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

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Objective—Previously we established that the vascular injury response was attenuated in ovariectomized wild-type rodents treated with 17β-estradiol (E_2). 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 E_2 modulates vascular injury in the CRPtg mouse.

Methods and Results—Intact (INT) or ovariectomized (OVX) CRPtg and NTG, treated with E_2 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 E_2. 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 E_2 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 neo-intimal expression of human CRP. E_2 reduces both neointima formation and neo-intimal expression of human CRP, suggesting that E_2 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, E_2), 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, E_2 inhibited this influx of cells. We also demonstrated E_2 inhibitable overexpression of proinflammatory cytokines in injured vessels, including the neutrophil-specific chemokine CINC and the monocyte chemotactant MCP-1. In separate but related studies using neutrophil-specific chemokine CINC and the monocyte chemotactant MCP-1. In separate but related studies using 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.
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 ovariectomy (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. Plus studies performed in OVX mice confirmed that supraphysiological amounts of E2 (80±35 pg/ml serum, measured by radioimmunoassay) are maintained for at least 4 weeks after implantation of a single E2 pellet. As controls, some OVX mice received placebo pellets, and intact mice (INT) were subjected to sham OVX and sham implantation procedures. Each of the 3 experimental groups (OVX+E2, OVX+placebo, sham OVX+sham implant) included 6 to 8 CRPtg and an equal number of NTG. Two days after pellet implantation mice were anesthetized and, to stimulate neointima formation, under a dissecting microscope the right common carotid artery was exposed through a midline cervical incision and ligated with an 8-0 silk suture just proximal to the bifurcation as described.13 The contralateral left common carotid artery was also exposed but not ligated and served as an internal control.

### Immunochemoanalysis and Morphometric Analyses

Twenty-eight days after injury mice were euthanized with an overdose of pentobarbital. The vasculature was immediately flushed 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), was identified by inspecting representative serial sections stained with hematoxylin and eosin (H&E). Additional serial sections of vessels 200, 500, 700, and 900 μm distal to the ligation site were then treated with Verhoeff’s elastin stain to enhance the elastic laminae. To visualize human CRP in arterial lesions, immunohistochemical analysis was performed on separate hematoxylin-stained sections as described9 using anti-human CRP antibody and the Vectastain ABC kit (Vector Laboratories). Serial sections of the unligated contralateral vessels were obtained and processed in the same fashion.

Computer-assisted morphometric analysis of digitized images captured from each arterial section was performed with image analysis software (Scion Image). Measurements of the 4 serial sections obtained for each vessel were averaged for statistical purposes. The cross-sectional area of the media, ie, the zone bounded by the external elastic lamina and the internal elastic lamina, and the area of the intima, ie, the zone between the internal elastic lamina and the lumen, were calculated. Neointima formation in the occluded carotid arteries is expressed as intimal area or as the intimal area/medial area ratio. All measurements were performed by a single examiner blinded to the genotype and treatment group of the mice.

### Laser Capture Microdissection and Real-Time RT-PCR

To assess local expression of human CRP within the neointimal and medial domains of injured arteries, a combination of laser capture microdissection (LCM) and real-time quantitative RT-PCR was used. Arteries were harvested, immersed in OCT compound, and frozen (−80°C). Thin tissue sections (8 μm) were sliced on a −20°C cryostat (30 sections per artery), mounted on glass slides, and lightly stained with H&E to aid visualization of tissues. The stained sections were placed under a dissecting microscope equipped with LCM hardware and software (Arcturus Engineering Inc). LCM transfer film was positioned to overlay the tissue section of interest and, when cells of interest were centered in the field-of-view, the spot-size (7.5 to 15 μm) and power (45 to 50 mW) of an infrared targeting beam was adjusted to melt the transfer film, thus bonding it to and capturing underlying cells. The transfer film with bonded cells was then recovered, leaving unwanted cells behind. In this way, from the 30 tissue 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 guanidium thiocyanate lysis solution, and RNA was extracted using the RNAqueous-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'-GCTGGAAGGGCAAGAT-3' and 5'-CGAACATGAAA-GATGGGA-3'; for human CRP, 5'-TTTACAGTGCTGGTCTCAAA-3' and 5'-CCACCCGAAAGATCTC-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...
Figure 1. Serum human CRP (A) and mouse SAP (B) in CRPtg mice measured 7 days after ovariectomy (OVX) or 7 days after surgery without ovariectomy (sham OVX), vs 2 days after subsequent subcutaneous implantation of pellets containing E2 (OVX + E2) or pellets containing placebo (OVX + placebo) or after surgery without implantation (sham OVX + sham E2). The horizontal lines indicate the upper and lower boundaries (mean ± 1 SEM) for baseline CRP and SAP measured before any treatments. The number of CRPtg mice in each treatment group is indicated. Insets, CRP and SAP serum levels achieved in the same mice 24 hours after intraperitoneal injection of LPS on day 27 after carotid artery ligation. The horizontal lines indicate the upper boundary (mean ± 1 SEM) for CRP and SAP on day 27. *P < 0.05 vs baseline levels; **P < 0.05 vs LPS-induced acute phase levels in OVX + E2 group (Student Newman Keul test).

Figure 2. Representative light micrographs of injured carotid arteries harvested 28 days after ligation injury from female CRPtg (A, C, E) and NTG (B, D, F) mice. Mice were studied INT or after OVX and treatment with E2. H&E with Verhoeff’s elastin stain.

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 also 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

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.
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 from 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 CRP mRNA is expressed in the neointima of injured vessels in CRPtg (Figure 5A). Importantly, although this difference did not achieve statistical significance, CRP mRNA expression in the neointima of injured vessels from E2-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).

Figure 3. Cross-sectional areas of neointima (A), media (B), and neointima/media ratio (C) of injured carotid arteries in female NTG and CRPtg mice measured 28 days after carotid artery ligation injury. Mice were studied INT or after OVX and implantation of E2. Data are shown as mean±SEM, and the sample size (number of mice) is indicated. *P<0.05 vs treatment-matched NTG; #P<0.05 vs genotype-matched INT.

Figure 4. Immunoreactive human CRP in injured carotid arteries of female CRPtg 28 days after carotid artery ligation injury. Human CRP (red staining) was evident in injured arteries of CRPtg OVX mice (A) and to a lesser degree in injured arteries of CRPtg OVX+E2 mice (C), but not in uninjured arteries of CRPtg (B and D). Only slight background staining, perhaps attributable to presence of endogenous mouse CRP, is observed in injured and uninjured vessels from NTG (E and F). Hematoxylin counterstain.

Discussion
Cardiovascular disease is the leading cause of death in women in developed societies, and the risk of cardiovascular disease increases substantially after menopause. This increased risk is thought to reflect loss of the multiple protective effects of E2 on the vasculature. Buoyed by supporting evidence from early observational studies indicating that treatment with various E2 preparations reduced cardiovascular disease risk by ≈50%, 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. 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. In light of these conflicting findings the use of menopausal hormone therapy is now being questioned, and controversy about its risks and benefits persist. 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.
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 shown to be a strong promoter of thrombosis in CRPtg mice.\textsuperscript{8} This indicates that the effects of menopausal hormone therapy are 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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