C-reactive protein does not relax vascular smooth muscle: effects mediated by sodium azide in commercially available preparations

Albert N. Swafford, Jr., Ian N. Bratz, Jarrod D. Knudson, Paul A. Rogers, Jennifer M. Timmerman, Johnathan D. Tune, and Gregory M. Dick

Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, Louisiana

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Swafford, Albert N., Jr., Ian N. Bratz, Jarrod D. Knudson, Paul A. Rogers, Jennifer M. Timmerman, Johnathan D. Tune, and Gregory M. Dick. C-reactive protein does not relax vascular smooth muscle: effects mediated by sodium azide in commercially available preparations. Am J Physiol Heart Circ Physiol 288:H1786–H1795, 2005. First published November 24, 2004; doi:10.1152/ajpheart.00996.2004.—C-reactive protein (CRP), an acute-phase protein and newly recognized indicator of cardiovascular risk, may have direct actions on the vascular wall. Previous studies suggest that CRP is a vasodilator that activates smooth muscle K⁺ channels. We examined the reported vasoactive properties of CRP and further explored its mechanisms of action. CRP decreased blood pressure in rats and increased coronary flow in open-chest dogs at a constant coronary perfusion pressure. CRP relaxed rat aortic rings and mesenteric small arteries that were contracted with phenylephrine. Relaxation was not affected by endothelial denudation or inhibition of nitric oxide (NO) synthase but was blocked by inhibition of soluble guanylate cyclase or K⁺ channels. CRP solutions remained effective, i.e., elicited vasodilation, even after boiling or enzymatic digestion, which suggests the presence of a nonprotein contaminant. Sodium azide (NaN₃, 0.1%) is the preservative used for commercially available CRP and a potential source of NO. NaN₃ elicited the same cardiovascular effects as CRP preparations at equal concentrations, and its actions were blocked by inhibition of guanylate cyclase and K⁺ channels. NaN₃-free CRP, prepared by gel-filtration centrifugation and confirmed by electrophoresis, had no effect on vascular tone. Inhibition of vascular smooth muscle catalase with 3-amino-1,2,4-triazole completely prevented the effects of NaN₃ and NaN₃-containing CRP solutions. We demonstrate that the acute vasoactive properties of commercially available CRP preparations are attributable to NaN₃ (and subsequent production of NO by catalase); therefore, this study suggests a reappraisal of the acute role of CRP in regulating vascular tone.

catalase; nitric oxide; soluble guanylate cyclase; potassium channel; guanosine 3′,5′-cyclic monophosphate

C-REACTIVE PROTEIN (CRP) is an acute-phase protein that is produced by the liver in response to interleukin-6. CRP plays a host-defense role by marking foreign substances and activating the classical complement pathway. In the acute-phase response, CRP concentrations can double every 8 h to reach a peak after ~48 h. With the resolution of infection or inflammation, CRP levels fall rapidly (~6 h half-life in plasma). Recent evidence, however, suggests that CRP remains chronically elevated in many clinical conditions including those associated with cardiovascular disease [e.g., hypertension, atherosclerosis, metabolic syndrome, and diabetes (5, 18)]. As a result, it has been suggested that CRP be added to the list of cardiovascular risk factors (15). Questions abound regarding why CRP remains elevated and, more importantly, whether CRP plays an active role in the development and progression of cardiovascular disease. Recent in vitro studies indicate that CRP has effects on cultured vascular cells and model systems consistent with an active role in cardiovascular disease. For example, CRP increases the expression of endothelial adhesion molecules (14), reduces nitric oxide (NO) production and inhibits angiogenesis (26), and promotes smooth muscle apoptosis (3). These in vitro findings with CRP correlate well with a variety of adverse cardiovascular outcomes associated with elevated levels of CRP (27). In a surprising twist, two recent studies (19, 22) suggest that CRP relaxes vascular smooth muscle via the activation of K⁺ channels. The physiological significance of CRP-induced vasodilation is not clear but could theoretically be involved in the hypotension and circulatory collapse that accompany septic shock, as the associated CRP levels are exceedingly high (16). Alternatively, it has been questioned whether CRP is truly vasoactive (24); therefore, significant controversy exists regarding the effects of CRP on vascular reactivity. We performed the present study to further examine the purported vasoactive properties of CRP and explore mechanisms of action.

METHODS

These studies were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Blood pressure measurements. Male Sprague-Dawley rats were anesthetized with pentobarbital sodium (65 mg/kg ip), intubated, and ventilated with room air. Carotid and jugular catheters were implanted for pressure measurement and drug infusion, respectively. Bolus injections of CRP and sodium azide (NaN₃) were given intravenously, and effects on arterial pressure were recorded. Coronary blood flow measurements. Male mongrel dogs were sedated with morphine (3 mg/kg sc), anesthetized with chloralose (100 mg/kg iv), intubated, and ventilated with room air supplemented with O₂ to maintain normal blood-gas parameters. The left anterior descending artery (LAD) was isolated distal to the first diagonal branch, and a stainless steel cannula was introduced. The LAD perfusion territory was supplied with blood from the femoral artery via a servo-controlled roller pump. Pressure was maintained at 100 mmHg, and coronary flow was measured with an in-line transducer (Transonic Systems; Ithaca, NY). Bolus intracoronary injections of CRP and NaN₃ were given, and effects on coronary blood flow were recorded. Isometric tension recordings. The rat thoracic aorta was dissected and cut into 5-mm rings for organ-bath studies. Rings were suspended from a force transducer (Kent Scientific; Torrington, CT) between two

Address for reprint requests and other correspondence: G. M. Dick, Dept. of Physiology, Louisiana State Univ. Health Sciences Center, 1901 Perdido St., New Orleans, LA 70112 (E-mail: gdick@lsuhsc.edu).

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stainless steel supports and were submerged in warmed Krebs solution aerated with 95% O2-5% CO2. Krebs solution contained (in mM) 132 NaCl, 25 NaHCO₃, 5 KCl, 2.5 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, and 10 glucose. Aortic rings were brought to optimal length (∼3 g passive tension; <10% change in active tension between passive tensions differing by 0.5 g) by repeated stretching and contracting with 60 mM K⁺.

Pressurized small artery studies. The mesenteric arcade was excised through a midline abdominal incision and placed in ice-cold physiological saline solution that contained (in mM) 129.8 NaCl, 5.4 KCl, 0.5 NaH₂PO₄, 0.83 MgSO₄, 19 NaHCO₃, 1.8 CaCl₂, and 5.5 glucose. Fourth-order branches were dissected free, cannulated, secured with 7-0 silk, and pressurized to 60 mmHg. Measurements of internal diameter were made using a charge-couple device camera and a video-tracking device (Living Systems; Burlington, VT). Vessels were superfused (5 ml/min) with a solution that contained (in mM) 140 NaCl, 4.7 KCl, 2 CaCl₂, 1.17 MgSO₄, 3 MOPS, 1.2 NaH₂PO₄, 5 glucose, 2 pyruvate, 0.02 EDTA, and 10 mg/ml albumin. The bath was warmed to 37°C, and arteries were allowed to equilibrate for 45 min before viability with phenylephrine (1 × 10⁻⁶ M) was assessed. Vessels were subsequently washed for 30 min and then constricted to 60% of baseline diameter. Once a stable level of constriction was achieved, CRP or NaN₃ was added to the bath. Passive diameter was determined of baseline diameter. Once a stable level of constriction was achieved, CRP or NaN₃ was added to the bath. Passive diameter was determined at the end of each experiment by relaxing the artery with a maximal concentration of sodium nitroprusside (100 μM).

CRP and other reagents. Human CRP purified from pleural fluid was purchased from Chemicon International (product no. AG723; Temecula, CA). Iberiotoxin, 4-aminopyridine, glibenclamide, NaN₃, N^{\text{ω}}-nitro-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazone[4,3-a]quinoxalin-1-one (ODQ), methylene blue, 3-amino-1,2,4-triazole, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). CRP was separated from NaN₃ using Micro Bio-Spin 6 chromatography columns with a 6-kDa cutoff (Bio-Rad; Hercules, CA). Filtered and unfiltered CRP samples were loaded on a Criterion 15% Tris·HCl gel (Bio-Rad) for electrophoresis and were stained with Coomassie blue for visualization.

Statistical analysis. Data are presented as means ± SE of n experiments. Comparisons were made by Student’s t-test or one-way ANOVA as detailed in the text for different experimental designs. The criterion for statistical significance was set as P ≤ 0.05.

RESULTS

Cardiovascular effects of commercial CRP. Mean arterial pressure in three rats averaged 106 ± 5 mmHg. Bolus intravenous injections of commercially available CRP produced transient reductions in blood pressure (Fig. 1A). Injecting 5 μl of a 2.3 mg/ml CRP solution reduced mean arterial pressure to 79 ± 5 mmHg (P < 0.05 by paired t-test). Coronary blood flow in the LAD perfusion territory of four open-chest dogs averaged 39 ± 6 ml/min. Perfusion pressure was held constant at 100 mmHg, and bolus intracoronary injections of commercially available CRP increased coronary flow in a concentration-dependent manner (Fig. 1B; P < 0.05 by one-way ANOVA). Commercially available CRP relaxed rat aortic segments (Fig. 1C) and mesenteric small arteries (Fig. 1D). Rat aortic segments were contracted with 1 μM phenylephrine and were relaxed by CRP (5 μl of a 2.3 mg/ml solution added to a 5-ml organ bath). Aortic relaxation was typically ~50–70%. Mesenteric small arteries (165 ± 9 μm) were contracted with 0.1–1 μM phenylephrine and relaxed with dilutions of CRP over several log orders. These results are consistent with two previous studies (19, 22) that indicated vasodilator properties...
of commercially available CRP in large arteries. Furthermore, the results extend the previous studies by showing CRP-induced arteriolar dilation, increased organ blood flow, and reduced arterial pressure.

**Mechanisms of CRP-induced vasodilation.** Relaxation of aortic segments was not blocked by l-NAME (300 μM), which is an inhibitor of NO production, or endothelium removal (Fig. 2A). Denudation of the endothelium was confirmed by lack of relaxation to 10 μM acetylcholine (1 ± 1%; n = 4). These findings indicate that relaxation is not endothelium dependent and suggest a direct smooth muscle action. Electromechanical coupling is an important determinant of smooth muscle tone, and K+ channel activation can produce vascular relaxation; therefore, we determined the effect of a variety of K+ antagonists. CRP-induced aortic relaxation was not blocked by 100 nM iberiotoxin [an inhibitor of Ca2+-dependent K+ channels (BKCa)], 3 mM 4-aminopyridine [an inhibitor of voltage-dependent K+ channels (Kv)], or 100 μM glibenclamide [an inhibitor of ATP-dependent K+ channels (KATP)]. Aortic relaxation by commercially available CRP was blocked by a high concentration (1 mM) of Ba2+, which is a nonselective K+ channel inhibitor, or precontraction with 100 mM extracellular K+, which reduces the electrochemical driving force for K+ efflux (Fig. 2B). The relaxation of aortic segments by commercially available CRP was completely blocked by inhibitors of soluble guanylate cyclase (10 μM ODQ and 100 μM methylene blue; Fig. 2A). These findings suggest that the production of NO in smooth muscle cells is responsible for vascular relaxation. NaN3, the preservative used in commercially available CRP preparations, is a potential source of NO via metabolism by catalase (12).

**Cardiovascular effects of NaN3.** Commercially available CRP preparations retained vasodilator properties after boiling or degradation with papain (Fig. 3), thus suggesting the presence of a vasoactive component that is not protein. The following experiments were performed to determine whether NaN3, a metabolic inhibitor used to preserve commercially available CRP, is the active component. Bolus intravenous injections of 0.1% NaN3 transiently reduced blood pressure (Fig. 4A). Mean arterial pressure in three rats was 119 ± 9 mmHg and was reduced to 75 ± 4 mmHg by injection of 5 μl of 0.1% NaN3. The NaN3 concentration (0.1% wt/vol) was identical to that used as a preservative for CRP as reported by the manufacturer. Bolus intracoronary injections of 0.1% NaN3 increased coronary flow in a concentration-dependent manner (average basal flow at 100 mmHg, 32 ± 7 ml/min; n = 3; Fig. 4B). NaN3 relaxed rat aortic segments (Fig. 4C) and mesenteric small arteries (Fig. 4D) that had been contracted with phenylephrine. These results are consistent with the idea that NaN3 is the vasoactive component of commercially available CRP.

**Mechanisms of NaN3-induced vasodilation.** The mechanisms of relaxation induced by NaN3 were identical to those of commercially available CRP (Fig. 5). NaN3-induced relaxation of rat aortic segments was not affected by 300 μM l-NAME or endothelium denudation. NaN3-induced aortic relaxation was not blocked by 100 nM iberiotoxin, 3 mM 4-aminopyridine, or 100 μM glibenclamide, which suggests that neither BKCa, Kv, or KATP channels are responsible. Aortic relaxation by NaN3 was largely blocked by a high concentration (1 mM) of Ba2+ or contraction with 100 mM extracellular K+, which suggests...
that unidentified K⁺ channels (or other hyperpolarizing influences) may be involved. Ba²⁺ inhibited NaN₃-induced relaxation in a concentration-dependent manner with an IC₅₀ of ≈60 µM (Fig. 6). The relaxation of aortic segments by NaN₃ was completely blocked by 10 µM ODQ or 100 µM methylene blue, thereby indicating a role for soluble guanylate cyclase. Removing NaN₃ from commercially available CRP abolished its effects on vascular relaxation (Fig. 7A). Gel-filtration microcentrifuge columns with a 6-kDa cutoff were used to remove NaN₃ from CRP. Protein gels confirmed that CRP was still present in the NaN₃-free preparation (Fig. 7B). These findings suggest that the NaN₃ found in commercial CRP preparations is a source of NO in vascular smooth muscle, perhaps through the action of catalase (12).

Critical role of smooth muscle catalase. Inhibiting catalase with 3-amino-1,2,4-triazole impairs relaxation of rat aortic smooth muscle in response to some nitrovasodilators that require metabolic conversion (11). Rings were treated for 1 h in the organ bath with 50 mM 3-amino-1,2,4-triazole, which was then washed out before experiments were started. Complete inhibition of catalase prevented aortic relaxation by CRP solution and authentic NaN₃ (Fig. 8). Treatment with 3-amino-1,2,4-triazole was not toxic to the vascular smooth muscle, as rings contracted in response to phenylephrine and relaxed to sodium nitroprusside and 8-bromo-cGMP. Relaxation in response to sodium nitroprusside (10 µM) averaged 88 ± 2% in treated rings (n = 8). Relaxation in response to 8-Br-cGMP (1 mM) averaged 99 ± 1% in treated rings (n = 8). These data are consistent with a critical role for catalase in smooth muscle relaxation in response to commercially available CRP solutions and NaN₃.

Metabolic inhibition, electromechanical coupling, and vascular relaxation. We tested whether metabolic inhibition might be a mechanism of NaN₃-induced vasodilation. Antagonists of NaN₃-induced relaxation were tested on responses to carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a powerful metabolic inhibitor that acts as a H⁺ ionophore to uncouple oxidative phosphorylation in mitochondria. Although high levels of K⁺ depolarization, Ba²⁺, or ODQ or inhibition of catalase with 3-amino-1,2,4-triazole effectively blocked NaN₃-induced relaxation, none of these agents prevented vasodilation in response to 10 µM FCCP (Fig. 9). These data suggest that metabolic inhibition is an unlikely mechanism of action for NaN₃-induced vasodilation. The data to this point do not allow us to clearly determine whether NaN₃-induced relaxation of smooth muscle depends on the nature of the precontraction (i.e., electromechanical vs. pharmacomechanical coupling). We determined whether NaN₃ could relax rat aortic rings that had been contracted with the thromboxane A₂ mimetic U-46619 (Fig. 10). Although NaN₃ readily relaxed aortic rings precontracted with 1 µM phenylephrine (59 ± 2%; n = 8; Fig. 10A), NaN₃ had little effect on rings precontracted with U-46619 (10 ± 3% relaxation; n = 12). The different nature of the contractions elicited by phenylephrine and U-44619 is demonstrated by disparate responses to 10 µM diltiazem, which is an L-type Ca²⁺ channel antagonist (Fig. 10, C and D). Diltiazem relaxed rings precontracted with phenylephrine by 56 ± 4% (n = 8). In contrast, rings precontracted with...
U-46619 relaxed only 6 ± 2% (n = 12) in response to diltiazem (P < 0.05 vs. phenylephrine by unpaired t-test). These findings also raise the possibility that NaN₃ may relax smooth muscle by directly inhibiting L-type Ca²⁺ channels rather than relaxing smooth muscle indirectly by causing membrane hyperpolarization. Direct inhibition of L-type Ca²⁺ channels is an unlikely mechanism of action, as agents such as ODQ, Ba²⁺, and 3-amino-1,2,4-triazole, which block NaN₃-induced relaxation, did not prevent relaxation in response to diltiazem (Fig. 11). Furthermore, evidence against nonspecific inhibition of contraction by NaN₃ (e.g., metabolic inhibition or block of L-type Ca²⁺ channels by NaN₃) comes from experi-

Fig. 5. Mechanisms of NaN₃-induced vascular relaxation. A: group data (n = 4–20 rats) for relaxation of aorta by NaN₃ in rings with intact endothelium, denuded of endothelium, or treated with 300 µM L-NAME. Inhibition of soluble guanylate cyclase with 10 µM ODQ or 100 µM methylene blue abolished NaN₃-induced relaxation. B: group data (n = 4–16 rats) for NaN₃-induced relaxation of aortic rings contracted with 1 µM phenylephrine in the absence or presence of specific K⁺ channel antagonists (100 nMiberitoxin, 3 mM 4-aminopyridine, and 100 µM glibenclamide). Ba²⁺ (1 mM) or contraction with K⁺ (100 mM) significantly attenuated NaN₃-induced relaxation.*P < 0.05 vs. control (intact treatment in A and phenylephrine administration in B) by one-way ANOVA with Holm-Sidak post hoc test.

Fig. 6. Representative isometric tension recordings show the concentration-dependent effects of Ba²⁺ to inhibit NaN₃-induced relaxation. A: 30 µM Ba²⁺. B: 100 µM Ba²⁺. C: group data (n = 4–12) demonstrate concentration-dependent effects of Ba²⁺ to inhibit NaN₃-induced relaxation. Aortic rings in the presence of the indicated Ba²⁺ concentrations were preconstricted with 1 µM phenylephrine and then treated with 0.1% NaN₃ (5 µl was added to a 5-ml organ bath).

Fig. 7. Azide-free CRP does not relax vascular smooth muscle: NaN₃ is the vasoactive component of commercially available CRP preparations. A: NaN₃-free CRP, prepared by gel filtration, did not elicit relaxation; rings were contracted with 1 µM phenylephrine. In contrast, authentic NaN₃ does cause relaxation (5 µl of 0.1% NaN₃ was added to a 5-ml organ bath). B: Coomassie-stained gel demonstrates that filtered CRP contains the same protein as the unfiltered starting material.

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ments with U-46619. Although neither NaN₃ nor diltiazem relaxed aortic segments that were precontracted with U-46619, metabolic inhibition by FCCP elicited profound relaxation (Fig. 12; 67 ± 7%; n = 8).

**DISCUSSION**

In this study, we demonstrate rapid vasodilator effects of commercially available CRP; however, the observed effects are an artifact due to the presence of NaN₃. That is, CRP has no acute vasoactive properties per se, but commercially available preparations do as a result of the preservative NaN₃. A variety of techniques were used to show that commercial CRP reduces blood pressure, increases organ blood flow, and relaxes arterial smooth muscle. Vasodilation does not depend on the endothelium but is likely mediated directly on smooth muscle via the activation of K⁺ channels. Characteristics of the candidate K⁺ channel(s) have not been fully determined, but the NaN₃-induced effects were not blocked by the selective antagonists iberiotoxin, 4-aminopyridine, or glibenclamide. In contrast, nonspecific K⁺ channel antagonism such as contraction with 100 mM extracellular K⁺ or 1 mM Ba²⁺ was required to inhibit vasodilation induced by commercially available CRP. Activation of these putative K⁺ channels (or other hyperpolarizing mechanism) is secondary to the activity of catalase and guanylate cyclase, as relaxation was prevented by 3-amino-1,2,4-triazole, methylene blue, or ODQ. Commercially available CRP preparations retained vasodilator properties after boiling or degradation by papain; therefore, the findings suggest the presence of a vasoactive component that is not a protein. NaN₃, a metabolic inhibitor used to preserve commercially available CRP, Activation of these putative K⁺ channels (or other hyperpolarizing mechanism) is secondary to the activity of catalase and guanylate cyclase, as relaxation was prevented by 3-amino-1,2,4-triazole, methylene blue, or ODQ. Commercially available CRP preparations retained vasodilator properties after boiling or degradation by papain; therefore, the findings suggest the presence of a vasoactive component that is not a protein. NaN₃, a metabolic inhibitor used to preserve commercially available CRP, fully accounts for the observed vasoactive properties (4). In the presence of O₂, NaN₃...
can be converted to NO by catalase (9, 12, 21). NO activates soluble guanylate cyclase and produces cGMP (2). Vasodilation produced by commercial CRP or authentic NaN₃ is likely mediated by the cGMP-dependent activation of smooth muscle K⁺ channels (28).

Three previous studies examined the effects of CRP on vascular reactivity and have come to remarkably different conclusions; therefore, the discrepancies deserve attention. The first study of CRP-induced vasorelaxation was published as an original report in 2002 (19), whereas the second was published as part of a review of vascular function in 2003 (22). Both studies used commercially available CRP to demonstrate vascular relaxation mediated by smooth muscle K⁺ channels. The third report, published in 2004 by van den Berg et al. (24), proposed that CRP-induced vasorelaxation was an artifact due to the presence of NaN₃ in commercial preparations. The data presented here agree completely with those provided by van den Berg et al. and further demonstrate a requisite role for smooth muscle catalase and cGMP-dependent signaling in the observed vascular relaxations. In an isometric tension study of human internal mammary arterial rings, Sternik et al. (19) showed that CRP relaxed arterial rings (by ≈80%) precontracted with endothelin-1. Triggle et al. (22) later demonstrated CRP-induced relaxations of the mouse aorta, mesenteric artery, and coronary artery that averaged between 50 and 80%. We report a comparable effect of commercial CRP preparations on rat aortic rings and mesenteric small arteries precontracted with phenylephrine. Our data agree with the previous studies in that relaxations induced by commercially available CRP are unaffected by denuding the endothelium or inhibiting NO synthase.

Fig. 10. Ability of NaN₃ to relax smooth muscle depends on the electromechanical nature of the precontraction. A: NaN₃ (1:1,000 dilution of 0.1%) relaxes an aortic ring contracted with 1 μM phenylephrine. B: NaN₃ has little effect on an aortic ring contracted with the thromboxane A₂ mimetic U-46619 (1 μM). C: L-type Ca²⁺ channel blocker diltiazem (10 μM) relaxes an aortic ring contracted with phenylephrine. D: diltiazem has little effect on an aortic ring contracted with U-46619.

Fig. 11. NaN₃-induced vasodilation is not due to a direct effect on L-type Ca²⁺ channels, as antagonists of NaN₃-induced relaxation do not block relaxation by the Ca²⁺ channel blocker diltiazem. A: diltiazem (10 μM), an L-type Ca²⁺ channel antagonist, relaxes an aortic ring treated with 1 mM Ba²⁺ and precontracted with 1 μM phenylephrine. B: diltiazem relaxes an aortic ring treated with 10 μM ODQ and precontracted with 1 μM phenylephrine. C: diltiazem relaxes an aortic ring treated for 1 h with 50 mM ATZ and then precontracted with 1 μM phenylephrine. Although Ba²⁺, ODQ, or ATZ blocked NaN₃-induced relaxation, these agents did not block relaxation by diltiazem.
Together, these data suggest that the effect of commercially available CRP to induce vasorelaxation is mediated directly on smooth muscle.

With that in mind, both Sternik et al. and Triggle et al. performed a logical next set of experiments with CRP and antagonists of smooth muscle $K^+$ channels or contractions induced by high extracellular $K^+$ concentration, which limits the ability of $K^+$ channels to hyperpolarize the membrane potential and cause relaxation. Sternik et al. (19) demonstrated that the effect of commercially available CRP was diminished by contracting with a high extracellular $K^+$ concentration. They also determined that 1 mM $\text{BaCl}_2$ and 0.1 mM tetraethylammonium greatly reduced relaxations, whereas 1 $\mu\text{M}$ glibenclamide (an inhibitor of $K_{\text{ATP}}$ channels) had no effect. These observations imply that $K^+$ channels, particularly $BK_{\text{Ca}}$ or perhaps inward rectifier $K^+$ channels, may produce CRP-induced relaxation in the human internal mammary artery. Triggle et al. (22) demonstrated that the effect of commercially available CRP on mouse arteries was inhibited by either 30 mM $K^+$ or a combination of 1 mM 4-aminopyridine and 10 mM tetraethylammonium. We show that specific $K^+$ channel blockers (100 nM iberiotoxin, 3 mM 4-aminopyridine, and 100 $\mu\text{M}$ glibenclamide) do not block relaxations produced by commercially available CRP. Relaxations in rat aorta were, however, blocked by high concentrations of extracellular $K^+$ (100 mM) or $\text{Ba}^{2+}$ (1 mM). Tissue- and species-specific differences in the vascular smooth muscle $K^+$ channels likely exist between our study and the two previous studies; however, based on our findings in rat aortic rings and mesenteric small arteries, we concur that $K^+$ channel activation (or some other hyperpolarizing mechanism) likely plays a major role in mediating the vascular relaxation produced by commercially available CRP. Our conclusion, based on the inhibitory effects of high $K^+$ or $\text{Ba}^{2+}$ concentrations (and lack of effect of specific inhibitors), is that CRP-$\text{NaN}_3$ may relax aortic rings by activating $K^+$ channels other than $BK_{\text{Ca}}$, 4-aminopyridine-sensitive $K_v$, or $K_{\text{ATP}}$ channels. Given the present uncertain nature of any $K^+$ channel(s) that may be involved, the possibility remains that $\text{NaN}_3$ may activate hyperpolarizing mechanisms other than $K^+$ channels in smooth muscle (e.g., $Na^+-K^+$-ATPase). Alternatively, $\text{NaN}_3$ may have direct inhibitory effects on some depolarizing or contractile mechanisms in smooth muscle; however, our data suggest that $\text{NaN}_3$-induced relaxation is not due to direct blocking of L-type $Ca^{2+}$ channels or metabolic inhibition. Regardless, the mechanisms for relaxation are acted upon by $\text{NaN}_3$ and not CRP.

Acute intravenous infusion of commercially available CRP produced an immediate and dramatic decrease in mean arterial pressure. In contrast, plasma CRP levels in humans correlate...
positively with the risk of developing hypertension, which suggests that hypertension is in some way itself an inflammatory disorder or that hypertension develops secondary to systemic inflammation (18). Intracoronary bolus injections of commercially available CRP increased coronary blood flow. In contrast, elevated levels of CRP are associated with reduced coronary blood flow and impaired coronary vasoreactivity in humans (17). These apparent discrepancies between the in vitro and clinical findings are best explained by the fact that CRP is not itself acutely vasoactive; rather, the preservative NaN₃ found in commercially available preparations of CRP produces vasodilation. This conclusion is also supported by the well-documented facts that NaN₃ causes hypotension (8, 23) and nitrovasodilators increase coronary blood flow (1).

In addition to acute changes in vascular reactivity, commercial CRP preparations have also been shown to activate and induce apoptosis in vascular smooth muscle cells (3, 6). Similarly, commercial CRP has been shown to attenuate cell survival and angiogenesis in endothelial progenitor cells (20, 25). The implications of such findings are striking given the association between chronically elevated plasma CRP and cardiovascular risk, disease, and mortality. It is possible that effects of commercial CRP on phenotypic changes [e.g., expression of adhesion molecules (14)] and programmed cell death could be mediated by NaN₃, not CRP. This possibility requires further investigation, particularly regarding whether the observed effects of prolonged exposure to NaN₃ may be due to catalase-dependent production of NO or metabolic inhibition. NaN₃, like cyanide, inhibits the terminal step in the mitochondrial respiratory chain, which is catalyzed by cytochrome c oxidase (10). By combining with the oxidized heme (Fe₃⁺) in cytochromes a and a₃, NaN₃ prevents the reduction of heme iron by electrons derived from reduced cytochrome c. As a result, NaN₃ prevents oxygen from reacting with cytochromes a and a₃ (7); therefore, mitochondrial respiration and energy production would be reduced and cell death might ensue. Given these effects of NaN₃ as an inhibitor of oxidative phosphorylation and a source of NO in the presence of catalase, studies reporting effects of commercial CRP preparations on biological systems should be interpreted cautiously. We recommend that experiments with commercial CRP be performed with controls including 1) CRP separated from NaN₃ (e.g., accomplished by gel-filtration centrifugation); 2) proteolysis and denaturation of CRP (e.g., papain treatment and boiling); 3) authentic NaN₃ at concentrations equal to those found in commercial preparations; and 4) whether inhibition of catalase (e.g., 3-amino-1,2,4-triazole) or soluble guanylate cyclase (e.g., ODQ) alters the response.

Using a variety of techniques, we demonstrate that commercially available CRP exerts a number of vascular and hemodynamic effects including hypotension, increased coronary flow, and endothelium-independent relaxation of arteries. Importantly, however, we emphasize that these results are not due to vasoactive properties of CRP per se; rather, all observations are fully explained by the presence of NaN₃ used as a preservative. Our results with commercially available CRP agree largely with those of Sternik et al. (19) and Triggle et al. (22); however, interpretation of the data and conclusions differ dramatically. Our findings and conclusions agree completely with those of van den Berg et al. (24), who first suggested that CRP-induced vasorelaxation is due to the presence of NaN₃ in commercial preparations. Our data extend their previous work and allow us to delineate a probable mechanism of action for dilation that explains previous studies (Fig. 13). CRP is not acutely vasoactive, as removing NaN₃ from commercial preparations abolished relaxation, whereas degrading CRP with papain had no effect on relaxation. NaN₃ found in commercially available CRP preparations is metabolized by catalase to produce NO. This step was abrogated by inhibiting catalase with 3-amino-1,2,4-triazole. Activation of soluble guanylate cyclase by NO leads to vasorelaxation that was blocked by ODQ or methylene blue. Vascular smooth muscle K⁺ channels appear to be involved in NaN₃-induced relaxation, as responses were reduced greatly by contracting tissues with a high extracellular K⁺ concentration or adding the nonspecific K⁺ channel blocker Ba²⁺. Thus all vasoactive effects of commercially available CRP were reproduced by equal concentrations of NaN₃ and used the same signaling pathways. Finally, given the known action of NaN₃ as a metabolic poison and a source of NO, we suggest that other in vitro studies of CRP effects (e.g., apoptosis and phenotypic changes) be performed with NaN₃-free preparations.

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**REFERENCES**


