C-reactive protein inhibits in vitro oxidation of low-density lipoprotein

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Abstract C-reactive protein (CRP) is elevated in cardiovascular disease and binds to oxidized phosphatidylcholine (oxPtC) in the low-density lipoprotein (LDL) surface. In the present study, we tested if CRP influences the susceptibility of LDL to oxidation. At physiological concentrations of 1–7 μg/ml, CRP strongly inhibited copper-mediated oxidation of LDL and phospholipid liposomes in a concentration-dependent manner. Similar concentrations of different monoclonal antibodies or albumin did not influence LDL oxidation. Antioxidant activity of CRP was inhibited by phosphocholine (PC), indicating that the observed activity involves binding of CRP to oxPtC. These results suggest that CRP may limit atherogenic oxidation of LDL in vivo.

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Keywords: Low-density lipoprotein; LDL; C-reactive protein; CRP; Copper-mediated oxidation; Oxidized phospholipids; Conjugated dienes

1. Introduction

C-reactive protein (CRP) is an acute phase reactant that is upregulated by microbial infections [1,2]. The protein consists of five identical subunits, each with a single binding site for phosphocholine (PC) [3]. Binding of CRP to the PC component of surface polysaccharides opsonizes invading microbes, leading to enhanced uptake of these microbes by phagocytic cells of the immune system. CRP binds to Fc-gamma-receptors [4–6] and to C1q [7], causing activation of the complement pathway. Thus, CRP serves as a critical component of the innate immune system.

Recent studies have shown that CRP also binds to the PC moiety of oxidized phosphatidylcholine (oxPtC) in the surface of apoptotic cells and oxidized LDL [8]. CRP does not bind to viable cells or non-oxidized LDL, in which the PC head groups of phosphatidylcholines are thought to be cryptic [8]. The oxPtC ligand of CRP is similar, if not identical, to the ligand of anti-PC auto-antibodies and to ligands of the macrophage scavenger receptors, CD36 and SR-B1. Hence, oxPtC appears to be a pattern recognition ligand that interacts with multiple pathways of the innate immune system [9]. Growing evidence suggests that these pathways have evolved to dispose of damaged molecular complexes, such as apoptotic cells and oxidized LDL [9].

The clearance of damaged, oxidized LDL is intimately tied to the development and progression of atherosclerosis [10]. Oxidation of LDL in the vascular intima generates a variety of bioactive lipids that induce the recruitment and differentiation of macrophages. These macrophages take up and neutralize oxidized LDL by scavenger receptor-mediated endocytosis, a process that may lead to the formation of foam cells, atherosclerotic plaques, and subsequent cardiovascular events. Thus, oxidized LDL has been identified as a major factor in the development and progression of atherosclerosis [10]. Likewise, CRP, which binds to the surface of oxidized LDL, has been linked to cardiovascular disease [11–14]. The plasma concentration of CRP correlates with the risk of myocardial infarction and stroke, and has recently emerged as a sensitive risk marker for cardiovascular events [13,14].

It is currently unclear if CRP plays an active role in the cardiovascular disease process [15,16]. Although early in vitro experiments have suggested that CRP itself is pro-atherogenic [17–20], recent studies indicate that many of the observed atherogenic events were induced by trace amounts of azide in the CRP preparation and not by CRP itself [21–24]. Furthermore, overexpression of human CRP in apolipoprotein E-deficient mice did not accelerate the development or progression of atherosclerosis [25]. Hence, a clear pro- or anti-atherogenic role of CRP remains to be established.

As CRP binds to oxPtC in the surface of oxidized LDL [8], and oxPtC-intermediates participate in oxidative chain reactions [26], we hypothesized that the binding of CRP to oxidized LDL may influence further oxidation of the lipoprotein particle. To test this hypothesis, we monitored the in vitro oxidation of LDL in the presence or absence of CRP. Our results demonstrate that physiological concentrations of CRP inhibit LDL oxidation, suggesting that the protein may limit the atherogenic oxidation of LDL in vivo.

2. Materials and methods

2.1. Materials

Human CRP (≥99% pure) was obtained from MP Biomedicals, LLC (Solon, OH). Bovine serum albumin (BSA), phosphocholine (PC), and monoclonal anti-PC antibodies (TEPC15) were purchased from Sigma Chemical Company (St. Louis, MO). Monoclonal antibodies against apolipoprotein B-100 (apoB) were purchased from Calbiochem (La Jolla, CA); 1-stearoyl-2-arachidonyl phosphatidylcholine (SAPtC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL).

Abbreviations: CRP, C-reactive protein; oxPtC, oxidized phosphatidylcholine; LDL, low-density lipoprotein; PC, phosphocholine; apoB, apolipoprotein B-100; SAPtC, 1-stearoyl-2-arachidonyl phosphatidylcholine
2.2. LDL purification

LDL (d = 1.019–1.063 g/mL) was isolated from the blood of normo-lipidemic subjects using sequential floatation ultracentrifugation [27], essentially as described by Schumaker and Puppione [28]. Purified LDL was dialyzed against phosphate-buffered saline (PBS) (i.e., 10 mM sodium phosphate, 150 mM NaCl, pH 7.4), stored under argon, and used within 7 days. Blood samples for LDL isolation were donated with informed consent under protocols that were approved by the Institutional Review Board of Virginia Commonwealth University.

2.3. Oxidation of LDL

Oxidation reactions were performed at 37 °C in 1 ml volumes of PBS, 0.5 mM CuCl₂, pH 7.4, and with purified LDL at a concentration of 50 μg protein/ml. Oxidation of LDL was catalyzed with 2 μM CuCl₂. Susceptibility of LDL to oxidation was determined by spectrophotometric detection of conjugated dienes at 234 nm, as described by Esterbauer et al. [29], by spectrophotometric detection of thiobarbituric acid-reactive substances (TBARS), as described by Morel et al. [30], and by determining the relative electrophoretic mobility of oxidized LDL in agarose gels.

Formation of conjugated dienes was monitored in quartz cuvettes, using a Lambda 20 spectrophotometer (Perkin–Elmer Corp., Norwalk, CT). The temperature of the cuvettes was maintained at 37 °C using a PTP-1 Peltier System (Perkin–Elmer Corp.). Background absorbance of PBS (containing 2 μM CuCl₂, and in some cases CRP, BSA, monoclonal antibodies, or PC) was adjusted to 0, after which LDL was added to start the oxidation reactions. Initial absorbance of unoxidized LDL (50 μg protein/ml) was between 0.2 and 0.3 AU. In experiments

Fig. 1. CRP inhibits copper-mediated oxidation of LDL in a concentration-dependent manner. In panels A–F, oxidation of LDL (50 μg protein/ml) was catalyzed with 2 μM CuCl₂. Data points represent single measurements. (A) Concentrations of 1–7 μg/ml CRP caused a progressive increase in oxidation lag time, a decrease in maximum oxidation rate, and an increase in time to reach maximum oxidation (see Table 1). (B) LDL preparations from different donors, oxidized in the absence (closed symbols) and presence (open symbols) of 4 μg/ml CRP. Open and closed symbols with the same shape refer to LDL from the same donor. (C) Triplicate incubations of a single LDL preparation, oxidized in the absence (closed symbols) and presence (open symbols) of 4 μg/ml CRP. (D) BSA, at concentrations of 10 and 20 μg/ml, had no influence on copper-mediated LDL oxidation. (E) Monoclonal antibodies against apoB (α-apoB) and phosphorylcholine (TEPC15) had no influence on copper-mediated LDL oxidation. (F) CRP inhibits the formation of TBARS (open symbols), as well as conjugated dienes (closed symbols). Circles: control incubations in the absence of additional protein; squares: control incubations in the presence of 7 μg/ml BSA; triangles: incubations in the presence of 7 μg/ml CRP.
addressing the influence of PC on CRP antioxidant activity, CRP was pre-incubated with PC for 1 h at 37 °C (in PBS) before oxidation was started by the addition of 2 μM CuCl₂ and 50 μg/ml LDL.

For TBARS assays, 500 μl of 20% trichloroacetic acid (TCA) were added to a 50 μl aliquot of LDL (50 μg/ml), either before or after oxidation in the presence or absence of BSA or CRP as described above. Samples with TCA were placed on ice for 5 min. After precipitation, 500 μl of a 1% thiobarbituric acid solution were added, and the samples were heated at 95 °C for 45 min, cooled down on ice for another 5 min, and centrifuged at 1000 × g for 20 min. Subsequently, the absorbance of the supernatant was measured at 532 nm.

Increased negative charge and electrophoretic mobility of oxidized LDL were determined in 0.5% agarose gels. LDL (~3 μg protein in a 10 μl sample) was subjected to electrophoresis for 30 min at 100 V in 0.1 M Tris-base, 0.09 M boric acid, 1 mM EDTA, pH 8.3. LDL was fixed and stained in the gel with glacial acetic acid:water:methanol (1:3:6), containing 7% Sudan Black.

2.4. Oxidation of phospholipid liposomes
SAPtC liposomes were prepared essentially as described by Barenholz et al. [31]. Briefly, 20 mg of SAPtC were dried under argon and subsequently subjected to vacuum for 2 h at 20 °C. Lipids were then resuspended in 2.4 ml of PBS, and sonified using a model 450 sonifier (Branson Ultrasonics Corp., Danbury, CT) in cycles of 2–3 min for not more than 30 min. The preparation was kept on ice for 1 min between cycles. The sonifier output control was set at 2 and the duty cycle was set at 30%. The resulting liposome suspension was subjected to centrifugation at 14,000 r.p.m. for 12 min in an Eppendorf microcentrifuge. The supernatant, containing a more homogenous liposome dispersion, was collected and assayed for phospholipid content using a spectrophotometric assay (Wako chemicals, Neuss, Germany). Copper-mediated oxidation of the liposome preparation was performed as described above for LDL, using 100 μg/ml of phospholipid and 2 μM CuCl₂ per sample.

3. Results

Fig. 1A and Table 1 show that CRP inhibits copper-mediated oxidation of LDL in a concentration-dependent manner, as measured by the formation of conjugated dienes. Physiological concentrations of 1–7 μg/ml CRP caused an increase in oxidation lag time, a decrease in maximum oxidation rate, and an increase in the time it takes to reach maximum levels of oxidation (Fig. 1A and Table 1). At a concentration of 7 μg/ml CRP, oxidation of LDL was dramatically reduced, and establishment of a typical oxidative propagation phase was completely prevented within the 350 min experimental timeframe.

The experiment of Fig. 1A was repeated multiple times with LDL preparations from different donors. Each of these experiments confirmed the dose-dependent antioxidative activity of CRP, and in each of the experiments a concentration of 7 μg/ml CRP prevented establishment of a propagation phase within the experimental timeframe (350 min). However, antioxidative kinetics of CRP concentrations lower than 7 μg/ml varied when assayed with LDL preparations from different donors (Fig. 1B). Among the three LDL samples of Fig. 1B, the increase in lag time due to the inclusion of 4 μg/ml CRP varied from 235% to 412%, the decrease in maximum oxidation rate varied from 32% to 42%, and the increase in the time it took to reach maximum levels of oxidation varied from 205% to 396%. When assayed on a single LDL sample, oxidative parameters were very reproducible, both in the absence and presence of CRP (Fig. 1C); standard deviations for the triplicate measurements in Fig. 1C ranged from 0.006 to 0.055 absorbance units.

In contrast to the strong antioxidant activity of 1–7 μg/ml CRP (Fig. 1A), BSA at concentrations of up to 20 μg/ml (Fig. 1D) and anti-apoB or anti-PC monoclonal antibodies at concentrations of 10 μg/ml (Fig. 1E) did not affect LDL oxidation. Fig. 1F shows that CRP but not BSA prevented the time-dependent formation of both conjugated dienes and TBARS when LDL was incubated with 2 μM CuCl₂. Similarly, CRP but not BSA prevented the formation of negative charges on the lipoprotein particle upon incubation with 2 μM CuCl₂ (Fig. 2).

To test whether the antioxidative activity of CRP requires the presence of apolipoprotein B-100 (apoB), i.e., the major protein component of LDL, we assessed the oxidation of protein-free SAPtC liposomes. Fig. 3 shows that, in the absence of CRP, copper-mediated oxidation of 100 μg/ml SAPtC does not have a well-defined lag phase. Instead, oxidation immediately enters the propagation phase and proceeds at a relatively constant rate until a maximum oxidation level is reached after approximately 550 min. In the presence of 2–6 μg/ml CRP, oxidation rates decreased in a concentration-dependent manner, and maximum oxidation levels were not reached within the 600 min experimental timeframe (Fig. 3A). At a CRP concen-

<table>
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<th>CRP (μg/ml)</th>
<th>Lag time (min)</th>
<th>Maximum oxidation rate (E234 nm/min)</th>
<th>Time to oxidation (max) (min)</th>
<th>Oxidation maximum (E234 nm)</th>
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<td>0.0006</td>
<td>ND</td>
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Quantitative parameters were derived from the oxidation curves of Fig. 1A. Maximum rate of oxidation [29]: slope of a straight line through the steepest part of the oxidation curve; Lag time [29]: time point at which a straight line through the steepest part of the curve (maximum rate) intersects a horizontal line through the starting value of conjugated dienes (E234 nm) in the sample (see Fig. 1A). ND: cannot be determined within the experimental time-frame.
oxidation of liposomes. (C) Monoclonal antibodies against apoB (α-apoB) or phosphorylcholine (TEPC15) had no influence on copper-mediated oxidation of liposomes. Data points in A–C represent single experiments; experiments were repeated at least 3 times with similar results.

Fig. 3. CRP inhibits copper-mediated oxidation of SAPtC liposomes in a concentration-dependent manner. Oxidation of SAPtC (100 μg/ml) was catalyzed with 2 μM CuCl₂. (A) Concentrations of 1–6 μg/ml CRP caused a progressive decrease in maximum oxidation rate, and an increase in time to reach maximum oxidation. (B) BSA, at concentrations of 10 and 20 μg/ml, had no influence on copper-mediated oxidation of liposomes. (C) Monoclonal antibodies against α-apoB (α-apoB) or phosphorylcholine (TEPC15) had no influence on copper-mediated oxidation of liposomes. Data points in A–C represent single measurements; experiments were repeated at least 3 times with similar results.

CRP strongly inhibited LDL oxidation (Fig. 4). However, a 25 μM concentration of PC strongly attenuated the inhibition, as evidenced by a decreased oxidation lag time, an increased maximum rate of oxidation, and a decreased time to reach the maximum level of oxidation (Fig. 4 and Table 2). Higher concentrations of PC (up to 100 μM), did not further inhibit the antioxidant activity of CRP (results not shown). Control incubations demonstrated that, in the absence of CRP, PC does not influence LDL oxidation rates (Fig. 4). Taken together, these results suggest that the observed antioxidant activity is specific and depends, at least in part, on the binding of CRP to oxPtC head groups in the LDL surface.

4. Discussion

Recent studies have shown that CRP is a sensitive risk marker for cardiovascular disease [13,14]. However, the precise role of CRP in the development or progression of atherosclerosis remains to be defined. The present study shows that CRP inhibits in vitro oxidation of LDL, suggesting that the protein may limit the atherogenic oxidation of LDL in vivo.

The molecular mechanism that underlies the observed antioxidant activity of CRP is unclear. However, the activity does not appear to require apoB or any minor protein component of LDL, as CRP also inhibits oxidation of a protein-free liposome preparation (Fig. 3). Furthermore, inhibition of the activity by PC (Fig. 4) suggests that CRP needs to interact with oxPtC in the LDL surface in order to exert its antioxidant effect. This suggestion is consistent with recent studies describing the binding of CRP to oxidized LDL [8]. The exact chemical nature of the oxPtC ligand is unknown [8]. Indeed, CRP may bind to a variety of oxPtC species, including reactive oxPtC intermediates that are involved in oxidative chain reactions, such as PtC-peroxiradicals and PtC-hydroperoxides [26]. Binding of CRP to these reactive oxPtC intermediates may slow their lateral movement within the LDL surface and, as a result, may limit further oxidative events. In addition, binding of CRP to reactive oxPtC species may shield these intermediates from interacting with unoxidized phospholipids, likewise, limiting the progression of oxidative chain reactions.

The concentrations of CRP used in the present study to inhibit LDL oxidation (1–7 μg/ml) are within the range of physiological concentrations that indicate increased cardiovascular risk [11,13]. In subjects without microbial infection, plasma CRP levels below 1 μg/ml indicate relatively low risk, while levels of 1–3 μg/ml indicate moderate risk, and levels above 3 μg/ml indicate high risk of cardiovascular disease [11,13]. The fact that CRP concentrations of 1–7 μg/ml inhibit LDL oxidation under aggressive in vitro conditions (Figs. 1 and 2) supports the notion that similar concentrations of the protein may be protective under milder in vivo conditions.

Consistent with an in vivo antioxidant role of CRP is the protein’s localization in atherosclerotic lesions [32–34]. Recent data show that vascular endothelial cells can be induced to express CRP [35], suggesting that the protein may be secreted directly into the extracellular environment in which LDL oxidation occurs. It is, therefore, tempting to speculate that mild oxidation of LDL induces the recruitment of CRP to the lipoprotein surface in order to prevent further oxidation of LDL, and to promote clearance of minimally oxidized lipoprotein particles by macrophages and dendritic cells. This pro-
measurements; experiments were repeated at least three times with similar results.

Quantitative parameters were derived from the oxidation curves of Fig. 4. (For definition of ‘lag time’ and ‘maximum oxidation rate’, see Table 1.)

<table>
<thead>
<tr>
<th>[CRP] (µg/mL)</th>
<th>[PC] (µM)</th>
<th>Lag time (min)</th>
<th>Maximum oxidation rate (E234 nm/min)</th>
<th>Time to oxidation maximum (min)</th>
<th>Oxidation maximum (E234 nm)</th>
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<td>25</td>
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<td>0.00720</td>
<td>240</td>
<td>1.1795</td>
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</tbody>
</table>

Quantitative parameters were derived from the oxidation curves of Fig. 4. (For definition of ‘lag time’ and ‘maximum oxidation rate’, see Table 1.)

...cess may in essence be beneficial, and only under prolonged or excessive conditions lead to atherosclerosis. Thus, high plasma concentrations of CRP, indicating high risk of cardiovascular disease [13,14], may indicate maximal engagement of a protective mechanism.

Future studies will need to establish whether the observed antioxidant activity of CRP indeed plays a significant role in cardiovascular disease and other inflammatory disorders.

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