

## Monomeric C-Reactive Protein Decreases Acetylated LDL Uptake in Human Endothelial Cells

Susanne B. Schwedler,<sup>1\*</sup> Thomas Hansen-Hagge,<sup>2</sup> Matthias Reichert,<sup>1</sup> Daniel Schmiedeke,<sup>1</sup> Reinhard Schneider,<sup>1</sup> Jan Galle,<sup>3</sup> Lawrence A. Potempa,<sup>4</sup> Christoph Wanner,<sup>1</sup> and János G. Filep<sup>5</sup>

<sup>1</sup> University of Würzburg, Department of Medicine I, Würzburg, Germany; <sup>2</sup> Institute for Microtechnology, Mainz, Germany; <sup>3</sup> Klinikum Lüdenscheid, Lüdenscheid, Germany; <sup>4</sup> Acphazin Inc., Deerfield, IL, USA; <sup>5</sup> Research Center, Maisonneuve-Rosemont Hospital, University of Montréal, Montréal, Canada. \* address correspondence to this author at: Department of Medicine I, University Hospital Würzburg, Josef-Schneider-Str. 2, D-97080 Würzburg, Germany. Fax +49-931-201-36502; e-mail pelleas@t-online.de.

**BACKGROUND:** C-reactive protein (CRP) is a risk marker for cardiovascular disease and has been implicated in atherogenesis. In atherosclerotic plaques, it colocalizes with oxidized LDL (oxLDL) and promotes oxLDL uptake by macrophages, suggesting an important crosstalk between CRP and lipid processing. A growing body of evidence indicates the existence of distinct configurations of human CRP, native pentameric (nCRP) and structurally modified monomeric (mCRP), that elicit opposing bioactivities in vitro and in vivo. Here, we tested the impact of mCRP and nCRP on the uptake of acetylated LDL (acLDL), which is internalized by receptors similar to those of oxLDL in human endothelial cells.

**METHODS:** We cultured human umbilical vein endothelial cells (HUVECs) for 8 h with mCRP or nCRP (10–100 mg/L) and measured the uptake of acLDL (10–100 mg/L) over a 20-h period by FACS analysis. To assess the receptors involved, we used function-blocking antibodies against Fc  $\gamma$  receptor CD16 (Fc $\gamma$ RIII) and CD32 (Fc $\gamma$ RII), and RT-PCR analysis of CD16, CD32, and the receptor for oxidized LDL (LOX-1). Uptake of acLDL and CRP isoforms was visualized by immunofluorescence.

**RESULTS:** Culture of HUVECs with mCRP, but not nCRP, decreased uptake of acLDL, which was not prevented by anti-CD16 or anti-CD32 antibodies. LOX-1, CD16, and CD32 were undetectable by RT-PCR. Immunofluorescence showed decreased cytoplasmic acLDL staining in human umbilical vein endothelial cells (HUVECs) treated with mCRP, but not with nCRP.

**CONCLUSIONS:** Monomeric CRP, but not nCRP, decreased acLDL uptake in human endothelial cells inde-

pendent of CD16, CD32, or LOX-1. Our data support a protective role of mCRP in cardiovascular disease.

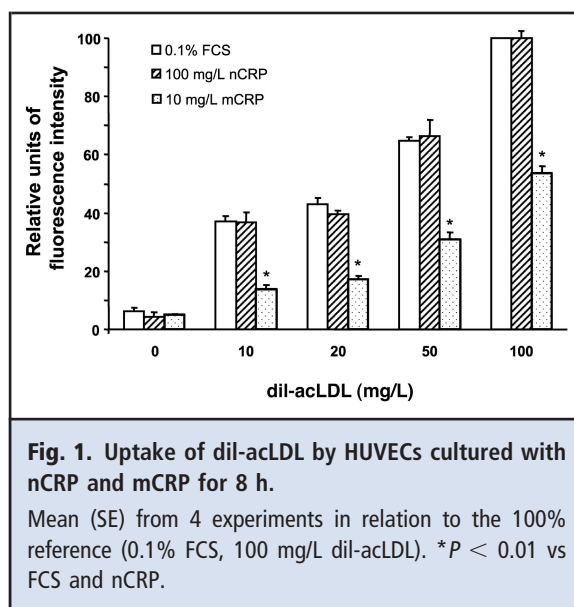
Atherosclerosis is characterized by a disorder of lipid deposition and inflammatory responses in the vessel wall. Macrophages accumulate lipids from retained LDL that undergo oxidative (oxLDL)<sup>6</sup> or enzymatic modification and are taken up via the scavenger receptor pathway [e.g., the lectin-like oxidized LDL receptor 1 (LOX-1)]. OxLDL has many proinflammatory properties. C-reactive protein (CRP) is a widely used marker of inflammation underlying human atherosclerotic disease. It may bind oxLDL by recognition of a phosphorylcholine moiety (1) and promotes uptake of oxLDL by rat macrophages in vivo (2).

Native pentameric CRP (nCRP) is found in the serum, whereas the conformationally rearranged subunit monomeric CRP (mCRP) is expressed in tissues and in the wall of normal human blood vessels (3). These distinct forms of CRP have been proposed to exert different biological activities in endothelial cells (4). We recently hypothesized that mCRP may possess antiatherogenic properties in vivo. Indeed, in *ApoE*<sup>-/-</sup> mice, a hypercholesterolemic mouse model with accelerated atherosclerosis, nCRP enhanced while mCRP prevented plaque formation in early stages of disease development (5). Endothelium-dependent relaxation was impaired in nCRP-treated but not in mCRP-treated *ApoE*<sup>-/-</sup> mice (6). CRP is thought to exert its biological effects predominantly via the Fc  $\gamma$  receptor CD32 (Fc $\gamma$ RII) (7–9), whereas the actions of mCRP can be partially blocked by anti-CD16 (Fc $\gamma$ RIII) antibodies (4). Li et al. (10) found that CRP may trigger the effects of oxLDL on endothelial cells by upregulating LOX-1. More recently, Fujita et al. (11) reported that CRP binds to LOX-1-expressing bovine endothelial cells parallel with induction of LOX-1 expression. These observations prompted us to investigate the involvement of CD16, CD32, and LOX-1 in mediating nCRP/mCRP regulation of acetylated LDL (acLDL) uptake. We chose acLDL as a model system because the effects of modified LDL (e.g., acLDL, oxLDL) may not

<sup>6</sup> Nonstandard abbreviations: oxLDL, oxidized LDL; LOX-1, lectin-like oxidized LDL receptor 1; CRP, C-reactive protein; nCRP native pentameric CRP; mCRP, structurally modified monomeric CRP; Fc $\gamma$ R, Fc  $\gamma$  receptor; acLDL, acetylated LDL; HUVEC, human umbilical vein endothelial cell; FCS, fetal calf serum; dil, 1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyaninperchlorat; dil-acLDL, dil-labeled acetylated LDL; DAPI, 4',6-diamine-2'-phenylindol-dihydrochloride; LPS, lipopolysaccharide; HAEC, human aortic endothelial cell; siRNA, small interfering RNA; HCAEC, human coronary artery endothelial cell; MAPK, mitogen-activated protein kinase; SR, scavenger receptor; SREC, SR expressed by endothelial cells; FEEL, fasciclin, epidermal growth factor (EGF)-like, laminin-type EGF-like, and link domain-containing scavenger receptor.

involve any classically defined receptor molecule. Indeed, we recently reported that oxLDL particles in lipophilic bilayers initiate an oxidative chain reaction without a mandatory aqueous phase interaction that would, for example, involve a receptor (12).

Native and monomeric CRP were generated and characterized as described (5). Pooled human LDL was obtained from healthy individuals and isolated by ultracentrifugation (13). We prepared acLDL according to Basu et al. (14). AcLDL is believed to be internalized by receptors similar to those of oxLDL, but it does not induce intracellular oxidative stress. In total, 3 different preparations of acLDL were used in the present study. We cultured human umbilical vein endothelial cells (HUVECs, passages 4–6) in accordance with the supplier's protocols (Cambrex). Confluent HUVEC monolayers were cultured for 8 h with recombinant human mCRP or nCRP [10 and 100 mg/L in 0.1% fetal calf serum (FCS)], washed, and cultured for an additional 20 h with dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyaninpercholol)-labeled acetylated LDL (dil-acLDL, 10–100 mg/L). We measured cellular uptake of acLDL in the FL2 channel of a flow cytometer (FACScalibur; BD Biosciences) and expressed the results as mean fluorescence intensity/cell after subtraction of nonspecific background. In additional experiments, HUVECs were pretreated for 30 min with anti-CD16 or anti-CD32 antibodies (Pharmingen, 2.5 mg/L) and cultured with mCRP (10 mg/L). As a control, HUVECs were cultured with lipopolysaccharide (LPS) (0.1–100  $\mu\text{g/L}$ , 24 h). We repeated all experiments at least 4 times. After 8 h of incubation, total cellular RNA was isolated from HUVECs using the RNeasy mini-kit (Qiagen) and reverse transcribed with the SMART<sup>TM</sup> PCR cDNA synthesis kit (Clontech Laboratories, Inc.). We performed PCR to amplify cDNAs using the following primers: 5'-TAG GGC TCC GGA TAT CTT TG-3'; 5'-TCT TGA GGG TCC TTT CTC CA-3' (CD16, 804 bp); 5'-TTG ACA GTT TTG CTG CTG CT-3'; 5'-GTC GTT GGG AGG AAG AGT CA-3' (CD32A, 996 bp); 5'-TTG CCA CTG AGA GTG ACT GG-3'; 5'-GGT CAC AGG CTT GGA TGA GT-3' (CD32B+C; 604 bp); 5'-ATG TTT GGC ACC CAA GTG AC-3'; and 5'-CGT GAC TGC TTC ACT CTC TCA-3' (LOX-1, 948 bp). The size of the PCR product was confirmed by agarose gel electrophoresis. In addition, positive controls for CD32A and LOX-1 were cloned and transformed in *E. coli* using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit; Qiagen) and sequenced. Sequence analysis showed complete amplification of CD32A and LOX-1. For immunofluorescence localization of CRP isoforms, we incubated HUVECs with mCRP or nCRP (10 mg/L, 8 h) for acLDL staining followed by an incubation with dil-acLDL (50 mg/L,



20 h), yielding a red fluorescence signal. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. After blocking with 10% goat serum, cells were incubated with mouse monoclonal antibodies directed against mCRP (clone 9C9) and nCRP (clone 1D6) as described (1 h, 37 °C) (15), followed by an incubation with goat antimouse Cy 2 (carbocyanin 1:200, 30 min at room temperature; Dianova) yielding a green fluorescence signal. Nuclei were stained with DAPI (4',6-diamine-2'-phenylindol-dihydrochloride, blue signal; Roche) added 1 h before the end of incubation. Controls included FCS-treated cells stained for CRP, nCRP-treated cells stained with the anti-mCRP antibody, and vice versa. We took photographs with identical exposure time for each staining. Corresponding DAPI and CRP staining was overlaid using Adobe Photoshop (Extended CS3; Adobe Systems). Data were expressed as mean (SE). We performed statistical evaluation using 1-way ANOVA followed by Tukey test for multiple comparisons. Values of  $P < 0.05$  were considered statistically significant.

We observed a dose-dependent uptake for acLDL by HUVECs. Culture of HUVECs with 10 mg/L nCRP (data not shown) and 100 mg/L nCRP (Fig. 1) did not produce significant changes in acLDL uptake ( $P > 0.37$ ). In contrast, culture with 10 mg/L mCRP resulted in a marked inhibition of acLDL uptake ( $P < 0.01$ ) (Fig. 1). LPS (0.1–100  $\mu\text{g/L}$ ), had no detectable effects (data not shown). Pretreatment of HUVECs with antibodies against CD16 and CD32 did not affect mCRP suppression of acLDL uptake (Table 1). Our RT-PCR assays did not detect expression of CD16, CD32A, or

**Table 1. Effect of antibodies against CD16 or CD32 on the impact of FCS and mCRP on the uptake of dil-acLDL.<sup>a</sup>**

	dil-acLDL, mg/L				
	0	10	20	50	100
FCS, 0.1%	5 (0)	49 (3)	56 (7)	76 (4)	100
+ anti-CD16	5 (0)	43 (5)	51 (9)	69 (9)	94 (4)
+ anti-CD32	4 (1)	39 (7)	44 (5)	75 (5)	98 (0)
mCRP, 10 mg/L	4 (1)	23 (5)	26 (6)	40 (4)	52 (4)
+ anti-CD16	4 (1)	23 (5)	26 (6)	40 (4)	52 (4)
+ anti-CD32	4 (1)	23 (3)	29 (5)	41 (3)	59 (5)

<sup>a</sup> Data are mean (SE) from 4 experiments in relation to the 100% reference (0.1% FCS, 100 mg/L dil-acLDL). *P* > 0.33 between groups of FCS, and *P* > 0.53 between groups of mCRP at various concentrations of dil-acLDL.

LOX-1 (see Supplemental Fig. 1, which accompanies the online version of this article at [www.clinchem.org/content/vol55/issue9](http://www.clinchem.org/content/vol55/issue9)) or CD32B or CD32C (data not shown). Immunofluorescence showed intensive acLDL staining with LDL-containing vesicles within the cytoplasm of control cells. Monomeric CRP, but not nCRP, produced an overall cytoplasmic reduction of acLDL signal. Untreated (control) and nCRP-treated HUVECs did not stain for mCRP or nCRP. In contrast, HUVECs cultured with mCRP stained positive for mCRP (online Supplemental Fig. 2).

Human atherosclerosis displays many of the characteristics of chronic inflammation. Because CRP has been reported to promote oxLDL uptake in vitro and in vivo, and distinct CRP isoforms exist, we hypothesized that mCRP and nCRP may have different effects on acLDL uptake by endothelial cells. We observed that mCRP reduced uptake of acLDL, whereas nCRP had no effects. How endothelial cells recognize and respond to CRP is a matter of debate. In the present study, function-blocking antibodies against CD16 and CD32 failed to affect mCRP effects, consistent with the lack of expression of CD16 and CD32 mRNA. These results contradict those of Devaraj et al. (7), who demonstrated CRP binding to both CD32 and CD64 on human aortic endothelial cells (HAECs). Liang et al. (9) found that a small interfering RNA (siRNA) against CD32 prevented CRP-induced upregulation of adhesion molecules in HUVECs and HAECs. Khreiss et al. (4) found that mCRP promoted a proinflammatory phenotype in human coronary artery endothelial cells (HCAECs) through a p38 mitogen-activated protein kinase (MAPK)-dependent mechanism, which could be partially attenuated by an anti-CD16 antibody. We could not detect LOX-1 mRNA in HUVECs. This is in

contrast to the observations of Li et al. (10), who reported that CRP enhanced LOX-1 mRNA concentrations in HAECs. A possible explanation for this apparent discrepancy is that mCRP may upregulate expression of other scavenger receptors, such as scavenger receptor (SR)-AI/II, SR-B1, CD36, CD68, SR expressed by endothelial cells (SREC), and FEEL [fasci- clin, epidermal growth factor (EGF)-like, laminin-type EGF-like, and link domain-containing scavenger receptor 1]. More recently, Fujita et al. (11) found that siRNAs for LOX-1 markedly, though never completely, attenuated binding of CRP to bovine endothelial cells. This would suggest CRP binding to other receptors or other molecules currently not defined as receptors. Further experiments disrupting caveolae and lipid rafts are needed to address the underlying mechanisms. The immunofluorescence experiments provide a potential explanation for the mCRP action. We found that mCRP attenuated acLDL staining, and most obvious reductions in staining occurred in areas close to the cellular membrane. Monomeric CRP staining localized to cellular membranes may indicate that mCRP prevents movement of modified LDL through the cell membrane. Because mCRP can directly insert into membranes (16), it may affect membrane fluidity and/or receptor-mediated endocytosis processes. Of note, Ji et al. (17) reported that mCRP-induced activation of HAECs and HCAECs was predominantly due to mCRP insertion into lipid rafts rather than direct binding to FcγRs or proteoglycans.

Our study has some limitations. We assessed CD16, CD32, and LOX-1 expression at the mRNA and not at the protein level. However, experiments with function-blocking antibodies would argue against the expression of functional CD16 and CD32 receptors. Also, specific membrane probes would be needed to localize the mCRP signal exactly to the membrane or intracellular or extracellular matrix. We cannot exclude the possibility that dissociation of nCRP into mCRP might have occurred during fixation, although the lack of staining for mCRP in HUVECs treated with nCRP would make this possibility unlikely. Finally, additional studies are needed to determine whether our observations can be extended to other endothelial cell types.

In conclusion, our results indicate that mCRP but not nCRP prevents uptake of modified LDL by endothelial cells, independent of CD16, CD32, and LOX-1. We suggest that endothelial cells exposed to mCRP may better resist entry of acLDL. These findings can help explain the effectiveness of mCRP treatment in reducing progression of atherosclerotic plaque in *ApoE*<sup>-/-</sup> mice.

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