GO6976 prevents TNF-α-induced suppression of adiponectin expression in 3T3-L1 adipocytes: Putative involvement of protein kinase C

Joong-Yeon Lim, Won Ho Kim, Sang Ick Park*

Division of Intractable Diseases, Center for Biomedical Sciences, National Institute of Health, 194 Tongillo, Eunpyeong-gu, Seoul 122-701, Republic of Korea

Received 10 June 2008; revised 19 August 2008; accepted 9 September 2008

Available online 17 September 2008

Edited by Laszlo Nagy

Abstract Adiponectin, one of the adipokines secreted by adipocytes, possesses insulin sensitizing and anti-atherosclerotic properties. Tumor necrosis factor-alpha (TNF-α) is known to suppress the expression and secretion of adiponectin in adipocytes; however, the underlying mechanism remains poorly understood. Here, we demonstrate that GO6976 (a selective inhibitor of conventional protein kinase C (PKC)) prevents TNF-α-induced suppression of adiponectin secretion and expression in fully differentiated 3T3-L1 adipocytes, accompanied by attenuation of c-Jun N-terminal kinase (JNK) activation. Additionally, the transcriptional activity of peroxisome proliferator-activated receptor-gamma (PPARγ) (a strong inducer of adiponectin) on the adiponectin promoter was inhibited in a PKC isomorph-specific manner. These results raise the possibility that PKC is involved in TNF-α-induced suppression of adiponectin in 3T3-L1 adipocytes.

© 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Adipocyte; Adiponectin; Tumor necrosis factor-alpha; Protein kinase C

1. Introduction

Adiponectin, also known as Acrp30, AdipoQ, apM1, and GBP28, is a 30-kD plasma protein. Initially, adiponectin was known to be exclusively expressed and secreted in adipose tissues; however, it is recently detected in non-adipose tissues as well [1,2]. Plasma concentration and mRNA expression of adiponectin are decreased in human patients and animal models that exhibit insulin resistance, which strongly correlates with conditions of metabolic syndromes such as type-2 diabetes and obesity [3]. In contrast, tumor necrosis factor-alpha (TNF-α), another adipokine released by adipose tissues, are markedly elevated in obese or diabetic animals and humans [4], and directly contribute to the reduction of adiponectin in adipocytes [5]. In a mouse model, adiponectin deficiency was associated with severe diet-induced insulin resistance and elevated TNF-α levels [6]. Conversely, chronic injection of adiponectin into insulin-resistant animals enhanced insulin sensitivity by activating glucose uptake and fatty acid oxidation, implying that adiponectin has an insulin sensitizing effect [7]. The reciprocal relationship between decreased adiponectin and increased TNF-α levels in plasma was also reported in chronic alcohol-fed animals, in which adiponectin treatment attenuates alcohol-induced liver disease [8,9]. Thus, adiponectin and TNF-α are reciprocally expressed in adipocytes and have opposing effects on the regulation of insulin resistance.

Although many investigators have tried to elucidate the molecular basis of TNF-α-induced downregulation of adiponectin expression in adipocytes, the underlying signaling mechanisms are not fully understood. TNF-α suppresses the expression and transcriptional activity of adipogetic transcription factors such as peroxisome proliferator-activated receptor-gamma (PPARγ), CCAAT/enhancer binding protein (C/EBP), and sterol regulatory element binding protein-1c (SREBP-1c), which are strong transcriptional inducers of adiponectin [10–12]. In addition, TNF-α activates c-Jun N-terminal kinase (JNK) to suppress adiponectin secretion [13].

Protein kinase C (PKC) plays a role in several TNF-α-induced biological functions. PKC mediates TNF-α-dependent induction of plasminogen activator inhibitor-1 (PAI-1) in 3T3-L1 adipocytes [14]. PKC also suppresses obese gene expression and leptin secretion in adipocytes [15]. Pretreatment with PKC inhibitors such as bisindolylmaleimide I (BIM) or 1-[(5-isoxoquinolinesulfonyl)-2-methyl-piperazine dihydrochloride (H-7) restores adipogetic gene expression suppressed by TNF-α. These observations indicate that PKC may be an important signaling mediator in TNF-α-induced biological functions in adipocytes.

In this study, we show that GO6976, a selective PKC inhibitor, prevents TNF-α-induced suppression of adiponectin secretion and expression as well as the transcriptional activity of PPARγ on adiponectin promoter. Moreover, overexpression of PKCα and PKCβ inhibited PPARγ-induced adiponectin promoter activity. Collectively, PKC might be involved in TNF-α-induced suppression of adiponectin expression in fully differentiated 3T3-L1 adipocytes.

2. Materials and methods

2.1. Cell culture and adipocyte differentiation

3T3-L1 preadipocytes and HEK293 cells were maintained in Dulbecco’s modified essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and supplemented with 100 U/
ml penicillin and 100 μg/ml streptomycin (growth medium) in a 5% CO₂ atmosphere at 37 °C. To induce adipocyte differentiation, 3T3-L1 cells were cultured as previously described with minor modification [16]. Briefly, cells were maintained in growth medium until 2 days after confluence, when differentiation was induced by incubation in growth medium containing 0.1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 10 μg/ml insulin for 3 days. After further incubation for 4–5 days in growth medium containing only insulin, more than 90% of cells differentiated into adipocytes exhibiting triglyceride accumulation.

2.2. Materials
DMEM, FBS, penicillin, and streptomycin were purchased from GIBCO BRL (Rockville, MD). Dexamethasone, IBMX, and insulin were from Sigma-Aldrich (St. Louis, MO). All chemical inhibitors including PKC inhibitor GO6976 were obtained from Calbiochem (San Diego, CA). Anti-adiponectin and anti-phospho-JNK antibodies were purchased from abCam (Cambridge, UK). Anti-actin, anti-p38MAPK, anti-ATF2, anti-ERK, anti-phospho-ERK, anti-c-Jun, and anti-phospho-c-Jun antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p38MAPK and anti-phospho-ATF2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Recombinant mouse TNF-α was obtained from R&D (Minneapolis, MN).

2.3. Transfection and luciferase assay
PPRE-luc and PPARγ expression vectors were kindly provided by Dr. JaeHun Cheong (Pusan National University, Korea). An adiponectin promoter-luc vector containing the mouse adiponectin promoter spanning –1500 to +50 bp was obtained from Dr. Hyun Bae Kim (National Institute of Health, Korea). PKC expression vectors were kindly provided by Dr. Jae-Won Soh (Inha University, Korea). For the transactivation assay, the luciferase reporter plasmid was co-transfected with β-galactosidase using lipofectamine (Invitrogen) according to the manufacturer’s protocol. Twenty hours post transfection, cells were lysed with reporter lysis buffer (200 μl/well, Promega), and luciferase activity was measured using the luciferase reporter assay system (Promega). β-Galactosidase activity was used to normalize transfection efficiency.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) and semi-quantitative PCR
First strand cDNA synthesis was performed with 1 μg of total RNA extracted using Trizol (Invitrogen) and reverse-transcriptase MMLV (Invitrogen). The oligonucleotide primers for semi-quantitative PCR were as follows: mouse adiponectin, 5’-TGGATCTGACGACAAA-3’ (forward) and 5’-CGAATGGGTACATTGGGAAC-3’ (reverse); mouse PAI-1, 5’-ATGAGATCAGTACTGCGGATGCC-3’ (forward) and 5’-AGAGACGGTGCTGCCATCAG-3’ (reverse);

![Fig. 1. PKC inhibitor GO6976 alleviates TNF-α-induced suppression of adiponectin secretion in 3T3-L1 adipocytes. (A) Fully differentiated 3T3-L1 adipocytes were serum starved for 6 h and then pretreated with various protein kinase inhibitors (SP, 50 μM SP600125; SB, 20 μM SB202190; GO, 1 μM GO6976; U, 10 μM U0126) for 1 h prior to further 24 h incubation without (−) or with TNF-α (+, 10 ng/ml). Secreted adiponectin was detected by immunoblotting using 40 μl culture medium. (B) After serum starvation for 6 h, 3T3-L1 adipocytes were pretreated with different concentrations of GO6976 (0.01–2 μM) for 1 h prior to addition of TNF-α (10 ng/ml). The figures show representative results of three independent experiments and the graph represents mean value of quantifications by densitometric analysis. **P < 0.01, *P < 0.05, compared with TNF-α alone-treated cells.](https://example.com)
glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an internal control), 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse). PCR products were separated by electrophoresis through 1% agarose at 100 V for 20 min, and ethidium bromide stained bands were detected using a UV transilluminator.

2.5. Protein isolation and immunoblotting
Protein isolation and immunoblotting were performed as described previously [17]. Briefly, cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 2 mM EGTA) containing a protease inhibitor cocktail of 1 mM Na3VO4, 1 mM NaF, and 1 mM PMSF (Sigma). Thirty μl aliquots of whole cell extract were separated by SDS–PAGE and transferred to nitrocellulose membranes by electroblotting for 2 h at 100 V. For detection of secreted adiponectin, 40 μl of culture medium was used for electrophoresis. Membranes were blocked with 5% non-fat dry milk dissolved in Tris-buffered saline (10 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature. The blots were probed with primary antibody for 2 h at room temperature or overnight at 4°C, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive bands were detected using an enhanced chemiluminescence detection kit (Amersham Biosciences).

2.6. Statistical analysis
All values are reported as means ± standard deviation (S.D.). Statistical comparison between experimental groups was performed using Student's t-test. P-values < 0.05 were considered statistically significant.

3. Results

3.1. TNF-α-induced suppression of adiponectin expression and secretion is attenuated by PKC inhibitor GO6976
Fully differentiated 3T3-L1 adipocytes exhibited oil red-positive staining (data not shown) and abundantly secreted a 30-kD protein corresponding to adiponectin. Treatment with TNF-α for 24 h dramatically suppressed the secretion of adiponectin (Fig. 1A). Pretreatment with GO6976, a selective inhibitor of conventional PKC isoforms, alleviated the TNF-α-induced suppression of adiponectin secretion with a greater effect than JNK inhibitor (SP600125), p38MAPK inhibitor (SB202190), or ERK inhibitor (U0126). Furthermore, the inhibitory action of GO6976 was dose-dependent; little effect was observed at concentrations <1 μM, but adiponectin secretion was restored to 76% of untreated controls with 2 μM GO6976 (Fig. 1B). Treatment with inhibitors alone did not alter adiponectin secretion levels (data not shown).

To investigate whether the effect of GO6976 on adiponectin secretion is due to its expresional regulation, we examined adiponectin mRNA and protein levels depending on various treatments. In parallel with the secretion pattern of adiponectin, GO6976 restored the TNF-α-induced suppression of adiponectin expression at both mRNA and protein levels (Fig. 2A). Similar to GO6976, SP600125 also showed the same effect, suggesting an involvement of JNK signaling as previously reported [13]. Additionally, we observed dose-dependent effect of GO6976 on both adiponectin mRNA and protein expression, showing maximum effect at 2 μM (Fig. 2B and C). This result suggests that the effect of GO6976 on adiponectin secretion result from the expresional regulation of adiponectin. Different from adiponectin, plasminogen activator inhibitor-1 (PAI)-1 is induced by TNF-α via PKC signaling [14]. GO6976 suppressed TNF-α-induced PAI-1 expression at mRNA level (Fig. 2B), suggesting that 3T3-L1 adipocytes used in this experiment normally response to PKC inhibitor GO6976.

3.2. PKC is involved in TNF-α-induced JNK activation
Activation of JNK 1/2 plays an important role in TNF-α-induced downregulation of adiponectin in 3T3-L1 adipocytes [13]. To investigate whether PKC functions upstream of JNK, we examined the effect of GO6976 on TNF-α-induced
JNK phosphorylation. Exposure to TNF-α for 1 h induced a substantial increase in JNK1/2 phosphorylation, which was blocked by pretreatment with GO6976 (Fig. 3A and B). TNF-α also increased the phosphorylation of other MAPKs including ERK1/2 and p38MAPK, but these activations were rarely altered by GO6976. To further investigate whether pretreatment with GO6976 could affect the activation of JNK downstream signaling, we examined phosphorylation of c-Jun and ATF2. TNF-α enhanced the phosphorylation of c-Jun and ATF2, which was attenuated by pretreatment with GO6976 (Fig. 3C and D). These results suggest that PKC could play a role as an upstream signaling mediator in TNF-α-induced JNK activation.

### 3.3. PKC inhibits the transcriptional activity of PPARγ on adiponectin promoter

It is well known that PPARγ plays a pivotal role in the induction of adiponectin as a transcription factor and that TNF-α suppresses the expression of adipogenic genes by inhibiting the transcriptional activity of adipogenic transcription factors including PPARγ. In this regard, we examined whether PKC could mediate the transcriptional activity of adiponectin promoter. We observed that TNF-α suppressed the transcriptional activity of PPARγ on adiponectin promoter (−1.5 kb) in PPARγ-overexpressed cells, which was significantly restored by pretreatment with GO6976 (Fig. 4A). Furthermore, PPARγ-dependent transcriptional activity was inhibited in a PKC isoform-dependent manner. Overexpression of PKCα or PKCβ, but not PKCδ or PKCζ, commonly suppressed PPARγ-dependent activation of adiponectin promoter (Fig. 4B) and PPARE (PPARγ-responsive element) reporter (Fig. 4C).

Taken together, these results raise the possibility that PKC might be involved in TNF-α-induced downregulation of adiponectin, at least in part, through inhibition of transcriptional activity of PPARγ.
4. Discussion

The expression and secretion of adiponectin is regulated by a variety of hormones and cytokines that influence insulin sensitivity in adipocytes. TNF-α, a cytokine that induces insulin resistance, functions as a major negative regulator of adiponectin and causes metabolic disorders by impairing the function of adipose tissues [18]. Due to the protective effect of adiponectin against insulin-associated metabolic diseases, TNF-α-induced reduction of adiponectin expression is considered a crucial event in the development of insulin resistance. Therefore, a precise understanding of the relationship between these adipokines and their regulatory mechanisms will be valuable in the development of treatments for insulin-related metabolic syndromes and alcohol-related liver injury.

In this study, we investigated the involvement of PKC in TNF-α-induced suppression of adiponectin expression in fully differentiated 3T3-L1 adipocytes, using GO6976 as a selective inhibitor of PKC. Pretreatment with GO6976 attenuated the suppressions of adiponectin secretion and expression induced by TNF-α. GO6976 is generally used as a PKC inhibitor; however, it is also known to inhibit other protein kinases including S6K1, MAPKAP-K1RSK2, MSK1, CHK1 and PHK [19]. Although we did not check the activities of these kinases and thus we can not completely exclude the association with them in the action of GO6976 at present, our data suggest that PKC is closely implicated in the adiponectin regulation in cultured adipocytes. Indeed, another PKC inhibitor, GO6983 also showed the similar effect (data not shown).

We observed that treatment with TNF-α rapidly activated JNK in cultured 3T3-L1 adipocytes, which is consistent with the previous report demonstrating the involvement of JNK in suppression of adiponectin expression [13]. Furthermore, it is known that the activation of c-Jun and ATF2 following JNK activation regulates PPARγ expression via Map4k4 stimulation, thereby contributing to suppression of adipogenesis [20]. Here, we showed that GO6976 suppressed TNF-α-induced activations of c-Jun and ATF2 as well as JNK, suggesting that PKC may play a role as an upstream signaling mediator of TNF-α-induced JNK activation for regulation of adiponectin expression.

PKCs play diverse roles in physiological functions including insulin signal transduction and differentiation of adipocytes; however, the precise role of the respective isoforms in adipocytes remains to be elucidated. Activation of atypical PKC isoforms (αPKC)/protein kinase B (PKB/AKT) is responsible for insulin-stimulated glucose transport through insulin receptor substrate-1 (IRS-1)-dependent PI3K signaling pathway, which is defective in type-2 diabetic and obese animal and human subjects [21]. TNF-α contributes to insulin resistance by inhibiting insulin-dependent activation of the IRS-PI3K-PKCβ/ζ signaling cascade [22]. Meanwhile, PKC-δ accounts for production of reactive oxygen species (ROS) inducing oxidative stress in high-fat diet-induced obese and insulin resistance mice [23]. Thus, the functional role of PKCs may vary depending on isoform and environmental conditions. In line with this, we observed PKCs differentially inhibit transcriptional activity of PPARγ in PKC isoform-specific manner. Transient expression

Fig. 4. Effects of PKCs on transcriptional activity of PPARγ. (A) Fully differentiated 3T3-L1 adipocytes were co-transfected with adiponectin promoter-luc (0.5 μg/well) and PPARγ (0.4 μg/well) with β-galactosidase (100 ng/well). After 24 h incubation, cells were pretreated with GO6976 (2 μM) for 1 h prior to treatment without (+) or with TNF-α (+, 10 ng/ml) for additional 24 h. Luciferase activity was measured and normalized to β-galactosidase activity. HEK293 cells were co-transfected with β-galactosidase (0.5 μg/well) and adiponectin promoter-luc (B) or PPRE-luc (C) (0.5 μg/well), together with PPARγ (0.5 μg/well) and each PKC isoform expression vector (0.3 μg/well). The total amount of DNA transfected was kept constant using pcDNA3.1 empty vector. Results shown are the mean value of three independent experiments. **P < 0.01, *P < 0.05, compared with PPARγ alone-transfected cells.
of PKC\(\alpha\) or PKC\(\beta\) attenuated PPAR\(\gamma\)-induced activations of adiponectin promoter and PPRE reporter, meanwhile PKC\(\delta\) and PKC\(\zeta\) did not show such inhibitory effect. Considering that PKC\(\alpha\) plays an important role in exerting the anti-adipogenic effect of PDGF in 3T3-L1 adipocytes [24], it is possible that PKC\(\zeta\) is involved in TNF-\(\alpha\)-induced suppression of adiponectin.

Taken together, our results suggest that PKC could be involved in TNF-\(\alpha\)-induced downregulation of adiponectin expression and secretion through regulation of transcriptional activity of PPAR\(\gamma\) in cultured 3T3-L1 adipocytes. These findings provide a new basis for the development of therapeutic agents for treating metabolic disorders such as diabetes and obesity.

**Acknowledgment:** This study was supported by a Korea National Institute of Health Intramural Research Grant (4800-4845-300-210-13).

**References**


