Rat C-Reactive Protein Causes a Charge Modification of LDL and Stimulates Its Degradation by Macrophages

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Abstract  We have previously shown the binding of low-density lipoprotein (LDL) to immobilized rat C-reactive protein (CRP) and the formation of a fluid-phase complex between these two proteins. In this report we used immunoelectrophoresis and agarose gel electrophoresis to show increased anodic migration of the LDL particle as a result of the modification of LDL by rat CRP. The degradation of the modified 125I-LDL by rat peritoneal macrophages was increased more than twofold in the presence of rat CRP. The increase in rat CRP–mediated 125I-LDL degradation by macrophages was dependent on the concentrations of 125I-LDL and rat CRP. This increased 125I-LDL degradation was inhibited by phosphorylcholine. In contrast, the degradation of 125I-acetyl-LDL by macrophages was not affected by rat CRP, although acetylated LDL inhibited the rat CRP-stimulated degradation of 125I-LDL. Increasing concentrations of LDL did not affect the degradation of rat 125I-CRP by the macrophages, which suggested that the rat CRP and the modified LDL did not enter the cell as a complex. Our results suggested that the increased degradation of 125I-LDL was caused by the charge modification of 125I-LDL by rat CRP, due to a fluid-phase complex formation between 125I-LDL and rat CRP, and that the degradation involved the scavenger receptor present on the macrophages. (Arterioscler Thromb. 1994;14:282-287.)

Key Words • C-reactive protein • LDL modification • LDL binding • macrophages

Materials

Carrier-free [125I]NaI was from DuPont. Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (D-PBS), fetal calf serum (FCS), and the penicillin-streptomycin solution used in the cell incubations were from GIBCO Laboratories. Rat CRP was purified from rat plasma by using a Sepharose-phenylphosphorylcholine affinity column. LDL (d=1.019 to 1.063 g/mL) were isolated from human serum by ultracentrifugation. FCS and lipoprotein-deficient serum were heat-inactivated before use by heating to 56°C for 30 minutes. The lipoprotein-deficient serum was defibrinated by thrombin treatment. Acetyl-LDL was prepared by treating LDL with acetic anhydride. LDL and acetyl-LDL were labeled with 125I (specific activity, 200 to 500 disintegrations per minute/ng) by using Iodo-Gen (Pierce Chemical Co). Dextran sulfate (molecular weight, 500 000) was from Sigma.

Immunoelectrophoresis and Agarose Gel Electrophoresis

Immunoelectrophoresis was performed on 1% agarose gel on glass plates (11×20 cm) by using 60 mmol/L barbital buffer
were obtained in each case. All data points represent the
washed with DMEM. Before the start of an experiment, the
cells, after which 1 mL medium A was added, and the cells
were incubated at 37°C in a 5% CO₂ humidified incubator.

Preparation of Macrophage Cells

Macrophage cells were harvested from rats by peritoneal
lavage with D-PBS. The peritoneal fluid, kept on ice, was
centrifuged at 400g for 10 minutes. The cell pellet was washed
once by centrifugation and resuspended in DMEM supple-
mented with 20% (vol/vol) heat-inactivated FCS and 1%
(vol/vol) penicillin-streptomycin solution (medium A). An
aliquot of the cell suspension was placed in a hemocytometer
to check cell viability after staining with trypan blue (0.04%).
A 1-mL aliquot containing 4x10⁶ cells was used to plate
six-well tissue-culture plates that were then incubated in a
humidified 5% CO₂ incubator for 2 hours at 37°C. The plates
were washed three times with DMEM to remove nonadherent
cells, after which 1 mL medium A was added, and the cells
were incubated at 37°C in a 5% CO₂ humidified incubator.

Measurement of Degradation of LDL, Acetyl-LDL,
and Rat CRP by Macrophages

125I-LDL or 125I-acetyl-LDL was incubated with macro-
phages (4x10⁶) at 37°C for up to 48 hours, either in the
presence or absence of rat CRP. The degradation of
125I-LDL and 125I-acetyl-LDL was determined by assaying the amount of
125I-labeled trichloroacetic acid (TCA)-soluble material
(125I-moniodotyrosine) that was formed by the cells and
released into the medium.²⁷,²⁸ Briefly, 400 µL of 25% (wt/vol)
TCA was added to a 400-µL aliquot of the incubation me-
dium, followed by 400 µL of a 5% (wt/vol) silver nitrate
solution. The mixture was vortexed, held on ice for 15 minutes,
and centrifuged at 12 800g for 7 minutes in a Beckman
microfuge. The supernatant was removed, and an aliquot (400
µL) was counted for radioactivity. Control incubations were
performed in the appropriate medium in the presence of
125I-LDL but without cells, and the radioactivity obtained from
these incubations was subtracted from the experimental val-
ues. The degradation is therefore expressed as nanograms
LDL degraded per milliliter of incubation medium. At the end
of the incubation the cells were thoroughly washed with 50
mmol/L tris(hydroxymethyl)aminomethane-HCl containing
0.15 mol/L NaCl and dissolved in 0.2 mol/L NaOH and used
for protein measurement. The protein content of lipoproteins
and solubilized cells was determined by the method of Lowry
et al²⁹ by using bovine serum albumin as standard. All experi-
ments were performed at least three times, and similar results
were obtained in each case. All data points represent the
average of duplicate incubations. In the experiment to study
the effect of increasing concentrations of LDL on the degra-
dation of 125I-CRP, the degradation of 125I-CRP was measured
by assaying the amount of 125I-labeled TCA-soluble material
that was formed in the incubation medium.

Results

We have previously shown,³⁰ by gel-filtration chroma-
tography, the formation of a fluid-phase complex be-
tween rat 125I-CRP and LDL. The formation of this
complex is Ca²⁺ dependent and involves the phospho-
rylcholine binding site on CRP. Here we showed, using
immunoelectrophoresis and agarose gel electrophore-
sis, an increased anodic migration of the LDL caused by
rat CRP.

Fig 1 (top) shows the electrophoretic migration pat-
terns of LDL by agarose immuno electrophoresis as de-
scribed in "Methods." Troughs were cut into the plate to which antiserum to LDL was added and allowed to develop overnight. B, Agarose gel electrophoresis of LDL (lane 1) and rat CRP (lane 7) and LDL in the presence of increasing concentrations of rat CRP (lanes 2 through 6; lane 2, 1:1 [molar ratios of LDL:CRP]; lane 3, 1:2; lane 4, 1:4; lane 5, 1:8; lane 6, 1:16). The samples were applied to the wells and electrophoresis was carried out as described in "Methods." The antigen-antibody reaction to develop.
LDL to immobilized rat CRP, which showed 1 molar equivalent of LDL bound to 4 molar equivalents of rat CRP. The increased migration of LDL toward the anode from the center of the well was dependent on CRP concentrations. This increased migration of LDL in the presence of rat CRP was interpreted as being most likely due to a net increase of negative charge on LDL caused by the formation of a complex with the negatively charged rat CRP, which has an isoelectric point of around 3.8. A similar result (Fig 1, bottom) was obtained when mixtures of increasing molar ratios of LDL/rat CRP were chromatographed on 1% agarose gel by using the Beckman Paragon electrophoresis system. Fig 1 (bottom) shows a gradually increased migration of LDL toward the anode in the presence of increasing concentrations of rat CRP.

It is well known that chemically modified LDL with an increased negative charge is more rapidly degraded by macrophages than native LDL. It has been previously shown that dextran sulfate or proteoglycans cause a net increase in the surface charge of LDL that results in the increased degradation of LDL by macrophages. In this study we compared the degradation of LDL modified by rat CRP with that of an LDL-dextran sulfate complex. To determine if there was any increased degradation of the rat CRP–modified LDL, rat peritoneal macrophages were incubated with 125I-LDL for zero through 48 hours. The degradation of 125I-LDL in the presence of rat CRP (LDL/CRP molar ratio, 1:25) was increased by 2.5-fold, whereas the degradation of LDL–dextran sulfate (LDL/dextran sulfate [Mr = 500 000] molar ratio, 1:50) in our hands resulted in about a threefold increase in 24-hour incubations (Fig 2). This result showed that rat CRP, even at a concentration of one-half that of dextran sulfate, can cause comparable stimulation of LDL degradation. The increased degradation of LDL in the presence of rat CRP seen in this study may have some physiological significance, since the stimulation is caused by a circulating acute-phase serum protein.

When the concentration of 125I-LDL (20 μg/mL) was kept constant, the addition of increasing amounts of rat CRP (up to 125 μg) caused a dose-dependent increase in the degradation of 125I-LDL by macrophages, as shown in Fig 3C.
in the degradation of $^{125}$I-LDL, after which no further increase in the degradation was observed (Fig 3A). The effect of a fixed amount of rat CRP on the degradation of $^{125}$I-LDL was also studied as a function of increasing concentrations of $^{125}$I-LDL, and a 2.5-fold increased degradation of $^{125}$I-LDL was observed compared with the degradation without rat CRP (Fig 3B).

To determine if LDL and rat CRP enter the macrophage as a complex, the degradation of $^{125}$I-CRP by macrophages was monitored as a function of increasing $^{125}$I-CRP concentration in the presence of different amounts of LDL (Fig 3C). An almost linear rate of degradation of $^{125}$I-CRP was obtained that was not affected by the presence of up to 20 µg LDL. In a separate experiment, this rate of degradation was unaffected even by higher concentrations of LDL (up to 80 µg; results not shown). Although it is clear that with increasing concentrations of CRP the degradation of $^{125}$I-CRP was increased, the presence of up to 80 µg LDL had no effect on the degradation of $^{125}$I-CRP. These results indicated that increased degradation of $^{125}$I-LDL due to CRP was not accompanied by increased degradation of CRP. It is therefore concluded that although CRP forms a complex with LDL and imparts increased negative charge to LDL, CRP and LDL do not enter the cell as a complex. Our results are opposite to those obtained from studies that show that LDL greatly increases the uptake of $[^{3}H]$dextran sulfate by macrophages, results that led the authors to conclude that LDL and dextran sulfate enter the cell as a complex.36

In a similar context, Lindstedt et al,37 in their studies on the effect of soluble heparin proteoglycans on LDL degradation by macrophages, observe that although proteoglycans stimulate LDL degradation by macrophages, the degradation of proteoglycans is not affected by LDL concentrations. Camejo et al34-3538 suggest that the complex LDL forms with proteoglycans is reversible and is dissociated before it is taken up by macrophages. Acetylation of LDL is known to convert a weakly anionic lipoprotein into a strongly anionic one that is taken up by the macrophages via the scavenger receptor (acetyl-LDL receptor).14 In contrast to the degradation of $^{125}$I-LDL, the degradation of $^{125}$I-acetyl-LDL by macrophages was not affected by rat CRP (Fig 4). It is reasonable to assume that the net charge alteration of acetyl-LDL by rat CRP will be minimal compared with that of native LDL by rat CRP. This minimal charge alteration would not be enough to produce increased degradation of $^{125}$I-acetyl-LDL in the presence of rat CRP.

To determine if the scavenger receptor on the macrophages is involved in the rat CRP-stimulated degradation of $^{125}$I-LDL, the degradation assays were performed in the presence of increasing amounts of acetyl-LDL. The addition of increasing amounts of acetyl-LDL reduced the rat CRP-stimulated degradation of $^{125}$I-LDL by 70% (Fig 5A). This suggested that acetyl-LDL and the $^{125}$I-LDL/rat CRP complex compete for a common site on the macrophage for degradation. On the basis of this result, the degradation of $^{125}$I-LDL in the presence of rat CRP appears to involve the acetyl-LDL receptor. Experiments to study the effect of increasing acetyl-LDL concentrations (0 to 20 µg/mL) on the binding of $^{125}$I-LDL to macrophages at 4°C in the presence of rat CRP showed
that acetyl-LDL at a concentration of 20 μg/mL inhibited the binding of 125I-LDL by as much as 35% (results not shown). In a separate experiment we also studied the effect of acetyl-LDL on the degradation of 125I-CRP by macrophages. Fig 5B shows that the degradation of 125I-CRP was not affected by up to 60 μg acetyl-LDL, which indicated that the degradation of 125I-CRP does not use the acetyl-LDL receptor pathway and therefore does not support the internalization of LDL-CRP complex. More recently, we have demonstrated the binding and degradation of 125I-CRP by macrophages.

We have previously shown that the phosphorylcholine binding site on rat CRP is involved in the fluid-phase complex formation with LDL, based on our observation that phosphorylcholine inhibited the formation of the fluid-phase complex between LDL and rat 125I-CRP. Addition of phosphorylcholine up to 5 μg/mL inhibited the degradation of 125I-LDL by 70% (Fig 6). This suggests that the CRP-mediated increased degradation of 125I-LDL is due to the fluid-phase complex formation between rat CRP and LDL.

Discussion

This study provided an example in which the scavenger receptor pathway is used for the clearance of charge-modified LDL. Recent reports have shown the involvement of the scavenger receptor in the clearance of LDL modified by soluble heparin proteoglycans and LDL-proteoglycan complexes from human atherosclerotic lesions as well as the negatively charged LDL from interstitial inflammatory fluid. In contrast, the clearance of LDL/dextran sulfate complex was shown to be cleared by an alternate pathway distinct from the scavenger receptor pathway. Although there are many reports of degradation by monocytes/macrophages of either chemically modified (for review, see References 14, 17, and 42) or oxidized LDL (for review, see References 33 through 45) or LDL-proteoglycan complexes, there is practically no information on the degradation of LDL modified by a circulating acute-phase serum protein. To the best of our knowledge this is the first report in which the charge modification of LDL by an acute-phase serum protein has been demonstrated by immunoelectrophoresis and agarose gel electrophoresis and the resulting increased degradation of the modified LDL by macrophages via the scavenger receptor pathway has been shown.

Two preliminary studies report the enhanced metabolism of LDL by macrophages in the presence of human CRP, but neither of them suggests a mechanism that may be involved in the process. Our study on the effect of rat CRP on LDL degradation showed that the enhanced LDL degradation may be due to charge modification of LDL by rat CRP.

In conclusion, our results with rat CRP demonstrated the possibility of increased susceptibility for the modification of LDL during the acute-phase response resulting in the increased uptake of the modified LDL by macrophages. This could be a potential mechanism for foam cell formation in macrophages during the acute-phase response.

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References


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