# Continuously-Infused Human C-Reactive Protein Is Neither Proatherosclerotic Nor Proinflammatory in Apolipoprotein E–Deficient Mice

Miguel A. Ortiz,[1] Gonzalo L. Campana, John R. Woods, George Boguslawski, Marcelo J. Sosa, Candace L. Walker, and Carlos A. Labarrere[1]

*Methodist Research Institute, Clarian Health, Indianapolis, Indiana 46202*

**Studies of human native C-reactive protein (nCRP) in mice have shown effects ranging from proatherogenic, to antiatherogenic, to no effect. It is likely that these disparities are related to (a) the use, in some studies, of contaminated nCRP, or to (b) variation in CRP levels associated with either its episodic administration or the use of CRP-transgenic mice. In our study, 12-week-old male apolipoprotein E–deficient (*apoE*$^{-/-}$) mice, maintained on a Western diet, received azide- and endotoxin-free nCRP ($n = 23$) or placebo ($n = 23$) continuously via osmotic pumps (20.4 μg/day) for 4 weeks. CRP-treated and control mice developed similar atherosclerotic lesions in whole aortas (nCRP: 10.4 ± 4.7% vs. controls: 11.7 ± 4.4%, $P = 0.76$) and aortic roots (nCRP: 65.0 ± 7.8% vs. controls: 64.7 ± 9.7%, $P = 0.94$). No differences were observed in macrophage or T-lymphocyte infiltrates and there was no meaningful change in VCAM-1 or IL-6 expression, in the levels of soluble VCAM-1, or in circulating proinflammatory (IL-1β, IL-6, IL-12p40, IL-12p70, TNF-α, and INF-γ), or anti-inflammatory (IL-4 and IL-10) cytokines. We conclude that continuous infusion of uncontaminated nCRP in *apoE*$^{-/-}$ mice is not associated with increased atherosclerosis, does not alter systemic or local inflammation, and does not affect endothelial activation. These observations suggest that alternative approaches to study CRP (perhaps using different pentraxins in the mouse model or using a rabbit model instead of a mouse model) are needed to evaluate the effects of pentraxins on atherosclerosis.** Exp Biol Med 234:624–631, 2009

**Key words:** C-reactive protein; atherosclerosis; aorta; inflammation; cell adhesion molecules

## Introduction

Atherosclerosis and its associated inflammatory events are the leading causes of death in developed countries (1, 2). It is, therefore, understandable that much of the current research on atherosclerosis has focused on the mechanisms of inflammation and its regulation in atherogenesis (3). C-reactive protein (CRP), a short pentraxin and an acute-phase reactant and marker of underlying systemic inflammation in humans (4, 5), has been shown to be elevated in patients with atherothrombosis and atherothrombosis-related events (6, 7). *In vitro* studies suggest that CRP also induces adhesion molecule expression in coronary artery endothelial cells (8), which has been linked to the development of atherosclerosis in humans and animals (6, 9).

Despite the evidence that CRP is a key contributor to the development of atherosclerosis in humans (5, 6), a complete understanding of its mechanistic role requires the use of suitable *in vivo* animal models. Unfortunately, experiments using atherosclerosis-prone murine models have produced contradictory findings. Some of these experiments have shown a proatherogenic effect of the pentameric form of human CRP (10, 11), while others have shown no effect (12–15) or even an antiatherogenic effect (16). These contradictory findings may have arisen because the CRP used in some of the studies was contaminated, or because the CRP levels were substantially different in some studies that administered exogenous CRP episodically (leading to transiently elevated CRP) compared to others that used CRP-transgenic mice (in which CRP was present at high levels continuously). Accordingly, we sought to determine whether human native CRP would produce a proatherogenic effect in apolipoprotein E–deficient (*apoE*$^{-/-}$) mice if it were verified to be azide- and endotoxin-free and administered as a continuous infusion.

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[1] To whom correspondence should be addressed at Methodist Research Institute, Clarian Health, 1800 N. Capitol Avenue, Suite E504A, Indianapolis, IN 46202. E-mail: mortiz@clarian.org (MAG) or clabarrere@clarian.org (CAL)

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## Materials and Methods

**Experimental Protocol.** We studied 46 *apoE*$^{-/-}$ male mice (Jackson Laboratory, Bar Harbor, ME). All animals were fed a high-fat, high-cholesterol Western diet (D12108, Research Diets Inc.) throughout the study. After a 1-week adaptation period, 12-week-old animals were weighed and randomly allocated to an experimental group that received nCRP ($n = 23$) or a control group that received a placebo solution ($n = 23$). The solutions were administered by continuous infusion using surgically implanted osmotic pumps (model Alzet-2004, DURECT Corp., Cupertino, CA) for 28 days. Body weight was determined weekly. At the end of the study, all animals were euthanized by $CO_2$ inhalation. Pumps, blood, and tissue samples were collected for laboratory analysis. Aortas were dissected and stained with Sudan IV to visualize the atherosclerotic lesions. Tissue samples were stored at −80°C until histological and immunohistochemical studies could be performed. The study followed national guidelines for the care and use of laboratory animals and all procedures were approved by the Institutional Animal Care and Use Committee from Clarian Health Partners.

**Selection of the Placebo Solution.** CRP-solvent buffer (see below) was used as placebo because initial studies in our laboratory investigating the necessity of using a protein solution as placebo showed no differences in atherosclerotic lesion formation in the whole aorta between mice treated with CRP-solvent solution ($n = 12$) or a solution containing bovine serum albumin ($n = 8$) (CRP solvent–treated: 8.7 ± 3.1% vs. bovine serum albumin–treated: 8.0 ± 2.9%, $P = 0.75$, $R^2 = 0.01$).

**Preparation of Uncontaminated CRP.** We used highly purified azide-free human nCRP (>95%) (Tri-Chem, West Chester, PA). The nCRP solution was dialyzed and passed through a polymyxin B column (Detoxi-Gel Endotoxin Removing Gel, Pierce, Rockford, IL) to render it endotoxin-free prior to administration. To maintain CRP stability, a buffer containing 0.2 mol/L NaCl, 0.002 mol/L $CaCl_2$, and 0.1 mol/L Tris, pH 7.5, was used as solvent. The CRP solution was concentrated using Centricon-20 devices (Millipore Corporation, Billerica, MA). The solution was sterilized by filtration through a sterile Millex-GV filter unit (0.22 μm PVDF, Millipore Corporation), and stored at 4°C. The final CRP concentration was 3.4 mg/ml. The endotoxin-free state was verified by the Pyrochrome Chromogenic amebocyte method (Associates of Cape Cod Inc., East Falmouth, MA). The level of endotoxin in the purified preparation was less than 0.1 EU/mg of CRP.

**CRP Administration.** Osmotic pumps were filled with sterile CRP solution (3.4 mg/ml) and the pumps were aseptically implanted subcutaneously under isoflurane anesthesia on the backs of the animals, slightly posterior to the scapulae, and connected through a catheter to the peritoneal cavity. The pumps delivered 6 μl of CRP solution per day (20.4 μg/day) by continuous infusion for 28 days.

**Determinations of nCRP in the Osmotic Pumps.** Stability of the human nCRP molecule in the osmotic pumps was confirmed at the beginning and at the end of the study using non-denaturing Western blotting. Human CRP from the osmotic pumps was subjected to electrophoresis under non-denaturing conditions (7% polyacrylamide gel and Tris-acetate buffer system from Invitrogen) and transferred to nitrocellulose membranes. The membranes were probed with mouse anti-CRP monoclonal antibody (CRP-8) and developed with sheep anti-mouse F(ab')$_2$ fragments conjugated to horseradish peroxidase. Control parallel membranes were analyzed by SDS-PAGE and Western blotting under reducing conditions (0.25% β-mercaptoethanol).

**Determinations of Serum Cytokine Levels.** Circulating levels of IL-1β, IL-4, IL-6, IL-10, IL-12p$_{40}$, IL-12p$_{70}$, TNF-α, and INF-γ were measured using the Search-Light Multiplex Assay System (Pierce Endogen, Rockford, IL).

**Tissue Harvesting and Quantitative Morphometry of the Atherosclerotic Lesions.** Aortic roots (4- to 6-mm blocks) were isolated and embedded in Optimum Cutting Temperature (OCT, Sakura Finetek, Torrance, CA) compound, frozen in liquid nitrogen, and stored at −80°C until processed. Atherosclerotic lesions were determined in serial cryosections (7 μm) from each aortic root. Only cryosections having complete aortic valve leaflets present were evaluated. Four serial cryosections per mouse stained with oil red-O were evaluated in order to minimize the possible variation associated with a particular section. Images were captured with a Leica microscope model DMR (×2.5).

Using an *en face* technique, atherosclerotic lesions were evaluated in pinned-out whole aortas stained with Sudan IV. Images of the aorta were captured with a Spot camera RT Color (Diagnostic Instruments, Inc., Model # 2.2.1) mounted to a Leica microscope model M26 (Nushbaum Inc., McHenry, IL), using Spot software 4.0.9 (Diagnostic Instruments Inc.), with ×0.63 magnification. The extent of the lesion area was calculated as a percentage of the total aortic area using Image-Pro Plus software (version 5.1).

**Immunohistochemistry.** Immunohistochemistry was performed on frozen sections of the aortic roots to assess the presence of VCAM-1 (BioLegend), IL-6 (Serotec), and human CRP (Abcam Inc.). To determine cell composition, we used antibodies specific for macrophages (MOMA-2, Serotec), CD4 (BioLegend), and CD8 T lymphocytes (BD Biosciences). Briefly, slides holding 7-μm cross sections were immersed in cold acetone, rehydrated in PBS (pH 7.4), and blocked with 3% $H_2O_2$ and normal rabbit serum. Tissue sections were incubated sequentially with primary antibodies and with the secondary biotinylated rabbit anti-rat IgG (H+L) mouse-adsorbed antibody (Vector Laboratories). When the rabbit monoclonal antibody against human CRP was used, it was detected with biotin-SP–conjugated AffinitiPure F(ab')$_2$ fragment goat anti-

rabbit IgG (Jackson ImmunoResearch Lab). Avidin-biotin horseradish peroxidase (Vector Laboratories) was used to visualize the primary antibodies. Vector hematoxylin was used as a nuclear counter stain. To evaluate VCAM-1, macrophage, and IL-6 expression in the aortic roots, we measured the ratio between the positive reactive area and the complete vascular area. Images were captured with a Leica microscope model DMR (×2.5).

**Statistical Analysis.** To control for differences in aorta size, the absolute lesion size was recalculated as the percentage of the total surface area of the complete aorta. To eliminate aortic root size as a factor, we calculated the extent of the atherosclerotic lesion in each slide as the ratio of the intimal region to the complete root area, and expressed it as the percentage of atherosclerotic lesion area. Results, expressed as mean ± SD, were analyzed by unpaired *t* test. In cases where within-group variation differed significantly between groups, as indicated by a significant *F* ratio, Welch's modified *t* test was used. Because of their high degree of variability, serum cytokine determinations were expressed as median and range and analyzed by the Mann-Whitney *U* test (GraphPad Instat; GraphPad Software Inc., San Diego, CA).

## Results

**Body Weight.** Body weights of nCRP-treated ($n = 23$) and control ($n = 23$) animals were not statistically different at the completion of the 4-week study (nCRP: 28.0 ± 1.6 g vs. controls: 28.0 ± 1.3 g, $P = 0.88$, $R^2 = 0.001$).

**Confirmation of Uncontaminated Human Pentameric nCRP Administration.** Residual CRP solution in the osmotic pumps at the end of the study consisted mostly of pentameric nCRP, although trace amounts of monomeric CRP were also identified (Fig. 1). This attests to the stability of the nCRP administered throughout the whole study. Likewise, only trace amounts of endotoxin (0.1 EU/mg) were detected in the CRP solution, confirming that we had administered endotoxin-free nCRP.

We had expected that the continuous infusion (3.4 mg/ml) we used, which delivered 20.4 μg/day, would have achieved CRP levels in mouse serum comparable to those found in the serum of at-risk humans. However, on evaluation of the mouse serum, we found much lower levels than this, even though the levels in the treated animals were 100-fold higher than those observed in the controls (nCRP: 463.6 ± 456.2 pg/ml vs. controls: 5.8 ± 4.0 pg/ml, respectively, $P = 0.0076$, $R^2 = 0.53$). We confirmed the delivery of human CRP into the treated mouse tissues using immunohistochemical techniques, as described in Materials and Methods (Fig. 2).

**Effect of nCRP Administration on Atherosclerosis in Whole Aortas.** Atherosclerotic lesions in whole aortas were similar in nCRP-treated ($n = 23$) and control ($n = 23$) animals (nCRP: 10.4 ± 4.7% vs. controls: 11.7 ± 4.4%, $P = 0.76$, $R^2 = 0.022$; Fig. 3), with lesions in both groups being more concentrated in the aortic arch and branches.

**Effect of nCRP Treatment on Aortic Root Atherosclerosis.** Atherosclerotic lesions examined in the aortic roots of a subset of nCRP-treated ($n = 11$) and control ($n = 11$) mice were similar (nCRP: 65.0 ± 7.8% vs. controls: 64.7 ± 9.7%, $P = 0.94$, $R^2 = 0.00035$; Fig. 4).

**Effect of nCRP Treatment on Cellular Composition of Aortic Root Lesions.** Atherosclerotic lesions were predominantly infiltrated by macrophage-derived foam cells. No significant group differences in macrophage reactivity were observed between nCRP-treated ($n = 11$) and control ($n = 11$) animals (nCRP: 16.4 ± 9.1% vs. controls: 17.6 ± 7.7%, $P = 0.75$, $R^2 = 0.006$). T-lymphocyte (CD4 and CD8) infiltration into the atherosclerotic lesions was very low and was found mostly in the adventitial area, with no differences observed between nCRP-treated and control animals (data not shown).

**Effect of nCRP Treatment on VCAM-1 and IL-6 Expression in the Aortic Roots.** Aortic roots of nCRP-treated and control animals showed similar VCAM-1 expression (nCRP: 38.2 ± 24.9% vs. controls: 35.4 ± 11.7%, $P = 0.75$, $R^2 = 0.008$). Accordingly, soluble VCAM-1 levels also were similar (nCRP: 625.7 ± 95.8 ng/ml vs. controls: 657.7 ± 85.5 ng/ml, $P = 0.41$, $R^2 = 0.03$). No significant differences in IL-6 expression were found (nCRP: 39.1 ± 19.2% vs. controls: 44.9 ± 26.1%, $P = 0.59$, $R^2 = 0.02$).

**Circulating Cytokine Levels.** To evaluate possible differences in systemic inflammation associated with CRP administration, we measured circulating levels of various cytokines in a subgroup of nCRP-treated ($n = 11$) and control ($n = 11$) animals. Neither serum levels of proinflammatory cytokines IL-1β, IL-6, IL-$12_{p40}$, IL-$12_{p70}$, TNF-α, and INF-γ nor the anti-inflammatory cytokine IL-10 showed significant group differences. IL-4 was not detectable in either nCRP-treated animals or controls (Table 1).

## Discussion

We hypothesized that azide- and endotoxin-free human nCRP, when continuously infused into *apoE*$^{-/-}$ mice fed a Western diet, would have an atherogenic effect similar to that seen in humans. We found, instead, that nCRP was not associated with increased development of atherosclerotic lesions either in complete aortas or in aortic roots and it had no effect on endothelial activation, or systemic or local inflammation.

CRP, a pentameric molecule belonging to the highly conserved pentraxin family, is the major acute-phase reactant in most mammalian species (4, 17). Its serum concentration in humans increases more than a thousand-fold during inflammation, and chronically elevated CRP is considered a risk factor for atherothrombotic disease and its associated events (4, 6). However, studies of the atherogenic

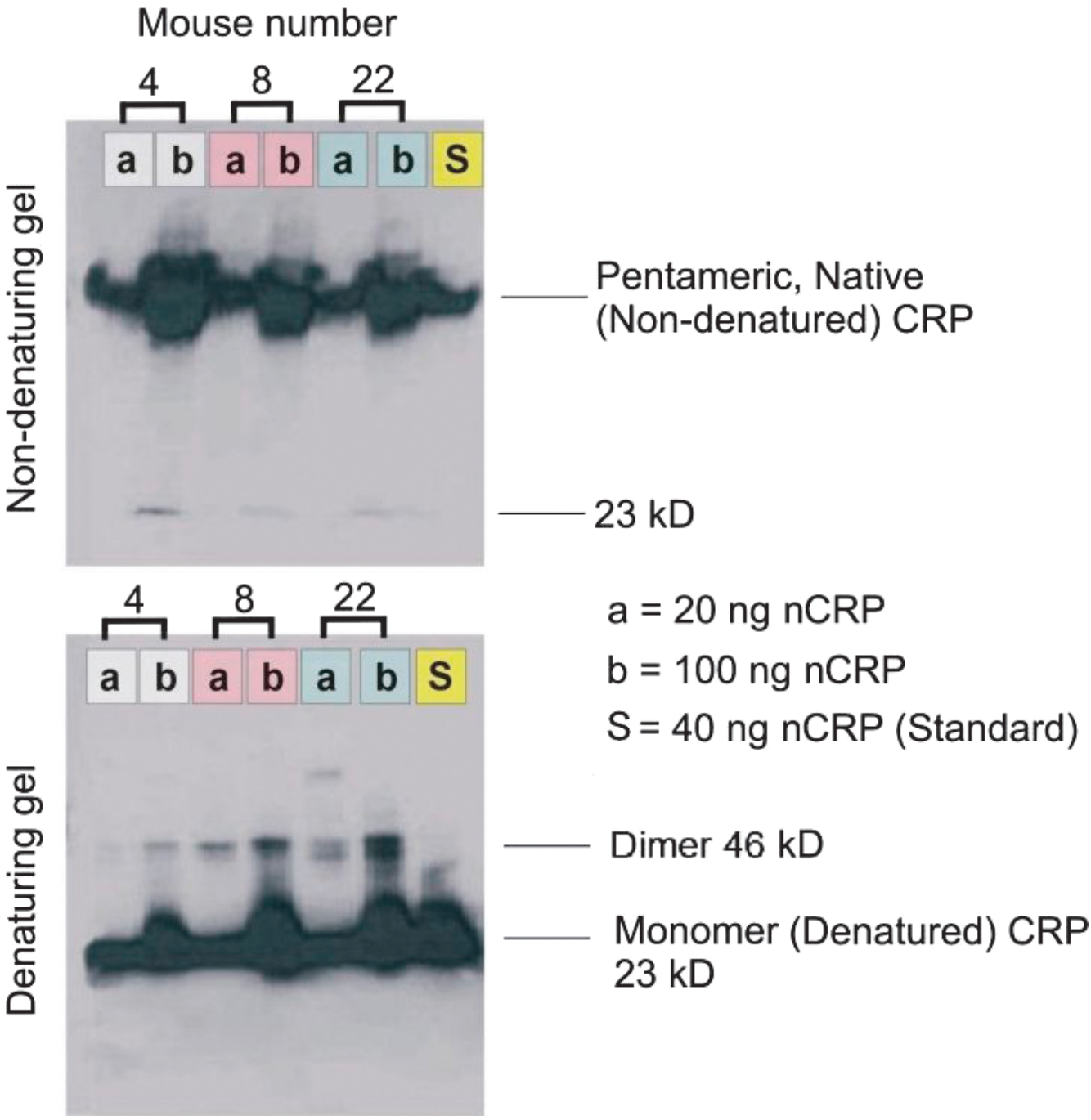


**Figure 1.** Pentameric native human CRP from the osmotic pumps. A non-denaturing gel (top) shows the presence of the intact nCRP molecule and only trace amounts of monomeric (23 kD) CRP in the remaining solution obtained from three different pumps at the end of the study (28 days). A denaturing gel (bottom) of the material from the same pumps shows the presence of a strong 23 kD band that corresponds to the monomeric form of human CRP.

role of human CRP in mouse models have produced contradictory results, perhaps due in part to the fact that CRP is *not* a major acute-phase reactant in mice as it is in humans. CRP blood levels increase only in trace amounts following inflammatory stimulation in mice (4). The main acute-phase reactant in the mouse is another member of the pentraxin family, serum amyloid P (SAP) component (4). Nevertheless, because of its clinical relevance, and given the current lack of alternative animal models, many studies evaluating the effects of human or rabbit CRP have been conducted in the mouse, generating confusion in the published literature.

Paul et al. (10) found accelerated progression of atherosclerosis in male (but not female) *apoE*$^{-/-}$ mice transgenic for human CRP, compared with CRP-nontransgenic controls. Others have reported that human CRP is not

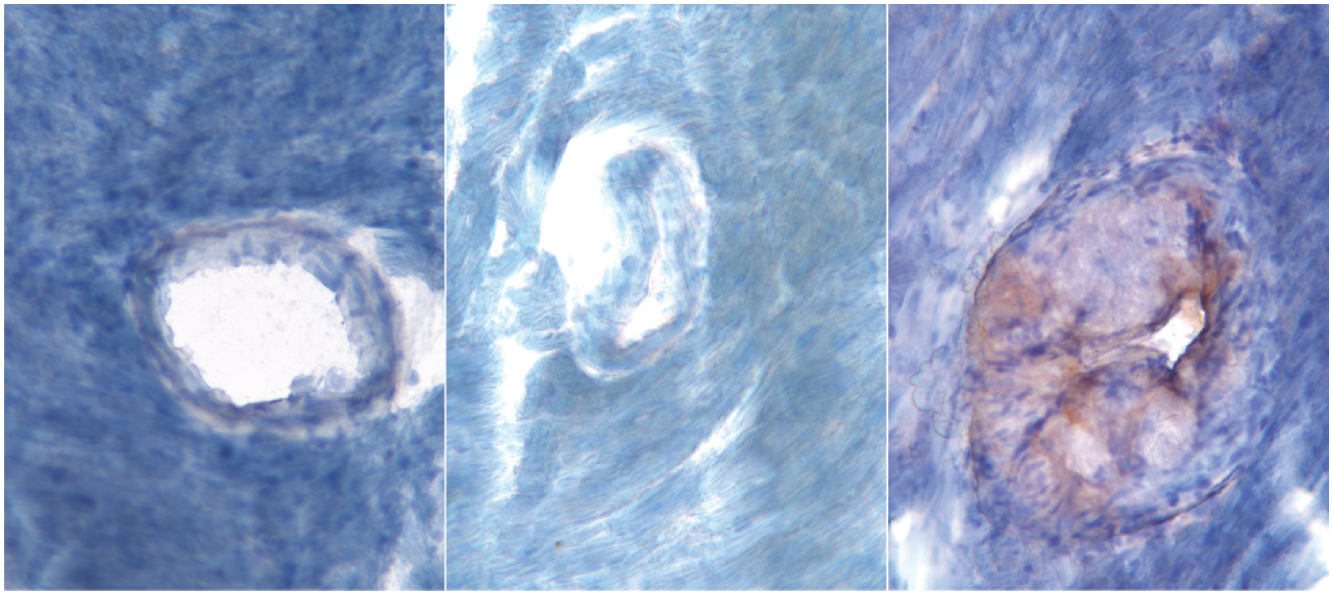

**Figure 2.** Immunohistochemical detection of human CRP in mouse tissues. Heart sections from mouse treated with human nCRP (right) show the presence of human CRP in the arterial wall of a coronary artery. Sections of mouse heart incubated with only secondary antibody (negative control, left) or treated with buffer followed by antibody specific for human CRP (center) show absence of reactivity. Original magnification ×320.

proatherogenic in *apoE*$^{-/-}$ (12, 18), apolipoprotein E*3 Leiden (13), or low-density lipoprotein (LDL) receptor–deficient (*Ldlr*$^{-/-}$) mice (14), nor does it have an atherogenic effect in rabbit CRP transgenic mice (18). Schwedler et al. (11) reported that pentameric nCRP increases, and monomeric CRP decreases, atherosclerosis in *apoE*$^{-/-}$ mice, while Tennent et al. (15) reported that transgenic human CRP is neither proatherogenic, proatherothrombotic, nor proinflammatory in *apoE*$^{-/-}$ mice. Kovacs et al. (16) found that CRP is antiatherogenic in CRP-transgenic *Ldlr*$^{-/-}$ apolipoprotein B100 (apoB100) mice, a finding supported by a recent study showing that enzymatically modified LDL bound to CRP impeded the transformation of macrophages into foam cells (19). Furthermore, the propensity of CRP to bind to oxidized LDL (ox-LDL) (16, 20) could facilitate the clearance of ox-LDL-CRP complexes before reaching the arterial intima, thereby limiting atherosclerotic lesion formation. This idea

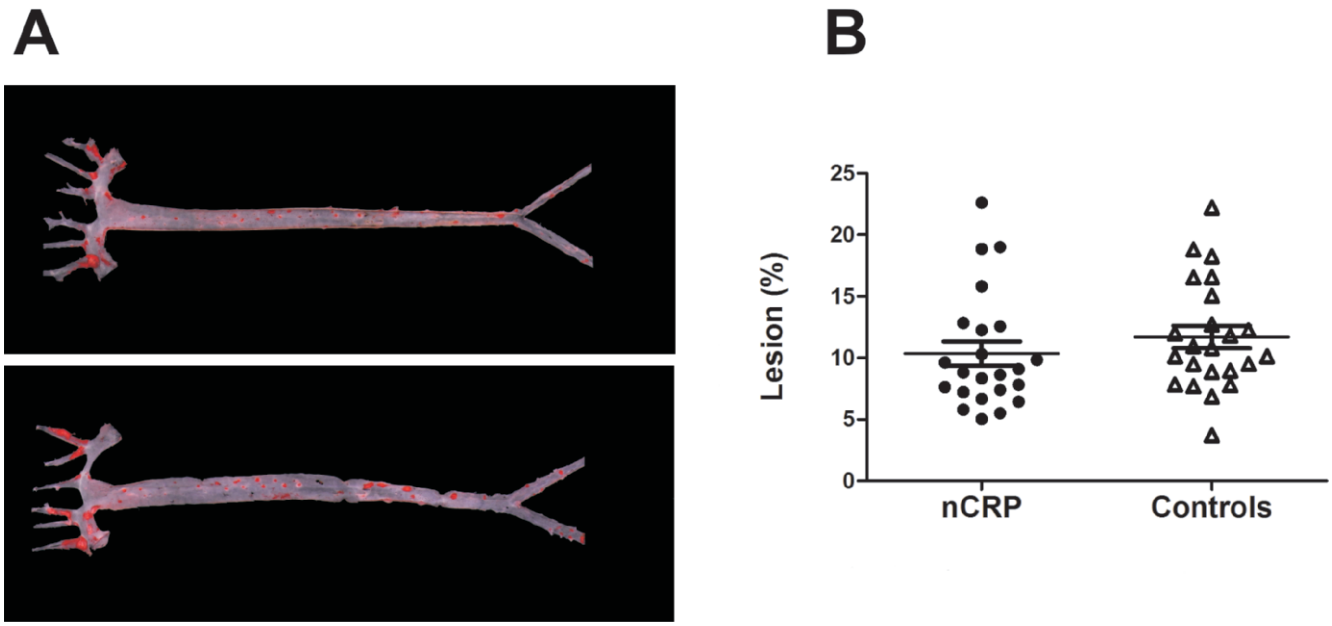


**Figure 3.** Atherosclerosis in complete aortas. A) Representative photographs of Sudan IV-stained aortas from 16-week-old nCRP-treated (top) and control (bottom) *apoE*$^{-/-}$ mice. Original magnification ×0.63. B) Evaluation of lesion formation in nCRP-treated and control *apoE*$^{-/-}$ mice. Symbols and horizontal bars indicate individual mice and group means ± SD, respectively.

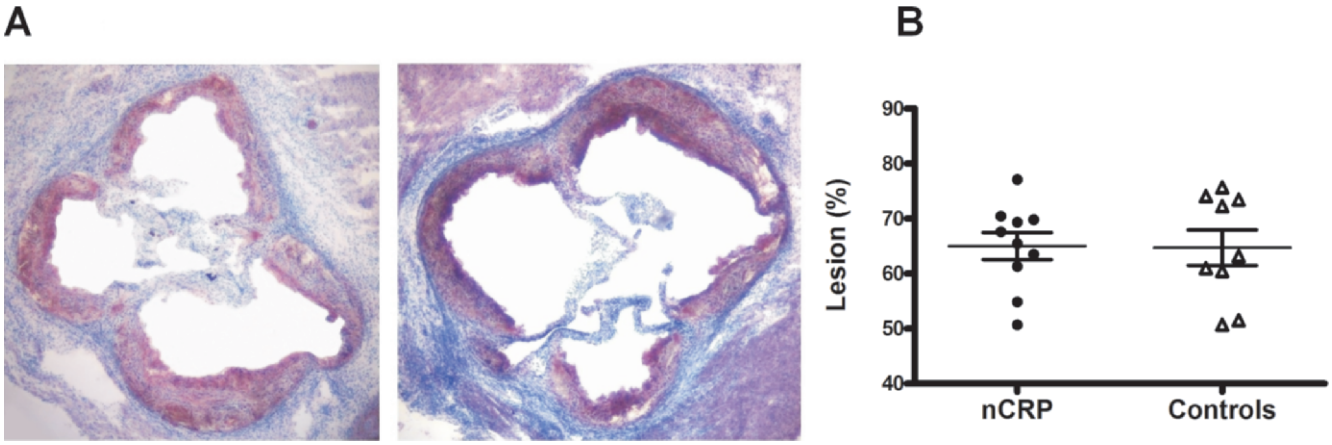


**Figure 4.** Atherosclerosis in aortic roots. A) Representative sections of oil red-O-stained aortic roots from 16-week-old nCRP-treated (left) and control (right) *apoE*$^{-/-}$ mice. Original magnification ×2.5. B) Lesion evaluation in nCRP-treated and control *apoE*$^{-/-}$ mice. Symbols and horizontal bars indicate individual mice and group means ± SD, respectively.

is consistent with the recent demonstration that ox-LDL removal from circulation results in the complete inhibition of atherosclerosis in *apoE*$^{-/-}$ mice (21).

Most animal studies have used transgenic mice for human CRP (10, 12, 13, 15, 16). In some of these studies, mice developed very high CRP levels (10), higher than the levels considered to be a risk factor in humans (3–6). Also, in contrast to the natural variability observed in humans (22), CRP levels in these CRP transgenic mice seem to have remained consistently high (12, 15, 16). Very high sustained CRP levels could produce unknown pathophysiologic changes in transgenic animals (23). Our study shows that the continuous infusion of uncontaminated pentameric human CRP has neither proatherogenic nor antiatherogenic effects in *apoE*$^{-/-}$ mice, supporting other studies in CRP-transgenic mice that also showed a lack of effect (12–15, 18).

Some previous studies may have used contaminated nCRP preparations, and this might explain some of the inconsistencies among studies. It has been suggested, for example, that the effects of CRP on endothelial cells *in vitro* might be due to endotoxin and/or sodium azide contamination of the CRP preparations (24, 25). Pepys et al. (26) suggested that the proinflammatory effect of bacterial recombinant human CRP observed in mice is caused by endotoxin contamination and not by CRP *per se*. However, these findings contradict those of another recent study showing that the biological effects of CRP are present in toll-like receptor 4 (TLR4) knockdown endothelial cells, which do not respond to high lipopolysaccharide (at a concentration as high as 1 μg/ml) treatment (27). We have shown here that when uncontaminated pentameric human CRP is administered continuously to *apoE*$^{-/-}$ mice, it is neither proatherogenic nor antiatherogenic.

Although CRP induces VCAM-1 expression on human arterial endothelial cells *in vitro* (8), *in vivo* studies in mice have produced inconsistent effects. Paul et al. (10) found increased VCAM-1 expression in CRP transgenic mice compared to controls, and Schwedler et al. (11) found increased endothelial VCAM-1 expression in plaque and nonplaque areas of nCRP-treated *apoE*$^{-/-}$ mice compared to animals treated with monomeric CRP or saline solution. On the other hand, Hirschfield et al. (12) found no differences in VCAM-1 expression between CRP transgenic and non-transgenic *apoE*$^{-/-}$ mice. Interestingly, several studies reported no differences in macrophage staining in

**Table 1.** Circulating Cytokine Levels in nCRP-Treated and Control *apoE*$^{-/-}$ Mice[a]

| Cytokines | nCRP ($n = 11$) | Controls ($n = 11$) | *P* value |
|---|---|---|---|
| **Proinflammatory** | | | |
| IL-1β (pg/mL) | 13.0 (2.6–123.2) | 12.1 (1.1–30.0) | 0.51 |
| IL-6 (pg/mL) | 72.6 (2.3–307.4) | 62.0 (21.2–199.2) | 0.83 |
| IL-$12_{p40}$ (pg/mL) | 28.0 (15.5–60.6) | 30.2 (18.7–42.8) | 0.98 |
| IL-$12_{p70}$ (pg/mL) | 5.6 (0.6–12.8) | 5.5 (2.5–8.5) | 1.00 |
| TNF-α (pg/mL) | 14.4 (6.2–25.8) | 10.7 (8.7–21.9) | 0.31 |
| INF-γ (pg/mL) | 7.8 (7.8–55.6) | 9.8 (2.4–117.6) | 0.75 |
| **Anti-inflammatory** | | | |
| IL-4 (pg/mL) | Not detectable | Not detectable | Not applicable |
| IL-10 (pg/mL) | 23.7 (10.6–52.0) | 26.6 (20.5–35.6) | 0.44 |

[a] Values are expressed as medians (min-max).

the lesions of CRP transgenic mice when compared to non-transgenic controls (12, 13, 16). We found no differences in aortic expression or circulating levels of VCAM-1, or in macrophage staining in atherosclerotic lesions of CRP-treated *apoE* $^{-/-}$ mice compared to controls, supporting the idea that human nCRP does *not* induce endothelial activation or accelerate atherosclerotic lesion formation in *apoE* $^{-/-}$ mice (12, 15).

There is strong evidence that CRP facilitates the release of cytokines and chemokines in humans, creating a proinflammatory milieu in the arterial intima (5). However, some studies in mice suggest that CRP alters the cytokine profile in a way that leads to downregulation of the inflammatory response (28–30). We found no differences between nCRP-treated animals and controls in circulating levels of either proinflammatory (IL-1β, IL-6, IL-12$p_{40}$, IL-12$p_{70}$, TNF-α, and INF-γ), or anti-inflammatory (IL-4 and IL-10) cytokines. The absence of a proinflammatory effect is consistent with other reports describing an absence of complement activation by human CRP in *apoE* $^{-/-}$ mice (15, 18). Our data support the idea that human nCRP has neither a proinflammatory nor anti-inflammatory effect in *apoE* $^{-/-}$ mice.

Our findings strongly suggest that using human nCRP in an atherosclerosis-prone mouse model to study the effects of CRP on atherosclerosis is not the best approach. If investigators continue to use the mouse as a model, an alternative approach would be to use *mouse* CRP rather than human CRP. However, since CRP is not a major acute-phase reactant in mice as it is in humans, it is unlikely that mouse CRP will produce the intended proinflammatory and atherogenic effects. A better alternative would be to study the effects of other members of the pentraxin family, such as mouse SAP or mouse long pentraxin 3, which are known to serve as acute-phase reactants in mice. It is more likely that these molecules would mimic the proinflammatory effects in mice that CRP is thought to produce in humans. Another option might be to use a rabbit model to study the effects of CRP considering that, in contrast to mice, CRP *is* an acute-phase reactant in rabbits.


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