C-Reactive Protein–Induced In Vitro Endothelial Cell Activation Is an Artefact Caused by Azide and Lipopolysaccharide

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Objective—C-reactive protein (CRP) has been proposed to be an independent risk factor for cardiovascular disease. In vitro studies investigating the mechanism behind this have used purified commercial CRP (cCRP) and endothelial cells. We investigated the role of contaminants in cCRP preparations.

Methods and Results—Human umbilical vein endothelial cells and the human endothelial cell line EA.hy926 were incubated with Escherichia coli–derived cCRP, in-house–generated azide-free recombinant, and ascites-purified CRP, azide, or lipopolysaccharide (LPS) equivalent to the concentration present in cCRP preparations. Cells were investigated for change in cell proliferation, morphology, apoptosis, and expression of endothelial NO synthase and intercellular adhesion molecule-1. Cell supernatants were assessed for monocyte chemoattractant protein-1 (MCP-1), interleukin-8, von Willebrand factor secretion, and pH change. Only cCRP was able to induce all activation events analyzed; however, this ability was lost on extensive dialysis, suggesting that low molecular weight contaminants were responsible for these events. Indeed, the effects of cCRP were mirrored by azide or LPS.

Conclusions—We investigated a wide range of effects on endothelial cells ascribed to CRP; however, azide and LPS, but never CRP itself, were responsible for the cell activation events. We conclude that CRP, per se, does not activate endothelial cells. (Arterioscler Thromb Vasc Biol. 2005;25:1225-1230.)

Key Words: C-reactive protein ■ endothelial cells ■ azide ■ LPS

C-reactive protein (CRP) has been suggested to be an independent risk factor for cardiovascular disease.1–3 The mechanism by which CRP might contribute to cardiovascular events is not known, and numerous in vitro studies have tried to address this question using commercial CRP (cCRP) and cultured endothelial cells (ECs). Reported effects of CRP on ECs include increased expression of adhesion molecules,4–8 decay accelerating factor (DAF),9 and secretion of chemokines,4–8 reduction in expression of endothelial NO synthase (eNOS),5,14,15 reduced secretion of von Willebrand Factor (vWF),7 inhibition of cell proliferation, reduction of cell viability, and increased apoptosis.14–17 How CRP initiates these events is unknown.

In vitro studies using cCRP preparations have been criticized for the lack of robust controls performed to assess whether the effects observed were not attributable to contaminants in the preparations.18,19 Because of the concern regarding the possible contamination of CRP with endotoxin and other additives, we generated our own recombinant CRP (rCRP; expressed in Chinese hamster ovary [CHO] cells)20 and also used CRP purified from human ascites (aCRP) using standard techniques.21 We reported previously that our own CRP preparations have the ability to bind to its natural ligands phosphorylcholine (PC) and complement component C1q.20 We were unable to reproduce the vasorelaxation reported by Sternik et al22 or the increased expression of DAF as reported by Li et al.9 In contrast, using E coli–derived cCRP, we were able to reproduce the effects reported by Sternik et al and Li et al, but we found that these effects were attributable totally to the presence of azide in the cCRP and could be mimicked by the addition of azide alone. Furthermore, the effects were lost on dialysis of the cCRP.20,23 Having acknowledged that some of the effects on cells of the vasculature attributed to CRP were caused by azide in the cCRP preparations, we carefully analyzed the literature and found only 2 studies in which it was specified that azide-free CRP was used. In these studies, no increased adhesion of monocytes to human umbilical vein ECs (HUVECs) was observed,24 and only a very small increase in interleukin-8 (IL-8) and MCP-1 secretion was found.25 Another study that aimed to remove endotoxin contamination using ion exchange chromatography unwittingly may also have removed...
the azide. In this study, the effect of CRP on IL-6 and MCP-1 secretion was almost completely abrogated.13

To assess whether the artifacts caused by azide, reported previously, were limited to increased vasorelaxation and increased expression of DAF or extended to other events attributed to CRP, we investigated the effects of cCRP and its possible contaminants azide and lipopolysaccharide (LPS) on a wide range of endothelial functions. We compared the effects of cCRP with in-house–generated azide-free rCRP and aCRP with extensively dialyzed cCRP. All the effects of cCRP on EC functions were removed on dialysis, and the majority of the effects could be mimicked by the addition of azide, whereas some effects could be mirrored by the addition of LPS.

Materials and Methods

Antibodies and Reagents

Commercial preparations of recombinant human E coli–derived CRP were obtained from Calbiochem. Suppliers of antibodies were Vaclav Horeji: monoclonal antibody (mAb) α-human intercellular adhesion molecule-1 (ICAM-1; MEM-111); Transduction Laboratories: mAb α-tubulin (CP06); DAKO: polyclonal antibodies α-vWF (A0082 and P02260) and goat anti-mouse Ig fluorescein isothiocyanate (GAM-FITC); R & D Systems: α-MCP-1 (ABAB79 and BAP279); and Jackson ImmunoResearch: goat α-rabbit Ig horseradish peroxidase (HRPO) and rabbit α-mouse Ig HRPO (RAM-HRPO). IL-8 ELISA kit was from Biosource and annexin V–FITC was from R & D Systems; LPS E coli 0111:B4 was from Sigma; and human tumor necrosis factor-α was from PeproTech. PBS was composed of: 145 mmol/L NaCl, 50 mmol/L NaF, 20 mmol/L Na4P2O7, 1 mmol/L CaCl2, 0.05% (wt/vol) azide. To remove the azide from the cCRP, 1 mg was dialyzed twice against 500 mL of the Tris/NaCl/CaCl2 buffer.20 All possible contaminants azide and lipopolysaccharide (LPS) on cCRP were similarly affected as the cCRP treated cells (data not shown). Cells were incubated in the presence of our own azide-free rCRP, aCRP, or dialyzed CRP (dCRP) and gate whether these effects were indeed induced by CRP, using polyclonal α-MCP-1 and polyclonal α-vWF as capture antibodies and biotinylated α-MCP-1 antibody or α-vWF followed by streptavidin HRPO; assays were developed with orthophenylenediamine (DAKO).

Alamar Blue

Cells were incubated in normal culture medium supplemented with 10% Alamar blue (Serotec) for 2 hours, after which cell supernatant was removed and fluorescence measured at 544 nm excitation and 590 nm emission.

Statistics

Statistical analysis was performed using the unpaired t test or by 1-way ANOVA, followed by the Tukey multiple comparison test, using a CI of 95%. Differences were considered significant at values of P<0.05. All experiments were performed ≥3 times in triplicate. Several studies reported that CRP inhibits cell proliferation, reduces cell viability, and induces apoptosis.14–16 To investigate whether these effects were indeed induced by CRP, cCRP, our own rCRP, aCRP, or dialyzed CRP (dCRP) and azide were investigated for their ability to modulate viability of HUVECs and EA.hy926.

Azide in cCRP Induces Change in Cell Morphology and Inhibition of Cell Proliferation and Viability

Although HUVECs cultured for 2 days at confluence in normal medium showed the typical cobblestone appearance, the cCRP made the cells appear flatter, whereas the nuclei became more elevated (Figure 1A). HUVECs cultured in the presence of our own azide-free rCRP, aCRP, or dCRP showed a similar morphology to control cells, whereas cells cultured in the presence of azide equivalent to that present in cCRP were similarly affected as the cCRP treated cells (data not shown). HUVECs, seeded at a low density and cultured for 6 days, showed a reduced cell density after culturing with
azide-containing samples, demonstrating that azide and not CRP inhibited cell proliferation (Figure 1B). Cells cultured at low density were less susceptible to the effects of azide than cells grown to confluence.

Analysis of annexin V–FITC binding by HUVECs after culturing for 2 days at confluence with CRP or azide, showed that azide, but not our rCRP or aCRP, induced a large increase in the percentage of annexin V–positive cells (Figure 1C). Analysis of these cells for uptake of PI, as a marker of cell death, showed that the majority of cells that bound the annexin V–FITC (88%) were also positive for PI, indicating that azide had induced cell death. (Figure 1D)

Figure 1. Azide in cCRP is responsible for reduced cell proliferation, change in cell morphology, and increased apoptosis and cell death. HUVECs (A through D) or EA.h926 cells (E) were incubated with various CRP preparations and azide. Incubations: control (medium alone; --); rCRP (R); aCRP (A); cCRP (C); azide (Az); or CRP and azide (R+Az). A, Phase contrast light microscopy of confluent HUVECs cultured for 2 days. B, Cell counts of HUVECs seeded at low density and cultured for 6 days. D indicates dCRP. C, Annexin V–FITC binding of HUVECs cultured for 2 days. D, PI uptake by HUVECs cultured for 2 days; E, pH of tissue culture supernatants of EA.h926 cells cultured for 1 day. Data points marked with asterisks were statistically different from the control treatment: **P < 0.01; ***P < 0.001.

A change in color of the cell supernatants of EA.h926 cells was observed when cells were cultured in the presence of azide-containing samples. When the pH of the supernatant was measured after overnight incubation with azide-containing samples, a 1-log decrease in pH was observed (Figure 1E).

Azide in cCRP Reduces eNOS Expression

eNOS plays an important role in EC functioning. Several studies reported a decrease in eNOS expression induced by CRP.5,14,15 Incubation of the EA.h926 cells with various CRP preparations with or without azide followed by Western blotting showed that after exposure to cCRP or azide, but not to our own azide-free rCRP or aCRP, eNOS expression was dramatically reduced, whereas the housekeeping protein α-tubulin remained unaltered (Figure 2). Azide also induced a 68% reduction in eNOS mRNA expression (Figure 2). Similar to the EA.h926 cells, azide-free CRP had no effect on eNOS protein expression in HUVECs, whereas azide

Figure 2. Azide in cCRP is responsible for reduced eNOS expression. EA.h926 cells and HUVECs were incubated for 24 hours with medium alone (--); 50 µg/mL of the following CRP preparations: rCRP (R); aCRP (A); cCRP (C); azide (Az; 50 µg/mL CRP equivalent); rCRP and azide (R+Az; 50 µg/mL CRP equivalent), phorbol 12-myristate 13-acetate (P; 10^{-7} M); or azide equivalent to that found in cCRP concentrations of 20, 40, 70, and 100 µg/mL, respectively. Cell lysates were analyzed by Western blotting (W.blot) using anti-eNOS and anti-α-tubulin. RT-PCR of EA.926 was performed using eNOS and GAPDH-specific primers.
added to the cells equivalent to 20 to 100 μg/mL cCRP nearly completely abrogated eNOS protein expression. eNOS in EA.hy926 cells was in the dimer form (molecular weight [Mr] 270 kDa), whereas eNOS in HUVECs was predominantly in the dimer form, but some monomer (Mr 135 kDa) was also detected (Figure 2).

LPS in cCRP Is Responsible for Increased ICAM-1 Expression

Several studies have reported an increase in expression of adhesion molecules, including ICAM-1 induced by cCRP.\textsuperscript{1–8} However, we only observed an increase in ICAM-1 expression on HUVECs with cCRP but not with our rCRP (Figure 3). Extensive dialysis of the cCRP removed the ICAM-1-inducing activity, but azide or a combination of azide and our rCRP did not induce ICAM-1. ICAM-1 induction was not found with all batches of cCRP, and when a batch of cCRP that induced ICAM-1 expression was analyzed for LPS content using the Limulus essay, it was found that this batch contained 16 ng/mL LPS, whereas our rCRP contained <55 pg/mL. When the amount of LPS equivalent to that found in cCRP was added to HUVECs, an increase in ICAM-1 expression was observed, which was further increased by the addition of azide equivalent to that present in this cCRP batch.

Azide in cCRP Decreases vWF Secretion, Whereas LPS Is Responsible for Increased IL-8 and MCP-1 Secretion

CRP has been reported to increase secretion of the chemokines IL-8 and MCP-1,\textsuperscript{6,10,12,13} whereas secretion of vWF has been reported to be reduced.\textsuperscript{7} Analyses of HUVEC supernatants after incubation with various preparations of CRP or azide showed that cCRP and azide reduced the expression of vWF, but that azide-free CRP had no effect (Figure 4A).

Analyses of these supernatants for secretion of MCP-1 (Figure 4B) and IL-8 (Figure 4C) showed that only cCRP induced secretion of these chemokines; however, this could not be mirrored by adding azide to the cells or adding azide to the rCRP. Dialysis of the cCRP, before addition to HUVECs, abolished the increased secretion of IL-8 and MCP-1, demonstrating that in this case, again, a low molecular weight contaminant was responsible for this observation. A possible candidate for this low molecular weight contaminant is LPS, and indeed, addition of LPS, equivalent to that found in the cCRP batch, did increase secretion of these chemokines by HUVECs.

Discussion

In this present study, we investigated the validity of the numerous responses of ECs attributed to CRP. We demonstrate here that none of the reported effects we investigated can be ascribed to CRP. We were only able to show effects of CRP preparations on ECs using E coli–derived cCRP, which is used in nearly all studies reporting an effect of CRP on ECs. These cCRP preparations contain high concentrations of azide (0.05% to 0.1%/mg CRP; as stated in manufacturer catalogs, websites, product information, and on the reagent tubes), and because they have been generated in E coli, they also may contain LPS and other bacterial products. Extensive dialysis of the cCRP abrogated all its effects on HUVECs or the EC line EA.hy926, demonstrating that all these events purportedly induced by cCRP can be ascribed to low molecular weight contaminants present in cCRP preparations. Our own rCRP and aCRP, which were purified in an identical manner to the cCRP, and dialyzed cCRP were indistinguishable from the undialyzed cCRP,\textsuperscript{20} excluding the possibility that changes in structure accounted for the inability of dialyzed cCRP or our rCRP and aCRP to induce EC activa-
tion. We show here that a large number of the effects induced by cCRP, including reduction in cell viability and cell proliferation, reduction in eNOS expression, and reduction in secretion of vWF, can be attributed to and mirrored by azide addition. Secretion of chemokines IL-8 and MCP-1 and expression of adhesion molecule ICAM-1 were also prevented by dialysis of cCRP; however, addition of azide did not induce the same events. Analysis of LPS contamination of a batch of cCRP that induced ICAM-1 (not all batches were able to do so) showed high levels of LPS, and addition to this concentration of LPS to HUVECs induced ICAM-1 expression and chemokine secretion.

Some studies specify the need for the presence of human serum in order for CRP to activate ECs5,4,10; however, most other published studies have been performed in the absence of human serum, and we did not observe a difference in experiments performed in the presence or absence of human serum. In our studies, we chose to use a concentration of 50 μg/mL of CRP, which is used in most studies. We also used higher (up to 200 μg/mL) and lower (10 μg/mL) concentrations, and only in the case of undialyzed cCRP effects on HUVECs and EA.hy926 were observed, which were dose dependent.

A study by Nagoshi et al aimed to separate possible endotoxin contamination from the cCRP by ion exchange chromatography.13 In this study, the azide may have been removed as well. The purified CRP in this study indeed lost its ability to induce IL-6 and MCP-1 secretion, which these authors ascribed to removal of LPS and which is in agreement with our findings (Figure 4B and 4C). Two studies (in addition to our previous reported studies) specify the use of azide-free CRP.24,25 When we heat-inactivated cCRP, it retained its ability to increase DAF expression, which we have shown could be attributed to azide.23 None of the studies ascribing an effect of cCRP on ECs performed a control experiment investigating the effect of the buffer in which the CRP was supplied on these cells.

LPS is a well-known activator of ECs, but how azide activates ECs is not clear. Azide is an inhibitor of cytochrome oxidase, and it affects metabolic functions of cells and is thus, in general, toxic for cells, which explains the inhibition of cell proliferation and induction of cell death. We have not investigated further the mechanism of how azide induces the events described in this article because it is irrelevant to how CRP affects endothelial function.

The question remains then: Does CRP really contribute to cardiovascular events, or is it merely a marker of ongoing inflammation? Several stages of atherosclerosis can be distinguished: the initiation of atherosclerosis, development of the plaque, and rupture of the plaque resulting in atherothrombosis and ischemia after occlusion, resulting in tissue damage. There is only clear evidence that CRP plays an enhancing role in ischemia-induced tissue damage. In vivo studies using rats showed that CRP enhances myocardial tissue damage and cerebral infarct size after ischemia and that this damage is dependent on complement.26,27 CRP has also been found to colocalize with complement-activation products after myocardial infarction.28 There is no clear evidence that CRP plays a role in any of the other stages of atherosclerosis. An in vivo study has shown that apolipoprotein E knockout mice expressing human CRP had enhanced aortic atherosclerosis but only after turpentine induced challenging, which constitutes a major inflammatory insult.29 Only a marginal difference in the atherosclerosis of unstimulated mice in this study was observed, suggesting that CRP does not play a significant role in this model. We suggest that CRP can indeed contribute to cardiovascular events but most likely only to acute events such as ischemia, and we suggest that experimental in vitro conditions using cultured cells have not met the conditions required for CRP “activation.” CRP can bind to a variety of substances; the best defined is probably PC.30 Although this is normally not exposed on the cell surface, in ischemia-damaged tissue and in the necrotic core of instable plaques, PC and other ligands (including, for example, oxidized low-density lipoprotein (LDL)) will be accessible to CRP. On binding to its ligand, CRP attains the ability to bind complement component C1q, which can initiate complement activation.31 In turn, complement activation products can then activate ECs, cells in the atherosclerotic plaque, and ischemic tissue, thus initiating and exacerbating cardiovascular events.32 CRP is found frequently in atherosclerotic plaques;33 however, after interaction with apoptotic cells or enzymatically modified LDL, CRP has also been shown to limit complement activation to the level of the C3-convertase, preventing the generation of the potential harmful membrane attack complex of complement.33,34 It was suggested that in this case, CRP may even have a cardioprotective role.34 In the in vitro investigations using cultured vascular cells, the initiating step of CRP binding to healthy cultured ECs has simply not been met, which explains the absence of any effect of CRP in our in vitro studies.

In conclusion, we have not found a single direct effect of CRP on ECs, and we therefore hypothesize that all other studies in which cCRP preparations have been used are most likely artifacts attributable to azide or LPS contamination. And indeed, in a recent study, it was shown that induction in smooth muscle cells of inducible NOS attributed to CRP was also caused by azide in cCRP preparations.35 The results of studies in which cCRP preparations have been used must be interpreted with great care; however, it is beyond our scope to reinvestigate them all.

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References


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