Interplay of pro- and anti-inflammatory cytokines to determine lipid accretion in adipocytes

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Abstract

Objective—Obesity is associated with an increase in various pro-inflammatory and anti-inflammatory cytokines, but the interplay of these cytokines is incompletely understood. We conducted experiments to test a broader hypothesis that a dynamic interplay of pro-inflammatory and anti-inflammatory cytokines controls lipid storage in adipocytes.

Design—Three experiments were designed to test the overall hypothesis that pro-inflammatory cytokine (e.g. TNF-α) inhibits anti-inflammatory cytokine (e.g. adiponectin) activity in an attempt to limit excess lipid accumulation in adipocytes.

Results—Experiment 1 showed that in pro-inflammatory animal models (ap2-P65, ob/ob and high fat diet-induced obese mice), the increase in TNF-α expression was associated with a decrease in adiponectin expression. Experiment 2 showed that in 3T3-L1 adipocytes, TNF-α significantly reduced lipid accumulation and glucose uptake induced by adiponectin, and increased lipolysis. Experiment 3 showed that in 3T3-L1 adipocytes, TNF-α reduced mRNA and protein expression of adiponectin. Adiponectin gene transcription and mRNA stability were both reduced by TNF-α. The expression of PPAR-γ, an activator of adiponectin gene promoter, was reduced by TNF-α. The inhibitory activity of TNF-α was blocked by chemical inhibitors of NF-κB and super suppressor IκBα (ssIκBα).

Conclusion—TNF-α opposes the action of adiponectin in the regulation of lipid metabolism, and inhibits adiponectin expression at transcriptional and post-transcriptional levels. The results suggest that pro-inflammatory cytokine inhibit anti-inflammatory cytokine in adipocytes to reduce lipid storage. This suggests a potential role of anti-inflammatory cytokines in the control of adipose tissue expansion.

Keywords

Adiponectin; mRNA stability; transcription; TNF-α; NF-κB; PPARγ

INTRODUCTION

Evidence suggests that the physiology of human body evolved to maintain adipose tissue levels within a range that is optimum for survival and reproduction. Too low as well as excessive adipose tissue content are associated with impaired reproductive potential for women [1-3] and men[4, 5] and survivability of progeny [6]. Extremes of adiposity are also associated with greater comorbidities, which may influence lifespan. In addition, adipose tissue and its products play an important role in the control of immune function [7]. These suggest that maintenance of optimum adiposity for energy store would be a high priority for body. Not surprisingly, adipose tissue products like adipokines appear to be engaged in maintenance of optimal adiposity as well as fertility [8].

The white adipose tissue as a primary energy store is regulated by pro-inflammatory and anti-inflammatory cytokines. Many pro-inflammatory cytokines (such as TNF-α and IL-1) inhibit adipogenesis and promote lipolysis. In contrast, anti-inflammatory cytokines (such as adiponectin) stimulate adipogenesis and lipid accumulation. We postulate that interplay between pro-inflammatory and anti-inflammatory cytokines is employed in the cellular mechanism to counter extremes of adiposity. Lower levels of adiposity is associated with preponderance of anti-inflammatory cytokines and less expression of pro-inflammatory cytokines [9, 10], which is in favor of fat accretion. Conversely, obesity is characterized by a state of chronic low-grade inflammation with pro-inflammatory cytokine elevation and anti-inflammatory cytokine reduction in circulation and adipose tissue [11, 12], which may collectively discourage further fat accumulation. This Yin and Yang effect of cytokines on adiposity may be illustrated with interaction of TNF-α and adiponectin, as representatives of the pro-inflammatory and anti-inflammatory cytokines, respectively.

Adiponectin is an example of anti-inflammatory adipokine expressed in mature adipocytes and is the most abundantly secreted protein from adipose tissue [13]. Adiponectin inhibits lipolysis in adipocytes [14], stimulates fatty acid oxidation in muscle cells, suppresses gluconeogenesis in hepatocytes, increases systemic insulin sensitivity, protects body from chronic inflammation and regulates food intake and body weight [15]. Although adipose tissue secretes adiponectin, its levels are reduced with excess adiposity, and increased with weight loss. The reduction of adiponectin is also associated with a suppression of insulin actions in the liver and muscle, known as insulin resistance in obesity.

On the other hand, TNF-α is a pro-inflammatory cytokine whose expression and circulating levels are increased with obesity and decreased with weight loss in obese mice [16, 17]. TNF-α

has numerous effects in adipose tissue, including the induction of apoptosis and lipolysis, inhibition of adipogenesis and insulin signaling [18, 19]. TNF-α triggers a signaling cascade (IKKβ/NF-kB) to regulates adipose tissue mass [18, 20]. In the control of adipogenesis, TNF-α regulates key transcription factors, such as peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT enhancer-binding protein-α (C/EBPα) [21]. An increase in TNF-α promotes the expression of other pro-inflammatory cytokines and reduces expression of anti-inflammatory cytokines, resulting in an overall pro-inflammatory state [22]. Thus, it can be conjectured that the preponderance of adiponectin and reduction of TNF-α activity may promote lipid accumulation in favor of adiposity. Whereas, an increase in TNF-α and a reduction in adiponectin will limit adipocyte expansion in favor of adipose tissue reduction.

The aim of this study was to use TNF-α and adiponectin as representatives to test the hypothesis that pro-inflammatory cytokines attempt to limit excess adipocyte expansion by reducing anti-inflammatory cytokine activity, which favors induction of energy expenditure in obesity. Specifically, we investigated TNF-regulation of adiponectin activity and expression in adipocytes.

METHODS

Each experiment is described in detail below, followed by detailed protocols in the Techniques and Assays (T&A) section.

Experiment 1: TNF-α and adiponectin mRNA expression in adipose tissue of pro-inflammatory mouse models

Male ob/ob mice (B6.V-Lepob/J, stock no. 000632), male C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The male C57BL/6 mice were fed a high-fat diet (HFD, 58% kcal from fat, D12331, Research Diets, New Brunswick, NJ) at 5 wk of age for diet-induced obesity (DIO). aP2-p65 transgenic mice were generated in a previous study [20]. The ob/ob mice and aP2-p65 mice were maintained on normal chow diet (12.8% kcal from fat). All of the mice were group housed with free access to water and diet in the animal facility at the Pennington Biomedical Research Center, which is with a 12:12-h light-dark cycle and constant temperature (22–24°C). All procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the Pennington Biomedical Research Center. At sacrifice, total RNA were isolated from mouse white adipose tissue samples as previously described [23], to determine TNF-α and adiponectin mRNA levels by quantitative real time PCR as described in T&A section.

Experiment 2: TNF-α inhibition of adiponectin metabolic activities in adipocytes

TNF-α was tested in the inhibition of adiponectin activities by examining lipid accumulation and glucose uptake in 3T3-L1 cells. The cells and differentiation were described elsewhere [18]. Forty-eight hours after initiating adipogenic induction, the cells were treated with TNF-α (Sigma#T6674) (20 ng/ml) and/or adiponectin (Sigma#SRP4902) (20 ng/ml or 100 ng/ml) for 7 days. Media containing TNF-α and adiponectin was refreshed every two days. Prior to changing media, old media was collected to determine glycerol output during

differentiation. At the end of 7 days, cells were fixed for Oil red O (ORO) staining to determine lipid accumulation. Cells were also processed for glucose uptake determination and collected for protein expression analysis of adipogenic and lipolytic genes by Western blotting (WB). All assays were performed with a minimum of three biological replicates. For all metabolic assays, 8-12 biological replicates were used, and normalized to protein content.

Experiment 3: Regulation of adiponectin expression by TNF-α

The luciferase reporter driven by adiponectin gene promoter (–1,300/+18) was kindly provided by Dr. Jianhua Shao (Graduate Center for Nutritional Sciences, University of Kentucky, Lexington, KY). The PPRE luciferase reporter was described elsewhere [18]. The super suppressor IκBα (ssIκBα) expression vector was originally described elsewhere [18]. For TNF-α and troglitazone treatment, the cells were kept in serum-free medium for 16 h and treated with TNF-α or troglitazone for 24 h before reporter assay. HEK 293 cells, 3T3-L1 fibroblasts transient transfection was conducted in triplicate in 12-well plates. The total DNA concentration was corrected in each well with a control plasmid, and the reporter activity was normalized by SV40-Renilla luciferase. Each experiment was repeated at least three times and triplicates were used at each point.

To determine signaling pathways of TNF-α, cells were also exposed to chemical inhibitor for JNK (SP600125, EI-305; Biomol, Plymouth Meeting, PA), the inhibitors for MEK (PD098059, p-215, Sigma, St. Louis, MO), and p38 (SB203580, s-8307, Sigma, St. Louis, MO), or IKK (15d-PGJ2, 538927, Calbiochem, San Diego, CA). Cells were pretreated with the inhibitor for 30-60 minutes and then followed by TNF-α treatment.

ssIκBα 3T3-L1 cell line is derived from 3T3-L1 cell line through stable transfection with a non-degradable mutant of NF-κB inhibitor IκBα (ssIκBα), in which S-A mutation was made at S32 and S36. This cell line was made and used in a previous study [18].

TECHNIQUES & ASSAYS

A. Western Blotting

Western blotting was conducted using a protocol described elsewhere [18]. Adiponectin antibody (MAB3832) was from Chemicon International and PPAR-γ (sc-7373x) antibody was purchased from the Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to tubulin (ab7291) and β-Actin (ab6276) were obtained from Abcam (Cambridge, UK). All of the experiments were conducted three or more times. The intensity of the western blotting signal was quantified using a computer program, PDQuest 7.1 (Bio-Rad).

B. Quantitative Real Time RT-PCR

RNA was isolated and purified using Trizol (T9424), chloroform (C2432), Isoproponal (I9516) from Sigma (St. Louis, MO). Tagman or cyber green methods were used in the quantitative real time RT-PCR to determine mRNA levels for TNF-α (Mm00443258_m1, Applied Biosystems), adiponectin (Mm00456425_m1, Applied Biosystems), PPAR-γ (Mm00440945-m1, Applied Biosystems), P65 (Mm00501346_m1, Applied Biosystems),

C/EBPα (5'-GCGAGCACGAGACGTCTATAGA-3';5'-GCCAGGAACTCGTCGTTGAA-3'), C/EBPβ (5'-AGCGGCTGCAGAAGAAGGT-3',5'-GGCAGCTGCTTGAACAAGTTC-3'), C/EBPδ (5'-CATCGACTTCAGCGCCTACAT-3', 5'-TGAAGAGGTCGGCGAAGAGT-3'). Mouse 18s was used as an internal control to normalize the mRNA levels in each sample. The reaction was conducted as described elsewhere [20]. The relative mRNA level was normalized over 18S ribosomal RNA gene.

C. Oil Red O Staining

At the end of 7 days post adipogenic induction, cells were fixed with 10% formalin for 1 hour and stained Oil red O dye (Fisher Biotech BP112-10) as described elsewhere [18]. Three biological replicates were analyzed for each treatment group and 4 technical replicates averaged for each biological replicate.

D. Glycerol output determination

The endogenous glycerol released in the media as a result of lipolysis was measured using a free glycerol determination kit (Sigma#FG0100) according to the manufacturer's instructions with some modifications.

E. Glucose uptake assay

Glucose uptake was conducted as described previously in detail[24, 25]. Briefly, cells in 6 well plates were serum starved for 2 hours, and then washed 2X with PBS before adding 900 μl KRP (136 mM NaCl, 4.7 mM KCl, 10 mM $NaPO_4$, 0.9 mM $CaCl_2$, 0.9 mM $MgSO_4$) for 15 minutes. One well was treated with 100 nM cytochalasin B (Sigma Aldrich, #C6762) for subtraction of nonspecific glucose uptake. 100 μl of 10X isotope solution was then added to each well for a final concentration of 100 nM cold 2-deoxy glucose and 0.5 μCi/ml [26]-2-Deoxyglucose (PerkinElmer #NEC720A250UC) for 5 minutes. Immediately following the incubation, cells were washed in ice-cold PBS. 1 ml of 0.05% SDS was then added to each well, after incubating for 30 min at 37°C, 900 μL of cell lysate was added to a scintillation vial by combining 4 wells and the remaining 50 μl was used for protein determination via BCA (bicinchoninic acid) assay. The scintillation values were normalized to protein content of each well.

F. Adiponectin ELISA

Mouse adiponectin ELISA kit (EZMADP-60K) from EMD Millipore (Billerica, Massachusetts 01821, USA) was used to detect adiponectin in the supernatant of 3T3-L1 adipocytes. The results were normalized to protein content in the supernatant.

Statistical Analysis—The data are presented as a mean ± SEM. The data were analyzed using two directional Student's t test with statistical significance considered at $p < 0.05$.

RESULTS

Experiment 1: TNFα and adiponectin mRNA in mice adipose tissue are negatively correlated

To gain insight into the relationship between pro-inflammatory and anti-inflammatory cytokines in adipose tissue, we investigated TNF-α and adiponectin expression in three mouse models of chronic inflammation including aP2-p65 mice, DIO mice, and ob/ob mice. aP2-p65 mouse is a transgenic mouse in which NF-kB subunit p65 (RelA) gene is overexpressed in adipose tissue under aP2-gene promoter [20]. The overexpression induced a high level of chronic inflammation in the adipose tissue of aP2-p65 mice compared to the wild type littermate. As shown in Figure 1, TNF-α mRNA was increased in the adipose tissue of transgenic mice (Fig. 1A). In the metabolic inflammatory models, the increase was also observed in the DIO mice and ob/ob mice relative to their lean controls (Fig. 1A). While, adiponectin mRNA was significantly reduced in the adipose tissue in all of those models (Fig. 1B). The results consistently suggest that TNF-α elevation is associated with adiponectin reduction in adipose tissue. The in vivo data provide physiological significance to the study of adiponectin regulation by TNF-α.

Experiment 2: TNF-α and adiponectin have opposing effects in 3T3-L1 adipocytes

We used adipogenesis to determine the interplay of TNF-α and adiponectin in regulation of lipid accumulation. 3T3-L1 preadipocytes were treated with TNF-α (20 ng/ml) during induction of adipogenesis for 7 days. Adipogenesis was measured by lipid accumulation in cells using oil red O (ORO) staining. TNF-α treatment blocked the cell differentiation (Fig. 2A) and resulted in significant reduction in lipid accumulation in these cells (p<0.001). An increase in lipolysis often leads to reduction in lipid accumulation in adipocyte. Glycerol concentration was measured in the cell culture supernatant and normalized to the cellular lipid content to determine the lipolysis activity in the cells. In TNF-treated cells, we observed increased glycerol content in the media (Fig. 2B), suggesting that lipolysis is induced by TNF-α to contribute to the inhibition of adipogenesis. TNF-α treatment also reduced glucose uptake in these cells (p=0.07; Fig. 2 C).

In this adipogenesis system, the anti-inflammatory cytokine adiponectin was tested in the regulation of lipid accumulation. Adiponectin treatment rescued the cells from TNF-induced block in adipogenesis and lipid accumulation. Adiponectin (20 ng/ml) significantly increased lipid content in the TNF-treated cells (Fig. 2A). Adiponectin treatment also reduced lipolysis (Fig. 2B) and significantly increased glucose uptake in the TNF-treated cells (Fig. 2C). In the presence of TNF-α, adiponectin dose-dependently increased lipid accumulation (p=0.05 and 0.01) (Fig. 2D). These data suggest that TNF-α and adiponectin antagonize each other in the regulation of lipid and glucose metabolism in adipocytes.

Experiment 3: Regulation of adiponectin expression by TNF-α

TNF-α inhibits adiponectin gene expression in mature 3T3-L1 adipocytes—To understand the mechanism of adiponectin suppression by TNF-α, we used the cellular model system to study adiponectin expression. Adiponectin mRNA was reduced by TNF-α (20 ng/ml) in the 3T3-L1 adipocytes (Fig. 3A). At the four time points with TNF-treatment, the

adiponectin mRNA was reduced by 50% (p<0.05) at 4 h, and the reduction was maintained at all of other time points thereafter (8, 16 and 24 h). Adiponectin protein was determined with ELISA in the supernatant and a reduction was observed at 8 h and thereafter in the presence of TNF-α (Fig. 3B, p<0.05). These data suggest that adiponectin expression is suppressed by TNF-α in 3T3-L1 adipocytes at mRNA and protein levels. The protein reduction is about 4 h late relative to the mRNA change.

TNF-α inhibits the adiponectin transcription and mRNA stability—Since both transcriptional and post-transcriptional events are able to influence mRNA level, we would like to determine which event is more important in the mechanism of adiponectin inhibition by TNF-α. We examined adiponectin gene promoter activity using a luciferase reporter, which contains -2.0 kb human adiponectin promoter. In the transient transfection, the reporter activity was enhanced 4.5 fold by troglitazone in 3T3-L1 adipocytes (Fig. 4A). The induction was completely blocked by TNF-α (20 ng/ml) (Fig. 4A). The data suggest that TNF-α inhibits the adiponectin promoter activity. For this inhibition, TNFα probably targets PPARγ.

To determine whether the posttranscriptional events are involved in adiponectin inhibition by TNF-α, we examined the stability of adiponectin mRNA. In the study, mRNA synthesis was blocked by actinomycin D (5 μg/ml) and the remaining adiponectin mRNA was measured at multiple time points (4, 8, 12 and 16 h) to determine the mRNA stability, which is indicated by the rate of mRNA reduction. The adiponectin mRNA had a long half-life over 16 h in the absence of TNF-treatment (Fig. 4B). The mRNA was reduced by less than 10% at 16 h, suggesting a long half-life (>16 h) of adiponectin mRNA. In the presence of TNF-α, the mRNA degradation was accelerated at all of the time points, and the mRNA half-life was reduced to 8 h (Fig. 4B). At 16 h, the mRNA was reduced by 70% (Fig. 4B). The data suggests that TNF-α induces degradation in adiponectin mRNA and shortens the mRNA stability. These results provide a new mechanism for adiponectin inhibition by TNF-α, which inhibits the adiponectin expression at both transcriptional and post-transcriptional levels.

TNF-α inhibits PPARγ gene expression through NF-κB pathway—PPARγ is an activator for adiponectin gene transcription, and likely a target of TNF-α in the inhibition of adiponectin expression. To test the possibility, we examined PPARγ expression in 3T3-L1 adipocytes after TNF-treatment. PPARγ mRNA was significantly decreased by TNF-α (20 ng/ml). The inhibition was observed at 4 h and maintained throughout the experiment of 24 h (Fig. 5A). PPARγ protein was also decreased by TNF-α (Fig. 5B). These data suggest that TNF-α suppresses PPARγ in mRNA and protein in adipocytes. In the study, we also examined the PPARγ mRNA stability. As shown in Figure 5C, the half-life of PPARγ mRNA was about 4 h and was not changed by TNF-α.

Several signaling pathways are activated by TNF-α, such as IKKβ/NF-κB pathway and MAPKs (ERK, JNK, and p38) pathways. To determine which pathway is required for the TNF-α activity, kinase-specific chemical inhibitors were used. These inhibitors include 15dPGJ2 (IKK2/IKKβ), SP600125 (JNK), SB203580 (p38), and PD98059 (MEK/ERK). 3T3-L1 adipocytes were pretreated with the inhibitors and then treated with TNF-α. IKKβ

inhibitor (15dPGJ2) was able to block the TNF-α activity (Fig. 5D). In addition to inhibition of IKKβ, 15dPGJ2 has an activity in the activation of PPARγ. It is possible that the effect of 15dPGJ2 is a result of PPARγ activation. To exclude this possibility, the experiment was repeated in ssIκBα adipocytes, in which IKKβ activity in the activation of NF-kB is blocked by supper suppressor IκBα [18]. In this cell line, the ability of TNF-α to inhibit PPARγ was blocked (Fig. 5E). The inhibitors to MAPKs were unable to block the TNF-activity (Fig. 5D). To ascertain the IKK/NF-κB activity in the regulation of PPARγ, we examined the PPARγ function using a luciferase reporter in 3T3-L1 cells. The reporter activity was induced by troglitazone (Fig. 5F). In the presence of TNF-α, the reporter response was reduced to 40% and the TNF-activity was completely blocked by the NF-kB inhibitor (ssIκBα) in a transient transfection. The data suggests that NF-κB mediates the TNF-α activity to inhibit PPARγ.

Adiponectin inhibition by IKK/NF-κB pathway—To determine the role of IKKβ/NF-κB pathway in the inhibition of adiponectin expression, we examined adiponectin mRNA in the same systems with NF-kB inhibition. The TNF-activity was blocked when IKK/NF-κB pathway, but not MAPK pathway was inhibited (Fig. 6A). The signaling pathways were suppressed by chemical inhibitors to IKK or MAPK (Fig. 6A). To ascertain the role of IKK/NF-κB pathway, we used the ssIκBα 3T3-L1 cells to determine the role of NF-kB in the adiponectin inhibition by TNF-α. Adiponectin was resistant to TNF-α treatment in ssIκBα 3T3-L1 adipocytes (Fig. 6B). Adiponectin was also examined during the adipogenesis of ssIκBα 3T3-L1 cells. Expression of adiponectin protein was completed blocked by TNF-α in the control cells, but not in ssIκBα 3T3-L1 cells during adipogenesis. These results suggest that IKKβ/NF-κB pathway is required by TNF-α to inhibit adiponectin gene expression.

Troglitazone antagonizes TNF-α activity by inhibition of NF-κB—We investigated the effect of troglitazone in antagonizing TNF-α activity. Troglitazone (3μM for 24 h) increased adiponectin expression in mRNA (Fig. 7A) and protein in the cell culture media (Fig. 7B). The troglitazone effect was in a dose-dependent manner at concentrations between 1-10 μM. The TNF-α activity was partly blocked by troglitazone (Fig. 7A). To determine whether troglitazone acts through inhibition of NF-κB activity, we examined NF-κB target genes (TNF-α and IκBα). Expression of IκBα and TNF-α was highly induced by NF-kB activation from TNF-treatment (Fig. 7, C and D). The induction was reduced by troglitazone. Troglitazone alone inhibited the basal level of gene expression as well. The data suggest that troglitazone antagonizes the TNF-α action through inhibition of NF-κB pathway.

DISCUSSION

In this study, we investigated the interplay of pro-inflammatory and anti-inflammatory cytokines in the regulation of adipocyte function. TNF-α and adiponectin were selected as representatives in each class of cytokines. We observed that in adipose tissue of mice with chronic inflammation, TNF-α induction was associated with adiponectin reduction in aP2-p65 mice, DIO mice and ob/ob mice. The results support the negative association of the expression of the two cytokine in human obesity [27]. The two cytokines, adiponectin and

TNF-α, exhibited opposite metabolic activities in the regulation of adipogenesis, lipolysis and glucose uptake in adipocytes, which is consistent with observations in other studies [18, 28]. Adiponectin increased lipid accumulation, reduces lipolysis and promotes insulin-induced glucose uptake. In contract, TNF-α decreased lipid accumulation, induced lipolysis, and attenuated insulin-induced glucose uptake. In addition, we observed that TNF-α repressed the adiponectin gene promoter activity, which is in line with observations in other reports [29, 30]. More importantly, we found that TNF-α promoted degradation of adiponectin mRNA, which was not reported in other studies [29, 30].

Inhibition of adiponectin expression by TNF-α was reported [31, 32]. However, post-transcriptional regulation of adiponectin mRNA was not examined in these studies. We observed that TNF-α inhibited adiponectin mRNA stability at the post-transcriptional level. Adiponectin mRNA exhibited a long half-life of more than 16 h. In response to TNF-α, the half-life was reduced to 8 h. The mRNA degradation was enhanced in response to TNF-α treatment. The observation provides a new mechanism for adiponectin regulation by inflammation. Adiponectin mRNA does not contain a classical AU-rich motif and poly (A) tail structure that are required for mRNA stability. Further studies are needed to explore the mechanism underlying the adiponectin mRNA stability.

At the transcriptional level, TNF-α inhibits adiponectin expression by targeting PPARγ, which is a transcriptional activator of adiponectin gene[33] and a master regulator of adipogenesis [35]. In this study, we observed that adiponectin gene promoter activity was enhanced by PPARγ ligand troglitazone, which is consistent with adiponectin mRNA expression in 3T3-L1 adipocytes in the response to troglitazone. The data suggests a critical role of PPARγ in the regulation of adiponectin transcription. A functional PPARγ response element (PPRE) was reported in the adiponectin gene promoter [33]. Our data suggests that PPRE is likely a target of TNF-α in the transcriptional suppression of adiponectin. In the mechanism, PPARγ expression was reduced and the mRNA stability was not altered by TNF-α. The PPARγ inhibition was dependent on activation of IKKβ/NF-kB pathway in our study. The NF-kB signaling pathway was reported to inhibit ligand-induced PPARγ activity in adipocytes through activation corepressor component histone deacetylase 3 [34].

Adiponectin may antagonize TNF-α activity by activating AMPK pathway. Adiponectin is able to attenuate the metabolic activities of TNF-α. The signaling pathway of adiponectin action was not investigated in this study. Adiponectin activates AMPK kinase through its cell membrane receptor [36]. This signaling pathway is likely involved in the inhibition of TNF-α activity. Specifically, AMPK inhibits mTOR/S6K pathway [37] that is required for activation of IKKβ/NF-kB signaling pathway by TNF-α [38]. Adiponectin was reported to inhibit NF-kB activity in endothelial cells [39]. Inhibition of IKKβ/NF-kB activity by adiponectin is a potential mechanism by which adiponectin antagonize the TNF-α activities.

Although cytokines are generally grouped as pro- or anti-inflammatory cytokines, the function and characteristics of individual cytokines may vary. Adiponectin expression pattern suggests that in general, preponderance of anti-inflammatory cytokines is dominant in adipose tissue at lower levels of adiposity. The biological significance is likely to promote energy accumulation and storage in adipose tissue. This point is supported by the phenotype

of adiponectin over-expressing mice [40], which exhibit super obesity without adipose chronic inflammation. Pro-inflammatory cytokines are increased in adipose tissue by obesity through a local hypoxia response [23]. The inflammation reflects a compensatory response in an effort to limit energy storage and adipose tissue expansion in obesity to maintain body fat within an optimal range [41]. Pro-inflammatory cytokine deficiency impairs this feedback response and leads to adulthood obesity. Phenotypes of many transgenic models suggest that when pro-inflammation is reduced by gene knockout, energy accumulation is enhanced in adipose tissue and the risk for obesity is increased. The phenotype has been reported in mice with global knockout for IL-1β [42, 43], IL-6 [44], and IL-18 [45]. In contract, an elevation in pro-inflammation in adipose tissue prevents energy accumulation and blocks diet-induced adipose tissue expansion. The conclusion is supported by the phenotypes of aP2-p65 mice or aP2-IKKβ mice [20, 34, 46], in which NF-κB-induced chronic inflammation represses adipocyte differentiation and adipose tissue expansion. The mice are resistant to diet-induced obesity and insulin resistance [20, 46]. These studies suggest that the interplay of pro-inflammatory and anti-inflammatory cytokines is important in the control of energy homeostasis and adipose tissue size.

In summary, we used TNF-α and adiponectin to demonstrate an interplay of pro-inflammatory and anti-inflammatory cytokines in the regulation of adipocyte activities. The result suggests that the interplay is important in the control of lipid accumulation in adipocytes. This study provides a new support to our hypothesis that in obesity, pro-inflammatory response serves as a feedback control of energy storage in adipose tissue [41, 47]. It opposes lipid accumulation by stimulating lipolysis and inhibiting adipogenesis as well as adiponectin expression. The chronic inflammation may represent a body's attempt to stimulate energy expenditure in the control of adiposity. As a result, inhibition of the inflammatory response by anti-inflammation agents may not yield a strong beneficial effect in the control of obesity and obesity-associated disorders. Although nearly two dozens of clinical trials have been conducted to test anti-inflammatory agents in the control of insulin resistance in type 2 diabetes patients, the results are not promising as most studies failed to yield the beneficial effects at the magnitude expected [48]. A better understanding of the physiological interplay of pro-inflammatory and anti-inflammatory cytokines may provide an explanation to the limited efficacy of anti-inflammatory therapies.

Acknowledgments

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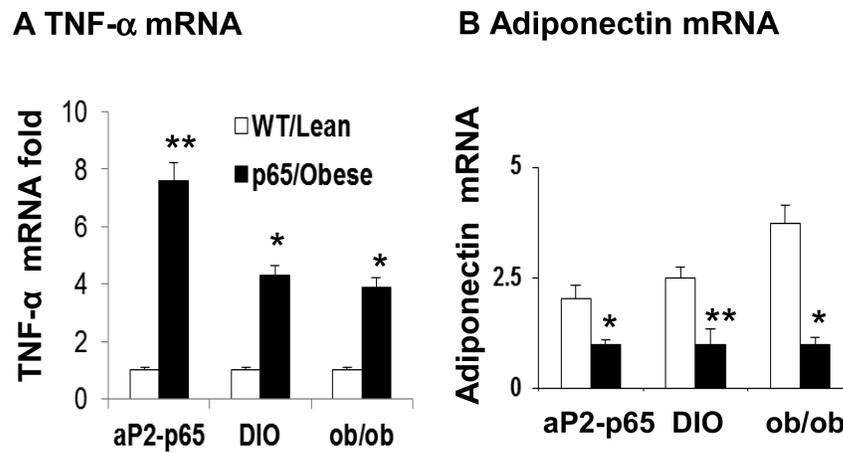
A TNF-α mRNA

B Adiponectin mRNA

Figure 1. Adiponectin and TNF-α expression in white adipose tissue
Epididymal fat tissues were collected at the age of 20 weeks from aP2-P65 mice, DIO mice and ob/ob mice. mRNA was determined in qRT-PCR. (A) TNF-α mRNA. (B) Adiponectin mRNA.
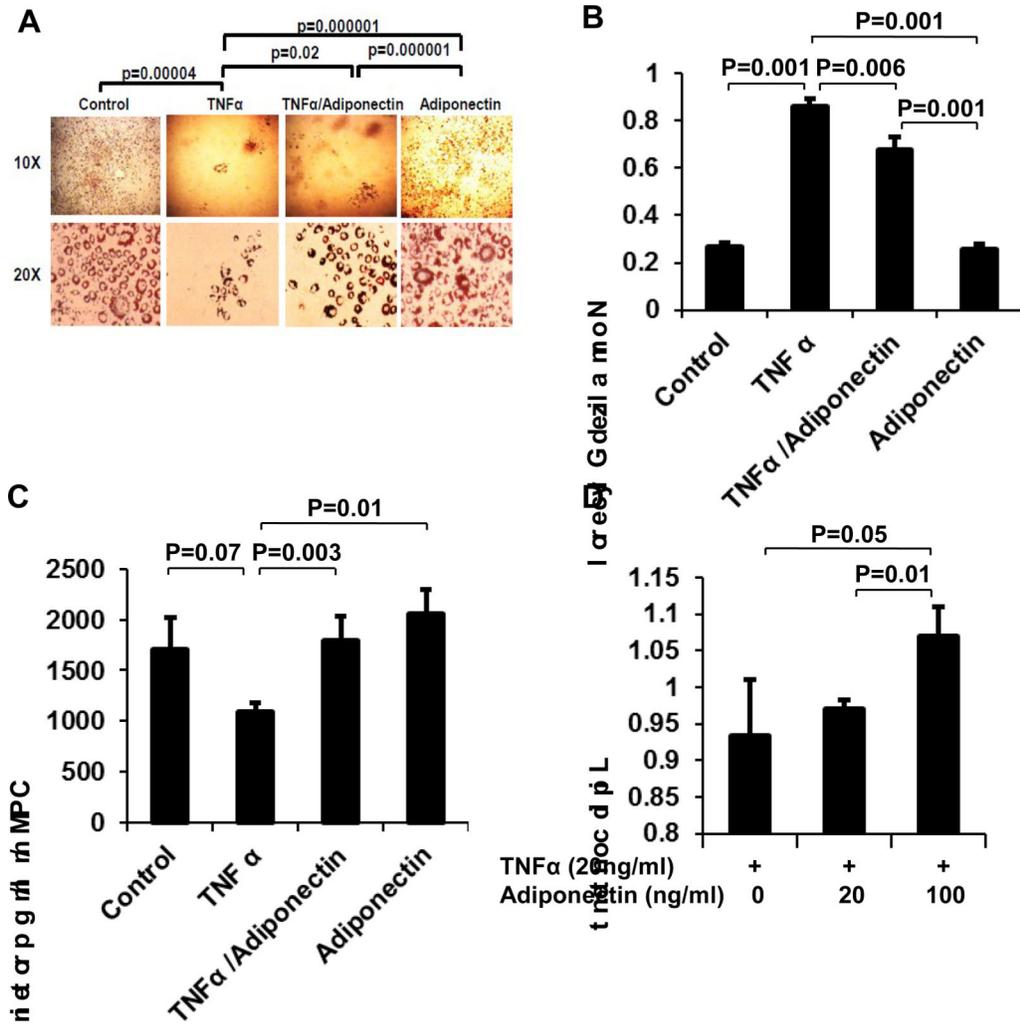
Figure 2. Dynamic balance between TNF-α and Adiponectin on adipogenesis
3T3-L1 cells were treated with TNF-α (20 ng/ml) and/or adiponectin after adipogenic induction for 7 days. TNF-α and adiponectin showed opposite activities in following aspects. (A) Adipocyte differentiation and lipid accumulation. Lipid is stained by oil red O. (B) Glycerol output by adipocytes. Glycerol was normalized by lipid content in cells. (C) Glucose uptake. Insulin-induced glucose uptake was measured in differentiated 3T3-L1 adipocytes with radiolabeled glucose. (D) Lipid content by oil red O staining and quantification. Adiponectin exhibited dose-dependent activity in antagonizing TNF-activity for lipid accumulation. The results are presented as mean ± SEM.
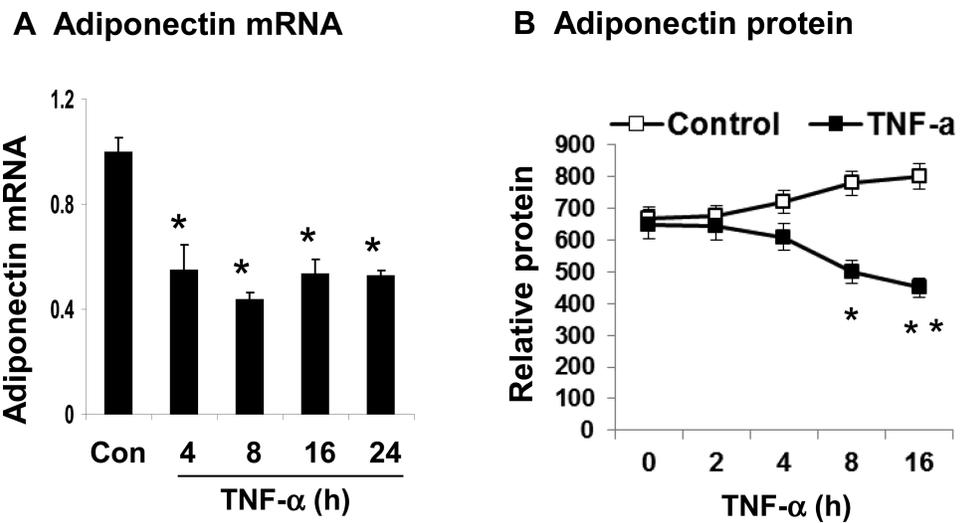
A Adiponectin mRNA

B Adiponectin protein

Figure 3. Inhibition of adiponectin expression by TNF-α in 3T3-L1 adipocytes
(A) Inhibition of adiponectin mRNA by TNF-α. The cells were serum-starved in DMEM containing 0.25% BSA overnight and then treated with TNF-α (20 ng/ml) for different times as indicated. The total RNA was extracted and subjected to qRT-PCR analysis for adiponectin mRNA. (B) Inhibition of adiponectin secretion by TNF-α. The cells were serum-starved overnight and treated with TNF-α for different times as indicated. Adiponectin in the culture medium of 3T3-L1 adipocytes was quantified by ELISA and the result was normalized with the supernatant protein content. In this figure, each bar represents mean ± SEM (n=3). *, P< 0.05. **, P<0.001.

A Adiponectin reporter

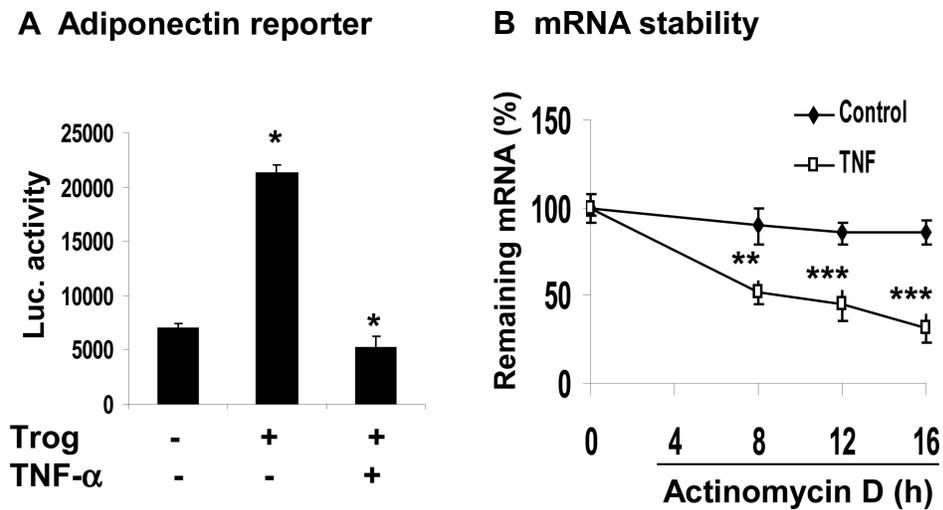| Trog | - | + | + |
| TNF-α | - | - | + |

B mRNA stability

Figure 4. Adiponectin transcription and mRNA stability
(A) Inhibition of adiponectin gene promoter by TNF-α. The transcriptional activity of mouse adiponectin promoter (–1,300/+18) was analyzed in 3T3-L1 adipocytes in a transient transfection. After 24 h transfection, the cells were serum-starved overnight and treated with troglitazone (10 μM) and TNF-α (20 ng/ml) for 16 h. (B) Influence of TNF-α on the adiponectin mRNA stability. De novo mRNA transcription was inhibited by actinomycin D (5 μg/ml) in 3T3-L1 adipocytes. The residual mRNA of adiponectin was quantified by qRT-PCR. Values represent percentage of residual mRNA versus mRNA level at time 0. Results are means ± SEM (n=3). *, P< 0.05. ***, P< 0.001.
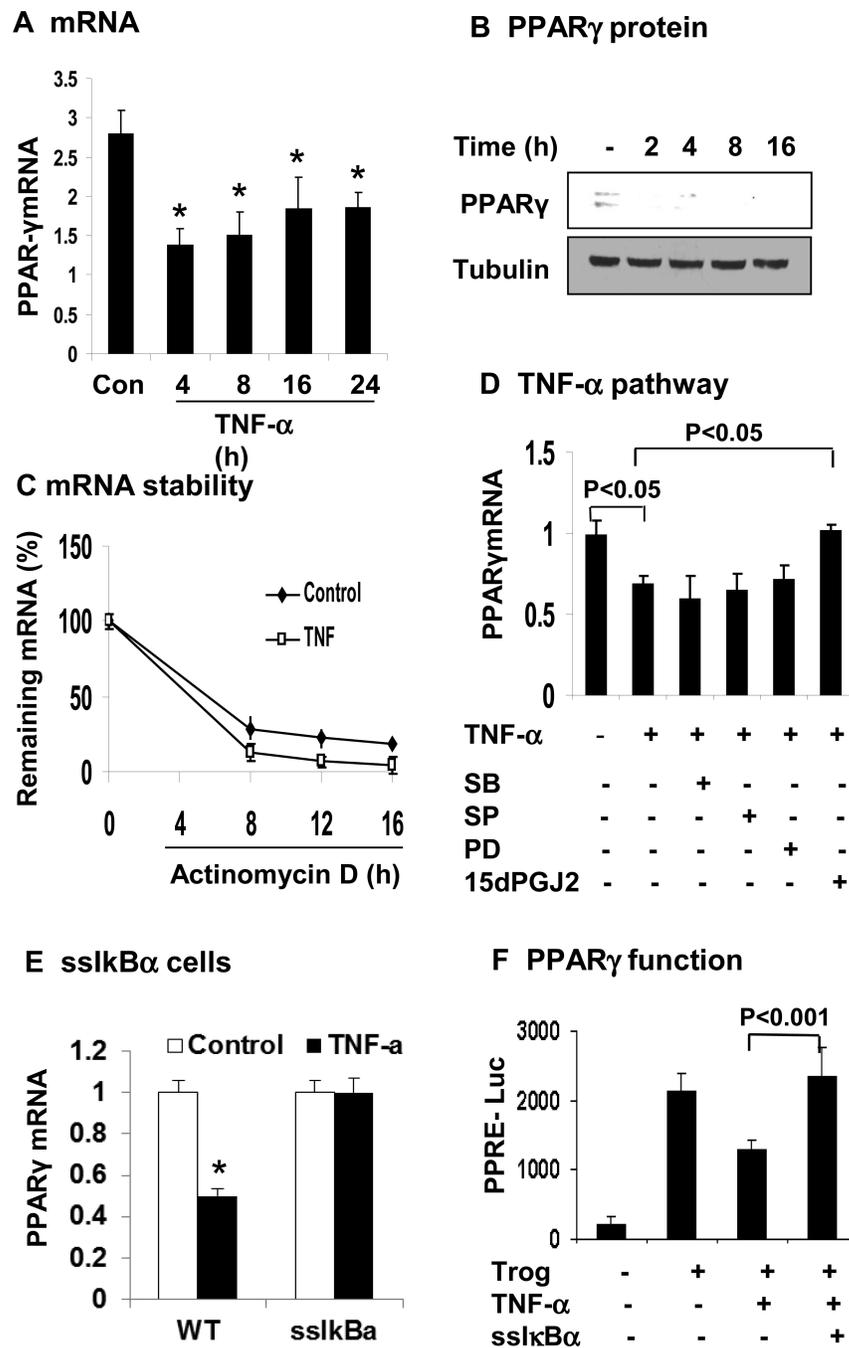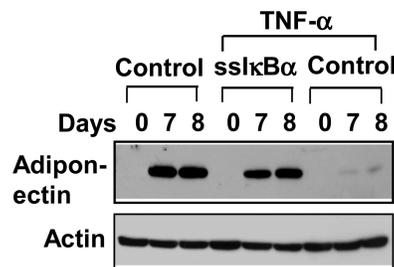
Figure 5. TNF-α inhibits PPARγ expression through NF-κB pathway
(A) Inhibition of PPARγ mRNA expression by TNF-α in 3T3-L1 adipocytes. The cells were serum-starved overnight and treated with TNF-α (20 ng/ml) for hours as indicated. PPARγ mRNA was determined in qRT-PCR. (B) Inhibition of PPARγ protein expression by TNF-α. PPARγ protein was determined in the whole cell lysate in a Western blot. (C) PPARγ mRNA stability. De novo mRNA transcription was inhibited by the addition of actinomycin D (5 μg/ml). The residual mRNA of PPARγ was quantified by qRT-PCR. Values represent percentage of residual mRNA versus mRNA level at time 0. (D) Blocking of TNF-α activity

by the NF-κB inhibitor. The 3T3-L1 adipocytes were pretreated with the pharmacological inhibitors for 30 min before addition of TNF-α. The inhibitors are SB203580 (SB, 10 μM), SP600125 (SP, 25 μM), PD98059 (PD, 40 μM), and 15dPGJ2 (15 μM). mRNA was determined in the cells after 16 h. (E) PPARγ mRNA was quantified in ssIκBα 3T3-L1 adipocytes after TNF-α treatment. (F) The transcriptional activity of PPARγ was analyzed in 3T3-L1 fibroblasts using the PPRE (3X)-luciferase reporter system in the transient transfection. Expression vector of ssIκBα was co-transfected to block NF-κB activation. At 24 h after transfection, the cells were serum-starved overnight and treated with troglitazone (10 μM) and TNF-α (20 ng/ml). In this figure, each bar represents mean ± SEM (n=3). *, P< 0.05.

A TNF-α pathway

TNF-α	-	+	+	+	+	+
SB	-	-	+	-	-	-
SP	-	-	-	+	-	-
PD	-	-	-	-	+	-
15dPGJ2	-	-	-	-	-	+
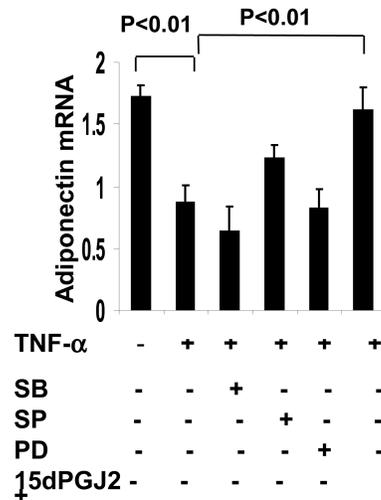
B ssIkBα blocks TNF-effect
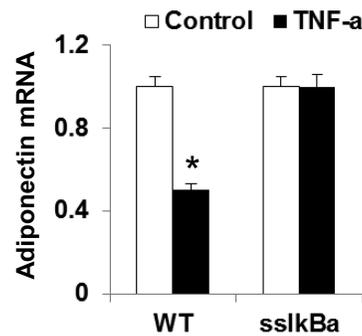
C 3T3- ssIκBα cells

Figure 6. IKK/NF-κB pathway in adiponectin inhibition

(A) Blocking of TNF-α activity by the NF-κB inhibitor. 3T3-L1 adipocytes were pretreated with the pharmacological inhibitors for 30 min before addition of TNF-α. The inhibitors are SB203580 (SB, 10 μM), SP600125 (SP, 25 μM), PD98059 (PD, 40 μM), and 15dPGJ2 (15 μM). mRNA was determined in the cells 16 h later. (B) Inhibition of TNF-α activity in 3T3-L1 ssIκBα stable cells. Wild type and ssIκBα 3T3-L1 adipocytes were serum starved overnight and treated with TNF-α (20 ng/ml) for 16 h. Adiponectin mRNA was determined by qRT-PCR. (C) Adiponectin expression during adipogenesis. The wild type and ssIαBa

3T3-L1 cells were treated with TNF-α (20 ng/ml) during adipogenesis. Adiponectin was determined in the whole cell lysate at days 7 and 8 of differentiation. In this figure, each bar represents mean ± SEM (n=3).

A Adiponectin mRNA

| | | P<0.05 | | | | |
| | P<0.05 | | | | P<0.05 | |

Trog (µM)   -   1   3   10   -   10
TNF-α   -   -   -   -   +   +

B Adiponectin protein

Trog (µM)   -   1   3   10   10   -
TNF-α   -   -   -   -   +   +

C IκBα mRNA

Trog (µM)   -   1   3   10   -   10
TNF-α   -   -   -   -   +   +

D TNF-α mRNA

Trog (µM)   -   1   3   10   -   10
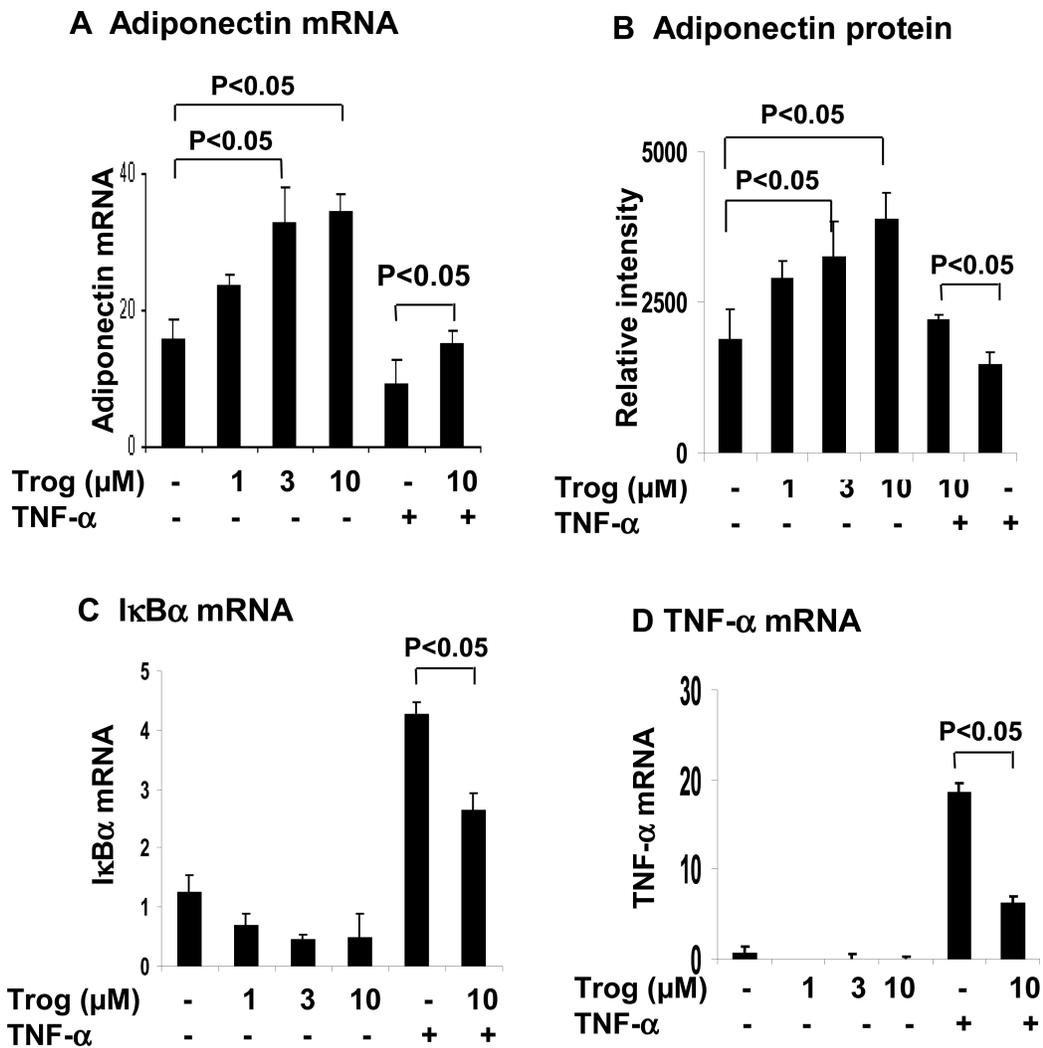TNF-α   -   -   -   -   +   +

Figure 7. Troglitazone increases adiponectin mRNA and protein through antagonizing NF-κB pathway

(A) and (B) Regulation of adiponectin expression by troglitazone in 3T3-L1 adipocytes. After serum-starved for overnight, the cells were treated with troglitazone (0, 1, 3, 10 µM) and TNF-α (20 ng/ml) for 24 h. Adiponectin mRNA was determined in qRT-PCR. The amount of adiponectin secreted into the medium was measured in Western blot. The adiponectin protein signal was determined by densitometry. (C) and (D) NF-κB target genes (IκBα, TNF-α) were measured after troglitazone and TNF-α treatment. mRNA was quantified in qRT-PCR. In this figure, each bar represents mean ± SEM (n=3). *, $P<0.05$.