C-reactive protein inhibits adiponectin gene expression and secretion in 3T3-L1 adipocytes

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Abstract

C-reactive protein (CRP) is considered as one of the most sensitive markers of inflammation. The aim of the present study is to investigate the effects of CRP on the production of adiponectin in 3T3-L1 adipocytes. Northern and western blot analysis revealed that CRP treatment inhibited adiponectin mRNA expression and secretion in a dose- and time-dependent manner. Co-incubation of adipocytes with rosiglitazone and CRP decreased induction of adiponectin gene expression by rosiglitazone. However, luciferase reporter assays did not show that CRP affected the activity of ~2.1 kb

adiponectin gene promoter, which was increased by rosiglitazone alone. Pharmacological inhibition of phosphatidylinositol (PI)-3 kinase by LY294002 partially reversed inhibition of adiponectin gene expression by CRP. These results collectively suggest that CRP suppresses adiponectin gene expression partially through the PI-3 kinase pathway, and that decreased production of adiponectin might represent a mechanism by which CRP regulates insulin sensitivity.

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Introduction

Adipose tissue is now recognized to be an important endocrine organ, secreting a variety of polypeptides (adipokines) that are involved in the regulation of energy metabolism, immune response, and cardiovascular tone (Chaldakov *et al.* 2003, Lyon *et al.* 2003). Adiponectin is an important adipokine exclusively secreted from adipose tissue (Berg *et al.* 2002). Growing evidence suggests that adiponectin is an insulin-sensitizing hormone with direct anti-diabetic, anti-atherogenic, and anti-inflammatory potentials (Berg *et al.* 2002, Chaldakov *et al.* 2003). Consistent with an insulin-sensitizing effect, transgenic ablation of adiponectin in mice induces insulin resistance (Kubota *et al.* 2002, Maeda *et al.* 2002), whereas transgenic overexpression (Yamauchi *et al.* 2002, Combs *et al.* 2004) improves insulin sensitivity. Interestingly, adiponectin also protects mice from atherosclerosis (Kubota *et al.* 2002, Yamauchi *et al.* 2003). Plasma levels of adiponectin correlate closely with systemic insulin sensitivity, and decreased adiponectin concentrations (hypoadiponectinemia) were observed in patients with type 2 diabetes, insulin resistance, or coronary heart disease (Hotta *et al.* 2000, Weyer *et al.* 2001). Several prospective studies suggest that a decline in plasma adiponectin concentration

preceded the decrease in insulin sensitivity, suggesting that adiponectin deficiency might be an important causative factor of insulin resistance (Hotta *et al.* 2001, Lindsay *et al.* 2002). Indeed, several insulin resistance inducing factors, such as tumor necrosis factor α , interleukin 6, dexamethasone and isoproterenol, have been shown to reduce adiponectin production (Fasshauer *et al.* 2001, 2002, 2003). On the other hand, the peroxisome proliferator-activated receptor (PPAR) γ agonists thiazolidinediones (TZD), which are used clinically as insulin sensitizing drugs, increased adiponectin expression and its plasma concentrations in rodents and human subjects (Maeda *et al.* 2001, Combs *et al.* 2002).

C-reactive protein (CRP) is one of the most sensitive inflammatory markers. An association of CRP to the development of atherosclerotic disease has been observed in experimental and epidemiological studies (Ridker *et al.* 2000, Libby *et al.* 2002). Recent studies have shown that elevation of CRP concentrations is an independent predictive parameter of type 2 diabetes mellitus (DM; Festa *et al.* 2002, Freeman *et al.* 2002), which is also strongly associated with various components of the metabolic syndrome (Frohlich *et al.* 2000, Aronson *et al.* 2004). A number of studies reported that serum highly sensitive CRP (hs-CRP) is negatively correlated with insulin sensitivity index (SI) and

serum adiponectin in some subjects (Ouchi *et al.* 2003, Schulze *et al.* 2004, Yuan *et al.* 2006). However, it has not been determined so far whether increased CRP level is a cause or an effect of insulin resistance and decreased adiponectin.

In the current study, we therefore examined the effect of CRP on adiponectin gene expression and secretion in 3T3-L1 adipocytes *in vitro*. We demonstrated that CRP suppressed adiponectin mRNA expression and secretion in a dose- and time-dependent manner. Furthermore, we found that pharmacological inhibition of phosphatidylinositol (PI)-3 kinase by LY294002 partially reversed inhibition of adiponectin gene expression by CRP. These results collectively suggest that CRP suppresses adiponectin gene expression partially through the PI-3 kinase pathway.

Materials and Methods

Materials

Insulin, dexamethasone, methyl-isobutyl-xanthine, anti-adiponectin antibody, AG490, PD98059, SB203580, and LY294002 were purchased from Sigma. Human recombinant CRP was obtained from Calbiochem (La Jolla, CA, USA). Rosiglitazone was generously provided by Shanghai Sunve Pharmaceutical Co., Ltd. Goat anti-rabbit IgG-HRP-conjugated secondary antibody was obtained from Dako (Glostrup, Denmark). Enhanced chemiluminescence (ECL) reagents were purchased from Pierce (USA). Trizol Reagent and lipofectamine 2000 were purchased from Invitrogen. DIG Northern Starter Kit was purchased from Roche. Dual luciferase reporter system and the reporter vectors pGL3-basic and pRL-SV40 are the products of Promega Corporation.

Cell culture and differentiation

3T3-L1 cells were maintained as subconfluent cultures in Dubecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and differentiated with DMEM supplemented with 5 mg/ml insulin, 0.5 mmol/l 1-methyl-3-isobutyl-xanthine, and 1 mmol/l dexamethasone 2 days after reaching confluence.

Analysis of adiponectin gene expression by northern blot

Total RNA was extracted from 3T3-L1 adipocytes using Trizol Reagents according to the manufacturer's instructions. Northern blot analysis was performed using non-isotopic DIG Northern Starter Kit as we previously described (Li *et al.* 2004). Target fragment (murine adiponectin) was cloned into PGEM-T easy vector and confirmed by automated sequencing. DIG-labeled probe was generated by

transcription with sp6 RNA polymerase using the DIG Northern starter kit. Ten micrograms of total RNA per lane were used for northern blot analysis.

Quantification of adiponectin concentration by western blot

Media adiponectin concentration was measured by western blot. Media of 7.5 µl were subjected to western blot to detect the amount of adiponectin that was secreted during CRP exposure. Proteins were separated by 12.5% SDS-PAGE gel, followed by electrophoretic transfer to a nitrocellulose membrane. Membranes were incubated overnight at 4 °C with anti-adiponectin antibody (1:2000), and incubated for at least 1 h at room temperature with goat anti-rabbit IgG-HRP-conjugated secondary antibody (1:2000). The signal was detected using the ECL system. The amount of adiponectin was normalized to cell number.

Cloning of human adiponectin promoter and construction of the luciferase reporter vector

A ~2.1 kb promoter segment corresponding to the range -2114 to +4 bp of the human adiponectin gene was amplified using the forward primer

CTGTAGGTACCGGGCATGAGGAATTAGAAAGCA and the reverse primer TAGAACTCGAGAATGGAAGTGAGGAGGAGATGG respectively. The DNA fragment was digested with *kpn*I and *Xho*I, and subcloned into PGL3-basic vector. The sequence of the cloned DNA fragment was confirmed by DNA sequencing.

Transient transfection and luciferase reporter assays

3T3-L1 adipocytes at day 7 after differentiation were transfected with the luciferase reporter vectors using Lipofectamine 2000 (Maeda *et al.* 2001, Xu *et al.* 2004). Luciferase assays were conducted using the Dual-Luciferase Reporter System (Promega). Transiently transfected cells were solubilized in 150 µl lysis buffer. After centrifugation to remove cell debris, 20 µl cell lysate was used to measure luciferase activity according to the manufacturer's instructions. In each measurement, luciferase units were normalized for background and transfection efficiency as determined by the 'Renilla' luciferase activity of the co-transfected pRL-SV40 plasmid.

Statistical analysis

Results were reproduced in at least three independent experiments. The results are presented as means of at least triplicate determinations $\pm$ s.d. Significance was determined by Student's *t*-test or one-way ANOVA. In all statistical comparisons, a *P* value of <0.05 was considered statistically significant.

Results

CRP inhibits adiponectin mRNA expression in a dose- and time-dependent manner

To evaluate the effect of CRP on adiponectin gene expression, we treated 3T3-L1 adipocytes with different concentrations of CRP for 24 h. Northern blot analysis revealed that CRP treatment inhibited adiponectin mRNA expression in a dose-dependent manner with significant 31% inhibition detectable with CRP concentrations at 25 µg/ml ($P<0.01$) and a maximal 52% decrease found at 50 µg/ml ($P<0.01$; Fig. 1A).

Furthermore, adiponectin mRNA expression was suppressed in a time-dependent manner with significant 42% inhibition detectable at 12 h of CRP treatment and a maximal 52% inhibition observed at 24 h after CRP addition ($P<0.01$; Fig. 1B).

Adiponectin secretion is inhibited by CRP in a dose- and time-dependent manner

Next, we determined whether changes in adiponectin mRNA expression would be paralleled by protein secretion. In fact, adiponectin secretion was suppressed in a dose-dependent manner with 19% inhibition detectable with CRP concentration at 25 µg/ml ($P<0.05$) and a 41% significant reduction found at 50 µg/ml ($P<0.01$; Fig. 2A). CRP-treatment inhibited adiponectin secretion also in a time-dependent manner with significant 29% inhibition detectable at 12 h of CRP treatment and a maximal 41% reduction found at 24 h ($P<0.01$; Fig. 2B).

Inhibition of adiponectin mRNA expression by CRP is partially mediated via PI-3 kinase pathway

Recently, the major steps in CRP signaling have been elucidated. Signaling proteins such as PI-3 kinase have been implicated in CRP signaling. We further tested whether signaling proteins such as Janus kinase 2 (Jak2), P42/44 mitogen-activated protein (MAP) kinase, P38 MAP kinase, and PI-3 kinase might play a role in the downregulation of adiponectin gene expression. For this purpose, 3T3-L1 adipocytes were pretreated with specific pharmacological inhibitors for 1 h before CRP (50 µg/ml) was added for 24 h. Each individual pharmacological inhibitor alone tended to decrease adiponectin mRNA expression (Fig. 3A). Again, adiponectin mRNA was decreased by 52% after 24 h of CRP treatment ($P<0.01$; Fig. 3B). Interestingly, inhibition of PI-3 kinase by LY294002 (10 µM) significantly reversed this inhibition and adiponectin expression recovered 77% of wild-type levels (Fig. 3B). In contrast, inhibition of Jak2, p44/42 MAP kinase, and p38 MAP kinase with AG490 (25 µM), PD 98059 (25 µM), and SB203580 (25 µM) respectively did not significantly influence the inhibition of adiponectin gene expression by CRP (Fig. 3B).

CRP and PPAR γ agonist affect adiponectin gene expression via distinct pathways

The expression of adiponectin has been shown to be under the control of the transcription factor PPAR γ . The PPAR γ agonists TZD was shown to be able to increase plasma adiponectin levels in humans and mice. Consistent with previous reports, we found that the PPAR agonist

Figure 1 CRP inhibits adiponectin mRNA expression in a dose- and time-dependent manner (A) Dose-dependent inhibition of adiponectin mRNA expression by CRP (24 h). (B) Time-dependent inhibition of adiponectin mRNA expression by CRP (50 µg/ml). Differentiated 3-L1 cells were serum-deprived overnight before CRP was added. Total RNA was extracted and subjected to northern blot as described in Materials and Methods. Adiponectin mRNA levels normalized to 18S rRNA expression were determined relative to untreated control cells (100%). Data represent means $\pm$ S.E.M. of three independent experiments. **Denotes $P<0.01$ comparing CRP-treated with non-treated adipocytes.

Figure 2 Adiponectin secretion is inhibited by CRP in a dose- and time-dependent manner. (A) Dose-dependent inhibition of adiponectin secretion by CRP (24 h). (B) Time-dependent inhibition of adiponectin secretion by CRP (50 µg/ml). Differentiated 3T3-L1 cells were serum-deprived overnight before CRP was added. 7.5 µl of the media was subjected to western blot to detect the amount of adiponectin that was secreted. The amount of adiponectin was normalized to cell number. Data are expressed relative to untreated control (Con) cells (100%) and represent means ± S.E.M. of three independent experiments. *Denotes $P < 0.05$, ** $P < 0.01$ comparing CRP-treated with untreated control cells.

rosiglitazone enhanced adiponectin mRNA expression by 1.7-fold (Fig. 4A), while 25 µg/ml CRP decreased adiponectin gene expression by 31%. Co-incubation of cells with CRP and rosiglitazone decreased induction of adiponectin gene expression by rosiglitazone (Fig. 4A).

Finally, we further investigated the effects of CRP on the activity of ~2.1 kb human adiponectin gene promoter, which includes the PPARγ responsive elements. The addition of 10 µM rosiglitazone significantly increased PPARγ activity (by 15-fold), whereas CRP (25 µg/ml) did

not suppress the activity of ~2.1 kb human adiponectin gene promoter that was increased by rosiglitazone (Fig. 4B), suggesting that CRP and PPARγ agonist affect adiponectin gene expression via distinct pathways.

Discussion

CRP is one of the most sensitive inflammatory markers. An association of CRP to the development of

Figure 3 Inhibition of adiponectin mRNA by CRP is partially mediated via PI-3 kinase. (A) After overnight serum-starvation, differentiated 3T3-L1 adipocytes were cultured in the presence or absence of the Janus kinase 2 (JAK2) inhibitor AG490 (AG, 25 µM), the P42/44 mitogen-activated protein (MAP) kinase inhibitor PD98059 (PD, 25 µM), the P38 MAP kinase inhibitor SB203580 (SB, 25 µM), or the phosphatidylinositol-3 (PI-3) kinase inhibitor LY294002 (LY, 10 µM) for 25 h. (B) After overnight serum-starvation, differentiated 3T3-L1 adipocytes were cultured in the presence or absence of 25 µM AG, 2.5 µM PD, 25 µM SB, or 10 µM LY for 1 h before CRP (50 µg/ml) was added for 24 h. After extraction of total RNA, northern blot was performed as described in Materials and Methods and adiponectin gene expression normalized to 18S rRNA levels is expressed relative to untreated control cells (100%). Results are means ± S.E.M. of three independent experiments. **Denotes $P < 0.01$ comparing specific pharmacological inhibitors or CRP treated with untreated, and #denotes $P < 0.05$ comparing LY294002 pretreated with CRP-treated cells respectively.

Figure 4 CRP and PPAR γ affect adiponectin gene expression via distinct pathways. (A) Differentiated 3T3-L1 cells were serum-deprived overnight before CRP (25 μ g/ml) and rosiglitazone (10 μ M) were added for 24 h. Extraction of total RNA and northern blot determining adiponectin mRNA levels normalized to 18S rRNA expression were done as described in Materials and Methods. Data are expressed relative to untreated control cells (100%) and represent means $\pm$ S.E.M. of three independent experiments. **Denotes $P < 0.01$ comparing with untreated control cells, and ##denotes $P < 0.01$ comparing Rosi-treated with Rosi + CRP-treated cells respectively. (B) Adiponectin-luciferase and pRL-SV40 vector were co-transfected into day 7-differentiated 3T3-L1 cells. For each 12-well dish, 1.5 μ g adiponectin-luciferase and 15 ng pRL-SV40 were used. Six hours after transfection, rosiglitazone (10 μ M) or CRP (25 μ g/ml) was supplemented to medium. At 24 h after transfection, cells were harvested, and firefly and Renilla luciferase reporter activity was measured as described in research and methods. Adiponectin-luciferase (firefly) activity was normalized by pRL-SV40 Renilla luciferase activity. Values are means $\pm$ S.E.M. from three independent experiments. **Denotes $P < 0.01$ compared with control values.

atherosclerotic disease has been observed in experimental and epidemiological studies (Ridker *et al.* 2000, Libby *et al.* 2002). Recent studies have shown that elevation of CRP concentrations is an independent predictive parameter of type 2 DM (Festa *et al.* 2002, Freeman *et al.* 2002), which is also strongly associated with various components of the metabolic syndrome (Frohlich *et al.* 2000, Aronson *et al.* 2004). Our group and others have reported that serum hs-CRP is inversely related to SI and serum adiponectin in certain defined subjects (Ouchi *et al.* 2003, Schulze *et al.* 2004, Yuan *et al.* 2006). However, to our knowledge, whether increased CRP level is a cause or an effect of insulin resistance or decreased adiponectin still remained unclear.

In the present study, we demonstrate for the first time that CRP suppresses adiponectin mRNA expression and secretion in a time- and dose-dependent manner. Adiponectin is a 244 amino acid adipose-specific protein that has been shown to possess anti-atherogenic and anti-inflammatory properties (Ouchi *et al.* 1999, Yokota *et al.* 2000) in addition to improving glucose tolerance and insulin resistance in mouse diabetic models (Yamauchi *et al.* 2001). Similarly, adiponectin is related to insulin resistance and adiposity in humans (Weyer *et al.* 2001). Furthermore, recent study suggests that adiponectin is a protective factor against later development of diabetes (Lindsay *et al.* 2002). Our findings indicate that increased CRP level may lead to decreased adiponectin, and that decreased production of adiponectin seems to be a mechanism by which CRP regulates insulin sensitivity.

Recently, the major steps in CRP signaling have been elucidated (Marnell *et al.* 2005). CRP binds to Fc γ R. Fc γ R

containing immunoreceptor tyrosine-based activation motifs (ITAM), such as Fc γ RI, Fc γ RIIA/C, and Fc γ RIIIA, are activated by clustering on the cell surface caused by ligand binding. This is followed by phosphorylation of the two tyrosines in the ITAM motif by Src-related tyrosine kinases such as Lyn, Fgr, and Hck. This leads to recruitment of Src homology 2-containing molecules such as Syk tyrosine kinase, which leads to a cascade of events: (1) phosphorylation of PI-3 kinase with the generation of PI(3,4,5)P $_3$, which promotes downstream signaling events, including phosphorylation of phospholipase C γ 2 (PL C γ 2), which produces (a) diacylglycerol (DAG), which activates phosphokinase C, which activates p38 transcription factor, and (b) calcium mobilization through inositol triphosphate (IP $_3$); (2) activation of Raf which binds Ras, phosphorylates MEK, which in turn phosphorylates ERK. Since pharmacological inhibition of PI-3 kinase by LY294002 partly reverses inhibition of adiponectin gene expression by CRP in the present study, PI-3 kinase is probably involved in adiponectin mRNA regulation by CRP. The PI-3 kinase is a ubiquitous, heterodimeric enzyme that plays a pivotal role in the regulation of many cellular processes, including motility, proliferation and survival, and carbohydrate metabolism. Bogan & Lodish (1999) reported that insulin stimulates adiponectin release within 1 h and inhibitors of PI-3 kinase block insulin-stimulated adiponectin secretion. It indicates that short-term insulin-induced release of stored adiponectin is also mediated by a PI-3 kinase-dependent pathway. In line with our finding, previous studies have found that inhibition of either PI-3 kinase or p70S6 kinase using their specific pharmacological inhibitors partially reversed insulin-mediated suppression of

adiponectin gene expression (Fasshauer *et al.* 2002, Xu *et al.* 2004), suggesting that PI-3 kinase/p70S6 kinase pathway is a suppressor of adiponectin gene expression.

The FOXO family of forkhead transcription factors is regulated by the PI-3 kinase-protein kinase B (PKB)/Akt pathway (Burgering & Kops 2002). A recent study (Davis *et al.* 2004) has shown that FoxC2 is associated with down-regulation of adiponectin in 3T3-L1 preadipocytes. Therefore, in addition to PI-3 kinase/p70S6 kinase, CRP inhibits adiponectin gene expression which may be also through PI-3 kinase-PKB/Akt-FOXO pathway.

The transcriptional events that underlie the adiponectin gene expression remain poorly understood. The transcription factor PPAR γ , a key regulator of adipogenesis, has been shown to transactivate adiponectin gene expression (Maeda *et al.* 2001). The PPAR γ agonist rosiglitazone increased adiponectin production in both animals and patients with diabetes (Combs *et al.* 2002). A more recent study has mapped the PPAR γ responsive elements to a DNA fragment between -285 and -273 of the human adiponectin gene. In line with recent studies (Maeda *et al.* 2001, Xu *et al.* 2004), rosiglitazone could increase adiponectin gene expression. The activity of a ~2.1 kb adiponectin gene promoter was increased by rosiglitazone. Co-incubation of adipocytes with rosiglitazone and CRP decreased induction of adiponectin gene expression while not affecting the activity of the adiponectin gene promoter induced by rosiglitazone. Our present study suggests that CRP and PPAR agonist affect adiponectin gene expression via distinct pathways.

Since adiponectin is an insulin-sensitizing hormone with direct anti-diabetic, anti-atherogenic, and anti-inflammatory potentials (Berg *et al.* 2002, Chaldakov *et al.* 2003), adiponectin deficiency appears to be an important causative factor of insulin resistance (Hotta *et al.* 2001, Lindsay *et al.* 2002). Hence, adiponectin replacement therapy may represent a novel strategy for the treatment of a variety of diseases, including insulin resistance, type 2 diabetes, and atherosclerosis (Berg *et al.* 2001, Fruebis *et al.* 2001, Yamauchi *et al.* 2001). Inhibition of CRP synthesis may be a strategy to increase endogenous adiponectin production.

In summary, our data clearly demonstrate that treatment of 3T3-L1 adipocytes with CRP significantly suppresses the expression and secretion of adiponectin in a dose- and time-dependent manner, which seems partially mediated via PI-3 kinase pathway.

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