

C-Reactive Protein–Mediated Low Density Lipoprotein Uptake by Macrophages : Implications for Atherosclerosis

Thomas P. Zwaka, Vinzenz Hombach and Jan Torzewski

Circulation. 2001;103:1194-1197

doi: 10.1161/01.CIR.103.9.1194

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2001 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:

<http://circ.ahajournals.org/content/103/9/1194>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Circulation* is online at:
<http://circ.ahajournals.org/subscriptions/>

C-Reactive Protein–Mediated Low Density Lipoprotein Uptake by Macrophages

Implications for Atherosclerosis

Thomas P. Zwaka, MD; Vinzenz Hombach, MD; Jan Torzewski, MD

Background—LDL and C-reactive protein (CRP) are important cardiovascular risk factors. Both LDL and CRP deposit in the arterial wall during atherogenesis. Stranded LDL is taken up by macrophages, causing foam cell formation. Because native LDL does not induce foam cell formation, we hypothesized that CRP may opsonize native LDL for macrophages.

Methods and Results—Monocytes were isolated from human blood and transformed into macrophages. CRP/LDL uptake was assessed by immunofluorescent labeling and the use of confocal laser scanning microscopy. Native LDL coincubated with CRP was taken up by macrophages by macropinocytosis. Uptake of the CRP/LDL coincubate was mediated by the CRP receptor CD32.

Conclusions—We conclude that foam cell formation in human atherogenesis may be caused in part by uptake of CRP-opsonized native LDL. (*Circulation*. 2001;103:1194-1197.)

Key Words: lipids ■ C-reactive protein ■ atherosclerosis

Several modifications of LDL (for example, acetylated LDL,^{1,2} oxidized LDL,³ and enzymatically modified LDL⁴) induce foam cell formation in vitro via so-called scavenger-receptor²-mediated pathways. However, the uptake of native LDL by macrophages in considerable amounts has never been demonstrated.

Recently, when inflammation was recognized as a major mechanism in atherosclerotic lesion formation,⁵ the involvement of the acute phase reactant C-reactive protein (CRP) became a matter of debate. CRP is an important cardiovascular risk factor⁶⁻⁹ and deposits in the arterial wall during atherogenesis, colocalizing with the terminal complement complex and foam cells.¹⁰⁻¹² CRP upregulates adhesion molecule expression on endothelial cells.¹³ It both opsonizes biological particles¹⁴ and binds to apolipoprotein B-containing lipoproteins (LDL and VLDL) at their Ca²⁺-dependent phosphorylcholine binding sites.¹⁵⁻²⁰ The major CRP-receptor on human macrophages has been identified as the low-affinity immunoglobulin receptor CD32.²¹ CRP-binding to CD32 is allele-specific.²²

Methods

CRP

Human CRP was purchased from Sigma. Purity and physical state were examined as described previously.¹² CRP preparations were tested by the Limulus endotoxin assay (Sigma).

LDL Uptake Assay

CRP at 900 mg/L was coincubated with native LDL (Sigma) at 1000 mg/dL in PBS containing CaCl₂ (0.132 g/L) and MgCl₂ (0.1 g/L) at room temperature for 15 minutes. The supernatant was then diluted in DMEM/10% AB-serum to a final concentration of 240 mg/L CRP and 250 mg/dL LDL. In control experiments, several lower CRP concentrations (down to 1 mg/L) were used. Before use in the LDL uptake assay, the CRP/LDL coincubate was again centrifuged at 15 000 rpm for 30 minutes to remove high molecular aggregates. A dilution with heat-inactivated 10% AB-serum (56°C for 30 minutes) was used as a control for a potential role of complement activation in our experiments. After a further 15 minutes, the coincubate was cooled to 4°C. Substitutions with PBS instead of CRP or LDL served as controls.

Monocyte Isolation

Monocytes were isolated from heparinized blood⁴ and adjusted with DMEM/10% human AB serum to a density of 1.0×10⁶ cells/mL. Cell suspensions of 50 μL per well were applied to a 4-chamber dish. Cells were cultured for 7 days at 37°C in 5%CO₂ and a medium containing 10% AB serum, which was renewed every 2 days. Macrophages were serum-starved for 12 hours, washed with PBS (4°C), and incubated with CRP/LDL coincubates or controls at 4°C for 30 minutes. The LDL uptake assay was performed by incubating cells at 37°C for stated time intervals. To block CRP-binding to CD32, control cells were incubated with aggregated IgG at 100 μg/mL.²¹ Aggregated IgG was prepared from human IgG (Sigma) by incubation at 63°C for 30 minutes at 10 mg/mL. The phosphatidylinositol₃-kinase inhibitor Wortmannin at 100 nmol/L, which is known to inhibit Fcγ receptor-dependent ingestion, was used as an additional control.

December 13, 2000; revision received January 11, 2001; accepted January 19, 2001.

From Internal Medicine II–Cardiology, University of Ulm, Ulm, Germany.

Correspondence to Jan Torzewski, MD, MPhil, Internal Medicine II, Cardiology, University of Ulm, Robert Koch-Str 8, 89081 Ulm, Germany. E-mail jan.torzewski@medizin.uni-ulm.de

© 2001 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

CD32 Polymorphism Analysis

For genetic analysis of CD32, genomic DNA was extracted from monocytes using QIAmp-Kit (Qiagen) and subjected to polymerase chain reaction using the following primers: sense, 5'-TTGGATAG-TACCTCTGAGACTG-3'; antisense, 5'-ACGTGAGGGGCTC-CAAGCTCT-3'. Genotype was assessed by DNA-sequencing of polymerase chain reaction products.

Flow Cytometry

Cells were stained for CD32 and the macrophage marker CD14 using monoclonal FITC-conjugated anti-CD32 and R-phycoerythrin-conjugated anti-CD14, both at a 1:20-dilution (Pharmingen). Cells were analyzed using Becton Dickinson FACSCalibur flow cytometer with CellQuest software. Forward and side scatter was used to gate cell population and to exclude cell debris. A minimum of 10 000 positively stained cells were analyzed. Irrelevant anti-mouse isotype-matched antibodies were used as controls.

Immunofluorescent Staining and Analysis

Monocytes were fixed in 4% formaldehyde for 20 minutes and permeabilized by 0.5% Triton X-100. Nonspecific binding was blocked with PBS/2% BSA. Cells were incubated with monoclonal anti-CRP (clone 8, Sigma) at 80 $\mu\text{g}/\text{mL}$ or with polyclonal goat anti-apoB-100 (Biosdesign) at 10 $\mu\text{g}/\text{mL}$. Cells were incubated with Indocarbocyanin-conjugated anti-mouse IgG (Jackson-Immuno-Research) at 15 $\mu\text{g}/\text{mL}$ or with Indocarbocyanin-conjugated anti-goat antibody (Alexis) at 20 $\mu\text{g}/\text{mL}$. Some samples were incubated with anti-CD32 FITC-conjugated mouse monoclonal antibody (DAKO) at 10 $\mu\text{g}/\text{mL}$ or TRITC or FITC-conjugated phalloidin (Sigma), both at 0.1 mg/mL. Finally, cells were mounted in Mowiol (Calbiochem) and visualized under confocal laser scan microscope (63 \times objective; Leica).

Results

In this study, CRP was coincubated with native LDL in the presence of calcium,^{15,16} and the coincubate was offered to human macrophages expressing the heterozygous phenotype of CD32.^{21,22} Lipoprotein uptake was assessed by confocal laser scanning microscopy. Figure 1A shows the kinetics of LDL (apolipoprotein B-100) staining. After 30 minutes, aggregates of LDL were observed under the ruffled macrophage membrane, indicating formation of LDL-containing vesicles. After 60 minutes, LDL complexes could be observed deeper within the cytoplasm, and they appeared to be more disseminated, suggesting further internalization and processing. Parallel filamentous-actin (f-actin) staining provided evidence for cytoskeletal reorganization in the region of vesicle formation. Time course and morphology of the vesicles suggested that vesicle formation was due to macropinocytosis.²³ In contrast, no vesicle formation was observed when cells were incubated at identical concentrations with native LDL alone or CRP alone. Incubation of cells with native LDL showed some background staining for LDL after 30 minutes (Figure 1B). Decrease in background stain after 60 minutes indicated intracellular LDL degradation^{1,2}.

To investigate whether CD32 is involved in vesicle formation, we analyzed CRP and CD32 staining at different time points after incubating cells with the LDL/CRP coincubate (Figure 2A). Figure 2A shows that CRP colocalizes with clusters of CD32 on cell surfaces after 10 minutes. This figure demonstrates extensive CRP capping on the macrophage surface, in analogy to the described interaction of CRP with Fc-receptors on lymphoid cells.²⁴

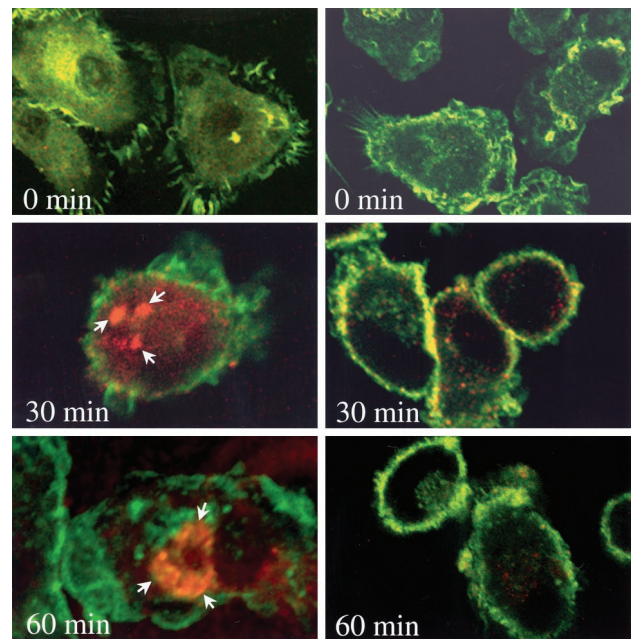


Figure 1. A, Apolipoprotein B-100 stain (red) and f-actin stain (green) after incubation of macrophages with CRP/LDL coincubate. Arrows indicate LDL-containing vesicles. After 30 minutes, vesicles can be observed under ruffled macrophage membrane. After 60 minutes, vesicles are visible deeper within cytoplasm and appear to be more disseminated. B, Apolipoprotein B-100 stain (red) and f-actin stain (green) after incubation of macrophages with LDL alone. After 30 minutes, there seems to be some disseminated apolipoprotein B-100 stain that decreases after 60 minutes, suggesting background uptake of LDL with further digestion. No vesicle formation is visible.

With further incubation, CRP/CD32 complexes become internalized (Figure 2A). The inset shows that CD32 is localized in the vesicle wall colocalizing with CRP to the vesicle lumen. This phenomenon does not occur after incubating cells with CRP alone (data not shown). Flow cytometric 2-color analysis of anti-CD32 and anti-CD14 revealed a 82.23% stain for CD32 and CD14 (with a 11.91% background stain) before incubation with CRP/LDL and a 23.63% stain for CD32 and CD14 (with a 7.82% background) after CRP/LDL incubation.

Finally, Figure 2B shows CRP and LDL staining 60 minutes after incubating cells with LDL/CRP coincubates. The figure demonstrates strict colocalization of CRP and LDL in the described vesicles. Because CRP is stained blue and LDL is stained red, vesicles containing CRP and LDL in colocalization are violet.

Further controls included heat inactivation of AB-serum and incubation in the presence of aggregated IgG or Wortmannin (data not shown). Both heat inactivation and aggregated IgG-preincubation abolished vesicle formation and CRP/LDL uptake. Wortmannin preincubation, however, markedly reduced but did not completely abolish vesicle formation. Furthermore, lower CRP concentrations (down to 1 mg/L) revealed significant reductions in but did not completely abolish vesicle formation.

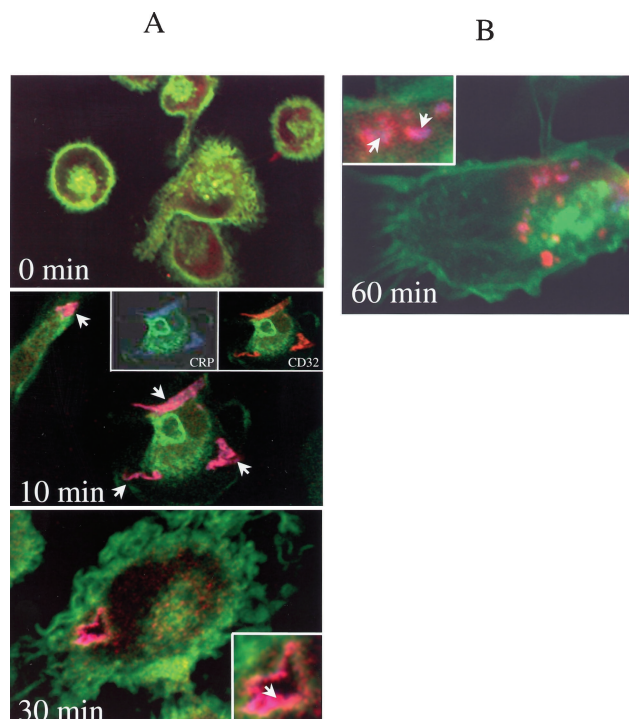


Figure 2. A, CRP-stain (10-minute inset, shown in blue) and CD32-stain (10-minute inset; shown in red) and f-actin stain (green) after incubating macrophages with CRP/LDL cocubate. Arrows indicate CRP/CD32 complexes (stained violet because of color overlap). After 10 minutes, clustering of CD32 and colocalization of CD32 clusters with CRP are visible on cell membrane. After 30 minutes, CRP/CD32 complexes have been internalized. Observed vesicles contain CD32 in vesicle wall and CRP to vesicle lumen (insert at 30 minutes). B, Apolipoprotein B-100 stain (red) and CRP stain (blue) after 60 minutes of incubating macrophages with CRP/LDL cocubate. Arrows indicate areas of CRP/LDL-colocalization. Vesicles containing both CRP and LDL in colocalization appear violet.

Discussion

In conclusion, our data describe a novel mechanism by which foam cell formation in human atherogenesis may occur. In contrast to the major former hypotheses on foam cell formation in atherogenesis,¹⁻³ our data suggest a mechanism for LDL uptake by macrophages without a need for biochemical modification of LDL. In our experiments, the acute phase reactant CRP mediated the uptake of native LDL. This effect was dependent on the presence of serum and was abolished by heat inactivation of the serum. Our finding is in line with former reports on CRP-mediated opsonization of biological particles¹⁴ and with a recent finding showing that CRP mediates its effects on endothelial adhesion molecule expression only in the presence of serum.¹⁴ Preliminary evidence from other investigators demonstrate that CD32 may cluster with other receptors, especially complement receptors (G. Schmitz, MD, unpublished observations, 2000). Because CRP is known to activate complement,¹⁰ this potentially important issue awaits further investigation. However, the involvement of other serum factors cannot be excluded.

Uptake of the CRP/LDL cocubate was mediated by CD32, as unequivocally demonstrated by colocalization of

CRP, CD32, and LDL in the vesicles and by flow cytometric analysis showing marked reduction of anti-CD32 binding with incubation time. FcR-dependence of vesicle formation is further supported by competitive inhibition through aggregated IgG. Because CRP influences reactive oxygen production by macrophages, CRP may also facilitate LDL oxidation in the atherosclerotic lesion.²⁵ The fact that CRP accumulates in lesions¹⁰⁻¹² suggests the presence of higher CRP concentrations in atherosclerotic tissue than in serum. However, CRP concentrations in the atherosclerotic lesion, which is the location of foam cell formation, are difficult to evaluate.

In view of the well-known property of CRP to opsonize biological particles for macrophages, our finding is in line with basic functions of the immune system. In light of the increasing evidence for CRP being an important cardiovascular risk factor, we suggest that CRP-binding to LDL in the human arterial wall may link LDL deposition to the onset of arteriosclerosis.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (SFB451/A4). We thank Dr D. Bowyer and Dr H. Fehling for critical reading of the manuscript.

References

1. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science*. 1986;232:34-47.
2. Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Ann Rev Biochem*. 1983;52:223-261.
3. Steinberg D, Parthasarathy S, Carew TE, et al. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med*. 1989;320:915-924.
4. Bhakdi S, Dorweiler B, Kirchmann R, et al. On the pathogenesis of atherosclerosis: enzymatic transformation of human low density lipoprotein to an atherogenic moiety. *J Exp Med*. 1995;182:1959-1971.
5. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med*. 1999;340:115-126.
6. Liuzzo G, Biasucci LM, Gallimore JR, et al. The prognostic value of C-reactive protein and serum amyloid A protein in severe unstable angina. *N Engl J Med*. 1994;331:417-424.
7. Haverkate F, Thompson SG, Pyke SD, et al. Production of C-reactive protein and risk of coronary events in stable and unstable angina. *Lancet*. 1997;349:462-466.
8. Ridker PM, Cushman M, Stampfer MJ, et al. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med*. 1997;336:973-979.
9. Koenig W, Sund M, Fröhlich M, et al. C-reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA Augsburg Cohort Study, 1984 to 1992. *Circulation*. 1999;99:237-242.
10. Torzewski J, Torzewski M, Bowyer DE, et al. C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. *Arterioscler Thromb Vasc Biol*. 1998;18:1386-1392.
11. Zhang YX, Cliff WJ, Schoeffl GI, et al. Coronary C-reactive protein distribution: its relation to development of atherosclerosis. *Atherosclerosis*. 1999;145:375-379.
12. Torzewski M, Rist C, Mortensen RF, et al. C-reactive protein in the arterial intima; role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. *Arterioscler Thromb Vasc Biol*. 2000;20:2094-2099.
13. Pasceri V, Willerson JT, Yeh ETH. Direct proinflammatory effects of C-reactive protein on human endothelial cells. *Circulation*. 2000;102:2165-2168.

14. Mortensen RF, Osmand AP, Lint TF, et al. Interaction of C-reactive protein with lymphocytes and monocytes: complement-dependent adherence and phagocytosis. *J Immunol.* 1976;117:774-778.
15. deBeer FC, Soutar AK, Baltz ML, et al. Low density lipoprotein and very low density lipoprotein are selectively bound by aggregated C-reactive protein. *J Exp Med.* 1982;156:230-242.
16. Pepys MB, Rowe IF, Baltz ML. C-reactive protein: binding to lipids and lipoproteins. *Int Rev Exp Pathol.* 1985;27:83-91.
17. Cabana GV, Gewurz H, Siegel JN. Interaction of very low density lipoprotein (VLDL) with rabbit C-reactive protein. *J Immunol.* 1982;128:2342-2348.
18. Cabana GV, Gewurz H, Siegel JN. Inflammation-induced changes in rabbit CRP and plasma lipoproteins. *J Immunol.* 1983;130:1736-1742.
19. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure Fold Des.* 1999;15:169-177.
20. Rowe IF, Soutar AK, Trayner IM, et al. Rabbit and rat C-reactive protein bind apolipoprotein B-containing lipoproteins. *J Exp Med.* 1984;159:604-616.
21. Bharadwaj D, Stein MP, Volzer M, et al. The major receptor for C-reactive protein on leukocytes is fcgamma receptor II. *J Exp Med.* 1999;190:585-590.
22. Stein MP, Edberg JC, Kimberly RP, et al. C-reactive protein binding to FcgammaRIIa on human monocytes and neutrophils is allele-specific. *J Clin Invest.* 2000;105:369-376.
23. Jones NL, Reagan JW, Willingham MC. The pathogenesis of foam cell formation. Modified LDL stimulates uptake of co-incubated LDL via macropinocytosis. *Arterioscler Thromb Vasc Biol.* 2000;20:773-781.
24. James K, Baum LL, Gewurz H. Interactions of C-reactive protein with lymphoid cells. *Ann N Y Acad Sci.* 1982;389:274-285.
25. Zeller JM, Sullivan BL. C-reactive protein selectively enhances the intracellular generation of reactive oxygen products by IgG-stimulated monocytes and neutrophils. *J Leukoc Biol.* 1992;52:449-455.