C-Reactive Protein Upregulates Angiotensin Type 1 Receptors in Vascular Smooth Muscle

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Background—Accumulating evidence suggests that C-reactive protein (CRP), in addition to predicting vascular disease, may actively facilitate lesion formation by inciting endothelial cell activation. Given the central importance of angiotensin type 1 receptor (AT_1-R) in the pathogenesis of atherosclerosis, we examined the effects of CRP on AT_1-R expression and kinetics in vascular smooth muscle (VSM) cells. In addition, the effects of CRP on VSM migration, proliferation, and reactive oxygen species (ROS) production were evaluated in the presence and absence of the angiotensin receptor blocker, losartan. Lastly, the effects of CRP (and losartan) on neointimal formation were examined in vivo in a rat carotid angioplasty model.

Methods and Results—The effects of human recombinant CRP (0 to 100 μg/mL) on AT_1-R transcript, mRNA stability, and protein expression were studied in cultured human VSM cells. AT_1-R binding was assessed with 125I-labeled angiotensin II (Ang II). VSM migration was assessed with wound cell migration assays, whereas VSM proliferation was determined with [3H]-incorporation and cell number. The effects of CRP (and losartan) on Ang II–induced ROS production were evaluated by 2’,7’-dichlorofluorescein fluorescence. Lastly, the effects of CRP (and losartan) on neointimal formation, VSM cell migration, proliferation, and matrix formation were studied in vivo in a rat carotid artery balloon injury model. CRP markedly upregulated AT_1-R mRNA and protein expression and increased AT_1-R number on VSM cells. CRP promoted VSM migration and proliferation in vitro and increased ROS production. Furthermore, CRP potentiated the effects of Ang II on these processes. In the rat carotid artery angioplasty model, exposure to CRP resulted in an increase in cell migration and proliferation, collagen and elastin content, and AT_1-R expression, as well as an increase in neointimal formation; these effects were attenuated by losartan.

Conclusions—CRP, at concentrations known to predict cardiovascular events, upregulates AT_1-R–mediated atherosclerotic events in vascular smooth muscle in vitro and in vivo. These data lend credence to the notion that CRP functions as a proatherosclerotic factor as well as a powerful risk marker. (Circulation. 2003;107:r53-r60.)

Key Words: C-reactive protein n atherosclerosis n smooth muscle n angiotensin

Elevated levels of C-reactive protein (CRP) have emerged as one of the most powerful independent predictors of myocardial infarction, stroke, and vascular death, with prognostic value exceeding that of LDL cholesterol.1–24 In addition to being a powerful risk marker, recent evidence suggests that CRP may directly participate in lesion formation through leukocyte activation and endothelial dysfunction.25–30 CRP, at concentrations known to predict diverse vascular insults, profoundly quenches nitric oxide (NO) synthesis, while augmenting the release of endothelin-1 (ET-1) and upregulating adhesion molecules and chemotactic chemokines, uncovering a proinflammatory and proatherosclerotic phenotype.30 Additionally, CRP facilitates endothelial cell apoptosis and inhibits angiogenesis30 while promoting activation of endothelial NFκB (Verma et al, unpublished observations, 2003). More recently, CRP has been demonstrated to inhibit bone marrow–derived endothelial progenitor cell survival and differentiation (Verma et al, unpublished observations), a key process in postnatal neovascularization. Therefore, CRP is not only a biomarker of atherosclerotic events but seems to function as an active partaker in plaque formation.

Angiotensin II (Ang II) is one of the most important bioactive factors involved in the development and progression of atherosclerosis. The majority of the proinflammatory effects of Ang II are mediated by the AT_1-R, whereas AT_2-R seems to confer vasculoprotection. The present study was conducted to evaluate (1) the direct effects of CRP on AT_1-R expression and kinetics in vascular smooth muscle (VSM) cells, and (2) to study the effect of CRP on AT_1-R–mediated processes, including VSM migration and proliferation, reac-
tive oxygen species (ROS) production, matrix formation, and neointimal formation. We herein report, for the first time, the direct effects of CRP to markedly upregulate AT$_1$-R number and expression, which incite increased ROS production and facilitate VSM migration and proliferation in vitro and in vivo.

Methods

Cell Culture
Human saphenous vein VSM cells were grown in IMDM and supplemented with 10% fetal bovine serum. VSM cells between passages 2 and 4 were used for the studies outlined below. Human recombinant CRP (Calbiochem) was used in all studies described. Given the concern surrounding the potential contamination of CRP with endotoxin, a number of control experiments were performed as described previously, and all studies were performed in the presence of polymyxin-B (50 μg/mL).

Angiotensin Receptor mRNA Expression
VSM cells were treated with CRP (50 μg/mL), and total cellular RNA was extracted by Trizol reagent. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed with the OneStep RT-PCR Kit (Qiagen). For each reaction, 1 μg of total RNA served as a template. For amplification, a primer pair specific for human AT$_1$-R (sense primer, 5'-GTCGATTTGCTTCTTATACATC-3'; antisense primer, 5'-AGCCAGGTATCGATCAATGCTGAGACATC-3') was used. The RT-PCR product was 304 bp. Reverse transcriptase was performed at 50°C for 30 minutes. For PCR, 35 cycles were used at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes. The RT-PCR products were visualized on 1% agarose gels with ethidium bromide. GAPDH was amplified as a reference for quantification of AT$_1$-R mRNA. Densitometric scanning to quantify amounts of RT-PCR product was performed with the use of the PDI Imageware System. The signal intensity of each AT$_1$-R band was normalized by GAPDH. The effects of CRP on mRNA stability were also evaluated. For this purpose, confluent endothelial cells were incubated with CRP in media containing 2.5 μg/mL actinomycin D. Culture media was removed at 0, 8, and 24 hours after actinomycin D treatment and RNA extracted for RT-PCR.

Ang II Production and Ang II Receptor Protein Expression
Human saphenous vein VSM cells (n=6 wells per group) were incubated with human recombinant CRP (5 to 100 μg/mL) for 24 hours. The level of Ang II in the supernatant was determined by ELISA (SPI-BIO). The effects of CRP on AT$_1$-R and AT$_2$-R protein expression was determined by Western blotting with the use of anti–AT$_1$-R and anti–AT$_2$-R polyclonal antibodies (Santa Cruz, Calif). Briefly, VSM cell lysates were fractionated through a 4% stacking and 10% running SDS-PAGE gel, and the fractionated proteins were transferred to polyvinyl difluoride membranes. Blots were blocked for 1 hour at room temperature with blocking buffer (5% nonfat milk in 10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20). Rabbit anti–AT$_1$-R and anti–AT$_2$-R polyclonal IgGs (Transduction Laboratories, Lexington, Ky), at a dilution of 1:2000, were reacted with the blots overnight at 4°C. After washing (2× for 15 minutes in TTBS), the blots were incubated with the secondary antibody (horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin antibody; Santa Cruz) at 1:2000 dilution for 1 hour at room temperature. Visualization was performed with the use of enhanced chemiluminescence. Densitometric analysis of Western blots was performed with the use of PDI Imageware System.

Assessment of AT$_1$-R Binding Sites
Confluent VSM cells were incubated with or without CRP (50 μg/mL, 24 hours) and AT$_1$-R binding was estimated by binding of $[^{125}I]$Sar$^1$,Ile$^8$-Ang II. In this method, 30 μL of binding buffer containing unlabeled Ang II was added to each well, after which 270 μL of buffer containing labeled $[^{125}I]$Sar$^1$,Ile$^8$-Ang II and binding was performed for 1 hour at room temperature. After termination of the reaction, radioactivity was determined and specific binding activity measured (total binding minus nonspecific binding by unlabeled Ang II). Scatchard plot analysis was performed and Bmax and Kd values calculated.

Cell Migration Assay
The effects of CRP on VSM cell migration were evaluated in vitro with the monolayer-wounding cell migration assay (wounding assay); VSM cells that had been grown to confluence in 60-mm plates were subjected to wounding as previously described. The cells were washed twice with PBS after wounding with a sterile, disposable rubber policeman. The wounded cells were then incubated in 10% FBS IMDM culture medium with CRP at final concentration of 5 to 50 μg/mL. Medium and CRP were changed every 48 hours. After 48 to 72 hours of incubation at 37°C, cells migrating from the wound edge were counted at 100× magnification under a phase-contrast microscope. Results were expressed as the mean number of migrating cells per field. In a separate group of experiments, VSM cells were incubated with CRP 25 μg/mL for 24 hours, followed by stimulation with Ang II (0.1 μmol/L) with or without N-acetylcyestein (100 μmol/L) or losartan (10 μmol/L). PDGF-BB (10 ng/mL) was used as a positive control.

Cell Proliferation Assessment
VSM cell proliferation was determined by [H]-thymidine incorporation and cell counting. Human VSM cells were incubated with CRP, Ang II (0.1 μmol/L), and losartan (10 μmol/L) for 24 hours. [H]-thymidine (1 μCi/mL, specific activity 20 Ci per mmol/L) was added during the last 4 hours of incubation. After washing with 10% trichloroacetic acid, the cells were digested in 0.5 N NaOH and radioactivity counted with a Beckman scintillation counter. Cell proliferation was also assessed by counting the cell number at 24 and 48 hours, respectively, in the presence and absence of CRP (50 μg/mL).

Measurement of Intracellular ROS
Intracellular ROS production was measured by 2',7'-dichlorofluorescein diacetate (DCF) fluorescence with confocal laser scanning microscopy techniques. Dishes of subconfluent cells were washed and incubated in the dark for 30 minutes in the presence of 10 μmol/L 2',7'-dichlorodihydrofluorescein diacetate (H,DCF-DA,Molecular Probes). Culture dishes were transferred to an inverted microscope with an MRC 1024 confocal imaging system (Bio-Rad), and ROS generation was detected as a result of the oxidation of H,DCF (excitation, 488 nm; emission long-pass LP515-nm filter set). Pixel images (512×512) were collected by single rapid scans. Pixel density was calculated.

Carotid Artery Balloon Angioplasty Model
Male Sprague-Dawley rats (weight 350 to 400 g; n=5 to 6 per group; Charles River) were anesthetized with ketamine and xylazine, and a 2F embolectomy balloon catheter (Baxter Health Care Corp) was introduced to the left common carotid artery by way of the external artery. Balloon angioplasty was performed as previously described. After balloon injury, an intra-arterial bolus of 200-μg CRP was injected through the same entrance at left external artery by a silicon tube, detained for 1 minute, and followed by an injection of 1 mL low-temperature gelling agarose (Type VII, Sigma) mixed with CRP at a concentration of 100 μg/mL around the carotid artery. In a subgroup, losartan in the drinking water (15 mg/kg per day) was started 3 days before balloon angioplasty and continued to the end of experiment. Rats were euthanized at various time points after injury, chosen as follows on the basis of previous studies elucidating the kinetics of the injury response. Cross-sectional rings (4 μmol/L) were cut at 5 mm before the bifurcation of internal and external carotid arteries and stained with hematoxylin and eosin. Trichromes stain, smooth muscle cell α-actin (DAKO), 5-bromo-2′-deoxyuridine (BrdU, DAKO), Picro-Sirius Red (for collagen stain-
ing, measured by confocal microscope). AT₁-R, CD45, histone H1, and monocyte and macrophage. Smooth muscle cell proliferation was measured in the media and intima with 100 mg of BrdU injected intraperitoneally 24 hours before sacrifice. Migration of cells from media to the intima was measured at 4 days as described previously. Development of the neointima was assessed by measuring the ratio of intimal/medial area. In addition, collagen and elastin content of the neointima was measured at 14 days. In the experiment of 4 weeks period, the low-temperature gelling agarose was replaced every 2 weeks.

Statistical Analysis
Group data are expressed as mean ± SEM. Data were compared between experimental groups by unpaired t test or one-way ANOVA. Differences between groups were further evaluated by the Fisher protected least-squares differences. Differences were considered significant at a value of *P* < 0.05.

Results

Effects of CRP on Angiotensin Receptor Expression and Receptor Binding
Figure 1A depicts the effects of CRP (50 µg/mL, 24 hours) on AT₁-R mRNA expression assessed by Northern blotting. CRP caused a significant increase in AT₁-R mRNA expression in VSM cells, ≈225% that of control, with no change in AT₁-R mRNA stability as assessed by actinomycin D studies (Figure 1C). The increased AT₁-R mRNA expression paralleled a net increase in AT₁-R protein expression, assessed by Western blotting (Figure 1B). CRP did not alter the expression of AT₂-R, nor did it influence Ang II release from VSM cells (not shown).

Figure 1D depicts the effects of CRP on AT₁-R binding sites as assessed by radiolabeled ligand binding assays. Scatchard plot analysis was performed and specific binding coefficients (Kd and Bmax) calculated. Binding to control VSM cells revealed a Kd of 4.38 nmol/L and a Bmax of 210 fmol/mg protein. Strikingly, in CRP-treated cells, there was a significant increase in Bmax (606 fmol/mg protein) with no change in Kd (4.82 nmol/L). These data suggest that CRP increased AT₁-R number without changing receptor affinity.

Effects of CRP on VSM Cell Migration
Figure 2 depicts the effects of CRP on VSM cell migration with the use of a wounding assay. CRP caused a significant increase in VSM cell migration at concentrations of both 25 and 50 µg/mL, which was ≈75% of that induced by PDGF (Figure 2B). The angiotensin receptor–blocker (losartan 10⁻⁵ mol/L) inhibited CRP-induced VSM cell migration (Figure 2B). We next evaluated the effects of CRP treatment on Ang II–induced VSM cell migration (Figure 2C). Ang II (0.1 µmol/L) increased VSM cell migration. Importantly, CRP treatment potentiated Ang II–mediated VSM cell migration (Figure 2C). The effects of CRP to increase Ang II–induced VSM cell migration were completely blocked by N-acetylcysteine, suggesting a role of increased ROS production in this response (Figure 2C). N-acetylcysteine also inhibited the effects of CRP (50 µg/mL) on VSM migration (not shown).
Effects of CRP on VSM Cell Proliferation

Figure 3 depicts the effects of CRP (Ang II and losartan) on VSM cell proliferation assessed by [3H]-thymidine incorporation and cell counts. CRP (50 μg/mL, 24 hours) caused a significant increase in [3H]-thymidine incorporation, an index of DNA synthesis. This effect was similar to that evoked by Ang II (0.1 μmol/L). However, in the presence of both CRP and Ang II, VSM proliferation was markedly stimulated (~250% that of control), suggesting that CRP potentiated the effects of Ang II on VSM cell DNA synthesis. These effects were attenuated by the angiotensin receptor–blocker losartan (10 μmol/L). VSM cell proliferation was also ascertained by cell counts at 24 and 48 hours after CRP incubation. Figure 3 (right panel) depicts the effects of CRP (50 μg/mL) to increase VSM cell number at both 24 and 48 hours, when compared with control cells.

Measurement of Intracellular ROS

Figure 4 depicts the effects of CRP (Ang II and losartan) on VSM cell ROS production, assessed by DCF fluorescence confocal microscopy (bright green). CRP (25 to 100 μg/mL) significantly augmented basal intracellular ROS production to a level similar to that seen with Ang II (0.1 μmol/L) alone. Notably, CRP markedly potentiated the effects of Ang II on ROS production; the combination of CRP and Ang II incited an ~3.5-fold increase in intracellular ROS production. These effects were dependent, in part, on AT1-R, because they were attenuated during losartan coincubation.

Effects of CRP on Neointimal Formation After Balloon Angioplasty

Both 2 and 4 weeks after carotid balloon angioplasty, CRP (50 μg/mL) induced a prominent increase in neointimal thickness, which was significantly greater than the control group (angioplasty alone, Figure 5A). Losartan-treated animals exhibited a decrease in neointimal formation under both control and CRP-treated conditions. In concert with increased neointimal formation, the CRP group exhibited significantly increased cell migration (Figure 5B) and cell proliferation, respectively.
assessed by number of BrdU-stained cells (Figure 5C). Importantly, increased neointimal formation was reflected in greater matrix formation, with the CRP-treated group exhibiting increased collagen and elastin content (Figures 6A and 6B). In keeping with the in vitro data, CRP increased AT1-R expression in the vessel wall after angioplasty, as evidenced by greater AT1-R staining in the neointima (Figure 6C).

Discussion
Over the past few years it has become increasingly clear that inflammation is at the root of atherosclerosis and its complications. In addition to playing a causal role in lesion formation, inflammation can yield predictive and prognostic information of considerable clinical utility. Amid the surge of inflammation research, one singular observation that has generated extraordinary interest is the acute-phase reactant CRP. Accumulating evidence suggests that circulating highsensitivity CRP represents one of the strongest independent predictors of vascular death in a number of settings. Indeed, CRP seems to be a stronger predictor than LDL cholesterol and adds prognostic value to conventional Framingham risk assessment. The link between CRP and atherosclerosis was initially suggested to be that of a biomarker versus a mediator of atherosclerosis. This dogma has been recently revisited, with observations from our group and others suggesting that CRP has a direct effect to promote atherosclerotic processes and endothelial cell inflammation. Human recombinant CRP, at concentrations known to predict vascular disease, elicits a multitude of effects on endothelial biology favoring a proinflammatory and proatherosclerotic phenotype. For

Figure 4. CRP increases ROS production under basal and Ang II-stimulated conditions. ROS were assessed by DCF fluorescence with confocal microscopy (see text for details). CRP increases ROS production to a level similar to that observed with Ang II (0.1 μg/mL) and markedly potentiates the effects of Ang II. These effects are blunted by losartan. The top panel is a representative example of ROS production by CRP at concentrations of 25 and 100 μg/mL (red arrow). *P<0.01, different from all groups; **P<0.01, different from all groups.

Figure 5. A, CRP significantly increases the intima/media ratio 14 and 28 days after angioplasty; an effect that is inhibited by losartan treatment. B, Carotid arteries exposed to CRP in vivo also demonstrated increased cell migration, and C, cell proliferation, as assessed by BrdU-stained cells. *P<0.05, **P<0.01, different from control; †P<0.01 different from the CRP group. The arrows indicate the position of the internal elastic lamina.
example, CRP potently downregulates eNOS transcript and destabilizes eNOS mRNA, with resultant decreases in both basal and stimulated NO release.\textsuperscript{30} In a synchronous fashion, CRP has been shown to stimulate endothelin-1 and interleukin-6 release, upregulate adhesion molecules, and stimulate monocyte chemotactic protein-1, while facilitating macrophage LDL uptake.\textsuperscript{25–28} More recently, CRP has been shown to facilitate endothelial cell apoptosis and inhibit angiogenesis\textsuperscript{30} while augmenting CD14-induced endothelial cell activation (Palusinski et al [Texas Heart Institute] and Verma et al [Toronto General Hospital], unpublished observations). Preliminary observations from our group indicate that CRP also potently upregulates NF-\textkappa{}B, a key nuclear factor facilitating transcription of numerous proatherosclerotic genes. The proatherosclerotic effects of CRP also seem to be modified by risk factors and treatment strategies. For example, hyperglycemia potentiates the effects of CRP on endothelial cell activation (Verma et al, unpublished observations), and pharmacological interventions with statins, glitazones, and bosentan attenuate these processes. In addition to direct effects on promoting endothelial cell activation, CRP seems to function in a fashion that inhibits bone marrow–derived endothelial progenitor cell survival and differentiation (Verma et al, unpublished observations). Endothelial progenitor cells have been suggested to play an important role in postnatal neovascularization, and the ability of CRP to inhibit progenitor cells may be an important mechanism inhibiting compensatory angiogenesis in chronic ischemia. Thus, CRP is not only an inflammatory marker of atherosclerosis/coronary events but also a mediator of the disease because it contributes to the substrate underlying lesion formation, plaque rupture, and coronary thrombosis by interacting with and altering the endothelial cell phenotype.

Ang II is one of the most important proinflammatory molecules, capable of promoting diverse proatherosclerotic processes at the level of the endothelium and VSM. Indeed, pharmacological treatment with angiotensin-converting enzyme inhibitors has emerged as a powerful risk reduction strategy in high-risk patients and patients with diabetes.\textsuperscript{36} The majority of Ang II is produced locally and mediates inflammatory and atherosclerotic actions via the AT\textsubscript{1}-R. The AT\textsubscript{1}-R is a key atherosclerotic switch facilitating Ang II–induced ROS production, VSM migration, proliferation, and vascular remodeling.\textsuperscript{37} Given the central importance of AT\textsubscript{1}-R in the development and clinical course of atherosclerosis, we evaluated the effects of CRP on AT\textsubscript{1}-R and associated pathophysiological processes.

Several novel observations emanate from this study (Figure 7). First, CRP potently upregulates AT\textsubscript{1}-R mRNA and protein and increases the number of AT\textsubscript{1}-R binding sites in VSM cells. This effect is not related to a change in AT\textsubscript{1}-R mRNA stability, because the half-life of AT\textsubscript{1}-R transcript was similar after incubation with actinomycin D. Second, CRP seems to exert direct effects on atherosclerotic processes in the VSM cell, in addition to the well-established proinflammatory effects on endothelial cells. In cultured VSM cells in vitro, CRP markedly stimulated cell migration and proliferation, with an effect approaching 75% of that noted with the prototypical stimulant PDGF. The effects of CRP on VSM cells seem to be closely related to the expression of AT\textsubscript{1}-R because they were inhibited by losartan, an angiotensin receptor blocker. Additionally, CRP augmented Ang II–induced VSM cell migration and proliferation, further supporting a functional relationship between CRP and Ang II in mediating VSM cell pathology. Third, CRP increased basal ROS production and potentiated the effects of Ang II on ROS formation. These effects were also inhibited by losartan, indicating that increased CRP-mediated ROS formation in VSM cells was in part related to increased AT\textsubscript{1}-R expression.
Further evidence of the role of Ang II–induced ROS production is provided by VSM cell migration studies, in which the effects of CRP were attenuated by NAC, a potent intracellular antioxidant. Lastly, in an in vivo model of carotid balloon angioplasty, CRP exposure facilitated AT1-R expression with resultant increases in neointimal formation, VSM migration, and proliferation, and promoted collagen and elastin production, key matrix proteins in the vessel wall. These effects were attenuated by angiotensin receptor blockade with losartan. Taken together, our data provide evidence to support direct proatherosclerotic effects of CRP at the level of the VSM in vitro and in vivo mediated in part, by increased AT1-R expression and signaling. No effect of CRP was found on AT2-R, which may be vasculoprotective in certain settings. Likewise, we did not observe an effect of CRP on Ang II release.

It is important to highlight that for the rat carotid artery injury experiments, we employed human (versus rat) CRP. Because human CRP is a potent activator of rat complement, but rat CRP does not activate rat complement, the use of human CRP in rat in vivo models has been considered appropriate. Griselli et al have demonstrated that the effect of human CRP in a rat myocardial infarction model was completely abrogated by in vivo complement depletion.38 We acknowledge that the study used VSM cells from saphenous veins, and the possibility that differences between venous and arterial smooth muscle cells exist cannot be excluded.

In summary, CRP, at concentrations known to predict cardiovascular events, upregulates AT1-R–mediated atherosclerotic events in VSM in vitro and in vivo. These data lend credence to the notion that CRP functions as a proatherosclerotic factor as well as a powerful risk marker.

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