mediated inflammatory response.

In summary, we found an important role of CRP-LOX-1 interaction in complement activation. The CRP-LOX-1 complex might be of physiologic significance in humans, where CRP activates the complement system. This warrants further study to obtain experimental evidence in higher animals, including humans.

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