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Macrophage Conditioned Medium Induces the Expression of C-Reactive Protein in Human Aortic Endothelial Cells

Potential for Paracrine/Autocrine Effects

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C-reactive protein (CRP) is a risk marker for cardiovascular events in apparently healthy persons. Cogent data show that, aside from the liver, CRP is produced in atherosclerotic lesions, kidney, neurons, and alveolar macrophages. Because several proatherogenic effects of CRP have been documented in endothelial cells, we examined human aortic endothelial cells (HAEC) for CRP production. We detected the presence of CRP mRNA by RT-PCR and *in situ* hybridization, intracellular protein by Western blot and secreted protein by ELISA. Coincubation with the cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor alone and in combination showed that the most potent agonist for CRP production from HAEC is the combination of IL-1 and IL-6 ($P < 0.05$). To mimic the *in vivo* situation, we examined whether vascular smooth muscle cell (VSMC) and/or macrophage conditioned media (MCM) could augment CRP production by HAEC. While VSMC-conditioned media had no effect, incubation with MCM resulted in a significant twofold increase in the synthesis of both intracellular and secreted CRP ($P < 0.05$). The effect of MCM could be reversed by inhibiting both IL-1 and IL-6. Thus, stimulated synthesis and secretion of CRP by cells in the atherosclerotic lesion by paracrine/autocrine loops could result in local concentrations of CRP far in excess of plasma concentrations and could contribute to proinflammatory, proatherogenic effects. *(Am J Pathol 2005, 166:1265–1271)*

C-reactive protein (CRP), the prototypic downstream stable marker for inflammation, is a predictor of future cardiovascular disease in apparently healthy persons. To date in numerous studies, CRP has been shown to predict myocardial infarction, cardiovascular death, stroke, peripheral arterial disease, and sudden death.[1–3] Human CRP, a non-glycosylated protein, is a member of the pentraxin family, comprising 5 subunits of non-covalently associated protomers (206 amino acids each) with a molecular weight of 118 kd, and is mapped to chromosome 1.[4] CRP synthesis in liver is induced by many factors including infection, tissue injury, and autoimmune disorders.[5,6] CRP production is mainly driven by interleukin (IL)-6 with a synergistic effect of IL-1 in hepatocytes.[7–12]

Recent data from several studies have now shown that CRP is not only produced by the liver but also by other tissues. Recently CRP was shown to be expressed in atherosclerotic lesions.[13–15] The demonstration of CRP mRNA abundance in human atherosclerotic lesions of 10-fold in excess of normal arteries implies that in lesions there could be possible microdomains with very high levels of CRP. Thus, the potential for autocrine and paracrine loops exist in the intima and could contribute to atherothrombosis. Also, alveolar macrophages, neuronal cells, and tubular epithelial cells have been shown to produce CRP.[16–18] To date the majority of the proatherogenic effects of CRP have been documented in endothelial cells.[19] However, there are no data on whether human aortic endothelial cells produce CRP. In this study we show that human aortic endothelial cells (HAEC) and human coronary artery endothelial cells (HCAEC) ex-

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press mRNA and protein for CRP, and this is augmented by cytokines as well as macrophage conditioned media.

Materials and Methods

Cell Culture

HAEC and HCAEC and endothelial cell supplements were obtained from Clonetics. These cells were subcultured in EGM-2MV media supplemented with growth factors and 2% serum and used between the second and sixth passages with 80 to 90% confluency.[20] These cells were incubated in serum- and growth factor-free media for the expression of CRP. IL-1 (10–50 ng/ml), IL-6 (5–50 ng/ml), and tumor necrosis factor (TNF)-α (10–50 ng/ml) were incubated with these cells in serum-free conditions for 24 or 48 hours and the RNA or protein/media were collected for CRP measurements, respectively.

For macrophage culture, following informed consent, fasting blood was obtained from healthy human volunteers. The monocytes were isolated and cultured as described previously.[21] These cells were cultured for 9 days and replenished with medium containing autologous serum (10%) on days 0, 3, and 6. On day 9, the conditioned medium was collected, centrifuged, filtered, and stored at −20°C in aliquots. These were added to endothelial cells in the dose range of 0 to 20%. For neutralizing experiments, IL-1 receptor antagonist (10–200 ng/ml), IL-1, or IL-6 neutralizing antibodies (0.1–2 μg/ml) and soluble TNF-receptor 1 (0.1−0.5 μg/ml) were preincubated for 1 hour with endothelial cells before the addition of 1% macrophage conditioned media for a period of 12 or 48 hours for RNA or protein isolation. The concentration of IL-1 and IL-6 in the media was checked by ELISA to determine efficacy of neutralization following precipitation of the antigen-antibody complex (data not shown).

CRP mRNA Expression

RNA was extracted from the cells using TRIzol (Invitrogen, Carlsbad, CA) reagent. The first strand of cDNA was synthesized using total RNA (1 μg/reaction). cDNA (100 ng) was amplified using primers (Integrated DNA Technologies, Coralville, IA) specific for CRP (forward, TCGTATGCCACCAAGAGACAAGACA; reverse, AACACTTCGCCTTGCACTTCATA-CT) and GAPDH (forward: 5′-CCA-CCCATGGCAAATTCCATGGCA-3′ and reverse: 5′-TCTAGACGGCAGGTCAGGTCCACC-3′). CRP was amplified for 35 cycles and GAPDH for 20 cycles. CRP yielded a band at 440 bp and GAPDH at 520 bp on 2% agarose gels.

In Situ *Hybridization*

Products from the above RT-PCR reactions were used for preparation of sense and antisense riboprobes. SP6/T7 promoters were added to the PCR products using the LigN′Scribe protocol from Ambion, Austin, TX, followed by *in vitro* transcription using fluorescein-12-UTP and reagents from Roche, Indianapolis, IN. RNA was then purified using the RNeasy MinElute cleanup columns from Qiagen, Valencia, CA. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X-100, and then pre-hybridized for 1 hour at 45°C followed by hybridization for 24 hours using the fluorescent labeled sense and antisense probes. The excess probes were then removed and cells washed with 2X salt sodium citrate solution (SSC) at room temperature for 15 minutes and then with 0.1X SSC at 60°C for 15 minutes. After washing in PBS, cells were examined under a fluorescent microscope.

CRP Protein Expression

The cells were collected in lysis buffer containing Tris 10 mmol/L, EDTA 1 mmol/L, EGTA 0.5 mmol/L, glucose 5 mmol/L, and protease inhibitor cocktail, after the incubations with different agents and sonicated briefly three times for 10 seconds each using a membrane dislocator (Fisher Scientific, Santa Clara, CA). The protein was estimated and 10 to 30 μg/lane was loaded on SDS-PAGE gels. Proteins were transferred to nylon membranes and blocked using 5% milk solution. Rabbit anti-human CRP (Calbiochem, San Diego, CA) antibody was used as primary antibody, and after washing six times with 1X TBS-Tween 20, the membranes were incubated with goat anti-rabbit IgG secondary antibody. After thorough washing the membranes were developed using an Enhanced Chemiluminescence kit (Amersham-Pharmacia, Piscataway, NJ). In all Western blot experiments, β-actin (mouse monoclonal antibody; Sigma Aldrich, St. Louis, MO) was used as an internal control.

CRP Assays

CRP levels in the supernatants were measured using an enzyme-linked immunosorbent assay (ELISA) kit specific for human CRP (Alpco Laboratories, Windham, NH) according to the manufacturer's instructions. The minimum detectable concentration of the assay was 0.95 ng/ml. The intra-assay CV of the assay was $<$10%. All media CRP levels are reported as ng/mg cell protein.

Statistical analyses was performed using GraphPad Prizm software. Analysis of variance followed by paired *t*-tests were used to determine significant differences between treatments, and significance was set at $P < 0.05$.

Results

As shown in Figure 1, both HAEC and HCAEC demonstrated mRNA and protein for CRP, which was identical to that of the liver cell line, HepG2 (Figure 1, A and B). However, we failed to detect any CRP mRNA or protein from HUVEC. To further confirm the presence of CRP in HAEC, *in situ* hybridization was performed using antisense riboprobes specific for CRP. Strong staining for CRP (Figure 1C) was evident with antisense but not sense riboprobes for CRP. It has been shown in many

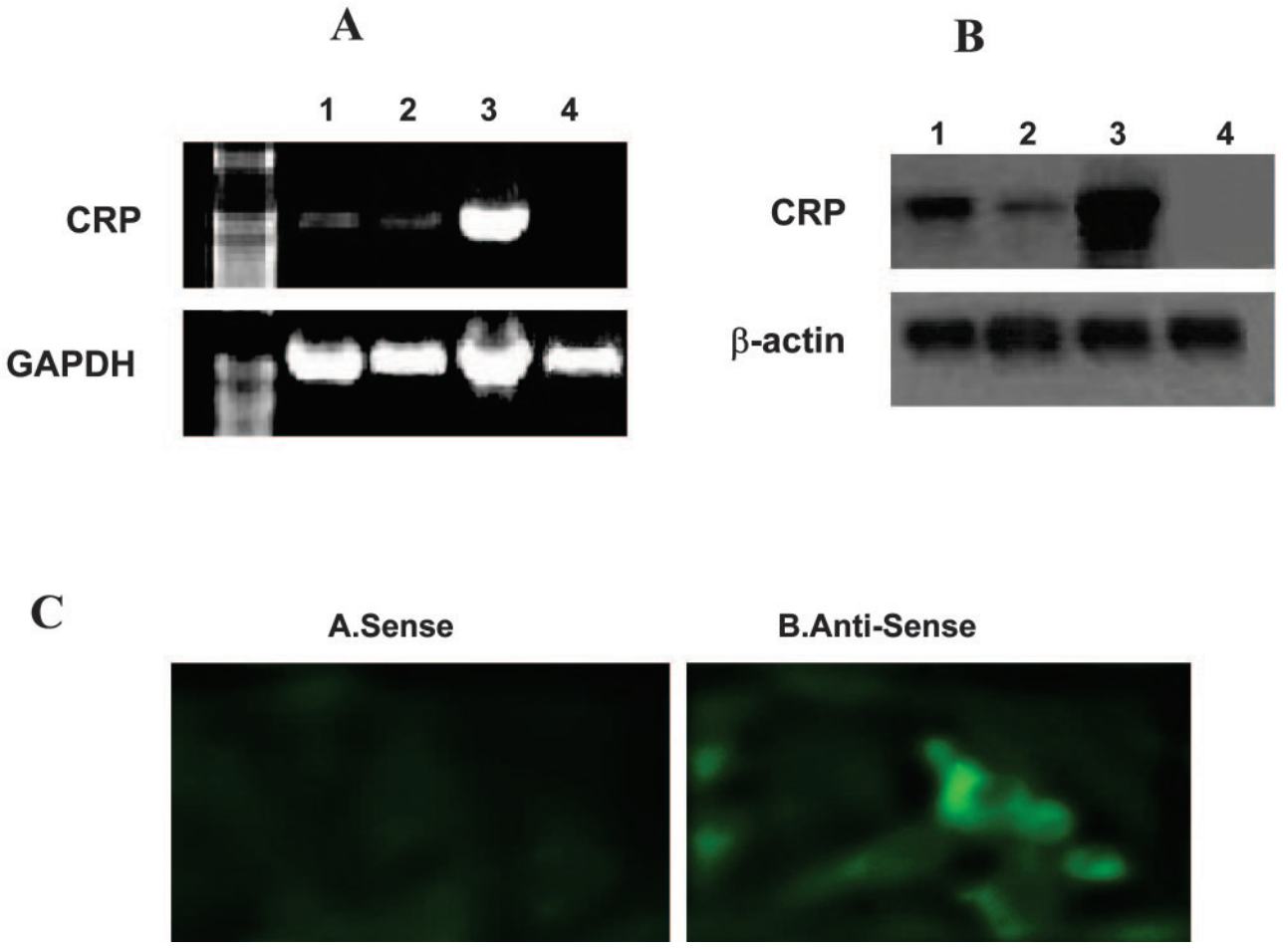

Figure 1. Expression of CRP mRNA (**A**) and protein (**B**) in HAEC (**lane 1**), HCAEC (**lane 2**), HepG2 cells (**lane 3**), and HUVEC (**lane 4**). **C:** *In situ* hybridization for CRP using sense (**A**) and antisense (**B**) riboprobes. Cells were cultured for 24 hours in serum-free media and RNA was isolated, and first strand of cDNA was synthesized and amplified by RT-PCR. GAPDH amplification was used as an internal control. For Western blots, the cells were collected after 48 hours and then Western blots were performed as described in Materials and Methods. β-Actin was used as internal control. *In situ* hybridization was carried out as described in Materials and Methods. Data presented are representative of five different experiments for **A** and **B** and three different experiments for **C**.

studies that proinflammatory cytokines induce CRP in hepatocytes. Hence, HAEC were incubated with proinflammatory cytokines IL-1β, IL-6, and TNF-α either for 12 hours or 24 hours and the RNA or protein was collected for RT-PCR or Western blots, respectively. While TNF had no significant effect, both IL-1 and IL-6 induced CRP mRNA and protein expression in HAEC (Figure 2). In HAEC the most potent stimulus was a combination of IL-1 and IL-6, despite the concentration of IL-1 being reduced by 50% when combined with IL-6. Also, CRP levels in the supernatant, as assessed by ELISA, were significantly increased with the combination of IL-1 and IL-6 compared to either alone or compared to control (control, 0.4 $\pm$ 0.2 ng/mg cell protein; IL-1, 1.0 $\pm$ 0.3* ng/mg cell protein; IL-6, 3.6 $\pm$ 0.8* ng/mg cell protein; IL-1 + IL-6, 5.1 $\pm$ 1.7* ng/mg cell protein, $n = 3$, $^*P < 0.05$ compared to control).

In atherosclerotic lesions, the endothelial cells are in proximity to both smooth muscle cells and macrophages. Hence, we examined the effect of different concentrations of macrophage and smooth muscle cell conditioned media (0 to 20%) on CRP expression in HAEC. While smooth muscle cells conditioned medium had no significant effect on CRP expression in HAEC (data not shown), macrophage conditioned medium (MCM) resulted in a significant increase in CRP as determined by Western blot (Figure 3A). Furthermore, the induction of CRP mRNA with MCM paralleled the increase in protein (Figure 3B). Also, incubation with MCM induced a substantial increase in secreted CRP protein and this effect was dose-dependent (Table 1).

As with hepatocytes, in HAEC, CRP is stimulated by both IL-1 and IL-6. These results suggest that the cytokines present in the macrophage conditioned media might be responsible for CRP expression in HAEC. Based on the regulation of CRP mRNA and protein in hepatocytes, we attempted to define the active moiety in macrophage conditioned medium that elicited CRP release. To this end, we performed experiments with neutralizing antibodies to IL-1 and IL-6 and also with IL-1R antagonist and sTNFR. We showed that both IL-1Ra and IL-6 antibodies independently decrease mRNA (Figure 4A) and protein for CRP (Figure 4B) in EC, while an irrelevant antibody was without effect (Figure 4, A and B). We were able to reverse this increase in CRP mRNA and protein with the combination of antibodies to IL-1 and IL-6

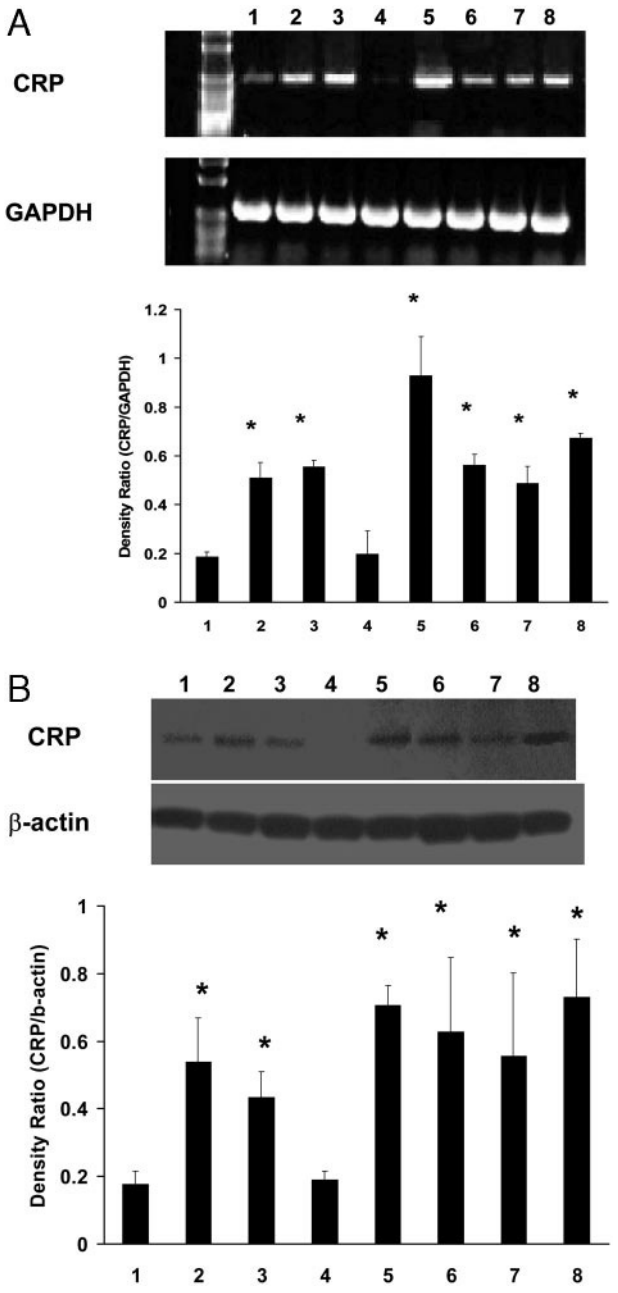

Figure 2. Effect of cytokines on the expression of CRP mRNA (**A**) and protein (**B**) in HAEC. **Lane 1**, control; **lane 2**, IL-1β (50 ng/ml); **lane 3**, IL-6 (10 ng/ml); **lane 4**, TNF-α (50 ng/ml); **lane 5**, IL-1β (25 ng/ml) + IL-6 (10 ng/ml); **lane 6**, IL-1β (25 ng/ml) + TNF-α (10 ng/ml); **lane 7**, IL-6 (10 ng/ml) + TNF-α (10 ng/ml); **lane 8**, IL-1β (25 ng/ml), IL-6 (10 ng/ml), TNF-α (10 ng/ml). $N = 4$ experiments for densitometric ratios, $^*P < 0.05$ compared to control

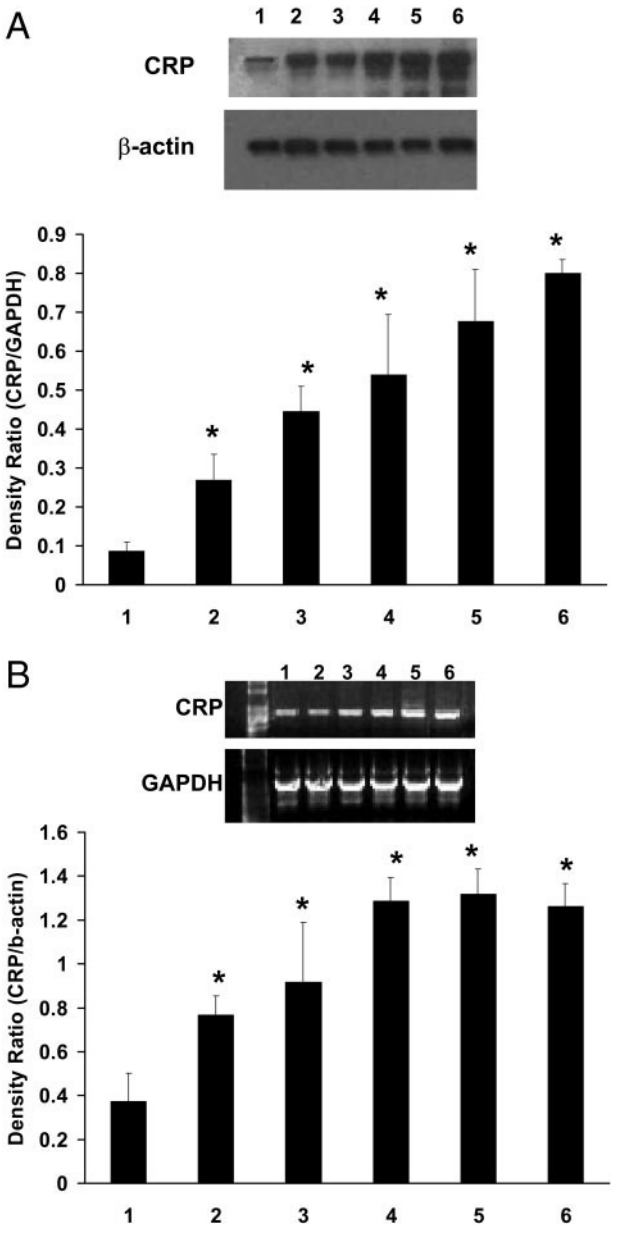

Figure 3. Effect of macrophage conditioned media on HAEC CRP expression by Western blots (**A**) and mRNA expression by RT-PCR (**B**). **Lane 1**, control; **lane 2**, MCM 1%; **lane 3**, MCM 2%; **lane 4**, MCM 5%; **lane 5**, MCM 10%; **lane 6**, MCM 20%. $N = 5$ experiments for densitometric ratios, $^*P < 0.05$ compared to control.

(Figure 4, A and B). Substituting IL-1 neutralizing antibodies for IL-1RA revealed a substantial decrease in MCM-induced CRP mRNA and protein alone and in combination with IL-6 antibodies (data not shown). Furthermore, incubation of MCM with sTNFR failed to significantly affect CRP expression (secreted CRP, MCM 1%, 6.6 $\pm$ 0.9 ng/mg cell protein; MCM 1% + S-TNFR, 7.1 $\pm$ 1.1 ng/mg cell protein, $n = 3$, $P > 0.05$).

Discussion

Inflammation is pivotal in all stages of atherosclerosis.[22–24] Numerous prospective studies have established that levels of C-reactive protein, an acute phase protein, predict future cardiovascular events. In addition, several recent studies show that CRP exerts proatherogenic, prothrombotic effects in smooth muscle cells, monocyte-macrophages, and endothelial cells.[16] We and others have shown that in endothelial cells, CRP down-regulates endothelial nitric oxide synthase (eNOS), and prostacyclin and up-regulates endothelin-1 (ET-1), plasminogen activator inhibitor-1 (PAI-1), IL-8, vascular cell adhesion molecule

Table 1. CRP Secretion from HAEC Following Incubation with MCM

HAEC media	CRP (ng/mg cell protein)
Control	0.5
MCM (1%)	7.3 ± 0.61*
MCM (2%)	16.8 ± 0.92*
MCM (5%)	52.6 ± 1.6**
MCM (10%)	83.4 ± 8.6**
MCM (20%)	142.8 ± 16.2**

*$P < 0.05$; **$P < 0.01$ compared to control. $n = 3$ experiments.

(VCAM), intercellular adhesion molecule (ICAM), monocyte chemotactic protein-1 (MCP-1), and monocyte-endothelial cell adhesion.[19] While the original research indicated that CRP is produced exclusively in the liver, several recent reports have shown that in fact, CRP is expressed in non-hepatic tissues such as atherosclerotic plaques, tubular epithelial cells, neurons, and alveolar macrophages.[13–18] In this report, we make the novel observation that human aortic endothelial cells also express mRNA and protein for CRP. Using a comprehensive approach of examining CRP mRNA by RT-PCR and *in situ* hybridization, CRP protein by Western blotting and secreted protein by ELISA, we document mRNA, intracellular and secreted CRP from human aortic endothelial cells. Initial observations documenting CRP in the vessel wall were reported by Reynolds and Vance,[15] who showed immunoreactivity for CRP in lesions in human autopsy aortic specimens. Subsequently, Zhang et al[25] demonstrated CRP immunofluorescence in atherosclerotic lesions, but not in normal human coronary arteries. However, Yasojima et al[13] made the seminal observation, using techniques of *in situ* hybridization, immunohistochemistry, RT-PCR, and Western blotting, that mRNA and protein for CRP is expressed in arterial plaque tissue and that both CRP mRNA and protein levels are 10-fold higher in plaque when compared to the normal artery, suggesting that CRP is produced in atherosclerotic lesions. The CRP mRNA was mainly localized to macrophages and smooth muscle like cells. Subsequently, Kobayashi et al[14] documented the presence of CRP mRNA and protein in directional coronary atherectomy specimens. Using double immunofluorescence, they showed that both macrophages and SMC expressed CRP. They ascribed their failure to document overlap of CRP with endothelial cell markers to the large amounts of endothelial cells lost or damaged during the procedure. Like Yasojima et al,[13] they failed to document secreted CRP from vascular cells. Burke et al[26] reported a strong correlation of serum levels of hs-CRP with immunohistochemical staining intensity for CRP and numbers of thin cap atheroma in patients dying suddenly with severe coronary artery disease. Sano et al[27] showed that in the setting of acute myocardial infarction, CRP is related to plaque rupture by IVUS. Jabs et al[28] have also shown that CRP is expressed in coronary artery venous bypass grafts by real-time PCR and immunohistochemistry. Vainas et al[29] have recorded a correlation between serum CRP and abdominal aortic aneurysmal size and documented CRP mRNA in aneurysmal aortic tissue. Again, neither the secretion of CRP nor the regulation of CRP was reported in the above studies. Calabro et al[30] have shown that human coronary

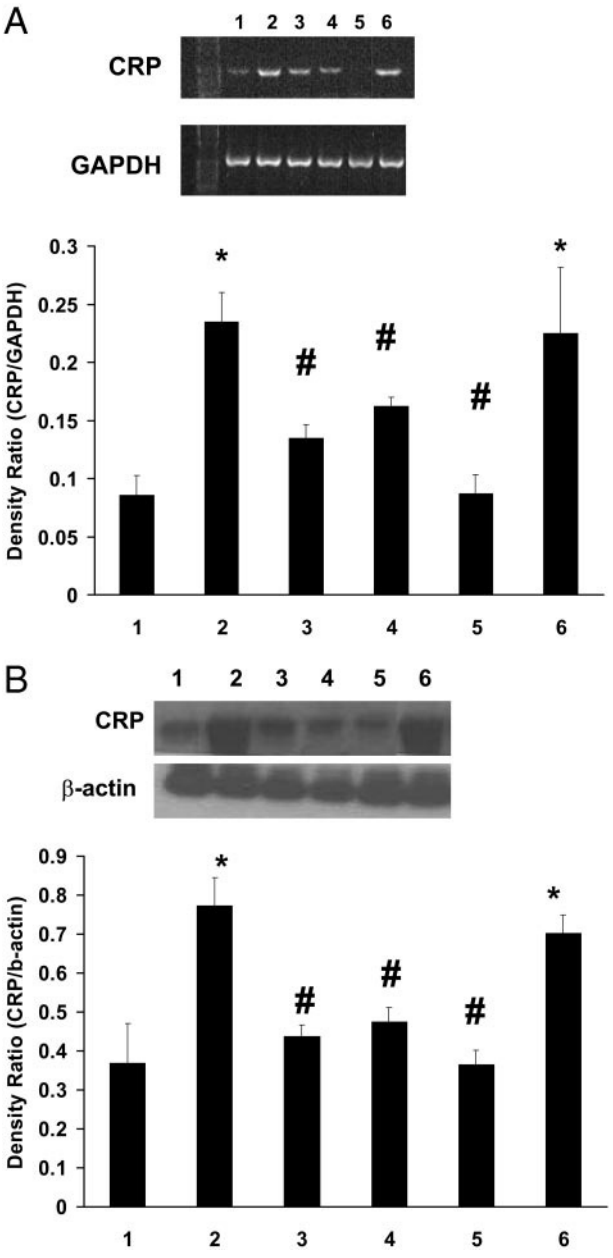

Figure 4. Effect of neutralizing antibodies to IL-1 and IL-6 as well as IL-1R antagonist on macrophage conditioned media induced CRP mRNA (**A**) and protein (**B**) expression in HAEC. **Lane 1**, control; **lane 2**, MCM 1%; **lane 3**, MCM 1% + IL-1Ra (10 ng/ml); **lane 4**, MCM 1% + IL-6 ab (0.1 μg/ml); **lane 5**, MCM 1% + IL-1Ra (10 ng/ml) + IL-6 ab (0.1 μg/ml); **lane 6**, MCM 1% + irrelevant antibody (1 μg/ml). $N = 5$ experiments for densitometric ratios, *$P < 0.05$ compared to control; #$P < 0.05$ compared to 1% MCM.

artery smooth muscle cells but not HUVEC (we also failed to observe any CRP expression in HUVEC), in culture express CRP by RT-PCR and ELISA. However, they did not use serum-free media or report on intracellular detection of CRP. Hence, the CRP detected by ELISA could have come from the external sources such as serum. The difficulty in all of the above-mentioned studies except that of Calabro et al[30] is that they could not measure the secreted CRP, since most of the studies examined tissues. Hence, it is not clear whether the expressed CRP is secreted and thus functional. This is critical to document since it is relevant to the thesis

that CRP exerts paracrine and autocrine effects in the intima. Furthermore, there is a paucity of data in HAEC, where the majority of the proatherogenic effects of CRP have been described. Our study clearly shows that the expressed CRP is secreted as measured by ELISA and this, together with CRP deriving from the liver and other sources, could result in much higher CRP levels in microdomains in the intima. Yasojima et al[13] have shown 10-fold excess mRNA for CRP in atherosclerotic lesions compared to normal arteries and 7.2-fold higher than in liver. None of the studies detailed above has systematically demonstrated the expression of CRP mRNA, intracellular protein, and secreted protein levels or examined the modulation of CRP message and protein in HAEC. This is the first complete study to demonstrate that C-reactive protein is expressed at RNA levels as determined by RT-PCR and *in situ* hybridization and at protein levels as shown by Western blots and ELISA for the secreted protein in human aortic endothelial cells. Furthermore, we hypothesize that the secreted CRP could be involved in the autocrine or paracrine loop to induce vascular dysfunction as shown previously.[19,31–33] While in agreement with Calabro et al[30] we fail to show CRP expression in HUVEC, it is important to note that the arterial tree is the primary site for atherosclerosis, and there may be differences between the arterial and venous beds with respect to CRP expression and its proatherogenic effects. This will be explored in future studies.

In addition, we show that the cytokines IL-1 and IL-6 in combination up-regulate CRP mRNA and protein. In the vessel wall, the endothelial cells are in the proximity of smooth muscle cells and macrophages. The secretions from these cells could affect the function of endothelial cells. Hence, we decided to examine the effect of conditioned media from smooth muscle cells and human macrophages on CRP expression in HAEC. As shown in our results, macrophage conditioned media significantly induced CRP mRNA, protein, and secreted protein but not smooth muscle cell conditioned media. Macrophage conditioned media, when added to HAEC, resulted in a significant increase in secreted CRP. This suggests the possibility of autocrine as well as paracrine loops for the actions of CRP in endothelial cells culminating in endothelial cell dysfunction: increased plasminogen activator inhibitor-1, increased cell adhesion molecules, decreased eNOS and prostacyclin. In the liver, proinflammatory cytokines such as IL-1 and IL-6 are shown to up-regulate CRP.[34–37] Our experiments show that among the different cytokines, IL-1 and IL-6, in combination, appear to be the most potent inducers of CRP mRNA and protein in HAEC. Furthermore, neutralization of both IL-1 and IL-6 was more effective than neutralization of either alone in inhibiting the MCM-induced CRP mRNA and protein. These results are in support of previous observations made by Calabro et al[30] in human coronary SMCs, where they showed maximal induction of CRP mRNA following the combined incubation with IL-1 and IL-6. Furthermore, in hepatocytes, the combination of IL-1 and IL-6, but not IL-1 alone, appears to drive maximal production of CRP.[10–12,35,37] Our future studies will be directed to explore the transcriptional regulation of endothelial cell CRP focusing initially on Rel proteins STAT3 and C/EBP.[12] These findings support our hypothesis that there are microdomains in the intima, where the local production of CRP results in higher levels than in the circulation, creating the potential for autocrine/paracrine loops among cells in the atherosclerotic lesion. This could lead to a vicious cycle culminating in plaque instability and rupture, since CRP is an index of plaque activity. Thus, in this study, we make two novel observations: aortic endothelial cells, in addition to expressing CRP mRNA and protein, secrete appreciable amounts of CRP; and macrophage conditioned media is a potent stimulant for CRP production via IL-1 and IL-6.

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