C-Reactive Protein Promotes Cardiac Fibrosis and Inflammation in Angiotensin II–Induced Hypertensive Cardiac Disease


Abstract—C-reactive protein (CRP) is a risk factor or biomarker for cardiovascular diseases, including hypertension. The present study investigated the functional importance of human CRP in hypertensive cardiac remodeling by a chronic infusion of angiotensin II (Ang II) into mice that express human CRP. Compared with the wild-type mice, although Ang II infusion caused an equally high systolic blood pressure, levels of human CRP were further elevated, and cardiac remodeling was markedly exacerbated in mice that express human CRP, resulting in a significant reduction in the left ventricular ejection fraction and fractional shortening and an increase in cardiac fibrosis (collagen I and III and α-smooth muscle actin) and inflammation (interleukin 1β and tumor necrosis factor-α). The enhancement in cardiac remodeling in mice that express human CRP was associated with further upregulation of the Ang II type I receptor and transforming growth factor-β1 and overactivation of both transforming growth factor-β/Smad and nuclear factor-κB signaling pathways. Furthermore, in vitro studies in cardiac fibroblasts revealed that CRP alone was able to significantly induce expression of the Ang II type I receptor, collagen I/III, and α-smooth muscle actin, as well as proinflammation cytokines (interleukin 1β and tumor necrosis factor-α), which was further enhanced by addition of Ang II. In conclusion, CRP is not only a biomarker but also a mediator in Ang II–mediated cardiac remodeling. Enhanced upregulation of the Ang II type I receptor and activation of the transforming growth factor-β/Smad and nuclear factor-κB signaling pathways may be the mechanisms by which CRP promotes cardiac fibrosis and inflammation under high Ang II conditions. (Hypertension. 2010;55:953-960.)

Key Words: CRP ■ hypertension ■ angiotensin II ■ cardiac fibrosis ■ inflammation ■ TGF-β/Smads

C-reactive protein (CRP), an acute-phase protein, is considered a biomarker or risk factor for cardiovascular diseases (CVDs), including hypertension.1,2 This is supported by the findings that serum levels of CRP predict the development of chronic heart failure and vascular complications in patients with hypertension and inversely correlate with left ventricular ejection fraction in chronic stable angina patients.3–6 However, the exact role of CRP in CVD remains largely unknown.

CRP is mainly produced in the liver and is released in response to acute injury, infection, and other inflammatory stimuli. Unlike its human counterpart, mouse CRP is synthesized only in trace amounts, and it is not an acute-phase reactant.7 However, human CRP in mice can activate complement (A, B, and C) and bind to mouse FcγRI and FcγRIIb.8 Thus, despite the fact that this is a xenogenic model, transgenic human CRP mice serve as a convenient and unique tool to investigate the biological activities of CRP in vivo, including experimental allergic encephalomyelitis,9 thrombosis,10 and atherosclerosis.11

Increasing evidence has shown that angiotensin II (Ang II) is a key mediator in hypertensive CVD. Treatment of patients with CVD by blocking Ang II with angiotensin-converting enzyme inhibitors or antagonists to Ang II type I receptor (AT1) decreases plasma CRP levels while improving cardiac function.12,13 These clinical findings raise the possibility that there is a functional link between Ang II and CRP in the progression of CVD. In the present study we tested the hypothesis that CRP may promote cardiac remodeling in Ang II–induced CVD. This was tested in mice that express human CRP (CRP Tg) with chronic infusion of Ang II via subcutaneous osmotic minipumps and in vitro in cardiac fibroblasts. Effects of CRP on blood pressure, cardiac function, and cardiac fibrosis and inflammation were determined, and the potential mode of action of CRP was investigated.
**Methods**

**Mouse Model of Ang II–Induced Hypertension**

CRP Tg mice, congenic to the C57BL/6 strain, were used in this study. Characterization of CRP Tg mice has been described previously. Hypertensive CVD was induced in genetically identical littermates of CRP Tg and wild-type (Wt) mice (n=6 to 8 males, aged 8 weeks, 24.80±0.22 g) by continuous infusion of Ang II at a dose of 1.46 mg/kg per day for 28 days via subcutaneous osmotic minipumps (model 2004; ALZA Corp), as described in the online Data Supplement Methods section.

**MRI**

Cardiac function was measured by MRI following the established protocol as described in the online Data Supplement Methods section.

**Immunohistochemistry**

Immunohistochemistry was performed in paraffin sections using a microwave-based antigen retrieval method, and the positive signals were quantitated as described previously. The detailed protocols were presented in the online Data Supplement Methods section.

**Real-Time PCR**

The ventricular total RNA was isolated using the RNeasy kit, according to the manufacturer’s instructions (Qiagen). The cDNA was synthesized, and real-time PCR was performed with the Opticon 2 Real-Time PCR machine (Bio-Rad) by using IQ SYBR green supermix reagent (Bio-Rad). The primers and details of real-time PCR analysis were presented in the online Data Supplement Methods section.

**Cardiac Fibroblasts Isolation and Cell Culture**

Mouse cardiac fibroblasts from male Wt mice (C57BL/6) were isolated by using Liberase Blendzyme 4 (Roche Applied Science) and were stimulated with 10 μg/mL of recombinant human CRP (Azide free, from R&D System) with or without Ang II (0.5 μmol/L) for examination of the AT1 receptor expression, fibrosis, and inflammation, as described in the online Data Supplement Methods section.

**ELISA**

Serum levels of human CRP and protein levels of inflammatory cytokines (interleukin [IL] 1β and tumor necrosis factor [TNF]-α) from the cultured supernatant were determined by commercial ELISA kits as described in as described in the online Data Supplement Methods section.

**Statistical Analyses**

Data obtained from this study were expressed as the mean±SEM. Statistical analyses were performed using 1-way ANOVA followed by Newman-Keuls multiple comparison test from GraphPad Prism 5.0 (GraphPad Software).
Results

Ang II Infusion Upregulated Serum Levels of CRP in CRP Tg Mice
Serum human CRP was detected in CRP Tg mice by human CRP-specific ELISA but was undetectable in Wt mice. After a chronic infusion of Ang II for 28 days, human CRP levels were elevated 5-fold in CRP Tg mice (from 1.63±0.44 to 9.01±1.19 μg/mL; *P<0.001), demonstrating that Ang II upregulated CRP expression in CRP Tg mice.

Elevated CRP Level Impairs Cardiac Function in Ang II-Infused Mice
Effect of CRP on systolic blood pressure and cardiac function was shown in Figure S1 (in the online Data Supplement). Ang II infusion significantly increased systolic blood pressure equally in both Wt and CRP Tg mice (Figure S1A). However, despite the equivalent blood pressures and heart rates in both Tg and Wt mice (not shown), MRI detected that CRP Tg mice exhibited more severe cardiac functional impairment than Wt mice, as demonstrated by a significant decrease in ejection fraction percentage and fractional shortening percentage (Figure S1B and S1C).

Cardiac Fibrosis Is Enhanced in CRP Tg Mice in Response to a Chronic Ang II Infusion
We next examined whether CRP is able to promote Ang II–induced cardiac fibrosis in CRP Tg mice. As shown in Figure 1, immunohistochemistry and quantitative real-time PCR demonstrated that Ang II infusion significantly increased collagen I mRNA and protein expression in cardiac tissues of Wt mice. These increases were more pronounced in CRP Tg mice, resulting in moderate-to-severe interstitial collagen I accumulation (Figure 1A through 1F). Similarly, Ang II significantly upregulated cardiac collagen III and α-smooth muscle actin (α-SMA) in both mRNA and protein levels in Wt mice, but again these responses were significantly enhanced in CRP Tg mice (Figure 1G through 1J).

Upregulated AT$_1$ Receptor and Enhanced Transforming Growth Factor-β/Smad Signaling May Be Mechanisms by Which CRP Promotes Cardiac Fibrosis in Response to Ang II Infusion
We then investigated the mechanisms by which CRP promotes Ang II–mediated cardiac fibrosis in CRP Tg mice by examining the Ang II-transforming growth factor (TGF)-β/Smad signaling pathway. As shown in Figure 2, compared with saline-treated mice, Ang II infusion upregulated cardiac AT$_1$ receptor mRNA and protein levels in Wt mice, which was further enhanced in cardiac tissues of CRP Tg mice, presumably fibroblasts, myocytes, and vascular cells (Figure 2A through 2F). The ability of CRP to directly stimulate AT$_1$ receptor expression was shown in Figure 2G, in that the addition of CRP to the cultured cardiac fibroblasts was able to upregulate AT$_1$ receptor mRNA expression, which was further increased in the presence of Ang II.

Chronic Ang II infusion also increased TGF-β1 expression and activation of Smad signaling (Smad2/3 phosphorylation) in cardiac tissues of fibroblasts, myocytes, and vascular cells of Wt mice, which were also largely enhanced in CRP Tg mice (Figure 3A and 3B). However, real-time PCR showed that, although Ang II infusion significantly upregulated cardiac angiotensin-converting enzyme and angiotensinogen, there was no difference between Wt and CRP Tg mice (Figure S2).
Cardiac Inflammation Is Promoted in Ang II–Induced Hypertensive CRP Tg Mice via the Nuclear Factor-κB–Dependent Mechanism

Immunohistochemistry showed that Ang II infusion upregulated proinflammatory cytokines, such as IL-1β and TNF-α, in all of the cardiac cells of Wt mice (Figure 4A, 4Bi, 4Biii, and 4Bv). This elevation was further increased in CRP Tg mice (Figure 4A, 4Bii, 4Biv, and 4Bv). However, although real-time PCR detected a >3-fold increase in IL-1β and TNF-α mRNA expression in CRP Tg mice in response to Ang II infusion, neither response was statistically significant when compared with the Wt mice (Figure 4Avi and 4Bvi). This may be because of relatively big variations in mRNA expression in some animals. In addition, Ang II infusion also significantly induced cardiac monocyte chemoattractant protein 1 mRNA expression in Wt mice (0.0017±2.6×10^{-4} versus 0.0003±4.3×10^{-5} in saline-Wt mice; P<0.001), which was further increased in CRP Tg mice (0.0024±3.4×10^{-4}, P<0.001 versus Tg-saline mice; P<0.05 versus Wt-Ang II mice).

We then tested the hypothesis that CRP might enhance Ang II–induced activation of the nuclear factor-κB (NF-κB) signaling pathway in the heart. As shown in Figure 5, immunohistochemistry revealed that, compared with saline-infused Wt mice, there was a marked activation of NF-κB/p65 in Ang II–infused Wt mice, as demonstrated...
by the increased nuclear localization of phosphorylated NF-κB/p65 in cardiac tissues (Figure 5A, 5C, and 5E). This response was significantly enhanced in CRP Tg mice (Figure 5B, 5D, and 5E), demonstrating that enhanced activation of the NF-κB signaling pathway may be a critical mechanism whereby cardiac inflammation was augmented in CRP Tg mice.

CRP Induces Cardiac Fibrosis and Inflammation Directly and Additively With Ang II In Vitro
To investigate whether CRP induces cardiac fibrosis and inflammation directly or additively with Ang II, cardiac fibroblasts isolated from the ventricles of WT mice were treated with CRP and/or Ang II. As shown in Figure 6, the addition of CRP alone was able to significantly induce collagen I and III, α-SMA, TNF-α, and IL-1β mRNA expression, which was further significantly upregulated in the presence of Ang II (Figure 6A through 6E). ELISA analysis also showed that CRP upregulated TNF-α and IL-1β protein production directly and additively with Ang II (Figure 6F and 6G). The bioactivities of CRP in cardiac fibrosis and inflammation were confirmed by the boiled inactivation of CRP (Figure 6).

Discussion
Increasing evidence shows that CRP is a risk factor or biomarker for CVD. The present study provides direct biological evidence for the pathogenic importance of CRP in hypertensive CVD. We found that chronic infusion of Ang II was able to elevate human CRP in CRP Tg mice, which promoted hypertensive cardiac disease, including a fall in ejection fraction and fractional shortening and enhanced cardiac fibrosis and inflammation. Moreover, we also identified that enhanced upregulation of the AT₁
response to Ang II may be a mechanism by which CRP Tg mice were promoting Ang II–mediated cardiac fibrosis and inflammation.

Fibrosis is a hallmark of CVD. It is clear that Ang II mediates cardiac fibrosis via the TGF-β1–dependent mechanism.19,20 This is further demonstrated by recent studies showing that Ang II activates the TGF-β/Smad signaling to mediate vascular fibrosis via both TGF-β–dependent and -independent pathways.21,22 A novel and significant finding in the present study was that Ang II–induced cardiac TGF-β1 expression and activation of TGF-β/Smad signaling were further enhanced in CRP Tg mice. The ability of the addition of CRP to directly induce collagen matrix and α-SMA expression in cardiac fibroblasts in vitro or to promote this fibrosis response in the presence of Ang II in vivo and in vitro indicates a pathogenic role of CRP in hypertensive cardiac remodeling.

Enhanced cardiac inflammation may be another mechanism by which CRP Tg mice exacerbate cardiac remodeling under high Ang II conditions. It has been demonstrated that CRP is capable of inducing production of proinflammatory cytokines, including IL-1β and TNF-α, in cultured monocytes or endothelial cells via the NF-κB–dependent mechanism.23–26 In the present study, we also demonstrated that CRP promoted Ang II–induced activation of NF-κB/p65 and expression of IL-1β and TNF-α in cardiac tissues of CRP Tg mice and in vitro in cardiac fibroblasts. Moreover, the finding that CRP alone was capable of inducing expression of proinflammatory cytokines, such as IL-1β and TNF-α, in the absence of Ang II revealed that CRP may act not only as a biomarker of inflammation but also as a mediator or, at least, as a cofactor of Ang II to mediate cardiac inflammation via the NF-κB–dependent mechanism.

Upregulation of the AT1 receptor may also be a mechanism by which CRP exacerbates Ang II–induced cardiac fibrosis and inflammation. It is reported that CRP is able to upregulate AT1 receptor expression on vascular smooth muscle cells in vitro and in a mouse model of atherosclerosis.11,27 This was consistent with the present study that upregulation of the AT1 receptor found in the cardiac tissues of Wt mice was further significantly increased in CRP Tg mice in response to Ang II. Furthermore, in vitro findings of the addition of CRP to directly induce the AT1 receptor expression on cardiac fibroblasts, which was further upregulated in the presence of Ang II, added new evidence for the close link between CRP and the AT1 receptor in the pathophysiological process. It is also possible that CRP may upregulate the AT1 receptor in cardiac tissues via induction of proinflammatory cytokines IL-1β and TNF-α.28,29 Thus, Ang II may, via its AT1 receptor, upregulate CRP, which, in turn, may enhance Ang II–mediated cardiac fibrosis and inflammation by upregulating the AT1 receptor. This may explain the finding that elevated human CRP in Tg mice promoted Ang II–mediated hypertensive cardiac remodeling in the present study.

However, it should be pointed out that the interaction between CRP and Ang II in upregulating the AT1 receptor and promoting cardiac inflammation and fibrosis in vitro...
on cardiac fibroblasts may not imply directly to the cardiac remodeling in vivo, because the process in cardiac remodeling in vivo is very complex, particularly under hypertensive conditions. In addition, compared with rabbit CRP Tg mice in which elevated systolic blood pressure was developed,\textsuperscript{30} the present study failed to detect high blood pressure in human CRP Tg mice. This discrepancy may be associated with much lower levels of CRP in human CRP Tg mice (1.63±0.44 μg/mL) when compared with rabbit CRP Tg mice (9 μg/mL).\textsuperscript{30} In addition, the use of far more sensitive radiotelemetry, instead of the tail-cuff method, as used in the present study, may also have contributed to the high blood pressure detected in rabbit CRP Tg mice.\textsuperscript{30}

**Perspectives**

Although increasing evidence demonstrates CRP as a risk factor in CVD, the pathogenic importance of this molecule in CVD remains largely unclear. The present study provides new evidence for a role of CRP in hypertensive cardiac remodeling in vivo and in vitro. We found that CRP is able to directly induce cardiac fibrosis and inflammation on cardiac fibroblasts and also promotes Ang II–mediated cardiac remodeling in vivo and in vitro by upregulating the AT\textsubscript{1} receptor and by enhanced activation of the TGF-β/Smad and NF-κB signaling pathways. Thus, results from the present study reveal that CRP may act not only as an inflammation marker but also as a mediator factor in hypertensive cardiac remodeling.

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**Disclosures**

None.

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C-reactive protein Promotes Cardiac Fibrosis and Inflammation in Angiotensin II-Induced Hypertensive Cardiac Disease

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METHODS

Mouse model of Angiotensin II Induced Hypertension

CRP Tg mice, congenic to the C57BL/6 strain, were used in this study. Characterization of CRP Tg mice has been described previously [1]. Hypertensive CVD was induced in genetically identical littermates of CRP Tg and wild type (Wt) mice (n=6-8, males, aged 8 weeks, 24.80±0.22g) by continuous infusion of Ang II at a dose of 1.46 mg/kg/day for 28 days via subcutaneous osmotic minipumps (Model 2004; ALZA Corp., Palo Alto, CA) as previously described [2]. Control animals (n=6) consisted of genetically identical littermates of CRP Tg and Wt mice followed the same experimental procedure, but received saline infusion via subcutaneous osmotic minipumps as described above. In addition, groups of 6 CRP Tg and Wt mice without treatment were used as age-matched controls. Systolic and diastolic blood pressure (BP) and resting heart rates before treatment and at days 3, 7, 14, 21, 28 days post Ang II infusion were measured by tail plethysmography using the BP2000 blood pressure analysis system (Visitech Systems, Apex, NC) in conscious mice. At day 28 post Ang II or saline infusion, mice were euthanized under anesthesia (pentobarbitone, 50mg/kg, ip). Blood samples were obtained by cardiac puncture and sera were then separated with BD Vacutainer. The left and right ventricles were collected for histology, immunohistochemistry, and real-time PCR analyses. The experimental procedures were approved by the Committee on the Use of Live Animals for Teaching and Research at the University of Hong Kong.

Magnetic resonance imaging (MRI)

Cardiac dimensions and function was measured by MRI as previously described [3]. Mice were anaesthetized and subjected to MRI before infusion and at 28 days post infusion. Briefly, ECG electrodes were attached to the front paws and the animals were placed prone over a respiratory sensor. Both ECG- and respiratory-triggering was used. FLASH cine sequence was employed to obtain four short-axis slices covering the whole heart. The sequence parameters were as follows: TR = 1 R-R interval, TE = 2.3 ms, cardiac frames = 12, matrix size = 192x192, flip angle = 30º, FOV= 4 cm², slice thickness=1.0 mm, slice gap=0.1 mm. Endocardium and epicardium contours were semi-automatically traced using Segment v1.45 software (http://segment.heiberg.se). End-diastolic volume (EDV) and end-systolic volume (ESV) were measured from the cine image set. Ejection fraction was defined as [(EDV-ESV)/EDV] and the fractional shortening was measured as (%FS=[(DD–SD)/DD] × 100%). All MRI experiments were performed on a high-field small-bore 7 Tesla rodent MRI scanner (70/16 PharmaScan, Bruker Biospin GmbH, Germany) at the University of Hong Kong.

Immunohistochemistry

The ventricular tissues were fixed in Methyl Carnoy's solution for histological and immunohistochemical examination. Immunohistochemistry was performed in paraffin sections using a microwaved-based antigen retrieval method as described previously [4]. Antibodies used in this study included: rabbit or goat polyclonal antibodies to collagen I and III (Southern Tech, Birmingham, AL), α-SMA (Sigma, St. Louis, MO), TGF-β1, phospho-Smad2/3, TNF-α, IL-1β,
phospho-NF-κB (p65), and AT1 (Santa Cruz Biotech Inc., Santa Cruz, CA). An irrelevant isotype rabbit or goat IgG was used as a negative control. The stained sections were developed with diaminobenzidine to produce brown products and counterstained with hematoxylin.

Quantitative analysis of immunostaining was performed as described previously [5]. Briefly, the number of positive cells for phospho-Smad2/3 and phospho-p65 was counted in entire LV tissues under high-power fields (x40) by means of a 0.025-mm² graticule fitted in the eyepiece of the microscope and positive cells were expressed as cells/cm². Expression of cardiac collagen types I and III, α-SMA, TGF-β, AT1, TNF-α and IL-1β was determined using the quantitative Image Analysis System (Carl Zesis Microimaging, Thornwood, NY). Briefly, the examined area of LV tissues was outlined and the positive staining patterns were identified, and the percent positive area was measured. Data were expressed as percentage of positive area examined. The analysis was performed in a blinded manner on coded slides.

Real-time PCR

The ventricular total RNA was isolated using the RNeasy kit, according to the manufacturer's instructions (Qiagen, Valencia, CA). The cDNA was synthesized and real-time PCR was performed with the Opticon®2 Real-Time PCR machine (Bio-Rad, Hercules, CA) by using IQ SYBR green supermix reagent (Bio-Rad, Hercules, CA) [5]. The primers used for collagen I, III, α-SMA, TGF-β1, TNF-α, IL-1β, MCP-1 and GAPDH were described previously [5], while AT1, forward 5'- CCATTGTCCACCCGATGAA-3' and reverse 5'-TGACTTTGGCCACCAGCAT -3', was used. Reaction specificity was confirmed by melting curve analysis. Housekeeping gene GAPDH was used as an internal standard. Ratios for mRNA/GAPDH mRNA were calculated using the ΔΔCt method (2-ΔΔCt) for each sample and expressed as the mean ± standard errors of the mean (SEM).

Cardiac Fibroblasts Isolation and Cell Culture

Mouse cardiac fibroblasts were isolated from male WT mice (C57BL/6) by using Liberase Blendzyme 4 (Roche Applied Science, Indianapolis, IN) as previously described [6]. Briefly, the sliced ventricle tissues were digested by using 0.1 mg/ml Liberase Blendzyme 4 for 1 hour, and then cell were washed and adhered onto uncoated plastic dishes for 1 hour for the attachment. The attached cells were then cultured in DMEM/F-12 (Invitrogen, Carlsbad, CA) containing 10% FBS (HyClone, Logan, UT). After being cultured for 5 days, more than 95% of cells with the fibroblast phenotype were identified as being positive for vimentin but negative for Factor VIII and desmin (data not shown). Cells at passage 2 and 3 were treated with 10 µg/ml of recombinant human CRP (Azide free, from R & D System) with or without Ang II (0.5 µM) for 24 hours. Inactive CRP which was treated by boiling at 100º C for 10 min was added as a negative control. Finally, fibroblasts were harvested for total RNA extraction for real-time PCR analysis as described above and the cell culture supernatant was collected for the Bio-plex cytokine assay. At least 4-independent experiments were performed.
ELISA

Serum levels of human CRP in CRP Tg and Wt mice with or without Ang II infusion were measured in duplicate by a commercial ELISA kit according to the manufacturer's instructions (R&D System, Minneapolis, USA). In addition, protein levels of inflammatory cytokines (IL-1β, TNF-α) from the cultured supernatant of cardiac fibroblasts stimulated with or without CRP (10μg/ml) and/or Ang II (0.5μM) were determined by Bio-Plex multiplex mouse cytokine assay according to the manufacturer’s protocols (Bio-Rad, Hercules, CA).

References


RESULTS

Figure S1. CRP Tg mice have equal levels of high systolic blood pressure as Wt mice, but impairs cardiac function in response to a chronic Ang II infusion. A. The systolic blood pressure (BP). Data represent the mean ± SEM for a group of 6-8 mice. *** p<0.001 vs the
baseline value. **B.** Representative MRI images of a short-axis slice of a mouse heart at (a) end diastolic (ED) and (b) end systolic (ES) cardiac phases. LV endocardium contours were defined by the area marked with [*]. Ejection fraction (EF) and fractional shortening are calculated as described in the Methods. **C.** LV EF and LV FS measured by MRI before (open bar) and at day 28 (hatched bar) after Ang II infusion. Each bar represents the mean ± SEM for a group of 6-8 mice. * p<0.05, *** p<0.001 vs normal before Ang II infusion;  # P<0.05 vs Wt mice at day 28 after Ang II infusion.

**Figure S2.** Effect of CRP on ACE and angiotensinogen mRNA expression in response to a chronic Ang II infusion. A. ACE mRNA expression. B. Angiotensinogen mRNA expression. Data represent the mean ± SEM for a group of 6 mice. *** p<0.001 vs saline-treated animals.