



HHS Public Access

Author manuscript

J Mol Endocrinol. Author manuscript; available in PMC 2015 August 01.

Published in final edited form as:

J Mol Endocrinol. 2014 October ; 53(2): 191–200. doi:10.1530/JME-14-0066.

Inhibition of HDAC3 promotes ligand-independent PPAR γ activation by protein acetylation

Xiaoting Jiang¹, Xin Ye¹, Wei Guo^{1,2}, Hongyun Lu^{1,3}, and Zhanguo Gao^{1,4}

¹Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana 70808

²Department of Pathology, Shanghai University of Traditional Chinese Medicine, Shanghai, China

³Department of Endocrinology & Metabolism, the Third/Fifth Affiliated Hospital Sun Yat-sen University, Zhuhai, Guangdong, China

⁴Department of Medical Tests, Xinxiang Medical University, Xinxiang, China

Abstract

PPAR γ (peroxisome proliferator-activated receptor gamma) is a nuclear receptor whose activation is dependent on a ligand. PPAR γ activation by exogenous ligands, such as thiazolidinediones (TZDs), is a strategy in the treatment of type 2 diabetes for the improvement of insulin sensitivity. In addition to a ligand, PPAR γ function is also regulated by posttranslational modifications, such as phosphorylation, sumoylation, and ubiquitination. Here, we report that PPAR γ protein is modified by acetylation, which induces the PPAR γ function in the absence of an external ligand. We observed that histone deacetylase 3 (HDAC3) interacted with PPAR γ to deacetylate the protein. In immunoprecipitation, the HDAC3 protein was associated with the PPAR γ protein. Inhibition of HDAC3 using RNAi-mediated knockdown or HDAC3 inhibitor increased acetylation of the PPAR γ protein. Furthermore, inhibition of HDAC3 enhanced expression of PPAR γ target genes such as adiponectin and aP2. The expression was associated with an increase in glucose uptake and insulin signaling in adipocytes. HDAC3 inhibition enhanced lipid accumulation during differentiation of adipocytes. PPAR γ acetylation was also induced by pioglitazone and acetylation is required for PPAR γ activation. In the absence of TZDs, the acetylation from HDAC3 inhibition was sufficient to induce the transcriptional activity of PPAR γ . Treating the Dio mice with HDAC3 inhibitor or pioglitazone for 2 weeks significantly improved high fat diet induced–insulin resistance. Our data suggest that acetylation of PPAR γ is a ligand-independent mechanism of PPAR γ activation. HDAC3 inhibitor is a potential PPAR γ activator for improvement of insulin sensitivity.

To whom correspondence should be addressed: Zhanguo Gao, Pennington Biomedical Research Center, Baton Rouge, Louisiana, USA. Tel.: 225-763-3023, gaoz@pbrc.edu.

Declaration of interest

The authors declare that they have no conflicts of interest concerning this article.

Keywords

type 2 diabetes; insulin sensitivity; metabolic syndrome; adipocytes; adipogenesis; PPAR γ ; posttranslational modifications; histone deacetylase; HDAC inhibitors; acetylation

1. Introduction

PPAR γ is a well-documented transcription factor that plays an important role in the control of glucose and fatty acid metabolism. In the mechanism, PPAR γ induces expression of adipocyte-specific genes and promotes differentiation of preadipocytes through transcriptional activation of target genes (Rosen and Spiegelman 2000). PPAR γ is also required in the maintenance of physiological function of mature adipocytes. Insufficient PPAR γ activity is associated with adipose tissue dysfunction and glucose disorders in metabolic syndrome (Fujiki, et al. 2009). At the molecular level, PPAR γ forms heterodimers with the retinoid X receptor (RXR) when it binds to the gene promoter DNA of target genes. The transcriptional activity of PPAR γ is regulated by ligands that determine PPAR γ interaction with coactivators and corepressors (Berger and Moller 2002). TZD is a synthetic PPAR γ ligand that has been widely used in clinical practice to improve insulin sensitivity in type 2 diabetes. In the absence of ligands, PPAR γ binds the corepressor that is formed by HDAC3 and SMRT/NCOR. Ligand binding leads to disassociation of the corepressor complex and induces recruitment of coactivators. Although TZDs are outstanding PPAR γ ligands with strong therapeutic activities in the treatment of type 2 diabetes, their side effects for the heart and bladder have caused alarm in clinical applications. It is urgent to identify a new PPAR γ activator to replace TZDs in the treatment of type 2 diabetes (Ye 2011). For this reason, we explored a new strategy of PPAR γ activation with a focus on HDAC3 inhibition.

Regulation of PPAR γ protein by direct acetylation is a new topic in the study of PPAR γ function. The PPAR γ function is regulated by posttranslational modifications such as phosphorylation (Hu, et al. 1996), sumoylation (Pascual, et al. 2005), ubiquitination (Anbalagan, et al. 2012; Christianson, et al. 2008; Floyd and Stephens 2002; Hauser, et al. 2000), and histone acetylation (Qiang, et al. 2012; Sugii and Evans 2011). Phosphorylation of PPAR γ at Serine 112 and 273 inhibits the PPAR γ transcriptional activity. Sumoylation of PPAR γ at lysine 107 in the AF1 region and at lysine 395 in the AF2 region (lysine 77 and 365 in PPAR γ 1, respectively) activates PPAR γ by blocking the interaction between the nuclear receptor corepressor of HDAC3 and PPAR γ . Ubiquitination of PPAR γ leads to protein degradation following PPAR γ activation by TZDs (Anbalagan et al. 2012; Christianson et al. 2008; Floyd and Stephens 2002; Hauser et al. 2000). It is largely unknown whether PPAR γ protein is acetylated and, if so, how the PPAR γ function is regulated by acetylation. In this study, we addressed this issue by analysis of PPAR γ protein acetylation.

HDAC3 belongs to the class I HDAC proteins, which play important roles in the regulation of histone protein acetylation in the process of chromatin remodeling and gene transcription. HDACs have three classes, class I (HDAC1, 2, 3, 8, 11), class II (HDAC4, 5, 6, 7, 9, 10) (Huang, et al. 2000), and class III (SIRT1-7) (Blander and Guarente 2004). TSA is a pan-

HDAC inhibitor for class I and class II HDACs. In our previous studies, we reported that HDAC inhibitors such as sodium butyrate and TSA promoted ligand-induced PPAR γ function in adipocytes in vitro (Gao, et al. 2006) and prevented high fat diet-induced obesity in mice (Gao, et al. 2009). HDAC3, a member of class I HDACs, has been reported by our and other laboratories to regulate PPAR γ function in adipocytes (Fajas, et al. 2002; Gao et al. 2006; Guan, et al. 2005; Miard and Fajas 2005). However, it is unknown whether HDAC3 inhibition is sufficient to activate PPAR γ in the absence of classical ligands.

In this study, we found that PPAR γ protein is acetylated. The acetylation was induced by a ligand and decreased by HDAC3. HDAC3 inhibition induced PPAR γ acetylation and activation in the absence of exogenous ligands. The current study suggests that HDAC3 inhibition is a new approach to activate PPAR γ in the absence of exogenous ligands.

2. Materials and methods

2.1. Mouse models and treatment

Diet-induced obesity (Dio) male C57BL/6J mice were purchased from the Jackson laboratory (Bar Harbor, ME) at 16-week-old, which had been fed a high fat diet (HFD, 60% calories as fat, Research Diets D12492) for 10 weeks. The mice were group-housed two to four mice per cage in the animal facility of the Pennington Biomedical Research Center with 12:12-h light-dark cycle and temperature of 22–24°C. The mice had free access to water and diet. The mice were treated with HDAC3 inhibitor by intraperitoneal injection at 10 μ g/kg body weight per day for 2 weeks. Pioglitazone at the dose of 10 mg/kg body weight per day was used as a positive control. The pioglitazone was administered into the diet, and this group of mice was injected with the same amount of DMSO in PBS by intraperitoneal injection every day. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center.

2.2. Cell culture and reagents

The cell lines 3T3-L1 (CL-173) and HEK293 (CRL-1573) were purchased from the American Type Culture Collection and maintained in 10% and 5% fetal bovine serum, Dulbecco's modified Eagle's medium in a 5% CO₂ incubator. The cells were starved in Dulbecco's modified Eagle's medium containing 0.25% fatty acid-free bovine serum albumin overnight before treatment with 150 nM of HDAC3 inhibitor. HDAC3 inhibitor (cat. #EB1003) was purchased from KeraFAST (Boston, MA). Pioglitazone (cat. #E6910) was purchased from Sigma.

2.3. Adipogenesis

3T3-L1 preadipocytes were grown into confluence in a six-well or 100-mm plate. Then they were differentiated into adipocytes using a standard protocol. The 3T3-L1 cells were incubated in the adipogenic cocktail (5 μ g/ml insulin, 0.5 mM isobutylmethylxanthine, and 10 μ M dexamethasone) for 2 days. This was followed by incubation in an insulin-supplemented medium for an additional 4 days. The normal medium was used at day 7 to maintain the adipocytes. Adipogenesis was quantified with oil red O staining, as described previously (Gao et al. 2006).

2.4. Glucose uptake

3T3-L1 preadipocytes (5×10^5 /well) were differentiated into adipocytes in a 12-well plate. After serum starvation in 0.25% BSA DMEM overnight, the cells were treated with HDAC inhibitors, and glucose uptake was measured as described elsewhere (Gao, et al. 2004).

2.5. Immunoblot

Whole cell lysates were prepared by sonication in lysis buffer and used in Western blots, as described elsewhere (Gao, et al. 2002). Antibodies to acetyl-lysine (ab21623), β -Actin (ab6276), HDAC3 (ab2379), and GFP (ab290) were purchased from Abcam (Cambridge, MA). Monoclonal PPAR γ (E-8, sc-7273x) and HA (sc-7392) antibodies were from Santa Cruz.

2.6. Immunoprecipitation (IP) and HDAC assay

IP was carried out using whole-cell lysates (400 μ g of total protein), 2–4 μ g of antibody, and 40 μ l of protein G-Sepharose beads (Amersham Biosciences), as described elsewhere (Gao et al. 2002). Histone deacetylase assay was conducted using a histone deacetylase assay kit (17–320; Upstate). Briefly, PPAR γ was immunoprecipitated and then was used as a substrate in HDAC assay. Recombinant HDAC3 protein (cat. #H00008841, ABNOVA) was added into the reactions as an enzyme in the assay.

2.7. HDAC3 inhibitor specificity test

HDAC3 inhibitor specificity was measured using a Fluor-de-Lys® HDAC3/NCOR1 fluorometric drug discovery kit (BML-AK531, Enzo Life Science) and a Fluor-de-Lys® HDAC1 fluorometric drug discovery assay kit (BML-AK511, Enzo Life Science) as described in the manufacturer's instructions.

2.8. Intraperitoneal insulin tolerance test (ITT)

Fourteen-week-old Dio mice, which had already been given a high fat diet (D12492) for 8 weeks, were purchased from JAX Lab (stock #000664). After quarantine, the mice were divided into three groups. Each group had 8 mice. For 2 weeks, 10 μ g/kg body weight/day of HDAC3 inhibitor was administrated by intraperitoneal injection. Control groups were given PBS with 0.1% DMSO (solvent). Pioglitazone was applied in the diet at the dose of 10 mg/kg body weight/day. ITT was conducted by intraperitoneal injection of insulin (I9278, Sigma) at 0.75 unit/kg of body weight in mice after a 4-h fast, as described elsewhere (Gao et al. 2009). Blood glucose was monitored in the tail vein blood using the FreeStyle blood glucose monitoring system (TheraSense, Phoenix, AZ).

2.9. Transfection and Luciferase Assay

Transient transfection was conducted in triplicate in 12-well plates. HEK293 cells (1.5×10^5 /well) were plated for 16 h and transfected with plasmid DNA utilizing Lipofectamine. The PPAR γ reporter system was constituted utilizing 0.2 μ g each of PPRE (3 \times)-luciferase, PPAR γ 2, and RXR α in each well (Gao, et al. 2006). The cells were treated with 1 μ M pioglitazone or 150nM HDAC3 inhibitor for 16 h to activate PPAR γ 2 after transfection for 24 h. The luciferase assay was conducted using the luciferase substrate system (Promega)

with a 96-well luminometer (Gao, et al. 2006). Each experiment was repeated at least three times.

2.10. Statistical analysis

All experiments were repeated independently at least three times with consistent results. For most figures, a representative bar graph showed the mean \pm S.E. of multiple independent experiments normalized to appropriate controls. Student's t-test or one-way analysis of variance was used as appropriate in statistical analyses of the data. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. PPAR γ is acetylated in adipocytes

PPAR γ function is regulated by posttranslational modifications, such as phosphorylation (Hu et al. 1996), sumoylation (Geiss-Friedlander and Melchior 2007; Yang and Gregoire 2006), and ubiquitination (Anbalagan et al. 2012; Christianson et al. 2008; Floyd and Stephens 2002; Hauser et al. 2000). However, whether PPAR γ can be regulated by acetylation is not well known. We examined PPAR γ acetylation in 3T3-L1 adipocytes before and after differentiation. The acetylation was examined in an immunoblot with the acetylation-specific antibody after PPAR γ isolation by immunoprecipitation (IP) (Fig. 1). In the undifferentiated cells, the purified PPAR γ protein exhibited no significant signal in the acetylation assay (Fig. 1). In the differentiated cells, there are two isoforms of PPAR γ , PPAR γ 1 and PPAR γ 2, with different sizes of molecular weight. Both isoforms of PPAR γ proteins expressed a strong signal of acetylation (Fig. 1). The levels of acetylation were identical between PPAR γ 1 and PPAR γ 2. The data suggest that PPAR γ protein is acetylated in the adipocytes.

3.2. HDAC3 regulates PPAR γ acetylation

It is generally believed that PPAR γ is associated with the nuclear receptor corepressor in the absence of a ligand. Disassociation of the corepressor complex is induced by the interaction of ligand PPAR γ , which is required for recruitment of coactivators and acetylation of histone proteins in the initiation of gene transcription. The corepressor contains HDAC3 and SMRT or NCoR. HDAC3 inhibits the transcription by deacetylating histone proteins (Gao, et al. 2005; Hu, et al. 2001; Krogsdam, et al. 2002; Treuter, et al. 1998; Zamir, et al. 1997). However, it is unknown whether HDAC3 regulates acetylation of PPAR γ protein. To address this issue, we examined HDAC3–PPAR γ interaction in cells through immunoprecipitation. In the study, GFP-tagged HDAC3 was expressed, together with HA-tagged PPAR γ , in a transient cotransfection of HEK293 cells. Antibodies to GFP and HA were used to isolate HDAC3 and PPAR γ in IP, respectively. We observed that the HDAC3 product contained PPAR γ and that PPAR γ product contained HDAC3 (Fig. 2A). The data suggest that HDAC3 physically interacts with PPAR γ . To test HDAC3 in deacetylation PPAR γ , we determined their enzyme and substrate relationship. In the assay, a recombinant HDAC3 protein (cat. #H00008841, ABNOVA) was used as the deacetylase enzyme. Acetylation was induced in HA-tagged PPAR γ 2 in HEK 293 cells with HDAC inhibitor TSA (200nM for 30 minutes). When HDAC3 was inhibited with a chemical inhibitor

(HD-75) in a deacetylation assay in test tube, PPAR γ acetylation was preserved by the inhibitor (Fig. 2B). The acetylation was reduced in the absence of the inhibitor (Fig. 2B). The data suggest that as a protein deacetylase, HDAC3 directly deacetylates PPAR γ protein.

We tested the specificity of HDAC3 inhibitor HD-75 (Fig. 2C) using a Fluor-de-Lys® HDAC3/NCOR1 fluorometric drug discovery kit and a Fluor-de-Lys® HDAC1 fluorometric drug discovery assay kit. HDAC1 assay kit was used as a control for the specificity of HDAC3 inhibitor. These kits are ideal for chemical library screening for candidate inhibitors. In the HDAC3 assay, the deacetylase activity of HDAC3 was inhibited by the HDAC3 inhibitor at IC₅₀ of 150nM. In this dosage, it only slightly inhibited HDAC1 activity (Fig. 2D). This suggested that HD-75 has specificity for HDAC3.

PPAR γ acetylation was examined by the inhibition of HDAC3 using HDAC3 inhibition in 3T3-L1 adipocytes. We tested that HD-75 has a strong induction of PPAR γ acetylation at the dose of 150nM in 3T3-L1 adipocytes. In the study, 3T3-L1 adipocytes were treated with 150nM of HDAC3 inhibitor for two hours. The cells were harvested and 500ug of the whole cell lysates protein was used in IP with anti-acetyl-lysine antibody. We detected a stronger PPAR γ signal in the IP product in the sample treated by the HDAC3 inhibitor. PPAR γ acetylation was significantly enhanced by HDAC3 inhibitor also in the blot (Fig. 2E). 40ug of protein from 500ug/500ul IP samples was used for the loading control indicated by the β -Actin in the immunoblot (Fig. 2E, lane 3). The data suggest that PPAR γ acetylation was regulated by the HDAC3 inhibitor in cells.

PPAR γ activation by the ligands was reported to induce degradation of the PPAR γ protein (Floyd and Stephens 2002; Hauser et al. 2000). It is unknown whether the ligand induces PPAR γ acetylation. We addressed this question by examining PPAR γ acetylation in pioglitazone-treated 3T3-L1 adipocytes. In cells, pioglitazone enhanced PPAR γ acetylation (Fig. 2F). The data suggest that pioglitazone induces PPAR γ acetylation. Acetylation of PPAR γ by inhibition of HDAC3 did not cause PPAR γ degradation. This suggests that acetylation of PPAR γ by inhibition of HDAC3 may be different from pioglitazone-induced acetylation of PPAR γ .

3.3. HDAC3 inhibitor induces PPAR γ Acetylation at multiple lysine sites

PPAR γ acetylation sites were analyzed by Mass Spectrometry. In the study, PPAR γ was expressed in a transient transfection of HEK293 cells. Antibody to PPAR γ was used to isolate PPAR γ in IP, respectively. PPAR γ in the IP product was purified by SDS-Page. These analyses were performed by the Proteomics Core facility at Applied Biomics, Inc. (23785 Cabot Blvd. Suite 311 Hayward, CA 94545). We used their service and detected four acetylated lysine residues in PPAR γ (Figure 3). Four acetylated lysine sites (K289, K386, K462 and K466) were detected by Mass Spectrometry (lower panel). The data confirmed that PPAR γ acetylation was induced by HDAC3 inhibitor. Further study is needed for the function of acetylated lysine sites in PPAR γ .

3.4. Acetylation induces PPAR γ function

To test acetylation modification in the regulation of PPAR γ function, we determined the transcriptional activity of PPAR γ by quantifying expression of the target genes. PPAR γ

acetylation was induced in 3T3-L1 adipocytes through inhibition of HDAC3 activity with gene knockdown. A vector-based GFP-positive RNAi to HDAC3 was delivered by adenovirus, which infected 3T3-L1 adipocytes with 90% efficiency (Fig. 4A). The efficiency was quantified for GFP-positive cells under the fluorescence microscope. Expression of PPAR γ target genes was determined in an immunoblot. In the control cells that were infected with the control virus, HDAC3 protein was observed with an abundant protein band. In the cells infected by RNAi virus, HDAC3 was reduced by 90% according to the decreased signal of HDAC3 protein band (Fig. 4B). Knockdown of HDAC3 significantly enhanced expression of PPAR γ responsive genes, including aP2 (FABP4) (Fig. 4B). The results suggest that HDAC3 inhibition promotes the transcriptional activity of PPAR γ in adipocytes.

HDAC3 knockdown promotes adipogenesis. PPAR γ induces expression of a variety of genes in the pathways for lipid biosynthesis and storage, which is required for differentiation of preadipocytes. Adipogenesis is often used to determine PPAR γ function. We conducted an adipogenesis to test the approach of HDAC3 inhibition in the regulation of PPAR γ function. HDAC3 was inhibited in 3T3-L1 preadipocytes by gene knockdown using the adenovirus RNAi delivery system (Fig. 4C).

Adipogenesis was induced at 48 h after virus infection. We observed the green fluorescence in cells from day 1 to day 8 in adipogenesis (data not shown). Lipid accumulation was quantified by oil red O staining in the differentiated cells 8 days later. HDAC3 knockdown increased the lipid content by 30% in this adipogenesis system (Fig. 4D). The data suggest that inhibition of HDAC3 promotes lipid accumulation in adipocytes. The result further supports that inhibition of HDAC3 is an approach to promote PPAR γ function.

3.5. HDAC3 inhibitor promotes adipogenesis

A chemical inhibitor of HDAC3 was tested in the regulation of adipogenesis in an effort to identify a new PPAR γ activator independent of TZDs. In the study, activation of PPAR γ by the HDAC3 inhibitor was tested in the adipocyte differentiation model. 3T3-L1 adipocytes were differentiated in the standard adipogenic cocktail. The HDAC3 inhibitor was added to the culture medium at 150 nM during adipogenesis. Pioglitazone was used as a positive control. At the end of differentiation, the degree of differentiation was determined by oil red O staining of intracellular lipids. The HDAC3 inhibitor enhanced lipid accumulation in the cells, as indicated by the results (Fig. 5A). Oil red O staining was enhanced by 50% in the inhibitor- and pioglitazone-treated groups (Fig. 5A lower panel). HDAC3 inhibitor has the same effect with pioglitazone in inducing adipogenesis. PPAR γ target genes including adiponectin and aP2 were enhanced by the inhibitor and pioglitazone in western blot (Fig. 5B). Comparing the HDAC3 inhibitor with pioglitazone, HDAC3 inhibitor enhanced more adiponectin expression. Adiponectin is known to promote insulin sensitivity. In reporter assay, HDAC3 inhibitor has the same effect with pioglitazone in inducing PPAR γ transcriptional activity (Fig. 5C). The data suggest that the HDAC3 inhibitor activates PPAR γ function in the absence of exogenous ligands and the potency of HDAC3 inhibitor is similar to that of pioglitazone.

3.6. Inhibition of HDAC3 enhances insulin sensitivity

To further investigate the effect of HDAC3 inhibitor in the activation of PPAR γ , first, we used glucose uptake to determine PPAR γ function, which may enhance glucose uptake by induction of IRS-2 and Glut4 in the insulin-signaling pathway. Pioglitazone was used as a positive control. The results indicated that PPAR γ ligand increased insulin-induced glucose uptake in 3T3-L1 adipocytes (Fig. 6A). The HDAC3 inhibitor exhibited the same activity in the promotion of glucose uptake (Fig. 6A). Insulin signaling activity was examined by Akt Serine 473 phosphorylation thereafter. Insulin-induced phosphorylation of Akt Serine 473 was enhanced by the HDAC3 inhibitor (Fig. 6B). To test that HDAC3 inhibitor enhances insulin sensitivity in vivo, we treated the Dio mice with HDAC3 inhibitor by intraperitoneal injection at 10 μ g/kg body weight per day for 2 weeks. The treatment was given to 16-week-old Dio mice, which had been fed a high fat diet for 10 weeks. In our previous studies, feeding the B6 mice for 12 weeks with a high fat diet induced insulin resistance. Pioglitazone at the dose of 10 mg/kg body weight per day was used as a positive control. The pioglitazone was administered into the diet, and this group of mice was injected with the same amount of DMSO in PBS by intraperitoneal injection every day. After 2 weeks of treatment, insulin sensitivity was evaluated by an insulin tolerance test after 4 h of fasting. HDAC3 inhibitor and pioglitazone both significantly reduced glucose levels and enhanced insulin sensitivity (Fig. 6C). Body weight and food intake were not changed in the mice by the 2 week treatment. The results suggest that the HDAC3 inhibitor enhanced PPAR γ function to serve as an insulin sensitizer in adipocytes.

4. DISCUSSION

Our data suggest that acetylation of PPAR γ is induced by pioglitazone. Pioglitazone induces PPAR γ activation through recruitment of coactivators and disassociation of corepressors. The coactivators contribute to the transcriptional activation by inducing histone acetylation, which is required for chromatin structure change. There has been little information about PPAR γ acetylation, though PPAR γ activity is regulated by protein modification such as phosphorylation (Hu et al. 1996), sumoylation (Pascual et al. 2005), and ubiquitination (Anbalagan et al. 2012; Christianson et al. 2008; Floyd and Stephens 2002; Hauser et al. 2000). In a recent study, it was reported that PPAR γ acetylation (Lysine 268 and 293) was induced by a ligand and decreased by SIRT1 in HEK293 cells (Qiang et al. 2012). Although the study suggests a role of acetylation in the regulation of PPAR γ function, it is not known whether the acetylation happens in the absence of a ligand. In that study, PPAR γ acetylation was regulated by SIRT1 and the effect was investigated in “browning” of white adipose tissue. The acetylation inhibits brown adipocyte differentiation in the white adipose tissue (inguinal fat) by blocking PPAR γ interaction with the coactivator Prdm16. They reported that inhibition of the acetylation promotes preadipocyte differentiation into brown adipocytes in response to cold challenge at 4°C cold room. Their study suggests that PPAR γ acetylation favors lipid accumulation and preadipocyte differentiation into white adipocytes, which un-favors brown adipocyte differentiation. HDAC1/HDAC3 was reported to modulate PPAR γ transcription through the sumoylated CEBPD in hepatic lipogenesis (Lai, et al. 2008). However, there is no report that PPAR γ was acetylated by HDAC1/HDAC3. Our data suggest that HDAC3 regulates PPAR γ acetylation and function directly. In this

current study, we observed that PPAR γ acetylation was induced by pioglitazone (Fig. 2F). PPAR γ acetylation was induced by inhibition of HDAC3 in the absence of an exogenous ligand. The ligand-independent acetylation enhanced the transcriptional activity of PPAR γ , as indicated by PPAR γ target gene expression, lipid accumulation in adipogenesis, and insulin-induced glucose uptake. Our results suggest that PPAR γ acetylation is a new approach to increase PPAR γ activity and this acetylation may occur in the absence of exogenous ligands. It is not known whether acetylation correlates with phosphorylation, sumoylation, and ubiquitination in PPAR γ protein.

In terms of the mechanism by which PPAR γ acetylation leads to PPAR γ activation, we would like to propose a model here. In this model, we suggest that there is a basal level of acetylation in the PPAR γ protein in the absence of a ligand. The corepressor removes the acetylation constantly to prevent PPAR γ activation without a ligand. The corepressor activity is abolished when it is disassociated from the PPAR γ protein in response to a ligand. When the corepressor activity is inhibited, the basal acetylation is accumulated in the PPAR γ protein in the absence of deacetylation activity. Acetylated PPAR γ induces recruitment of acetyltransferases (HATs), such as p300/CBP, to induce gene transcription, which in turn induces histone acetylation in PPAR γ responsive genes (Freedman 1999; Graves, et al. 1992; Rosen and Spiegelman 2000; Rosen, et al. 2000). This model explains the role of PPAR γ acetylation in PPAR γ activation in the absence of a ligand. Our data suggest that PPAR γ acetylation is coupled with histone acetylation. Histone acetylation is required for gene transcription, but histone acetylation is likely a consequence of PPAR γ acetylation. This possibility remains to be tested.

Our data suggest that HDAC3 inhibitor is a new PPAR γ activator that exhibits potency comparable to pioglitazone. TZDs are the most powerful insulin sensitizer in the treatment of T2DM (Tontonoz and Spiegelman 2008). TZD-based medicines include pioglitazone (Actos by Takeda Pharmaceuticals) and rosiglitazone (Avandia by GlaxoSmithKline). Although TZD-based medicines have outstanding therapeutic activities, the adverse effects, such as heart attacks and bladder cancer, have significantly reduced their applications in the treatment of T2DM (Cariou, et al. 2012; Ferrara, et al. 2011; Rosen 2010; Tseng 2012). We believe that PPAR γ activation remains as an excellent approach in the treatment of type 2 diabetes. All of the TZD-based medicines activate PPAR γ . However, their side effects are different for the heart and bladder, suggesting that the side effects are not due to PPAR γ activation. It is likely that the side effects are the off-target activities of the medicine. Our data suggest that HDAC3 inhibitor is a potential new generation of PPAR γ activator and an insulin sensitizer. Inhibition of HDAC3 is beneficial in preventing neuronal death (Bardai, et al. 2012), improving beta-cell function (Chou, et al. 2012), and having anticancer effects (Bhaskara, et al. 2008; Escaffit, et al. 2007). Moreover, inhibition of HDAC3 promotes the transcriptional activities of myocyte enhancer factor 2 (MEF2) (Gregoire, et al. 2007; Naya and Olson 1999), implying that inhibition of HDAC3 may protect heart function. In our previous study, pan-inhibitors of HDACs prevented HFD-induced obesity and insulin resistance in mice (Gao et al. 2009). These findings suggest that HDAC3 inhibitor may be able to avoid the side effects of synthetic PPAR γ ligands in vivo.

In conclusion, we report that the transcription factor PPAR γ is modulated by acetylation in response to ligands. The acetylation is sufficient to induce PPAR γ function in the absence of exogenous ligands. HDAC3 inhibitor is able to activate PPAR γ in a ligand-independent manner. These observations indicate that HDAC3 inhibitor may be a ligand-independent PPAR γ activator. Inhibition of HDAC3 may present a new approach to improve insulin sensitivity in the treatment of type 2 diabetes.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health COBRE grant (2P20RR021945), American Diabetes Association Junior Faculty Award 1-09-JF-17 and the National Natural Science Foundation of China (Grant no. 81370915).

The abbreviations used are

| | |
|--------------------------------|---|
| PPARγ | peroxisome proliferator- activated receptor, gamma |
| aP2 | fatty acid binding protein 4 |
| TZDs | thiazolidinediones |
| HDAC3 | histone deacetylase 3 |
| RXR | retinoid X receptor |
| SMRT | silencing mediator for retinoid and thyroid hormone receptors |
| NCoR | nuclear receptor corepressor |
| ITT | intraperitoneal insulin tolerance test |
| IP | immunoprecipitation |
| T2DM | type 2 diabetes mellitus |

REFERENCES

- Anbalagan M, Huderson B, Murphy L, Rowan BG. Post-translational modifications of nuclear receptors and human disease. *Nucl Recept Signal*. 2012; 10:e001. [PubMed: 22438791]
- Bardai FH, Price V, Zaayman M, Wang L, D'Mello SR. Histone deacetylase (HDAC1) is a molecular switch between neuronal survival and death. *J Biol Chem*. 2012
- Berger J, Moller DE. The mechanisms of action of PPARs. *Annu Rev Med*. 2002; 53:409–435. [PubMed: 11818483]
- Bhaskara S, Chyla BJ, Amann JM, Knutson SK, Cortez D, Sun ZW, Hiebert SW. Deletion of histone deacetylase 3 reveals critical roles in S phase progression and DNA damage control. *Mol Cell*. 2008; 30:61–72. [PubMed: 18406327]
- Blander G, Guarente L. The Sir2 family of protein deacetylases. *Annu Rev Biochem*. 2004; 73:417–435. [PubMed: 15189148]
- Cariou B, Charbonnel B, Staels B. Thiazolidinediones and PPAR γ agonists: time for a reassessment. *Trends Endocrinol Metab*. 2012; 23:205–215. [PubMed: 22513163]
- Chou DH, Holson EB, Wagner FF, Tang AJ, Maglathlin RL, Lewis TA, Schreiber SL, Wagner BK. Inhibition of histone deacetylase 3 protects beta cells from cytokine-induced apoptosis. *Chem Biol*. 2012; 19:669–673. [PubMed: 22726680]
- Christianson JL, Nicoloso S, Straubhaar J, Czech MP. Stearoyl-CoA desaturase 2 is required for peroxisome proliferator-activated receptor gamma expression and adipogenesis in cultured 3T3-L1 cells. *J Biol Chem*. 2008; 283:2906–2916. [PubMed: 18032385]

- Escaffit F, Vaute O, Chevillard-Briet M, Segui B, Takami Y, Nakayama T, Trouche D. Cleavage and cytoplasmic relocalization of histone deacetylase 3 are important for apoptosis progression. *Mol Cell Biol.* 2007; 27:554–567. [PubMed: 17101790]
- Fajas L, Egler V, Reiter R, Hansen J, Kristiansen K, Debril MB, Miard S, Auwerx J. The retinoblastoma-histone deacetylase 3 complex inhibits PPARgamma and adipocyte differentiation. *Dev Cell.* 2002; 3:903–910. [PubMed: 12479814]
- Ferrara A, Lewis JD, Quesenberry CP Jr, Peng T, Strom BL, Van Den Eeden SK, Ehrlich SF, Habel LA. Cohort study of pioglitazone and cancer incidence in patients with diabetes. *Diabetes Care.* 2011; 34:923–929. [PubMed: 21447664]
- Floyd ZE, Stephens JM. Interferon-gamma-mediated activation and ubiquitin-proteasome-dependent degradation of PPARgamma in adipocytes. *J Biol Chem.* 2002; 277:4062–4068. [PubMed: 11733495]
- Freedman LP. Increasing the complexity of coactivation in nuclear receptor signaling. *Cell.* 1999; 97:5–8. [PubMed: 10199396]
- Fujiki K, Kano F, Shiota K, Murata M. Expression of the peroxisome proliferator activated receptor gamma gene is repressed by DNA methylation in visceral adipose tissue of mouse models of diabetes. *BMC Biol.* 2009; 7:38. [PubMed: 19589179]
- Gao Z, Chiao P, Zhang X, Lazar MA, Seto E, Young HA, Ye J. Coactivators and corepressors of NF-kappaB in IkappaB alpha gene promoter. *J Biol Chem.* 2005; 280:21091–21098. [PubMed: 15811852]
- Gao Z, He Q, Peng B, Chiao PJ, Ye J. Regulation of nuclear translocation of HDAC3 by IkappaBalpha is required for tumor necrosis factor inhibition of peroxisome proliferator-activated receptor gamma function. *J Biol Chem.* 2006; 281:4540–4547. [PubMed: 16371367]
- Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J. Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *J Biol Chem.* 2002; 277:48115–48121. [PubMed: 12351658]
- Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M, Cefalu WT, Ye J. Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes.* 2009; 58:1509–1517. [PubMed: 19366864]
- Gao Z, Zhang X, Zuberi A, Hwang D, Quon MJ, Lefevre M, Ye J. Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3T3-L1 adipocytes. *Mol Endocrinol.* 2004; 18:2024–2034. [PubMed: 15143153]
- Geiss-Friedlander R, Melchior F. Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol.* 2007; 8:947–956. [PubMed: 18000527]
- Graves RA, Tontonoz P, Spiegelman BM. Analysis of a tissue-specific enhancer: ARF6 regulates adipogenic gene expression. *Mol Cell Biol.* 1992; 12:1202–1208. [PubMed: 1545801]
- Gregoire S, Xiao L, Nie J, Zhang X, Xu M, Li J, Wong J, Seto E, Yang XJ. Histone deacetylase 3 interacts with and deacetylates myocyte enhancer factor 2. *Mol Cell Biol.* 2007; 27:1280–1295. [PubMed: 17158926]
- Guan HP, Ishizuka T, Chui PC, Lehrke M, Lazar MA. Corepressors selectively control the transcriptional activity of PPARgamma in adipocytes. *Genes Dev.* 2005; 19:453–461. [PubMed: 15681609]
- Hauser S, Adelmant G, Sarraf P, Wright HM, Mueller E, Spiegelman BM. Degradation of the peroxisome proliferator-activated receptor gamma is linked to ligand-dependent activation. *J Biol Chem.* 2000; 275:18527–18533. [PubMed: 10748014]
- Hu E, Kim JB, Sarraf P, Spiegelman BM. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. *Science.* 1996; 274:2100–2103. [PubMed: 8953045]
- Hu X, Li Y, Lazar MA. Determinants of CoRNR-dependent repression complex assembly on nuclear hormone receptors. *Mol Cell Biol.* 2001; 21:1747–1758. [PubMed: 11238912]
- Huang EY, Zhang J, Miska EA, Guenther MG, Kouzarides T, Lazar MA. Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. *Genes Dev.* 2000; 14:45–54. [PubMed: 10640275]
- Krogdram AM, Nielsen CA, Neve S, Holst D, Helledie T, Thomsen B, Bendixen C, Mandrup S, Kristiansen K. Nuclear receptor corepressor-dependent repression of peroxisome-proliferator-

- activated receptor delta-mediated transactivation. *Biochem J.* 2002; 363:157–165. [PubMed: 11903058]
- Lai PH, Wang WL, Ko CY, Lee YC, Yang WM, Shen TW, Chang WC, Wang JM. HDAC1/HDAC3 modulates PPARG2 transcription through the sumoylated CEBPD in hepatic lipogenesis. *Biochim Biophys Acta.* 2008; 1783:1803–1814. [PubMed: 18619497]
- Miard S, Fajas L. Atypical transcriptional regulators and cofactors of PPARgamma. *Int J Obes (Lond).* 2005; 29(Suppl 1):S10–S12. [PubMed: 15711575]
- Naya FJ, Olson E. MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. *Curr Opin Cell Biol.* 1999; 11:683–688. [PubMed: 10600704]
- Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG, Glass CK. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature.* 2005; 437:759–763. [PubMed: 16127449]
- Qiang L, Wang L, Kon N, Zhao W, Lee S, Zhang Y, Rosenbaum M, Zhao Y, Gu W, Farmer SR, et al. Brown Remodeling of White Adipose Tissue by SirT1-Dependent Deacetylation of Ppargamma. *Cell.* 2012; 150:620–632. [PubMed: 22863012]
- Rosen CJ. Revisiting the rosiglitazone story--lessons learned. *N Engl J Med.* 2010; 363:803–806. [PubMed: 20660395]
- Rosen ED, Spiegelman BM. Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol.* 2000; 16:145–171. [PubMed: 11031233]
- Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. *Genes Dev.* 2000; 14:1293–1307. [PubMed: 10837022]
- Sugii S, Evans RM. Epigenetic codes of PPARgamma in metabolic disease. *FEBS Lett.* 2011; 585:2121–2128. [PubMed: 21605560]
- Tontonoz P, Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem.* 2008; 77:289–312. [PubMed: 18518822]
- Treuter E, Albrektsen T, Johansson L, Leers J, Gustafsson JA. A regulatory role for RIP140 in nuclear receptor activation. *Mol Endocrinol.* 1998; 12:864–881. [PubMed: 9626662]
- Tseng CH. Pioglitazone and bladder cancer in human studies: is it diabetes itself, diabetes drugs, flawed analyses or different ethnicities? *J Formos Med Assoc.* 2012; 111:123–131. [PubMed: 22423665]
- Yang XJ, Gregoire S. A recurrent phospho-sumoyl switch in transcriptional repression and beyond. *Mol Cell.* 2006; 23:779–786. [PubMed: 16973431]
- Ye J. Challenges in Drug Discovery for Thiazolidinedione Substitute. *Yao Xue Xue Bao.* 2011; 1:137–142. [PubMed: 22427718]
- Zamir I, Zhang J, Lazar MA. Stoichiometric and steric principles governing repression by nuclear hormone receptors. *Genes Dev.* 1997; 11:835–846. [PubMed: 9106656]

IP: PPAR γ
IB: Acetyl-lysine

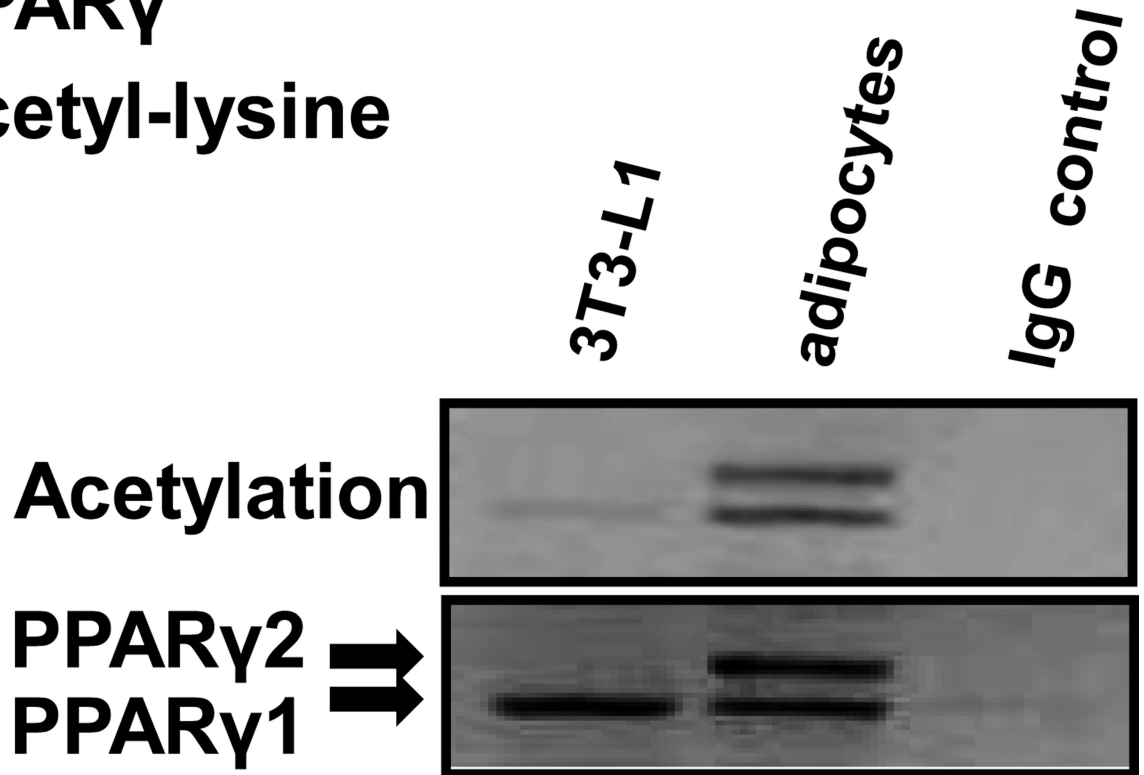
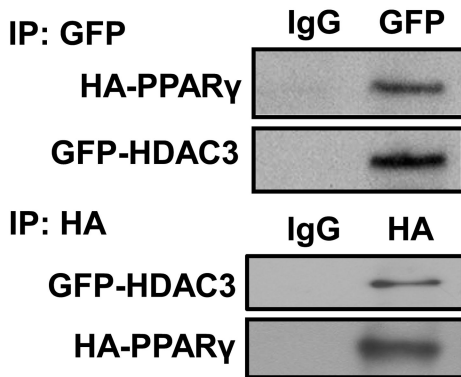


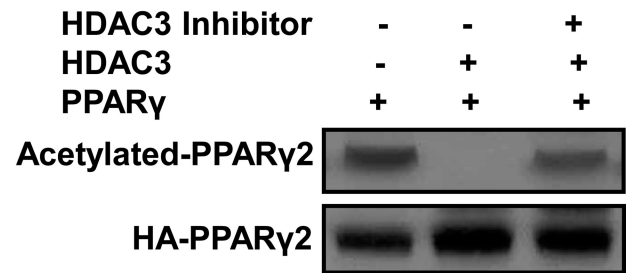
Figure 1.

PPAR γ is an acetylated protein. PPAR γ was precipitated with a monoclonal PPAR γ antibody (E-8, sc-7273x, Santa Cruz) using 500 μ g of protein from 3T3-L1 preadipocytes and fully differentiated 3T3-L1 adipocyte lysates. Mouse IgG was used as a negative control with the adipocyte lysates. The acetylation was detected by using rabbit acetyl-lysine antibody.

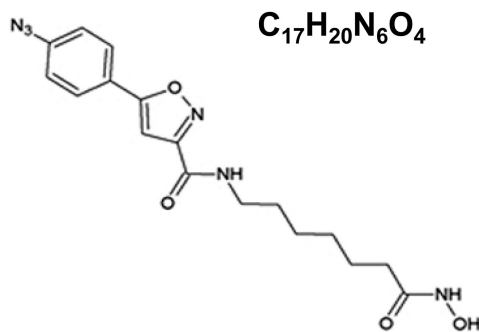
A. HDAC3-PPAR γ interaction



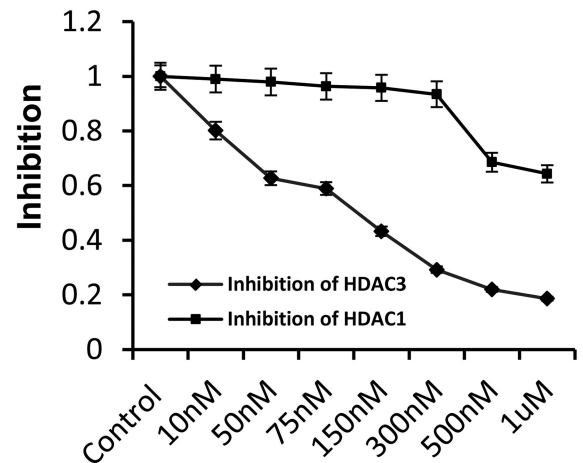
B. HDAC assay



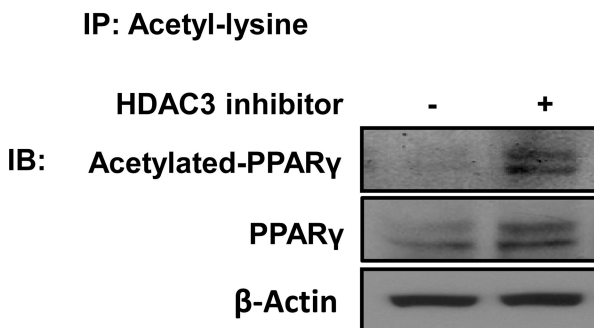
C. HDAC3 Inhibitor (HD-75) structure



D. HDAC3 specificity test



E. Inhibiting HDAC3 induces PPAR γ acetylation



F. Pioglitazone induces PPAR γ acetylation and degradation

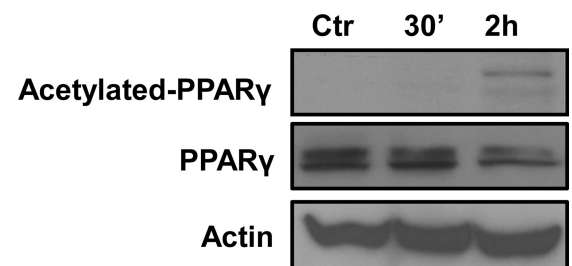


Figure 2. HDAC3 regulates PPAR γ acetylation. A. Physical interaction of PPAR γ with HDAC3. Co-IP of HA-tagged PPAR γ or GFP-tagged HDAC3 in HEK293 cells. B. HDAC3 deacetylates PPAR γ in HDAC assay. HA-PPAR γ 2 plasmids were transfected into HEK293 cells. The

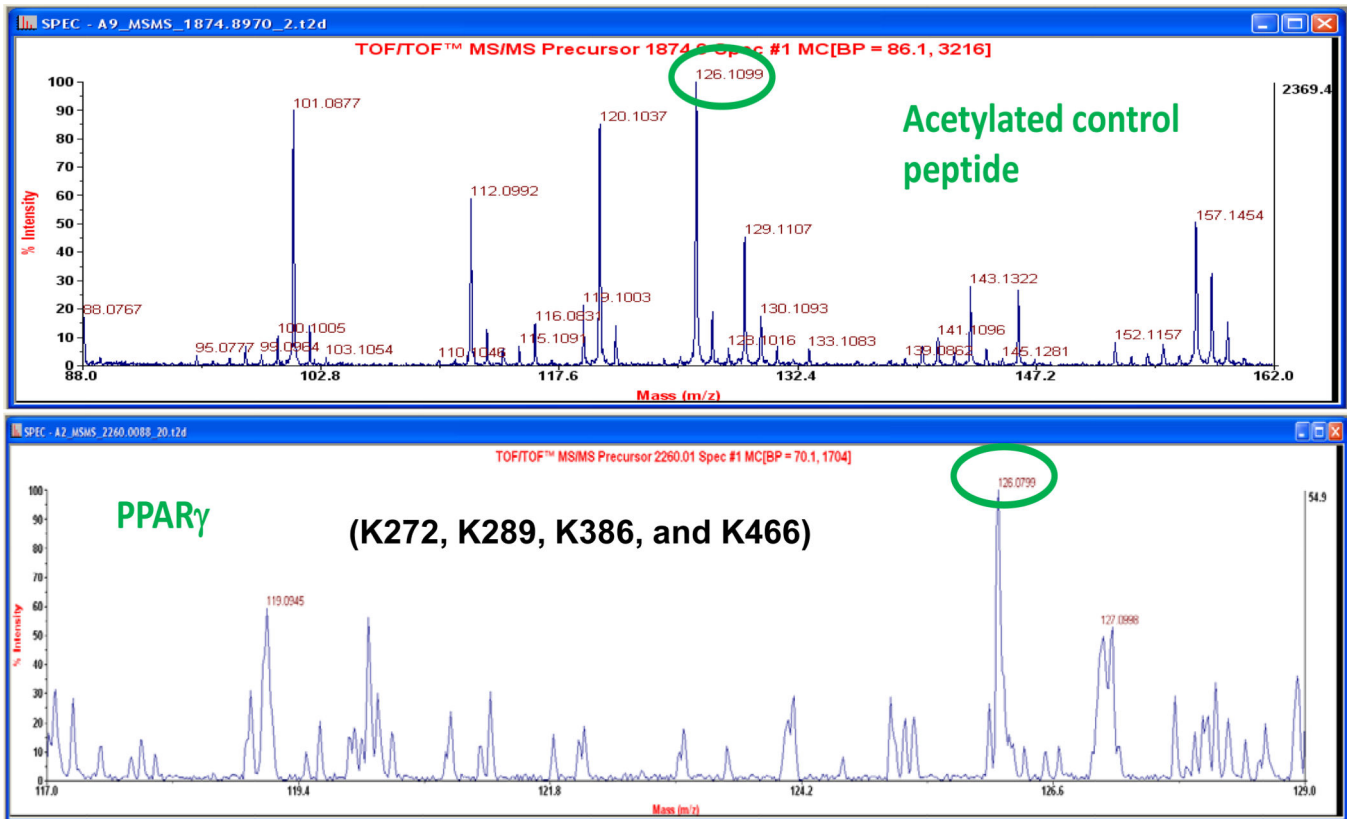
cells were treated with 200 nM TSA for 30 minutes to induce protein acetylation. PPAR γ was immunoprecipitated on beads, and the IP product on beads was served as a substrate in HDAC assay. C. HDAC3 inhibitor structure. D. HDAC3 specificity test. 10ng of HDAC3 protein was used in the HDAC3 assay. E. Inhibiting HDAC3 induces PPAR γ acetylation. 3T3-L1 adipocytes were treated with 150 nM of HDAC3 inhibitor for 2 h before being harvested. 500 μ g of protein was used in IP with anti-acetyl-lysine antibody. E. Pioglitazone induces PPAR γ acetylation in adipocytes.

Author Manuscript

Author Manuscript

Author Manuscript

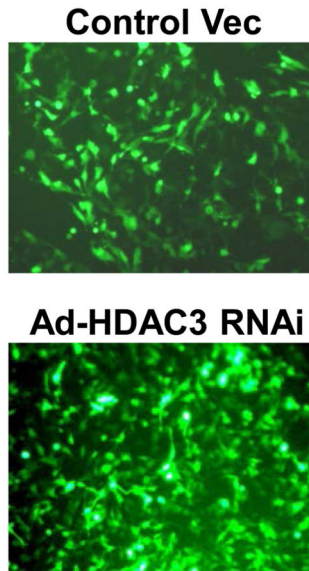
Author Manuscript



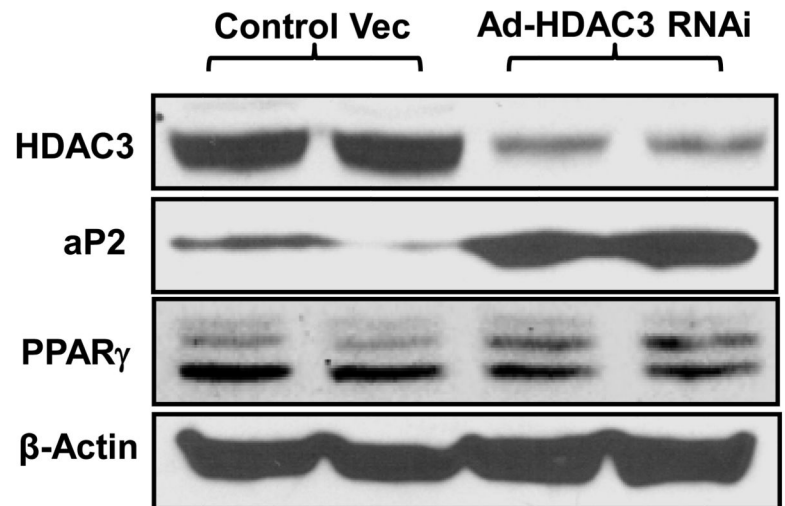
- K272** **DKSPFVIYD**
- K289** **ILTGKTTDK**
- K386** **SLRKPFQDF**
- K466** **QLFAKVLQK**

Figure 3. Acetylation sites analysis by Mass Spectrometry. Acetylated control peptide by Mass Spectrometry was generated a 126.1 peak (top panel). Four acetylated lysine sites (K289, K386, K462 and K466) were detected by MS (lower panel). The MS figure shows one of the four acetylated lysine sites.

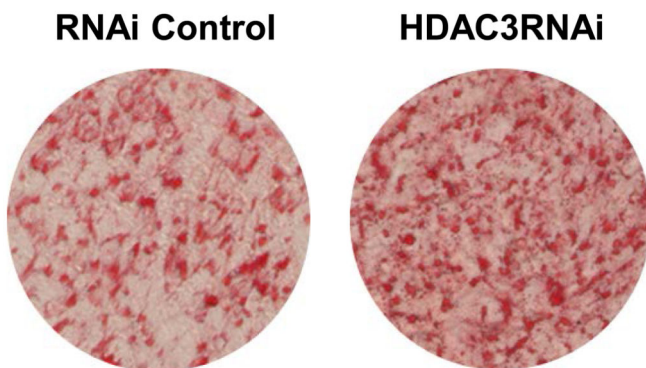
A. Ad-HDAC3RNAi infection in 3T3-L1 adipocytes



B. Knockdown efficiency in 3T3-L1 adipocytes



C. Oil red O staining



D. Quantification of Oil red O staining

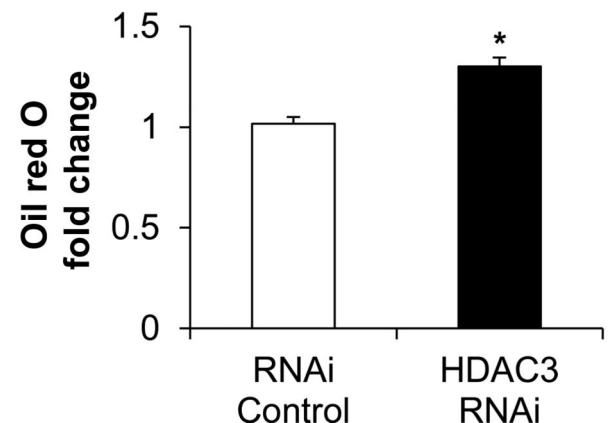
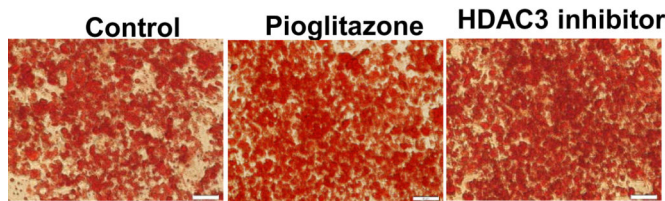


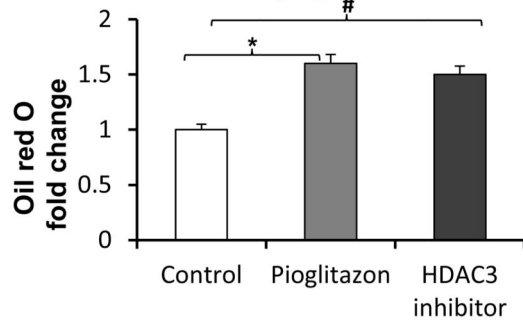
Figure 4.

Acetylation induces PPAR γ activation. A. 3T3-L1 adipocytes were infected with adeno-GFP-HDAC3 RNAi virus. B. Knockdown efficiency of HDAC3 in 3T3-L1 adipocytes with a 24-h infection and PPAR γ target gene aP2 were measured in the immunoblot. C. Knockdown HDAC3 promotes adipogenesis. 3T3-L1 preadipocytes were infected with 50 μ l of adenovirus in a 10-cm cell dish for 24 h, and then the cells were induced for adipogenesis with a standard protocol. After 8 days of induction, the cells were stained with oil red O. Adeno-RNAi empty vector virus was used as a negative control. D. Adipogenesis was quantified with a microscope and color absorbance. Data presented with triplicates by student's t-test, *, $p < 0.05$.

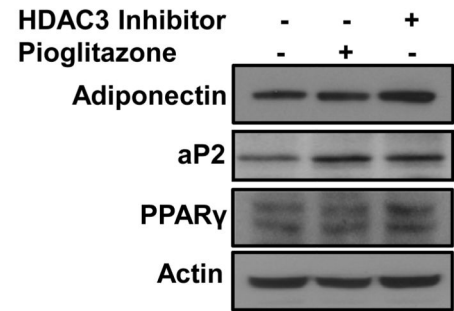
A. Adipogenesis



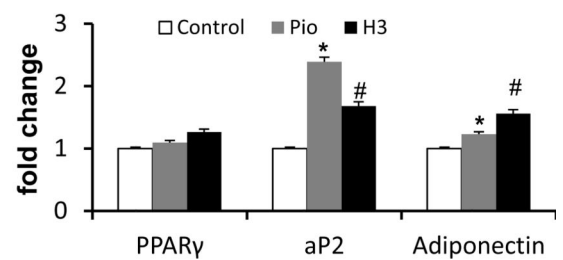
Quantification of adipogenesis



B. PPAR γ target gene



Quantification of immunoblot



C. PPRE-luc reporter assay

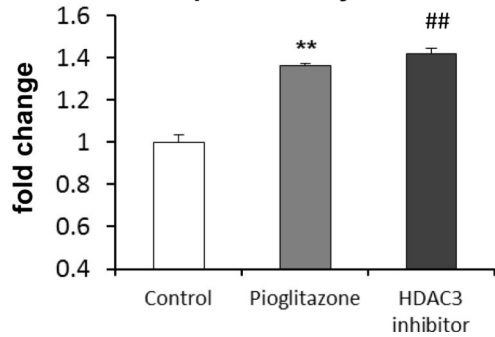
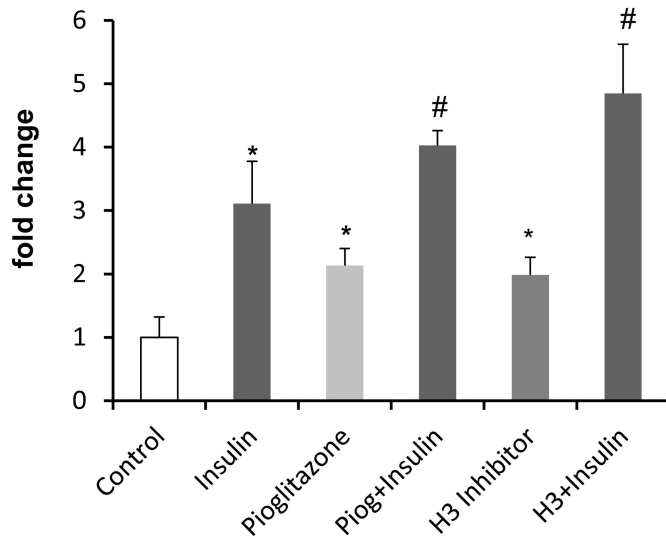
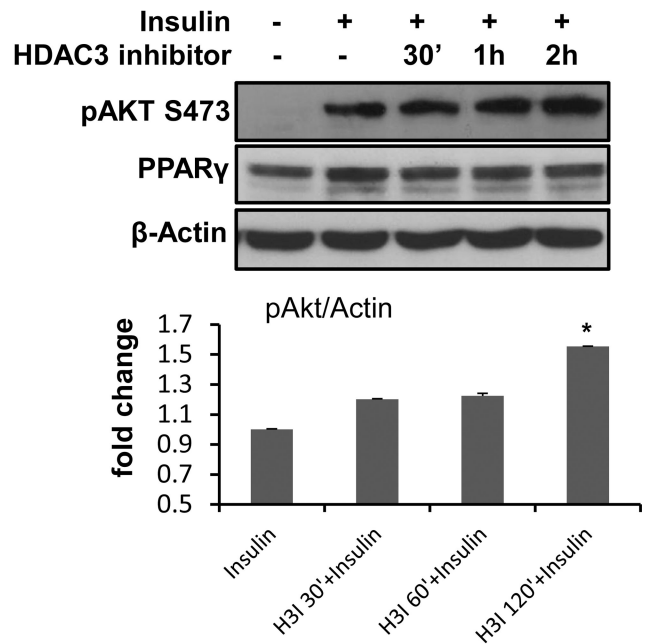


Figure 5. Inhibiting HDAC3 promoted PPAR γ function. A. Adipogenesis. HDAC3 inhibitor (150nM) was added into a standard inducing cocktail, and adipogenesis was induced in a 12-well plate. Adipogenesis was evaluated using oil red O staining methods. The experiments were conducted at least 3 times, and each trial had constant results. B. PPAR γ target gene expression by western blot and quantification. C. PPRE-Luc reporter assay. #, HDAC3 inhibitor vs. control, p<0.05, ##, p<0.001; *, Pioglitazone vs. control, p<0.05, **, p<0.001 by student's t-test. Pio: pioglitazone; H3: HDAC3 inhibitor.

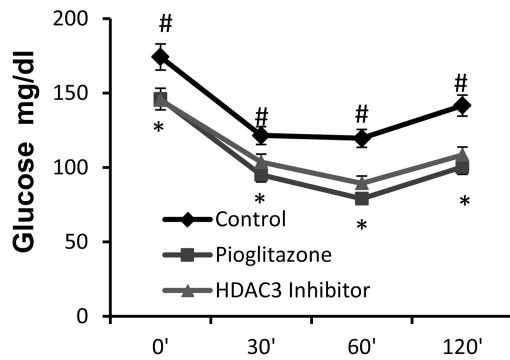
A. Glucose uptake



B. Insulin signaling



C. ITT



D. Area under the curve

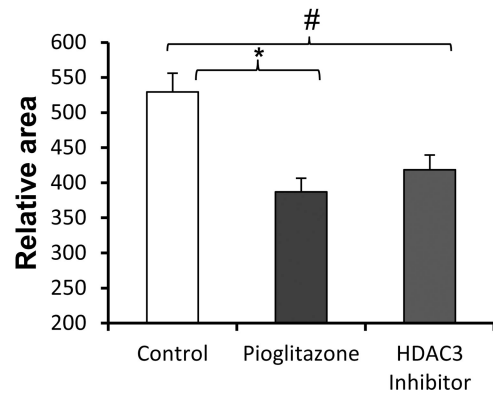


Figure 6.

Inhibiting HDAC3 increased glucose uptake and insulin sensitivity. A. Glucose uptake. Fully differentiated 3T3-L1 adipocytes were treated with HDAC3 inhibitor overnight, and then glucose uptake was measured. *, drug vs. control; #, drug+insulin vs. insulin; $p < 0.05$ by student's t-test. H3I: HDAC3 inhibitor. B. Insulin signaling. C. Insulin tolerance test. D. Area under the curve of ITT. #, HDAC3 inhibitor vs. control; *, Pioglitazone vs. control; *, #, $p < 0.05$ by student's t-test, $n = 8$.