Proapoptotic, Antimigratory, Antiproliferative, and Antiangiogenic Effects of Commercial C-Reactive Protein on Various Human Endothelial Cell Types In Vitro

Implications of Contaminating Presence of Sodium Azide in Commercial Preparation


Abstract—Recent experimental studies suggest C-reactive protein (CRP) may be a potential mediator of atherosclerosis and its complications. However, there is growing criticism of in vitro CRP studies that use commercial CRP preparations containing biologically active contaminants. The effects of commercial CRP, dialyzed commercial CRP (dCRP) to remove azide, and sodium azide (NaN₃) alone at equivalent concentrations to the undialyzed preparation were tested at varying concentrations on human umbilical vein endothelial cells (HUVEC), circulating endothelial outgrowth cells (EOC), and endothelial progenitor cells (EPC) in vitro. CRP and NaN₃ alone exhibited equivalent concentration-dependent, proapoptotic effects on HUVEC, EOC, and EPC (P<0.01 versus control), whereas dCRP had no such effect. Similarly, CRP and NaN₃ alone caused equivalent concentration-dependent decreases in migration, proliferation, and matrigel tube formation (P<0.01 versus control) in EOC and HUVEC, whereas dCRP had absolutely no effect on these biological functions at any of the concentrations used. We conclude that proapoptotic, antiproliferative, antimigratory, and antiangiogenic effects of this commercial CRP preparation on a number of endothelial cell phenotypes in culture may be explained by the presence of sodium azide in this preparation. This study has implications for interpretation of in vitro studies using CRP preparations containing azide at equivalent or higher concentrations. (Circ Res. 2005;97:135-143.)

Key Words: CRP ▪ sodium azide ▪ endothelial progenitor cells ▪ apoptosis

C-reactive protein (CRP), an acute phase reactant, belongs to the highly conserved pentraxin family of plasma proteins and serves as a pattern recognition molecule for innate immunity.¹ There is increasing clinical and emerging experimental evidence for CRP, not only as a strong independent predictor of cardiovascular events,²⁻⁶ but also as a potential mediator of atherosclerosis and its complications.⁷⁻⁸ For instance, CRP has recently been shown to upregulate expression of proatherogenic molecules such as interleukin (IL)-6, endothelin-1,⁹ and monocyte chemoattractant protein in endothelial cells,¹⁰ and to mediate low-density lipoprotein (LDL) uptake in macrophages.¹¹ Nevertheless, there continues to be debate as to the mechanism whereby CRP contributes to the pathology of cardiovascular disease. Moreover, there is emerging criticism of the use of commercial preparations of CRP for in vitro studies when such preparations are incompletely defined and frequently contain biologically active contaminants.¹²⁻¹⁴

The purpose of the current study was to evaluate specific biological effects of commercially available CRP on human endothelial cells and human endothelial progenitor cells (EPC) in culture. A second aim was to determine whether the effects of CRP on apoptosis, migration, proliferation, and in vitro endothelial tube formation were mediated specifically by CRP or by a contaminant in the commercial CRP preparation. We report here that the in vitro proapoptotic, antimigratory, antiproliferative, and antiangiogenic effects of a well-described commercial CRP are caused by the presence of sodium azide in this preparation.

Materials and Methods

Materials

Commercial recombinant human CRP at a concentration of 1 mg/mL was purchased from Calbiochem (236608) supplied in 20 mmol/L Tris-HCl pH 7.5, 140 mmol/L NaCl, 2 mmol/L CaCl₂, and 0.05% sodium azide (NaN₃). To remove NaN₃ from the commercial CRP preparation, 1 mL of the CRP was dialyzed twice against 500 mL of the same buffer (Tris/NaCl/CaCl₂, without NaN₃) at 4°C using a slide-a-lyzer (Pierce 66425) with a cutoff of 10 000 Da. The concentrations of CRP in the commercial and dialyzed CRP (dCRP)
preparations used for all experiments were 0, 1, 5, 10, 50, or 100 μg/mL for some assays. NaN₃, obtained from Sigma (s-2002), was prepared in the same buffer as the commercial CRP (Tris/NaCl/CaCl₂) at a concentration of 0.05% NaN₃. The concentrations of NaN₃, used for all experiments were equivalent to those in the commercial CRP preparation after serial 0, 1, 5, 10, 50, 100 μL/mL dilutions of the starting 0.05% NaN₃ preparation.

For mononuclear cell (MNC) isolation, ficoll-plaque Plus (17-1440-02) from Amersham Biosciences was used. Immunofluorescent staining and FACS analysis of human EOC, endothelial outgrowth cells (EOC), and human umbilical venous endothelial cells (HUVEC) was performed using the following primary antibodies to CD34 (Santa Cruz, SC-7324): CD31 (Santa Cruz, SC-8006), VECAD (Santa Cruz, SC-66458), Flik-1 (Santa Cruz, sc-6251), Tie-2 receptor (Santa Cruz, SC-324), endothelial nitric oxide synthase (eNOS; Transduction Laboratory, N30020), von Willebrand Factor (vWF; Dako, A0082), and anti-mouse Alexa488 (Molecular Probes, A11001). Anti-rabbit (Molecular probes, A11008) or anti-goat FITC (Dako, F0250) antibodies were used for secondary detection. For each of the primary antibodies, isotype specific mouse IgG, rabbit IgG, and goat IgG were used as a control (R&D Systems, MAB002, AB-105-C, AB-108-C). AcLDL and isoctenin GS-IB₄ were purchased from Molecular Probes (L-3484 and I-21411), and CRP (Sigma, C1688) were used for either secondary or primary detection. For secondary detection, goat anti-mouse IgG, rabbit horseradish peroxidase (HRP) antibodies (Calbiochem, 401215 and 401393) were used for secondary detection.

Methods

Quantification and Functional Analysis of Commercial and Dialyzed CRP Solutions

The concentration of CRP before and after dialysis was measured with a highly sensitive method on a Hitachi 912 chemistry analyzer by a polystyrene particle enhanced immunoturbidimetric assay, using the manufacturer’s instructions. The pre- and postdialyzed CRP (dCRP) at specific dilutions was also semi-quantified on SDS-PAGE gel with the commercial CRP as standard. The function of commercial CRP and dCRP preparations was evaluated by the phosphorylcholine precipitation method previously described. The same quantities of eluted CRP and dCRP from PC-beads were mixed with protein loading buffer, boiled for 5 minutes, and run on a 12% SDS-PAGE gel. The gel was Coomassie Brilliant Blue stained for a visualization of specific protein bands.

In a separate experiment, the relative quantities of pentameric versus monomeric CRP were determined in commercial and dialyzed CRP preparations using 7% nonreducing PAGE with analysis via Coomassie staining and Western blot analysis.

Quantification of Endotoxin in Commercial and Dialyzed CRP Solution

The level of endotoxin in CRP preparations before and after dialysis was determined as previously described by using Limulus Amebocyte Lysate pyrogen (Bio-Whittaker, N189-125) following manufacturer’s instructions.

Quantification of Sodium Azide in Commercial and Dialyzed CRP Solution

Commercial CRP and sodium azide preparations at equivalent azide concentrations to those present in the commercial CRP preparation were analyzed for NaN₃ content by ion-exchange chromatography (West Coast Analytical). Dialyzed CRP preparations were similarly analyzed for NaN₃ content.

Isolation of Human EOC

MNC were isolated from 50 mL of peripheral blood buffy coat from each healthy donor using a ficoll gradient centrifugation method. After isolation, MNC were seeded at 10 million cells per well on collagen type I coated 12-well plates and cultured for 3 to 4 weeks in EGM-2 (Clonetics) media. Typical cobblestone shaped colony forming units were identified at 3 to 4 weeks after initial seeding. EOC were expanded by passage, and cells were used for experiments between passage 4 to 6.

Isolation and Immunophenotyping of Human EPC

Human EPC were grown in culture from human blood MNC as previously described. Immunophenotyping by FACS analysis was performed on day 3 after plating as previously described. Additional staining with Dil-AcLDL, isocotenin, and DAPI was also performed according to established methodology.

Immunophenotypic Characterization of Human EOC and HUVEC

Morphological appearance and indirect immunofluorescence, as well as FACS analysis, were used to characterize human EOC and HUVEC as previously described. Expression of intracellular eNOS was also determined by Western blot analysis of cell lysates.

Apoptosis Assays

The proapoptotic effects of CRP, dCRP, and NaN₃ on EOC and HUVEC were determined by Western blot analysis of procaspase 3 and 9, and XIAP. EOC and HUVEC were placed in 6-well plates in 5% FBS–enriched EGM-2 for 24 hours and then treated with CRP, dCRP, and NaN₃, as described above in EGM-2 containing 2% FBS for another 48 hours. Treated cells were lysed in modified RIPA lysis buffer. The protein extracts were size-separated on 12% SDS-PAGE and then Western blot analysis for procaspase 3 and 9 and XIAP was performed.

In a separate analysis, apoptotic morphological characteristics of cells incubated in the presence or absence of CRP, dCRP, or NaN₃ on NUNC plates (4×10⁶ cells per well) were determined by 4',6-diamidino-2-phenylindole (DAPI) staining after 4% formaldehyde fixation. The percentage of apoptotic nuclei was quantitated using the established morphological criteria. The typical condensed apoptotic nuclei were counted as a percentage of the total nuclei using a Zeiss Axioplan microscope.

Cell Number and Apoptosis Assays for Human EPC

Human MNC were plated on fibronectin-coated plates as previously described in the presence of CRP, dCRP, or NaN₃ on NUNC plates (4×10⁶ cells per well) were determined by 4',6-diamidino-2-phenylindole (DAPI) staining after 4% formaldehyde fixation. The percentage of apoptotic nuclei was quantitated using the established morphological criteria. The typical condensed apoptotic nuclei were counted as a percentage of the total nuclei using a Zeiss Axioplan microscope.

Matrigel Tube Formation Assay

A matrigel tube formation assay was used to determine the effects of the CRP, dCRP, and NaN₃ on the in vitro angiogenesis potential of EOC and HUVEC. After CRP, dCRP, or NaN₃ pretreatment for 24 hours, EOC and HUVEC on standard culture plates were resuspended in EGM-2 with varying concentrations of CRP, dCRP, or NaN₃ and added onto matrigel-coated wells at an approximate seeding density of 4×10⁶ cells per well. Cells were incubated for 6 hours at 37°C and tube formation was assessed (counting the number of tubes per low powered field) by grid counting method and photographed with a phase contrast microscope.

Cell Proliferation

The rate of cell proliferation of EOC and HUVEC was determined using a BrdU incorporation assay kit (Roche, 1647229). EOC and HUVEC were placed on 96-well plates at a seeding density of 3×10⁴ cells per well in EGM-2 for 24 hours, and were then treated with CRP, dCRP, or NaN₃ for another 24 hours before applying BrdU
labeling solution. BrdU incorporation was detected according to manufacturer’s instructions.  

**Migration Assay**

Cell migration was determined using a scratch assay as previously described. EOC and HUVEC were seeded on 6-well plates to reach 100% confluence within 24 hours and then treated with CRP, dCRP, or NaN3. Subsequently, a similarly sized scratch was made across the center of each well and immediately imaged at baseline, 12, 24, and 36 hours, respectively, under a KS400 light microscope. The rate of cell migration was determined by comparing the sizes of scratch area using an Image J program.

**Statistical Analysis**

All data are presented as the mean ± SEM. Comparison between groups was made using one-way ANOVA. A probability value of P ≤ 0.05 was considered statistically significant. All experiments were performed in triplicate on at least 3 separate occasions.

**Results**

**Immunophenotyping of HUVEC and EOC**

EOC and HUVEC were grown in culture as described previously. EOC grew out from peripheral blood MNC as polygonal cell clusters with endothelial morphology at ≈ 21 days after initial culture of blood MNC on collagen type I matrix (Figure 1A and 1B). These endothelial outgrowth colonies were expanded and stained positive for CD31 and
VECAD (Figure 1C through 1F) and showed surface antigen expression of c-kit, eNOS, Flk-1, CD34, and Tie-2 receptor (Figure 1K). Similarly, HUVEC showed strongly positive immunoreactivity for CD31, VECAD, eNOS, Flk-1, CD34, and Tie-2 and were weakly positive for c-kit (Figure 1G through 1J and 1L).

Quantitation of Sodium Azide and CRP and Characterization of CRP Conformation and Function in Experimental CRP Preparations

To determine that similar concentrations of sodium azide were present in the commercial CRP and sodium azide preparations, quantitative analysis of sodium azide was performed by direct measurement in solution using ion exchange chromatography. Sodium azide analysis of commercial CRP and sodium azide preparations showed near identical azide concentrations on chromatography (Figure 2A and 2C). The concentrations of azide in each preparation were within 10% of the coefficient of variation in the mmol concentration range (6.8 mmol to 7.3 mmol; Figure 2A and 2C). However, sodium azide was universally undetectable in dialyzed CRP preparations when measured by ion exchange chromatography (Figure 2B).

To determine that equal concentrations of CRP were present in commercial and dialyzed CRP preparations, analysis of CRP in each solution was performed using a highly sensitive immunoturbimetric assay. Near identical CRP concentrations were detected in random equivalent dilutions of both preparations (Figure 2D). Moreover, subsequent 500-fold dilution of starting concentrations revealed equivalent bands on Western blot analysis (Figure 2E).

To ensure that dialysis of the commercial CRP preparation had no negative effects on the CRP protein, the function of commercial CRP and dCRP preparations was analyzed by phosphorylcholine precipitation method as described previously. Both commercial and dialyzed CRP preparations showed equivalent ability to bind completely to a natural ligand such as phosphorylcholine (Figure 2F). Moreover, on nonreducing PAGE, all CRP in the commercial and dialyzed preparations was in the pentameric form with no detection of monomeric CRP in either preparation by Coomassie staining of the native gel or Western analysis (Figure 2G). The level of endotoxin detected in commercial and dialyzed CRP preparations was <10 IU/mL for both solutions.

CRP Preparation Effects on Indices of Apoptosis

Apoptosis of EOC and HUVEC was analyzed by morphological criteria and use of DAPI staining to identify chromatin condensation and evidence of apoptotic nuclei. Commercial CRP preparations caused a dose-dependent increase in apoptosis of EOC and HUVEC (Figure 3A through 3D). Importantly, this proapoptotic effect was completely abrogated in the CRP preparations after dialysis to remove sodium azide (Figure 3A through 3D). In addition, the proapoptotic effect of the commercial CRP preparations could be completely recapitulated in a dose-dependent manner using identical...
concentrations of sodium azide as contained in the commercial preparation (Figure 3A through 3D).

In a second analysis of CRP apoptosis effects, procaspase 3 and 9 immunoreactivity on Western blot was evaluated in EOC and HUVEC treated with commercial CRP preparations, dCRP, and NaN₃ alone. A dose-dependent decrease in procaspase 3 and 9 immunoreactivity on Western blot was detected in commercial CRP and sodium azide alone treated EOC and HUVEC after exposure to both commercial CRP and NaN₃. However, dCRP has no effect at any concentration on intracellular eNOS, XIAP, procaspase 3 or 9 protein levels. Equal protein loading is confirmed by β-actin controls in all immunoblots. The concentrations of NaN₃ are 0, 0.5, 2.5, 5, 25 ng/mL, respectively, in A and C and none in B.

**Figure 4. Effect of CRP/dCRP/Nan₃ on procaspases, XIAP, and eNOS protein.** Western blot demonstrating similar dose-dependent decrease in eNOS, procaspase 3 and 9, and XIAP in human EOC and HUVEC after exposure to both commercial CRP and NaN₃. However, dCRP has no effect at any concentration on intracellular eNOS, XIAP, procaspase 3 or 9 protein levels. Equal protein loading is confirmed by β-actin controls in all immunoblots. The concentrations of NaN₃ are 0, 0.5, 2.5, 5, 25 ng/mL, respectively, in A and C and none in B.

**Effects of CRP on Human EPC**

EPC were cultured on fibronectin as previously described and analyzed at day 3. These cells can be identified by the presence of a number of immunophenotypic markers (Figure 5A). To test the effects of commercial CRP, dialyzed CRP, and sodium azide preparations on EPC, human blood MNC were seeded on fibronectin in the presence or absence of all 3 preparations. Commercial CRP caused a dose-dependent reduction in the number of EPC identified at 3 days after initial MNC seeding (Figure 5B). Dialyzed CRP had no such effect on EPC numbers (Figure 5B). Importantly, sodium azide at similar concentrations to those found in the crude commercial CRP preparation induced a similar dose-dependent reduction in EPC numbers (Figure 5B). Crude commercial CRP preparation also caused a dose-dependent increase in apoptosis of EPC (by morphological and caspase criteria), and this was recapitulated by sodium azide used at identical concentrations to those found in the commercial preparation (Figure 5C and 5D). Importantly, in EPC, dialyzed CRP again had no such effect on either index of cellular apoptosis in EPC (Figure 5C and 5D).

**CRP Effects on In Vitro Tube Formation on Matrigel**

Matrigel tube formation assays are an established model for study of in vitro angiogenic capacity of endothelial-like cells. To assess the effects of CRP on angiogenesis in vitro, a matrigel tube formation assay was performed on EOC and HUVEC incubated with EGM-2 in the presence or absence of varying concentrations of commercial CRP, dCRP, and NaN₃ alone. Capillary tube formation was potently inhibited in a dose-dependent manner by commercial CRP preparation (Figure 6A, 6B, 6F, and 6G), and a similar effect was noted for equivalent concentrations of the azide alone preparation when compared with control treatments (Figure 6A, 6D, 6F, and 6I). However, dCRP lacking sodium azide had no such effect on EOC or HUVEC tube formation in vitro (Figure 6C and 6H).

**Effects of CRP on EOC and HUVEC Migration and Proliferation**

To assess the effects of CRP on EOC and HUVEC migration, a scratch assay was used as described previously. Crude commercial CRP preparations decreased EOC and HUVEC migration in a dose-dependent manner, and this was recapitulated by sodium azide preparations at equivalent azide concentrations (Figure 7A and 7C). Similarly, commercial CRP and sodium azide alone caused a dose-dependent downregulation in XIAP in both cell types (Figure 7A and 7C). Finally, commercial CRP and azide alone preparations caused a dose-dependent reduction in eNOS expression in both cell types (Figure 7A and 7C). However, after dialysis to remove sodium azide, the dialyzed CRP preparations had absolutely no effect at any concentration on procaspase 3 and 9, XIAP, or eNOS expression in either EOC or HUVEC (Figure 4B).

**Figure 4.**
Discussion

There is considerable clinical evidence supporting a strong association between elevated CRP levels in human subjects and increased risk of cardiovascular events.2–6 What remains less clear is whether CRP acts simply as a marker of vascular disease burden and activity or indeed participates in the development, progression, and complications of atherosclerosis. In support of this latter concept, an increasing number of in vitro studies have implicated CRP as exerting adverse and ultimately harmful effects on endothelium and therefore acts as a potential initiator and mediator of atherosclerosis.9,16,22,27–31 However, a number of recent studies have used commercial CRP preparations that remain poorly characterized and indeed contain known quantities of biologically active contaminants. Moreover, active working dilutions of CRP used in these studies were sufficiently concentrated as not to preclude persistent contamination with significant quantities of functionally active contaminants such as sodium azide.

In this study, we demonstrate that CRP from a well described commercial preparation can induce apoptosis and inhibit migration, proliferation, and tube formation, in addition to decreasing eNOS expression in human vascular endothelial cells and endothelial progenitor cells in culture. However, the adverse biological effects of the commercial CRP preparation can be completely abrogated by dialysis to remove sodium azide.13 Moreover, sodium azide used at concentrations equivalent to those found in the initial commercial CRP preparation completely recapitulated all the commercial preparation effects on human endothelial cells and endothelial progenitor cells in vitro. Together, these data indicate that the proapoptotic, antiproliferative, antimigratory, and antiangiogenic effects of the CRP commercial preparation, at least as used in the present study, are likely artifactual, and instead are mediated by the sodium azide present in the CRP preparation.

In this study, we have ensured by multiple quantitative analyses that the only difference between the commercial and dialyzed CRP preparations in our experiments was the presence or absence of sodium azide in each respective solution. First, quantitative differences between the CRP in the commercial and dialyzed preparations were excluded by determination of identical CRP concentrations on immunoturbimetric and immunoblot analysis. Second, functional differences between the CRP in both preparations were unlikely, as the commercial and dialyzed CRP solutions were both prepared

Figure 5. Human EPC from buffy coat MNC at day 3 after initial seeding. A, EPC co-stain with Dil-AcLDL (red) and Lectin (green) (merged-yellow) and show positive immunoreactivity on FACS for CD34, Flk-1, and vWF. The number of EPC was dose-dependently reduced in CRP- (black bar) and NaN3 (gray bar)-treated groups compared with control treatment (*P<0.01 vs control), but dCRP (white bar) did not induce any changes in the number of EPC (B). (D) Western blot shows similar decrease in XIAP and procaspases 3 and 9 expression in EPC on exposure to both commercial CRP and NaN3 in a dose-dependent pattern, but dCRP does not. Equal protein loading is confirmed by β-actin controls. C, The quantification of apoptosis determined by DAPI staining (blue) for typical condensed apoptotic nuclei (arrows) showing a dose-dependent increase in apoptotic nuclei in both CRP (black bar) and NaN3 (gray bar), but not dCRP (white bar) treated groups compared with control treatment (*P<0.01 vs control). The concentrations of NaN3 are 0, 0.5, 2.5 ng/mL in B and 0, 2.5, 25 ng/mL in C and D, respectively. A and C, scale bar=20 μm.

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at 4°C for the same amount of time, and both CRP solutions bound with identical efficiency to a natural CRP ligand (phosphorylcholine). We also excluded the possibility of loss of pentameric conformation of commercial CRP during dialysis by determining the absence of monomeric and presence of equal quantities of pentameric CRP by Coomasie and Western analysis in both pre- and postdialysis solutions. Third, detection of endotoxin in the commercial and dialyzed CRP preparations at equivalent and low concentrations below the threshold for biologic activity excluded this contaminant as a confounding variable.32,33 Finally, the absence of azide in dCRP preparations, and the presence of identical concentrations of azide in the commercial CRP and NaN3 alone preparations, suggested that the only remaining difference between each preparation relates to sodium azide concentration.

These data may have important implications for understanding the effects of CRP on endothelial biology. Two recent publications have reported adverse unsalutory effects of CRP on human endothelial cell and endothelial progenitor cell biology in vitro22,27 using either an identical commercial preparation to that described in the current study or another preparation containing similar or greater quantities of azide. Neither of these studies has, to the best of our knowledge, specifically addressed the azide issue, either by removing sodium azide or vitiating the effects of sodium azide. Given the absence of specific biological effects of dialyzed CRP in our study and notably the recapitulation of the commercial CRP preparation effects by equivalent concentrations of sodium azide alone, we recommend circumspection in interpretation of proapoptotic, antimigratory, and antiproliferative effects of commercial CRP on endothelial cells in vitro.

In light of our present findings, it is germane to underscore the established adverse effects of azide. Sodium azide prevents oxidative phosphorylation and can suppress nitric oxide.12 Azide also inhibits the terminal step of the mitochondrial respiratory channel, which is catalyzed by cytochrome c-oxidase34 preventing oxygen reaction with cytochromes A and A3. By combining with oxidized heme (Fe3+), azide also prevents reduction of heme ion by electrons derived from reduced cytochrome C12. This series of biologic interactions markedly reduces mitochondrial respiration and energy production and may promote cell death.35

Study Limitations
Our study specifically investigated the effects of CRP on endothelial progenitor and adult endothelial cell apoptosis, proliferation, migration, and tube formation in vitro. We did not address the proinflammatory effects of CRP on endothelial cells which have been well described in the absence of sodium azide contamination.36 Moreover our study should not be interpreted as the only mechanism whereby CRP interacts with cultured endothelial cells, given the extensive literature that supports in vitro proinflammatory CRP effects. Previous in vivo studies have also shown CRP to be proatherogenic in an apoE knockout mouse overexpressing the CRP transgene, although the precise mechanism underlying this effect is still unclear.37 Our data does not exclude a potential in vivo effect for CRP on endothelial cells but rather underscores the need for methodological parameters in endothelial cell culture studies using commercial CRP preparations.

In summary, our data suggest that the adverse effects of commercial CRP preparations on endothelial cell apoptosis, proliferation, migration, and tube formation in vitro may be
mediated by contaminating sodium azide in the initial preparation. We suggest that future studies with commercial CRP be performed using controls which include CRP separation from sodium azide, authentication of biological findings with use of sodium azide concentrations equal to those found in commercial preparation, or alternatively, use of a recombinant CRP source that does not involve azide contamination.

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