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Effects of C-reactive protein on adipokines genes expression in 3T3-L1 adipocytes

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ABSTRACT

Adipose tissue is now recognized to be an important endocrine organ, secreting a variety of adipokines that are involved in the regulation of energy metabolism, insulin resistance and metabolic syndrome. C-reactive protein (CRP) is considered as one of the most sensitive markers of inflammation. A number of studies have shown that elevation of CRP concentrations is an independent predictive parameter of type 2 diabetes mellitus, which is also strongly associated with various components of the metabolic syndrome. The aim of the present study is to investigate the effects of CRP on adipokines genes expression in 3T3-L1 adipocytes. Quantitative real-time PCR analysis revealed that CRP inhibited adiponectin, leptin and peroxisome proliferator-activated receptor-gamma (PPAR-γ) genes expression and raised tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) mRNA levels in matured 3T3-L1 adipocytes in a dose and time-dependent manner. Pharmacological inhibition of phosphatidylinositol (PI)-3 kinase by wortmannin partially reversed the effects of CRP on adiponectin, TNF-α and leptin genes expression. These results collectively suggest that CRP regulates adiponectin, TNF-α, leptin, IL-6 and PPAR-γ genes expression, and that might represent a mechanism by which CRP regulates insulin resistance, obesity and metabolic syndrome.

1. Introduction

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 [1,2]. Numerous studies have shown that elevation of CRP concentrations is an independent predictive parameter of type 2 diabetes mellitus (DM) [3,4], which is also strongly associated with various components of the metabolic syndrome [5,6], and serum highly sensitive CRP (hs-CRP) is negatively correlated with insulin sensitivity index (SI) in some subjects [7,8]. However, it has not been determined so far whether increased CRP level is a cause or an effect of insulin resistance or metabolic syndrome.

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, insulin resistance and metabolic syndrome [9,10].

A number of studies have shown that tumor necrosis factor-α (TNF-α) and interleukin-6(IL-6) are major inflammatory adipokines that have been linked to the development of insulin resistance and type 2 diabetes [11–13].

Leptin, which is mainly secreted by adipocytes, serves as a major "adipostat" by repressing food intake and promoting energy expenditure. Predictably, animals and humans with mutations in either leptin or the leptin receptor are obese [14,15]. Recent study has shown that the glucose/leptin ratio can be used in addition to glucose/insulin ratio, quantitative insulin-sensitivity check index, and homeostasis model assessment to accurately assess insulin resistance in subjects with hyperglycemia [16].

Adiponectin is an important adipokine exclusively secreted from adipose tissue [17,18]. Growing evidence suggests that adiponectin is an insulin-sensitizing hormone with direct anti-diabetic, anti-atherogenic and anti-inflammatory potentials [17,18].

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) belongs to the nuclear hormone receptor superfamily. It has been identified as a key regulator of adipocyte differentiation and glucose metabolism [19]. It appears to function as both a direct regulator of many fat specific genes and also as a "master" regulator that can trigger the entire program of adipogenesis [20].

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In our previous study, northern and western blot analysis revealed that CRP-treatment inhibited adiponectin mRNA expression and secretion in 3T3-L1 adipocytes [21]. But there is no information about the effects of CRP on the genes expression of TNF-α, IL-6, leptin and PPAR-γ. In the present study, we demonstrate that CRP increases TNF-α and IL-6 mRNA and suppresses leptin and PPAR-γ genes expression in a dose- and time-dependent manner. Furthermore, we found evidence that CRP regulates adiponectin, TNF-α and leptin genes expression partially through the PI-3 kinase pathway.

2. Materials and methods

2.1. Materials

Insulin, dexamethasone, methyl-isobutyl-xanthine, AG490, PD98059, SB203580 and wortmannin were purchased from Sigma (Saint Louis, MO, USA). Human recombinant CRP was obtained from Calbiochem (La Jolla, CA, USA). Rosiglitazone was generously provided by Shanghai Sunve Pharmaceutical Co., Ltd. Trizol Reagent was purchased from Invitrogen (Carlsbad, CA, USA). Superscript II reverse transcriptase was purchased from BD bioscience (Palo Alto, CA).

2.2. 3T3-L1 cell culture and differentiation

3T3-L1 cells (American Type Culture Collection, Manassas, VA) were maintained as subconfluent cultures in Dubecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) 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. On day 2 and thereafter, DMEM containing 10% FBS and insulin (5 mg/ml) only was subsequently replaced every 2 days. In general, by day 8, 90% of preadipocytes differentiated into adipocytes as determined by lipid accumulation visualized with Oil Red O staining.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from 3T3-L1 adipocytes using Trizol reagent. First strand cDNA synthesis was performed with 1 μg of total RNA using Superscript II reverse transcriptase.

2.4. Real-time PCR

In a florescent temperature cycler (LightCycler, Roche Diagnostics Ltd, Lewes, UK), 10% of each RT reaction was amplified in a 20 μl PCR containing 4 mM $MgCl_2$, 4 pM each primer and 1× LightCycler DNA Master SYBR Green 1 mix (Roche Diagnostics Ltd, Lewes, UK). Samples were incubated in the LightCycler for an initial denaturation at 94 °C for 30 s, followed by 40 PCR cycles. Each cycle consisted of 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 12 s. The oligonucleotide primers for the experiment are indicated in Table 1. To confirm amplification of specific transcripts, melting curve profiles (cooling the sample to 65 °C for 15 s and heating slowly to 95 °C with continuous measurement of fluorescence) were produced at the end of each PCR. The threshold cycles (Ct) were measured in separate tubes and in duplicate. The identity and purity of the amplified products were checked by electrophoresis on 2% agarose gel. To ensure the quality of the measurements, each assay included a negative control for each gene. The amount of mRNA for each adipokine was normalized according to the amount of mRNA encoding β-actin. The ΔCt values were calculated in every sample for each gene of interest as follows: $Ct_{\text{gene of interest}} - Ct_{\text{reference gene}}$ with β-actin as the reference gene.

Table 1
Gene sequences used as forward (F) and reverse (R) primers for real-time PCR.

Gene	Sequence	Accession No.	Amplicon (bp)
Adiponectin	F: GTCAGTGGATCTGACGACACAA R: ATGCCTGCCATCCAACCTG	NM-009605	171
TNF-α	F: GTTCTATGGCCCAGACCCTCAC R: GGCACCACTAGTTGGTTGTCTTTG	NM-013693	144
IL-6	F: CCACTTCACAAGTCGGAGGCTTA R: GCAAGTGCATCATCGTTGTTCATAC	NM-031168	115
Leptin	F: ACCTGTCTACTCATGCCGCACTC R: CTGTCCTGCAGCCTGTTTG	NM-008493	125
PPAR-γ	F: TGTCGGTTTCAGAAGTGCCTTG R: TTCAGCTGGTCGATATCACTGGAG	NM-011146	122
β-Actin	F: AGCGAGCATCCCCCAAAGTT R: GGGCACGAAGGCTCATCATCATT	NM-007393	139

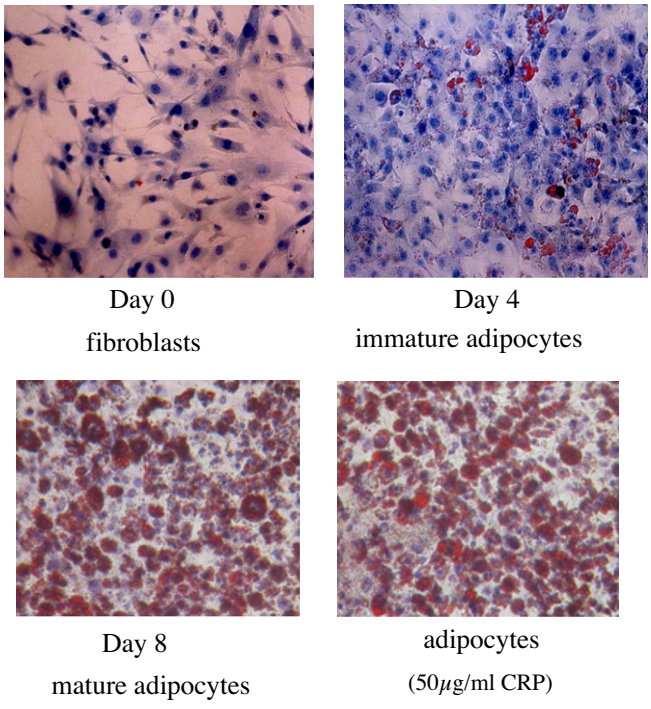

Fig. 1. Lipid accumulation during adipocyte differentiation. 3T3-L1 fibroblast cells were cultured in DMEM with 10% fetal bovine serum, and differentiated to adipocytes by adding insulin, dexamethasone, and 3-isobutyl-1-methylxanthine. In a series of experiments, CRP was included as indicated in Section 2. Cells were stained with Red-Oil.

Relative changes in the expression level of one specific gene (ΔΔCt) were calculated as ΔCt of the test group minus ΔCt of the control group and then presented as $2^{-\Delta\Delta Ct}$ [22].

2.5. Statistical analysis

Results were reproduced in at least three independent experiments. The results are presented as means ± SEM. Significance was determined by Student's *t* test or one-way ANOVA. In all statistical comparisons, a *p* value of less than 0.01 was considered statistically significant.

3. Results

3.1. Differentiation of 3T3-L1 preadipocytes to adipocytes

The 3T3-L1 cellular line was differentiated from fibroblasts to adipocytes with a differentiation cocktail, more than 90% of the

cells showed marked multiple vesicles with lipid accumulation (Oil Red O staining) after 8 days of differentiation. The presence of CRP apparently did not affect the lipid accumulation or the architecture of the cells (Fig. 1).

3.2. CRP inhibits adiponectin, leptin and PPAR-γ genes expression in a dose- and time-dependent manner

To evaluate the effect of CRP on adipokines genes expression, we treated different concentrations of CRP for 24 h. In our previous study, northern and western blot analysis revealed that CRP-treatment inhibited adiponectin mRNA expression and secretion in 3T3-L1 adipocytes [21]. In the present study, quantitative real-time PCR analysis revealed that CRP-treatment inhibited adiponectin, leptin and PPAR-γ mRNA expression in a dose-dependent manner with significant 33%, 52%, 28% inhibition detectable at 25 μg/ml ($p < 0.01$), and a maximal 45%, 62%, 54% decrease found at 50 μg/ml, respectively ($p < 0.01$) (Fig. 2A–C). Consistent with previous reports, we found that the PPAR-γ agonist rosiglitazone (RSG) enhanced adiponectin mRNA expression by 1.7-fold while it inhibited leptin and PPAR-γ mRNA expression with 59% and 52%.

Furthermore, we treated 3T3-L1 adipocytes with CRP concentrations at 50 μg/ml for 0, 2, 4, 8, 12, 24 h. Results showed that adiponectin, leptin and PPAR-γ mRNA expression were suppressed in a time-dependent manner with significant 38%, 49%, 33% inhibition detectable at 12 h of CRP treatment and a maximal 45%, 62%, 54% inhibition observed at 24 h, respectively ($p < 0.01$) (Fig. 2D–F).

3.3. CRP raises TNF-α and IL-6 gene expression in a dose- and time-dependent manner

As illustrated in Fig. 3(A, B), we found TNF-α and IL-6 mRNA levels were increased in a dose-dependent manner with significant 39% and 29% promotion detectable at 25 μg/ml ($p < 0.01$), and a maximal 83% and 161% increase found at 50 μg/ml, respectively

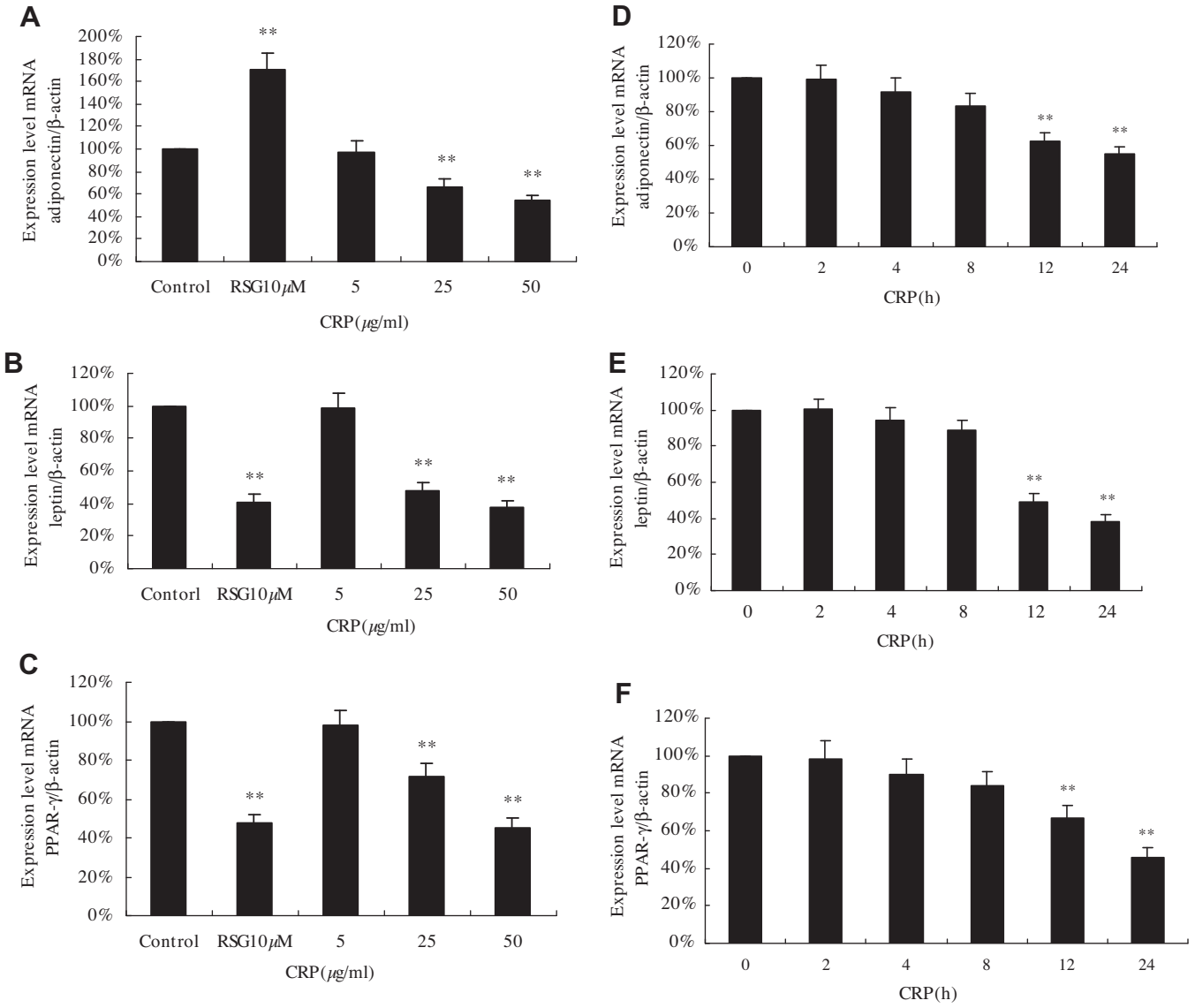

Fig. 2. CRP (24 h) inhibits adiponectin (A), leptin (B) and PPAR-γ (C) mRNA expression in a dose-dependent manner and CRP (50 μg/ml) inhibits adiponectin (D), leptin (E) and PPAR-γ (F) mRNA expression in a time-dependent manner. Differentiated 3T3-L1 cells were serum-deprived overnight before CRP was added. Total RNA was extracted and subjected to a quantitative real-time PCR as described in Section 2. Adiponectin, leptin and PPAR-γ mRNA levels normalized to β-actin expression were determined relative to untreated control cells (100%). Data represent means ± SEM of three independent experiments. **Denotes $p < 0.01$ comparing treated with non-treated adipocytes.

($p < 0.01$). Consistently we found that RSG inhibited TNF-α and IL-6 mRNA expression with 26% and 31%.

Also, we found TNF-α and IL-6 mRNA levels were increased in a time-dependent manner with significant 41% and 42% promotion detectable at 12 h of CRP treatment, and a maximal 83% and 161% increase at 24 h, respectively ($p < 0.01$) (Fig. 3C, D).

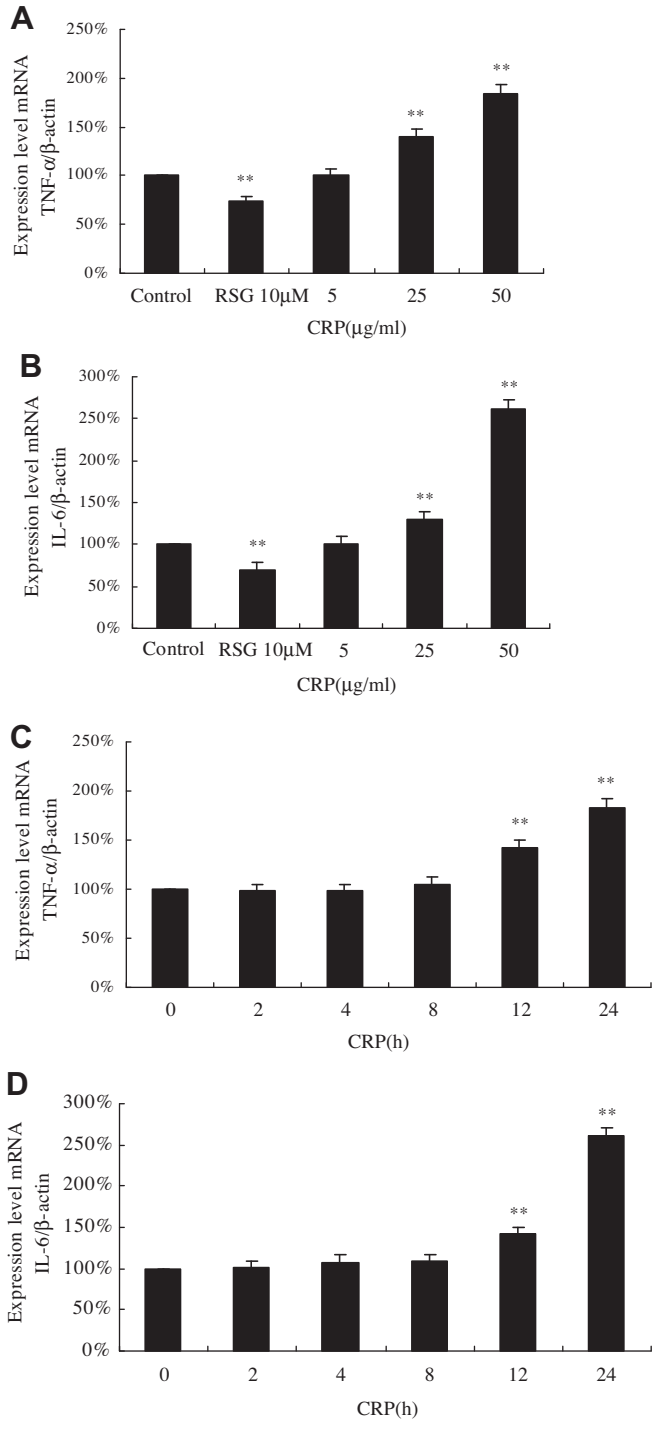

Fig. 3. CRP (24 h) raises TNF-α (A) and IL-6 (B) mRNA expression in a dose-dependent manner and CRP (50 μg/ml) raises TNF-α (C) and IL-6 (D) mRNA expression in a time-dependent manner. Differentiated 3T3-L1 cells were serum-deprived overnight before CRP was added. Total RNA was extracted and subjected to a quantitative real-time PCR as described in Section 2. TNF-α and IL-6 mRNA levels normalized to β-actin expression were determined relative to untreated control cells (100%). Data represent means ± SEM of three independent experiments. **Denotes $p < 0.01$ comparing treated with non-treated adipocytes.

3.4. Effects of CRP on adiponectin, TNF-α and leptin genes expression were partially mediated via PI-3 kinase pathway

We further tested whether signaling proteins such as Janus kinase2(Jak2), P42/44 mitogen activated protein (MAP) kinase, P38 MAP kinase, and PI-3 kinase might play a role in the downregulation of adipokines genes 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. In our previous study, pharmacological inhibition of PI-3 kinase by LY294002 partially reversed inhibition of adiponectin gene expression by CRP [21]. In the present study, wortmannin, another PI-3 kinase inhibitor was used. As a result, 100 nM wortmannin partially rebacked the effects of 50 μg/ml CRP on adiponectin, TNF-α and leptin mRNA expression ($p < 0.01$). However, wortmannin did not reversed the effects of CRP on IL-6 and PPAR-γ genes expression ($p > 0.05$) (Fig. 4). 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 effects of adiponectin, TNF-α, leptin, IL-6 and PPAR-γ genes expression by CRP (data not shown).

4. Discussion

CRP, an acute phase reactant, is associated with systemic inflammation. Many studies have demonstrated that CRP levels have important prognostic implications for patients.

Adipose tissue is now recognized to be an important endocrine organ, secreting a variety of polypetides (adipokines), such as adiponectin, TNF-α, IL-6 and leptin, that are involved in the regulation of energy metabolism, insulin resistance and metabolic syndrome [9,10].

Adiponectin is a 244 amino acid adipose-specific protein that has been shown to possess anti-atherogenic and anti-inflammatory properties [23] in addition to improving glucose tolerance and insulin resistance in mouse diabetic models [24]. Similarly, adiponectin is related to insulin resistance and adiposity in humans [25]. Furthermore, adiponectin is a protective factor against later development of diabetes [26]. In contrast, TNF-α and IL-6 are major inflammatory adipokines that have been linked to the development of insulin resistance and type 2 diabetes [11–13]. TNF-α and IL-6 may impair insulin signaling by stimulating serine phosphorylation of the insulin receptor substrate-1 (IRS-1) and by diminishing insulin-induced tyrosine phosphorylation, subsequently blocking the next steps of insulin signaling, where IRS-1 is associated with PI3 kinase and glucose transporter type 4 (GLUT-4) translocation, resulting in insulin resistance [27,28].In the present study, we demonstrate that CRP suppresses adiponectin mRNA and increases TNF-α and IL-6 genes expression in 3T3-L1 cells in vitro. In line with our findings, a recent study shows that human CRP overexpression facilitates the development of insulin resistance in association with adiponectin down-regulation and enhancement of expression of pro-inflammatory cytokines, such as TNF-α in epididymal adipose tissue [29]. Evidence suggests that adiponectin, TNF-α and IL-6 also influence CRP gene expression and secretion. Previous reports have shown that IL-6 is a powerful inducer of CRP production in the liver. TNF-α, has been reported to regulate the hepatic CRP synthesis and induce IL-6 expression [30]. Recent studies have demonstrated that adiponectin suppresses CRP synthesis and secretion from endothelial and liver cells [31,32]. Based on the findings mentioned above, it seems that there is a vicious cycle, whereby elevated CRP level may lead to insulin resistance via inhibiting the expression of adiponectin and enhancing TNF-α and IL-6 genes expression, which in turn prompting an even higher level of circulating CRP.

In our study, we also demonstrate that CRP suppresses leptin mRNA in a dose- and time- dependent manner. Leptin, which is mainly secreted by adipocytes, serves as a major "adipostat" by repressing food intake and promoting energy expenditure [33]. In addition to its well-described role in energy balance, leptin has notable effects on glucose homeostasis, as it reverses hyperglycaemia in *ob*/*ob* mice before body weight is corrected. Similarly, pair feeding *ob*/*ob* mice to match control animals does not restore glucose tolerance as well as exogenous leptin does [34]. Leptin also improves glucose homeostasis in lipodystrophic mice, and in humans with lipodystrophy or congenital leptin deficiency [35,36]. Recent study has shown that the glucose/leptin ratio can be used in addition to glucose/insulin ratio, quantitative insulin-sensitivity check index, and homeostasis model assessment to accurately assess insulin resistance in subjects with hyperglycemia [16]. Therefore, it seems that inhibition of leptin gene expression might be a mechanism by which CRP regulates metabolic syndrome.

PPAR-γ acts as a transcriptional activator of many adipocyte-specific genes involved in lipid synthesis, handling and storage of lipids, growth regulation, insulin signaling and adipokine production [19]. PPAR-γ binds to peroxisome proliferator response elements (PPREs) as a heterodimer with members of the retinoid X receptor (RXR) subfamily, provoking target gene expression. Moreover, PPAR-γ has been shown to inhibit the expression of proinflammatory cytokines, such as TNF-α and IL-6 [37]. Since CRP inhibits PPAR-γ gene expression in 3T3-L1 adipocytes in vitro in the present study, we suggest that increased CRP level may lead to insulin resistance or metabolic syndrome via suppressing

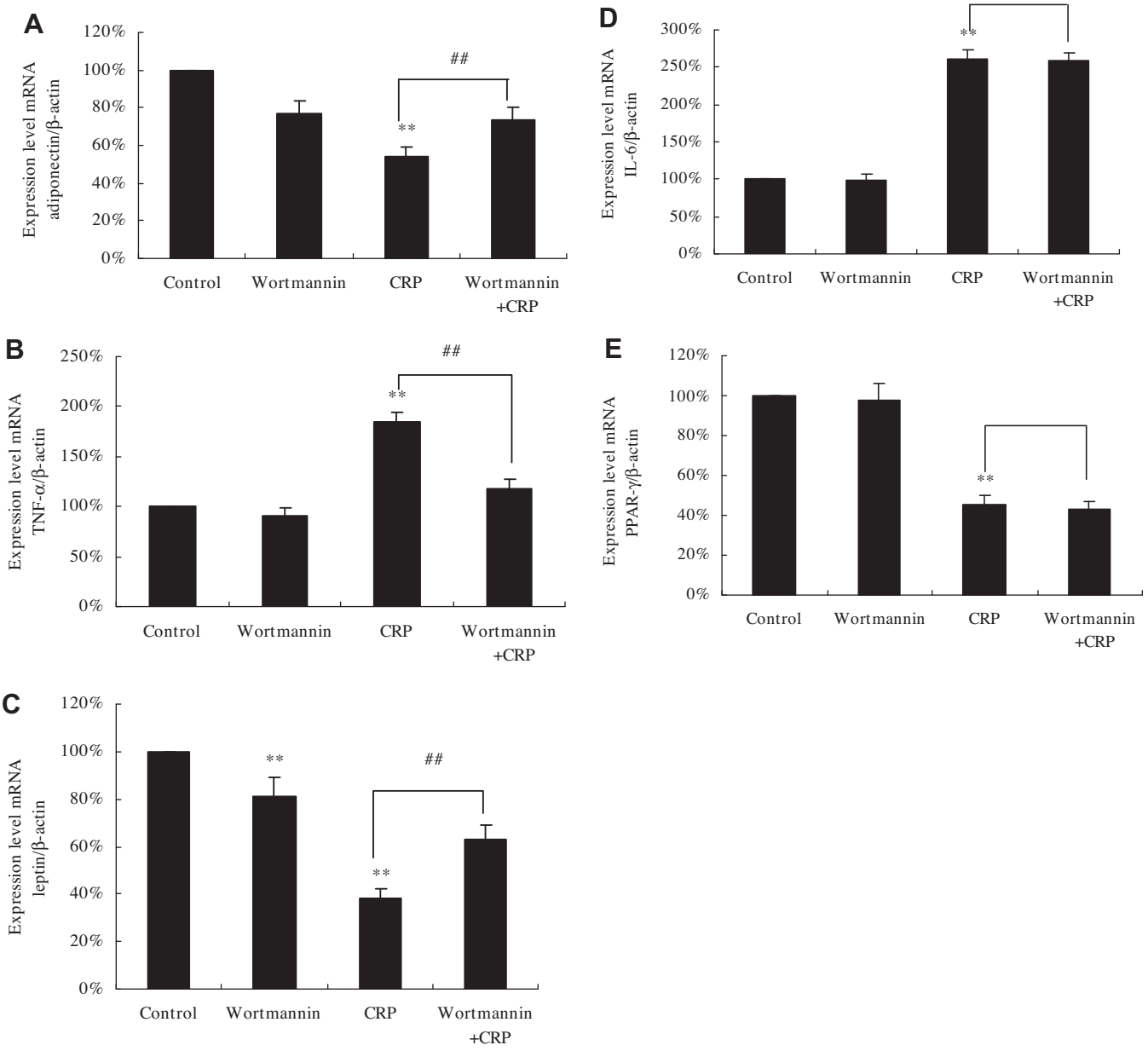

Fig. 4. Regulation of adiponectin, TNF-α and leptin mRNA expression by CRP is partially mediated via PI-3 kinase. After overnight serum-starvation, 3T3-L1 adipocytes were pretreated with specific pharmacological inhibitors for 1 h before CRP (50 μg/ml) was added for 24 h. Total RNA was extracted and subjected to a quantitative real-time PCR as described in Section 2. Adiponectin (A), TNF-α (B), leptin (C), IL-6 (D) and PPAR-γ (E) mRNA levels normalized to β-actin expression were determined relative to untreated control cells (100%). Data represent means ± SEM of three independent experiments. **Denotes $p < 0.01$ comparing CRP-treated and wortmannin-treated with non-treated adipocytes, and ##denotes $p < 0.01$ comparing CRP-treated with wortmannin + CRP-treated cells.

PPAR-γ gene expression, and the inhibition of PPAR-γ might mediate the effects of CRP on adipokines genes expression.

Recently, the major steps in CRP signaling have been elucidated [38]. 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)P_3, which promotes downstream signaling events, including phosphorylation of phospholipase Cγ2 (PL Cγ2) which produces (a) DAG which activates phosphokinase C which activates p38 transcription factor, (b) calcium mobilization through IP_3, (2) activation of Raf which binds Ras, phosphorylates MEK which in turn phosphorylates ERK. Since pharmacological inhibition of PI-3 kinase by wortmannin partly rebacked adiponectin, TNF-α and leptin genes expression by CRP in out study, PI-3 kinase is probably involved in adiponectin, TNF-α and leptin mRNA regulation by CRP. However, in the present study, pharmacological inhibition of PI-3 kinase by wortmannin did not reverse the effects of CRP on IL-6 and PPAR-γ genes expression. Recent studies have shown that there are interrelations among adipokines. Each having an impact on the expression of the other [39]. Adiponectin reduces LPS-mediated increase in mRNA IL-6 expression in pig adipocytes by attenuating NF-κB activation [40]. In another study, adiponectin shows similar effects on TNF-α and IL-6 expression in porcine macrophages [41]. Besides, extra cellular IL-6 counter-regulates adiponectin gene expression and secretion in 3T3-L1 adipocytes [42]. There is evidence that PPAR-γ expression is controlled by adiponectin and vice versa [40,43]. Therefore, the effect of CRP could also be due to disturbances in the expression of one of the adipokines, thus changing the expression of the others, as mentioned above.

Taken together, our result collectively suggests that CRP affects adiponectin, TNF-α, IL-6, leptin and PPAR-γ genes expression, and that might represent a mechanism by which CRP regulates the occurrence of insulin resistance, obesity and metabolic syndrome.

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