Estrogen Treatment Abrogates Neointima Formation in Human C-Reactive Protein Transgenic Mice

Dajun Wang, Suzanne Oparil, Yiu-Fai Chen, Mark A. McCrory, Gregory A. Skibinski, Wenguang Feng, Alexander J. Szalai

Objective—Previously we established that the vascular injury response was attenuated in ovariectomized wild-type rodents treated with 17β-estradiol (E2). We also showed that the response to acute vascular injury in transgenic mice expressing human C-reactive protein (CRPtg) is exaggerated compared with their nontransgenic (NTG) counterparts. Herein we tested the hypothesis that E2 modulates vascular injury in the CRPtg mouse.

Methods and Results—Intact (INT) or ovariectomized (OVX) CRPtg and NTG, treated with E2 or vehicle, had their right common carotid artery ligated. Resultant neointima formation was exaggerated in CRPtg compared with NTG, whether INT or OVX, but was prevented in both genotypes by E2. Expression of human CRP protein (immunohistochemical analysis) and mRNA (laser microdissection followed by real-time quantitative RT-PCR) was detected in the neointima of OVX CRPtg and was greatly diminished by E2 treatment. CRP was not detected in uninjured arteries or in the media of injured arteries, and blood CRP level was consistently low.

Conclusions—The exaggerated response to vascular injury in CRPtg is associated with increased neointimal expression of human CRP. E2 reduces both neointima formation and neointimal expression of human CRP, suggesting that E2 is vasoprotective. (Arterioscler Thromb Vasc Biol. 2005;25:2094-2099.)

Key Words: C-reactive protein ■ estrogen ■ hormone replacement therapy ■ cardiovascular disease ■ inflammation

Recently we demonstrated using rats that the inflammatory phenotype of the injured carotid artery is susceptible to modulation by estrogen (17β-estradiol, E2), a sex hormone known to affect cardiovascular disease risk in humans. In the rat model we observed that within 24 hours after artery injury, inflammatory cells (neutrophils, monocytes, macrophages) transited the adventitia in large numbers and entered the vessel wall. Importantly, E2 inhibited this influx of cells. We also demonstrated E2 inhibitable overexpression of proinflammatory cytokines in injured vessels, including the neutrophil-specific chemokine CINC and the monocyte chemoattractant MCP-1. In separate but related studies using human C-reactive protein transgenic mice (CRPtg) we examined the impact of endogenously expressed human CRP on vascular damage and repair. Because native mouse CRP circulates only in trace amounts and its blood level does not change appreciably during inflammation, and because expression of the human CRP gene in CRPtg mice mirrors that in humans, and CRP and their nontransgenic littermates (NTG) provided an especially convenient and appropriate tool to study the cardiovascular biology of human CRP. These studies showed that CRPtg experienced much faster and higher rates of complete thrombotic occlusion after vascular injury than NTG, and that human CRP accelerates aortic atherosclerosis in CRPtg/ApoE−/− mice compared with ApoE−/−. Despite the recognized species differences between CRPtg mice and humans, these in vivo findings suggest an active and direct role of CRP in both acute and chronic vascular injury.

Because in addition to affecting cardiovascular disease risk, E2 is known to affect blood CRP level in humans, in the present study we used CRPtg mice to test the hypothesis that this hormone modulates CRP mediated exacerbation of acute vascular injury.

Methods

Animals

Ten- to 12-week-old female CRPtg and wild-type (NTG) littermates with a C57BL/6J background (The Jackson Laboratory, Bar Harbor, Me) (reviewed in ref. 7) were studied. Details of the human transgene and its human-like expression in CRPtg have been described. Importantly, the flanking sequences of the transgene include all of the human cis-acting regulatory elements (ie, the entire human promoter) responsible for both tissue specificity and acute phase inducibility of human CRP, and all of the trans-acting factors required for correct regulation of the transgene are conserved from mice to humans. Thus, as in humans, in CRPtg human CRP is expressed mostly (but not exclusively) in the liver, it is present at low levels in the serum under steady-state conditions, and it is present at high levels in the serum during the acute phase response. In
comparison, endogenous mouse CRP is always present at low levels in the serum and it is not an acute phase protein.12 All mice were maintained at constant humidity (60±5%) and temperature (24±1°C) with a 12 hour light cycle (6 AM to 6 PM) and fed a standard mouse pellet diet (Ralston Purina Diet). The average body weights of mice used in the study did not differ significantly among the various treatment groups. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 96-01, revised 1996).

**Carotid Artery Injury**

At the start of the experiment, mice were anesthetized with ketamine (80 mg/kg IV; Abbott Laboratories) and xylazine (5 mg/kg IV; Rompun, Bayer Corp) and subjected to ovarioectomy (OVX), followed by subcutaneous implantation of 17β-estradiol (E2) 60-day release pellets (0.5 mg per pellet releasing 8.3 µg E2/d). Innovative Research of America 7 days later. Pilot studies performed in OVX mice confirmed that supraphysiological amounts of E2 (80 µg per mouse) were detectable in serum collected 18 days after E2 implantation with 0.1 mol/L sodium phosphate buffer (pH 7.3) and perfused with 4% paraformaldehyde. Both carotid arteries were excised, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. The ligation site (hereafter, the section at 0 mm distal to the ligation site) was identified by Verhoeff’s elastin stain to enhance the elastic laminae. To visualize cells captured from the 30 tissue sections taken from each carotid artery, we used a laser capture microdissection (LCM) and real-time quantitative RT-PCR was carried out on separate sections taken from injured and uninjured arteries to capture ~600 cells from the neointima. A similar procedure was carried out on separate sections taken from each carotid artery, we captured ~600 cells from the neointima. A similar procedure was carried out on separate sections taken from injured and uninjured arteries to capture ~600 cells from the media of each vessel. Captured cells were placed in a 500 µL tube filled with guanidine thiocyanate lysis solution, and RNA was extracted using the RNeasy Micro kit from Ambion.

For real-time quantitative RT-PCR, protein- and DNA-free RNA was reverse transcribed to cDNA using the SYBR Green RT-PCR kit (Applied Biosystems) and specific primers (for ribosomal protein S9, 5’TGGGTAGAGGGCGAAGATG-3’ and 5’- CGAACAATGAAA-GATGGGAT-3’; for human CRP, 5’T-TTATTACHTTGGTGTCTC-CAA-3’ and 5’-CCACCGAAGGAATCTTG-3’). cDNA was amplified by PCR in the iCycler for 40 cycles, and relative RNA level was calculated with iCycler software (Applied Biosystems). Unknowns were normalized against ribosomal protein S9 mRNA (Rp) because expression of this housekeeping gene has been shown to remain stable in a carotid injury model.

**Measurement of Circulating Human CRP and Mouse Serum Amyloid P-Component**

Blood (50 µL) was collected from the retro orbital plexus of anesthetized mice on day 0, 7 days after OVX or sham surgery, and 2 days after E2 or placebo pellet implantation. To verify that the CRP transgene remained fully responsive after the various surgeries, on day 27 after carotid injury each animal was injected intraperitoneally with 25 µg of lipopolysaccharide (LPS), a standard stimulus we and others have shown elicits a robust human CRP and mouse serum amyloid P-component (SAP) acute phase response in CRPtg.5–7,11,14 LPS-induced acute phase human CRP and mouse SAP was assayed using sera collected on day 28. Transgenic human CRP was measured by ELISA; the assay does not detect mouse CRP and has a lower limit of detection of 20 ng of human CRP per ml of mouse serum. The endogenous mouse acute phase protein SAP was detected by rocket immunoelectrophoresis.15

**Statistical Analysis**

Serum CRP and SAP values and results of morphometric analyses are expressed as the mean±SEM without transformation. All statistical analyses were performed using the SigmaStat software package (SigmaStat, Jandel Scientific). Comparisons among experimental groups were performed with 1-way ANOVA followed by pair-wise multiple comparisons using the Student–Newman–Keul test. Differences were considered significant if the associated probability value was <0.05.

**Results**

Serum human CRP levels in CRPtg varied within 1 standard deviation of baseline values throughout the duration of the experiment (Figure 1A); thus serum CRP did not differ substantially in mice subjected to OVX compared with OVX followed by E2, nor from that in their respective sham or placebo-treated controls. Even on day 27 after carotid artery ligation serum CRP was not elevated, despite a robust CRP transgene response to LPS-induced inflammation in all CRPtg mice (Figure 1A, inset). In contrast in the same CRPtg mice, OVX elicited significant elevation of the endogenous acute phase protein mouse SAP (Figure 1B) and significantly
heightened LPS-induced acute phase SAP (Figure 1B, inset). The combined data show that for female CRPtg, OVX and subcutaneous replenishment of E2 does not significantly alter baseline or acute phase expression of transgenic human CRP but does alter expression of endogenous mouse SAP.

The architecture of injured right and uninjured left carotid arteries was examined 28 days after ligation. In uninjured left carotid arteries from all mice examined the intima was a single cell layer thick, the internal elastic lamina was intact, and the external elastic lamina was in full contact with the adventitia (data not shown). Also there was no difference in the medial area (wall thickness) of uninjured left carotid arteries among the different experimental groups. These findings indicate that the anatomy of uninjured carotid arteries is not significantly changed after either OVX or subsequent E2 replenishment. In stark contrast, the ligation-injured right arteries had extensive neointima formation (Figure 2). Morphometric analyses of injured arteries from INT and OVX mice revealed a significant nearly 2-fold increase in the neointimal area in CRPtg compared with treatment-matched NTG (Figure 2A and 2C versus 2B and 2D, respectively, and Figure 3A); in contrast, the medial area was not remarkably different among the treatment groups (Figure 3B). As a consequence the neointima/media ratio in injured right arteries was nearly 2-fold greater in CRPtg compared with NTG mice, the difference achieving statistical significance in the OVX group (Figure 3C). Remarkably, in OVX mice replenished with E2 there was less neointima formation than in INT mice and no significant difference between CRPtg and NTG (Figure 2E versus 2F and Figure 3D).
3C). Thus compared with genotype-matched OVX mice, OVX mice given E2 had an \(~85\%\) reduction in neointima formation whereas the media was unaffected.

Immunohistochemical analysis (Figure 4) revealed the presence of human CRP mainly in the neointima in injured vessels of CRPtg. Despite the consistently low level of serum CRP in CRPtg, there was more intense and widespread CRP immunoreactivity in injured vessels from placebo treated OVX animals (Figure 4A) than in those from OVX animals replenished with E2 (Figure 4C). On the other hand, little or no human CRP was detected in arteries from uninjured CRPtg (Figure 4B and Figure 4D). LCM and real-time RT-PCR confirmed that, akin to humans, a small amount of human \(\text{CRP}\) mRNA is expressed in the neointima of injured vessels in CRPtg (Figure 5A). Importantly, although this difference did not achieve statistical significance, \(\text{CRP}\) mRNA expression in the neointima of injured vessels from \(\text{E}_2\)-treated OVX CRPtg was \(~50\%\) lower than that detected in placebo-treated OVX CRPtg (Figure 5C). Unlike the neointima, CRP mRNA was not detected in the media of injured arteries (Figure 5B) or uninjured ones (data not shown).

**Discussion**

Cardiovascular disease is the leading cause of death in women in developed societies, and the risk of cardiovascular disease increases substantially after menopause.\(^{16,17}\) This increased risk is thought to reflect loss of the multiple protective effects of \(\text{E}_2\) on the vasculature.\(^{18}\) Buoyed by supporting evidence from early observational studies indicating that treatment with various \(\text{E}_2\) preparations reduced cardiovascular disease risk by \(~50\%\), \(^{16,17,19}\) use of postmenopausal hormones became a very popular therapy. Challenging these findings is a more recent meta-analysis that adjusted for socioeconomic status and other confounding risk factors, showing no cardiovascular disease risk reduction in women taking menopausal hormones.\(^{20}\) Indeed, randomized controlled trials of menopausal hormone treatment, including the Women’s Health Initiative, showed either no benefit or increased coronary heart disease risk in elderly women with no obvious cardiovascular disease at baseline, randomized to hormones compared with placebo treatments.\(^{3,4,21}\) In light of these conflicting findings the use of menopausal hormone therapy is now being questioned, and controversy about its risks and benefits persist.\(^{4}\) A recent hypothesis has arisen that offers resolution to this paradox, ie, that early initiation of hormone therapy in women at inception of their menopause will delay cardiovascular disease; this is the driving force behind the Kronos Early Estrogen Prevention Study.\(^{22}\)
Circulating CRP is known to be an independent and accurate predictor of cardiovascular events in women\textsuperscript{23–25} and was shown to be a strong promoter of thrombosis in CRPtg mice.\textsuperscript{8} Furthermore, oral menopausal hormone therapy is associated with both increased plasma levels of CRP\textsuperscript{26,27} and increased risk of venous thromboembolism,\textsuperscript{28} suggesting a potential mechanism by which ovarian hormones might influence cardiovascular disease risk. Importantly, the hormone-induced increase in CRP is not accompanied by elevation of interleukin (IL)-6, the major regulator of CRP under inflammatory conditions, or of other acute phase reactants.\textsuperscript{26,27} This indicates that the effects of menopausal hormones on CRP do not represent a generalized inflammatory effect mediated through the upstream cytokine IL-6, but rather are related to a different mechanism. Supporting this conjecture is the observation that in contrast to orally-administered estrogen, transdermal E\textsubscript{2} does not elevate circulating CRP levels\textsuperscript{26,27} and does not increase the risk of thromboembolism.\textsuperscript{28}

The results of our current animal study provide compelling indirect evidence that human CRP may be directly involved in mediating the vascular response to injury, at least in CRPtg mice. We first established that presence of the human CRP transgene correlates to exacerbated neointima formation after acute vascular injury induced by carotid artery ligation in female CRPtg. Importantly, this detrimental association with CRP is strengthened and its impact worsened by OVX, and it is abrogated by subsequent treatment with E\textsubscript{2}. We documented accumulation of human CRP in the injured vessels of CRPtg in the absence of significant fluctuation in serum human CRP and confirmed that, as in humans,\textsuperscript{29} human CRP mRNA is expressed in the CRPtg vasculature. These findings are in concert with the results of clinical studies suggesting that E\textsubscript{2} is vasoprotective and that transdermal E\textsubscript{2} does not elevate circulating CRP levels.

Although the mere presence of the protein in the injured arteries does not mean CRP actually participates in the injury process, the current findings are in concert with our earlier observation of CRP accumulation in atherosclerotic arteries.\textsuperscript{9} Together, these independent lines of evidence underscore the probable biological importance of locally expressed CRP. Importantly, because human CRP is expressed endogenously by CRPtg, none of the effects we observed can be attributed to incidental chemical or other contamination of the human protein. The current data indicate that at least part of the modulating effect of E\textsubscript{2} in the CRPtg model of acute vascular injury could be a consequence of decreased local expression of CRP in the injured artery, independent of detectable changes in blood CRP. By extension, any protective effect of E\textsubscript{2} in humans might also be associated with reduced accumulation of CRP at the site of injury, probably independent of blood CRP level. At present the exact mechanism(s) by which E\textsubscript{2} modulates CRP gene expression and/or its downstream inflammatory actions in the vasculature remain unknown. We cannot rule out the contribution of other gonadal hormones in this process, but our preliminary results point toward an E\textsubscript{2}→FcγR→CRP axis of vascular inflammation.

In conclusion, our finding of significantly increased neointima formation in female CRPtg subjected to carotid artery ligation shows that endogenous expression of human CRP coincides with a worsened vascular injury response. Subcutaneous administration of E\textsubscript{2} can abrogate this effect, a beneficial outcome which coincides with decreased local expression of CRP in the neointima despite stable blood CRP level. Although we have not yet investigated whether orally-administered E\textsubscript{2} has the same or a different effect, the already available data suggest that the negative impact of CRP on the vasculature might be overcome with a clinically relevant therapy.

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References


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