C-Reactive Protein-Induced In Vitro Vasorelaxation Is an Artefact Caused by the Presence of Sodium Azide in Commercial Preparations

Carmen W. van den Berg, Karolina E. Taylor, Derek Lang

Objective—Although C-reactive protein (CRP) is increasingly recognized as an independent risk factor for acute myocardial events, recent evidence suggests that it can directly induce vasorelaxation. This study aimed to investigate the mechanism of this CRP-induced response.

Methods and Results—Isometric tension recordings were used to measure endothelium-dependent and endothelium-independent vascular smooth muscle relaxation in isolated rabbit aortic rings. CRP generated in-house by genetic engineering and expressed in Chinese hamster ovary cells, CRP purified from ascites, and CRP obtained from commercial sources were assessed for vasorelaxing properties. Only the commercial CRP preparation induced vasorelaxation; more than half maximal relaxation was observed at 0.025 μg/mL and maximum relaxation attained at 0.25 μg/mL. Commercial CRP contains high levels of sodium azide, a well-known vasorelaxant. Removal of this agent by dialysis abolished the vasodilatory effect of commercial CRP. Sodium azide alone at concentrations equivalent to that present in the commercial CRP produced a near-identical relaxation pattern to the undialyzed commercial product.

Conclusions—CRP has no vasorelaxant properties per se, and the reported vasorelaxant ability of CRP is an artifact caused by sodium azide present in commercial preparations of this agent. (Arterioscler Thromb Vasc Biol. 2004;24:e168-e171.)

Key Words: C-reactive protein ■ sodium azide ■ vasorelaxation

C-reactive protein (CRP) is an acute phase protein, the expression of which can be increased 1000-fold in acute inflammatory conditions like sepsis, although increased levels have also been found in chronic inflammatory conditions such as rheumatoid arthritis. Recently, elevated levels of CRP have been found to be associated with increased risk of acute myocardial events, and CRP is now considered to be an independent risk factor for cardiovascular events.1–5 The mechanism by which CRP contributes to the pathology of cardiovascular disease is poorly understood. Recently, in an in vitro study using human internal mammary artery rings, Sternik et al reported that CRP induces vasorelaxation, a response that is independent of the endothelium and inhibited by potassium channel blockers.6 In vitro studies investigating the role of CRP in cardiovascular disease have been criticized for the use of commercial preparations of CRP, which may be incompletely defined and may contain contaminants. Furthermore, few robust specificity controls have been performed.7 Therefore, the aim of the present study was to assess whether CRP produced by mammalian cells and purified in-house was able to induce vasorelaxation of preconstricted rabbit aortic rings.

Materials and Methods

Recombinant human Escherichia coli derived CRP was obtained from Calbiochem. CRP was generated in-house by genetic engineering, the entire cDNA encoding human CRP was cloned from interleukin-6 stimulated HEP-3B cells and the cDNA identical to the sequence deposited in Genbank (accession no. NM_000567) was expressed in Chinese hamster ovary cells.8 CRP was purified from human ascites and from cell supernatants of CRP expressing Chinese hamster ovary cells by phosphorylcholine (PC) chromatography using immobilized p-aminophenyl phosphoryl choline gel (Pierce PC-agarose) as described by Volanakis et al.9 The commercial recombinant E. coli-derived Calbiochem CRP preparation was supplied in 20 mmol/L Tris, 140 mmol/L NaCl, 2 mmol/L CaCl₂, pH 7.5, and 0.05% (wt/vol) sodium azide. Our recombinant CRP and CRP purified from ascites were dialyzed against the same buffer.
(although without sodium azide) using a dialysis slide (Pierce) with a cutoff of 10 000 Da. To remove the sodium azide from the commercial CRP, this was dialyzed twice against 500 mL of the Tris/NaCl/CaCl₂ buffer. Sodium azide and phenylephrine were obtained from Sigma.

### Isometric Tension Recordings

The thoracic aortae of male New Zealand White rabbits (2 to 2.5 kg) were removed into fresh Kreb’s buffer (1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 24 mmol/L NaHCO₃, 15 mmol/L glucose, 1.5 mmol/L CaCl₂, 5.3 mmol/L KCl, 138 mmol/L NaCl), gassed with 95% O₂/5% CO₂ at 37°C. For isometric tension recording, 2- to 3-mm-wide endothelium-intact (+E) or endothelium-denuded (−E) rings were mounted in tissue baths containing fresh Kreb’s buffer with a resting tension set at 2 grams. 10

In the first series of experiments, all tissues (+E) were preconstricted with a submaximal concentration of phenylephrine (PE) (1 μmol/L), followed by exposure to increasing concentrations of either “in-house” recombinant CRP, CRP purified from human ascites, commercial recombinant CRP, dialyzed commercial CRP, all from 0.0025 to 2.5 μg/mL, or sodium azide at concentrations equivalent to that in the commercial CRP (1.92×10⁻⁴ to 1.92×10⁻¹ M).

In the second series of experiments, all tissues (−E) were again preconstricted with a submaximal concentration of PE (1 μmol/L), followed by exposure to increasing concentrations of either commercial recombinant CRP or dialyzed commercial CRP at the same concentrations as mentioned.

### CRP-C1q Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay microtiter plates were coated with 1 μg/mL CRP, blocked with 1% bovine serum albumin in phosphate-buffered saline, and incubated with serial dilutions of purified C1q (a kind gift from Dr Mark Griffith, Department of Medical Biochemistry, Cardiff). Wells were washed, incubated with rabbit anti-human C1q (Calbiochem), washed, incubated with goat antirabbit Ig horseradish peroxidase (Jackson), and developed with 1,2-phenylenediamine dihydrochloride (OPD) (Dako).

### Phosphorylcholine Precipitation of CRP

Forty microliters of CRP (250 μg/mL) was incubated with 40 μL PC-agarose beads (Pierce) in 20 mmol/L Tris, 140 mmol/L NaCl, 2 mmol/L CaCl₂, pH 7.5 for 1 hour at room temperature. Beads were centrifuged and the supernatant removed. Beads were washed twice and bound protein was eluted with 20 mmol/L Tris, 140 mmol/L NaCl, 10 mmol/L EDTA, pH 7.5. Samples were mixed 1:1 with SDS-PAGE sample buffer, heated at 95°C for 10 minutes and run on 12.5% SDS-PAGE under nonreducing conditions, and gels were stained with Coomassie Brilliant Blue.

### Statistical Analysis

Student unpaired t tests were used to compare PE-induced constriction values when appropriate. For aortic ring experiments, maximum relaxation response (Rmax) values for each concentration of sodium azide and bindin concentration-response curve were calculated using GraphPad Prism software, and a 1-way analysis of variance followed by Student Newman Keuls test were used to compare these values when appropriate. Differences were considered significant when P<0.05. All data are expressed as mean±SEM (n=3). Relaxation responses are expressed as a percentage of the appropriate PE-induced constriction.

### Results

Rabbit aortic rings sections were preconstricted with PE, resulting in mean constriction values of 7.68±0.52 grams (in-house recombinant CRP); 7.62±0.38 grams (ascites CRP); 8.06±1.01 grams (Calbiochem CRP); 8.35±0.13 grams (dialyzed CRP); and 8.44±0.43 grams (azide), respectively, in the appropriate endothelium-intact tissues, and 8.853±0.3604 grams (Calbiochem CRP) and 8.047±0.4526 grams (dialyzed CRP), respectively, in the appropriate endothelium-denuded tissues. No statistical differences between groups were observed.

In tissues exposed to increasing concentrations of either our own recombinant CRP or CRP purified from ascites, only minimal relaxation was observed at the highest concentrations of CRP used (Figure 1A). However, when CRP from a commercial source (recombinant E. coli produced from Calbiochem) was used, relaxation was readily observed in the presence of 0.025 μg/mL, which is a concentration well below that found in healthy individuals (<0.8 μg/mL).

The commercial CRP preparation used in this study contained sodium azide (0.05% sodium azide/mg CRP), which is a well-known vasorelaxant. 12,13 To investigate the discrepancy between the results obtained using the commercial CRP and our in-house recombinant and ascites-derived sodium azide-free CRP, the commercial CRP was dialyzed twice using a dialysis slide (Pierce) with a cutoff of 10 000 Da. When PE-preconstricted tissues were exposed to increasing concentrations of this dialyzed commercial CRP, the relaxation response seen with the intact commercial product was almost completely abolished (Figure 1B). Strikingly, the relaxation response to the undialysed commercial CRP was mirrored by the relaxation response to increasing concentrations of sodium azide. There was no statistical difference between these responses (Figure 1B). Figure 2 demonstrates that the relaxation response to the commercial CRP was not dependent on the presence of an intact endothelium.

To assess if the dialyzed recombinant E. coli-derived CRP retained its well-described biological characteristics, ie, binding to its natural ligands complement component C1q and PC, 14 a CRP-C1q sandwich enzyme-linked immunosorbent assay and affinity precipitations using PC-agarose beads were performed. As can be seen in Figure 3A, all CRP preparations tested had the ability to bind its natural ligand C1q (Figure 3B). Furthermore, all CRP preparations retained their ability to bind to PC (Figure 3B).

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**Figure 1.** Relaxation responses of endothelial-intact rabbit aortic rings to CRP. A, Relaxation responses to in-house recombinant CRP (●), CRP purified from human ascites (▲), and commercial recombinant CRP (Calbiochem) (■) in phenylephrine (1 μmol/L) precontracted endothelium-intact rings. **P<0.001 cf. in-house recombinant and ascites derived CRP. B, Relaxation responses to commercial recombinant CRP (Calbiochem) (●), dialyzed commercial CRP (○), and sodium azide (1.92×10⁻⁴ to 1.92×10⁻¹ M, concentrations equivalent to that found in the commercial CRP, ●) in phenylephrine (1 μmol/L) precontracted endothelium-intact rings. **P<0.001 cf. ascites derived CRP.
We have investigated the vasorelaxant properties of various CRP preparations. The data presented here demonstrate conclusively that the relaxation responses to the commercial CRP are caused by the sodium azide present in this preparation and not to CRP itself. Our in-house recombinant and ascites-derived sodium azide-free CRP are without effect on the precontracted rabbit aortic rings, whereas concentrations of sodium azide equivalent to those found in commercial CRP preparations mirrored the relaxation response to increasing commercial CRP concentrations. Dialysis of the commercial CRP to remove sodium azide also largely removes the vasorelaxing capabilities of this preparation. The relaxation observed at high concentrations of this intervention is most likely caused by residual sodium azide that could not be removed by the dialysis procedure. In the present study, sodium azide is shown to be a potent vasodilating agent even at low concentrations. The dialyzed CRP and the recombinant and in-house generated and purified CRP all retained their well-defined biological functions, ie, binding of C1q and PC, eliminating the possibility that the inability of these CRP preparations to induce vasorelaxation was caused by structural changes.

In the studies of Sternik et al, who first reported the vasorelaxant properties of CRP, CRP was obtained from the same commercial source as in our study. Sternik et al using human internal mammary arteries suggested that this CRP caused vasorelaxation at concentrations well below plasma levels found in healthy individuals. If CRP does indeed possess such vasorelaxant properties, then under acute and chronic inflammatory conditions and even in healthy individuals, CRP-induced hypotension would be a serious problem. Hypotension is a major side effect of sodium azide toxicity.

Sternik et al also reported that the vasorelaxant properties of CRP were independent of an intact endothelium, which suggested a direct effect of CRP on the smooth muscle cell layer. When the endothelium is intact, it is difficult to envisage how a molecule the size of CRP (125 kDa) can directly act on the smooth muscle cells. Although it has been suggested that CRP can bind to CD32 on certain endothelial cells, carefully controlled studies have shown that CRP does not bind to CD32, and furthermore that CRP does not bind to a healthy endothelium (our unpublished observations).

The discrepancy between the lack of vasorelaxation to our in-house recombinant or ascites-derived CRP and the dialyzed commercial CRP compared with that of the commercial CRP used here and by Sternik et al can be completely explained by the presence of sodium azide in the commercial CRP preparations. Although Sternik et al do indicate that the CRP preparation was purified by 2-stage PC affinity chromatography, it is not clear if the authors removed the azide from their CRP preparation. It is a well-established fact that sodium azide is a potent vasorelaxant and that the most commonly reported health effect from azide exposure is hypotension. Furthermore, sodium azide inhibits cytochrome oxidase resulting in a decrease of cellular ATP. Such an effect may result in the increased opening of ATP-dependent potassium channels and hyperpolarization-induced vascular smooth muscle relaxation. In the study of Sternik et al, CRP-induced relaxation was inhibited by potassium channel blockers, an observation in complete accordance with our finding that sodium azide, and not CRP, is the vasorelaxing agent in commercial CRP preparations.

Although the study of Sternik et al used a human arterial preparation and the present study used a rabbit arterial preparation, it is highly unlikely that a species effect will account for the differences in results or interpretation. That CRP is a highly conserved protein and that human CRP has been shown to be active in a variety of animal models support this rationale. The main conclusion of the present study is that CRP has no vasorelaxant properties per se and that studies using commercial preparations of CRP should be interpreted with care to avoid possible artifactual observations.
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References
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