Adiponectin Decreases C-Reactive Protein Synthesis and Secretion From Endothelial Cells
Evidence for an Adipose Tissue-Vascular Loop

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Background and Objective—Inflammation is pivotal in atherosclerosis. C-reactive protein (CRP), in addition to being a cardiovascular risk marker, may also be proatherogenic. We have previously shown that in addition to the liver, human aortic endothelial cells (HAECs) synthesize and secrete CRP. Whereas CRP levels are increased in obesity, metabolic syndrome, and diabetes, levels of adiponectin are reduced in these conditions. We tested the hypothesis that adiponectin reduces CRP synthesis and secretion in HAECs under normoglycemic (5.5 mmol/L glucose) and hyperglycemic conditions (15 mmol/L glucose).

Methods and Results—Adiponectin dose-dependently reduced CRP mRNA and protein from HAECs. Adiponectin treatment of HAECs significantly decreased IκB phosphorylation and NFκB binding activity. There was no effect of adiponectin on STAT or C/EBP transcriptional activity. Adiponectin also activated AMP kinase resulting in decreased NFκB activity and decreased CRP mRNA and protein. These effects of adiponectin were mimicked by AICAR, an activator of AMPK, and reversed by inhibition of AMPK. Thus, adiponectin reduces CRP synthesis and secretion from HAECs under hyperglycemia via upregulation of AMP kinase and downregulation of NFκB. Similar findings were observed in rat primary hepatocytes.

Conclusions—Thus, in obesity and diabetes, the hypoadiponectinemia could exacerbate the proinflammatory state by inducing CRP production. (Arterioscler Thromb Vasc Biol. 2008;28:1368-1374)

Key Words: CRP ■ adiponectin ■ endothelium ■ adipose

Inflammation is pivotal in all phases of atherogenesis.1,2 C-reactive protein (CRP), the prototypic marker of inflammation in man, has been shown in several studies to be a cardiovascular risk marker with high levels of CRP predicting cardiovascular events.1,3 Much recent data challenge the dogma that CRP is exclusively produced by the liver.3 Indeed, cogent data suggest that it is produced in atherosclerotic lesions, the kidney, neurons, and alveolar macrophages.4,5 mRNA and protein for CRP is expressed in arterial plaque tissue, and 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.6 Furthermore, we showed that human aortic endothelial cells synthesize and secrete CRP.7 The most potent agonist for CRP production from HAECs is the combination of interleukin (IL)-1 and IL-6.8 In addition, the secretion of CRP is augmented 100-fold in presence of macrophage conditioned media (MCM).7 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. This could contribute to the poorer prognosis in patients with elevated CRP levels and acute coronary syndromes.9,10 Furthermore, the adipose tissue, previously thought to be an inert triglyceride depot, has been shown to produce numerous adipokines including adiponectin.

See accompanying article on page 1219

Adiponectin is a potent adipocytokine.11–16 Decreased levels of adiponectin are found in obesity, type 2 diabetes, and coronary artery disease. Low levels of adiponectin are inversely associated with high levels of CRP. Furthermore, adiponectin has been shown to upregulate eNOS and decrease cytokine and chemokine synthesis from HAECs. Recent studies have shown that recombinant globular adiponectin, a proteolytic cleavage product of total adiponectin is pharmacologically active, upregulates eNOS in vascular endothelial cells, and decreases atherosclerosis in apo E−/− mice.13,16,17 Also, there is emerging data indicating that CRP impairs insulin signaling and adiponectin is able to improve insulin sensitivity.18,19 A strong and inverse correlation has also
been documented between CRP and adiponectin mRNA in human adipose tissue. Thus, adiponectin could be regulating CRP. However, there is a paucity of data examining the effect of adiponectin on CRP synthesis and secretion from HAECs. In this study, we provide novel data that CRP synthesis and secretion from HAECs is augmented under hyperglycemia and that pretreatment with adiponectin significantly downregulates synthesis and secretion of CRP from HAECs.

Methods

Cell Culture

HAECs were used between passages 3 to 5 and cultured in normoglycemic medium (NG, 5.5 mmol/L glucose) in the presence or absence of globular adiponectin (0 to 10 μg/mL) in 12-well culture plates. Also, total adiponectin was used in some studies. Both total and globular adiponectin were obtained from Peprotech Inc. HAECs were incubated in serum- and growth factor–free media for the expression of CRP under normoglycemic conditions (5.5 mmol/L glucose) or hyperglycemic conditions (15 mmol/L) for 24 or 48 hours, and RNA or protein/media were collected for CRP measurements, respectively. Mannitol was used as a hyperosmolar control. All media were endothoxin-free, and adiponectin was passed through Detoxigel.

For experiments with adiponectin, endothelial cells were pretreated with adiponectin, (0, 2.5, 5, and 10 μg/mL) for 2 hours before incubation in hyperglycemic conditions. Concentrations of adiponectin that have been used are physiological as reported previously.

CRP Protein Expression

The cells were collected in M-PER (Pierce Biotechnologies). Western blotting for CRP was performed as described previously7 using rabbit anti-human CRP (Calbiochem) antibody. Western blotting for phospho p65, phospho p38MAPK, and phospho AMPK was performed with monoclonal antibodies (Santa Cruz Biotechnology and Abcam Inc, respectively) and β-actin (mouse monoclonal antibody; Sigma Aldrich) or the respective unphosphorylated total antibody (anti p65, anti p38 MAPK from Santa Cruz Biotechnology and anti-AMPK antibody from Abcam Inc) was used as an internal control.7

Secreted CRP by ELISA

CRP levels in the supernatants of HAECs (pooled from 3 different wells and concentrated) were measured using an enzyme-linked immunosorbent assay (ELISA) specific for human CRP (Alpcor Laboratories) as described previously and reported as ng/mg cell protein.7

CRP mRNA

CRP mRNA was assessed by semi-quantitative RT-PCR and real-time RT-PCR. RNA was isolated from the cells using TRIzol (Invitrogen). For the RT reaction, 1 μg of total RNA was used based on the standard curve to synthesize the cDNA. For semi-quantitative PCR, the primers used for human CRP were forward: 5’-TCG TAT GCC ACC AAC AGA CAA GAC A 3’ and reverse: AAC ACT TCG TCT GTC ACT trichloroacetic acid (TCA) TAC T 3’ and 18s RNA primer pair was obtained from R&D. PCR reaction was performed using Invitrogen master mixes as previously described.7,21 using 50 ng of cDNA. CRP and 18s RNA yield a band between 400 to 600 bp, were resolved on a 2% agarose gel. Band intensities were determined using Image quant software (GE Healthcare and Biosciences).7 For real-time RT-PCR, we used 25 ng of cDNA for PCR reaction. Human CRP (amplicon size 808bp, assay id# Hs00357041_m1) and 18s (amplicon size 187bp; assay id#Hs99999901-s1) gene expression assays were purchased from Applied Biosystems and followed the recommended protocol. No RT, no template, and positive controls were run in parallel. The optimal amount of RNA was determined by generating a standard curve using serial dilutions of control RNA (concentration of RNA versus Ct values) with fixed number of cycles, and subjecting the PCR products to gel electrophoresis for further confirmation using 18s RNA as the reference. Data were calculated using the 2ΔΔCT method and presented as CRP/18s mRNA ratio normalized to mannitol/low glucose.

NFκB, STAT, C/EBP Binding Activity Assay

Activation of NFκB, STAT, C/EBP in nuclear extracts was determined using TransAM assay (Active Motif). Nuclear extracts were suspended in TransAM lysis buffer and nuclear proteins (5 μg total protein) were incubated with immobilized oligonucleotides containing the NFκB consensus DNA-binding site (5’-GGGACTTTCC-3’) for 1 hour at room temperature. After washing, 100 μL of p65 subunits monoclonal antibody (1:1000 dilutions) were added for 1 hour at room temperature. After 3 washes, 100 μL of horseradish-peroxidase–conjugated secondary antibody (1:1000 dilutions) were added to each well for 1 hour at room temperature. The absorbance at 450 nm was determined using a standard for NFκB. STAT and C/EBP activities were examined similarly using the respective Trans-AM kits.

Luciferase Transactivation Assay

Deletion promoter constructs and mutants were obtained from the Samols laboratory. HAECs (50% to 80% confluent) were transfected with 2 μg of DNA (1 μg luciferase reporter-CRP promoter construct and 1 μg transcription factor expression vector). Luciferase transactivation assays were performed using CRP promoter constructs with mutated NFκB sites to evaluate their role in mediating the effect of HG or adiponectin. After 24 hours, luciferase assays were performed (Promega). Luciferase activity was normalized to the protein concentration of the extract measured by using a BioRad DC protein assay kit.

Isolation of Rat Primary Hepatocytes

Primary cultures of rat hepatocytes were prepared by the method of Gores et al. Briefly, rats were anesthetized, and isolated livers were perfused via the portal vein with Ca2+- and Mg2+-free HEPES buffer. Subsequent perfusion included 1.0 mmol/L Ca2+ and 0.02% collagenase. The livers were then gently raked, the cell suspension was centrifuged, and the resulting cell pellet was resuspended in DMEM containing 0.1% BSA, 200U/mL penicillin, and 200 mg/mL streptomycin. The cells were plated at a density of 5×10⁶ cells on rat tail collagen-coated plates in absence and presence of adiponectin and challenged with IL-1+IL-6 as described previously.

Statistical analyses was performed using GraphPad Prism 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

Adiponectin Decreases CRP Synthesis and Secretion Induced by HG in HAECs

We demonstrate that CRP mRNA and protein are significantly increased under hyperglycemic (HG) conditions and that CRP protein and mRNA are inhibited by pretreatment with globular adiponectin (Figure 1a and 1b). Furthermore, secreted CRP is significantly increased in HG conditions, and treatment with both globular (dose-dependently) and total adiponectin inhibited CRP secreted protein induced by hyperglycemia (Figure 1c).
Effect of Adiponectin on Transcriptional Factors Involved in CRP Synthesis

Because the promoter for CRP in hepatocytes contains binding elements for NFκB, STAT, and C/EBP, we examined the effect of HG on these 3 transcriptional factor activities, in absence and presence of adiponectin. Whereas HG upregulated STAT-1 (Figure 2a), C/EBP-β (Figure 2b), and NFκB (Figure 2c) activities in nuclear extracts of HAECs, adiponectin failed to affect STAT and C/EBP-β activities. However there was a significant downregulation of NFκB transcriptional activity after pretreatment with adiponectin (Figure 2c).

Furthermore, in nuclear extracts, adiponectin pretreatment of HAECs (0 to 10 μg/ml for 24 hours) significantly decreased phosphorylation of p65 induced by HG (Figure 2d).

Loss of NFκB Abrogates HG-Induced CRP Synthesis in HAECs

To confirm the role of NFκB, we transfected HAECs with dominant negative NFκB or control vector. When NFκB was inhibited, there was significant decrease in CRP synthesis and secretion with HG (Figure 3a and 3b). Furthermore, luciferase transactivation assays performed using CRP promoter constructs with mutated NFκB sites (PC3) resulted in loss of CRP promoter activity in presence of HG indicating the critical involvement of NFκB in CRP transcription in HAECs (please see http://atvb.ahajournals.org for supplemental materials).

Effect of Adiponectin on MAPK in HAECs

Because HG activated p38MAPK but not extracellular signal regulated kinase (ERK)/JNK, we tested the effect of adiponectin on HG-induced phosphorylation of p38MAPK and adiponectin failed to have any significant effect (data not shown).

Adiponectin Upregulates AMPK Activity in HAECs

Several reports indicate that adiponectin also activates AMP kinase, we tested the effect of adiponectin on AMPK activity and subsequent CRP release under HG conditions. Adiponectin pretreatment resulted in significant upregulation in AMPK activity, and this was mimicked by AICAR, 250 μmol/L (AICAR is a cell-permeable adenosine analogue that can be phosphorylated to ZMP, an AMP analogue and known AMPK activator; Figure 4a). In presence of Compound C, 100 nmol/L (AMPK inhibitor, Calbiochem), there was a significant reversal of the effects of adiponectin on AMP kinase phosphorylation, NFκB activity and CRP mRNA and protein expression (Figure 4a through 4d). Also pretreatment with either adiponectin or AICAR resulted in decreased NFκB binding (Figure 4d) and decreased CRP mRNA and protein (Figure 4b and Figure 4c, respectively), and this was reversed with Compound C (Figure 4b through 4d).

Adiponectin Decreases CRP Synthesis in Primary Rat Hepatocytes

Lastly, because a large part of transcriptional regulation of CRP in hepatocytes has been studied in hepatoma cell lines (HepG2 or Hep3B cells), we isolated primary rat hepatocytes and examined the effect of adiponectin on IL-1- and IL-6-mediated CRP synthesis and secretion in these primary cells.
As shown in Figure 5a, the combination of IL-1 and IL-6 resulted in significant upregulation in secreted CRP. This was abrogated in presence of adiponectin. Similar results were observed for CRP mRNA (data not shown). Furthermore, the combination of IL-1/IL-6 resulted in significant increase in C/EBP-β, STAT 3, and NFκB activities, and adiponectin preincubation decreased NFκB activity (Figure 5b).

**Discussion**

We report 2 novel findings in this article. First, high glucose results in increased synthesis and secretion of CRP from human aortic endothelial cells, and this appears to be via upregulation of NFκB. Secondly, we demonstrate that adiponectin abrogates the HG-induced CRP synthesis and secretion via upregulation of AMP kinase and downregulation of NFκB activity. Furthermore, we confirm these inhibitory effects of adiponectin on CRP synthesis in primary rat hepatocytes.

Conditions associated with diabetes and obesity exhibit increased cardiovascular morbidity and mortality. Thus, it is important to clarify the molecular pathways between fat accumulation and vascular disease. Whereas adiponectin is expressed largely in adipose tissue, circulating levels of adiponectin are significantly decreased in obesity, diabetes, metabolic syndrome, and CAD.11–17 Conversely, high sensitivity CRP (hsCRP) levels are higher in these conditions of hypoadiponectinemia. High levels of CRP are associated with increased cardiovascular events.2,3 Also, CRP is produced

![Figure 2. Effect of Adiponectin on HG-induced nuclear transcription factor activity: HAECs were incubated with NG/HG/adiponectin. a, STAT1 and STAT3; b, C/EBP-β; c, NFκB. *P<0.01 vs control. **P<0.001 vs NG and #P<0.01 vs HG. d, A representative blot of phospho p65 expression with densitometric ratios of pp65/p65 (n=3).](image)

![Figure 3. Loss of NFκB abrogates HG-induced CRP: HAECs were transiently transfected with control or dominant negative IκB vector for 24 hours in presence of NG or HG. a, Secreted CRP. b, Representative RT-PCR gel of CRP with ratios. *P<0.001 compared to mannitol/NG; #P<0.01 vs HG, n=3.](image)
locally in vascular tissue, and this leads to concentrations 10-fold excess of that in plasma.\textsuperscript{1,4,20} CRP has been shown to be produced in macrophages, in neurons, and in the kidney and adipocytes. We have previously shown that CRP is synthesized and secreted by HAECs and that this is augmented several fold in presence of macrophage conditioned media, indicating that there is cross-talk between vascular cells, ie, macrophages and endothelial cells resulting in increased inflammation.\textsuperscript{7} Ouchi has previously shown a strong inverse correlation between CRP and adiponectin mRNA.\textsuperscript{20} However, this does not imply cause and effect. In a recent report, it has been suggested that CRP inhibits adiponectin secretion in 3T3 cells.\textsuperscript{24} Because there are many contaminants associated with CRP and the authors do not appear to purify their CRP, this finding needs to be interpreted with caution. If confirmed, it will suggest that both adiponectin and CRP can modulate each other’s secretion and thus account for the proinflammatory burden of obesity and diabetes. We were unable to confirm the inhibitory effect of CRP on adiponectin using carefully purified CRP\textsuperscript{25} (data not shown).

Diabetes and metabolic syndrome are associated with increased inflammation.\textsuperscript{26–28} Both IL-1 and IL-6 are potent inducers of CRP synthesis and secretion.\textsuperscript{8} In this study, we show that under HG conditions, HAECs synthesize and secrete increased levels of CRP compared to normoglycemia. In addition, we demonstrate that adiponectin significantly abrogates HG-induced synthesis and secretion of CRP.

Adiponectin modulates endothelial function, improves endothelial NO, and has an inhibitory effect on proliferation of vascular smooth muscle cells induced by growth factors, inhibits collagen-induced platelet aggregation, suppresses foam cell formation via inhibiting scavenger receptor A, and has direct effects on improving liver and muscle insulin sensitivity.\textsuperscript{10–15,29} Overexpression of human adiponectin attenuated plaque formation in ApoE\textsuperscript{-/-} mice. Thus, adiponectin could have a protective effect on atherosclerosis, and it may be the link between obesity, type 2 diabetes, and atherosclerosis. Yamauchi et al\textsuperscript{13,30} showed that globular adiponectin, a proteolytic cleavage product of adiponectin, has a greater potency in reversing insulin resistance than native uncleaved adiponectin. In endothelial cells, globular adiponectin inhibits expression of cytokines, chemokines, intracellular cell adhesion molecule, vascular cell adhesion molecule, upregulates eNOS, and results in decreased monocyte-endothelial cell adhesion and NF\textsuperscript{κ}B.\textsuperscript{13,16,17,30} Although there appears to be a reciprocal relationship between levels of adiponectin and CRP,\textsuperscript{20} especially in patients with the metabolic syndrome or diabetes, there exist no data on the regulation of CRP synthesis and secretion in ECs with adiponectin. Also, previously another adipokine, leptin has been shown to upregulate CRP synthesis and secretion in cultured HAECs.\textsuperscript{31} Here, we present novel data that adiponectin downregulates CRP synthesis and secretion in HAECs.

In liver-derived cell lines, the main regulators of cytokine induced CRP synthesis are C/EBP-\(\beta\), STAT 3, and NF\textsuperscript{κ}B.
transcriptional regulation in HAECs has not been delineated previously. Here, we show that among the transcription factors affecting the CRP promoter, in presence of HG, there is increased STAT1, C/EBP-β, and NFκB activity. In addition, using promoter deletion constructs for CRP, we show that CRP transcription under HG conditions in HAECs is NFκB-centric. Furthermore, transfection of HAECs with dominant negative NFκB abrogated the HG-induced CRP synthesis and secretion, confirming the crucial role of NFκB in HG-induced transcription of CRP. Previously, adiponectin has been shown to downregulate NFκB activity in endothelial cells resulting in decreased expression of the cell adhesion molecules and IL-8. In this study, we provide evidence that adiponectin downregulates CRP synthesis and secretion in HAECs under HG conditions via inhibition of NFκB.

AMPK is a highly conserved heterotrimeric signaling kinase responsive to hypoxia, exercise, and cellular stress. Adiponectin metabolic signaling in liver, skeletal muscle, and adipose has been shown to be mediated via AMPK activation. Also, adiponectin has been shown to increase nitric oxide synthesis via activation of AMPK. Under HG conditions, apoptosis in ECs as well as diminished ability of insulin to activate Akt was prevented by activation of AMPK by AICAR, suggesting that AMPK may play a key role in protecting ECs from the adverse effects of HG.

In support of these studies, we also show that adiponectin upregulates AMPK phosphorylation in HAECs under HG conditions, these effects are mimicked by AICAR, a known activator of AMPK activity, and inhibited by addition of compound C, a specific inhibitor of AMPK phosphorylation. Adiponectin decreases macrophage phagocytic capacity through cross-talk between AMPK and NFκB signaling pathways.

We show in HAECs that adiponectin upregulates AMPK, resulting in decreased NFκB activity, and subsequent synthesis and secretion of CRP under HG conditions.

The liver is the primary source of CRP synthesis, thus we also examined the effect of adiponectin in primary rat hepatocytes that were induced to produce CRP with the combination of IL-1 and IL-6, because such treatment has previously been shown to induce CRP synthesis in Hep3B and HepG2 cells. We provide novel data that adiponectin decreases IL-1- and IL-6-induced CRP synthesis and secretion in primary rat hepatocytes and that this may be attributable to downregulation of C/EBP and NFκB activities. Further studies in this model will elucidate the molecular pathways by which adiponectin exerts these effects.

In addition to the growing antiatherogenic and antidiabetic properties of adiponectin, we provide novel evidence that adiponectin abrogates HG-induced CRP synthesis and secretion via upregulation of AMPK and downregulation of NFκB. Our observations provide a fundamental mechanism for the link between adiposity and endothelial dysfunction and suggest that adiponectin upregulation could be beneficial in modulating the proinflammatory/prothrombotic effects of CRP.

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**Disclosures**

None.

**References**

9. Yip HK, Hung CL, Fang CY, Hsieh YK, Yang CH, Hung WC, Wu CJ. Level of high-sensitivity C-reactive protein is predictive of 30-day...


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Cells were transiently transfected with the promoter deletion constructs (1μg) along with luciferase reporter and luciferase activity was performed as described in Methods.

Promoter Construct 1(PC1)- has all 3, NFκb, C/EBP and STAT 3 promoter elements; PC2- C/EBP deletion construct; PC3- NFκb deletion construct

*p<0.001 compared to HG+PC1 and HG+PC2.