C-Reactive Protein Increases Plasminogen Activator Inhibitor-1 Expression and Activity in Human Aortic Endothelial Cells

Implications for the Metabolic Syndrome and Atherothrombosis

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Background—Inflammation plays a pivotal role in atherosclerosis. In addition to being a risk marker for cardiovascular disease, much recent data suggest that C-reactive protein (CRP) promotes atherogenesis via effects on monocytes and endothelial cells. The metabolic syndrome is associated with significantly elevated levels of CRP. Plasminogen activator inhibitor-1 (PAI-1), a marker of atherothrombosis, is also elevated in the metabolic syndrome and in diabetes, and endothelial cells are the major source of PAI-1. However, there are no studies examining the effect of CRP on PAI-1 in human aortic endothelial cells (HAECs).

Methods and Results—Incubation of HAECs with CRP results in a time- and dose-dependent increase in secreted PAI-1 antigen, PAI-1 activity, intracellular PAI-1 protein, and PAI-1 mRNA. CRP stabilizes PAI-1 mRNA. Inhibitors of endothelial NO synthase, blocking antibodies to interleukin-6 and an endothelin-1 receptor blocker, fail to attenuate the effect of CRP on PAI-1. CRP additionally increased PAI-1 under hyperglycemic conditions.

Conclusions—This study makes the novel observation that CRP induces PAI-1 expression and activity in HAECs and thus has implications for both the metabolic syndrome and atherothrombosis. (Circulation. 2003;107:398-404.)

Key Words: inflammation endothelium thrombosis

Inflammation plays a critical role in all stages of atherosclerosis from the nascent lesion to acute coronary syndromes.1 C-reactive protein (CRP) is a prototypic marker of inflammation and has been shown in several prospective studies to predict cardiovascular events (CVEs).2–6 Although CRP is clearly a risk marker, data are evolving to suggest that CRP also promotes atherogenesis.7–14 To date, it has been shown in monocytes that CRP induces the production of inflammatory cytokines and promotes monocyte chemotaxis and tissue factor expression.7–9 In endothelial cells, CRP increases the expression of cell adhesion molecules, chemokines, and endothelin-1 (ET-1), decreases endothelial NO synthase (eNOS) expression and activity, and augments monocyte-endothelial cell adhesion.10–14 Also, it is present in the foam cells in atherosclerotic lesions and colocalizes with activated fragments of the complement system.7

Plasminogen activator inhibitor-1 (PAI-1) has a molecular mass of 50 000 and belongs to the superfamily of serine protease inhibitors.15 It is a marker of impaired fibrinolysis and atherothrombosis,15–22 PAI-1 is a key regulator of fibrinolysis by inhibiting tissue plasminogen activator (tPA). Decreased fibrinolysis, primarily attributable to increased PAI-1 activity, has been demonstrated in patients with coronary artery disease (CAD), and there is considerable evidence for elevated PAI-1 levels in CAD, but its status as a factor is still unclear. The role of PAI-1 as a CAD risk marker was first described by Hamsten et al17 in survivors of myocardial infarction. Increased PAI-1 levels have been shown to enhance thrombosis, and antibodies directed against PAI-1 prevented the progression of thrombosis.18–22 Clinical studies have demonstrated an association between high PAI-1 levels and MI or CAD, recurrence of MI, or CVEs in the metabolic syndrome.23–27 Schneiderman et al28 have reported increased PAI-1 gene expression in human atherosclerotic arteries, and there was a clear trend with the degree of atherosclerosis. All of these factors point to the crucial role of PAI-1 in atherothrombosis in humans.18–28 It has been proposed that increased PAI-1 in the vessel wall can promote formation of plaques with lipid-laden cores and thin fibrous caps, which are more prone to rupture.29 Furthermore, PAI-1 deficiency protects against atherosclerotic progression in the mouse carotid artery.30 Recent exciting data demonstrate that transgenic mice that express a stable form of human PAI-1 develop coronary arterial thrombosis.31

PAI-1 levels have been shown to correlate with many variables that cosegregate with the metabolic syn-
CRP levels are significantly increased in patients with features of the metabolic syndrome.\textsuperscript{12,13} Although PAI-1 is expressed in platelets, adipocytes, hepatocytes, monocytes, and smooth muscle cells, endothelial/hepatic PAI-1 is primarily responsible for PAI-1 levels found in plasma.\textsuperscript{29,34} We have recently shown that CRP exerts a direct proinflammatory effect by decreasing eNOS activity and enhancing monocyte adhesion to human aortic endothelial cells (HAECs).\textsuperscript{14} However, there are no studies examining the effect of CRP on PAI-1 expression in HAECs. To additionally understand the effect of CRP on mediators of atherothrombosis, we tested the effect of CRP on PAI-1 expression and activity in HAECs.

**Methods**

For all the experiments, HAECs (Clonetics) were used between 3 to 5 passages. Purity of recombinant human CRP (Calbiochem) was checked by SDS-PAGE, yielding a single band when 1 \( \mu \)g was loaded on the gel. Endotoxin was removed from CRP with Detoxigel column (Pierce Biochemicals) and found to be <0.125 EU/mL (<12.5 pg/mL) by Limulus assay (BioWhittaker), as described previously.\textsuperscript{14} All media were tested for endotoxin and found to have <0.125 EU/mL.

HAECs (1\( \times \)10\(^6\) cells/mL) were used for all assays and incubated with different concentrations of CRP (ranging from 0 to 50 \( \mu \)g/mL) for the different times (3 to 24 hours). Cell viability, assessed by the MTT assay, was >95% with this dose range of CRP.

PAI-1-secreted antigen levels in the cell supernates were measured by sandwich ELISA using a mouse monoclonal anti-PAI-1 IgG (American Diagnostica). This ELISA measures free and complexed human PAI-1. Furthermore, levels of PAI-1 have been shown to significantly correlate with PAI-1 activity.\textsuperscript{35,36} PAI-1 activity was assessed by the Spectrolyse assay using reagents from American Diagnostica, which uses a chromogenic substrate assay in which plasminogen, tPA, and the chromogenic substrate are incubated and the interassay and intra-assay CV for these assays was <10%.

Also, Western blotting for intracellular PAI-1 in HAECs was performed. Cells were lysed, and 20 \( \mu \)g protein per well was loaded and transferred to membranes. Membranes were blocked with 5% milk and then incubated with either rabbit anti-human PAI-1 antibody (1:200 dilution, Santa Cruz Biochemicals, Santa Cruz, Calif) or as a control, anti-human \( \beta \)-actin antibody (Sigma-Aldrich, St Louis, Mo). After washing and incubation with anti-rabbit HRP-conjugated secondary antibodies, the membranes were developed with ECL (Amersham-Pharmacia), as described previously.\textsuperscript{14} PAI-1 mRNA was assessed by first-strand cDNA synthesis followed by reverse transcriptase–polymerase chain reaction (RT-PCR), and the ratio of PAI-1/GAPDH was analyzed. Briefly, RNA was isolated using Trizol (Invitrogen), and 5 \( \mu \)g RNA was used for first-strand cDNA synthesis (Invitrogen). cDNA (100 ng) was amplified using primers (Integrated DNA Technologies) specific for PAI-1 (forward: 5' -GCA CAA TCC CCC ATC CTA CG-3'; reverse: 5'-GTCCT CTC CAC CTC TGA AA-3') and GAPDH (forward: 5'-CCC CATGGCAATTCGTTGCA-3'; reverse: 5'-TCT AGA CGG CAG GTC AGG TCA ACC-3'). PAI-1 was amplified for 30 cycles and GAPDH for 20 cycles. PAI-1 mRNA stability experiments were conducted using actinomycin D (10 \( \mu \)g/mL) as described previously.\textsuperscript{14}

To determine if CRP uptake in HAECs is receptor-mediated, HAECs were incubated with 10 to 50 \( \mu \)g/mL CRP for up to 120 minutes at 4\( ^\circ \)C in PBS with 0.1% azide, which blocks internalization. At the respective time points, FITC-labeled antibodies to CD32 and CD64 (Caltag, Pharmingen) were added, and an additional incubation was undertaken.\textsuperscript{37} The cells were analyzed by flow cytometry to determine the abundance of CD32 and CD64. Irrelevant isotype controls were added to check for nonspecific binding.
Figure 4. Effect of CRP on intracellular PAI-1 protein levels in HAECs. HAEC were incubated with CRP (5 to 50 μg/mL) for 12 hours. Western blotting for intracellular PAI-1 protein or β-actin (as loading control) was performed as described in Methods (A). Lane 1, control; lane 2, CRP 5 μg/mL; lane 3, CRP 10 μg/mL; lane 4, CRP 25 μg/mL; and lane 5, CRP 50 μg/mL. Intracellular PAI-1 protein/β-actin ratio is provided in panel B. *P<0.01 compared with control.

Figure 5. Effect of CRP on PAI-1 mRNA levels in HAECs. HAECs were incubated with CRP (5 to 50 μg/mL) for 6 hours. RT-PCR for PAI-1 mRNA or GAPDH mRNA (as loading control) was performed as described in Methods (A). Lane 1, control; lane 2, CRP 5 μg/mL; lane 3, CRP 10 μg/mL; lane 4, CRP 25 μg/mL; and lane 5, CRP 50 μg/mL. PAI-1/GAPDH ratio is provided in panel B. *P<0.01 compared with control.
All experiments except the receptor binding were performed on at least 3 occasions in duplicate or triplicate. Data are presented as mean±SD. ANOVA was performed to assess significant differences with different doses of CRP. Wilcoxon signed-rank tests were used to compute differences in the variables, and the level of significance was set at \( P<0.05 \).

**Results**

Incubation of HAECs with CRP at different time points (3, 6, 12, and 24 hours) resulted in a maximum increase in secreted PAI-1 antigen levels at 12 hours (Figure 1). Also, PAI-1 activity was significantly increased at 12 hours after incubation with CRP (Figure 2). Boiling of CRP (100°C for 1 hour) abolished its effect on secreted PAI-1 antigen (data not shown). Furthermore, coinubation of CRP with polymyxin B (25 \( \mu \)g/mL) did not abrogate its effect on PAI-1, whereas trypsinization of CRP abrogated its effect on PAI-1, suggesting that this effect was attributable to CRP but not lipopolysaccharide (Figure 3). Also, lipopolysaccharide (up to 100 \( \mu \)g/mL) failed to stimulate secreted PAI-1 antigen in HAECs. Western blotting for intracellular PAI-1 protein showed that CRP (5 to 50 \( \mu \)g/mL) caused a dose-dependent increase in intracellular PAI-1 protein, which was maximal at 12 hours with no change in \( \beta \)-actin levels (Figure 4). Also, incubation of HAECs with CRP resulted in a dose-dependent increase of PAI-1 mRNA levels as determined by PAI-1 RT-PCR using GAPDH as internal control (Figure 5). PAI-1 mRNA was maximally increased at 6 hours. Furthermore, CRP significantly increased PAI-1 mRNA stability (control, \( t_{1/2} \): 15 hours; CRP 50 \( \mu \)g/mL, \( t_{1/2} \): 18 hours; \( P<0.05 \), \( n=3 \) experiments).

Because we had earlier shown that CRP decreases eNOS\(^{14}\) and, furthermore, it has been shown that CRP activates ET-1 and IL-6 in human saphenous vein endothelial cells,\(^{12}\) we tested the effects of these mediators on PAI-1 expression augmented by CRP. Inhibition of eNOS with \( L \)-NMMA (1 mmol/L) while decreasing eNOS in HAECs failed to affect PAI-1 expression (Figure 6). Similarly, the ET-1 receptor blocker (bosentan, 10 \( \mu \)mol/L) failed to have any effect on PAI-1 expression; blocking antibodies to IL-6 (5 \( \mu \)g/mL) did not have any effect on PAI-1 expression (Figure 6) but decreased IL-6 levels. In preliminary experiments (\( n=2 \)), we show that CRP binds to both CD32 and CD64 in HAECs. Binding was maximum at 90 minutes and saturable at CRP levels of 50 to 100 \( \mu \)g/mL.

Because PAI-1 is increased in the metabolic syndrome and diabetes,\(^{26,27,38–42}\) we examined the effect of CRP under high-glucose conditions (25 mmol/L) on PAI-1 expression. CRP significantly increased secreted PAI-1 levels additionally under hyperglycemic conditions (C-765±149 ng/mL; CRP 50 \( \mu \)g/mL to 1183±171 ng/mL; CRP 50 \( \mu \)g/mL plus HG 25 mmol/L to 1455±174 ng/mL; \( P<0.005 \) by ANOVA; Figure 7).

**Discussion**

In addition to being a risk marker for cardiovascular disease, several lines of evidence point to a proatherogenic role for CRP.\(^{2–14}\) CRP has been shown to exert proinflammatory effects in endothelial cells. Endothelial PAI-1 seems to be primarily responsible for PAI-1 levels in plasma.\(^{26,34}\) CRP levels and PAI-1, a marker of atherothrombosis, are increased...
in subjects with the metabolic syndrome and diabetes. Furthermore, both PAI-1 and CRP levels seem to be elevated and cosegregate with the different features of the metabolic syndrome.26,27,32,33,38–42 However, there are no studies examining the effect of CRP on PAI-1 in HAECs or adipocytes.

Because we have previously shown that CRP decreases eNOS in HAECs14 and previous work has shown that CRP stimulates cell-adhesion molecules, monocyte chemotactic protein-1, and monocyte-endothelial cell adhesion, in the present study, we tested the hypothesis that CRP could promote expression and activity of PAI-1 in HAECs.

We first tested the effect of CRP on secreted PAI-1 antigen levels as well as activity in HAECs. CRP significantly increased secreted and intracellular PAI-1 antigen as well as activity in HAECs in a dose-dependent manner. Also, it is important to note that all reagents and media used were free of endotoxin (<12.5 pg/mL); addition of polymixin B did not affect the of effects CRP on PAI-1. Ballou et al9 have previously shown that CRP induces monocyte proinflammatory cytokine release and that addition of polymixin B did not abrogate its effect, thus ruling out the effect of endotoxin contribution to the proatherogenic effects of CRP. Furthermore, lipopolysaccharide (100 pg/mL) at a concentration far in excess of any contamination present in our CRP preparations (<12.5 pg/mL) failed to stimulate PAI-1 levels in HAECs. The effect of CRP on PAI-1 antigen and activity levels was maximal at 12 hours. It has previously been reported by Kooistra et al43 that PAI-1 released from endothelial cells is rapidly inactivated because of production of substrate tPA by endothelial cells. Furthermore, aortic endothelial cells produce 20 times more PAI-1 than HUVECs, and the amount of PAI-1 produced by HAECs increases from passages 1 to 444; thus HAECs are a good model to study the regulation of PAI-1. All of our experiments were conducted within 5 passages of cells.

It seems that the effect of CRP on PAI-1 levels is at the transcriptional level. Our studies show that CRP augments the stability of PAI-1 mRNA. Previously, insulin and cytokines have been shown to augment PAI-1 release via increasing mRNA stability in BAECs.45,46

To obtain mechanistic insights into the effects of CRP on PAI-1 in HAECs, we performed inhibitor studies. CRP has been shown to augment endothelin-1 (ET-1) and interleukin 6 (IL-6)12 and thereby contribute to increased ICAM-1, VCAM-1, and MCP-1 in human saphenous vein endothelial cells. In our system (HAECs), in addition to CRP failing to augment ET-1/IL-6 levels, an ET blocker or IL-6 blocking antibodies failed to affect PAI-1 expression and activity. Thus, it is clear that different mechanistic pathways operate in different cell systems, ie, venous versus aortic endothelium. Because the aortic endothelium is the primary site for atherosclerosis, it is prudent to study the effects of CRP in these cells. Because CRP decreases eNOS expression and activity in HAECs,14 we examined the effect of L-NMMA on PAI-1 expression. L-NMMA, although decreasing eNOS protein, failed to have any effect on PAI-1 expression. Fc receptors have been shown to be the major receptors for CRP on leukocytes and are absent in venous endothelium.47 Thus, the reported effects on venous endothelium may not be receptor-
mediated. In preliminary data, we show that CD32 and CD64 seem to be the receptors for CRP in HAECS, and future detailed studies will delineate the major receptor accounting for the effect of CRP on HAECS.

The metabolic syndrome seems to be a major risk factor for cardiovascular disease, and numerous studies have now confirmed that CRP levels are elevated in patients with the metabolic syndrome and diabetes.32,33 In the Insulin Resistance Atherosclerosis Study (IRAS),27 PAI-1 and CRP showed strong correlations with development of diabetes. Unlike fibrinogen and CRP, the association of PAI-1 to incident diabetes was particularly strong and independent of other known factors associated with diabetes. The authors suggested that both PAI-1 levels and CRP levels may be common antecedents for the metabolic syndrome and atherosclerosis and may do so by promoting chronic inflammation (common soil hypothesis). Increased levels of PAI-1 have been shown to be correlated with chronic inflammation (common soil hypothesis). Increased levels of PAI-1 have been shown to be correlated with insulin resistance, so that increased plasma PAI-1 levels are now considered one of the features of the metabolic syndrome.38,48,49 Chronic hyperglycemia is associated with increased PAI-1 localization in the aortic wall,39,40 and Sobel et al41 have found greater PAI-1 content in atheroma specimens of diabetics. Thus, it is very interesting that in our studies, in presence of high glucose, PAI-1 expression and activity is augmented additionally by CRP in HAECS. Thus, given that both CRP and PAI-1 are present in the atherosclerotic lesion, augmentation of PAI-1 by CRP, especially under hyperglycemic conditions, could have a negative impact on vascular remodeling.29

The present study points to a pivotal role for inflammation as assessed by increased CRP and increased PAI-1 levels, which seems to be the underpinning of atherothrombosis, especially in the metabolic syndrome and the diabetic state. Future studies will unravel other mechanisms by which CRP orchestrates this novel biological effect in endothelial cells.

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