Human C-reactive protein promotes oxidized low density lipoprotein uptake and matrix metalloproteinase-9 release in Wistar rats

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Abstract C-reactive protein (CRP) is present in the atherosclerotic plaques and appears to promote atherogenesis. Intraplaque CRP colocalizes with oxidized low density lipoprotein (OxLDL) and macrophages in human atherosclerotic lesions. Matrix metalloproteinase-9 (MMP-9) has been implicated in plaque rupture. CRP promotes OxLDL uptake and MMP induction in vitro; however, these have not been investigated in vivo. We examined the effect of CRP on OxLDL uptake and MMP-9 production in vivo in Wistar rats. CRP significantly increased OxLDL uptake in the peritoneal and sterile pouch macrophages compared with human serum albumin (huSA). CRP also significantly increased intracellular cholesteryl ester accumulation compared with huSA. The increased uptake of OxLDL by CRP was inhibited by pretreatment with antibodies to CD32, CD64, CD36, and fucoidin, suggesting uptake by both scavenger receptors and Fc-γ receptors. Furthermore, CRP treatment increased MMP-9 activity in macrophages compared with huSA, which was abrogated by inhibitors to p38 mitogen-activated protein kinase, extracellular signal-regulated kinase (ERK), and nuclear factor (NF)-κB but not Jun N-terminal kinase (JNK) before human CRP treatment. Because OxLDL uptake by macrophages contributes to foam cell formation and MMP release contributes to plaque instability, this study provides novel in vivo evidence for the role of CRP in atherosclerosis.—Singh, U., M. R. Dasu, P. G. Yancey, A. Afify, S. Devaraj, and I. Jialal. **Human C-reactive protein promotes oxidized low density lipoprotein uptake and matrix metalloproteinase-9 release in Wistar rats. *J. Lipid Res.* 2008.** 49: **1015–1023.**

Supplementary key words mechanistic insights • macrophages • sterile pouch

Increasing evidence supports the involvement of inflammation in the pathogenesis of atherosclerosis and acute coronary syndromes (ACSs) (1). Monocyte/macrophages are pivotal cells in atherosclerosis and participate in all stages, from initiation of the fatty streak to plaque rupture. C- reactive protein (CRP), the prototypic marker of inflammation, in addition to being a risk marker, appears to be an active participant in atherosclerosis (2). Much data are accumulating to suggest that CRP also promotes atherothrombosis (3). Also, CRP is known to be present in atherosclerotic lesions and is significantly higher in the plaque of patients with unstable angina pectoris than in those with stable angina pectoris (4). To date, it has been shown that in monocytes, CRP induces the production of inflammatory cytokines and promotes monocyte chemotaxis, reactive oxygen species, and tissue factor expression (3, 5, 6). CRP has also been reported to bind to oxidized low density lipoprotein (OxLDL) (7, 8) and promote its uptake in the monocyte/macrophage-like U937 cell line (8). Importantly, it has been reported that intraplaque CRP colocalizes with OxLDL and macrophages in human atherosclerotic lesions (9).

Plaque rupture and erosion are believed to be the key events that trigger the formation of thrombus and subsequent ACSs (10). Levels of matrix metalloproteinase-9 (MMP-9) are increased in patients with heart failure and ACS/unstable angina (11) and exhibit a strong correlation with high CRP levels. Also, a causal role of MMP-9 is supported by genetic studies showing that functional promoter variations of the MMP-9 gene are related to the presence and severity of cardiovascular disease (12). Our group also earlier reported a significant increase in MMP-9 in subjects with high CRP versus low CRP (13). Importantly, MMP-1 (a collagenase), MMP-2 (a gelatinase), and MMP-3 (a stromelysin) are predominantly secreted by endothelial cells (14), whereas the major MMP secreted from macrophages is MMP-9 (a gelatinase) (15). Also, CRP has been reported to induce the release of various different forms of MMP from the cells involved in the process of atherogenesis (16–21).

Manuscript received 20 November 2007 and in revised form 14 January 2008 and in re-revised form 1 February 2008.

Published, JLR Papers in Press, February 2, 2008.
DOI 10.1194/jlr.M700535-JLR200

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Despite all of the in vitro reports cited above, there is a paucity of in vivo data to support the proinflammatory effects of CRP. A valid animal model to test the effect of human CRP (hCRP) has been an issue in the literature. The data concerning hCRP transgene in apolipoprotein E-deficient mice have been largely inconsistent (3). However, hCRP administration in rats is reported to promote myocardial infarct size and cerebral infarct in coronary artery ligation (22) and cerebral artery occlusion (23) models, respectively. Furthermore, Pepys et al. (24) recently validated the rat as an appropriate model to test the effect of hCRP by blocking CRP's effects on infarct size with a low molecular weight inhibitor.

Based on these data, we explored the effect of CRP in vivo on rat macrophages. Macrophages appear to be important not only because they are the predominant cell type in atherosclerotic lesions and the main source of MMP-9 but also because CRP and OxLDL have been reported to be colocalized in these cells. Thus, the present study was designed to investigate the effect of CRP on OxLDL uptake and MMP-9 release in vivo.

MATERIALS AND METHODS

Gelatin gels for zymography were obtained from Invitrogen. Coomassie Brilliant Blue R-250 was from Sigma Chemical Co. MMP-2 and tissue inhibitor of metalloproteinase-1 (TIMP-1) ELISA were from R&D Biosystems. SB203580 [a p38 mitogen-activated protein kinase (MAPK) inhibitor], PD098059 [an extracellular signal-regulated kinase (ERK) 1/2 inhibitor], caffeic acid phenehthyl ester (CAPE) (an NF-κB inhibitor), SP600125 [a Jun N-terminal kinase (JNK) inhibitor], human serum albumin (huSA), and fucoidin were from Sigma. CD32, CD36, and CD64 as well as isotype control antibodies were obtained from BD Biosciences (San Jose, CA), Cell Sciences (Canton, MA), and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. 2,2-Azobis-2-amidino-propane dihydrochloride (AAPH) was from WAKO. The fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′ tetramethylindocarbocyanide perchlorate (DiI) was from Molecular Probes (Eugene, OR). The NF-κB activity kit was from Active Motif (Carlsbad,CA). All of the reagents used were tested for endotoxin, and the levels were <0.06 ng/ml consistently.

hCRP was purified from human ascitic/pleural fluids as described by Du Clos, Zlock, and Marnell (25). Recently, we showed that our in-house purified, dialyzed CRP mediates its inflammatory effects in TLR4 knockdown cells, providing further cogent data that CRP-mediated effects are not attributable to endotoxin contamination (26).

LDL preparation, oxidation, and labeling

Native LDL (density, 1.019–1.063) was isolated from plasma obtained from healthy donors by sequential density gradient ultracentrifugation (27) and desalted through Econopak columns using PBS. Oxidation of LDL was performed by incubating native LDL (1 mg protein/ml) at 37°C with freshly prepared AAPH (5 mM final concentration). The extent of oxidation was determined by the amount of thiobarbituric acid-reactive substances (28 ± 8 nmol malondialdehyde (MDA) equivalents/mg protein). Also, LDL was oxidized with cupric sulfate (5 μM in PBS) (28) and myeloperoxidase (MPO; 30 nM) and water (100 μM) for 8 h (29). Although the degree of oxidation with copper (27 ± 5 nmol MDA equivalents/mg protein) was similar to that with AAPH, MPO-modified LDL was oxidized to a lesser extent (15 ± 6 nmol MDA equivalents/mg protein). OxLDL was labeled with DiI as described previously (27). The specific activity of DiI-labeled OxLDL used in our experiments was 13.1 μmol/mg protein (n = 3 different LDL preparations).

CRP and DiI-OxLDL uptake by human monocyte-derived macrophages

Experiments were first performed with human monocyte-derived macrophages (HMDMs). Mononuclear cells were isolated from healthy human volunteers by density-gradient centrifugation using Ficoll-Hypaque. The cells were plated and allowed to differentiate into HMDMs as described previously (30). On the 7th day of culture, HMDMs were incubated at 37°C for 24 h with DiI-OxLDL prepared using copper, AAPH, and MPO (50 μg protein/ml) along with huSA/hCRP (25 μg/ml). After the incubation, cells were washed with PBS and analyzed for DiI uptake by flow cytometry. The results are expressed as mean fluorescence intensity (MFI) per 10^5 cells. All experiments were performed in duplicate and repeated three to five times with pooled human mononuclear cells from three healthy human volunteers per experiment.

Animal treatment

Male hooded Wistar rats (weighing 125–150 g) were obtained from Charles River Laboratories. Animals were housed in a controlled environment at an ambient temperature of 21 ± 2°C on a 12 h light/dark cycle. Food and water were provided ad libitum. The rats were acclimatized to animal housing conditions for 1 week. The protocol was approved by the animal committee of the University of California at Davis. Two different routes of administration for hCRP (hCRP test protein and huSA control protein) in rats were used to examine its effects in vivo: *i*) intraperitoneal model, CRP administered intraperitoneally; and *ii*) sterile pouch model, CRP administered in a sterile air pouch. For all in vivo experiments, AAPH OxLDL was used.

Intraperitoneal model. hCRP/huSA (20 mg/kg body weight for 2 days, n = 4 in each group) was injected in the peritoneal cavity of the rats. Also, for OxLDL uptake experiments, DiI-OxLDL (500 μg of total protein) was injected along with hCRP/huSA on the 2nd day of injection. The rats were euthanized on the 3rd day by overdose of pentobarbital, and peritoneal macrophages were isolated as described previously (31) and used for further analysis. The total number of cells in the peritoneal cavity was ~5–7 × 10^6 cells. A total of 75–80% of the peritoneal aspirate contained CD68-positive cells.

Sterile air pouch model. This model has been used widely to examine inflammatory responses (32, 33). The formation of an air pouch provides an optimal tissue cavity into which inflammatory stimuli can be introduced. This model offers several advantages (33), such as a site of administration of stimuli to study local cellular responses, and potentially permits the administration of substances in much lower amounts compared with intraperitoneal administration. It is important to emphasize that isolation of CRP from pleural/ascitic fluids free of potent interfering contaminants such as endotoxin is not only laborious but expensive as well. Thus, we chose to use the confined sterile pouch model, because it is cost-effective to investigate the mechanistic events involved in CRP-mediated OxLDL uptake and MMP-9 production.

Sterile air pouch formation

The backs of the rats were shaved and cleaned with alcohol swabs under mild isoflurane sedation. The sterile air pouches

were made on the dorsal surface of the rats using a slight modification of an established protocol (32) at ~1.5 inches from the mid neck subcutaneously by injecting 20 ml of sterile air with a 23 gauge needle syringe. hCRP/huSA (25 μg/ml intrapouch) was injected directly into the pouch cavities of lightly restrained (hand-held) conscious animals on the 3rd day after formation of the pouch. For CRP-mediated OxLDL experiments, DiI-OxLDL (50 μg/ml intrapouch) was injected in the pouch along with CRP. The rats were euthanized the next day by overdose of pentobarbital. The washouts of the pouch were made by deflating the pouch, irrigating with normal saline, and aspiration. The volume and total number of cells in the pouch fluid remained fairly constant (4–5 ml and 3×10^6 cells, respectively) over the time period, and 76% of the cells were macrophages. The cells were washed twice with PBS and resuspended at a concentration of 1×10^5 cells/ml in RPMI for analysis.

Validation of the air pouch model

The pouch exudates were used to examine the known proinflammatory effects of hCRP to validate the air pouch model. The release of various cytokines [interleukin-1 (IL-1), tumor necrosis factor (TNF), IL-8, and IL-6] in the pouch fluid was measured by ELISA (R&D Systems), and the results are expressed per milligram of cell protein.

CRP and DiI-OxLDL uptake

We aimed to examine whether CRP enhances OxLDL uptake in vivo. To achieve this aim, we injected DiI-OxLDL along with hCRP/huSA intraperitoneally in the rats and performed the assays described below.

Di-OxLDL uptake

The isolated peritoneal macrophages were plated on 24-well plates (1×10^4 cells) and allowed to adhere for 2 h. At the end of the incubation, the cells were washed two times with PBS. DiI accumulation in the cells was measured by its extraction with isopropanol and the determination of fluorescence at excitation/emission wavelengths of 532/564 nm, respectively, as described (34).

Analysis of intracellular cholesteryl ester accumulation

The cells were allowed to adhere to 12-well plates (1×10^6 cells/well) for 2 h in duplicate from each rat. At the end of the incubation, the cells were washed two times with PBS and air-dried under the hood so that there was no residual moisture in the wells. The cells isolated from rats injected with vehicle only (no OxLDL) served as a control. Both total and free cholesterol were quantitated using gas chromatography (35). The accumulation of cholesteryl esters was determined by subtracting the free cholesterol amount from total cholesterol. The results are expressed per milligram of cell protein. Additionally, pouch macrophages were also examined for intracellular cholesteryl ester accumulation.

Involvement of the receptor type in CRP-mediated OxLDL uptake

The pouch model was used to explore the specific receptor type involved in CRP-mediated OxLDL uptake. We first investigated the effect of hCRP compared with huSA (n = 5 rats per group) on DiI-OxLDL uptake by cells present in the pouch. To further delineate the receptor type involved, blocking antibodies (10 μg/ml intrapouch) to CD32, CD64, CD36, or irrelevant IgG either alone or in combination (CD32 + CD64, CD32 + CD36) were injected into the rats (three to five per group) at 2 h before hCRP injection along with DiI-OxLDL. Additionally, fucoidin, a scavenger receptor-A agonist (10 μg/ml intrapouch), was also injected either alone or in combination with anti-CD36 antibody. Rats were euthanized 12–16 h later as described, and pouch exudates were collected. DiI-OxLDL uptake was measured in the pouch cells using flow cytometry in the phycoerythrin channel. The results were expressed as MFI per 10^5 cells. Cell viability by trypan blue exclusion was >90%. The antibodies or fucoidin concentrations used were not toxic to the animals.

Determination of CRP's effect on MMP-9 production

The peritoneal macrophages isolated from rats of the intraperitoneal model were lysed in mammalian protein extraction reagent including protease inhibitors as described (19) and used for MMP-9 activity. To 10 μg of cell lysates was added 5× SDS sample buffer without reducing agent. The unheated samples were then applied to gelatin zymography gels. Electrophoresis was run at 100 V in a cold room. After electrophoresis, the gels were washed at room temperature with renaturing buffer for 30 min to remove the SDS and subsequently incubated at 37°C overnight in developing buffer to allow proteinase digestion of its substrate. The gel was rinsed in distilled water, stained with 0.25% Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (30:10:60, v/v/v) on a shaker, and destained with methanol-acetic acid-water (10:10:80, v/v/v). The gel was then photographed on a light box. Proteolysis was detected as a white zone of digested gelatin against a dark blue background of stained gelatin.

Mechanistic insights for CRP-induced MMP-9 release

These experiments were performed using a pouch model. The rats were divided into six groups (n = 4 rats per group) as follows: group 1, huSA; group 2, hCRP; group 3, SB203580 (1 mg/kg body weight) + hCRP; group 4, PD098059 (0.5 mg/kg body weight) + hCRP; group 5, SP600125 (1 mg/kg body weight) + hCRP; group 6, CAPE (0.2 mg/kg body weight) + hCRP. On the 3rd day after the formation of pouches, various inhibitors were injected into the pouches at specific concentrations as described in the literature for in vivo use (36–38). huSA or hCRP was injected at 2 h after the injection of inhibitors, followed by euthanization of rats 12–16 h later. The pouch exudates (20 μl) were used for gelatin zymography as described previously. Additionally, the cell lysates were run on SDS-PAGE for Western blotting using 20 μg of protein for loading on gels and using primary antibody for MMP-9 (goat anti-rat MMP-9 IgG, 1:200) and donkey anti-goat IgG as the secondary antibody (1:500) followed by chemiluminescence with ECL reagent. The β-actin protein was used as a loading control, which was detected with monoclonal anti-human actin IgG and is cross-reactive with rat actin.

Measurement of MMP-2 and TIMP-1 levels

Pouch exudates were also used to measure MMP-2 using the MMP-2 human ELISA kit, as this kit has cross-reactivity with rat MMP-2 as well. The TIMP-1 concentration in the pouch exudates was measured by a specific enzyme immunoassay kit.

CRP and NF-κB activity

CRP has been shown to induce NF-κB activity in human aortic endothelial cells (3) as well as human monocytes (39), and MMP-9 is reported to have an NF-κB element in its promoter. Thus, in the present study, we also measured p65 NF-κB binding activity in the nuclear fractions of the cells isolated from pouch exudates. The results are expressed per milligram of cell protein of nuclear fractions.

Statistical analysis

All experiments were performed at least three times in duplicate. The comparisons between group means were analyzed using ANOVA. The experimental results are presented as means ± SD. Paired *t*-tests were used to compute differences in the variables, and the level of significance was set at $P < 0.05$.

RESULTS

The uptake of all three forms of OxLDL by HMDMs was significantly greater when coincubated with hCRP compared with huSA ($P < 0.05$) (**Fig. 1A**). For all subsequent experiments, AAPH OxLDL was used.

Intraperitoneal model

Administration of hCRP compared with huSA in Wistar rats led to significantly higher circulating levels of hCRP (18 mg/l vs. undetected, hCRP vs. huSA group, respectively; $P < 0.001$).

CRP and DiI-OxLDL uptake studies. huSA injection to rats resulted in 1.3-fold increased DiI-OxLDL uptake compared

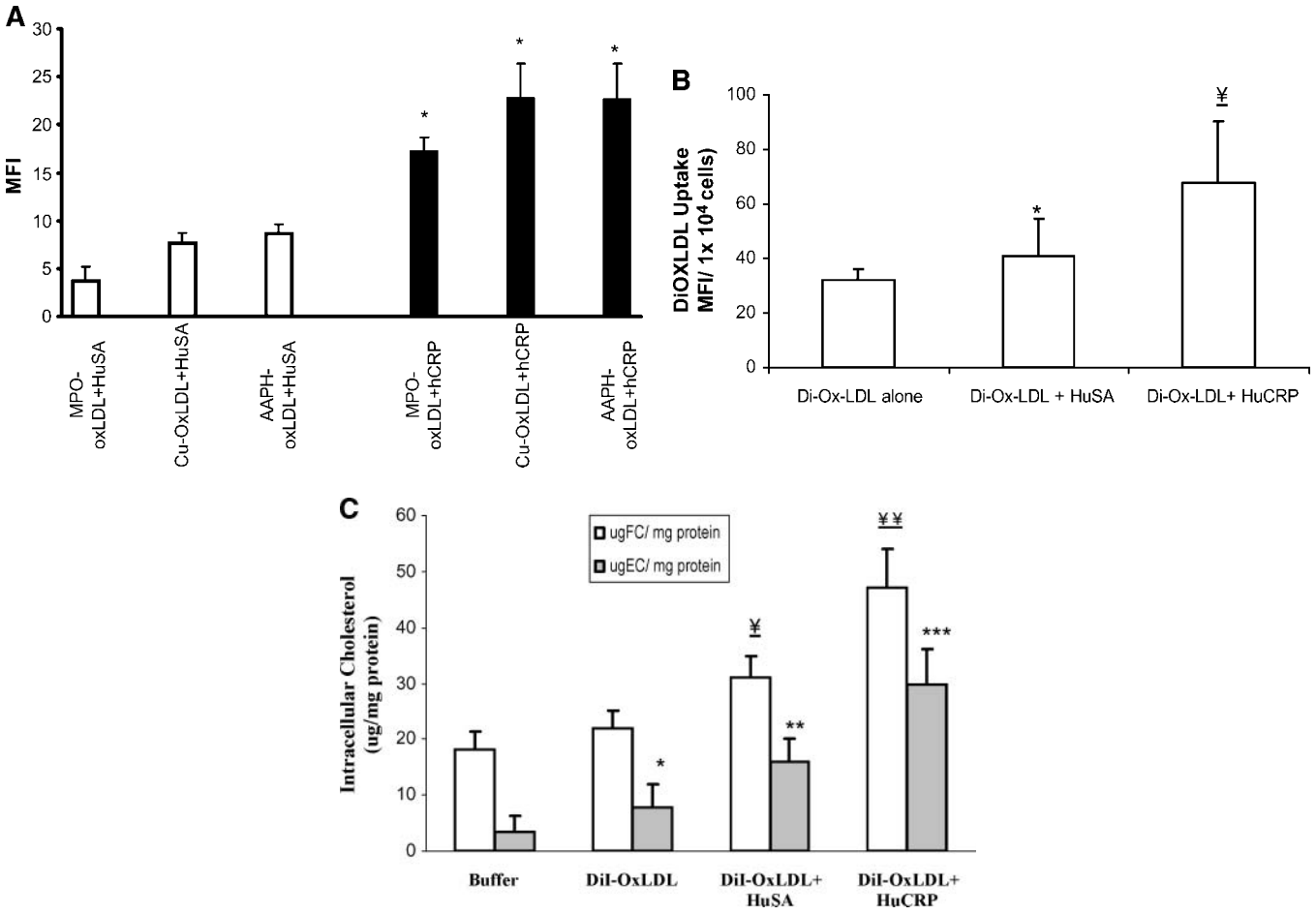

Fig. 1. A: Effects of human C-reactive protein (hCRP) compared with human serum albumin (huSA) on 1,1′-dioctadecyl-3,3,3′,3′ tetramethylindocarbocyanide perchlorate (DiI) oxidized low density lipoprotein (OxLDL) uptake in human monocyte-derived macrophages (HMDMs). LDL was oxidized using 2,2-azobis-2-amidino-propane dihydrochloride (AAPH), copper (Cu), and myeloperoxidase (MPO) as described in Materials and Methods. HMDMs were cultured for 7 days and treated with DiI-OxLDL alone or in the presence of huSA/hCRP. DiI uptake was measured as described in Materials and Methods. * $P < 0.05$ compared with DiI-OxLDL + huSA (n = 3 experiments). MFI, mean fluorescence intensity. B: Effects of intraperitoneal hCRP compared with huSA administration along with DiI-OxLDL in Wistar rats on DiI uptake. The rats were injected with hCRP/huSA for 2 days at 20 mg/kg body weight. On the 2nd day, DiI-OxLDL was injected along with hCRP/huSA. Peritoneal macrophages were isolated and plated on 24-well plates. After adherence to the surface, DiI was extracted with isopropanol and fluorescence was measured as described in Materials and Methods. DiI uptake is expressed as MFI per 10^4 cells. * $P = 0.049$ compared with Di-OxLDL alone; ¥ $P = 0.03$ compared with huSA + DiI-OxLDL (n = 4 rats/group). C: Effects of intraperitoneal hCRP compared with huSA administration along with DiI-OxLDL in Wistar rats on free cholesterol (FC) and intracellular cholesteryl ester (EC) accumulation. The rats were injected with hCRP/huSA for 2 days at 20 mg/kg body weight. On the 2nd day, DiI-OxLDL was injected along with hCRP/huSA. Peritoneal macrophages were isolated and plated on 12-well plates. Total and free cholesterol were quantitated, and cholesteryl ester content was determined as detailed in Materials and Methods. * $P < 0.05$ compared with buffer, ** $P < 0.05$ compared with OxLDL alone, *** $P < 0.003$ compared with DiI-OxLDL + huSA, ¥ $P < 0.05$ compared with OxLDL alone, ¥¥ $P < 0.01$ compared with DiI-OxLDL + huSA (n = 4 rats/group). Values shown are means ± SD.

with DiI-OxLDL alone ($P = 0.049$). Furthermore, there was a significantly increased DiI-OxLDL uptake in peritoneal macrophages of hCRP-injected rats compared with huSA treatment in vivo (1.7-fold; $P = 0.03$) (Fig. 1B).

Intracellular cholesteryl ester accumulation. The measurement of intracellular cholesterol by gas chromatography revealed significantly higher amounts ($P < 0.003$) of cholesteryl esters and free cholesterol in peritoneal macrophages isolated from rats administered OxLDL/hCRP compared with OxLDL/huSA (Fig. 1C). Similar to DiI-OxLDL uptake, there was increased cholesteryl ester with OxLDL/huSA compared with either buffer alone (5-fold) or OxLDL alone (2-fold). Furthermore, there was a significant upregulation of cholesteryl esters with OxLDL/hCRP compared with OxLDL/huSA (1.9-fold), OxLDL alone (3.8-fold), and buffer alone (9-fold). These results reveal that CRP promotes OxLDL uptake, leading to an increased accumulation of cholesteryl esters in the macrophages in vivo.

Sterile air pouch model

Validation of the proinflammatory effects of CRP in the pouch model. We validated the pouch model by examining the known proinflammatory effects of CRP in vitro, as reported previously in the literature. The levels of the various proinflammatory cytokines IL-1β (huSA, 14,518 ± 1,567 vs. hCRP, 39,682 ± 6,033 pg/mg protein; $P < 0.05$), IL-6 (huSA, 13,942 ± 1,131 vs. hCRP, 55,919 ± 2,922 pg/mg protein; $P < 0.05$), IL-8 (huSA, 5,263 ± 200 vs. hCRP, 19,058 ± 556 pg/mg protein; $P < 0.05$), and TNF-α (huSA, 12,470 ± 1,567 vs. hCRP, 153,591 ± 12,176 pg/mg protein; $P < 0.05$) were increased significantly in pouch exudates from hCRP-administered compared with huSA-administered rats. Thus, these results validate the pouch model to examine various proinflammatory effects of CRP. Hence, the pouch model was used to examine CRP-mediated events in depth to be cost-effective, because this model required less CRP compared with the intraperitoneal model.

CRP-mediated OxLDL uptake by macrophages in vivo includes multiple receptors. The uptake of DiI-OxLDL also was increased significantly ($P < 0.05$) in cells when DiI-OxLDL was administered in the rat pouches in the presence of hCRP compared with huSA (**Fig. 2A**). Several cell surface receptors were examined to determine the involvement of CRP-mediated OxLDL uptake by macrophages. The increased uptake of OxLDL by CRP was inhibited by pretreatment for 1–2 h with antibodies to CD32 (65%), CD64 (46%), CD36 (55%), and fucoidin (61%) as well as the combination of CD32 + CD36 (75%) and CD36 + fucoidin (68%) (Fig. 2B). The combination of antibodies to CD32 and CD64 (38%) was not additive to either alone. Pretreatment with isotype control IgG failed to have any significant effect on CRP-mediated OxLDL uptake (24.5 ± 1.5 vs. 25.7 ± 2.2 MFI per 10^5 cells). Furthermore, similar results were observed with human macrophages (data not shown). Also, pouch macrophages after OxLDL/hCRP injection revealed significant increases in intracellular cholesteryl ester accumulation (OxLDL/huSA, 1.5 ± 0.4 vs. OxLDL/hCRP, 2.8 ± 1.2 μg/mg cell protein; $P < 0.05$).

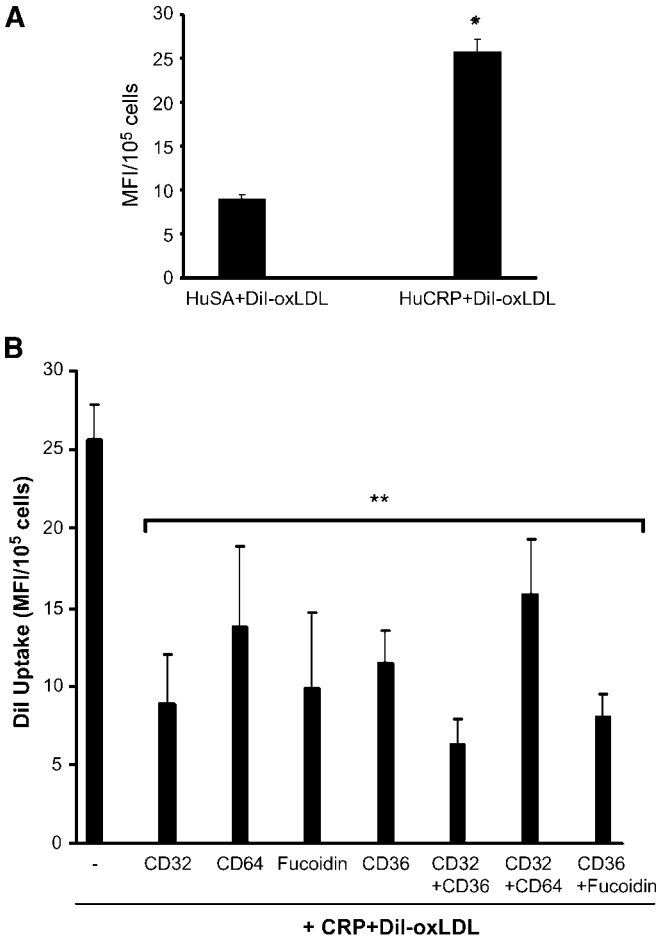

Fig. 2. A: Effects of hCRP/huSA intrapouch administration (25 μg/ml) on DiI-OxLDL uptake. Sterile pouches were made in rats, and hCRP/huSA was injected in the pouch cavities of Wistar rats on the 3rd day after the formation of the pouch. For CRP-mediated OxLDL uptake experiments, DiI-OxLDL (50 μg/ml protein) was injected in the pouch along with CRP. The next day, the pouch fluid was aspirated as described in Materials and Methods and the cells isolated were used for DiI uptake. * $P < 0.05$ compared with huSA + DiI-OxLDL (n = 5 rats/group). B: Demonstration of the involvement of various receptors involved in CRP-mediated DiI-OxLDL uptake. Rats were injected with hCRP/huSA as described above in the pouch cavities of Wistar rats on the 3rd day after the formation of the pouch. Antibodies to CD32, CD64, CD36, and control isotype as well as the combinations CD32 + CD36, CD32 + CD64, and CD36 + fucoidin were also injected at 1–2 h before hCRP treatment. DiI uptake was analyzed by flow cytometry as described in Materials and Methods. ** $P < 0.05$ versus DiI-OxLDL + CRP (n = 3–5 rats/group). Values shown are means ± SD.

CRP and MMP-9 induction. Zymography analysis revealed a significant ($P < 0.01$) induction of MMP-9, and there was a trend toward increased MMP-2 in the rat peritoneal macrophages from hCRP-injected rats compared with huSA-injected rats (**Fig. 3**). However, there was no change in MMPs revealed on a casein gel, which detects MMP-1 and MMP-3 (data not shown).

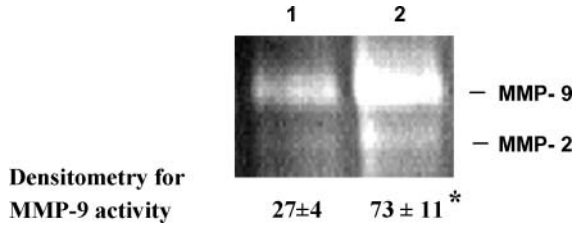

Fig. 3. Effects of CRP on matrix metalloproteinase-9 (MMP-9) production in peritoneal macrophages of Wistar rats administered huSA or hCRP (20 mg/kg body weight/day for 2 days). Peritoneal macrophages were isolated, and lysates were run on a SDS-PAGE gelatin gel for MMP-9 zymography at 4°C. After electrophoresis, the gel was subjected to washing, development, and staining as described in Materials and Methods. Active MMP-9 was visualized as proteolytic (white) bands on a dark background. Column 1, huSA; column 2, hCRP. The gel shown is representative of samples from four different rats. * $P < 0.01$ compared with huSA.

Mechanistic insights for CRP-induced MMP-9. The pouch fluids were run on SDS-PAGE gelatin gels to reveal MMP-9 activity. Also, pouch cell lysates were run on SDS-PAGE gels for MMP-9 total protein mass by Western blot. Our attempts to explore the mechanistic events in CRP-mediated MMP-9 induction revealed that p38 MAPK, ERK, and NF-κB pathways were involved, as the inhibitors used for all of these pathways attenuated CRP-mediated MMP-9 activity (**Fig. 4A**) as well as its total mass induction (Fig. 4B). However, it is clear that the JNK pathway was not involved, because SP600125 injection before CRP treatment failed to affect CRP-mediated MMP-9 release.

CRP and nuclear factor-κB activity. Because the NF-κB inhibitor (CAPE) used in the present study attenuated CRP-mediated MMP-9 induction, we also measured the NF-κB DNA binding activity in the nuclear extracts of cells isolated from pouch exudates. Our results revealed that CRP treatment significantly upregulated p65 NF-κB DNA binding activity in pouch cells compared with huSA treatment (huSA, 32 ± 1.2 vs. hCRP, 44 ± 5.8 ng/mg nuclear protein; $P < 0.05$ compared with huSA injection).

CRP, MMP-2, and TIMP-1 levels. MMP-2 levels measured in the pouch exudates were not significantly different in hCRP-administered rats compared with huSA treatment (692 ± 267 vs. 821 ± 309 ng/mg protein, respectively; $P > 0.05$). Furthermore, TIMP-1 levels were also unchanged with CRP treatment (data not shown).

DISCUSSION

Inflammation plays an important role in the pathogenesis of atherosclerosis and plaque instability. Monocyte/macrophages are recognized as key promoters of acute and chronic inflammatory responses. Furthermore, high levels of CRP are associated with an increased risk for cardiovascular disease. Importantly, the colocalization of CRP

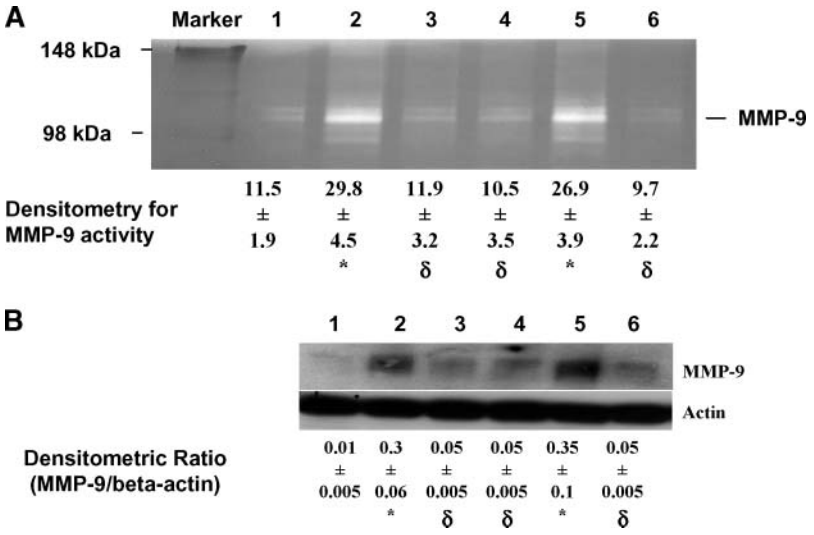

Fig. 4. A: Effects of pretreatment with various inhibitors to p38, extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), and NF-κB in the rat sterile air pouch model in Wistar rats on MMP-9 activity. Column 1, huSA; column 2, hCRP; column 3, p38 MAPK inhibitor + hCRP; column 4, ERK inhibitor + hCRP; column 5, JNK inhibitor + CRP; column 6, NF-κB inhibitor + CRP. The pouch fluids were run on SDS-PAGE gelatin gels for zymography as described in Materials and Methods. The gel shown is representative from four rats per group. Densitometric values (means ± SD) are given below the gel in arbitrary units. * $P < 0.001$ compared with huSA, $^{\delta}$ $P > 0.05$ compared with hCRP. B: MMP-9 total protein mass as described in Materials and Methods. Column 1, huSA; column 2, hCRP; column 3, p38 MAPK inhibitor + hCRP; column 4, ERK inhibitor + hCRP; column 5, JNK inhibitor + CRP; column 6, NF-κB inhibitor + CRP. The pouch cell lysates were run on SDS-PAGE gelatin gels for MMP-9 total protein mass as described in Materials and Methods. The gel shown is representative from four rats per group. Densitometric ratios (means ± SD) are given below the gel. * $P < 0.05$ compared with huSA; $^{\delta}$ $P > 0.05$ compared with hCRP.

and macrophages has been demonstrated in advanced human atherosclerotic plaque (40). The mechanistic link between CRP and atherosclerosis has been the focus of much research. However, there is a paucity of in vivo data to support the proinflammatory effects of CRP.

A valid animal model to test the effect of hCRP has been an issue in the literature. As reviewed recently (3), CRP is not a major acute phase protein in mice. With regard to exploring the role of CRP in vivo, in the rat carotid artery angioplasty model, CRP administration has been shown to increase neointimal proliferation (41). Recently, Pepys et al. (24) further validated the rat model as an appropriate model to test the effect of hCRP. By blocking CRP's effects with a low molecular weight inhibitor, the increase in infarct size after coronary artery ligation was prevented. Thus, the rat model is considered appropriate and relevant to study the effect of hCRP. In the present study, we explore two major effects of CRP in vivo, plaque formation (OxLDL uptake) and plaque instability (MMP) at ~18 mg/l CRP concentration. Ridker and Cook (42) have shown that CRP levels >20 mg/l predict future cardiovascular events. Importantly, CRP levels of ~50 mg/l have been reported in patients with ACSs (3, 43). Thus, the levels of CRP attained in Wistar rats and shown to promote OxLDL uptake and MMP-9 production can clearly be attained in patients. Therefore, it is likely that local CRP levels in atherosclerotic lesions, from either the circulation or its local synthesis by cells present in arterial lesions, might promote OxLDL uptake and MMP-9 induction by macrophages.

To further strengthen our in vivo reported findings and explore CRP-mediated events in depth, we exploited an in vivo rat sterile pouch model because this offers the advantage of having less CRP being injected in a local environment; thus, it is cost-effective. Also, this model has been used by various investigators for the simultaneous measurement of cellular and mediator components of the inflammatory exudates. Because of the many advantages this system provides, we chose to use it in the present study to investigate the local inflammatory effects of hCRP, with the aim of identifying the mechanisms of action of hCRP in vivo. Injection of hCRP resulted in increased proinflammatory cytokines in pouch exudates, confirming the validity of this model. Thus, we believe that this model serves as an ideal model to study CRP effects in vivo.

Previously, Chang et al. (7) reported that CRP binds to OxLDL and not native LDL by recognition of a phosphorylcholine moiety that becomes accessible as a result of the oxidation of a phosphatidylcholine molecule. Earlier, Meuwissen et al. (9) reported the colocalization of intraplaque CRP and OxLDL in the plaque. Furthermore, a recent report has demonstrated strong associations between antibodies to OxLDL and CRP in patients with ACS (44). All of these reports provide an emerging concept for the involvement of CRP in the formation of foam cells by opsonizing OxLDL particles. Although these data suggest that CRP enhances foam cell formation, this has not been confirmed in vivo. We demonstrate here that CRP enhances the uptake of DiI-OxLDL by rat macrophages in vivo. Also, CRP treatment in vivo led to an increased accumulation of intracellular cholesteryl esters, the hallmark of foam cells.

CRP is known to signal through Fc-γ receptors type I (CD64) and type II (CD32) (3, 18, 45). We have shown that CRP's effects are mediated via CD32 and CD64 in human aortic endothelial cells (HAECs) (3). Zwaka, Hombach, and Torzewski (46) suggested a role for Fc-γ receptor II (CD32) in the CRP-mediated uptake of native LDL in circulating monocytes that were transformed into macrophages, whereas Fu and Borensztajn (47) observed that the uptake by U937 macrophages of complexes of aggregated CRP and LDL was CD32-independent. Additionally, it has been reported that in the presence of CRP, the binding of OxLDL to cells is mainly via CRP-Fc-γ receptor interaction (8). We report in the present study that the uptake of OxLDL in macrophages occurs via CRP-Fc-γ receptor interaction as well as scavenger receptors, because blocking either Fc-γ receptors or the scavenger receptors led to the attenuation of CRP-induced DiI-OxLDL uptake. Thus, two pathways exist for the routing of CRP-OxLDL: Fc-γ receptor and scavenger receptors.

Furthermore, MMPs are reported to play an important role in the pathogenesis of cardiovascular disease, owing to their major role in vascular remodeling (10). In this regard, MMP-9 is highly expressed in the vulnerable regions of atherosclerotic plaques and has been suggested to be causally involved in the remodeling processes associated with atherogenesis and plaque rupture (11). Moreover, human monocyte-derived macrophages, which harbor MMPs, have been shown to induce collagen breakdown in fibrous caps in vivo (15). Also, CRP in vitro is reported to promote the production of several MMPs, such as MMP-9 in mononuclear cells (16) and rat peritoneal macrophages (17) as well as MMP-1 in U937 histiocytes and human monocyte-derived macrophages (18). CRP-mediated MMP induction has been reported at multiple levels, such as p38 MAPK and the synergistic action of p38 and JNK for MMP-1 and MMP-10, respectively, in HAECs and human cornorary artery endothelial cells (19), the MAPK pathway for MMP-2 in human vascular smooth muscle cells (20), ERK for MMP-1 in U937 histiocytes (18), NADPH oxidase for MMP-9 release in rat macrophages (17), and the CD40-CD40L signaling pathway for the induction of MMP-2 and MMP-9 (21). Additionally, the use of function-blocking antibodies to TNF-α and IL-1β has been reported to significantly inhibit the induction of MMP-9 in human mononuclear cells (16).

In the present study, we elucidated the potential mechanism for CRP-induced MMP-9 production in vivo and report that this effect is mediated via p38 MAPK, ERK and NF-κB activation. Additionally, the MMP-9 gene is reported to have an NF-κB element in its promoter (48). We also demonstrate significantly increased NF-κB DNA binding activity in the nuclear extract of pouch macrophages from hCRP-treated compared with huSA-treated rats. Our observation that CRP activates the NF-κB pathway in vivo is appealing. NF-κB appears to be an upstream event in CRP-mediated MMP-9 induction. Also, the NF-κB pathway has

been reported to be involved in upregulating IL-8, intracellular adhesion molecule, vascular cell adhesion molecule, and plasminogen activator inhibitor-1 by CRP (3) in HAECs as well as a downstream target for MAPKs. Furthermore, macrophage-specific inhibition of NF-κB has been shown to result in decreased foam cell formation (49). Thus, our findings suggest that CRP may have direct effects on many biological and pathological processes. Additionally, a number of biological mediators, such as cytokines, reactive oxygen species, and cholesterol, have been shown to enhance the expression of MMPs and their endogenous inhibitors, TIMPs, in atherosclerotic plaques (50). However, CRP failed to have any effect on TIMP-1 production concomitant with increased MMP-9 activity in the present study, in agreement with the findings reported by other investigators. Based on the increased ratio of MMP-9 to TIMP-1, CRP may promote plaque instability.

To conclude, in the present study, we make the novel observation that in vivo, CRP enhances OxLDL uptake and cholesteryl ester accumulation and stimulates MMP-9 release, which could contribute to plaque rupture. This could have implications for ACS patients, because higher levels of CRP portend a poor prognosis (51). Thus, strategies aimed at decreasing CRP (24) may prove to be beneficial in preventing atherothrombotic events and reducing atherosclerosis-related events.

This work was supported by National Institutes of Health Grant RO1 HL-074360 to I. J.

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