Inhibition of HDAC3 promotes ligand-independent PPARγ activation by protein acetylation

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Abstract

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor whose activation is dependent on a ligand. PPARγ activation by exogenous ligands, such as thiazolidinediones (TZDs), is a strategy in the treatment of type 2 diabetes mellitus for the improvement of insulin sensitivity. In addition to a ligand, PPARγ function is also regulated by posttranslational modifications, such as phosphorylation, sumoylation, and ubiquitination. Herein, we report that the PPARγ protein is modified by acetylation, which induces the PPARγ function in the absence of an external ligand. We observed that histone deacetylase 3 (HDAC3) interacted with PPARγ to deacetylate the protein. In immunoprecipitation assays, the HDAC3 protein was associated with the PPARγ protein. Inhibition of HDAC3 using RNAi-mediated knockdown or HDAC3 inhibitor increased acetylation of the PPARγ protein. Furthermore, inhibition of HDAC3 enhanced the expression of PPARγ 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. PPARγ acetylation was also induced by pioglitazone and acetylation was required for PPARγ activation. In the absence of TZDs, the acetylation from HDAC3 inhibition was sufficient to induce the transcriptional activity of PPARγ. Treating diet-induced obesity mice with HDAC3 inhibitor or pioglitazone for 2 weeks significantly improved high-fat-diet-induced insulin resistance. Our results indicate that acetylation of PPARγ is a ligand-independent mechanism of PPARγ activation. HDAC3 inhibitor is a potential PPARγ activator for the improvement of insulin sensitivity.

Key Words
- type 2 diabetes
- insulin sensitivity
- metabolic syndrome
- adipocytes
- adipogenesis
- PPARγ
- posttranslational modifications
- histone deacetylase
- HDAC inhibitors
- acetylation

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Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ) is a well-documented transcription factor that plays an important role in the control of glucose and fatty acid metabolism. In this mechanism, PPARγ induces the expression of adipocyte-specific genes and promotes differentiation of preadipocytes through transcriptional activation of target genes (Rosen & Spiegelman 2000). PPARγ is also required for the maintenance of physiological function of mature adipocytes. Insufficient PPARγ activity is associated with adipose tissue dysfunction and glucose disorders in metabolic syndrome (Fujiki *et al.* 2009). At the molecular level, PPARγ forms heterodimers

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with the retinoid X receptor (RXR) when it binds to the promoter DNA of target genes. The transcriptional activity of PPARγ is regulated by ligands that determine PPARγ interaction with coactivators and corepressors (Berger & Moller 2002). Thiazolidinedione (TZD) is a synthetic PPARγ ligand that has been widely used in clinical practice to improve insulin sensitivity in type 2 diabetes mellitus (T2DM). In the absence of ligands, PPARγ binds the corepressor that is formed by histone deacetylase 3 (HDAC3) and silencing mediator for retinoid and thyroid hormone receptors (SMRT)/nuclear receptor corepressor (NCoR). Ligand binding leads to disassociation of the corepressor complex and induces recruitment of coactivators. Although TZDs are outstanding PPARγ ligands with strong therapeutic activities in the treatment of T2DM, their side effects on the heart and bladder have caused alarm with regard to clinical applications. It is an urgent priority to identify a new PPARγ activator to replace TZDs in the treatment of T2DM (Ye 2011). For this reason, we explored a new strategy of PPARγ activation with a focus on HDAC3 inhibition.

Regulation of PPARγ protein by direct acetylation is a new topic in the study of PPARγ function. PPARγ function is regulated by posttranslational modifications such as phosphorylation (Hu *et al*. 1996), sumoylation (Pascual *et al*. 2005), ubiquitination (Hauser *et al*. 2000, Floyd & Stephens 2002, Christianson *et al*. 2008, Anbalagan *et al*. 2012), and histone acetylation (Sugii & Evans 2011, Qiang *et al*. 2012). Phosphorylation of PPARγ at serine 112 and 273 inhibits PPARγ transcriptional activity. Sumoylation of PPARγ at lysine 107 in the AF1 region and at lysine 395 in the AF2 region (lysine 77 and 365 in PPARγ1 respectively) activates PPARγ by blocking the interaction between the NCoR of HDAC3 and PPARγ. Ubiquitination of PPARγ leads to protein degradation following PPARγ activation by TZDs (Hauser *et al*. 2000, Floyd & Stephens 2002, Christianson *et al*. 2008, Anbalagan *et al*. 2012). It is largely unknown whether PPARγ protein is acetylated and, if so, how PPARγ function is regulated by acetylation. In this study, we addressed this issue by analysis of PPARγ 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, and 11), class II (HDAC4, 5, 6, 7, 9, and 10) (Huang *et al*. 2000), and class III (SIRT1–7) (Blander & Guarente 2004). Trichostatin-A (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 PPARγ function in adipocytes *in vitro* (Gao *et al*. 2006) and prevented high-fat-diet-induced obesity (DIO) in mice (Gao *et al*. 2009). HDAC3, a member of the class I HDACs, has been reported by our and other laboratories to regulate PPARγ function in adipocytes (Fajas *et al*. 2002, Guan *et al*. 2005, Miard & Fajas 2005, Gao *et al*. 2006). However, it is not known whether HDAC3 inhibition is sufficient to activate PPARγ in the absence of classical ligands.

In this study, we found that PPARγ protein is acetylated. The acetylation was induced by a ligand and decreased by HDAC3. HDAC3 inhibition induced PPARγ acetylation and activation in the absence of exogenous ligands. The results of this study indicate that HDAC3 inhibition could be a new approach to activate PPARγ in the absence of exogenous ligands.

Materials and methods

Mouse models and treatment

DIO male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 16-weeks-old, and had been fed a high-fat diet (HFD, 60% calories as fat, Research Diets (New Brunswick, NJ, USA) 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 a 12 h light:12 h darkness cycle and at a temperature of 22–24 °C. The mice had free access to water and feed. The mice were treated with HDAC3 inhibitor by i.p. injection at dose of 10 μg/kg body weight per day for 2 weeks. Pioglitazone at a 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 received the same amount of DMSO in PBS by i.p. injection every day. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center.

Cell culture and reagents

The cell lines 3T3-L1 (CL-173) and HEK293 (CRL-1573) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in 10 and 5% fetal bovine serum, DMEM in a 5% CO_2 incubator. The cells were starved in DMEM containing 0.25% fatty-acid–free BSA overnight before treatment with 150 nM of HDAC3 inhibitor. HDAC3 inhibitor (cat. no. EB1003) was purchased from KeraFAST (Boston, MA, USA). Pioglitazone (cat. no. E6910) was purchased from Sigma.

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Adipogenesis

3T3-L1 preadipocytes were grown to 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 μg/ml insulin, 0.5 mM isobutylmethylxanthine, and 10 μm 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).

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).

Immunoblot

The whole-cell lysates were prepared by sonication in lysis buffer and used in western blotting analysis, 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, USA). Monoclonal PPARγ (E-8, sc-7273x) and HA (sc-7392) antibodies were from Santa Cruz.

Immunoprecipitation and HDAC assay

Immunoprecipitation (IP) was carried out using whole-cell lysates (400 μg of total protein), 2–4 μg of antibody, and 40 μl of protein G-Sepharose beads (Amersham Biosciences), as described elsewhere (Gao *et al*. 2002). The HDAC assay was conducted using a HDAC assay kit (17-320; Millipore (Billerica, MA, USA)). Briefly, PPARγ was immunoprecipitated and then was used as a substrate in HDAC assay. Recombinant HDAC3 protein (cat. no. H00008841, ABNOVA (Walnut, CA, USA)) was added into the reactions as an enzyme in the assay.

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 (Farmingdale, NY, USA)) and a Fluor-de-Lys HDAC1 fluorometric drug discovery assay kit (BML-AK511, Enzo Life Science) as described in the manufacturer's instructions.

I.p. insulin tolerance test

Fourteen-week-old DIO mice, which had already been given a HFD (D12492) for 8 weeks, were purchased from Jackson Laboratory (stock #000664). After quarantine, the mice were divided into three groups. Each group consisted of eight mice. For 2 weeks, 10 μg/kg body weight per day of HDAC3 inhibitor was administrated by i.p. injection. The 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 per day. An insulin tolerance test (ITT) was conducted by i.p. injection of insulin (I9278, Sigma) at 0.75 units/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, USA).

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 using Lipofectamine. The PPARγ reporter system was constituted using 0.2 μg each of PPRE (3×)-luciferase, PPARγ2, and RXRα in each well (Gao *et al*. 2006). The cells were treated with 1 μM pioglitazone or 150 nM HDAC3 inhibitor for 16 h to activate PPARγ2 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.

Statistical analysis

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

Results

PPARγ is acetylated in adipocytes

PPARγ function is regulated by posttranslational modifications, such as phosphorylation (Hu *et al*. 1996),

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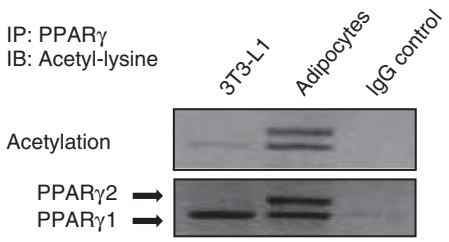

Figure 1
PPARγ is an acetylated protein. PPARγ was precipitated with a monoclonal PPARγ antibody (E-8, sc-7273x, Santa Cruz) using 500 μg of protein from lysates of 3T3-L1 preadipocytes and fully differentiated 3T3-L1 adipocyte. Mouse IgG was used as a negative control with the adipocyte lysates. The acetylation was detected by using rabbit acetyl-lysine antibody.

sumoylation (Yang & Gregoire 2006, Geiss-Friedlander & Melchior 2007), and ubiquitination (Hauser *et al*. 2000, Floyd & Stephens 2002, Christianson *et al*. 2008, Anbalagan *et al*. 2012). However, whether PPARγ can be regulated by acetylation is not well known. We examined PPARγ acetylation in 3T3-L1 adipocytes before and after differentiation. Acetylation was examined using an immunoblot with the acetylation-specific antibody after PPARγ isolation by IP (Fig. 1). In the undifferentiated cells, the purified PPARγ protein exhibited no significant signal in the acetylation assay (Fig. 1). In the differentiated cells, there are two isoforms of PPARγ, PPARγ1 and PPARγ2, with different molecular weights. Both isoforms of PPARγ proteins expressed a strong signal of acetylation (Fig. 1). The levels of acetylation were identical between PPARγ1 and PPARγ2. The results indicate that PPARγ protein is acetylated in the adipocytes.

HDAC3 regulates PPARγ acetylation

It is generally believed that PPARγ is associated with the corepressor in the absence of a ligand. Disassociation of the corepressor complex is induced by the interaction of ligand PPARγ, 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 transcription by deacetylating histone proteins (Zamir *et al*. 1997, Treuter *et al*. 1998, Hu *et al*. 2001, Krogsdam *et al*. 2002, Gao *et al*. 2005). However, it is not known whether HDAC3 regulates acetylation of PPARγ protein. To address this issue, we examined HDAC3–PPARγ interaction in cells through IP. In the study, GFP-tagged HDAC3 was expressed, together with HA-tagged PPARγ, in a transient cotransfection of HEK293 cells. Antibodies to GFP and HA were used to isolate HDAC3 and PPARγ in IP respectively. We observed that the HDAC3 product contained PPARγ and that PPARγ product contained HDAC3 (Fig. 2A). The results indicate that HDAC3 physically interacts with PPARγ. To test HDAC3 in deacetylation of PPARγ, we determined their enzyme and substrate relationship. In the assay, a recombinant HDAC3 protein (cat. no. H00008841, ABNOVA) was used as the deacetylase enzyme. Acetylation was induced in HA-tagged PPARγ2 in HEK 293 cells with the HDAC inhibitor TSA (200 nM for 30 min). When HDAC3 was inhibited with a chemical inhibitor (HD-75) in a deacetylation assay in test tube, PPARγ acetylation was preserved by the inhibitor (Fig. 2B). The acetylation was reduced in the absence of the inhibitor (Fig. 2B). The results indicate that as a protein deacetylase, HDAC3 directly deacetylates PPARγ protein.

We tested the specificity of the 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. A HDAC1 assay kit was used as a control for the specificity of the HDAC3 inhibitor. These kits are ideal for chemical library screening of candidate inhibitors. In the HDAC3 assay, the deacetylase activity of HDAC3 was inhibited by the HDAC3 inhibitor at IC_{50} of 150 nM. At this dosage, it only slightly inhibited HDAC1 activity (Fig. 2D). This indicates that HD-75 has specificity for HDAC3.

PPARγ acetylation was examined using the inhibition of HDAC3 in 3T3-L1 adipocytes. We tested whether HD-75 causes a strong induction of PPARγ acetylation at a dose of 150 nM in 3T3-L1 adipocytes. In the study, 3T3-L1 adipocytes were treated with 150 nM of HDAC3 inhibitor for 2 h. The cells were harvested and 500 μg of the whole-cell lysates protein was used in IP with anti-acetyl-lysine antibody. We detected a stronger PPARγ signal in the IP product in the sample treated with the HDAC3 inhibitor. PPARγ acetylation was significantly enhanced by the HDAC3 inhibitor and also in the immunoblots (Fig. 2E). Briefly, 40 μg of protein from 500 μg/500 μl IP samples was used for the loading control indicated by the β-actin in the immonoblot (Fig. 2E, bottom panel). The results indicate that PPARγ acetylation was regulated by the HDAC3 inhibitor in cells.

PPARγ activation by the ligands has been reported to induce degradation of the PPARγ protein (Hauser *et al*. 2000, Floyd & Stephens 2002). It is not known whether the ligand induces PPARγ acetylation. We addressed this question by examining PPARγ acetylation in pioglitazone-treated 3T3-L1 adipocytes. In cells, pioglitazone enhanced PPARγ acetylation (Fig. 2F). The results indicate that

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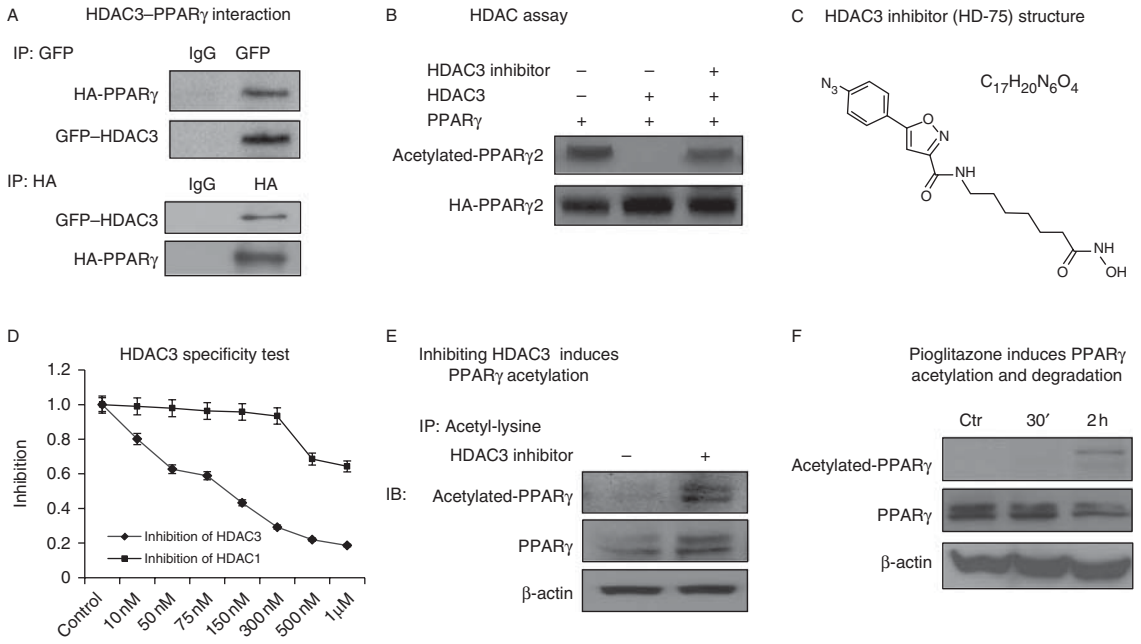

Figure 2

HDAC3 regulates PPARγ acetylation. (A) Physical interaction of PPARγ with HDAC3. Co-IP of HA-tagged PPARγ or GFP-tagged HDAC3 in HEK293 cells. (B) HDAC3 deacetylates PPARγ in HDAC assay. HA-PPARγ2 plasmids were transfected into HEK293 cells. The cells were treated with 200 nM TSA for 30 min to induce protein acetylation. PPARγ was immunoprecipitated on beads, and the IP product on beads served as a substrate in the HDAC assay. (C) HDAC3 inhibitor structure. (D) HDAC3 specificity test. Ten nanograms of HDAC3 protein was used in the HDAC3 assay. (E) Inhibiting HDAC3 induces PPARγ acetylation. 3T3-L1 adipocytes were treated with 150 nM of HDAC3 inhibitor for 2 h before being harvested. 500 μg of protein was used in IP with anti-acetyl-lysine antibody. (F) Pioglitazone induces PPARγ acetylation in adipocytes.

pioglitazone induces PPARγ acetylation. Acetylation of PPARγ by the inhibition of HDAC3 did not cause PPARγ degradation. This indicates that acetylation of PPARγ by the inhibition of HDAC3 may be different from pioglitazone-induced acetylation of PPARγ.

HDAC3 inhibitor induces PPARγ acetylation at multiple lysine sites

The PPARγ acetylation sites were analyzed by mass spectrometry. In the study, PPARγ was expressed in a transient transfection of HEK293 cells. Antibody to PPARγ was used to isolate PPARγ in IP. PPARγ 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, USA). We used their service and detected four acetylated lysine residues in PPARγ (Fig. 3). The four acetylated lysine sites (K289, K386, K462, and K466) were detected by mass spectrometry (lower panel). The data confirmed that PPARγ acetylation was induced by the HDAC3 inhibitor. Further study is needed of the function of acetylated lysine sites in PPARγ.

Acetylation induces PPARγ function

To test acetylation modification in the regulation of PPARγ function, we determined the transcriptional activity of PPARγ by quantifying the expression of the target genes. PPARγ acetylation was induced in 3T3-L1 adipocytes through the 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 a fluorescence microscope. The expression of PPARγ target genes was determined using an immunoblot. In the control cells that were infected with the control virus, HDAC3 protein was observed to correspond to an abundant protein band. In the cells infected by RNAi virus, HDAC3 was reduced by 90% according to the decreased signal of the HDAC3 protein band (Fig. 4B). Knockdown of *HDAC3* significantly increased expression of PPARγ-responsive genes, including aP2 (fatty acid-binding protein 4 (FABP4); Fig. 4B). The results indicate that HDAC3 inhibition promotes the transcriptional activity of PPARγ in adipocytes.

HDAC3 knockdown promotes adipogenesis. PPARγ induces the expression of a variety of genes in the pathways

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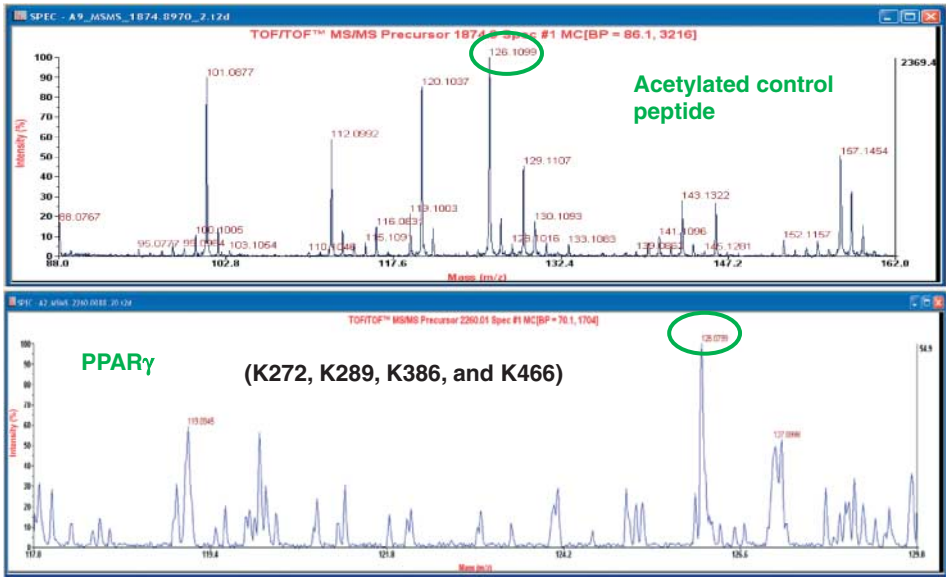

Figure 3

Acetylation sites analysis by mass spectrometry. Acetylated control peptide was generated by mass spectrometry at 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 full colour version of this figure is available at http://dx.doi.org/10.1530/JME-14-0066.

for lipid biosynthesis and storage, which is required for the differentiation of preadipocytes. Adipogenesis is often used to determine PPARγ function. We induced adipogenesis to test the effects of HDAC3 inhibition on the regulation of PPARγ 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 days 1 to 8 during 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 results indicate that inhibition of HDAC3 promotes lipid accumulation in adipocytes. The result further supports the hypothesis that inhibition of HDAC3 could be an approach to promote PPARγ function.

HDAC3 inhibitor promotes adipogenesis

A chemical inhibitor of HDAC3 was tested for the regulation of adipogenesis in an effort to identify a new PPARγ activator independent of TZDs. In the study, activation of PPARγ by the HDAC3 inhibitor was tested in the adipocyte differentiation model. The 3T3-L1 adipocytes were differentiated in the standard adipogenic cocktail. The HDAC3 inhibitor was added to the culture medium at a concentration of 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). The HDAC3 inhibitor had the same effect as pioglitazone in inducing adipogenesis. The PPARγ target genes including adiponectin and aP2 were enhanced by the inhibitor and pioglitazone in the western blotting analysis (Fig. 5B). Comparing the HDAC3 inhibitor with pioglitazone, the HDAC3 inhibitor generated a greater increase in adiponectin expression. Adiponectin is known to promote insulin sensitivity. In a reporter assay, the HDAC3 inhibitor had the same effect as pioglitazone in inducing

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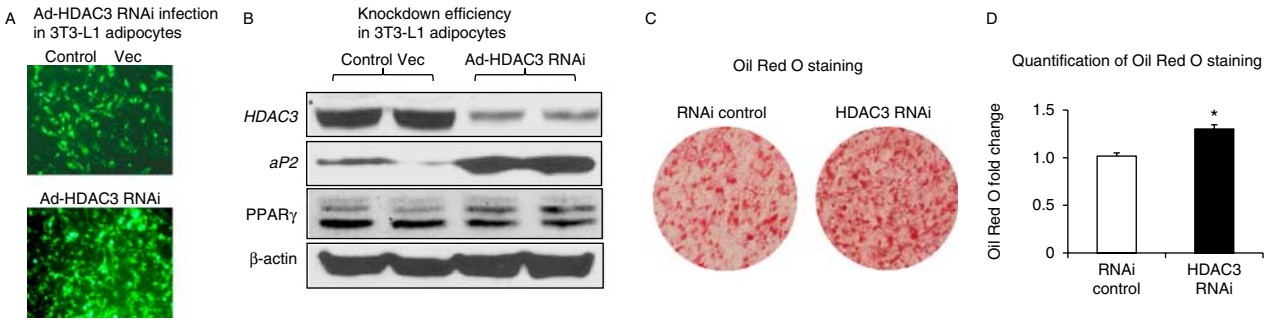

Figure 4

Acetylation induces PPARγ 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 expression of the PPARγ target gene *aP2* were measured using an immunoblot. (C) Knockdown of *HDAC3* promotes adipogenesis. 3T3-L1 preadipocytes were infected with 50 μl 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 using a microscope and color absorbance. Data presented from triplicates analysed by Student's *t*-test. $^{*}P<0.05$. A full colour version of this figure is available at http://dx.doi.org/10.1530/JME-14-0066.

PPARγ transcriptional activity (Fig. 5C). The results indicate that the HDAC3 inhibitor activates PPARγ function in the absence of exogenous ligands and that the potency of the HDAC3 inhibitor is similar to that of pioglitazone.

Inhibition of HDAC3 enhances insulin sensitivity

To further investigate the effect of HDAC3 inhibitor in the activation of PPARγ, first, we used glucose uptake to determine PPARγ function, which may enhance glucose uptake by induction of IRS2 and Glut4 in the insulin-signaling pathway. Pioglitazone was used as a positive control. The results indicated that PPARγ 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. Insulin-induced phosphorylation of AKT serine 473 was enhanced by the HDAC3 inhibitor (Fig. 6B). To test whether HDAC3 inhibitor enhances insulin sensitivity *in vivo*, we treated DIO mice with HDAC3 inhibitor at 10 a dose of μg/kg body weight per day for 2 weeks. The treatment was given to 16-week-old DIO mice, which had been fed a HFD for 10 weeks. In our previous studies, feeding B6 mice for 12 weeks with a HFD induced insulin resistance. Pioglitazone at the dose of 10 mg/kg body weight per day was used as a positive control. The pioglitazone was

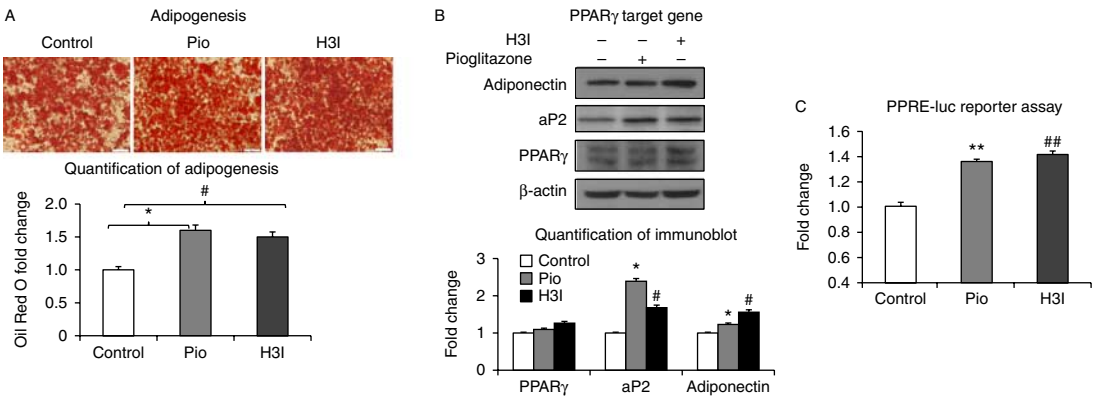

Figure 5

Inhibiting HDAC3-promoted PPARγ function. (A) Adipogenesis. H3I (150 nM) 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 three times, and each trial had consistent results. (B) PPARγ target gene expression by western blotting and quantification. (C) PPRE-Luc reporter assay. H3I vs control, $^{\#}P<0.05$, $^{\#\#}P<0.001$; Pio vs control, $^{*}P<0.05$, $^{**}P<0.001$ by Student's *t*-test. Pio, pioglitazone; H3I, HDAC3 inhibitor. A full colour version of this figure is available at http://dx.doi.org/10.1530/JME-14-0066.

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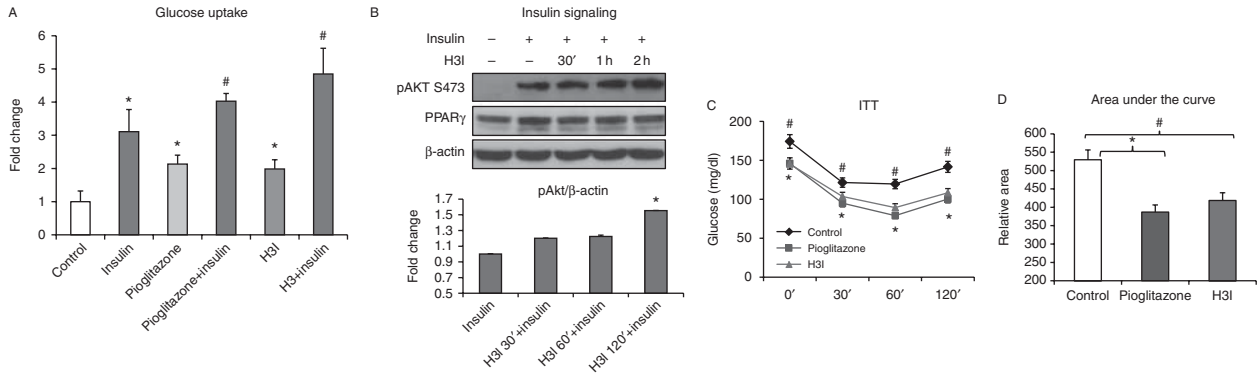

Figure 6

Inhibition of HDAC3 increased glucose uptake and insulin sensitivity. (A) Glucose uptake. Fully differentiated 3T3-L1 adipocytes were treated with H3I 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. #H3I vs control; *pioglitazone vs control; *, #$P<0.05$ by Student's *t*-test, $n=8$.

administered into the diet, and this group of mice received the same amount of DMSO in PBS by i.p. injection every day. After 2 weeks of treatment, insulin sensitivity was evaluated by an ITT after 4 h of fasting. The HDAC3 inhibitor and pioglitazone both significantly reduced glucose levels and enhanced insulin sensitivity (Fig. 6C and D). Body weight and food intake were not changed in the mice by the 2-week treatment. The results indicate that the HDAC3 inhibitor enhanced PPARγ function, serving as an insulin sensitizer in adipocytes.

Discussion

Our results indicate that acetylation of PPARγ is induced by pioglitazone. Pioglitazone induces PPARγ 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 available about PPARγ acetylation, though PPARγ activity is regulated by protein modification such as phosphorylation (Hu *et al*. 1996), sumoylation (Pascual *et al*. 2005), and ubiquitination (Hauser *et al*. 2000, Floyd & Stephens 2002, Christianson *et al*. 2008, Anbalagan *et al*. 2012). In a recent study, it was reported that PPARγ acetylation (lysine 268 and 293) was induced by a ligand and decreased by SIRT1 in HEK293 cells (Qiang *et al*. 2012). Although the results of that study indicated a role of acetylation in the regulation of PPARγ function, it was not known whether the acetylation happens in the absence of a ligand. In that study, PPARγ 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 the interaction of PPARγ with the coactivator PRDM16. It was reported that inhibition of the acetylation promoted preadipocyte differentiation into brown adipocytes in response to cold challenge at 4 °C in a cold room. The results indicated that PPARγ acetylation favors lipid accumulation and preadipocyte differentiation into white adipocytes, which disfavors brown adipocyte differentiation. HDAC1/HDAC3 was reported to modulate PPARγ transcription through the sumoylated CEBPD in hepatic lipogenesis (Lai *et al*. 2008). However, there is no report that PPARγ was acetylated by HDAC1/HDAC3. Our results indicate that HDAC3 regulates PPARγ acetylation and function directly. In this current study, we observed that PPARγ acetylation was induced by pioglitazone (Fig. 2F). PPARγ acetylation was induced by the inhibition of HDAC3 in the absence of an exogenous ligand. The ligand-independent acetylation enhanced the transcriptional activity of PPARγ, as indicated by PPARγ target gene expression, lipid accumulation in adipogenesis, and insulin-induced indicate uptake. Our results indicate that PPARγ acetylation could be a new approach to increase PPARγ activity and that this acetylation may occur in the absence of exogenous ligands. It is not known whether acetylation correlates with phosphorylation, sumoylation, and ubiquitination of the PPARγ protein.

In terms of the mechanism by which PPARγ acetylation leads to PPARγ activation, we would like to propose a model here. In this model, we suggest that there is a basal level of acetylation of the PPARγ protein in the absence of a ligand. The corepressor removes the acetylation constantly to prevent PPARγ activation without a ligand.

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The corepressor activity is abolished when it is disassociated from the PPARγ protein in response to a ligand. When the corepressor activity is inhibited, the acetylation accumulates in the PPARγ protein in the absence of deacetylation activity. Acetylated PPARγ induces the recruitment of acetyltransferases (HATs), such as p300/CBP, to induce gene transcription, which in turn induces histone acetylation in PPARγ-responsive genes (Graves *et al*. 1992, Freedman 1999, Rosen & Spiegelman 2000, Rosen *et al*. 2000). This model explains the role of PPARγ acetylation in PPARγ activation in the absence of a ligand. Our results indicate that PPARγ acetylation is coupled with histone acetylation. Histone acetylation is required for gene transcription, but histone acetylation is probably a consequence of PPARγ acetylation. This possibility remains to be tested.

Our resuls indicate that the HDAC3 inhibitor is a new PPARγ activator that exhibits potency similar to that of pioglitazone. The TZDs are the most powerful insulin sensitizer in the treatment of T2DM (Tontonoz & Spiegelman 2008). TZD-based medicines include pioglitazone (Actos by Takeda Pharmaceuticals (Chuo-ku, Osaka, Japan)) 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 (Rosen 2010, Ferrara *et al*. 2011, Cariou *et al*. 2012, Tseng 2012). We believe that PPARγ activation remains an excellent approach in the treatment of T2DM. All of the TZD-based medicines activate PPARγ. However, their side effects are different for the heart and bladder, indicating that the side effects are not due to PPARγ activation. It is likely that the side effects are the off-target activities of the medicine. Our results indicate that the HDAC3 inhibitor is a potential new generation of PPARγ activator and an insulin sensitizer. Inhibition of HDAC3 is beneficial in preventing neuronal death (Bardai *et al*. 2012), improving β-cell function (Chou *et al*. 2012), and having anticancer effects (Escaffit *et al*. 2007, Bhaskara *et al*. 2008). Moreover, inhibition of HDAC3 promotes the transcriptional activities of myocyte enhancer factor 2 (Naya & Olson 1999, Gregoire *et al*. 2007), 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 indicate that HDAC3 inhibitor may be able to avoid the side effects of synthetic PPARγ ligands *in vivo*.

In conclusion, we report that the transcription factor PPARγ is modulated by acetylation in response to ligands. The acetylation is sufficient to induce PPARγ function in the absence of exogenous ligands. A HDAC3 inhibitor is able to activate PPARγ in a ligand-independent manner. These results indicate that the HDAC3 inhibitor may be a ligand-independent PPARγ activator. Inhibition of HDAC3 may represent a new approach for improving insulin sensitivity in the treatment of T2DM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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