Tumor necrosis factor alpha inhibits peroxisome proliferator-activated receptor gamma activity at a post-translational level in hepatic stellate cells.

Chin K. Sung, Hongyun She, Shigang Xiong and Hidekazu Tsukamoto

Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089-9141; Department of Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA 90073

Please address correspondence and reprint requests to:
Hidekazu Tsukamoto, D.V.M., Ph.D.
Department of Pathology
Keck School of Medicine of the University of Southern California
1333 San Pablo Street, MMR 412
Los Angeles, CA 90089-9141
TEL: (323) 442-5107
FAX: (323) 442-3126
E-mail: htsukamo@usc.edu
Diminished activity of peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) is implicated in activation of hepatic stellate cells (HSC), a critical event in development of liver fibrosis. In the present study, we investigated PPAR$\gamma$ regulation by tumor necrosis factor $\alpha$ (TNF$\alpha$) in a HSC cell line designated as BSC. In BSC, TNF$\alpha$ decreased both basal and ligand (GW1929)-induced PPAR$\gamma$ mRNA levels without changing its protein expression. Nuclear extracts from BSC treated with TNF$\alpha$ showed decreased binding of PPAR$\gamma$ to PPAR-responsive element (PPRE) as determined by electrophoretic mobility shift assay. In BSC transiently transfected with a PPAR$\gamma$1 expression vector and a PPRE-luciferase reporter gene, TNF$\alpha$ decreased both basal and GW1929-induced transactivation of the PPRE promoter. TNF$\alpha$ increased activation of extracellular signal-regulated kinases (ERK) 1/2 and c-Jun NH$_2$-Terminal Kinase (JNK), previously implicated in phosphorylation of Ser$^{82}$ of PPAR$\gamma$1 and resultant negative regulation of PPAR$\gamma$ transactivity. In fact, TNF$\alpha$ failed to inhibit transactivity of a Ser$^{82}$Ala PPAR$\gamma$1 mutant in BSC. TNF$\alpha$-mediated inhibition of PPAR$\gamma$ transactivity was not blocked with a Ser$^{32}$Ala/Ser$^{36}$Ala mutant of inhibitory NF$\kappa$B$\alpha$ (IxB$\alpha$). These results suggest that TNF$\alpha$ inhibits PPAR$\gamma$ transactivity in cultured HSC at least in part by diminished PPAR$\gamma$-PPRE (DNA) binding and ERK1/2-mediated phosphorylation of Ser$^{82}$ of PPAR$\gamma$1, but not via the NF$\kappa$B pathway.
KEYWORDS

Tumor necrosis factor α, hepatic stellate cells, peroxisome proliferator-activated receptor γ
INTRODUCTION

Hepatic stellate cells (HSC) are vitamin A-storing, perisinusoidal pericytes that serve as the major source of extracellular matrices in liver fibrosis (9; 23). In liver fibrosis, HSC undergo phenotypic changes from quiescent cells to actively proliferating myofibroblastic cells (9; 16). This process of HSC activation is associated with upregulation of various activation markers such as collagen, TGFβ, and α-smooth muscle actin (24; 25). Several in vivo and in vitro studies have demonstrated that natural or synthetic ligands for peroxisome proliferator-activated receptor γ (PPARγ) could ameliorate HSC activation and even liver fibrosis, implicating the importance of PPARγ in maintenance of the quiescent state of HSC (13; 24; 25).

PPARγ is a nuclear receptor that heterodimerizes with retinoid X receptor (RXR) (7; 22; 34). PPARγ binds to PPAR response elements (PPRE) of target gene promoters and transactivates them upon binding by its ligands such as 15-deoxy-Δ^{12,14}-prostaglandin J2 (15dPGJ2) and synthetic thiazolidinediones (7; 34). Lately, PPARγ ligands have been reported to render various biological effects including increased insulin sensitivity, anti-inflammation and macrophage differentiation (7; 22; 26). A critical role of enhanced PPARγ function by its ligands or its overexpression has been best demonstrated in fat cell differentiation (26; 34). Previously, we and others have reported that PPARγ mRNA expression was significantly decreased in HSC isolated from various animal models of liver fibrosis (i.e. toxins and bile duct ligation) (24; 25). During HSC activation in culture, a well-established in vitro model of HSC activation, PPARγ mRNA expression also decreased (24; 25). More recently, forced expression of PPARγ in culture-activated HSC was shown to revert its morphology to less activated (i.e. more quiescent)
phenotype (17). These reports suggest an important role of PPARγ in maintenance of the quiescent HSC phenotype.

In response to liver injury, platelets and liver macrophages (i.e. Kupffer cells) produce and secrete increased amounts of cytokines and growth factors (8; 9; 23). TNFα is one such cytokine that is known to play pleiotropic functions in cells. Intracellular signaling pathways for TNFα are extremely complex and can lead to multiple cell responses including cell proliferation, inflammation, or cell death (3; 14). Currently, TNFα upon its interaction with its receptor is known to utilize two distinct intracellular signaling pathways including the NFκB pathway and mitogen-activated protein (MAP) kinase pathway (JNK and p38 kinase) (14; 33). Here, we investigated molecular mechanisms underlying TNFα-mediated regulation of PPARγ activity employing a HSC cell line BSC. We report that TNFα inhibits PPARγ binding to PPRE (DNA). In BSC, TNFα stimulates ERK1/2 and JNK and inhibits PPARγ transactivity in a manner dependent on Ser^{82} of PPARγ.
MATERIALS AND METHODS

Materials. PPARγ ligand GW1929 was a gift from T. Willson (Glaxo Wellcome R & D, Research Triangle Park, NC). TNFα was purchased from R & D Systems Inc. (Minneapolis, MN); Targefect F-2 transfection reagent (Targeting system, San Diego, CA) from Targeting System (San Diego, CA); Trizol reagent from Invitrogen (Carlsbad, CA); and α-32P-labeled dCTP (3000Ci/mmol) from PerkinElmer Life Sciences, Inc. (Boston, MA) from Cell Signaling Technology (Beverly, MA). Antibodies to phosphorylated ERK1/2 (Thr202/Tyr204), ERK1/2 and PPARγ were from Santa Cruz Biotech (Santa Cruz, CA); antibodies to phosphorylated JNK (Thr183/Tyr185) and JNK from Cell Signaling Technology (Beverly, MA).

PCDNA3 empty and pCDNA3-human PPARγ1 expression vectors were gifts from V.K.K. Chatterjee (Univ. of Cambridge, Cambridge, UK). PStud-mouse PPARγ1 and pStud-mouse Ser82Ala-PPARγ1 expression vectors were gifts from S.R.Tafuri (Warner-Lambert C., Ann Arbor, MI). NF-κB luciferase construct containing five repeats of the consensus NF-κB binding sequence was purchased from Stratagene (La Jolla, CA). Other chemicals were from Sigma (St. Louis, MO), unless specified otherwise.

Cell culture. BSC cells were originated from rat HSC isolated from experimental biliary liver fibrosis rendered by 18 day bile duct obstruction (35). The spontaneously immortalized HSCs were maintained in DMEM plus 10% FBS and passed at a dilution of 1:3 every week until characterization was performed. The colonization activity and cell viability of BSC were evaluated and subclones were established by the limiting dilution method on the 63rd passage. Trypsinized BSC were diluted and replated onto 96-well microtiter plates (Costar, Corning, NY).
with 1 cell/per well in 200 µl DMEM supplemented with 10% FBS, antibiotics and 10% cell-free conditioned medium from original BSC culture. After 3 weeks of culture the number of colonies from single cells were counted and growing clones were trypsinized with 0.05% trypsin-EDTA (GIBCO, Carlsbad, CA) and subcultured in DMEM containing 10% FBS and antibiotics in 24-well plates, subsequently transferred to 6-well plates (Costar, Corning, NY) for expansion. Total seventy-four clonal cell lines were obtained in this manner. One of the clones, designated BSC-C10, was characterized by immunofluorescent microscopy and Northern Blot analysis or RT-PCR for expression of HSC markers at protein and mRNA levels. The BSC-C10 clone expresses markers for HSC such as glial acidic fibrillary protein, desmin, α-smooth muscle actin, synaptophysin, vascular cell adhesion molecule, neural cell adhesion molecule. BSC were maintained in low glucose DMEM supplemented with 10% FBS, 100 mg/ml streptomycin, and 10,000 units/ml penicillin and 25µg/ml amphotericin B.

Isolation of HSC. Hepatic stellate cells (HSC) were isolated from normal male Wister rats as previously described (24) by the Non-Parenchymal Liver Cell Core of the USC-UCLA Research Center for Alcoholic Liver and Pancreatic Diseases. The purity of isolated HSC was examined by phase contrast microscopy and UV-excited fluorescence microscopy and viability based on trypan blue exclusion. Freshly isolated rat HSC were cultured on 100 mm culture dishes in low glucose DMEM supplemented with 10% FBS.

Real Time reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from BSC treated for 18 hrs with either TNFα (20 ng/ml) or GW1929 (1 µM) or both using Trizol reagent. Total RNA (50 ng each for PPARγ and 5 ng each for GAPDH) was
reverse-transcribed at 48 C for 30 min followed by 40 cycles of PCR using TaqMan Gold RT-PCR kit (Applied Biosystems, Foster city, CA). Probes used were 5, 6-carboxyfluorescein amidite labeled at the 5′ end and black hole quencher-1 labeled at the 3′ end (Biosearch Technologies Inc., Novato, CA). ABI PRISM 7900 HT machine (Molecular Biology Core, USC Liver Center) was used to detect the threshold cycle (Ct). Each C_t value for PPARγ was first normalized to the respective C_t value for GAPDH and subsequently to a control (i.e. reference) sample.

**Electrophoretic mobility shift assay.** BSC were treated in serum free DMEM containing 0.1 % BSA for 18 hrs with either TNFα (20 ng/ml) or GW1929 (1 μM) or both. Nuclear extracts were then prepared as previously described by Schreiber et al (29). Nuclear extracts (15 μg each) were preincubated on ice for 15 min in a reaction mixture containing 20 mM HEPES (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 2 mM DTT, 20 % glycerol, 200 μg/ml poly (dI-dC) and incubated for 30 min with 1-2 ng of α^{32}P-labeled double stranded ARE-7 (the PPRE from aP2 gene). For a supershift assay, antibodies to PPARγ were added to reaction mixture and incubated for additional 20 min. The reaction mixture was then resolved by a 6% polyacrylamide gel electrophoresis followed by autoradiography.

**Transient Transfection and luciferase reporter assay.** For PPRE promoter activity assays, BSC in 6-well plates (100,000 cells per well) were transfected with PPRE-luciferase construct (tk-PPRE X 3-luciferase; 1 μg per well) and expression vectors (1 μg per well), either empty vector or PPARγ1 containing vector. For NFκB promoter activity assays, BSC were transfected with a NFκB-luciferase construct (0.5 μg), pCDNA3-PPARγ1 expression vector (0.5 μg) and
pCMX expression vector, either empty vector (1 µg) or Ser\textsuperscript{32}Ala/Ser\textsuperscript{36}Ala mutant of IκBα containing vector (0.5-1 µg). For the assessment of transfection efficiency, renilla phRL-TK vector (2 ng per well) was used (Promega, Madison, WI). A non-lipid cationic Targefect F2 reagent (Targeting Systems, San Diego, CA) was used at 1:1 molar ratio of total DNA (µg) and volume of F2 (µl). After 24 hrs of transfection, the cells were treated in serum free DMEM containing 0.1 % BSA for 16-18 hrs with or without TNFα (20 ng/ml) in the presence or absence of a selective PPARγ ligand GW1929 (1 µM). Next, the cells were washed in PBS three times and solubilized in 1X passive lysis buffer (Promega, Madison, WI) and cell lysates were assayed for both firefly (PPRE-luciferase or NFκB-luciferase) and renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Ratios of firefly luciferase activity and renilla luciferase activity were calculated.

**Preparation of cell lysates and Western blot analysis.** To assess PPARγ protein levels, BSC in 100 mm dishes were treated for 18 hrs with TNFα (20 ng/ml) or GW1929 (1 µM) or both in serum free DMEM containing 0.1% BSA, washed in PBS three times and solubilized for 30 min in modified RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1 % SDS, 1% NP-40, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 1 mM DTT, 2 mM Na\textsubscript{3}VO\textsubscript{4}, 10 mM NaF) containing a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). To assess phosphorylation and protein levels of ERK and JNK, BSC in 100 mm dishes were treated for 5-10 min with or without TNFα (20 ng/ml). After centrifugation in a microcentrifuge (15,000 x g, 15 min), soluble supernatants (i.e. cell lysates) were transferred into new tubes and resolved by Western blot analysis. Briefly, cell lysates (25 µg) were resolved by SDS-PAGE followed by electrophoretic transfer of proteins onto nitocellulose membranes. The membranes were then probed with appropriate antibodies and
signal was detected by hydrogen peroxide-enhanced chemiluminescence method using the Pierce ECL kit (Amersham, Arlington Heights, IL) as previously described (18).

**Statistical Analysis.** Data are presented as mean ± SEM of several experiments. Statistical analyses were performed by two-tailed Student’s t-test.
RESULTS

TNFα decreases basal and GW1929-induced PPARγ mRNA levels without altering PPARγ protein expression in BSC. We have previously demonstrated that TNFα treatment of primary rat HSC cultured for 3-4 days decreased mRNA levels of PPARγ as assessed by RT-PCR (25). In order to quantify TNFα-induced changes in PPARγ mRNA in primary HSC, we used one day old HSC that were previously shown to express a higher level of PPARγ mRNA (23; 24), treated with TNFα for 24 hr, and performed real time RT-PCR on extracted RNA samples. Results from this experiment demonstrated a 85% reduction in PPARγ mRNA levels by the cytokine (Fig. 1A).

To ascertain this finding in BSC, a HSC-derived cell line, we treated BSC for 18 hrs with TNFα in the presence and absence of a selective PPARγ ligand GW1929 and measured PPARγ mRNA levels by real time RT-PCR. Treatment of BSC with GW1929 increased PPARγ mRNA level about two fold (Fig. 1B). TNFα decreased both basal and GW1929-induced PPARγ mRNA levels by 90-95 %. These data suggest that BSC responds to TNFα in a similar manner to primary HSC.

To determine whether the decreased PPARγ mRNA level by TNFα is accompanied by decreased PPARγ protein expression, we examined PPARγ protein levels by Western blot analysis (Fig. 1C). Interestingly, TNFα treatment for 18 hrs did not affect PPARγ protein levels in spite of diminished PPARγ mRNA levels (Fig. 1C).
TNFα decreases basal and GW1929-induced PPARγ-PPRE (DNA) binding in BSC. PPARγ is a transcription factor that binds to PPRE of target gene promoters in nucleus, leading to regulation of gene expression (7; 22; 34). To determine whether TNFα alters PPARγ -DNA binding in BSC, we prepared nuclear extracts from BSC treated for 18 hrs with TNFα in the presence and absence of GW1929 and performed electrophoretic mobility shift assay using a labeled double stranded oligonucleotides containing the (ARE7) PPRE as described in Materials and Methods. The (ARE7) PPRE preferentially binds PPARγ over PPARα (21). Treatment of BSC with GW1929 enhanced PPARγ-PPRE binding and TNFα decreased both basal and GW1929-induced PPARγ-PPRE binding (Fig. 2). Specificity of PPARγ in the protein-PPRE (DNA) complex was confirmed by disappearance of the band when the reaction mixture was incubated with antibodies to PPARγ (Fig.2). Control rabbit IgG failed to affect PPARγ-PPRE complex formation.

TNFα decreases transcriptional activity of PPARγ in BSC. To determine whether TNFα affects PPARγ-mediated transactivation of PPRE in BSC, we transiently transfected the cells with a PPRE-luciferase reporter construct and a PPARγ1 expression vector. After 24 hrs of transfection, the cells were treated for 18 hrs with TNFα in the presence and absence of GW1929. In BSC co-transfected with an empty expression vector, TNFα significantly decreased GW1929-induced transactivity of endogenous PPARγ (Fig. 3). Overexpression of PPARγ in BSC increased the PPRE promoter activity five fold and GW1929 further increased this activity three fold. In these cells, TNFα treatment significantly decreased both basal (GW1929-independent) and GW1929-induced PPRE promoter activity by 32 % and 42 %, respectively
(Fig. 3). These data suggest that TNFα inhibits PPRE transactivation by both endogenous and exogenous PPARγ in the presence and absence of the PPARγ ligand GW1929. These inhibitory effects of TNFα are in agreement with those on PPARγ-PPRE (DNA) binding as shown in Fig. 2.

**TNFα increases activation-specific phosphorylation of ERK1/2 and JNK in BSC.** The A/B domain of PPARγ contains a single consensus MAP kinase phosphorylation site that becomes phosphorylated by ERK1/2 and JNK, resulting in decreased transcriptional activity of PPARγ (1; 4; 20). To assess this possibility, we first examined whether these MAP kinases are phosphorylated by TNFα as the evidence of their activation in BSC. We treated the cells with TNFα for 5-10 min and cell lysates were resolved by Western blot analysis. TNFα increased activation-specific phosphorylation of both ERK1/2 (Thr²⁰²/Tyr²⁰⁴) and JNK (Thr¹⁸³/Tyr¹⁸⁵) in BSC (Fig. 4). TNFα, however, failed to affect phosphorylation of p38 MAP kinase in these cells (Data not shown). These data suggest that TNFα potentially phosphorylates PPARγ in BSC via activation of ERK1/2 and JNK that may lead to inhibition of transactivation of PPARγ.

**TNFα fails to inhibit PPRE transactivation by Ser⁸²Ala mutant form of PPARγ in BSC.** To ascertain the role of PPARγ phosphorylation by TNFα-induced MAP kinase on PPRE transactivation, we performed transient transfection experiments employing a Ser⁸²Ala-PPARγ1 mutant expression vector together with the PPRE-luciferase reporter construct. It should be noted that HSC express predominantly PPARγ1 isoform, but not adipocytes-specific PPARγ2 isoform (25). Serine at 82 amino acid of the mouse PPARγ1 (equivalent to Serine at 84 amino acid of the
human PPARγ1) is the single MAP kinase consensus recognition site that is indeed phosphorylated by ERK1/2 (4) and JNK (5), resulting in inhibition of PPARγ1 activity. In BSC, overexpression of Ser82Ala-PPARγ1 mutant achieved higher PPRE promoter activity than the wild type PPARγ1 (the first set of bars in Fig. 5). GW1929 enhanced the PPRE promoter activity mediated by both mutant and wild type PPARγ and the mutant still displayed greater PPRE transactivation (the second set of bars). In these cells, TNFα, however, failed to inhibit transactivation of Ser82Ala-PPARγ1 while the inhibition was reproduced in the cells overexpressing wild type PPARγ1. These data indicate that Ser82 phosphorylation of PPARγ1 is important for inhibitory action of TNFα on ligand-induced PPARγ activity in BSC.

**Inhibitory action of TNFα on PPARγ transactivation is independent of the NFκB-pathway in BSC.** In addition to the MAP kinase cascade, NFκB pathway has been reported to be another major TNFα signaling pathway in cells (33). To determine whether the NFκB pathway plays a role in inhibitory action of TNFα in PPARγ activity in BSC, we employed Ser32Ala/Ser36Ala mutant of NFκB inhibitory protein α (IκBα) (4). This phosphorylation mutant form of IκBα fails to be phosphorylated by IκB kinase and thus constitutively binds to NFκB in the cytoplasm preventing nuclear translocation of NFκB, resulting in inhibition of NFκB signaling pathway (14). First, we determined effects of Ser32Ala/Ser36Ala IκBα mutant on NFκB signaling pathway by assessing NFκB promoter activity. In BSC, expression of Ser32Ala/Ser36Ala IκBα mutant inhibited NFκB promoter activity reaching maximal 80-90 % inhibition at 0.5-1 μg of construct (Fig. 6B). Next, we co-transfected cells with the PPRE-luciferase construct, and expression vectors for PPARγ1 and Ser32Ala/Ser36Ala IκBα, and measured PPRE luciferase activity
following treatment of cells with TNFα and GW1929. In control BSC transfected with pCMX empty expression vector for Ser\textsuperscript{32}Ala/Ser\textsuperscript{36}Ala IκBα, TNFα inhibited both basal and GW1929-induced PPRE promoter activity as previously described (Fig. 6A). Expression of Ser\textsuperscript{32}Ala/Ser\textsuperscript{36}Ala IκBα (i.e. inhibition of the NFκ B pathway) in BSC moderately increased basal PPRE promoter activity. In these cells, TNFα could still inhibit both basal and GW1929-induced PPRE promoter activity to the similar extent as in control cells (Fig. 6A). These data suggest that inhibitory action of TNFα on PPARγ transactivation in BSC is not altered by inhibition of the NFκB pathway. Increased basal PPRE promoter activity by expression of Ser\textsuperscript{32}Ala/Ser\textsuperscript{36}Ala IκBα (i.e. inhibition of the NFκ B pathway) suggests that the NFκB pathway may play a negative role in basal transactivity of PPARγ.
DISCUSSION

Our present study suggests that in BSC, a HSC-derived cell line, TNFα inhibits PPARγ transactivity at a post-translational level at least in part by inhibition of PPARγ-PPRE (DNA) binding and also by a mechanism involving phosphorylation of PPARγ at the consensus MAP kinase phosphorylation site (Ser82 of PPARγ1) mediated by TNFα-induced ERK1/2 activation. Since phosphorylation of PPARγ does not affect the ability of PPARγ to bind DNA (i.e. PPRE) (1; 4), these two actions of TNFα seem to be operative in parallel in BSC. Our data also suggest that inhibitory action of TNFα on PPARγ transactivity in these cells is not affected by inhibition of the NFκB pathway, a major TNFα signaling pathway in cells. It should be noted, however, that inhibition of basal NFκB signaling in BSC increased basal PPARγ-mediated PPRE promoter activity, suggesting the negative role of NFκB signaling in PPARγ transactivity in control cells.

Chronic injury leading to fibrosis in liver occurs in response to various insults including viral hepatitis, alcohol abuse, drugs, metabolic diseases due to overload of iron and copper, and autoimmune attacks of hepatocytes or bile duct epithelium (8; 9; 23). HSC is now well established as the key cellular element involved in the development of hepatic fibrosis (8; 9; 23). In response to various insults, normally quiescent vitamin A storing HSC undergoes a phenotypic transformation termed “activation” or “transdifferentiation” to become actively proliferating myofibroblast-like cells. HSC activation involves the coordinated activity of several key transcriptional regulators of the HSC genome including NFκB, AP-1, Kruppel-like transcription factor 6, C/EBP and E-box transcription factors (23). In contrast to these transcription factors associated with the activated phenotype of HSC, PPARγ has been identified as a transcription factor that is active in quiescent HSC, but its activity decreases in activated
HSC (24; 25). In both primary HSC from cholestatic liver fibrosis animal model (25) and
culture-activated HSC (24; 25), PPARγ mRNA expression and PPRE binding were greatly
diminished when compared with control quiescent HSC. Treatment of activated HSC with
PPARγ ligands restored PPARγ expression and diminished various biochemical markers
associated with fibrosis (13; 25). Moreover, oral administration of synthetic PPARγ ligands,
thiazolidinediones, in animal models of liver fibrosis (toxin administration or bile duct ligation)
was shown to retard fibrosis in vivo (13). These studies strongly support the therapeutic potential
of PPARγ ligands in liver fibrosis.

Since decreases in PPARγ expression level and its activity are correlated with and seem to
precede HSC activation (i.e. phenotypic changes and increases in fibrogenic activation markers),
it is important to understand how PPARγ is regulated in HSC. In response to liver injury, liver
macrophages are activated to release various cytokines including TNFα. Thus, we initiated this
study to determine molecular mechanisms underlying TNFα-mediated PPARγ regulation in
HSC. In the present study, we demonstrated that inhibitory action of TNFα on PPARγ activity in
BSC was at the post-translational level. Although TNFα greatly diminished mRNA levels of
PPARγ in BSC, it failed to alter PPARγ protein level. This discrepancy between mRNA and
protein levels of PPARγ was rather puzzling. To this end, complex regulation of PPARγ
expression by growth factors and cytokines has recently been reported. In particular, TGFβ was
shown to exert a biphasic effect on PPARγ mRNA expression in human aortic smooth muscle
cells (early stimulation and late repression) (11). A short-term treatment (1-2 hrs) of these cells
with TNFα was also shown to rather increase PPARγ expression (10). PDGF regulation of
PPARγ was more complex in that PPARγ expression was increased at 2 hrs but declined to some
extent at 6 hrs, and rose again at 12 hrs to achieve a second peak that was sustained for 72 hrs (12). To make the matter even more complex, Hauser et al reported that PPARγ protein levels are significantly decreased in adipose cells and fibroblasts in response to specific ligands such as thiazolidinediones, which involves proteasome-mediated PPARγ degradation (15). All these reports indicate that complex regulation of PPARγ expression occurs in cells in response to various signals. It is noteworthy that discrepancy between levels of mRNA and protein has been previously reported for NADH cytochrome p450 oxidoreductase following thyroid hormone treatment in liver (2) and for the small RING-finger protein SNURF/RNF4 in testis (28).

Counter-acting potential of TNFα on PPARγ function has previously been reported. TNFα causes insulin resistance in insulin target cells whereas treatment of insulin resistant animals with PPARγ ligands ameliorates insulin resistance (19; 26; 27). TNFα has also been reported to inhibit fat cell differentiation whereas PPARγ is a potent positive regulator of this function and PPARγ overexpression facilitates fat cell differentiation (30; 31). Our present study suggests that TNFα may exert some of its action to counter-act PPARγ activity at a post-translational level in cells.

PPARγ has two isoforms, γ1 and γ2, that originate from the same gene but result from differential promoter usages with 30 amino acid difference at the NH2 terminus (26; 36). HSC express predominantly PPARγ1 but not adipocytes-specific PPARγ2 (25). The amino-terminal A/B domain of these receptors contains a single consensus MAP kinase site (Ser82 and Ser112 of mouse PPARγ1 and γ2, respectively). Upon their phosphorylation by growth factor-activated ERK1/2, their transcriptional activities decrease (1; 4; 20). Mutation of this phosphorylation site has been reported to greatly augment PPARγ transcriptional activity (1; 4; 20) and to enhance fat cell differentiation (20). The mutated PPARγ was also reported to be resistant to growth factor-
mediated inhibition of its transcriptional function. Moreover, purified PPARγ protein was directly phosphorylated in vitro by recombinant activated ERK2 (4). Later, JNK was also shown to efficiently phosphorylate PPARγ at the same Ser82 site (5). In BSC, we found that TNFα stimulates both ERK1/2 and JNK, and inhibitory action of TNFα on transactivation of PPARγ is abolished when the cells express Ser82Ala-PPARγ1 mutant, suggesting importance of PPARγ phosphorylation in the inhibitory action of TNFα. Now the question arises as to whether both ERK1/2 and JNK play a role in PPARγ phosphorylation and resultant inhibition of its function in BSC. Our preliminary study with the MEK/ERK inhibitor PD98059 suggests that ERK1/2 may be responsible for PPARγ phosphorylation and subsequent inhibition of its transcriptional activity although the role of JNK in PPARγ regulation cannot be completely ruled out (data not shown). Further analysis with dominant negative forms of these enzymes would be needed to confirm our interpretation. In BSC, TNFα also inhibits PPARγ-PPRE (DNA) binding. However, this is most likely independent of PPARγ phosphorylation since PPARγ phosphorylation has been reported not to alter PPARγ-DNA binding (1; 4). These results led us to propose two levels of PPARγ regulation by TNFα in BSC: one level at PPARγ phosphorylation and the other level at decreased PPARγ-DNA binding.

Additional support for TNFα-induced inhibition of PPARγ-DNA binding being independent of PPARγ phosphorylation comes from the recent study with mesenchymal stem cell line ST2 where PPARγ was reported to form a complex with NFκB that in turn blocked PPARγ-DNA binding following TNFα treatment (32). Overexpression of NFκB-inducing kinase (i.e. increased NFκB level in nucleus) inhibited ligand-induced transactivation of PPARγ. This inhibitory action of NFκB-inducing kinase on PPARγ transactivation was still observed when cells are co-
transfected with Ser$^{112}$Ala-PPAR$\gamma$2 mutant (equivalent to Ser$^{82}$Ala PPAR$\gamma$1) (32). These data may be interpreted that overexpression of NF$\kappa$B could inhibit PPAR$\gamma$ activity regardless of its phosphorylation status. In order to determine contribution of the NF$\kappa$B signaling to PPAR$\gamma$ activity in BSC, we took a different approach to decrease endogenous NF$\kappa$B signaling by expression of the phosphorylation mutant form of I$\kappa$B$\alpha$ (to increase I$\kappa$B$\alpha$-NF$\kappa$B complex formation in cytoplasm). In these cells, we observed increased basal transcriptional activity of PPAR$\gamma$ that is consistent with a negative role of NF$\kappa$B in PPAR$\gamma$ activity. In these cells, TNF$\alpha$ was capable of inhibiting PPAR$\gamma$ activity as well as in control cells possessing a higher level of NF$\kappa$B signaling. Our data together with results of Suzawa et al (32) suggest that TNF$\alpha$ could exert its inhibitory action on PPAR$\gamma$ activity by either PPAR$\gamma$ phosphorylation or inhibition of PPAR$\gamma$-DNA binding that is independent of each other. Depending on magnitude of NF$\kappa$B signaling in cells, TNF$\alpha$ may differentially utilize one mechanism over the other.

Taken together, we propose a working model for TNF$\alpha$-mediated regulation of PPAR$\gamma$ activity in HSC (Fig. 7). Treatment of the cells with TNF$\alpha$ causes activation of ERK and JNK following the known complex formation of TNF receptor with various intracellular adaptor proteins (6). Ser$^{82}$ phosphorylation of PPAR$\gamma$ by ERK or/and JNK results in decreased PPAR$\gamma$ transcriptional activity and this molecular change underlies activation of HSC. TNF$\alpha$ also diminishes PPAR$\gamma$-DNA binding to further decrease PPAR$\gamma$ activity. In summary, TNF$\alpha$ inhibits PPAR$\gamma$ activity at a post-translational level in the HSC-derived cells. TNF$\alpha$ inhibits PPAR$\gamma$ activity at least in part by inhibition of PPAR$\gamma$-PPRE (DNA) binding and appears to also involve ERK1/2-mediated phosphorylation of Ser$^{82}$-PPAR$\gamma$1, but not the NF$\kappa$B pathway.
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FIGURE LEGENDS

**Figure 1A. TNFα decreases PPARγ mRNA levels in early primary culture of HSC.** Rat HSC were cultured on 100 mm dishes for one day. HSC attached to culture dishes were then treated with TNFα (20 ng/ml) for 24 hrs in DMEM containing 1 % fetal bovine serum. Total RNA was then extracted and used for real time RT-PCR to measure mRNA for PPARγ and GAPDH as described in Materials and Methods. The ratio of PPARγ mRNA/GAPDH mRNA was calculated. Data are presented as the percent of control and are mean ± SEM of 4 experiments. *, p < 0.05 by student’s t-test.

**Figure 1B. TNFα decreases basal and GW1929-induced PPARγ mRNA levels in BSC.** The cells in 100 mm dishes were treated for 18 hrs with TNFα (20 ng/ml), GW 1929 (1 μM) or both in serum free DMEM containing 0.1 % BSA. Total RNA was then extracted with TriZol reagent (Invitrogen, Carlsbad, CA) and used for real time RT-PCR to measure mRNA for PPARγ and GAPDH as described in Materials and Methods. The ratio of PPARγ mRNA/GAPDH mRNA was calculated. Data are presented as the percent of control and are mean ± SEM of 3-4 experiments. *, p < 0.05 by student’s t-test.

**Figure 1C. TNFα does not alter PPARγ protein levels in BSC.** After treatment of cells for 18 hrs with TNFα (20 ng/ml), GW 1929 (1 μM) or both, cells were solubilized in modified RIPA buffer and cell lysates were resolved by Western blot analysis with anti-PPARγ antibody as described in Materials and Methods.
Figure 2. TNFα decreases basal and GW1929-induced PPARγ-PPRE (DNA) binding in BSC. The cells were first treated for 18 hrs with TNFα (20 ng/ml), GW1929 (1 μM) or both. Nuclear extracts (15 μg protein per sample) prepared from these cells were incubated with 32P-labeled (ARE7) PPRE followed by polyacrylamide gel electrophoresis and autoradiography to assess electrophoretic mobility shift of protein-DNA complex as described in Materials and Methods. Specificity of PPARγ in protein-DNA complex was confirmed with anti-PPARγ antibody. Control rabbit IgG did not affect the binding pattern of protein-DNA complex.

Figure 3. TNFα inhibits PPARγ-dependent and GW1929-induced PPRE promoter activity in BSC. Cells in 6-well plates were first co-transfected with the PPRE-luciferase reporter construct (1 μg) and pCDNA3-PPARγ1 expression vector (1 μg). 24 hrs later, the cells were treated for 18 hrs in serum free DMEM with TNFα (20 ng/ml), GW1929 (1 μM) or both. Cell lysates were assayed for luciferase activity using dual luciferase reporter assay system (Promega Corp., Madison, WI) as described in Materials and Methods. PPRE-driven luciferase activities were normalized for transfection efficiency using renilla luciferase activities. Data are presented as the percent of the basal and are mean ± SEM of five experiments. *, p < 0.05 by student’s t-test.

Figure 4. TNFα increases activation-specific phosphorylation of ERK1/2 and JNK in BSC. After 18 hrs of serum starvation, the cells were treated with or without TNFα (20 ng/ml) for 5
min and 10 min. Cell lysates were then resolved by Western blot analysis with antibodies to phosphorylated ERK1/2 (Thr202/Tyr204) (Fig. 4A) and phosphorylated JNK (Thr183/Tyr185) (Fig.4B) as described in Materials and Methods.

**Figure 5. TNFα fails to inhibit Ser82Ala PPARγ1-dependent PPRE promoter activity in BSC.** Cells in 6-well plates were first co-transfected with the PPRE-luciferase construct (1 μg), and pStud-PPARγ1 or pStud-Ser82Ala PPARγ1 expression vector (1 μg). After 24 hrs, the cells were treated for 18 hrs with TNFα (20 ng/ml) and GW1929 (1 μM) and solubilized for luciferase activity assays as described in Fig. 3. Data are presented as the percent of basal in wild type PPARγ1 (pStud-PPARγ1) expressing cells and are mean ± SEM of three experiments. N.S., not significant; WT, wild type PPARγ1; S82A, Ser82Ala mutant of PPARγ1.

**Figure 6A. Inhibition of the NFκB pathway does not affect inhibitory action of TNFα on PPRE promoter activity in BSC.** The cells in 6-well plates were co-transfected with the PPRE-luciferase construct (1 μg), pCDNA3-PPARγ1 expression vector (0.5 μg), and either pCMX empty or pCMX-Ser32Ala/Ser36Ala IκBα expression vector (0.5 μg). 24 hrs later, cells were treated for 18 hrs with TNFα (20 ng/ml) or GW1929 (1 μM) or both. Cell lysates were next assayed for luciferase activities as described in Materials and Methods. Data are presented as the percent of basal and are mean ± SEM of three experiments. *, p < 0.05; and #, p < 0.1 by student’s t-test. pCMX-S32A/S36A-IκBα, pCMX-Ser32Ala/Ser36Ala-IκBα.

**Fig. 6B. Inhibition of NFκB-promoter activity by expression of Ser32Ala/Ser36Ala IκBα in BSC.** Cells were co-transfected with the NFκB-luciferase construct (0.5 μg), Ser32Ala/Ser36Ala-IκBα,
IκBα expression vector (0.5 μg, 1 μg), and pCDNA3-PPARγ1 expression vector (0.5 μg). After 24 hrs, cells were lysed and cell lysates were assayed for luciferase activities. Data are presented as the percent of control and are mean ± SEM of four experiments. *, p < 0.05 vs control by student’s t-test. N.S., not significant.

**Fig. 7. Proposed mechanisms of TNFα-mediated regulation of PPARγ activity in HSC.** The treatment of HSC with TNFα activates ERK and JNK that may phosphorylate Ser^{82} of PPARγ, resulting in a decrease of PPARγ transcriptional activity. TNFα also inhibits PPARγ-DNA binding that further contributes to reduced PPARγ transcriptional activity that in turn leads to activation of HSC.
REFERENCE LIST


Fig 1A

PPARγ mRNA (Relative unit)

TNFα -  +

*
Fig 1B

PPARγ mRNA (Relative unit)

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<tr>
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<th>TNFα</th>
<th>GW1929</th>
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<td>+</td>
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* Indicates significant difference.
Probe Only
Cont
+TNFα
+ GW
+ GW + TNFα
+GW + anti-PPARγ
+ GW + Cont-IgG
Fig 4

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TNFα → ↑ERK/JNK Activity → P-PPARγ → ↓PPARγ-DNA Binding → ↓PPARγ Activity → HSC Activation