REGULATION OF NUCLEAR TRANSLOCATION OF HDAC3 BY IKBα IS REQUIRED FOR TNF-INHIBITION OF PPARγ FUNCTION

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Running title: Regulation of PPARγ by TNF-α

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Inhibition of PPARγ function by TNF-α contributes to metabolic disorder in glucose and fatty acids under inflammation and cancer, but the molecular mechanism remains to be fully understood. In this study, we demonstrate that nuclear translocation of HDAC3 is regulated by TNF-α, and this event is required for inhibition of the transcriptional activity of PPARγ by TNF-α. HDAC3 is associated with IkBα in the cytoplasm. After IkBα degradation in response to TNF-α, HDAC3 is subject to nuclear translocation leading to an increase in HDAC3 activity in the nucleus. This event leads to subcellular redistribution of HDAC3. Knockout of IkBα, but not p65 or p50, leads to disappearance of HDAC3 in the cytoplasm. This is associated with HDAC3 enrichment in the nucleus. The data suggests that inhibition of PPARγ by TNF-α is not associated with a reduction in the DNA-binding activity of PPARγ. These results support that IkBα-dependent nuclear translocation of HDAC3 is responsible for PPARγ inhibition by TNF-α.

PPARγ is a nuclear receptor in the family of peroxisome proliferator-activated receptor (PPAR) that includes PPARα, PPARγ, and PPARδ (PPARβ) (reviewed in (1,2). PPARγ is a master transcriptional regulator of lipid and glucose metabolism (reviewed in (1-3). Inhibition of PPARγ function by inflammatory cytokines may contribute to the loss of insulin sensitivity in obese subjects and loss of fat storage in cancer patients under cachexia. Although TNF-α is known to inhibit the ligand-dependent transcriptional activity of PPARγ, the precise mechanism remains to be fully understood (4-8). In this study, we addressed this issue by analyzing the molecular mechanism of TNF-α action on PPARγ.

The transcriptional activity of PPARγ is controlled by DNA-binding activity and nuclear receptor cofactors that include corepressors and coactivators. PPARs form heterodimers with the retinoid X receptor (RXR), which is activated by 9-cis retinoic acid (9). It is generally believed that the heterodimer is associated with the nuclear receptor corepressor complex in the absence of PPARγ ligand. Upon activation by a ligand, the corepressor complex is replaced by coactivators leading to transcriptional initiation of target genes. The corepressor for PPARγ is a protein complex containing HDAC3 (histone deacetylase 3) and SMRT (silencing
mediator for retinoic and thyroid hormone receptors) or N-CoR (nuclear corepressor). RIP140 (receptor-interacting protein) may also be a component in the corepressor complex (10-13). The coactivators of PPARγ include the well-established cofactors such as p300/CBP, p160 and PGC-1 (PPARγ coactivator-1) (reviewed in (14), as well as the relative new coactivators TRAP220 (Thyroid hormone Receptor-Associated Protein 220 or PBP, PPARγ-Binding Protein) (15,16), ARA70 (Androgen Receptor-Associated protein) (17) and PRIP (PPARγ-interacting protein, ASC-2/RAP250 /TRBP/NRC) (18-21). The coactivator p160 has three isoforms: SRC-1 (steroid receptor coactivator 1, NCoA-1), SRC-2 (NCoA-2/TIF2/GRIP1) and SRC-3 (NCoA-3/pCIP/AIB-1/ACTR/RAC-3/TRAM-1) (22).

It has been well documented that PPARγ activity is inhibited by TNF-α. The inhibition can be divided into two types on the basis of PPARγ gene expression. First, PPARγ expression is reduced at mRNA level (5,6). This is observed in 3T3-L1 adipocytes treated with TNF-α for 24 hours or longer. Second, PPARγ expression is not changed and the inhibition is observed in cells transfected with a PPARγ expression vector (4,7,8). In the second model, the ligand-dependent transcriptional activity of PPARγ is reduced as a result of loss of DNA-binding activity. However, both types of inhibition are dependent on activation of IKK/NF-kB pathway as the TNF-α activity was abolished by the super repressor IkBα (Inhibitor kappa Bα) (6). NF-kB is a transcription factor that stays in the cytoplasm in the absence of activators. It is generally believed that IkBα inhibits NF-kB by maintaining NF-kB in the cytoplasm (reviewed in (23). IkBα degradation is controlled by a phosphorylation-mediated and proteasome-dependent mechanism that is initiated by activation of IKK2 (24). In the TNF-α signaling pathway, although ERK and JNK (c-JUN NH2 terminal kinase) were reported to inhibit the transcriptional activity of PPARγ through phosphorylation of serine residues in PPARγ protein (25,26), the role of these MAPKs remains to be further characterized.

In this study, TNF-induced inhibition of the transcriptional activity of PPARγ is analyzed with a focus on IkBα. Our results demonstrate that IkBα controls the nuclear translocation of HDAC3, which is required for the suppression of PPARγ activity by TNF-α. This study supports a new mechanism by which TNF-α inhibits PPARγ activity by targeting the nuclear receptor corepressor.

**Experimental Procedures**

**Reagents** - The PPRE luciferase reporter was constructed utilizing the pGL3 basic luciferase vector. In this vector, the luciferase gene is driven by the thymidine kinase (TK) promoter (-105/+51) of herpes simplex virus. The PPARγ-specific reporter was generated by inserting three copies of the PPRE element of the rat acyl-coA synthase gene (-583 CCTTTCCGAACGTCATTGGTCCTGGTCCCATTTGTGCT -544) (27) at the upstream of TK promoter. Mammalian expression vectors for PPARγ2, RXRα, and IKK2, have been described elsewhere (28-30). The super suppressor IkBα expression vector, and p65−/− MEFs were originally obtained from Dr. Inder M. Verma (Salk Institute). P50−/− MEFs was made from p50 knockout (p50−/) embryo of 13 days. Antibodies to IkBα
GLUT4 (sc-7938), PPARγ (sc-7373X), Smp3 (sc-644X), SMRT (sc-1610), N-CoR (sc-8994) and Pol II (sc-9001) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin (ab6276), and HDAC3 (ab2379) antibodies were obtained from Abcam (Cambridge, UK). Antibodies to HDAC1 (H 6287) and adiponectin (MAB3832) were from Sigma and Chemicon international, respectively. RNAi Expression vectors for SMRT, NCoR, HDAC1, HDAC2, and HDAC3 were described previously (31,32).

Transfection and luciferase assay - Cell lines including HEK293, and 3T3-L1 are purchased from the American Type Culture Collection and maintained in cell culture according to the guidelines recommended by the provider. Transient transfection was conducted in triplicate in 12-well plates. Cells (1.5 X 10^5/well) were plated for sixteen hours and transfected with plasmid DNA utilizing Lipofectamine2000. The PPARγ reporter system was constituted utilizing 0.2 µg each of PPRE (3x)-luciferase, PPARγ2, and RXRα in each well. In cotransfection assay, 0.2 µg of plasmid DNA was used unless indicated in the figure legend. The cells were treated with 1 µM troglitazone for 16 hours to activate PPARγ2 after transfection for 24 hours. For TNF- treatment, the cells were treated with troglitazone in serum-free medium for 16 hours, followed by TNF-treatment for 5 hours. In all of the transient transfection experiments, the internal control was 0.1 µg/well of SV40-renilla luciferase reporter plasmid, and the total DNA concentration was corrected in each well with a control plasmid. The luciferase assay was conducted using the dual luciferase substrate system (Promega) with a 96-well luminometer. The luciferase activity was normalized with the internal control Renilla luciferase, and a mean value together with a standard error of the triplicate samples was used to determine the reporter activity. Each experiment was repeated at least three times.

sslkBα cell line and 3T3-L1 adipocytes - To make the sslkBα stable cell line, 3T3-L1 fibroblast was infected with pBabe retrovirus that carries the Flag-sslkBα expression cassette. The positive clone was selected by culturing the infected cells in puromycin-containing medium for two days and followed by a screening of flag epitope in the whole cell lysate in an immunoblot. 3T3-L1 adipocytes were obtained by differentiation of the fibroblasts in the standard adipogenic cocktail as described elsewhere (33). The control cells were made by infection of 3T3-L1 fibroblasts with the empty retrovirus.

Immunoblotting (IB) and Co-immunoprecipitation (Co-IP) - The whole cell lysate, nuclear and cytoplasmic extracts were made as described elsewhere (29). In IP, the condition is as follow: the cell extract (400 µg), 2-4 µg antibody, and 20 µl protein G sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ), and incubation for 3-4 hours at 4 ºC. The immune complex was washed five times in a cell lysis buffer before being used for immunoblotting. The product was resolved in 7% SDS-PAGE and transferred onto PVDF membrane for immunoblotting. Blotting of the membrane was conducted in a milk buffer with first antibody for 1-24 hours and HRP-conjugated secondary antibody for 30 minutes. To detect multiple signals from one membrane, the membrane was treated with a stripping buffer (59mM Tri-
Cytoplasmic and nuclear extracts - The cytoplasmic and nuclear proteins were prepared as described in an earlier study (34). IB and Co-IP were conducted according to protocols used previously (29).

Electrophoretic mobility shift assay (EMSA) - The HEK293 cells (2 x10^6) were plated in the 100 mm cell culture plate, and transfected with PPARγ2 plasmid DNA (1 µg) with lipofectamine2000 as indicated. In cotransfection experiments, 1 µg of each test plasmid was used unless otherwise indicated. The nuclear extract was made from 3T3-L1 adipocytes or HEK293 cells transfected with expression vectors for PPARγ2 and RXRα. The EMSA probes for PPARγ contains the PPRE sequence in the acyl-coA synthase promoter. The assay was conducted as described elsewhere (34). The EMSA probes for PPARγ contains the PPRE element in the acyl-coA synthase promoter. The probe was labeled with 32-P and used in EMSA with the nuclear extract. For oligonucleotide competition and antibody supershift experiments, a 50-fold excess of unlabelled oligonucleotide probe and 2 µg IgG were used at each point, respectively.

Chromatin immunoprecipitation (ChIP) - ChIP protocol was developed from a published study (35). Cells were maintained in 100 mm cell culture plate, treated with TNF-α (10 ng/ml) after serum-starvation overnight, and collected after formaldehyde treatment. The chromatin DNA was extracted, broken into fragments of 400-1200bp in length by sonication, and immunoprecipitated with antibodies to the target, such as PPARγ, HDAC3, SMRT or and Pol II. IgG was used in IP as a control for non-specific signal. DNA in the IP product was amplified in PCR with the ChIP assay primers that cover the two PPRE sites (ARE6 and ARE7) in the aP2 gene promoter (36): Forward 5' GGAATCAGGTAGCTGGAGAATCGC 3'; reverse 5' GGCTTGATTGTTACAAGGCAAGGAA 3'. This pair of primer has an annealing temperature of 59 °C, and yields a product of 280 bp. The image of PCR product is presented in reversed black/white in which DNA band is in black. The PCR products were quantitated for the signal intensity.

Quantitative real time RT-PCR - mRNA level of aP2 (fatty acid binding protein 4) was determined using Taqman quantitative real time RT-PCR. The total RNA was extracted using the Trizol protocol. The real time RT-PCR reaction was conducted in triplicates using Taqman probe and primers set for aP2 (Mm00445880_m1, Applied Biosystems). β-actin was used as a control for normalization of aP2 signal. The mean value of the triplicates was used to indicate mRNA level of aP2.

Statistical analysis - Each experiment was conducted at least three times with consistent result. The representative gel or blot is presented in this manuscript. In the PPARγ reporter assay, a mean value and standard deviation of the triplicates is used to represent the reporter activity. The data was analyzed using student’s T-test with significance p<0.05.

RESULTS
ssIkBα abolishes TNF-inhibition of PPARγ function in reporter assay - It is well known that TNF-α induces lipolysis in mature adipocytes, and inhibits differentiation of preadipocytes. Since PPARγ controls transcription of a variety of genes for lipid biosynthesis and adipogenesis, PPARγ has been a target in search for the molecular mechanism of TNF-α activity. In addition to the inhibition of PPARγ expression, TNF-α may reduce the transcriptional activity of PPARγ through two possibilities: (a) Phosphorylation of PPARγ by activation of serine kinases (ERK or JNK) (4,25,26,37); (b) Suppression of DNA-binding of PPARγ by activation of NF-kB (7). In the regulation of PPARγ function, TNF-α activity was investigated with a focus on the role of IkBα in this study. The initial observation was made using a PPARγ reporter system in transient transfection of HEK293 cells, in which the reporter activity was induced by the synthetic ligand troglitazone (Fig. 1A). TNF-α led to 40% reduction in the PPARγ reporter. The reduction was completely blocked by expression of the super suppressor IkBα (ssIkBα), a non-degradable mutant of IkBα (IkBαS32A,S36A). A similar result was observed when TNF-α was replaced by IKK2 in the reporter system (Fig. 1B). Since ssIkBα is not degradable, these results suggest that the TNF-α activity is dependent on degradation of IkBα protein. In support of this possibility, the proteasome inhibitor MG132 that blocks IkBα degradation also eliminated activity of TNF-α or IKK2 (Fig. 1C). The experiment was repeated using 3T3-L1 cells and a similar result was obtained (Fig. 1D), suggesting that the role of IkBα is not influenced by cell types. Of interest, ssIkBα was able to enhance the reporter activity in the absence of TNF-α (Fig. 1A). Therefore, the molecular basis of ssIkBα activity was further investigated in this study.

Nuclear corepressor is involved in TNF-α activity - Transactivation by PPARγ is regulated by events such as the association of nuclear receptor coactivator and the DNA-binding activity of PPARγ. The coactivator association is determined by the corepressor that is composed of HDAC3/SMRT or HDAC3/NCoR. The corepressor is associated with PPARγ in the absence of PPARγ ligand (38,39). To investigate how PPARγ function is repressed after IkBα degradation, the DNA binding activity of PPARγ was examined using EMSA assay. The assay was done using the nuclear extract of 293 cells transfected with PPARγ. The result shows that the PPARγ DNA-binding activity was not reduced by either TNF-α or ssIkBα (Fig. 2A), suggesting that the suppression of PPARγ activity by TNF-α is not a result of loss of DNA-binding activity. Since ssIkBα did not change the DNA-binding activity, the enhancement of PPARγ activity by ssIkBα in the absence of TNF-α may be achieved through a DNA-independent mechanism. These data suggest that TNF-α can modulate PPARγ function through a mechanism independent of DNA-binding. Nature of the PPARγ/DNA complex was confirmed in oligo competition and supershift assay (Fig. 2B).

To examine the role of corepressor complex, we tested HDAC1, HADC2, HDAC3, SMRT, and NCoR with RNAi-mediated gene knockdown. The specificity and efficacy of the RNAi vectors used here were all confirmed in previous studies by determining the target protein in the whole cell lysate (31,32,40). The data
for HDAC3 RNAi is presented here as an example to show that the knockdown is significant in this experimental system (Fig. 2D). To save space, the knockdown results for HDAC1, HDAC2, SMRT and NCoR are not shown here. We focused on HDAC3 and SMRT in this study since significant effects were observed with knockdown by these two RNAi. Inhibition of either HDAC3 or SMRT led to abolishment of TNF-α activity in the suppression of PPARγ (Fig. 2C), suggesting that HDAC3 and SMRT are the major corepressor proteins for PPARγ. A decrease in NCoR also led to partial protection of PPARγ function. In the same condition, inhibition of HDAC1 and HDAC2 failed to generate the effect. Since inhibition of the corepressor protein HDAC3, SMRT or NCoR led to protection of PPARγ activity, the data suggest that TNF-induced inhibition of PPARγ is dependent on the corepressor function.

**TNF-α induces recruitment of the corepressor proteins to PPARγ** - It is believed that association of the corepressor with PPARγ is required for inhibition of the transcriptional activity of PPARγ. Above data suggest that TNF-α may act by enhancing recruitment of the corepressor to PPARγ. To test this possibility, the association of the corepressors with PPARγ was examined in the aP2 (FABP-4) gene promoter using ChIP assay. In mature 3T3-L1 adipocytes, the signal of HDAC3 was increased in the gene promoter after TNF-treatment (Fig. 3A). This effect of TNF-α was reduced by Troglitazone (Trog), which decreased the HDAC3 signal and increased the signal for RNA polymerase II (Pol II), an indicator of transcription initiation. Similarly, SMRT signal was also increased by TNF-α (Fig. 3A), and the increase was also attenuated by Troglitazone. This line of evidence suggests that TNF-α acts through increasing association of the corepressor HDAC3/SMRT with PPARγ. Although the corepressor association was modulated by TNF-α, the DNA-binding activity of PPARγ was not changed before and after TNF-treatment (Fig. 3A). In the EMSA assay, DNA-binding activity of PPARγ was examined in the adipocyte nuclear extract. Similar to that observed in 293 cells, no significant reduction was observed for PPARγ (Fig. 3B). These data consistently support that corepressors, but not DNA-binding, was involved in the PPARγ inhibition by TNF-α.

**HDAC3 is a component of the IkBα/NF-κB complex** - The data above suggest that the recruitment of HDAC3 and SMRT may be responsible for PPARγ inhibition by TNF-α. We hypothesized that the recruitment might be related to IkBα degradation since the TNF-α activity was abolished by ssIkBα. IkBα degradation may contribute to the enhanced recruitment of corepressors by changing total protein or intracellular distribution of HDAC3/SMRT. To test these possibilities, the total protein for HDAC3 and SMRT was determined in the whole cell lysate of 3T3-L1 adipocytes. The result does not support a change in the protein abundance after TNF-α treatment (Fig. 4A). Intracellular distribution of HDAC3 and SMRT was determined by examining their protein abundance in the cytoplasmic and nuclear extracts. Before TNF-treatment, the HDAC3 protein was detected in both cytoplasmic and nuclear extracts (Fig. 4B). With TNF-treatment, HDAC3 level was significantly decreased in the cytoplasm, but increased in the nuclear extracts at 15 minutes (Fig. 4B). Such a quick and correlated HDAC3 change in the two different compartments...
of cell suggests a role of nuclear translocation in the regulation of HADC3 redistribution. The peak time of HDAC3 in the nucleus is correlated to that of HDAC3 recruitment in the aP2 promoter, which was observed in the ChIP assay. In the experiment, HDAC1 and SMRT were only detected in the nucleus, and their subcellular distribution was not influenced by TNF-α (Fig. 4B). According to these data, we hypothesized that HDAC3 might be important in the inhibition of PPARγ by TNF-α.

HDAC3 translocation may be controlled by IkBα since TNF-α activity is dependent on IkBα degradation. To test this possibility, the study was focused on HDAC3-IkBα interaction since the nuclear translocation may be a consequence of IkBα degradation. The association of HDAC3 with IkBα was investigated using co-immunoprecipitation (Co-IP) (Fig. 4C). The experiment was conducted using the cytoplasmic extract of 293 cells. As expected, HDAC3 was found in the IP product of IkBα antibody, and IkBα was identified in the IP product of HDAC3 antibody. These data suggest that IkBα and HDAC3 coexist in the same protein complex in cells. However, in this experiment, SMRT was not detected in the IP product of either IkBα or HDAC3, suggesting that HDAC3 does not associate with SMRT in the cytoplasm. This is consistent with the observation that SMRT was not detected in the cytoplasm (Fig. 4B).

IkBα controls nuclear translocation of HDAC3 - The functional significance of IkBα-HDAC3 association may be retention of HDAC3 in the cytoplasm. If this hypothesis is correct, HDAC3 should stay in the nucleus in the absence of IkBα. To test this possibility, IkBα−/− MEF cells were compared with the wild type (WT) MEFs in this study. In the wild type MEFs, the HDAC3 protein was found in both cytoplasmic and nuclear extracts by immunoblotting (Fig. 5, A and B). With TNF-treatment, the decrease in cytoplasmic HDAC3 was associated with IkBα degradation (Fig. 5A). Corresponding to the decrease in the cytoplasm, HDAC3 was increased in the nucleus. This was accompanied by nuclear translocation of NF-kB p65 (Fig. 5B). As a control, HDAC1 was not detected in the cytoplasmic, and its abundance was not changed in the nucleus after TNF-α treatment. In IkBα−/− MEF cells, the HDAC3 protein was not detectable in the cytoplasm (Fig. 5, A and B), but only detected in the nuclear extract. The nuclear abundance of HDAC3 was significantly higher than that of WT cells. In IkBα−/− cells, TNF-α was unable to increase the nuclear abundance of HDAC3. The data suggest that IkBα is required for the cytoplasmic localization of HDAC3. In the IkBα null cells, HDAC1 abundance was reduced. This suggests that IkBα also regulates HDAC1 activity. Since HDAC1 has not been reported in the regulation of PPARγ function, HDAC1 was not further investigated here. Quality of the cytoplasmic and nuclear extracts is indicated by the control signals such as actin in the cytoplasm and Sp3 in the nucleus.

Distribution of HDAC3 was corrected in IkBα−/− MEFs by reconstitution of IkBα. Regulation of HDAC3 by IkBα was further examined using a GFP-HDAC3 fusion protein (Fig. 5C). In the wild type MEFs, GFP-HDAC3 was detected in both cytoplasmic and nuclear compartments after transient transfection with a GFP-HDAC3
expression vector. GFP-HDAC3 was enriched in the cytoplasm by coexpression of ssIkBα. In transient transfection of IκBα−/− MEFs, GFP-HDAC3 was concentrated in the nucleus, and was not observed in the cytoplasm. Coexpression of ssIkBα led to GFP-HDAC3 decrease in the nucleus, and an increase in the cytoplasm. These data further support the role of IκBα in the control of subcellular distribution of HDAC3. The IκBα activity was also observed in 3T3-L1 cells that were engineered with Flag-ssIkBα. The cytoplasmic abundance of HDAC3 was significantly increased in the Flag-ssIkBα cells in comparison to the control 3T3-L1 cells (Fig. 5D). This change was associated with a decreased in nuclear HDAC3. As expected, TNF-induced nuclear translocation of HDAC3 was not detectable in this stable cell line. Collectively, the data consistently support that IκBα regulates nuclear translocation of HDAC3.

 ssIkBα promotes lipid accumulation in 3T3-L1 adipocytes - The observations above suggest that ssIkBα should be able to promote PPARγ function by limiting HDAC3 access to PPARγ. If this is correct, the ssIkBα-3T3-L1 cells should exhibit an accelerated lipid accumulation during adipogenesis, and become resistant to the inhibitory activity of TNF-α. To test these possibilities, adipogenesis was induced in the control and ssIkBα cells (3T3-L1) in the absence or presence of TNF-α. Without TNF-α, both cell lines are well differentiated into adipocytes after exposure to the adipogenic cocktail (Fig. 6A). However, the lipid droplets were much bigger in the ssIkBα cells as indicated in the picture under a microscope. In the presence of TNF-α, differentiation of ssIkBα-cells was not inhibited as indicated by the expression of molecular markers of adipocytes, such as adiponectin, aP2, PPARγ, and GLUT4 (Fig. 6B). Expression of these markers was significantly reduced by TNF-α in the control cells, but not in the ssIkBα cells, suggesting that ssIkBα cells are resistant to TNF-α. In this study, cells were exposed to TNF-α along the course of adipogenesis for 7 days. This chronic treatment led to inhibition of PPARγ protein expression as indicated by its protein level (Fig. 6B, PPARγ), suggesting that TNF-α may block differentiation of preadipocytes into adipocytes through suppression of PPARγ expression. This activity of TNF-α might be dependent on activation of the NF-κB pathway since inhibition of NF-κB pathway by ssIkBα leads to resistance to TNF-α. HDAC3 translocation may contribute to this inhibition.

To test TNF-α inhibition of the ligand-dependent activity of PPARγ, the interaction of HDAC3 and PPARγ was examined in 3T3-L1 adipocytes using ChIP assay. In the absence of TNF-α, the interaction was detectable in the control adipocytes, but significantly reduced in the ssIkBα adipocytes (Fig. 6C). In the presence of TNF-α, the interaction was significantly enhanced by TNF-α in the control cells (Fig. 6C). In the ssIkBα-cells, the TNF-mediated enhancement was remarkably reduced. The change in HDAC3-PPARγ interaction was correlated to the Pol II signal that was reduced in the control cells, but not in the ssIkBα cells. DNA-binding activity of PPARγ was not changed by TNF-α in either cell line as PPARγ signal was not reduced. Since nuclear translocation of HDAC3 was blocked by ssIkBα, these results suggest that in adipocytes, HDAC3 translocation is...
responsible for the enhanced interaction between HDAC3 and PPARγ, and ssIkBα is able to block the translocation induced by TNF-α.

The interaction of HDAC3 and PPARγ is reflected in the transcriptional suppression of PPARγ target gene. aP2 is a well-established target gene for PPARγ. The aP2 expression was reduced by TNF-α in the control adipocytes, but not in the ssIkBα adipocytes (Fig. 6D), suggesting that HDAC3 is required for TNF-mediated inhibition of PPARγ target gene expression. Collectively, these data suggest that the TNF-α uses two different mechanisms in the inhibition of adipogenesis and adipocyte de-differentiation. In the inhibition of adipogenesis, TNF-α blocks gene expression of PPARγ leading to a low level of PPARγ protein. In the mature adipocytes, TNF-α induces de-differentiation through inhibition of the ligand-dependent PPARγ activity. Nuclear translocation of HDAC3 is required for the latter activity of TNF-α. HDAC3 translocation may be involved in both mechanisms since IkBα degradation is required in either case.

DISCUSSION

IKK is a major kinase mediating TNF-regulation of PPARγ function. TNF-α is able to activate several serine kinases that have been reported to regulate the ligand-dependent activity of PPARγ. Three of the kinases (IKK, ERK and JNK) were shown to inhibit (4,7,8,25,26,37), but one (p38) to enhance the function of PPARγ (41-44). Although all of the four serine kinases are activated by TNF-α (45), results from this and other studies suggest that IKK is a dominant kinase in the regulation of PPARγ function by TNF-α (7,8). The key evidence is that inhibition of PPARγ function by TNF-α is completely blocked by inactivation of IKK or its downstream event. The IKK/NF-kB signaling pathway also represents a major avenue for IL-1 inhibition of PPARγ function (5,7,8,46). ERK and JNK were shown to inhibit PPARγ function by a direct phosphorylation of serine residues in PPARγ (4,25,26,37), such as Ser112 in PPARγ2 (4). Since ERK and JNK are the major kinases in the signaling pathways of EGF (epidermal growth factor) and FGF (fibroblast growth factor) (47), these kinases may play an important role in the inhibition of adipogenesis by EGF and FGF (48-50).

HDAC3 is required for the inhibition of ligand-dependent activity of PPARγ by TNF-α. Our data support that TNF-α is able to inhibit PPARγ activity at two different levels. In the chronic (>16 hrs) treatment, TNF-α reduces expression of PPARγ in adipocytes. In the acute treatment, TNF-α inhibits the ligand-dependent activity without decreasing PPARγ expression or its DNA-binding activity. Results from this and other studies suggest that both chronic and acute inhibition are dependent on the IKK/NF-kB pathway (6,8). Regarding the acute effect, NF-kB was reported to reduce the DNA-binding activity of PPARγ (7). Protein-protein interaction between NF-kB and PPARγ was proposed to mediate the inhibition, and PGC-2 was shown to be required for the NFkB-PPARγ interaction. Our data from ChIP and EMSA assays demonstrated that the DNA-binding activity of PPARγ was not changed by TNF-α in the acute treatment. The data is consistent in adipocytes and 293 cells. The only change induced by TNF-α was an
increased association of PPARγ with HDAC3/SMRT. When this change was blocked by sIskBα, TNF-α lost its inhibitory activity in PPARγ. In 293 cells, knockdown of either HDAC3 or SMRT led to abolishment of TNF-α activity. These data suggest that the nuclear receptor corepressor is the target of TNF-α. Since TNF-α induces nuclear translocation of HDAC3, TNF-α may regulate the corepressor function through HDAC3. Recently, IKK has been shown to modify SMRT activity through direct phosphorylation of SMRT protein (51). This event may also contribute to TNF-inhibition of PPARγ, but our data suggests that HDAC3 nuclear translocation is required for the inhibition. We observed that the TNF-activity was completely blocked by sIskBα, which should not influence IKK activity if phosphorylation of SMRT is indeed induced by IKK. It is not clear if TNF-α can modify the phosphorylation status of HDAC3. If this does happen, nuclear translocation of HDAC3 is still necessary. The role of nuclear corepressor is also supported by our observation that the TNF-α inhibition was attenuated by overexpression of the nuclear receptor coactivators (Gao Z and Ye J, unpublished data). Overexpression of the coactivators is able to rescue PPARγ function in the presence of TNF-α. It is known that coactivator is able to antagonize corepressor activity through increasing histone acetylation.

Nuclear translocation of HDAC3 is controlled by IkBα in the IkB-HDAC3 model (Fig. 7A). HDAC3 contains both nuclear export signal (180-313 aa in the central portion), and the nuclear localization signal (312-428 aa in the C-terminal) (52). The protein structure suggests that HDAC3 may shuttle between the cytoplasm and nucleus by itself. However, the molecular events that initiate the shuttling are not clear. Our data suggests that IkBα is important in the control of HDAC3 shuttling. When IkBα is degraded, HDAC3 enters the nucleus; when newly-synthesized IkBα is available in the nucleus, HDAC3 is bound to IkBα and subject to nuclear export. This molecular model is supported by four lines of evidence: (a) Nuclear translocation of HDAC3 is coupled with IkBα degradation; (b) in IkBα−/− cells, HDAC3 is exclusively located in the nucleus; (c) sIskBα retains HDAC3 in the cytoplasm and reduces the nuclear abundance of HDAC3; (d) HDAC3 associates with IkBα through the ankyrin repeat domain of IkBα (53). Since HDAC3 is able to regulate transcription of a variety of genes (54,55), the IkB-HDAC3 model may provide new explanation to many phenomena that have been reported for IkBα, such as death of newborn mice with IkBα knockout (IkBα−/−) (56), inhibition of limb development by IkBα in drosophila (57), and enhancement of transcriptional activity of non-kB transcription factors by IkBα (53,58).

The role of transcription factor NF-kB in the inhibition of PPARγ activity remains to be investigated. HDAC3 was reported to form a complex with NF-kB p65, and this leads to inhibition of the transcriptional activity of NF-kB through deacetylation (40,59-61). However, it is not clear what mediates the HDAC3-p65 association (59,61). Our data suggest that IkBα may be required for the association since IkBα is able to interact with both proteins. This raises a possibility about control of HDAC3 nuclear translocation by p65. If this is true, we would observe a change in HDAC3 distribution in the absence of p65. However, our data does
not support this possibility. The pattern of intracellular distribution of HDAC3 was not changed in p65−/− MEFs (Fig. 7B). However, p65 may contribute to PPARγ inhibition by blocking IkBα-HDAC3 association in the nucleus (53). The pattern of intracellular distribution of HDAC3 was not changed in p50−/− MEFs (Fig. 7B). These data suggest that p65 and p50 are not required for HDAC3 localization in cells.

In summary, TNF-α inhibits PPARγ activity through two mechanisms. One is dependent on inhibition of PPARγ expression. The other is suppression of the transcriptional activity of PPARγ by nuclear corepressor. IkBα-regulation of corepressor function may be required in both mechanisms. In this study, TNF-α is shown to inhibit the transcriptional activity of PPARγ through a pathway in which DNA-binding activity of PPARγ is not reduced. IkBα plays an important role in this pathway by regulating nuclear translocation of HDAC3. Our observation also supports a new function of IkBα.

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References

**FIGURE LEGEND**

Fig. 1 *Inhibition of PPARγ reporter activity by TNF-α* – The transcriptional activity of PPARγ was analyzed in HEK293 cells and 3T3-L1 fibroblasts using the PPRE(3x)-luciferase reporter system in the transient transfection. The transfection and data analysis were performed as stated in the methods. (A) Inhibition of TNF-α activity by ssIkB in HEK293 cells. The reporter activity was induced with Troglitazone (Trog, 1 µM). TNF-α treatment was conducted at a final concentration of 10 ng/ml unless indicated specifically. (B) Inhibition of IKK activity by ssIkBα in HEK293 cells. (C) Inhibition of TNF-α and IKK activities by proteasome inhibitor MG-132 at a final concentration of 15 µM. (D) Inhibition of TNF-α activity by ssIkBα or MG-132 in 3T3-L1 adipocytes.

Fig. 2 *HDAC3 and SMRT in TNF-induced PPARγ inhibition* - (A) EMSA for DNA-binding activity of PPARγ. The assay was done with the nuclear extract of HEK293 cells that were transfected with the PPARγ2 expression vector. ssIkBα expression vector was co-transfected at dosages (µg) indicated at the top of each lane. The nuclear extract was made after two hour treatment with TNF-α (10 ng/ml). (B) Confirmation of PPARγ-DNA complex in supershift assay. (C) Analysis of corepressors with RNAi-mediated gene knockdown in the PPARγ reporter assay. RNAi-expression vectors for HDAC1-3, NCoR and SMRT were co-transfected with the reporter system into HEK293 cells at 0.2 µg/point. The reporter was induced by Troglitazone (Trog) and inhibited by TNF-α. (D) Test of RNAi effect on HDAC3 expression in an immunoblot. HDAC3 protein level was determined in HEK293 cells transfected with control vector or RNAi expression vector. The dosage (µg) of each vector was shown at the top of each lane.

Fig. 3 *HDAC3 interaction with PPARγ in adipocytes* - (A) ChIP assay for the interaction of HDAC3 and PPARγ in aP2 gene promoter. The assay was conducted in 3T3-L1 mature adipocytes after TNF-α treatment (10 ng/ml, 30 minutes). (B) DNA-binding activity of PPARγ in EMSA. The nuclear extract was made from the mature 3T3-L1 adipocytes after TNF-α treatment at different times as indicated at the top of each lane. Unlabelled PPRE probe or AP-1 probe was used in oligo competition. In these two experiments, Troglitazone (Trog) was used at a final concentration of 1 µM for 24 hour treatment.

Fig. 4 *Nuclear translocation of HDAC3 induced by TNF-α* - (A) The total protein of HDAC3 in 3T3-L1 adipocytes. The mature 3T3-L1 adipocytes were serum-starved overnight and treated with TNF-α for different times as indicated. The whole cell lysate was made and used in the immunoblot. (B) Immunoblot of the cytoplasmic and nuclear extracts of mature 3T3-L1 adipocytes. The cytoplasmic (EC) and nuclear (NE) extracts were made as stated in the experimental procedure. The protein abundance of HDAC3 and SMRT was examined in an immunoblot. HDAC1 and actin were controls in the nuclear and cytoplasmic extracts, respectively. (C) Association of IkBα and HDAC3 in coimmunoprecipitation. The cytoplasmic extract was made from 3T3-L1 adipocytes and used in the Co-IP. IgG was a control for non-specific signal.
Fig. 5 Regulation of nuclear translocation of HDAC3 by IkBα - (A) Immunoblot of the cytoplasmic extract of wild type (WT) and IkBα−/− MEFs. The cells were treated with TNF-α for 15 minutes as indicated. HDAC3 protein was determined in the cytoplasmic extract in an immunoblot. HDAC1 and Sp3 were used as negative controls since they are nuclear proteins. IkBα and actin were positive controls in the cytoplasmic extract. (B) Immunoblot of the nuclear extract of wild type and IkBα−/− MEFs. HDAC1, p65 and Sp3 were positive controls in the nuclear extract. Actin was a negative control. (C) Intracellular distribution of GFP-HDAC3 in WT and IkBα−/− MEFs. The cells were transiently transfected with expression vectors for GFP-HDAC3 and ssIkBα. The pictures were made 48 hour later under a fluorescence microscope with oil lens (100 X). (D) Immunoblot of the cytoplasmic (CE) and nuclear extracts (CE) from the control and ssIkBα cell lines.

Fig. 6 Inhibition of TNF-α activity by ssIkBα in 3T3-L1 adipocytes - (A) Lipid droplet in adipocytes from the control and ssIkBα cells. The picture was made at day 7 of differentiation under a microscope at 400 times of magnification. The large lipid droplets are highlighted by the arrows. (B) Molecular markers of adipocytes in immunoblot. The cells were treated with TNF-α during adipogenesis. Adipogenesis was determined by the adipocyte-specific markers in the whole cell lysate at day 7 and 8 of differentiation. The markers include adiponectin (ApN), fatty acid binding protein 4 (aP2), PPARγ and glucose transporter 4 (GLUT4). (C) HDAC3-PPARγ interaction in adipocytes. Mature adipocytes were obtained by differentiation of 3T3-L1 fibroblasts in the adipogenic cocktail for 7 days. ChIP assay was conducted to determine the interaction in adipocytes after TNF-α treatment for 30 minutes. The cells were serum-starved for 4 hours before exposure to TNF-α (10 ng/ml). (D) Inhibition of aP2 mRNA expression by TNF-α in mature adipocytes. mRNA of aP2 was determined by quantitative real time RT-PCR in 3T3-L1 adipocytes after overnight TNF-α treatment.

Fig. 7 Regulation of HDAC3 cytoplasm-nucleus shuttling by IkBα - (A) IkB-HDAC3 model. HDAC3 stays in the cytoplasm through association with IkBα. After IkBα degradation, HDAC3 enters the nucleus where it inhibits the transcriptional activity of PPARγ through histone deacetylation. When the newly-synthesized IkBα is available, it associates with nuclear HDAC3 and transfers HDAC3 into the cytoplasm. HDAC3 may enter and leave the nucleus through this mechanism. In this model, the role of IkBα is highlighted in the control of cytoplasm-nucleus shuttling of HDAC3. (B) HDAC3 distribution in p65−/− or p50−/− MEFs. HDAC3 abundance was determined in the cytoplasmic and nuclear extracts of the wild type and the knockout MEFs in an immunoblot.
Fig. 1 (Gao)

A. TNF-α

B. IKK2

C. MG132

D. 3T3-L1
Fig. 2 (Gao)

A. EMSA

B. Supershift

C. TNF+RNAi

D. HDAC3 RNAi

Fig. 2 (Gao)
Fig. 3 (Gao)

A. ChIP in aP2

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B. EMSA

- TNF-α

- Probe
- Control
- Trog
- Trog+30′
- Trog+60′
- Cold probe
- AP-1

- PPARγ
- Free probe
- Non-specific
**Fig. 4 (Gao)**

### A. Total protein

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### C. Co-IP

- IP
  - IgG
  - IkBα
  - HDAC3
- IB
  - 1
  - 2
  - 3
Fig. 5 (Gao)

A. CE of IkBα−/−

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B. NE of IkBα−/−

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C. GFP-HDAC3 in IkBα−/−

D. ssIkB-3T3L1

Control          ssIkBα
Fig. 6 (Gao)

### A. Lipid

- **Control**
- **ssIkBα**

- **Oil Red** -stained
- **Unstained**

### B. Adipogenic marker

**TNF-α**
- **Days**
  - 0
  - 7
  - 8
  - 0
  - 7
  - 8
- **Control**
- **ssIkBα**

- **ApN**
- **aP2**
- **PPARγ**
- **GLUT4**
- **Actin**

### C. ChIP

- **Control**
- **ssIkBα**

- **HDAC3**
- **Pol II**
- **PPARγ**
- **IgG**
- **Input**

### D. aP2 mRNA

- **Control**
- **ssIkBα**

- **aP2 mRNA**
  - **P<0.001**
Fig. 7 (Gao)

A. IkB-HDAC3 Model

B. p65/- MEF
Regulation of nuclear translocation of HDAC3 by IkBalpha is required for TNF-inhibition of PPARgamma function
Zhangou Gao, Qing He, Bailu Peng, Paul Chiao and Jianping Ye

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