

C-Reactive Protein Upregulates Complement-Inhibitory Factors in Endothelial Cells

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Background—Because complement-mediated vascular injury participates in atherosclerosis and C-reactive protein (CRP) can activate the complement cascade, we sought to determine whether CRP affects the expression of the protective complement-inhibitory factors on the cell surface of endothelial cells (ECs).

Methods and Results—Human coronary artery or human saphenous vein ECs were incubated with CRP (0 to 100 $\mu\text{g/mL}$, 0 to 72 hours), and the expression of the complement-inhibitory proteins decay-accelerating factor (DAF), membrane cofactor protein (CD46), and CD59 were measured by flow cytometry. Incubation with CRP resulted in a significant increase in the expression of all 3 proteins. CRP-induced upregulation of DAF required increased steady-state mRNA and de novo protein synthesis. The increased expression of complement-inhibitory proteins was functionally effective, resulting in significant reduction of complement-mediated lysis of antibody-coated human saphenous vein ECs.

Conclusions—These observations provide evidence for a possible protective role for CRP in atherogenesis. (*Circulation*. 2004;109:833-836.)

Key Words: endothelium ■ atherosclerosis ■ proteins

The inflammatory marker C-reactive protein (CRP) represents one of the strongest independent predictors of vascular death in several settings.^{1,2} Originally suggested to play the role of a biomarker, CRP seems to be a mediator of atherosclerosis.³ CRP elicits a multitude of effects on endothelial biology that favor a proatherosclerotic phenotype, such as decreasing NO release,⁴ upregulating adhesion molecules,⁵ stimulating vascular smooth muscle cell proliferation and migration,⁶ and activating the complement system.⁷

The complement system is a complex cascade of enzymes and regulatory proteins that normally participate in host defenses against microorganisms via opsonization, chemotaxis of leukocytes, cell lysis, and cell activation.⁸ However, complement activation also has been proposed to participate in both the initiation and progression of atherosclerosis.⁹ This is supported by the presence of activated complement components in atherosclerotic plaques, such as the membrane attack complex (MAC, C5b-9), which promotes cellular activation, upregulates adhesion molecules, stimulates chemokine secretion, and can cause cell lysis.¹⁰

To prevent host cell damage, nucleated cells have developed membrane-bound regulators of complement activation. Among these regulatory proteins is decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), which binds to and facilitates the degradation of C3b and

C4b, and CD59, which inhibits C5b-9.¹¹ DAF prevents the formation and accelerates the decay of the C3 and C5 convertases that act early within the complement cascade,¹¹ functioning to maintain vascular integrity as a key protector against complement-mediated cell lysis.¹²

The classical pathway of complement may be activated by CRP bound to enzymatically degraded low-density lipoprotein.¹³ The effect of CRP on the expression of complement-inhibitory factors is unknown. Because complement-inhibitory proteins protect the endothelium from complement-mediated injury and CRP is proatherogenic, in part because of its ability to activate complement, we hypothesized that the detrimental effect of CRP may involve the downregulation of these protective proteins. Instead, we show that CRP stimulates DAF expression on endothelial cells and thus may protect these cells from complement-mediated cell injury.

Methods

Cell Culture

Human saphenous vein endothelial cells (HSVECs) were isolated from vein segments obtained from patients undergoing bypass surgery and grown in MCDB-131 complete medium (VEC Technologies Inc) supplemented with 10% FBS (Gibco). Approval was obtained from the University of Toronto Research Ethics Board. Human coronary artery endothelial cells (HCAECs), purchased from Clonetics, were cultured in EGM-2 media (Clonetics) and used at

Received July 8, 2003; de novo received October 30, 2003; accepted December 31, 2003.

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

DOI: 10.1161/01.CIR.0000117087.27524.0E

passages 2 to 3. Human recombinant CRP (Trichem Resources Inc) was used in all studies as described.

Flow Cytometry

The effects of CRP on DAF, CD46, and CD59 protein expression were determined using flow cytometry. HSVECs with or without CRP treatment were detached using nonenzymatic cell dissociation solution (Sigma) and stained for DAF, CD46, and CD59 using monoclonal R-phycoerythrin-conjugated anti-human DAF, anti-human CD46, or anti-human CD59 (DAKO). Cells were analyzed using a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. The fluorescence intensity of 10 000 cells for each sample was quantified. Unstained cells were used as controls. Mean fluorescence intensity from each group was calculated and represented as percent above control.

DAF mRNA Analysis

Total cellular RNA was isolated using the RNeasy mini kit (Qiagen). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Qiagen OneStep RT-PCR Kit. Total RNA 1 μ g served as template for each reaction. For amplification, a primer pair specific for human DAF (sense primer, 5'-TGATCTGCCTTAAGGGCAGTCAATGGT-3'; antisense primer, 5'-TACAATAAATAGAGTGCTCTCCAATCA-3') was used. RT was performed at 50°C for 30 minutes. For PCR, 35 cycles were used at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The RT-PCR products were visualized on 1% agarose gels using ethidium bromide. GAPDH was amplified as a reference. To determine the dependence of DAF expression on gene transcription, HSVECs were pretreated with actinomycin D (5 μ g/mL, Sigma) for 30 minutes before the addition of CRP (50 μ g/mL). To investigate whether CRP-induced DAF gene transcription required synthesis of a transactivating factor, HSVECs were preincubated for 30 minutes with cycloheximide (3 μ g/mL, Sigma) before the addition of CRP (50 μ g/mL). In both cases, DAF expression, as an indicator for mRNA stability and synthesis, was analyzed using flow cytometry.

Cell Lysis Assays

HSVECs were cultured overnight in 24-well plates (1×10^5 /well) at 37°C before the addition of CRP (50 μ g/mL) or medium alone for 24 hours. The cells were then incubated for 30 minutes at 37°C in medium containing 7 μ mol/L calcein-acetoxymethyl ester (Calcein AM, Molecular Probes). After washing in M199/1% BSA, HSVEC monolayers were opsonized with anti-Endoglin (CD105) Ab (DAKO) for 30 minutes at 37°C and then incubated with 5% to 20% baby rabbit complement (Serotec) in MCDB-131 with or without CRP (50 μ g/mL) for 45 minutes at 37°C. The supernatant from each well was then transferred to a 96-well plate (Costar). After washing in M199/1% BSA, the calcein AM remaining in the cells was released by incubation with 250 μ L of M199/1% BSA/0.1% Triton X-100. The lysate was then transferred to a 96-well plate, and the calcein AM released by complement and detergent was estimated using a CytoFluor 4000 fluorescence plate reader (PerSeptive Biosystems). The percentage of specific lysis in triplicate wells was calculated as complement-mediated release/maximal release multiplied by 100%, where maximal release is the complement-mediated release plus detergent-mediated release of calcein AM.

Statistical Analysis

Group data are expressed as mean \pm SEM. Data were compared between experimental groups by the unpaired *t* test or by 1-way ANOVA followed by Tukey honest significance difference. Differences were considered significant at a value of $P < 0.05$.

Results

CRP Upregulates the Expression of Complement-Inhibitory Factors on Human Endothelial Cells

Exposure of HSVECs or HCAECs to CRP resulted in a significant increase in DAF expression over baseline. For

HSVECs, a significant increase in DAF expression was observed after treatment with CRP at concentrations of 50 and 100 μ g/mL (Figure 1A). In contrast, for HCAECs, a significant upregulation of DAF expression was obtained at a lower CRP dose, 5 μ g/mL, which more closely resembles physiological CRP levels (Figure 1B). To determine the kinetics of CRP-induced DAF expression, HSVECs were cultured in the presence of CRP (50 μ g/mL) for up to 72 hours. After CRP treatment of HSVECs, DAF mRNA was first detected after 6 hours, with mRNA levels peaking at 9 hours (Figure 1C). By 24 hours after stimulation with CRP, DAF mRNA levels returned to baseline. A significant increase in DAF expression was first detected at 16 hours after incubation with CRP, being 2-fold above baseline levels after 72 hours (Figure 1D). CRP also had an effect on CD46 and CD59 expression. Incubation of HSVECs with CRP resulted in a significant increase in the levels of CD46 and CD59 on the cell surface (Figure 1E).

CRP-Induced DAF Expression Requires Increased mRNA and De Novo Protein Synthesis

The increase in DAF expression after treatment with CRP depends on gene transcription. Pretreatment of HSVECs with actinomycin D, which inhibits transcription, completely inhibited CRP-induced cell-surface DAF expression (Figure 2A). CRP-induced DAF expression requires synthesis of a transactivating factor, as indicated by impaired DAF mRNA and cell-surface expression after pretreatment of HSVECs with cycloheximide (3 μ g/mL) for 30 minutes (Figures 2B and 2C). Incubation with cycloheximide alone led to a superinduction of steady-state DAF mRNA, consistent with previous reports.¹² Addition of both CRP and cycloheximide led to a rise in steady-state DAF mRNA similar to that observed with cycloheximide alone, which was significantly lower than DAF mRNA levels after only CRP treatment (Figure 2B). Similar results were obtained when examining cell-surface DAF expression (Figure 2C). Together, these observations suggest that CRP upregulates DAF expression through de novo protein synthesis by stimulating gene transcription that depends on the synthesis of 1 or more intermediary proteins.

CRP-Induced Expression of Complement-Inhibitory Factors Protects HSVECs From Complement-Mediated Injury

To assess whether the elevated levels of DAF expression conferred protection from complement-mediated injury, both treated and untreated cells were exposed to complement after opsonization. Opsonization of HSVECs with a mAb against endoglin, which is highly expressed on the surface of HSVECs, provides complement with a target to bind to and become activated. Before exposure to baby rabbit serum, the HSVECs were loaded with calcein AM, the release of which serves as a marker for cell lysis. Pretreatment of HSVECs with CRP for 24 hours resulted in a significant reduction in complement-mediated cell lysis at all serum concentrations (Figure 2D). These results suggest that the increased levels of cell-surface complement inhibitors observed in response to CRP may provide protection against complement-mediated injury.

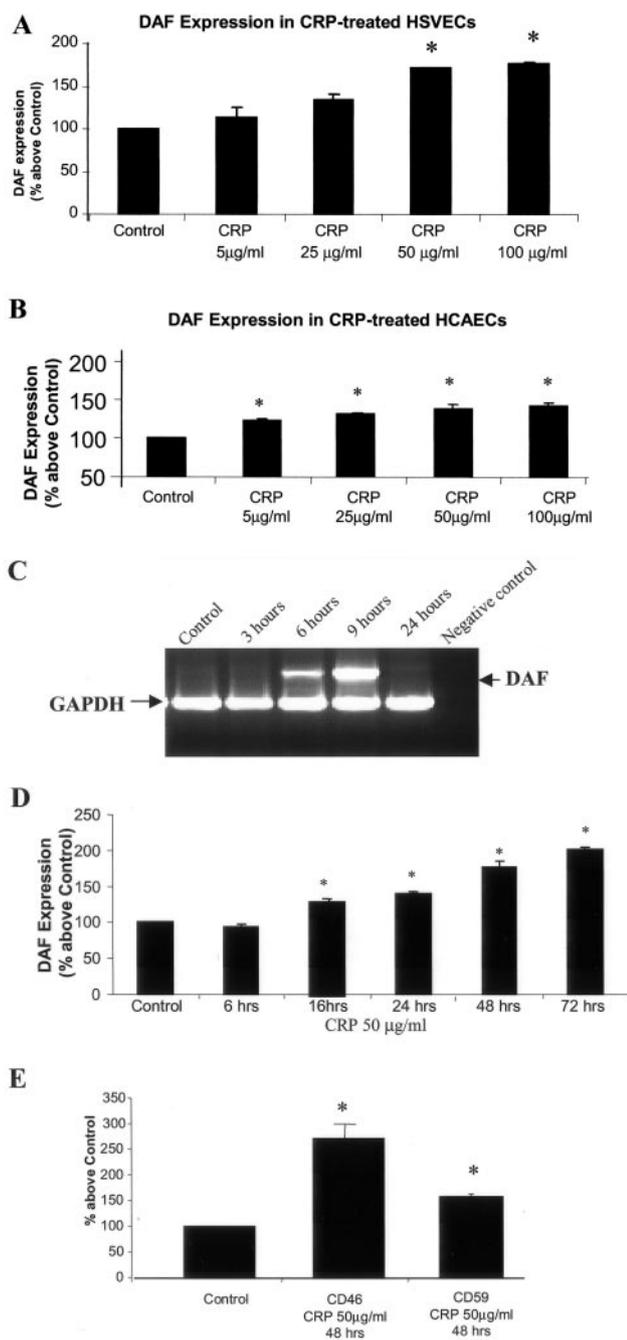


Figure 1. Analysis of DAF, CD46, and CD59 expression on ECs after stimulation by CRP. A, HSVECs were incubated for 24 hours with CRP at the concentrations shown and analyzed by flow cytometry for DAF expression. B, HCAECs were incubated for 48 hours with CRP at the concentrations shown and analyzed for DAF expression. C, HSVECs were incubated with CRP (50 µg/mL) for 3, 6, 9, and 24 hours. Total RNA was isolated, and DAF mRNA expression was analyzed by RT-PCR. Results are representative of 3 independent experiments. Negative control represents the RT-PCR reaction without the addition of total RNA. D, HSVECs were incubated for up to 72 hours with CRP (50 µg/mL). DAF expression was analyzed by flow cytometry. E, After the stimulation of HSVECs with CRP (50 µg/mL) for 48 hours, the expression of CD46 and CD59 was detected by flow cytometry. * $P < 0.05$ vs control. All data are expressed as mean \pm SEM; $n = 3$ per group.

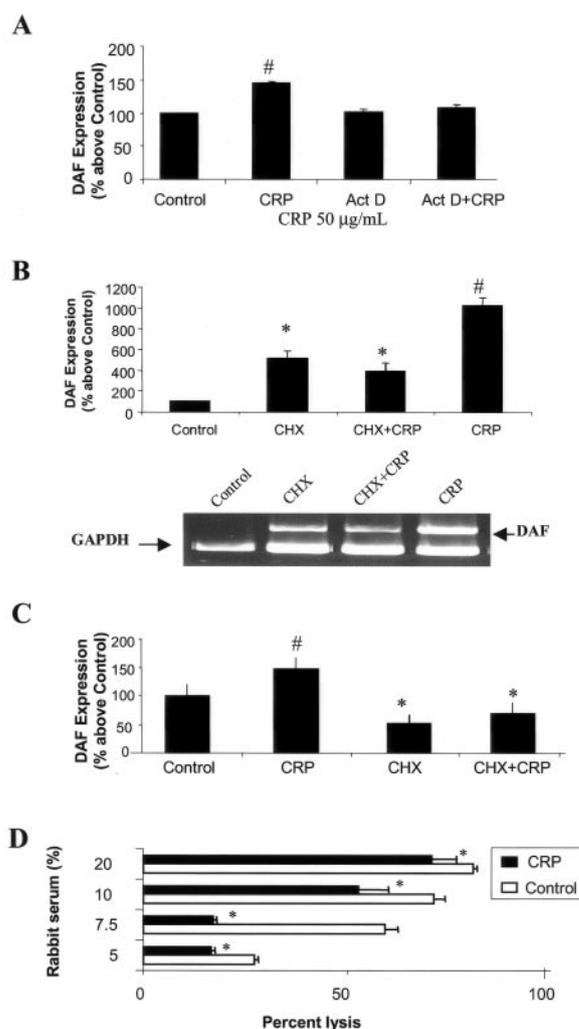


Figure 2. A, HSVECs, preincubated with or without actinomycin D (Act D, 5 µg/mL) before the addition of CRP, were cultured for up to 24 hours. DAF expression was analyzed by flow cytometry. B, HSVECs were incubated with cycloheximide (CHX, 3 µg/mL) in the presence and absence of CRP for 9 hours. Total RNA was isolated, and DAF mRNA expression was analyzed by RT-PCR. Results are representative of 3 independent experiments. C, HSVECs were preincubated with or without CHX (3 µg/mL) before the addition of CRP and cultured for up to 24 hours. DAF protein expression was assessed by flow cytometry. D, HSVECs were incubated for 24 hours with CRP before conducting the cell lysis assay. Percent cell lysis was calculated. CRP was added at a concentration of 50 µg/mL for all experiments. Values are mean \pm SEM, $n = 3$ to 4. * $P < 0.05$ vs control; # $P < 0.05$ vs all other groups.

Discussion

CRP seems to be not only a biomarker for atherosclerosis but also a mediator of plaque formation.³ By binding to enzymatically degraded low-density lipoprotein, CRP is able to activate the classical pathway of complement,¹³ serving as a potential link between complement activation and atherosclerosis.^{9,10} To protect against complement-mediated cell lysis, nucleated cells express complement inhibitor proteins on their surface. By upregulating the expression of these proteins in endothelial cells, CRP may serve to protect ECs from complement-mediated injury.

The ability of CRP to bind to nucleated cells and cause complement activation without cytolysis¹⁴ has been largely attributed to its ability to recruit the inhibitory plasma protein factor H.¹⁵ However, our results indicate that CRP may play a more active, protective role by stimulating the expression of DAF, CD46, and CD59 in endothelial cells. The kinetics of DAF expression were analyzed in greater detail because DAF seems to be the most useful molecule in terms of protecting cells from complement-mediated injury.¹⁶

The temporal changes observed in DAF mRNA and protein expression after culture with CRP were similar to the changes reported after stimulation with vascular endothelial growth factor,¹⁷ tumor necrosis factor- α , and interferon- γ .¹⁸ Our data suggest that CRP-induced expression of DAF depends on the synthesis of an intermediate transcriptional activator and that the observed increase in DAF expression is functionally relevant, as indicated by the reduction in complement-mediated cell lysis. However, unlike vascular endothelial growth factor, tumor necrosis factor- α , interferon- γ , or statins,^{12,17,18} CRP was also able to induce the expression of the additional complement inhibitors CD46 and CD59, suggesting that the changes mediated by CRP arise through a combination of signaling pathways. Although not tested, the ability of CRP to activate mitogen-activated protein kinases and the transcription factors nuclear factor- κ B and activator protein-1,¹⁹ linked to increased complement inhibitor expression,^{12,17,18} may be responsible for the increase in DAF, CD46, and CD59 observed after CRP stimulation. DAF and other complement-inhibitory proteins have been identified in atherosclerotic lesions, although there is no increase in their expression when complement activation occurs.²⁰ Thus, despite the apparent protective role of CRP in the face of complement-mediated lysis, the overall detrimental effects of CRP on several facets of atherosclerotic lesion initiation and progression seem to dominate. Another limitation to the present study is that deposition of complement proteins on cells has effects other than cell lysis, including calcium mobilization and enzyme release. The influence of CRP on these processes was not assessed.

Furthermore, debate about the role of CRP as a marker versus a mediator of disease still remains. The concentration of CRP at 50 μ g/mL, used to elicit a response in the present study, is greater than that used for clinical risk prediction. However, it is important to point out that comparisons between circulating levels of CRP and concentrations of CRP used in vitro cannot be made easily because plasma CRP concentrations do not indicate what occurs at the cellular level. Recently, it has been suggested that concentrations of CRP may be higher locally in the vessel wall and thus be at a sufficient concentration to promote atherogenesis.²¹

In conclusion, CRP upregulates complement-inhibitory proteins and protects ECs from complement-mediated cell injury. A balance of proatherogenic and antiatherogenic

effects of CRP on the vessel wall may be important in the development of atherosclerosis.

Acknowledgments

This study was supported by the Heart and Stroke Foundation of Ontario (to Drs Mickle, Li, and Verma) and the Canadian Institutes of Health Research (to Drs Mickle and Verma). Dr Wang is supported by Chang Gung Memorial Hospital, Taiwan.

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