Oxidized LDL Receptor LOX-1 Binds to C-reactive Protein and Mediates its Vascular Effects

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BACKGROUND: C-reactive protein (CRP) exerts biological activity on vascular endothelial cells. This activity may promote atherothrombosis, but the effects of this activity are still controversial. Lectin-like oxidized LDL receptor-1 (LOX-1), the oxidized LDL receptor on endothelial cells, is involved in endothelial dysfunction induced by oxidized LDL.

METHODS: We used laser confocal microscopy to examine and fluorescence cell image analysis to quantify the binding of fluorescently labeled CRP to cells expressing LOX-1. We then examined the binding of unlabeled CRP to recombinant human LOX-1 in a cell-free system. Small interfering RNAs (siRNAs) against LOX-1 were applied to cultured bovine endothelial cells to analyze the role of LOX-1 in native cells. To observe its in vivo effects, we injected CRP intradermally in stroke-prone spontaneously hypertensive (SHR-SP) rats and analyzed vascular permeability.

RESULTS: CRP bound to LOX-1–expressing cells in parallel with the induction of LOX-1 expression. CRP dose-dependently bound to the cell line and recombinant LOX-1, with significant binding detected at 0.3 mg/L CRP concentration. The Kd value of the binding was calculated to be 1.6 × 10⁻⁷ mol/L. siRNA against LOX-1 significantly inhibited the binding of fluorescently labeled CRP to the endothelial cells, whereas control RNA did not. In vivo, intradermal injection of CRP-induced vascular exudation of Evans blue dye in SHR-SP rats, in which expression of LOX-1 was greatly enhanced. Anti–LOX-1 antibody significantly suppressed vascular permeability.

CONCLUSIONS: CRP and oxidized LDL–receptor LOX-1 directly interact with each other. Two risk factors for ischemic heart diseases, CRP and oxidized LDL, share a common molecule, LOX-1, as their receptor.

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C-reactive protein (CRP)⁷ is an acute-phase plasma protein that is synthesized by hepatocytes in response to inflammation and tissue damage; the latter can cause a 1000-fold or more increase in the human plasma concentrations of high-sensitivity CRP (hsCRP). For this reason hsCRP has long been used as an inflammatory biomarker (1). CRP recognizes phosphocholine (2) and other various ligands, including phosphoethanolamine, chromatin, histones, fibronectin, and oxidized LDL (3, 4). Recent epidemiological studies have shown that even the slightest increase in serum concentration of hsCRP can be a major risk indicator for ischemic heart disease (5–7). Activation of the classical complement pathway through direct interaction with C1q is an established function of CRP. It is reported that administration of human CRP in a rat model of myocardial infarction activates complement systems, leading to increases in the size of myocardial infarction (8), and that the chemical blockade of CRP prevents deleterious effects and suppresses the myocardial infarction (9). In addition, a number of recent reports have shown that CRP induces endothelial activation/dysfunction leading to atherothrombosis (10). Because the earliest CRP reports may have reflected the effects of contaminants such as bacterial lipopolysaccharide and azide rather than CRP, there have been subsequent heated debates concerning CRP functions and actions.

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CRP outside of complement activation postulated to be the receptors that mediate the effects of CRP outside of complement activation. Fc receptors are postulated to be the receptors that mediate the effects of CRP outside of complement activation (12–14).

Lectin-like oxidized LDL receptor 1 (LOX-1) was originally found and identified as an endothelial receptor for oxidized LDL (15). Activation of LOX-1 in endothelial cells induces the generation of superoxide, a reduction in the release of nitric oxide, and the expression of proatherogenic molecules such as endothelin-1, monocyte chemoattractant protein 1, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1 (16–18). The overexpression of LOX-1 in mice enhances oxidative stress and the expression of adhesion molecules in blood vessels, accelerating atheroma-like lipid deposition in intramyocardial vessels (19). Deletion of LOX-1 in mice preserves endothelial function, leading to reduction in atherogenesis (20). In addition to oxidized LDL, LOX-1 binds various ligands, e.g., apoptotic cells, activated platelets, leukocytes, and bacteria (21–25). Related functions of LOX-1 include involvement in inflammation, myocardial infarction, and intimal thickening after balloon catheter injury (23, 26–29).

Because the changes induced by CRP and by LOX-1 activation overlap, we investigated the physical interactions between CRP and LOX-1 that may be related to cardiovascular pathophysiology.

Materials and Methods

CRP
Human CRP purified from pleural fluid was purchased from Chemicon (AG723). Sodium azide in the solution was removed by dialysis performed 3 times against a 3000-fold volume of Dalbecco’s PBS (Wako). Gram-negative bacterial endotoxins were undetectable by limulus amebocyte lysate (Associates of Cape Cod), which can detect as low as 30 endotoxin units/L of endotoxins. CRP preparations from 2 other distributors, one purified from human plasma (C4063, Sigma) and the other from human serum (#236603, Calbiochem), were also subjected to cell-free analyses.

FLUORESCENTLY LABELED CRP
CRP was fluorescently labeled with an Alexa Fluor 546 protein-labeling kit (Invitrogen) and dialyzed 3 times against a 3000-fold volume of PBS.

CELL LINE EXPRESSING HUMAN LOX-1 (HLOX-1-CHO) DRIVEN BY TETRACYCLINE-INDUCIBLE PROMOTER
CDNA encoding the human LOX-1 (Genbank NM002543) was subcloned into pTRE2hyg (Clonetech). CHO-K1 Tet-On cells (Clonetech) were transfected with pTRE2hyg-human LOX-1 by Lipofectamin-2000 transfection reagent (Invitrogen) according to the manufacture’s instructions. The stable transformants were selected with 400 mg/L of hygromycin B (Wako). The resistant clones that express LOX-1 in response to doxycycline (Calbiochem) were selected for use in these experiments. The LOX-1 expression was induced with doxycycline at the indicated concentration in Ham’s F-12 medium (Gibco)/10% fetal bovine serum 24 h before the experiments. Cells were washed twice with Ham’s F-12/10 mmol/L HEPES and chilled on ice for 30 min. Then, the medium was replaced with the indicated concentration of Alexa 546-CRP–containing ice-cold Ham’s F-12/10 mmol/L HEPES, and cells were incubated on ice for 1 h. After being washed with ice-cold PBS, the cells were fixed with phosphate-buffered formalin (Wako). The expression of LOX-1 was visualized by immunostaining with anti-human LOX-1 antibody (TS92) (30) combined with Alexa 488-antihuman IgG (1:2000) (Invitrogen). Then, the specimens were subjected to microscopic analysis with confocal laser microscope (TCS SP5, Leica), and quantitative fluorescence cell image analysis with the IN Cell analyzer 1000 system (GE Healthcare).

TRANSIENT GENE EXPRESSION ASSAY
COS7 cells maintained with DMEM/10% fetal bovine serum were seeded 1 day before transfection. The cells at 80%–90% confluency were transfected with indicated plasmid by use of Lipofectamin 2000 transfection reagent (Invitrogen). After 24 h, the cells were chilled on ice for 30 min and washed with ice-cold PBS. Then, the medium was replaced with the indicated concentration of Alexa 546-CRP–containing ice-cold DMEM/10 mmol/L HEPES, and cells were incubated on ice for 1 h. After being washed with ice-cold PBS, the cells were fixed with phosphate-buffered formalin (Wako). The expression of each receptor was assessed by immunostaining with anti-V5 antibody (1:1000) (Nacalai Tesque) combined with Alexa 488-antimouse IgG (1:2000) (Invitrogen). Then the specimens were subjected to microscopic analysis with confocal laser microscope (TCS SP5, Leica), and quantitative fluorescence cell image analysis with the IN Cell analyzer 1000 system (GE Healthcare).

RECOMBINANT LOX-1
cDNA encoding extracellular domain of human LOX-1 (61–273) was subcloned into pcDNA4 with a chicken IgG light chain leader peptide in the N-terminal and V5-6xHis tag in the C-terminus. The plasmid was transfected into FreeStyle 293-F cells (Invitrogen). After 4 days, the recombinant protein was purified from culture supernatant with Ni-NTA superfllow (Qiagen) according to the manufacturer’s instructions.
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CRP-LOX-1 INTERACTION ASSAY BY ELISA
Recombinant human LOX-1 (0.1 μg) or BSA (0.1 μg, Sigma) was immobilized to each well of 384-well plates (Maxisorp, Nunc) by incubation overnight at 4 °C in PBS. After 2 washes with PBS, the plates were blocked with 80 μL of 20% ImmuNoBlok (DS Pharma)/PBS at 4 °C for 8 h. After 2 washes with PBS, CRP in the reaction buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L CaCl2, 1% BSA, pH 7.0) was added to each well, and incubated at 4 °C overnight. The plates were then washed 3 times with PBS and incubated for 2 h with horseradish peroxidase– conjugated antihuman CRP antibody (1:5000) (Bethyl) in PBS, 1% BSA. After 5 washes with PBS, peroxidase activity was determined with a TMB Peroxidase EIA Substrate kit (Bio-Rad). For the analyses of the binding of heat-denatured CRP, CRP solution in PBS was heated in boiling water for 5 min before use. The immunoreactivity of the denatured CRP to the anti-CRP antibody was examined by ELISA of immobilized CRP. The indicated amounts of CRP, denatured CRP, or BSA were immobilized, and blocking was performed as above. Then, the immobilized proteins were detected by the horseradish peroxidase– conjugated antihuman CRP antibody as above.

BIACORE ANALYSES
The Kd value of the CRP binding to LOX-1 was measured by surface plasmon resonance on a BIACORE 2000 (GE Healthcare). Recombinant LOX-1 was immobilized on a research-grade CM5 sensor chip (GE Healthcare) by use of an Amine coupling kit (GE Healthcare) according to the manufacturer’s instructions. BSA was immobilized on a sensor chip by the same method described above, and used as a reference. We then injected 30 μL of the analytes (CRP 0.45, 0.89, 1.78, and 3.56 μmol/L) in 10 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L CaCl2, 1% BSA (pH 7.0) at a flow rate of 20 μL/min for 120 s, and dissociation in the same buffer was monitored for 120 s. After each analysis, the sensor chip was regenerated with 5 μL of 50 mmol/L NaOH at a flow rate of 60 μL/min. We calculated Kd values by using a 1:1 Langmuir binding model with BLAevaluation software version 3.0 (GE Healthcare).

RNA INTERFERENCE
We designed small interfering RNA (siRNA) duplex oligoribonucleotides targeting the bovine LOX-1 coding region (Genbank NM174132) by using the Block-iT RNAi (RNA interference) designer program from the Invitrogen website. The stealth RNAi negative control duplex (Invitrogen) was used as a negative control. The siRNA sequences are as follows: si1 for LOX-1 (204–228), 5’-UUCUUUAUGAGAUCA GAGACCUGGG-3’ and si2 for LOX-1 (396–420), 5’-ACUUCUUGGAGAUUCAGGUUCUGGC-3’.

Bovine aortic endothelial cells (BAEC) and bLOX-1-CHO (Chinese hamster ovary cells stably expressing bovine LOX-1) (15) were maintained with DMEM (Gibco)/10% fetal bovine serum/1% (vol/vol) Antibiotic-Antimycotic (Gibco) and Ham’s F-12 medium/10% fetal bovine serum containing 10 mg/L blasticidin (Kaken Pharmaceutical), respectively. The cells were seeded 1 day before transfection. The following day, the cells at 50%–60% confluency were transfected with siRNA oligos or the control siRNA by use of Lipofectamin RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s instructions. After incubation at 37 °C for 24 h, the effects of downregulation of expression of the LOX-1 gene [oxidized low density lipoprotein (lectin-like) receptor 1] were examined. Suppression of LOX-1 expression was confirmed by the immunostaining with anti–LOX-1 antibody (TS20) combined with Alexa488–antimouse IgG (1:2000) (Invitrogen) and quantitative analysis with the IN Cell analyzer. All transfections were performed in triplicate.

ANIMALS
All protocols were approved by the Institutional Animal Care and Use Committee of the National Cardiovascular Center. Male Wistar Kyoto (WKY) rats and stroke-prone spontaneously hypertensive (SHR-SP) rats (SLC) were used at 8 weeks of age for experiments.

IMMUNOHISTOCHEMICAL ANALYSIS
For the analysis by confocal laser microscope, rat dermal tissue was snap-frozen in liquid nitrogen and sectioned at 10 μm with a cryostat (Leica). The sections were fixed by 4% formaldehyde for 15 min, blocked with 20% Blockace (Snow brand) for 1 h at room temperature, and then stained with 2 mg/L Alexa633 anti–LOX-1 antibody (TS20) or 0.2 mg/L R-phycoerythrin– conjugated anti-Rat CD31 antibody (TLD-3A12, BD). Labeling of the anti–LOX-1 antibody was performed with an Alexa Fluor 633 protein-labeling kit (Invitrogen) according to the manufacturer’s instructions.

FLUORESCENT MILES PERMEABILITY ASSAY
The rats were anesthetized with Nembutal (50 mg/kg body weight, administered intraperitoneally), and warmed on the thermal control plate set at 37 °C (HI1220, Leica). Hairs in the dorsal skin region were shaved for fluorescence analyses. CRP, BSA, or vascular endothelial growth factor (Sigma) dissolved in 10 μL of PBS were intradermally injected to the shaved lumbar area of dorsal skin. In some experiments, anti–LOX-1 antibody (TS20, 3 μg) or nonimmune mouse IgG (3 μg, Sigma) was coinjected with CRP. Thirty
minutes later, 2% Evans blue (Wako) in saline (Otsuka Pharma) was injected via the tail vein at a dose of 20 mg/kg weight of the rats. Vascular permeability was assessed by the exudation of Evans blue into the animal dorsal skin, detected with the Maestro Imaging System (CRi). A bandpass filter from 575–605 nm was used for excitation, and the fluorescence intensity at 680 nm was measured for quantitative analysis. The fluorescein-labeled CRP binds to LOX-1.

Fig. 1. Fluorescently labeled CRP binds to LOX-1.

(A) Analysis of hLOX-1-CHO (Chinese hamster ovary cell expressing human LOX-1 driven by tetracycline-inducible promoter) (upper panels) and control CHO cells (lower panels) stained with anti-LOX-1 antibody (green) or incubated with Alexa546-CRP (10 mg/L, red) by laser confocal microscopy. The nuclei of the cells were counterstained with DAPI (blue). The merged image of Alexa546-CRP and LOX-1 indicates colocalization of these molecules on the cell surface. (B, C) Alexa546-CRP (10 mg/L) binding (B) to hLOX-1-CHO in response to the induction of the expression of LOX-1 (C) by increasing dose of doxycycline (0–300 μg/L). (D) Dose-dependent binding of Alexa546-CRP (0–30 mg/L) to hLOX-1-CHO that were pretreated with 100 μg/L of doxycycline. hLOX-1-CHO or control CHO cells were pretreated with indicated concentration of doxycycline to induce the expression of human LOX-1. Then, cells were incubated with Alexa546-CRP for 1 h at 4 °C. The binding of Alexa546-CRP and the expression of the introduced protein were analyzed by IN Cell analyzer. The asterisks indicate significant difference vs CHO (*P < 0.05, **P < 0.01). MFI, mean fluorescence intensity.
cence of the uninjected area in the shaved dorsal skin was assumed as background, and the integral of the fluorescence intensity of the leaked dye minus the background value was calculated by use of Image J software (NIH). The fluorescence was measured at 3 h after the injection of CRP. To minimize individual variation among the animals, the effects of CRP on vascular permeability were expressed relative to that of 1 ng vascular endothelial growth factor.

STATISTICAL ANALYSIS

All data are presented as mean (SE). Statistical analyses were performed with the Student t-test. P values <0.05 were considered to be statistically significant.

Results

We examined the binding of Alexa Fluor 546-labeled CRP (Alexa546-CRP) to a LOX-1–expressing cell line. To ensure the specificity of the binding, we used hLOX-1-CHO (Chinese hamster ovary cell expressing human LOX-1 driven by tetracycline-inducible promoter). After treatment with 100 μg/L of doxycycline, hLOX-1-CHO clearly bound to Alexa546-CRP, whereas control CHO cells did not (Fig. 1A). Analysis by confocal laser microscope showed LOX-1 protein and Alexa546-CRP colocalized on the cell surface (Fig. 1A). The binding of Alexa546-CRP increased in parallel with the expression of LOX-1 in hLOX-1-CHO depending on the dose of doxycycline (0–300 μg/L), whereas Alexa546-CRP did not significantly bind to control CHO cells regardless of doxycycline dose (Fig. 1B, C). After treatment with 100 μg/L of doxycycline, Alexa546-CRP bound to hLOX-1-CHO cells in a dose-dependent manner (0–30 mg/L), whereas control CHO cells did not show significant binding even at an Alexa546-CRP concentration of 30 mg/L of (Fig. 1D). Significant binding of Alexa546-CRP was clearly observed at concentrations as low as 0.3 mg/L.

We then used the transient expression system in COS7 cells to compare LOX-1 with the known receptors for CRP, CD32, and CD64. Expression level of the transfected cDNA was monitored by V5-tag fused to the C-terminus of each protein. Alexa546-CRP binding to CD32- and CD64-transfected cells was detected as reported (13, 14) at concentrations of 10 and 30 mg/L of Alexa546-CRP. Significant binding of Alexa546-CRP was again observed in COS7 cells transiently expressing LOX-1 from the concentration of 1 mg/L (Fig. 2).

We further characterized CRP–LOX-1 interaction in a cell-free system, to eliminate the possibility that the observed CRP binding might be due to indirect effects of LOX-1 expression. In this system, the recombinant extracellular domain of LOX-1 prepared with a mammalian cell line was coated to an ELISA plate, and CRP binding was detected by anti-CRP antibody. Therefore, with this sandwich ELISA, the possibility of artifactual detection of the binding of non-CRP proteins is lower. CRP bound to LOX-1 protein in a dose-dependent manner (0.3–30 mg/L) (Fig. 3A). Similar results were obtained by using another anti-CRP antibody for detection (data not shown). CRP preparations from 2 different commercial sources also showed significant binding to LOX-1 (data not shown), indicating that the CRP binding is independent of its type of preparation. The CRP that was heat denatured in boiling water lost its binding ability to LOX-1, although it was still recognized by anti-CRP antibody (Fig. 3A, B). All the preparations of CRP showed a single band in SDS-PAGE (data not shown). Supplementation of IgG up to 1 g/L did not affect the binding of CRP at the concentration of 3 mg/L (data not shown). Significant binding of CRP was clearly observed at 1 mg/L in the case of the Alexa546-CRP binding to LOX-1 expressed on the cells. To analyze the kinetics of CRP binding to LOX-1, we used a surface plasmon resonance detection system (Biacore). We observed specific binding of CRP to the

Fig. 2. Quantitative analyses of Alexa 546-CRP binding to COS7 cells expressing LOX-1 and Fcγ receptors CD32 and CD64.

cDNAs encoding human LOX-1 (Genbank NM002543), CD32 (Genbank NM021642), and CD64 (Genbank NM000566) subcloned into the pcDNA6.2/V5/GW/D-TOPO expression vector (V5) (Invitrogen) were transfected into COS7 cells. The binding of Alexa546-CRP and the expression of the introduced protein were analyzed by IN Cell analyzer. pcDNA3.1/V5-His/lacZ (Invitrogen) was used as a negative control. Signals observed in the cells transfected with pcDNA3.1/V5-His/lacZ were considered as nonspecific background. The asterisks indicate significant difference vs 0 mg/L of CRP (*P < 0.05, **P < 0.01).

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LOX-1–coated flow cell. With this system, $K_a$ was calculated to be $1.6 \times 10^{-7}$ mol/L.

To examine whether LOX-1 works as the receptor for CRP in native cells, we then examined BAEC, in which LOX-1 is expressed at a high level (15). We designed 2 different siRNAs for bovine LOX-1, which suppressed bovine LOX-1 expression in CHO cells and the binding of Alexa546-CRP (Fig. 4A). In BAEC, the siRNAs suppressed the binding of Alexa546-CRP to the cells more than 50% (Fig. 4B). The residual binding of Alexa546-CRP might be due to its binding to other receptors for CRP such as CD64 and CD32 or anionic phospholipids exposed on the surface of activated/dead cells.

Then, we investigated whether or not LOX-1 mediates the in vivo effects of exogenous CRP. It has been reported that administration of human CRP to rats yields an appropriate model for the analyses of the actions of human CRP, because human CRP activates both rat and human complement systems whereas endogenous rat CRP does not (8). Therefore, we injected human CRP intradermally into rats and analyzed the changes in vascular permeability at the site of the injection by monitoring the exudation of Evans blue dye. Because the expression of LOX-1 in SHR-SP rats is much higher than in normotensive WKY rats in CD31-positive endothelial cells and interstitial cells (Fig. 5A), we compared the responses to CRP in these strains. We observed that 1 and 5 ng of vascular endothelial growth factor increased vascular permeability observed by Evans blue dye exudation. In good agreement with the higher expression of LOX-1 in SHR-SP rats, SHR-SP rats showed significantly larger responses of Evans blue dye exudation around the sites of CRP injection compared with WKY rats at doses of 1 and 3 g (Fig. 5B). In SHR-SP rats intradermal injection of CRP dose-dependently induced the exudation of Evans blue dye, and coinjection of anti–LOX-1 antibody with CRP significantly suppressed the increase in vascular permeability (Fig. 5C). These results suggest that LOX-1 not only binds to CRP but also plays a key role in the vascular mechanisms of CRP-induced pathophysiology.

Discussion

In the present study we identified LOX-1 as a novel receptor for CRP. Fcγ receptors, CD64 and CD32, are also known receptors for CRP. In our experiments CRP bound to LOX-1, but also bound to those Fcγ receptors.

Expression patterns of LOX-1 and Fcγ receptors (a) are controlled in different manners, (b) exhibit different histological distributions, and (c) have different
original functions. There have been numerous reports that the biological activity of CRP in vitro, except for its complement activation, is mediated by Fc receptors (12). One reported study, however, showed that CRP did not bind to cells that express Fc receptors unless IgG was added to medium (31). It is also known that, in the presence of excess amounts of IgG or serum, CRP binding to Fc receptors is suppressed. These previous in vitro data indicate that the interaction of CRP and Fc receptors might be modulated by environmental IgG.

Because the gene for LOX-1 is an immediate early gene, LOX-1 expression is highly induced by various stimuli (25). Within endothelial cells such stimuli include oxidized LDL and various inflammatory cytokines. In proatherogenic states such as hypertension, hyperlipidemia, and diabetes, LOX-1 expression soars (25, 32). In cardiomyocytes, LOX-1 expression is enhanced by ischemia-reperfusion. On the other hand, Fc receptors are expressed constitutively with a distinct expression pattern, particularly in leukocytes, and there are reports that they also function in vascular endothelial cells and vascular smooth cells. Considering this difference in expression pattern, it can be assumed that LOX-1 plays a key role particularly at an acute phase of pathological progression. It can also be assumed that compared to Fc receptors LOX-1 functions strongly at the lumen of endothelial cells, where it is in direct contact with blood and therefore with abundant IgG.

We originally found and identified the LOX-1 molecule as the receptor for oxidized LDL (15). FcγRII was been reported to bind to oxidized LDL (33). Therefore a reasonable course of deduction suggests that both LOX-1 and Fcγ receptors bind to CRP, considering the possibility of structural similarity in their ligand-recognizing regions.

Furthermore, it is known that CRP also binds to oxidized LDL (4). Both CRP and LOX-1 bind to such molecules as anionic phospholipids, apoptotic cells, and bacteria (3, 21, 24, 34), although no common structural basis of LOX-1 ligands including CRP is known. LOX-1 has also been reported to be involved in the process of antigen cross-presentation in dendritic cells (35). These observations suggest that LOX-1 and CRP may play significant roles together in innate immunity. The reason that oxidized LDL is well recognized by these molecules could be a result of their response as stimulated by the innate immune system reaction to oxidized LDL as a foreign body stemming from endogenous tissues.

Until recently, the biological function of CRP other than its role in complement activation has often been attributed to the influence of some impurity mixed in the solution (11, 36). To avoid any such influence we made sure to use a CRP solution that gave a single band in SDS-PAGE and had a lipopolysaccharide level below the detectable limit. Human CRP was purchased from 3 different sources, and all 3 solutions regardless of the manufacturer demonstrated binding to LOX-1. The possibility of a minute amount of impure ingredient existing in the solution that affected the CRP binding cannot be totally excluded, but regardless of the influence of impurities, our results from using the cell-free system demonstrated that LOX-1 and CRP form a complex. It is important to assess the interaction between 2 very pure substances in a system that introduces no other impurities.
Fig. 5. CRP enhanced vascular permeability via LOX-1 in vivo.
(A) LOX-1 expression (green) in dermal blood vessels of SHR-SP (upper) and WKY rats (lower). Blood vessels were visualized by the staining of an endothelial marker CD31 (red). Higher expression of LOX-1 was observed in blood vessels and in nonvascular cells of SHR-SP compared with WKY rats. (B) Extravasation of Evans blue induced by intradermal injection of CRP (1, 3, and 10 μg) in SHR-SP (upper left) and WKY rats (lower left) 3 h after the injection. For negative control, 10 μg of BSA was injected. For positive control, 1 and 5 ng of vascular endothelial growth factor (VEGF) was injected. Evans blue was detected by its fluorescence by se of a Maestro Imaging System (CRi). Semiquantitative analysis of the exudation of Evans blue dye (right). Data are expressed as mean (SE) of 8 rats each injected at 4 points for each dose of CRP. The asterisks indicate significant differences between SHR-SP and WKY rats (* P < 0.05; ** P < 0.01). (C) Effects of anti-LOX-1 antibody on CRP-induced enhancement of vascular permeability. CRP was coinjected with 3 μg of anti-LOX-1 antibody (IgG) or control mouse IgG intradermally to lumbar area in dorsal skin of SHR-SP rats. Fluorescence of the Evans blue was quantitatively analyzed by Maestro Imaging System (CRi). Evans blue exudation induced by the injection of 1 ng of vascular endothelial growth factor in the same rat was used to normalize the permeability. Data are expressed as mean (SE).m. (n = 8). The asterisks represent significant differences between IgG and anti-LOX-1 antibody groups (** P < 0.01).
Such a system helps clarify the physicochemical properties of the pure substances. To understand the biological properties of CRP and its receptors, however, monitoring them in their natural and biological context in the presence of numerous impurities is necessary. There are actually many potential molecules that can be ligands to CRP and CRP receptors in vivo. We found in this study that CRP induces changes in blood vessel permeability and that LOX-1 appears to mediate these changes. Pepys et al. reported that administration of CRP to normal animals does not yield any significant vascular effects (36), whereas CRP administration to rat models of myocardial infarction increases infarct size (8, 9). In the present study, we have made it possible to monitor the vascular response to CRP by using hypertensive rats in which LOX-1 expression is greatly enhanced. In a rat model of myocardial infarction, increases in vascular permeability observed in the present study might lead to retention of lipoproteins in the vascular wall, a process that is critical to promoting atherogenesis (37).

To determine the $K_d$ value for the binding of CRP to LOX-1 we used surface plasmon resonance analysis, which can more accurately calculate the binding constants. $K_d$ was calculated to be $1.6 \times 10^{-7}$ mol/L (18 mg/L). The hsCRP cutoff value for the risk for ischemic heart disease is reportedly $0.9 - 2.6 \times 10^{-8}$ mol/L (1–3 mg/L) (7). In response to inflammatory stimuli, hsCRP concentrations can increase to more than $10^{-6}$ mol/L. Therefore, the $K_d$ value of CRP is in the relevant range to explain the pathophysiological significance of CRP. Actually, in both cell-based and cell-free assay systems in the present study, CRP-binding was clearly observed at 1 mg/L.

It is commonly known that 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, statins, reduce the risk of cardiovascular diseases by reducing plasma LDL-cholesterol. Recent findings suggest that pleiotropic effects of the statins in addition to their cholesterol-reducing functions may also contribute to the beneficial cardiovascular effects (38). Statins reportedly reduce the expression of oxidized LDL receptor LOX-1 (39). In a recent epidemiological study, JUPITER (Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin), the observed reduction in cardiovascular risk even among those with low LDL-cholesterol demonstrated that patients with increased CRP benefit from statin treatment (40). Thus, pharmacological approaches targeting cardiovascular risk factors are phenomenologically overlapping. In this study, we have demonstrated a direct molecular link connecting the major cardiovascular risk factors, which may be an initial step that leads to further novel studies and therapeutic approaches against cardiovascular diseases.
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