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

Guoyue Yuan1,2,*; Xia Chen1,*; Qinyun Ma1,*; Jie Qiao1; Rongying Li1; Xuesong Li1; Shengxian Li3; Jinfeng Tang1; Libin Zhou1; Huaidong Song1; and Mingdao Chen1

1 State Key Laboratory of Medical Genomics, Shanghai Institute of Endocrine and Metabolic Diseases, Center of Molecular Medicine, Ruijin Hospital, Shanghai Jiaotong University Medical School, 197, Ruijin Road II, Shanghai 200025, China
2 Department of Endocrinology, The Affiliated Hospital of Jiangsu University, Zhenjiang, Jiangsu 212001, China
3 Department of Endocrinology, Renji Hospital, 1630, Dongfeng Road, Pudong, Shanghai 210127, China

*G Yuan, X Chen and Q Ma contributed equally to this work

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. Journal of Endocrinology (2007) 194, 275–281

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 (hypo-adiponectinemia) 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 form 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 Dubeco’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-xanthin, and 1 mmol/l dexamethazone 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 CTGTTAGGTACCGGCGATGAGGAATTAGAAAGCA and the reverse primer TAGAACTCGAGAATGGAAGTTAGGAGAGATGG respectively. The DNA fragment was digested with kpn1 and XhoI, 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 ± 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 PPARγ agonist affect adiponectin gene expression via distinct pathways

The expression of adiponectin has been shown to be under the control of the transcription factor PPARγ. The PPARγ 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](https://www.endocrinology-journals.org/JE/3/194/275-281/Figure1.png)

**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 ± S.E.M. of three independent experiments. **Denotes $P<0.01$ comparing CRP-treated with non-treated adipocytes.
Rosiglitazone enhanced adiponectin mRNA expression by 1.7-fold (Fig. 4A), while 25 \(\mu\)g/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 PPAR\(\gamma\) responsive elements. The addition of 10 \(\mu\)M rosiglitazone significantly increased PPAR\(\gamma\) activity (by 15-fold), whereas CRP (25 \(\mu\)g/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 PPAR\(\gamma\) 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
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 FcγR. FcγR containing immunoreceptor tyrosine-based activation motifs (ITAM), such as FcγRI, FcγRIIA/C, and FcγRIIIA, 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)P3, which promotes downstream signaling events, including phosphorylation of phospholipase Cγ2 (PL Cγ2), which produces (a) diacylglycerol (DAG), which activates phosphokinase C, which activates p38 transcription factor, and (b) calcium mobilization through inositol triphosphate (IP3); (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 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 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.

Figure 4 CRP and PPARγ affect adiponectin gene expression via distinct pathways. (A) Differentiated 3T3-L1 cells were serum-deprived overnight before CRP (25 μg/ml) and rosiglitazone (10 μM) 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 ± 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 μg adiponectin-luciferase and 15 ng pRL-SV40 were used. Six hours after transfection, rosiglitazone (10 μM) or CRP (25 μg/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 ± S.E.M. from three independent experiments. **Denotes P<0.01 compared with control values.
adiponectin gene expression (Fashhauer 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 PPARγ, a key regulator of adipogenesis, has been shown to transactivate adiponectin gene expression (Maeda et al. 2001). The PPARγ agonist rosiglitazone increased adiponectin production in both animals and patients with diabetes (Combs et al. 2002). A more recent study has mapped the PPARγ 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.

Acknowledgements

This work was jointly supported in part by grants from the Foundation for the Author of National Excellent Doctoral Dissertation of People’s Republic of China (200154), the National Natural Science Foundation of China (30470816, 30530370, 30670987), the Education Commission of Shanghai (05BZ28), and the Science and Technology Commission of Zhenjiang City, Jiangsu Province (SG 2001094). All the funding mentioned in this article was supported by the government of China. There is no conflict of interest that would prejudice the impartiality of the research or a potential conflict of interest that is not fully declared within the text of the article.

References


Hotta K, Funahashi T, Bodkin NL, Ornstein HK, Arita Y, Hansen BC & Matsuzawa Y 2001 Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *Diabetes* 50 1126–1133.


Marniell L, Mold C & Du Clos TW 2005 C-reactive protein: ligands, receptors and role in inflammation. *Clinical Immunology* 107 104–111.


