Effect of C-reactive protein on gene expression in vascular endothelial cells

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Wang, Qingwei, Xiaojun Zhu, Qin Xu, Xia Ding, Yuqing E. Chen, and Qing Song. Effect of C-reactive protein on gene expression in vascular endothelial cells. Am J Physiol Heart Circ Physiol 288: H1539–H1545, 2005. First published December 9, 2004; doi:10.1152/ajpheart.00963.2004.—C-reactive protein (CRP) is significantly associated with the risk of ischemic cardiovascular disease in epidemiological studies. To explore if CRP has a functional role, we investigated its effect on the gene expression profile of vascular endothelial cells. Human vascular endothelial cells (umbilical vein endothelial cells and human aortic endothelial cells) were incubated with CRP at various concentrations (0–10 μg/ml). Microarray analysis showed that a total of 11 genes increased (IL-8, core promoter element binding protein, activin A, monocyte chemoattractant protein 1, Exostoses 1, Cbp/p300-interacting transactivator with Glu/Asp-rich COOH-terminal domain 2, plasminogen activator inhibitor 1, fibronectin-1, gravin, connexin43, and sorbip-related receptor-1) and 6 genes decreased (methionine adenosyltransferase 2A, tryptophan-rich basic protein, reticulocalbin 1, membrane-associated RING-CH protein VI, cytoplasmic dynein1, and annexin A1) by more than twofold for their mRNA levels. IL-8 was the most significantly upregulated gene (13.6-fold), which demonstrated a clear dose- and time-dependent pattern revealed by quantitative real-time PCR. Cell adhesion assay showed that CRP enhanced the monocyte adhesion to endothelial cell monolayer by 2-fold (P < 0.01), which was partially blocked by an anti–IL-8 antibody (34.2% inhibition, P < 0.01). Inhibition of ERK MAPK pathway using U0126 prevented CRP-induced IL-8 upregulation, and Western blot analysis revealed a rapid activation of ERK1/2 after CRP stimulation. These data showed that CRP can significantly influence gene expressions in vascular endothelium. The CRP-responsive genes suggested that CRP may have a broad functional role in cell growth and differentiation, vascular remodeling and solid tumor development.

C-reactive protein (CRP) is an acute-phase reactant during infection, inflammatory disease, cancer, and tissue injury (16, 45). CRP consists of five identical nonglycosylated 21-kDa subunits that are synthesized mainly in the liver (46). CRP is an ancient and evolutionarily conserved protein that is present at a low level (below 10 μg/ml) in humans normally. In response to acute-phase stimuli, plasma CRP concentration can increase rapidly and dramatically up to 1,000-fold and then return to normal levels (≤1 μg/ml) with resolution of the disease (46). In patients at risk of atherosclerotic disease, the CRP level is minimally elevated (≤10 μg/ml) and remains elevated for many months to years (48).

Recent epidemiological studies have sparked extensive research interests on CRP in the cardiovascular field (7, 8, 20, 27, 29, 33, 49–51, 54, 59, 61). It was reported that individuals in the higher CRP quartile have a higher risk of ischemic cardiovascular disease than individuals in the lower CRP quartile (7, 8, 20, 27, 29, 33, 49–51, 54, 59, 61). Although CRP emerged as a strong and reliable predictor of ischemic cardiovascular events, it is still unclear whether CRP is an innocent risk marker or a functional risk mediator in the pathological process (4, 62).

Atherosclerosis is the major cause for ischemic cardiovascular events (32, 53). It has been noticed that increased CRP concentration in the plasma is significantly associated with the presence of macrophages and T lymphocytes in plaques in high-grade carotid stenosis patients (2). CRP is also associated significantly with the angiographically documented coronary atherosclerotic disease in hyperalpliphoproteinemia patients (55) and carotid intima-media thickness in elderly individuals (60). Animal studies showed that rats receiving human CRP developed significantly larger cerebral infarcts after middle cerebral artery occlusion than control rats receiving human serum albumin (19). CRP may also contribute to the formation of foam cells in atherosclerotic lesions by causing the aggregation of LDL molecules that are then taken up by macrophages through a CD32-independent pathway (15). It has been suggested that the major receptor for CRP on phagocytic cells is FcγRIIa (CD32) (3); however, the receptor that mediates the CRP effect on vascular endothelial cells (ECs) has not been identified.

In this study, we hypothesized that CRP may directly interact with vascular ECs and alter their susceptibility to developing atherosclerotic lesions through changing its expression profile. We carried out experiments to systematically define the influence of CRP on the gene expression of human vascular ECs. Our results implied a broad biological role of CRP in cardiovascular disease and tumorigenesis and showed that gene expression in vascular ECs is very sensitive to increase of CRP concentration within its physiological range under 10 μg/ml.

METHODS

Cell culture and treatments. Human umbilical vein ECs (HUVECs, passage 1, pooled, Cambrex) and human aortic ECs (HAECs, passage 3, pooled, Cambrex) were cultured in complete endothelial growth medium with 2% FBS (EGM-2, Cambrex). Cells (passages 2–6) at 90% confluence were made quiescent by serum-free EGM-2 at 37°C for 24 h before the addition of recombinant human CRP (0–10 μg/ml, Calbiochem). CRP was purified with a Detoxi-Gel Endotoxin Removal Column (Pierce Biochemicals); endotoxin contamination was <0.05 EU/ml as determined by a QCL-1000 Limulus assay (Cambrex). Specific inhibitors for ERK (U0126, 10 μM, BioMol Research Laboratory) and p38 (SB203580, 25 μM, BioMol Research Laboratory) were added at 60 min before CRP incubation. Actinomycin D

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(0.2–2 μg/ml, Sigma), a potent RNA polymerase II inhibitor, was added at 30 min before CRP incubation. Cell viability was assessed by microscopy observations and quantitative 3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide (MTT) assays (ATCC 30-1010K). The above treatments were found to be not toxic.

Microarray analysis. HUVECs were incubated in 10 μg/ml CRP for 24 h. Total RNA was extracted using a RNeasy kit (Qiagen). Reverse transcription was performed with 3 μg RNA to generate [α-32P]dCTP-labeled cDNA probes, which were then hybridized with microarray GeneFilters (GF211, Invitrogen) following the manufacturer’s instructions. Images were acquired with a Cyclone Phosphorimager and analyzed with Pathways 3 software (Invitrogen). Experiments were performed in duplicate. Housekeeping genes, including GAPDH and β-actin (each has two spots in the microarray filter), were used as controls. Consistency was also monitored by several other genes that have multiple spots in this filter, e.g., tubulin-α2 and spermidine/spermine N1-acetyltransferase (SAT).

Quantitative real-time PCR. Reverse transcription was performed with 1 μg total RNA using a Superscript First-Strand Synthesis Kit (Invitrogen). IL-8 and GAPDH mRNA levels were measured by quantitative real-time PCR using a LightCycler thermocycler and SYBR green kit (Roche Diagnostics). IL-8 mRNA levels were measured by 30 cycles of quantitative real-time PCR (95°C/0 s, 63°C/5 s, and 72°C/16 s) using primers (5'-CAGTCCATGCCATCAC-3' and 5'-CTGAGGATGGACACCGAC-3') and 1 μCi [33P]dCTP-labeled cDNA probes, which were then hybridized with this filter. A total of 11 genes increased and 6 genes were also upregulated, which is consistent with previous reports (9, 10, 24, 42). CRP (<10 μg/ml) induced IL-8 gene upregulation to the same degree in the presence or absence of serum (data not shown), indicating that serum cofactors are not essential in mediating the IL-8 response to CRP.

In addition, several other genes were revealed for the first time to be significantly regulated by CRP (Table 1). The upregulated genes included core promoter element binding protein (ZF9/COPEB; 7.1-fold), inhibitin-βA (activin A, activin ABα polypeptide, 5.9-fold), Cbp/p300-interacting transactivator with Glu/Asp-rich COOH-terminal domain 2 (CITED2; 2.9-fold), fibroactin 1 (FN1; 2.2-fold), gravin (A-kinase anchor protein 12, 2.2-fold), connexin43 (Cx43; gap junction protein α1, 2.1-fold), and sortilin-related receptor (SORL1/LR11/LRP9, 2.0-fold). The CRP-downregulated genes included methionine adenosyltransferase IIa (MAT2A; 0.32-fold), reticulocalbin 1 (RCN1; 0.40-fold), tryptophan-rich basic protein (WRB; 0.41-fold), membrane-associated RING-CH protein VI (TEB4; 0.46-fold), cytoplasmic dynein (DNCL1 or PIN; 0.47-fold), and annexin A1 (ANXA1; 0.5-fold).

Dose- and time-dependent pattern of CRP induction. Because the plasma CRP level is quantitatively related to cardiovascular risk, we tested the dose-dependent relationship of the response of gene expression to CRP treatment. Plasma CRP levels of <1, 1–3, and 3–10 μg/ml are used to denote the low, intermediate, and high risks of cardiovascular disease (48); therefore, we focused on CRP concentrations ≤10 μg/ml in this study. We found that the degree of IL-8 upregulation was most upregulated (13.6-fold; Fig. 1). Monocyte chemoattractant protein 1 (MCP-1; 3.9-fold, P < 0.001) and plasminogen activator inhibitor 1 (PAI-1; 2.3-fold, P < 0.001) were also upregulated, which is consistent with previous reports (9, 10, 24, 42). CRP (<10 μg/ml) induced IL-8 gene upregulation to the same degree in the presence or absence of serum (data not shown), indicating that serum cofactors are not essential in mediating the IL-8 response to CRP.

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CRP enhances the monocyte adherence to the EC monolayer. A monocyte-EC adherence assay was performed to explore the biological consequence of CRP elevation. The results showed that a 24-h preincubation of vascular ECs with CRP (10 μg/ml) significantly increased the number of monocytes attached to the EC monolayers by twofold (P < 0.01 compared with the control group; Fig. 2). This effect was partially blocked by an


anti-human IL-8 monoclonal antibody (34.2% inhibition of CRP-stimulated monocyte-EC adhesion, \( P < 0.01 \) compared with the 10 \( \mu \)g/ml CRP group and the control group; Fig. 2). An excess amount of the IL-8 blocking antibody could not completely block the CRP-induced augmentations of monocyte attachment.

**CRP increases IL-8 expression via the ERK1/2 MAPK pathway in HUVECs.** To explore the signaling pathways that mediate the CRP effect, we preincubated HUVECs with various specific inhibitors and monitored the IL-8 response to CRP. U0126 (an inhibitor of MEK, the enzyme that activates ERK1/2 MAPK) could significantly inhibit CRP-induced IL-8 upregulation (\( P < 0.01 \); Fig. 3A), whereas SB203580 (an inhibitor of the p38 pathway) had no effect (Fig. 3A). Western blot analysis showed that CRP (10 \( \mu \)g/ml) stimulated a time-dependent phosphorylation of the 44/42-kDa doublet of ERK1/2 MAPK (Fig. 3B).

**CRP increases IL-8 expression at the transcriptional level.** To explore whether CRP affected the vascular endothelial gene expression at the transcriptional level, we used actinomycin D, a potent RNA polymerase II inhibitor, and monitored the IL-8 response to CRP. Our result showed that actinomycin D could completely abolish the IL-8 response (Fig. 4), indicating that the regulation of IL-8 expression by CRP occurred at the transcriptional level.

**DISCUSSION**

Epidemiological studies have found that plasma CRP level is associated with ischemic cardiovascular/cerebrovascular risk among apparently healthy individuals (7, 8, 20, 27, 29, 33, 49–51, 54, 59, 61). To explore the underlying molecular mechanisms through which CRP increases cardiovascular risk, we investigated the effects of CRP on gene expression in vascular ECs. We found that CRP could significantly induce a change of the gene expression profile in human vascular ECs. Moreover, these CRP-regulated genes are mainly involved in cell growth and differentiation, atherosclerosis, vascular remodeling, and solid tumor growth.

The vascular endothelium plays a pivotal role in the initiation of atherosclerosis lesions. In vascular ECs, it was reported that CRP could stimulate productions of MCP-1, PAI-1, ICAM-1, VCAM-1, and E-selectin (10, 42, 43). In this study, we systematically analyzed the gene expression profile in response to CRP treatment. We found that 11 genes were upregulated and 6 genes were downregulated by more than twofold after a 24-h CRP (10 \( \mu \)g/ml) incubation (Table 1). Among these genes, IL-8 was the most upregulated gene (13.6-fold; Fig. 1). Previous studies have showed that IL-8 is present in human atherosclerotic lesions (26). It is essential for tethering and rolling monocytes to adhere firmly onto the

| Table 1. CRP-regulated genes in HUVECs |

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus ID</th>
<th>Fold</th>
<th>Gene Function (Reference)</th>
</tr>
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<tbody>
<tr>
<td>IL-8</td>
<td>3576</td>
<td>13.6</td>
<td>Chemokine; firm adhesion of monocytes to vascular endothelium during atherosclerosis (17)</td>
</tr>
<tr>
<td>MCP1</td>
<td>6347</td>
<td>3.86</td>
<td>Chemokine; firm adhesion of monocytes to vascular endothelium during atherosclerosis (17)</td>
</tr>
<tr>
<td>EXT1</td>
<td>2131</td>
<td>2.91</td>
<td>Heparan sulfate polymerizing enzyme; morphogenesis, cell growth and differentiation, and tumor suppressor gene (1, 21)</td>
</tr>
<tr>
<td>CITED2</td>
<td>10370</td>
<td>2.86</td>
<td>Transcriptional repression of HIF-1α (14, 65), which is a key regulator of cellular response to hypoxia, energy metabolism, tumor angiogenesis, apoptosis, heart disease, and stroke (6, 18, 37, 38)</td>
</tr>
<tr>
<td>PAI-1</td>
<td>5054</td>
<td>2.32</td>
<td>Serine protease inhibitor; the key regulator of fibrinolysis in atherothrombosis by inhibiting tPA (23)</td>
</tr>
<tr>
<td>Fibronectin 1</td>
<td>2335</td>
<td>2.22</td>
<td>Cell adhesion and migration and is essential for heart and blood vessel development (58)</td>
</tr>
<tr>
<td>Gravin</td>
<td>9590</td>
<td>2.16</td>
<td>Located the action site of protein kinase A, brain angiogenesis, and tight junction formation (31)</td>
</tr>
<tr>
<td>Connexin43</td>
<td>2697</td>
<td>2.05</td>
<td>Cell-cell signaling, heart development and muscle contraction, and atherosclerotic plaque formation (30)</td>
</tr>
<tr>
<td>SORL-1</td>
<td>6653</td>
<td>2.00</td>
<td>LDL receptor (also called LR11 or LRP9) cholesterol metabolism, lipid transport, atherosclerosis and arterial remodeling, and cancer invasion (57, 66)</td>
</tr>
<tr>
<td>Annexin A1</td>
<td>311</td>
<td>0.50</td>
<td>Solid tumor growth (40)</td>
</tr>
<tr>
<td>DNCL1</td>
<td>8655</td>
<td>0.47</td>
<td>Also called PIN, a protein inhibitor of nNOS (22)</td>
</tr>
<tr>
<td>TEB4</td>
<td>10299</td>
<td>0.46</td>
<td>Marker of invasive capacity of malignant melanomas (52)</td>
</tr>
<tr>
<td>WRB</td>
<td>7485</td>
<td>0.41</td>
<td>Nuclear protein with unknown function; a candidate gene for congenital heart disease in Down syndrome (12)</td>
</tr>
<tr>
<td>RCN1</td>
<td>5954</td>
<td>0.40</td>
<td>Calcium-binding protein located in the lumen of the endoplasmic reticulum from a human transitional carcinoma cell line (41)</td>
</tr>
<tr>
<td>MAT2A</td>
<td>4144</td>
<td>0.32</td>
<td>Cell rapid growth or dedifferentiation (36)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>2597</td>
<td>0.94</td>
<td>Critical for energy yielding in carbohydrate metabolism</td>
</tr>
<tr>
<td>β-Actin</td>
<td>60</td>
<td>1.15</td>
<td>Cytoskeletal actin involved in cell motility processes</td>
</tr>
</tbody>
</table>

CRP, C-reactive protein; HUVECs, human umbilical vein endothelial cells; COPEB, core promoter transactivator element binding protein; MCP, monocyte chemoattractant protein; CITED, Cbp/p300-interacting transactivator with Glu/Asp-rich COOH-terminal domain; HIF, hypoxia-inducible factor; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; SORL, sortilin-related receptor; DNCL, cytoplasmic dynein; nNOS, neuronal nitric oxide synthase; WRB, tryptophan-rich basic protein; RCN, reticulocalbin; MAT, methionine adenosyltransferase.
vascular endothelium from the bloodstream to lesion-prone sites of large arteries, a key initial step during the formation of atherosclerosis (17, 47, 56). Targeted disruption of IL-8 receptors reduced intimal macrophage accumulation and atherosclerotic lesions in atherosclerosis-prone mice (5). We further tested whether elevated CRP can promote monocyte adhesion and whether it is mediated by the IL-8 pathway. Our results demonstrated directly that CRP (10 μg/ml for 24 h) pretreatment of ECs could significantly increase the number of monocytes attached to the pretreated EC monolayers (2-fold, \( P < 0.01 \); Fig. 2). This effect could be partially blocked by an anti-IL-8 monoclonal blocking antibody (34.2% inhibition of CRP-stimulated monocyte-EC adhesion, \( P < 0.01 \); Fig. 2), indicating that IL-8 mediated CRP-enhanced monocyte adhesion. Because excess amounts of the IL-8 antibody could not further reduce the CRP-stimulated monocyte adhesion (data not shown), other factors may be also involved in this process. These data strongly support the notion that CRP may have a direct pathological role during atherosclerosis and the development of cardiovascular disease.

A series of epidemiological studies have shown that the baseline (≤10 μg/ml) plasma CRP level is quantitatively related to cardiovascular risk (7, 8, 20, 27, 29, 33, 49–51, 54, 59, 61). In humans, the population distributions of CRP are as follows: quartile 1, ≤1.5 μg/ml; quartile 2, 1.5–3.7 μg/ml; quartile 3, 3.8–7.3 μg/ml; and quartile 4, ≥7.3 μg/ml (49).
Plasma CRP levels of ≤1, 1–3, and 3–10 μg/ml have been proposed to denote the low, intermediate, and high risks of cardiovascular disease (48). Because major infections, trauma, and acute hospitalizations can elevate CRP levels (100-fold or more), 10 μg/ml has been widely used as a criterion in most clinical settings for the CRP assessment in the evaluation of cardiovascular risk; levels higher than 10 μg/ml will be ignored and tests will be repeated at a future date when the patient is clinically stable (48). Accordingly, we designed our experiments to be mainly focused on the physiological range of CRP ≤10 μg/ml. We found that the degree of IL-8 response was very sensitive to the level of CRP concentration within this range (Fig. 1, A and B). IL-8 began to respond to CRP at as low as 1.25 μg/ml; the IL-8 response continued to increase with the CRP concentration within this range (Fig. 1). Considering that IL-8 is a key player in atherosclerosis, the dose-dependent relationship may explain at least partially the divergent cardiovascular risk among individuals with different CRP levels. The CRP level is stable over a long period of time within individuals, and it is not affected by food intake and has no circadian variation (48). In our results, the IL-8 upregulation started as early as 0.2 μg/ml incubation and continued to rise over the 24-h period of CRP stimulation (Fig. 1C); this observation raised the question of how long requires for the CRP elevation to affect the cardiovascular risk in patients.

Besides IL-8, MCP-1, and PAI-1, we observed for the first time that a list of genes involved in arterial remodeling and tumorigenesis responded significantly to CRP stimulation (Table 1). We noticed that most of the CRP-upregulated genes are involved in vascular remodeling and atherogenesis (Table 1), whereas a majority of CRP-downregulated genes are involved in tumor growth and invasion (Table 1). By influencing the expression of these genes within vascular ECs, elevated CRP may have a broad spectrum of functional roles in vascular remodeling, angiogenesis and atherosclerosis, and solid tumor development. These effects may be achieved through controlling either cell growth/differentiation or cell-cell signaling.

Interestingly, CRP could significantly increase the gene expression of CITED2 (Table 1), a transcriptional repressor of hypoxia-inducible factor 1α (HIF-1α) (14, 65) that is a well-known key regulator of tissues in sensing and responding to hypoxia stress during tumor development, heart disease, and stroke (6, 18, 37, 38). In addition, CRP significantly reduced the gene expression of a protein inhibitor (DNCL1; Table 1) of neuronal nitric oxide (NO) synthase (nNOS) (22). It is well known that nNOS-derived NO is neurotoxic during the initial phase of ischemia, and inhibition of nNOS could reduce the ischemic injury of neurons after stroke (25). This observation is in accordance with clinical observations in which a higher CRP concentration can predict a worse outcome and a lower survival rate in stroke patients (11, 39, 64). These data suggest that CRP may be related to cell tolerance to hypoxia and ischemia in heart disease, stroke, and solid tumors via its influences on HIF-1α and nNOS.

Identification of the molecular pathway is critical for the elucidation of CRP functions and for clinical interventions to prevent/reverse the CRP-induced higher disease risk. In this study, we demonstrated evidence that the IL-8 pathway constitutes one of the pathways that mediates pathological effects of CRP elevation on cardiovascular disease. Furthermore, using actinomycin D, a potent RNA polymerase II inhibitor that completely abolished the IL-8 mRNA increase (Fig. 4), we demonstrated that the CRP-induced IL-8 increase is achieved at the transcriptional level. Because both ERK1/2 and p38 MAPK pathways have been reported to be involved in the regulation of IL-8 production in different cell types (35, 63), we tested these pathways in the IL-8 response to CRP stimulation. We found that an ERK blocker (U0126) completely

Fig. 3. ERK1/2 mediated CRP-induced IL-8 upregulation. A: U0126 (a MEK/ERK inhibitor) blocked the CRP-induced IL-8 upregulation in HUVECs. SB, SB203580. B: Western blot analysis of phospho-ERK1/2 and total ERK1/2, which demonstrates the CRP-induced ERK activation in HUVECs.

Fig. 4. CRP increased IL-8 mRNA expression at the transcription level. HUVECs were preincubated with actinomycin D for 30 min before CRP stimulation, and the IL-8 mRNA levels were determined by quantitative real-time PCR and normalized to GAPDH mRNA levels. Data are presented as means ± SE; n = 3.
blocked this effect, whereas a p38 blocker (SB203580) had no effect (Fig. 3). Further experiments showed that ERK1/2 was activated by CRP incubation (10 μg/ml) in HUVECs (Fig. 3). These results provide the first definitive evidence that CRP could activate the MEK/ERK MAPK signaling pathway and could trigger gene regulation at the transcriptional level.

In summary, in concert with the epidemiological observations that the baseline CRP level is associated with increased cardiovascular risk in human populations, our study demonstrated a clear dose-dependent influence of CRP on gene expression of human vascular ECs. This effect occurred at the transcription level and was mediated through the MEK/ERK-dependent signaling pathway. A panel of novel CRP-responsive genes reported in this study suggested novel biological and pathological roles of CRP in cell growth and differentiation, atherosclerosis, vascular remodeling, hypoxic and ischemic responses, and solid tumor development. Unraveling the underlying molecular mechanisms of CRP may provide new directions for the prevention of ischemic cardiovascular events.

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GRANTS

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