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Adiponectin reduces C-reactive protein expression and downregulates STAT3 phosphorylation induced by IL-6 in $HepG_2$ cells

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Abstract By playing a direct proatherogenic role, C-reactive protein (CRP) is a potent independent predictor of future cardiovascular events. CRP is predominately synthesized by hepatocytes when stimulated by interleukin-6 (IL-6). In response to IL-6, the signal transducer and activator of transcription 3 (STAT3) becomes phosphorylated on tyrosine and serine residues. Phosphorylated STAT3 then activates CRP gene transcription. In obesity-related disorders such as diabetes, metabolic syndrome, and cardiovascular diseases, the circulating levels of CRP and adiponectin are inversely correlated, suggesting that these two factors might reciprocally regulate each other. We investigated the possibility that adiponectin inhibits CRP production in $HepG_2$ cells elicited by IL-6. CRP gene expression was determined using ELISA and semi-quantitative RT-PCR, and the phosphorylation of STAT3 was investigated with western blot. Adiponectin reduced IL-6-induced CRP mRNA levels in $HepG_2$ cells and CRP protein secretion. Preincubating $HepG_2$ cells with adiponectin led to a decline in STAT3 phosphorylation on both tyrosine and serine residues. Our results demonstrated that adiponectin suppresses CRP synthesis and secretion from $HepG_2$ cells and suggested that the suppression may be mediated through inhibition of the STAT3 pathway. The finding provides a novel insight into the molecular linkage between obesity and vascular diseases.

Keywords Adiponectin · C-reactive protein · IL-6 · STAT3 · $HepG_2$

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Introduction

Atherosclerosis is believed to be a chronic inflammation vascular disease, and the inflammatory process is involved in all phases of atherogenesis [1, 2]. As the prototypic marker of inflammation, C-reactive protein (CRP) has been shown to be a potent independent predictor of future cardiovascular events [3–6]. Beyond this predictive role, CRP can play a direct proatherogenic role. In apolipoprotein E-deficient mice, CRP accelerates the progression of atherosclerosis [7]. Mediated by activated platelets, pentameric CRP molecules dissociate to monomers, which then stimulate generation of reactive oxygen species and induce enhanced monocyte chemotaxis, activation, and adhesion [8], a pivotal step in the initiation of atherogenesis [9]. Furthermore, CRP impairs the endothelial glycocalyx, resulting in endothelial dysfunction [10].

As the most abundant adipokine, adiponectin is a multifunctional protein with antiinflammatory, antiatherogenic, and cardiovascular protective effects. Acting via calreticulin/CD91 receptors, adiponectin can increase the removal of early apoptotic bodies by macrophages and modulate the process of inflammation and autoimmunity [11]. By binding to C_1q, adiponectin can activate the classical complement pathway [12]. Also, adiponectin inhibits pro-inflammatory signaling in human macrophages

independent of interleukin-10 [13]. In addition, adiponectin inhibits endothelial synthesis of interleukin-8 (IL-8) [14], which exerts proangiogenic, proinflammatory, and proatherogenic effects. The inhibition of IL-8 synthesis by adiponectin suggests that adiponectin possesses properties of being antiinflammatory and antiatherogenic. Furthermore, adiponectin suppresses the expression of adhesion molecules, such as E-selectin and VCAM-1, and attenuates leukocyte–endothelium interactions, thus negatively modulating the proatherogenic process [15]. Local adiponectin treatment reduces atherosclerotic plaque size [16]. It was also shown that adiponectin protects against the development of systolic dysfunction following myocardial infarction [17] and myocardial ischemia–reperfusion [18].

It is found that decreased levels of plasma adiponectin are associated with elevated circulating CRP levels [19], and that there is a strong negative correlation between CRP and adiponectin mRNA levels in human adipose tissues [20]. Collectively, these data indicate that CRP and adiponectin might regulate each other's expression. Although Devaraj et al. demonstrated that adiponectin inhibits CRP production in human aortic endothelial cells and primary rat hepatocytes [21], the molecular mechanisms underlying the inhibition remain poorly understood. CRP is predominately synthesized in hepatocytes, and the most potent agonist for CRP production from hepatoma cell line is interleukin-6 (IL-6) [22]. Signal transducer and activator of transcription 3 (STAT3) participates in the induction of CRP gene transcription by IL-6 and interleukin-1 [23]. We thus hypothesized that adiponectin might inhibit the STAT3 pathway to repress CRP gene expression. In this study, we demonstrated that adiponectin suppresses IL-6-induced CRP gene expression in $HepG_2$ cells and the suppression may be mediated via disruption of the STAT3 pathway.

Materials and methods

Reagents

Human recombinant IL-6, full-length adiponectin, and enzyme-linked immunosorbent assay (ELISA) kits for CRP were purchased from R & D systems (Minneapolis, MN, USA). Anti-STAT3, anti-p-Tyr-STAT3, and anti-p-Ser-STAT3 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-IL-6Rα and anti-gp130 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

$HepG_2$ cells were maintained in DMEM medium (HyClone, Logan, USA) containing 10% fetal bovine serum, pencillin (100 μg/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2.

Secreted CRP measurement by ELISA

$HepG_2$ cells were cultured to about 80% confluence on 24-well plates in normal culture medium and then incubated in serum-free medium for 24 h. After washed with PBS, cells were treated with IL-6 at 25 ng/ml and adiponectin at indicated concentrations for 24 h. CRP levels in the supernatants of treated cell cultures were measured using the ELISA kits according to the manufacturer's protocol. The amount of CRP detected was calculated from a standard curve prepared with CRP standard.

mRNA extraction and semi-quantitative RT-PCR

$HepG_2$ cells were seeded onto 24-wells plates. After starving in serum-free medium, cells were treated with IL-6 and adiponectin for 6 or 24 h. Total cellular RNA was extracted using TRIzol (Invitrogen, Beverly, USA) reagent. For RT reaction, 1 μg of total RNA was used based on the standard curve to synthesize the cDNA. For PCR, 20 ng cDNA was applied. And the PCR reaction system is 20 μl system, the reagent addition as follows:

10× buffer	2.0 μl	Primers	20 pmol, respectively
$Mgcl_2$	25 mM 1.5 μl	Taq DNA polymerase	0.5 u
dNTP(Mix)	10 mM 0.5 μl	cDNA	20 ng
Water	Added to 20 μl		

and then 94°C 5 min, 94°C 30 s 60°C 30 s 72°C 30 s for 29 cycles, 72°C 5 min.

The primers applied specific for CRP were: 5′-TCGT ATGCCACCAAGAGACAAG-3′ (forward) and 5′-CACT TCGCCTTGCACTTCATAC-3′ (reverse); β-actin primers: 5′-CCCAGCACAATGAAGATCAAGATCAT-3′ (forward) and 5′-ATCTGCTGGAAGGTGGACAGCGA-3′ (reverse). Both RT and PCR reaction were performed using Promega master mixes (Madison, Wisconsin, USA). And CRP cDNAs were amplified for 35 cycles, and β-actin for 29 cycles. CRP and β-actin yielded a band at 438 and 101 bp, respectively, on 2% agarose gels. Band intensities were determined using Photoshop CS3 as detailed below.

(1) Open the scanned image in Photoshop.
(2) Under Image > Mode, check the grayscale option if it is not already selected.
(3) Under Image > Adjustments, select Invert. Now the dark parts of the film are light, and the light parts are dark.

(4) Choose the lasso tool from the tool palette.
(5) On the first band, use the lasso tool to draw a line all the way around the edges of the first band.
(6) Click on the small arrow in a circle in the upper right corner of the histogram window, and choose "expanded view" to show the values for the selected area.
(7) The histogram information includes a "Mean" value and a "Pixels" value. Record these two numbers.
(8) Multiply the Mean value by the Pixel value for each band. This gives an integrated measure of the intensity and size of the band, which is referred to as the absolute intensity.
(9) Repeat steps 5–8 for other bands.
(10) Relative Intensity is calculated using the internal control as the common point of comparison. This is done by dividing the absolute intensity of each sample band by the absolute intensity of the control band.

Western blot analysis

To study the effects of adiponectin on transcription factor STAT3, $HepG_2$ cells were cultured on 6-well plates. After starving in serum-free medium overnight, cells were pretreated with adiponectin for 3 h, and then exposed to IL-6 and adiponectin for additional 30 min. And to study the effects of adiponectin on IL-6Rα and gp130, after starvation in serum-free medium overnight, $HepG_2$ cells were treated with 1.0 μg/ml adiponectin for 3, 6, and 24 h. Cells were collected in ice-cold radioimmunoprecipitation (RIPA) lysis buffer and phosphatase inhibitor cocktail. Total protein concentrations were estimated using UV quantification assay. Western blotting was performed as previously described [24]. Rabbit anti-human STAT3, anti-p-Tyr-STAT3, anti-p-Ser-STAT3, anti-IL-6Rα, and anti-gp130 antibodies were used as primary antibody. After incubating with goat anti-rabbit IgG secondary antibody and thorough washing, proteins were visualized using an Enhanced HRP-DAB kit (Tiangen, Beijing, China). In all western blot experiments, β-actin was used as an internal control. And densitometry was carried out in Photoshop CS3 as detailed above.

Statistics analysis

All results were expressed as mean ± SD. Statistics analyses were performed by one-way classification ANOVA with Student–Newman–Keuls test, and $P < 0.05$ was considered to be significant.

Results

Adiponectin decreases IL-6-induced CRP release in $HepG_2$ cells

$HepG_2$ cells treated with IL-6 at 25 ng/ml alone for 24 h secreted twice as much CRP as the unstimulated cells (Fig 1). However, when incubated with adiponectin together, IL-6-stimulated CRP secretion was significantly reduced. Various concentrations of adiponectin applied (0.5, 1, and 2 μg/ml) appeared to inhibit CRP secretion by a similar magnitude.

Adiponectin reduces CRP mRNA levels in $HepG_2$ cells

In agreement with the stimulatory effect on CRP secretion, IL-6 treatment of $HepG_2$ cells significantly upregulated CRP mRNA levels at both 6 and 24 h (Fig. 2). As with the

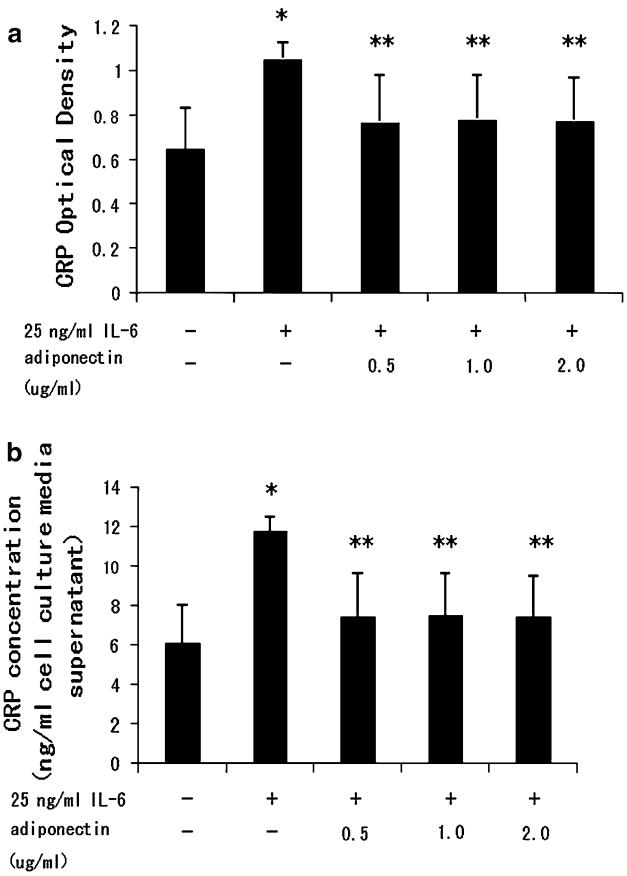

Fig. 1 Effect of adiponectin on IL-6-induced CRP secretion. CRP levels were measured by ELISA in supernatants of $HepG_2$ cells cultured for 24 h in serum-free media, stimulated by 25 ng/ml IL-6 alone or with different concentrations of adiponectin (0.5, 1.0, and 2.0 μg/ml). Results shown ($n = 5$, in triplicate) are CRP optical density (**a**) and CRP concentrations in the supernatant of $HepG_2$ cell culture medium (**b**) (mean ± SD, * compared to control $P < 0.05$; ** compared to IL-6 $P < 0.05$.)

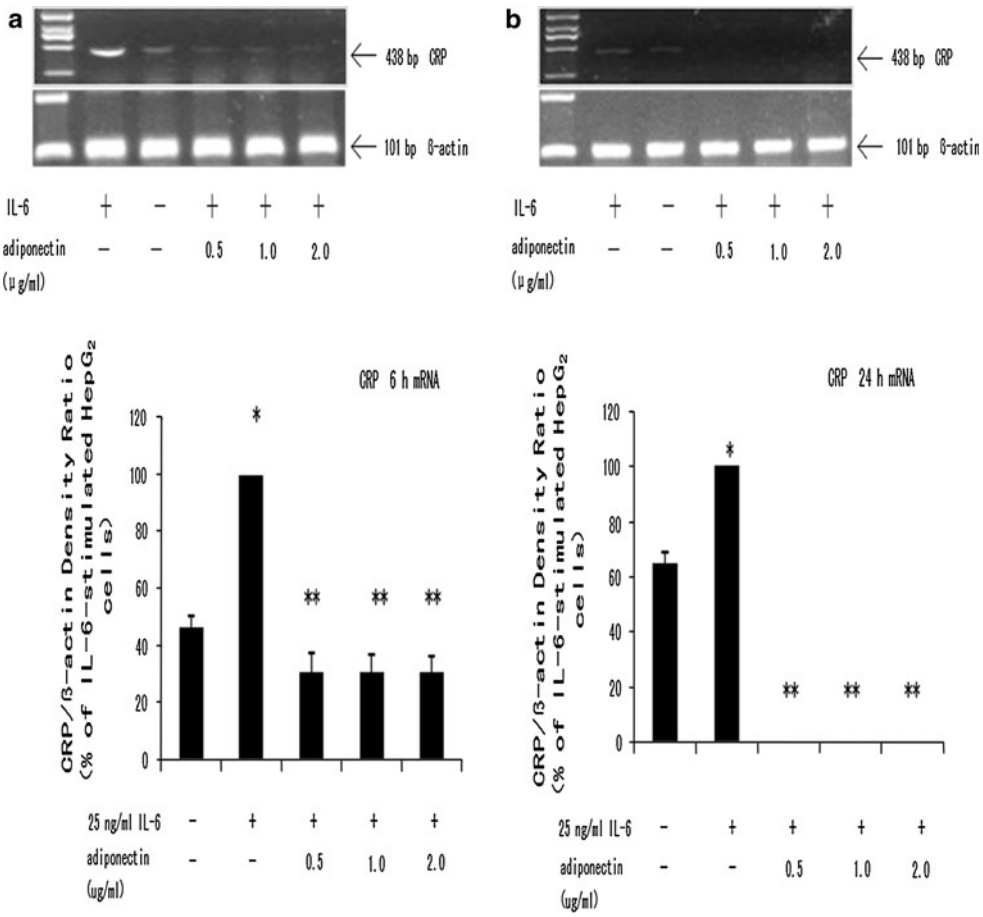

Fig. 2 Effect of adiponectin on IL-6-induced CRP mRNA expression. $HepG_2$ cells were cultured in serum-free media, and activated by 25 ng/ml IL-6 alone or with indicated concentrations of adiponectin for 6 h (**a**) or 24 h (**b**). Total cellular mRNA was extracted and measured by RT-PCR. $n = 5$ for densitometric ratios. * Compared to control $P < 0.05$; ** compared to IL-6 $P < 0.05$

case of CRP secretion, adiponectin strongly repressed IL-6-induced CRP upregulation, largely in a concentration-independent manner. Treatment with adiponectin for 24 h completely suppressed CRP expression (Fig. 2b).

Adiponectin downregulates STAT3 activity in $HepG_2$ cells

We investigated the underlying molecular mechanisms of adiponectin-induced suppression of CRP gene expression. Via its phosphorylation, STAT3 participates in transcriptional activation of CRP gene by IL-6 [25]. It was observed that phosphorylation of STAT3 on both tyrosine and serine residues reached maximum levels after exposing $HepG_2$ cells to IL-6 for 30 min (Fig. 3a). However, pretreating $HepG_2$ cells with 1 μg/ml adiponectin for 3 h strongly inhibited IL-6-induced phosphorylation of STAT3 on both tyrosine and serine residues (Fig. 3b). Neither IL-6 nor adiponectin affected total STAT3 levels. Finally, we demonstrated that the reduction of STAT3 phosphorylation by adiponectin does not result from the reduction of IL-6 receptor expression, because we did not observe any changes in IL-6Rα and gp130 expression levels, after adiponectin treatment for up to 24 h (Fig. 3c).

Discussion

In this study, we demonstrated that adiponectin inhibited IL-6-induced CRP expression in $HepG_2$ cells at the transcript level, and the inhibition was associated with a reduction in STAT3 phosphorylation.

Obesity is a high risk factor for metabolic syndrome, hypertension, atherosclerosis, and heart disease [26, 27]. With increasing accumulation of body fat, especially the visceral fat, the circulating adiponectin levels become somehow progressively lower [28]. The plasma levels of this adipose tissue-derived adipokine are reduced in obesity-related disorders: diabetes, metabolic syndrome, and cardiovascular disease [29–35]. Correspondingly, circulating levels of CRP are elevated in these hypoadiponectinemic conditions. Quchi et al. reported a strong inverse correlation between adiponectin and CRP mRNA levels in human adipose tissue [20]. These data indicate that adiponectin and CRP might regulate each other's expression. Devaraj et al. has previously shown that adiponectin decreases CRP synthesis and secretion from endothelial cells through downregulating NF-κB activity [21]. In the present study, we provide evidence that adiponectin reduces CRP expression in $HepG_2$ cells, likely via attenuating

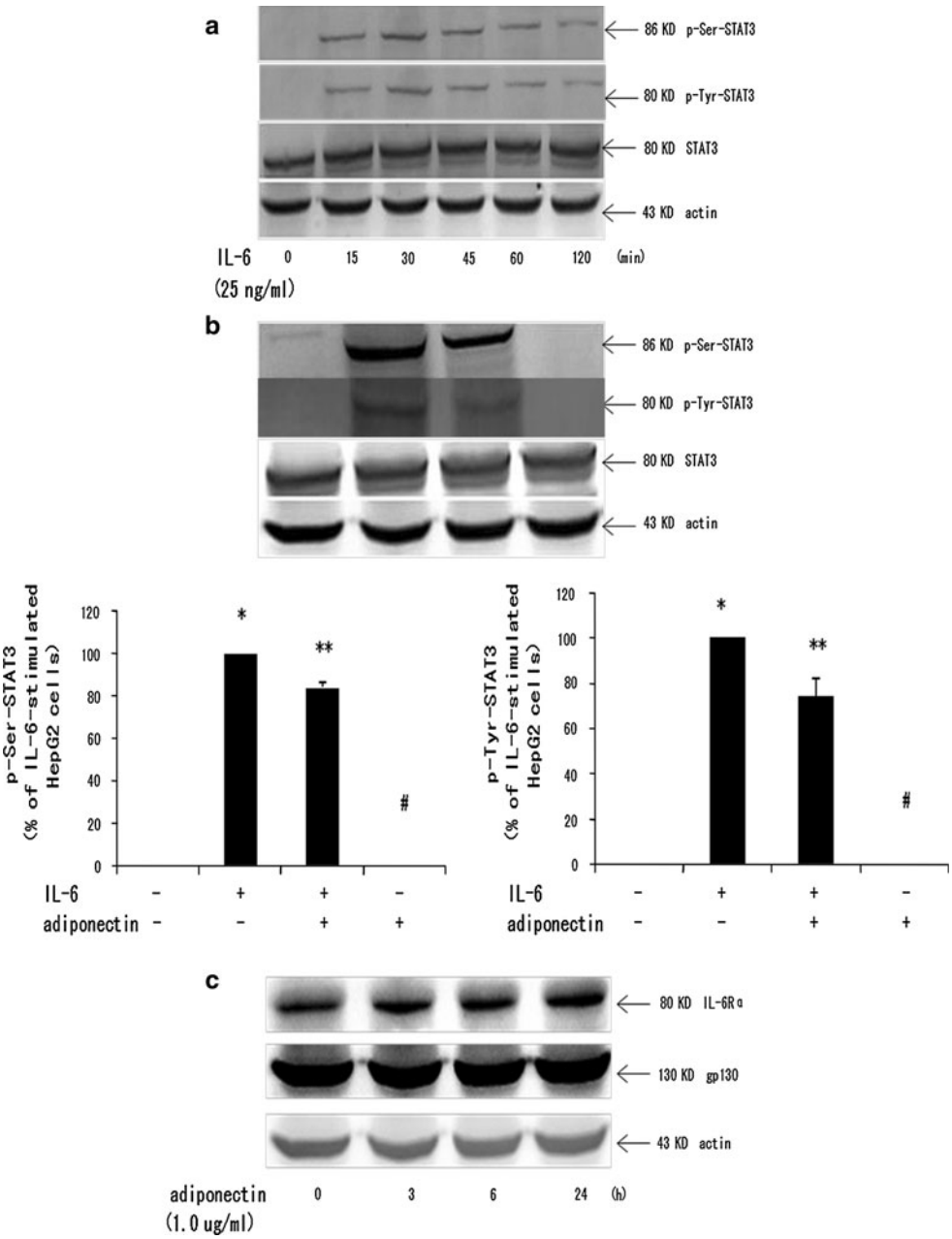

Fig. 3 Effect of adiponectin on IL-6-induced STAT3 phosphorylation. **a** $HepG_2$ cells were stimulated with 25 ng/ml IL-6 for 0, 15, 30, 45, 60, and 120 min. **b** After 3 h pretreatment with 1.0 μg/ml adiponectin, $HepG_2$ cells were exposed to 25 ng/ml IL-6 alone or with adiponectin for 30 min. Expression of STAT3, p-Ser-STAT3, and p-Tyr-STAT3 were obtained by western blots. $n = 5$ for densitometric ratios. * Compared to control $P < 0.05$; ** compared to IL-6 alone $P < 0.05$, # compared to IL-6 + adiponectin $P < 0.05$. **c** $HepG_2$ cells were treated with 1.0 μg/ml adiponectin for 3, 6, and 24 h. Expression of IL-6Rα and gp130 were determined by Western blotting

STAT3 activity. We previously demonstrated that CRP inhibited adiponectin synthesis in human and rat primary adipocytes [36], and other investigators obtained similar conclusion [37]. Together, these findings indicate that adiponectin and CRP reciprocally regulate each other, forming a double-negative feedback loop.

As one of the main regulators of cytokine-induced hepatic acute phase protein synthesis, STAT3 is involved in IL-6-induced CRP gene transcription through phosphorylation on tyrosine and serine residues [25]. The signaling pathways leading to STAT3 phosphorylation on tyrosine and serine residues differ from each other. The tyrosine kinase activity of stimulated IL-6 receptor complex (IL-6 Ra/gp130/JAK) mediates the tyrosine residue phosphorylation [38, 39], whereas IL-6-induced serine phosphorylation of STAT3 involves Vav, Rac-1, MEKK, and SEK-1 [40, 41]. In this study, we showed that adiponectin inhibits STAT3 phosphorylation on both tyrosine and serine residues, which is consistent with previous findings [13, 42], and adiponectin does not affect the IL-6 receptor complex. Therefore, we hypothesized that adiponectin reduces IL-6-induced STAT3 phosphorylation on tyrosine residue probably by modulating JAKs phosphorylation, although this possibility remains to be confirmed. Further studies are necessary to elucidate the molecular pathways by which adiponectin inhibits STAT3 phosphorylation.

Previous studies demonstrated that CRP is a robust and independent risk marker for cardiovascular disease [3–6]. Besides being a risk marker, CRP indeed is an active participant in atherogenesis [7, 8, 10]. A recent study showed that CRP enhances tissue factor (TF) expression by vascular smooth muscle cells [43]. Since it is known that TF initiates blood clotting, this suggests that CRP is associated with increased risk of myocardial infarction. Thus, the decrease in CRP expression by adiponectin may prevent cardiovascular incidence. Therapeutic regimen targeting CRP inhibition and/or adiponectin stimulation may hold promise for use in primary prevention of cardiovascular diseases.

In conclusion, we demonstrated that adiponectin represses CRP hepatic gene expression, thus potentially reducing the circulating CRP level. This provides a novel molecular mechanism for the linkage between obesity and vascular diseases.

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