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Article

The Small Molecule Harmine Is an Antidiabetic Cell-Type-Specific Regulator of PPARγ Expression

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SUMMARY

PPARγ is the master regulator of adipogenesis and the molecular target of the thiazolidinedione antidiabetic drugs. By screening for compounds that promote adipogenesis, we identified a small molecule that targets the PPARγ pathway by a distinct mechanism. This molecule, harmine, is not a ligand for the receptor; rather, it acts as a cell-type-specific regulator of PPARγ expression. Administration of harmine to diabetic mice mimics the effects of PPARγ ligands on adipocyte gene expression and insulin sensitivity. Unlike thiazolidinediones, however, harmine does not cause significant weight gain or hepatic lipid accumulation. Molecular studies indicate that harmine controls PPARγ expression through inhibition of the Wnt signaling pathway. This work validates phenotypic screening of adipocytes as a promising strategy for the identification of bioactive small molecules and suggests that regulators of PPARγ expression may represent a complementary approach to PPARγ ligands in the treatment of insulin resistance.

INTRODUCTION

Type 2 diabetes is a major cause of morbidity and an important risk factor for cardiovascular disease, cerebral infarction, blindness, and kidney failure. The prevalence of type 2 diabetes is increasing worldwide, and it is estimated that more than 366 million people will be affected by 2030 (Smyth and Heron, 2006). The primary factor underlying this increase is a rapid rise in the prevalence of obesity and obesity-related insulin resistance. At present, thiazolidinediones (TZDs) are the principal class of drugs available for improvement of insulin sensitivity in obese diabetic subjects (Smyth and Heron, 2006). TZDs are potent agonists for peroxisome proliferator-activated receptor γ (PPARγ), a central regulator of adipocyte gene expression and differentiation (Lehmann et al., 1995; Tontonoz et al., 1994). Although their precise mechanism of action is still the subject of intense research, TZDs are believed to exert their antidiabetic action in large measure by inducing the expression of adipocyte PPARγ target genes involved in lipid and glucose metabolism such as *CD36* and adiponectin (*Adipoq*) (Lehrke and Lazar, 2005).

Despite their efficacy, the development and clinical use of PPARγ ligands is limited by adverse effects such as fluid retention, weight gain, congestive heart failure, liver toxicity, and the potential for carcinogenesis (Berger et al., 2005; Lehrke and Lazar, 2005; Nesto et al., 2003; Yki-Jarvinen, 2004). Alternative approaches to the treatment of insulin resistance are therefore needed. Current attempts to develop safer insulin sensitizers are focused largely on the optimization of PPAR ligands that possess partial or gene-selective agonistic activity or the ability to bind multiple PPAR family members (Berger et al., 2005; Moller and Kaufman, 2005; Yki-Jarvinen, 2004). Since PPARγ agonists ameliorate obesity-related insulin resistance by inducing PPAR target genes in adipocytes, we speculated that small molecules that promote adipocyte differentiation and/or regulate PPARγ target genes by distinct mechanisms could be candidates for novel insulin sensitizers.

We describe here the development and validation of a high-throughput screen for the identification of bioactive small molecules based on an in vitro adipocyte differentiation assay. Using this approach, we identified an adipogenic small molecule, harmine, that regulates expression of the key adipogenic factor PPARγ. Moreover, we demonstrate that the ability of harmine to control PPARγ expression in adipocytes correlates with antidiabetic action in vivo. Harmine mimics the effects of PPARγ ligands on PPARγ-dependent gene expression in white adipose tissue and improves glucose tolerance in diabetic mice. Small-molecule regulators of PPARγ expression may represent a complementary approach to PPARγ ligands in the treatment of insulin resistance.

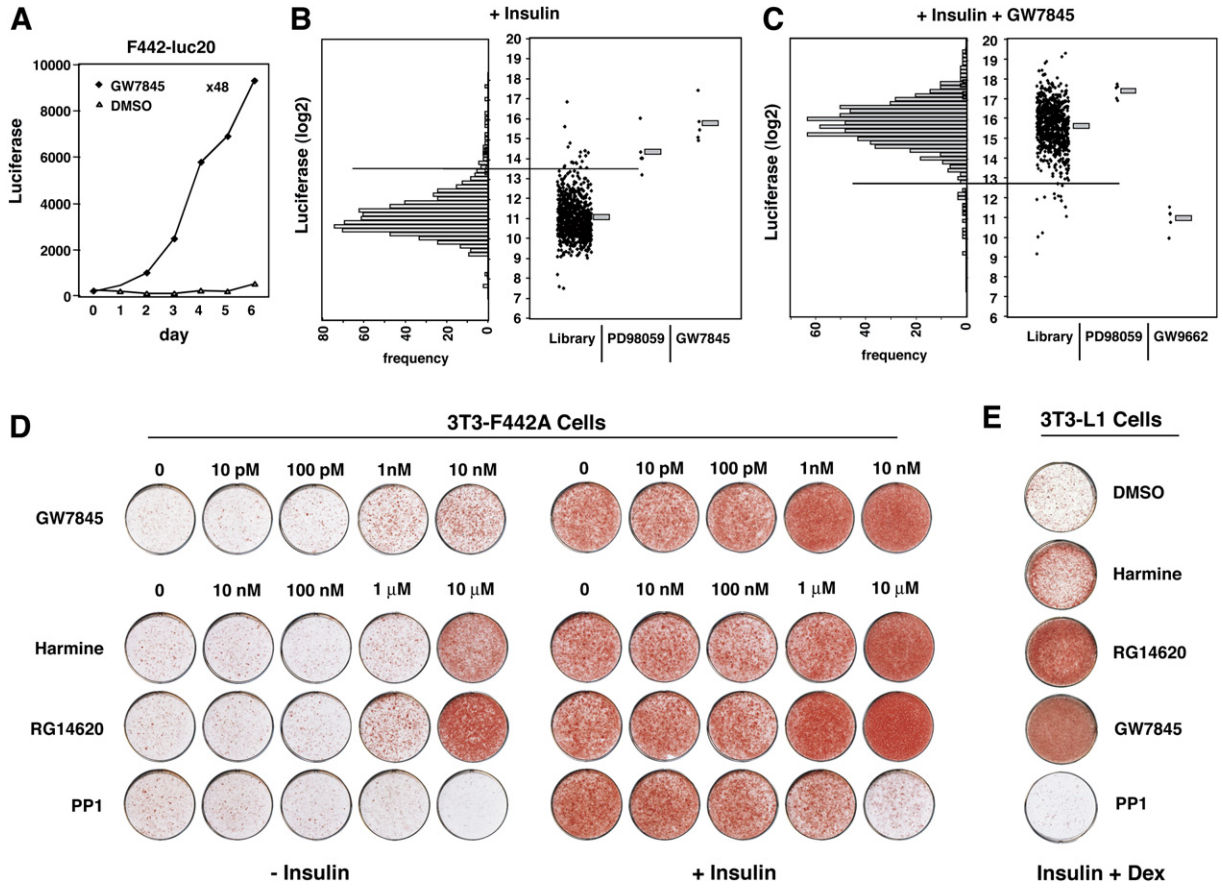

Figure 1. Identification of Small-Molecule Regulators of Adipogenesis by High-Throughput Cell-Based Screening

(A) Induction of luciferase activity during the differentiation of a stable reporter cell line, F442-luc20. Luciferase activity was determined after stimulation of F442-luc20 cells with the PPARγ ligand GW7845 (1 μM) as indicated.

(B and C) Histogram (left) and scatter plot (right) of the results from screening of the BIOMOL library in 96-well format for activators (B) or inhibitors (C) of differentiation. The library and insulin (5 μg/ml) were added to F442-luc20 cells 1 day before and on the day of confluence, respectively. For the inhibitor screen (C), GW7845 (10 nM) was added to the cells along with insulin. Luciferase was measured on day 5 after confluence and is shown in log scale. PD98059 and GW7845 were used as controls for activators, and GW9662 was used as a control for inhibitors. Compounds with activity more than 2.5 standard deviations from the mean (cutoff indicated by horizontal line) were chosen as initial positives.

(D and E) Oil red O staining of 3T3-F442A (D) and 3T3-L1 cells (E) after stimulation of differentiation by GW7845, harmine, RG14620, or PP1 at the indicated concentrations for 6 days with or without insulin (5 μg/ml) or dexamethasone (1 μM, first 2 days only) as indicated. In 3T3-L1 differentiation (E), GW7845 was used at 10 nM concentration, and all other compounds were used at 10 μM.

RESULTS

A Cell-Based Screen for Small-Molecule Regulators of Adipogenesis

To identify small-molecule inducers of differentiation, we generated a 3T3-F442A reporter cell line (F442-luc20) that stably expressed luciferase under the control of the differentiation-dependent −5.4 kb aP2 promoter (Ross et al., 1990). Luciferase activity in this cell line was induced up to 50-fold following stimulation of differentiation by the PPARγ agonist GW7845 (Willson et al., 2000) for 6 days (Figure 1A). This cell line was a sensitive reporter of proadipogenic small molecules such as GW7845 and PD98059 (Hu et al., 1996), as well as antiadipogenic molecules such as the PPARγ antagonist GW9662 (Willson et al., 2000) when assayed in 96-well or 384-well format (Figures 1B and 1C and data not shown). We used F442-luc20 cells to screen chemical libraries in conjunction with the UCLA Molecular Screening Shared Resource. Results from a BIOMOL library of 504 bioactive compounds screened for activator and repressor activities are shown graphically in Figures 1B and 1C. Secondary screening identified two compounds, harmine and RG14620, as positive regulators and one previously unknown negative regulator, the kinase inhibitor PP1 (see Figures S1A–S1C in the Supplemental Data available with this article online). Of note, a number of previously characterized inhibitors were also identified in the repressor screen, including various retinoids and prostaglandin F analogs, validating our approach. Adipocyte differentiation assays by oil red O staining and morphology in both 3T3-F442A and 3T3-L1 cells confirmed the pro- or

antiadipogenic activity of these compounds (Figures 1D and 1E and data not shown).

Since the activity of the luciferase reporter used in our screen is highly dependent on PPARγ, it was reasonable to suspect that some of the hits we obtained might be direct ligands for the receptor. However, harmine was inactive in transfection assays for PPARγ and RXR ligand activity using chimeric PPARγ-GAL4 or RXRα-GAL4 receptors and a luciferase reporter containing GAL4 binding sites (Forman et al., 1995) (Figure S2). Harmine also had no effect on the response of PPARγ to GW7845 in this assay, indicating that it is not an antagonist of the receptor (data not shown). RG14620 activated PPARγ very weakly at high doses, but this activity appears insufficient to account for its adipogenic action.

Harmine: A Small-Molecule Regulator of PPARγ Expression

At this stage, harmine was selected for further studies. Gene expression analysis confirmed increased expression of the adipocyte differentiation-dependent PPARγ target genes *aP2*, *LPL*, and *CD36* in 3T3-F442A preadipocytes treated with either harmine or GW7845 (Figure 2A). Rapid induction of these genes by GW7845 on day 1 was consistent with direct activation of PPARγ. However, the effects of harmine were delayed in onset, suggestive of a more indirect effect. PPARγ does not regulate its own expression directly (Tontonoz et al., 1994), and therefore, treatment with GW7845 led to only modest increases in *PPARγ* mRNA expression secondary to increased differentiation. Surprisingly, *PPARγ* mRNA and protein expression were strongly induced by harmine (Figures 2A and 2B). Consistent with their effects on lipid accumulation (Figure 1), induction of *aP2* expression by either harmine or GW7845 was dose dependent (Figure 2C). In contrast to harmine, GW7845 had only marginal effects on *PPARγ* mRNA expression, even at high doses.

Time-course experiments revealed that the induction of *PPARγ* (both isoforms 1 and 2) by harmine occurred as early as 4 hr after treatment (Figure 2D). Notably, harmine did not alter the expression of *C/EBPβ* or *C/EBPδ*, two transcription factors reported to be upstream of PPARγ in the adipocyte differentiation cascade (Wu et al., 1995, 1996). On the other hand, the standard differentiation induction cocktail (dexamethasone, IBMX, and insulin; DMI) upregulated the expression of *C/EBPβ* or *C/EBPδ* but did not induce *PPARγ* expression at these time points in 3T3-F442A cells (Figure 2D). Harmine had no effect on other known positive regulators of differentiation, including KLF5 and EBF1 (data not shown). Harmine was also observed to increase *PPARγ* expression in fully differentiated F442A adipocytes (Figure 2E), although the magnitude of this induction was smaller than in preadipocytes. The induction of *PPARγ* mRNA by harmine is the result of increased transcription, since it was blocked by actinomycin D treatment (Figure 2F). Consistent with the effect of harmine on 3T3-L1 differentiation, *PPARγ* was also induced by harmine in 3T3-L1 cells (Figure 2G). In order to confirm that the magnitude of *PPARγ* induction by harmine was sufficient to alter target gene expression in adipocytes, we ectopically expressed *PPARγ* in 3T3-L1 cells. Indeed, modest increases in *PPARγ* expression in preadipocytes led to significant increases in both lipid accumulation and *aP2* expression (Figure 2H).

Interestingly, we found that the effect of harmine on PPARγ was cell-type specific. In primary mouse hepatocytes, harmine had no effect on expression of either *PPARγ* or its target genes (*aP2* and *LPL*), while GW7845 induced *aP2* and *LPL* but not *PPARγ* (Figure S3A). On the other hand, harmine did induce expression of both *PPARγ1* and *PPARγ2* in primary mouse bone-marrow-derived macrophages (Figure S3B), suggesting that harmine might promote the anti-inflammatory action of PPARγ in this cell type (see below).

Harmine has previously been reported to interact with several cell-surface receptors, including monoamine oxidase A (MAO-A) (Kim et al., 1997), serotonin receptor 2A (5-HT2A) (Glennon et al., 2000), imidazoline receptors (I1 and I2 sites) (Husbands et al., 2001), and cyclin-dependent kinases (CDK1, 2, and 5) (Song et al., 2004). However, none of these appear to be the target for the metabolic actions of harmine in adipocytes. Highly specific ligands for MAO-A and MAO-B (clorgyline and deprenyl), 5-HT2A (α-methyl-5-hydroxytryptamine), imidazoline receptor I1 and I2 sites (clonidine and 2-BFI), and CDKs (roscovitine) did not promote adipocyte differentiation or *PPARγ* expression (Figure S4 and data not shown).

We also tested the effect of a combination of harmine and a maximal dose of GW7845 (100 nM). 3T3-F442A cells treated with both harmine and GW7845 showed greater differentiation than cells treated by either compound alone (Figure 2I). Harmine and the PPARγ agonist also showed an additive effect on the expression of the PPARγ target genes *aP2* and *Adipoq*, but not *PPARγ* itself. Together, the above results demonstrate that harmine acts via a mechanism distinct from that of previously described adipogenic chemicals, including PPARγ agonists, cAMP inducers, insulin, and glucocorticoids.

Harmine Is an Inhibitor of Wnt Signaling in Preadipocytes

Since harmine did not appear to alter known pathways that promote differentiation, we tested its effect on inhibitory pathways. Harmine had no effect on expression of Pref-1 and Notch1 (data not shown) but showed prominent effects on Wnt signaling in preadipocytes. The Wnt signaling pathway is a particularly important inhibitor of differentiation (Ross et al., 2000). Wnt activation blocks adipocyte differentiation, whereas suppression of the Wnt pathway promotes differentiation. Activation of TCF/LEF transcription factors by β-catenin plays a critical role in the canonical Wnt pathway (Moon et al., 2002). We found that harmine suppressed the activation of the TOPFLASH reporter by LEF1 and active β-catenin in both 293T cells and preadipocytes (Figure 3A). Harmine also suppressed the activation of LEF1 triggered by the GSK3β inhibitor LiCl or Wnt-3a (Figure 3B). Furthermore, harmine blocked the induction of the endogenous Wnt target

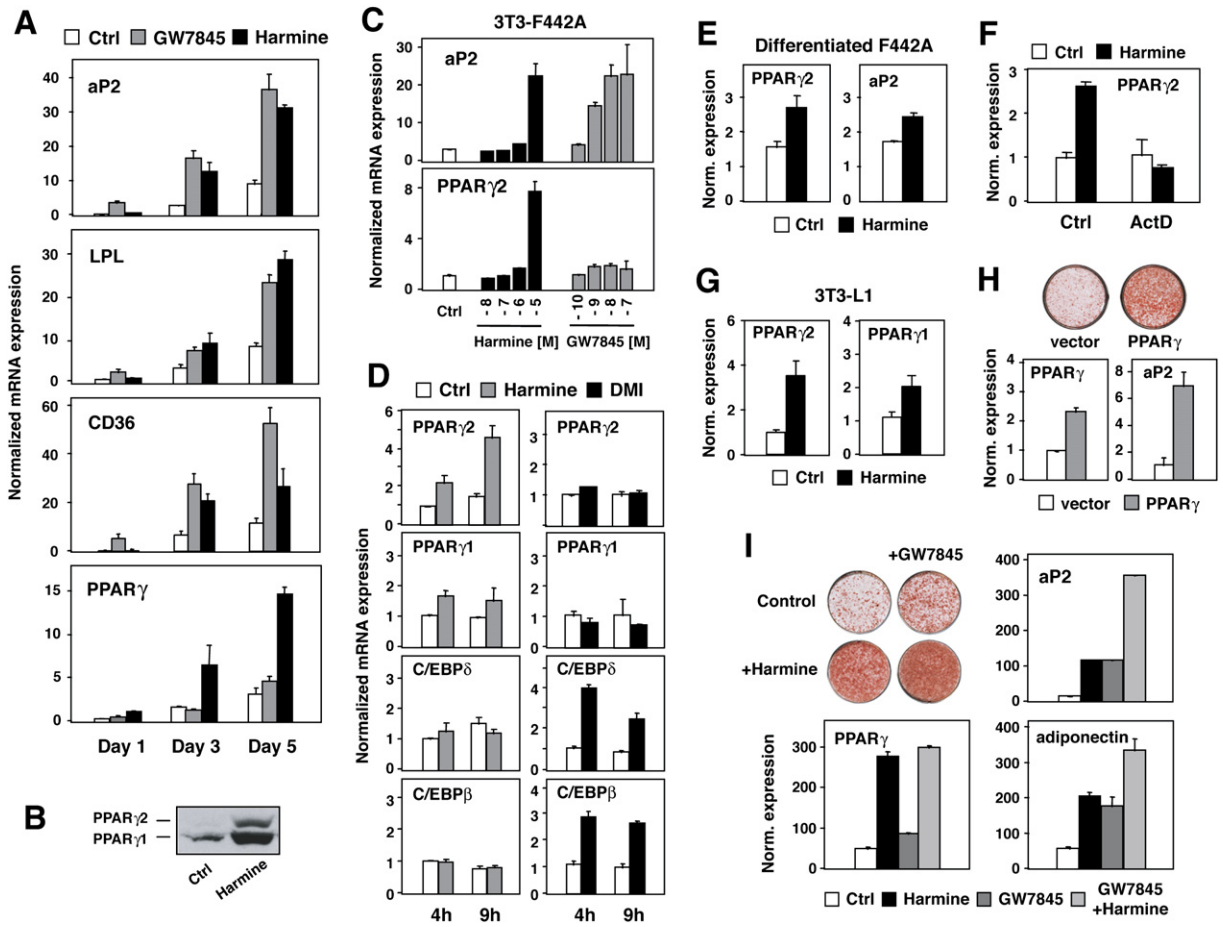

Figure 2. Harmine Is a Specific Inducer of PPARγ Expression in Preadipocytes

(A) Gene expression profile during the differentiation of 3T3-F442A cells treated with GW7845 (20 nM) or harmine (10 μM). Cells were treated with chemicals and insulin (5 μg/ml) at confluence. In this and all other figures, error bars represent SEM.

(B) Induction of PPARγ protein expression by harmine. 3T3-F442A cells were treated with harmine (10 μM) and insulin (5 μg/ml) for 6 days. Protein samples were collected and analyzed by western blotting.

(C) Selective and dose-dependent induction of *PPARγ2* expression by harmine. 3T3-F442A cells were treated with harmine or GW7845 at the indicated concentration for 5 days.

(D) Comparison of the effects of harmine and the standard differentiation induction cocktail (dexamethasone, IBMX, and insulin; DMI) on early gene expression change in 3T3-F442A cells. Cells were treated with harmine (10 μM) or DMI (1 μM, 0.5 mM, and 5 μg/ml, respectively) for the indicated time 1 day after confluence.

(E) Induction of *PPARγ* expression in 3T3-F442A cells by 48 hr harmine treatment 7 days after induction of differentiation.

(F) Actinomycin D treatment blocks the induction of *PPARγ2* expression by harmine. One day after confluence, 3T3-F442A cells were pretreated with actinomycin D (5 μg/ml) 1 hr before harmine treatment (10 μM). RNA was isolated after 12 hr.

(G) Induction of *PPARγ* expression by harmine in 3T3-L1 cells. Cells were treated with harmine (10 μM) in the presence of insulin (5 μg/ml) 1 day after confluence for 48 hr.

(H) Induction of *aP2* expression and lipid accumulation in 3T3-L1 cells overexpressing *PPARγ*. 3T3-L1 cells stably overexpressing *PPARγ* were generated as described previously (Tontonoz et al., 1994). Gene expression was measured at confluence. The plate was stained with oil red O on day 7.

(I) Additive effect of harmine and GW7845 on adipocyte differentiation and PPARγ target gene expression. Top left: oil red O staining of 3T3-F442A cells differentiated for 6 days with harmine, a maximal dose of GW7845 (100 nM), or both. Note: Higher doses of GW7845 do not further increase differentiation in this assay (data not shown). mRNA was collected from duplicate cultures on day 3, and expression of *PPARγ*, *aP2*, and *Adipoq* was determined. Gene expression was analyzed by real-time PCR in all panels.

genes *axin2*, *wisp2*, and *nkd2* by Wnt-3a in preadipocytes (Figure 3C and data not shown) (Jackson et al., 2005). Reciprocally, harmine reversed Wnt suppression of *PPARγ* as well as its target genes *aP2*, *Adipoq*, and *CD36* (Figure 3C and data not shown). In line with the effects on gene expression, harmine overcame the inhibitory effect of Wnt-3a or LiCl on adipocyte differentiation as assessed by oil red O staining and morphology (Figures 3D and 3E).

To further establish that harmine regulation of *PPARγ* is mediated through inhibition of Wnt signaling, we conducted limited structure-function analysis. We assayed

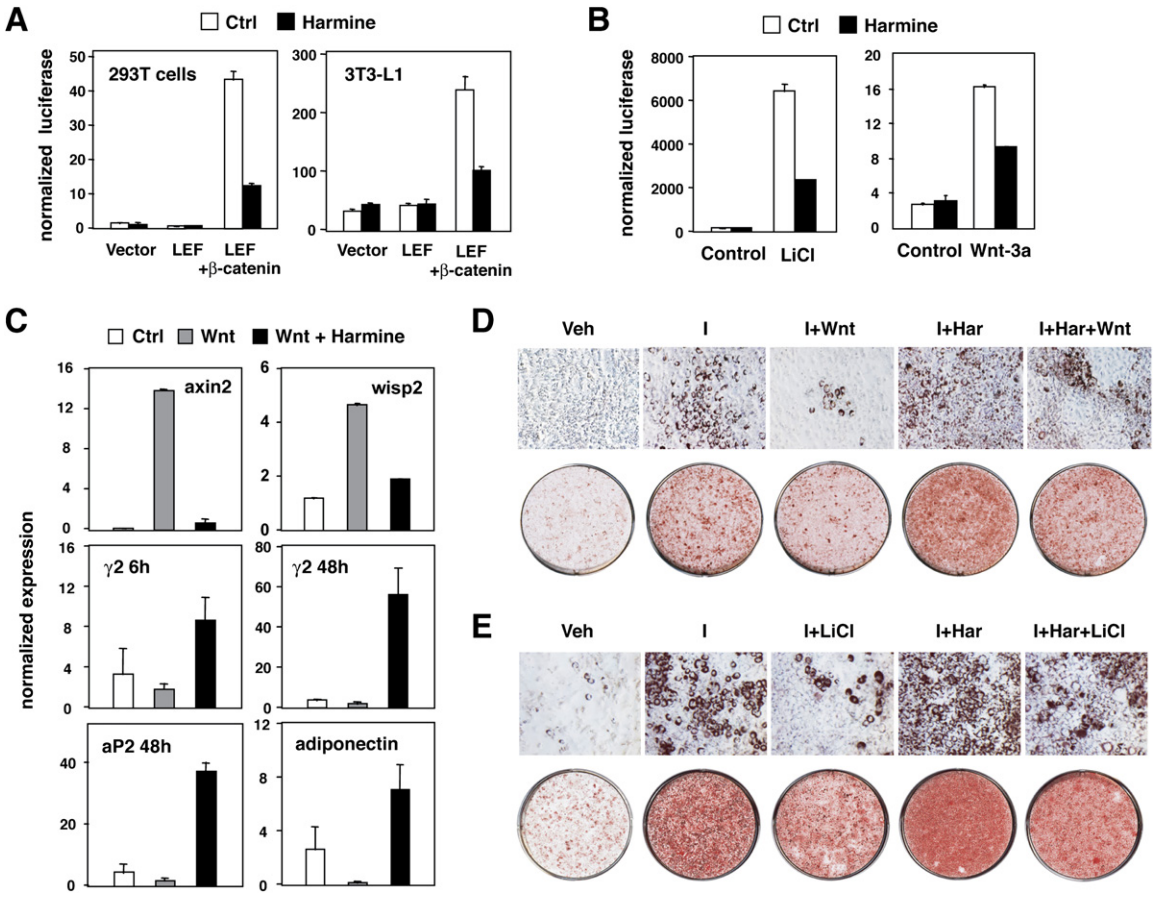

Figure 3. Inhibition of the Wnt Signaling Pathway by Harmine

(A) Harmine (10 μM) suppresses activation of the TOP-FLASH reporter by LEF1 and active β-catenin in 293T cells (left) and 3T3-L1 cells (right).

(B) Harmine suppresses activation of the TOP-FLASH reporter by the GSK3β inhibitor LiCl (25 mM) or Wnt-3a-conditioned medium in 293T cells.

(C) Reversal of Wnt-3a effects on Wnt target genes (top), PPARγ (middle), and PPARγ target genes (bottom) by harmine in 3T3-F442A cells. Cells were treated with Wnt-3a (10 ng/ml) and harmine (10 μM) 1 day after confluence.

(D and E) Harmine reverses the inhibitory effect of Wnt-3a (D) and LiCl (10 mM) (E) on adipocyte differentiation of 3T3-F442A. Top, microscopic view; bottom, oil red O staining. I, insulin.

six derivatives of harmine to determine the correlation between adipogenic and Wnt-inhibitory activity (Figure 4A). Most of these derivatives were inactive in both assays. Only 6-methoxyharman induced adipogenesis (Figure 4B) and *PPARγ* expression (Figure 4C), and this was also the only derivative that showed an inhibitory effect in the TOP-FLASH assay (Figure 4D). Finally, the magnitude of induction of *PPARγ* expression and lipid accumulation in response to harmine was significantly reduced in 3T3-F442A preadipocytes expressing a dominant-negative TCF construct (Figures 4E and 4F). Note: This dominant-negative construct reduces, but does not abolish, Wnt-stimulated gene expression in these cells (data not shown). Therefore, the residual effect of harmine could result from effects on alternative pathways or simply from suppression of residual Wnt activity. Together, the data of Figure 3 and Figure 4 demonstrate that the adipogenic activity of harmine results, at least in part, from its ability to block Wnt signaling in adipocytes.

Harmine Mimics the Effects of PPARγ Agonists in Diabetic Mice

Next, we investigated the effect of harmine in vivo. We treated C57BL/6 mice with vehicle (saline) or harmine (30 mg/kg) twice per day for 3 days. Consistent with the in vitro results, expression of *PPARγ* in white adipose tissue (WAT) was induced by harmine treatment (Figure S5). Furthermore, expression of the PPARγ target genes *aP2*, *CD36*, and *Adipoq* was also elevated in WAT of harmine-treated mice. Doses higher than 10 mg/kg were required to observe significant induction of *PPARγ* expression (data not shown). No differences in hepatic *PPARγ* expression were observed, however, suggesting that the effects of harmine on gene expression are tissue specific (data not shown; see also below).

Given the ability of harmine to mimic TZDs in their ability to regulate PPARγ target genes in adipose tissue, we asked whether harmine displayed antidiabetic activity. We treated obese *db/db* mice (C57BLKS/J background)

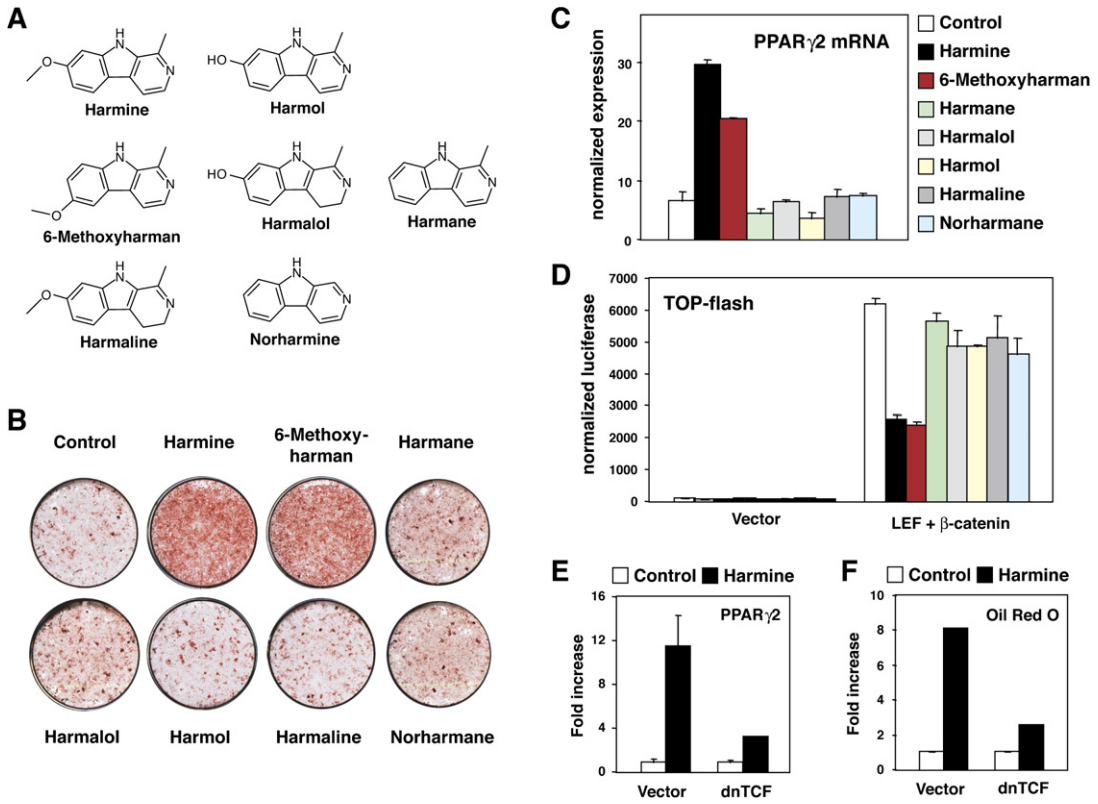

Figure 4. Structure-Function Analysis of Harmine Derivatives in Adipogenesis and Wnt Signaling

(A) Chemical structures of harmine derivatives.

(B and C) Effect of harmine derivatives (all at 10 μM) on adipocyte differentiation and *PPARγ* mRNA expression in 3T3-F442A cells.

(B) Oil red O staining after 6 days.

(C) PPARγ2 expression after 48 hr.

(D) Inhibition of LEF1 and active β-catenin-induced TOP-FLASH activity by harmine derivatives in 293T cells.

(E and F) Dominant-negative TCF blunts harmine effects on *PPARγ* expression and differentiation. 3T3-F442A cells stably expressing vector or a dominant-negative TCF protein (dnTCF) were generated by retroviral transduction.

(E) *PPARγ* expression 24 hr after harmine or vehicle treatment was determined by real-time PCR.

(F) Oil red O staining of cells on day 5 of differentiation was quantitated by measuring the dye extracted in isopropanol with a colorimetric assay (510 nm).

with either vehicle or harmine for 6 weeks. Body weight, body composition, and food intake were not different between groups (Figures 5A–5C). At the start of the experiment, the mice were not hyperglycemic, but, as expected, vehicle-treated mice progressed to diabetes over the following 30 days (Figure 5D). Within 2 weeks of treatment, harmine-treated mice exhibited reduced blood glucose levels compared to controls, and their development of hyperglycemia was delayed. Harmine-treated mice also showed lower levels of both free fatty acids (FFAs) and triglycerides (Figure 5E) after 26 days of harmine treatment. Moreover, both glucose tolerance and response to insulin were improved in animals receiving harmine, as determined by glucose (Figure 5F) and insulin (Figure 5G) tolerance tests. Since harmine has been reported to have CNS effects at doses of ~2 mg/kg (Pennes and Hoch, 1957; Zetler et al., 1972), we tested whether this dose, which does not induce *PPARγ* expression (data not shown), was sufficient to affect glucose metabolism. Mice treated with 2 mg/kg harmine did not show significant decreases in fasting glucose or increases in glucose tolerance (Figure 5H and data not shown). We also tested the effect of a combination of harmine (30 mg/kg) and rosiglitazone (4 mg/kg). Consistent with our in vitro observations, harmine and the PPARγ agonist showed an additive effect on fasting blood glucose levels in vivo (Figure 5I).

Harmine Regulates Metabolic and Inflammatory Gene Expression In Vivo

The beneficial effects of harmine on metabolic parameters in *db/db* mice correlated with beneficial effects on both metabolic and inflammatory gene expression in WAT. Expression of *PPARγ1* and *γ2* and their downstream target genes involved in lipid storage and glucose metabolism such as *aP2*, *CD36*, *LPL*, *GLUT4*, *Lipin-1a* and *-1b*, *C/EBPα*, and *Adipoq* was increased in WAT of harmine-treated animals compared to controls (Figure 6A and Figure S6). Furthermore, harmine reduced expression of the inflammatory

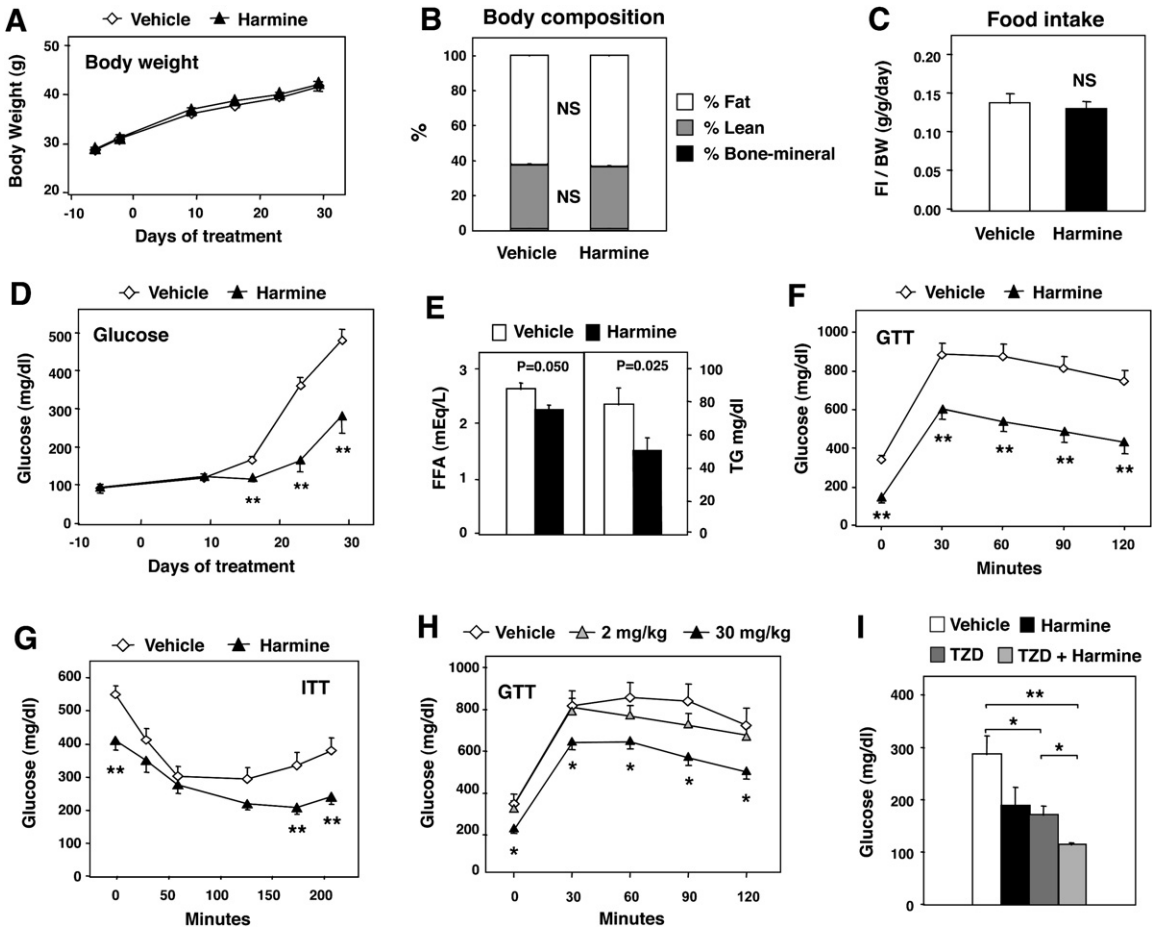

Figure 5. Harmine Treatment Ameliorates Insulin Resistance in *db/db* Mice

(A) Body weight of vehicle- (saline-) and harmine-treated mice. n = 10–11 per group.

(B) Whole-body composition of *db/db* mice treated with harmine was determined by dual-energy X-ray absorptiometry (DEXA scan) on day 19 of treatment. n = 12–14 per group.

(C) Food intake presented as amount consumed per mouse per day divided by body weight. n = 10–11 per group.

(D) Time course of glucose levels (16 hr fasting) in *db/db* mice treated with vehicle or harmine (30 mg/kg, i.p.). n = 10–11 per group.

(E) Free fatty acid and triglyceride levels on day 26 of treatment. n = 10–11 per group.

(F) Glucose tolerance test (GTT) performed on day 23 of treatment. n = 10–11 per group.

(G) Insulin tolerance test (ITT) performed on day 37 of treatment. n = 10–11 per group.

(H) Dose-dependent effect of harmine on GTT performed after 22 days of treatment. n = 8–9 per group.

(I) Additive effect of harmine and rosiglitazone on fasting glucose levels. Glucose levels were measured after 16 hr fasting on day 29 of harmine treatment (30 mg/kg) and day 11 of rosiglitazone treatment (4 mg/kg). n = 7–9 per group. $^{**}p < 0.01$; $^{*}p < 0.05$.

mediators *TNFα*, *IL-1β*, and *iNOS*, as well as the macrophage markers *F4/80* and *Mac1*, suggesting that adipose tissue inflammation was decreased. Immunohistological analysis confirmed that macrophage accumulation in WAT, as assessed by the number of crown-like structures (Cinti et al., 2005), was indeed reduced by harmine (Figures 6B and 6C). The suppressive effect of harmine on inflammatory gene expression and macrophage accumulation is consistent with previous work showing that PPARγ ligands suppress inflammatory gene expression and reduce macrophage numbers in WAT (Xu et al., 2003).

We also investigated the link between Wnt signaling and the tissue specificity of harmine on *PPARγ* expression in vivo. We found that expression of the Wnt target gene *wisp2* was significantly reduced in adipose tissue of mice treated with harmine (Figure 6D), consistent with the in vitro inhibition of Wnt signaling by harmine (Figure 3). The suppression of *wisp2* by harmine, however, was not restricted to WAT but was also seen in other tissues such as skeletal muscle and liver (Figure 6D). This observation suggests that the tissue-specific effects of harmine on *PPARγ* expression cannot be explained by tissue-specific inhibition of the Wnt pathway. We next investigated the tissue specificity of Wnt effects on *PPARγ* expression. Treatment of 3T3-F442A preadipocytes, primary hepatocytes, and C2C12 myocytes with Wnt-3a resulted in induction of the Wnt target genes *wisp2*, *axin2*, and *nkd2* in all three cell types (Figure 6E). However,

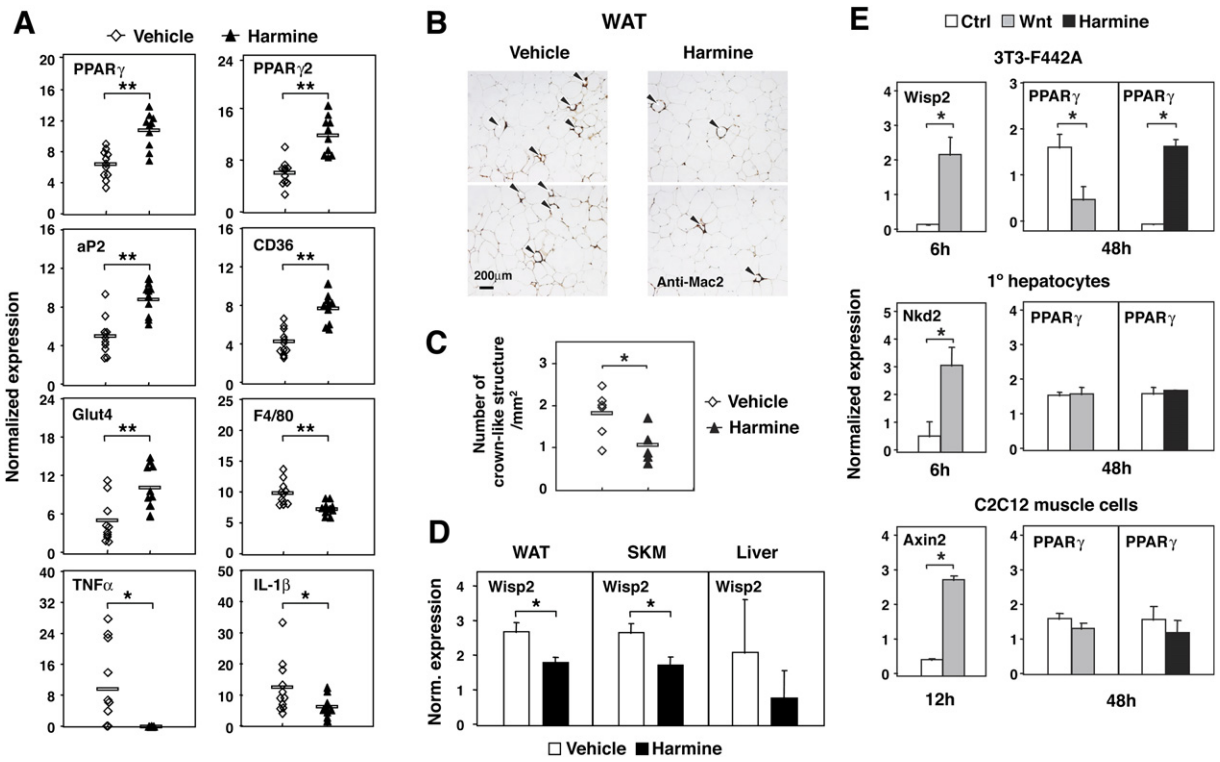

Figure 6. Harmine Treatment Induces Expression of PPARγ and Its Target Genes and Suppresses Adipose Tissue Inflammation In Vivo

(A) Gene expression in epididymal white adipose tissue (WAT) was determined by real-time PCR on day 43. n = 10–11 per group. Dots and bars in scatter plots represent individual mice and the average, respectively.

(B) Immunohistochemistry of epididymal WAT of *db/db* mice treated with harmine (day 43) with anti-Mac2 antibody. Arrowheads indicate crown-like structures, necrotic adipocytes surrounded by macrophages (Cinti et al., 2005).

(C) Quantification of the number of crown-like structures in epididymal WAT. Data are presented as number per mm^2. n = 6 per group.

(D) Expression of the Wnt target gene *wisp2* in WAT, skeletal muscle (SKM), and liver of mice treated with harmine (30 mg/kg, i.p., twice per day) for 3 days was analyzed by real-time PCR. n = 10 per group.

(E) Effect of Wnt or harmine treatment on *PPARγ* expression in 3T3-F442A cells, primary hepatocytes, and C2C12 myocytes. Cells were treated with either Wnt-3a-conditioned medium or harmine (10 μM) for the indicated time. Gene expression was analyzed by real-time PCR. $^{**}p < 0.01$; $^{*}p < 0.05$.

Wnt activation suppressed PPARγ expression only in preadipocytes, and harmine induced PPARγ expression only in preadipocytes. These data strongly suggest that the tissue-specific effect of harmine on *PPARγ* expression results from tissue-selective effects of the Wnt pathway on the *PPARγ* gene.

Harmine Promotes Fatty Acid Oxidation and Energy Expenditure

Interestingly, harmine also increased expression of *PGC-1α* and PPAR target genes involved in fatty acid oxidation in WAT, such as acyl-CoA oxidase (*Acox1*), *LCAD*, *MCAD*, and *CTP-1b*, as well as genes involved in energy dissipation and adoptive thermogenesis, such as *UCP1* and *UCP3* (Figure S6). All of these gene expression changes also occur in response to PPARγ ligands, further emphasizing the similarity of harmine and PPARγ ligand effects (Way et al., 2001). Previous studies have shown that induction of these genes by PPARs and PGC-1α is associated with increased energy expenditure (Kamei et al., 2003; Picard et al., 2002; Rothwell et al., 1987; Tanaka et al., 2003; Uno et al., 2006; Wang et al., 2003). Consistent with the induction of these genes by harmine, indirect calorimetry demonstrated increased oxygen consumption and heat production in harmine-treated mice, but no change in physical activity (Figure 7). Harmine treatment did not significantly alter expression of a number of other genes examined, including *C/EBPβ*, *C/EBPδ*, *PPARδ*, and *UCP2* (Figure S6 and data not shown). Expression of *resistin* (Steppan et al., 2001) was not altered by harmine, and *RBP4* (Yang et al., 2005) was increased, suggesting that these factors are not mediators of harmine effects on systemic metabolism.

In contrast to the multiple effects observed in WAT, harmine had little effect on gene expression linked to glucose metabolism in other key metabolic tissues including liver, brown adipose tissue (BAT), and skeletal muscle (Figures S7–S9). There was a trend toward increased *PPARγ2* expression in BAT, but this did not achieve statistical significance. Harmine did not alter genes involved in hepatic

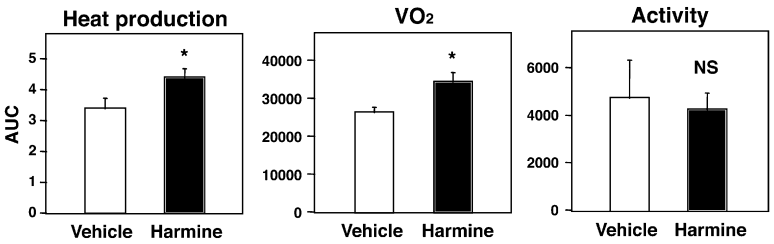

Figure 7. Effect of Harmine Administration on Energy Expenditure in Diabetic Mice

Oxygen consumption, heat production, and activity were measured over 3 days by indirect calorimetry using the Oxymax Lab Animal Monitoring System. Mice (five 10-week-old *db/db* males per group) were individually housed and allowed to acclimate in the experimental cages with free access to food and water. Animals were maintained on 12 hr light/dark cycles. Mice were treated daily with vehicle or 30 mg/kg harmine for 2 weeks prior to the experiment, and treatment continued during the experiment. Data on heat production (calculated from gas exchange data; kcal/mass/time), VO_2 (volume of oxygen consumed; ml/mass/time), and activity (XYZ coverage; total activity counts) were analyzed using the OxymaxWin v3.32 software package. Statistical comparisons of repeated measurements were made using ANOVA. For ease of presentation, data are expressed as area under the curve (AUC) calculated for the light and the dark period over the 3 recording days; statistical comparison of these totals was made using Student's t test. *$p < 0.05$.

glucose production, did not increase expression of thermogenic genes in BAT, and did not increase expression of genes involved in glucose uptake or fatty acid oxidation in skeletal muscle. The lack of significant harmine effects on these pathways is consistent with WAT being the primary target of its metabolic actions.

Harmine Does Not Share Hepatic and Renal Side Effects of Thiazolidinediones

The tissue specificity of harmine action suggested that this compound might not share some of the established side effects of TZDs. One of undesirable side effects of TZD treatment is weight gain. Unlike TZD treatment, we did not observe a significant difference in body weight within the time frame of this study (Figure 5A). Recent data indicate that fluid retention caused by activation of PPARγ in the kidney accounts for a significant fraction of short-term weight gain in TZD-treated mice (Guan et al., 2005; Zhang et al., 2005). Harmine did not alter the expression of *PPARγ* or PPARγ target genes including *aP2*, *LPL*, and *ENaCγ* in the kidney (Figure S10A). TZDs have also been shown to increase the number of small adipocytes in WAT, presumably by promoting differentiation (Okuno et al., 1998). On histological examination, WAT from PPARγ ligand-treated mice exhibited reduced adipocyte size as expected; however, harmine did not have a significant influence on adipocyte size in our studies (data not shown). The lack of an obvious effect of harmine on differentiation in vivo despite effects on glucose tolerance is consistent with modest activation of the PPARγ pathway: low-dose treatment with the PPARγ ligand pioglitazone (10 mg/kg) or a partial PPARγ agonist is sufficient to improve glucose tolerance without inducing adipocyte differentiation in vivo (Berger et al., 2003; Kubota et al., 2006; Rocchi et al., 2001). TZDs have also been shown to promote hepatic triglyceride accumulation in diabetic mice due to activation of PPARγ in the liver (Chao et al., 2000; Matsusue et al., 2003). In line with the inability of harmine to regulate *PPARγ* expression in liver (Figure S10B), however, harmine did not promote hepatic lipid accumulation in *db/db* mice, an established side effect of TZDs (Figure S10C).

DISCUSSION

The development of new drugs for the treatment of insulin resistance is an important goal of metabolic research. In this paper, we have outlined a cell-based strategy for the identification of metabolic regulators. We developed an assay for adipocyte differentiation that is amenable to high-throughput screening, and we used this assay to identify a particularly interesting compound, harmine. The idea that stimulators of adipogenesis could be beneficial in diabetes is somewhat counterintuitive given the association of obesity with insulin resistance. However, strong support for this idea came from the unexpected discovery that the antidiabetic thiazolidinediones were ligands for the adipogenic transcription factor PPARγ (Lehmann et al., 1995). We have now provided a second example of a small molecule that both promotes adipocyte differentiation in vitro and improves glucose tolerance in vivo. The biological activities of harmine and TZDs underscore the fact that links between adipose tissue and insulin resistance are complex and are likely to involve not only changes in adipose tissue differentiation and cell number but also changes in adipocyte function. Our results validate phenotypic screening for modulators of adipogenesis as a promising approach to the discovery of new metabolic drugs.

Historically, deciphering the mechanism of action of small molecules identified through phenotypic screening has often brought new insights into biology (Schreiber, 2003). Such was the case for TZDs, which were first identified as compounds that improved insulin sensitivity in rodents and only later recognized to be agonists for PPARγ (Lehmann et al., 1995; Schreiber, 2003). This discovery led to the elucidation of PPARγ as a key player in the pathophysiology of insulin resistance (Evans et al., 2004; Lehrke and Lazar, 2005). Our characterization of the biological activities of harmine has shed light on the relationship between Wnt signaling, *PPARγ* expression, and glucose metabolism. To our knowledge, cell-type-selective small-molecule regulators of *PPARγ* expression have not been described previously. Although most drug discovery efforts in this area are focused on the identification of new receptor ligands, previous studies using

mouse genetic models have supported the concept that alteration of *PPARγ* expression levels can also impact insulin sensitivity (He et al., 2003; Kubota et al., 1999; Miles et al., 2000). Our results extend this idea by showing that a small-molecule regulator of *PPARγ* expression can improve glucose metabolism in diabetic animals. The discovery that *PPARγ* expression in adipose tissue can be regulated pharmacologically opens the door to new strategies for the development of metabolic drugs.

Although the precise protein target remains to be identified, considerable evidence suggests that the metabolic effects of harmine in diabetic mice result at least in part from activation of the PPARγ pathway. First, harmine induces expression of PPARγ and its target genes in multiple preadipocyte cell lines without affecting the expression of other adipogenic factors such as *C/EBPβ* and *KLF5*. Second, harmine induces expression of *PPARγ* in adipose tissue in vivo, an activity not shared with any other small molecule to our knowledge. Third, the effects of harmine on gene expression in adipose tissue are virtually identical to the effects of TZDs (Way et al., 2001). Harmine and TZDs regulate the expression of the same battery of PPARγ target genes involved in fatty acid metabolism (transport, storage, synthesis, and oxidation) and energy dissipation (Way et al., 2001). Fourth, harmine mimics the effects of TZDs on inflammation in adipose tissue. Similar to TZDs, harmine attenuates inflammatory gene expression and macrophage accumulation in adipose tissue, which has been proposed to contribute to obesity-induced insulin resistance (Hotamisligil, 2006; Neels and Olefsky, 2006; Xu et al., 2003). Fifth, harmine induces energy expenditure in diabetic mice as previously reported for TZDs. Sixth, harmine's efficacy at lowering glucose levels and regulating adipocyte gene expression is consistent with other weak activators of the PPARγ pathway such as partial agonists (Berger et al., 2003; Rocchi et al., 2001). Seventh, regulation of PPARγ target gene expression in adipose is known to be critical for the actions of TZDs on glucose and lipid metabolism (Evans et al., 2004). Therefore, the metabolic effects of harmine are consistent with well-established links between adipocyte gene expression and systemic metabolism. Finally, the metabolic effects of harmine cannot be explained by changes in gene expression in liver or skeletal muscle. Thus, regulation of the PPARγ pathway in adipose tissue provides a highly plausible explanation for the metabolic effects of harmine.

Structure-function studies and gene expression analysis suggest that harmine exerts its metabolic effects at least in part through inhibition of Wnt signaling. Harmine inhibits Wnt-dependent gene expression in adipocyte cell lines and adipose tissue and reverses the inhibitory effect of Wnt agonists on differentiation and *PPARγ* expression. Interestingly, the tissue specificity of harmine's effects on *PPARγ* expression appears to result from cell-type-specific effects of Wnt signaling. For example, harmine inhibits Wnt signaling in muscle as well as fat, but Wnt does not control *PPARγ* expression in muscle. These observations suggest that other inhibitors of the Wnt signaling pathway could be also candidates for antidiabetic drugs. Furthermore, since a number of different transcription factors and signaling pathways are likely to converge on the regulation of *PPARγ* expression in adipocytes, it may be possible to identify additional small-molecule regulators of *PPARγ* expression with distinct mechanisms of action.

Harmine is a β-carboline alkaloid that was first isolated in 1847 from seeds of *Peganum harmala* (Syrian rue) and *Banisteriopsis caapi*, both of which have traditionally been used for ritual and medicinal preparations in the Middle East, Central Asia, and South America (Sourkes, 1999). It is also present in common plant-derived foods and in human tissues (Guan et al., 2001). To our knowledge, the ability of harmine to modulate glucose metabolism and/or diabetes has not been explored previously. Known pharmacologic effects of harmine include hallucinogenesis (Sourkes, 1999), convulsive or anticonvulsive actions (Aricioglu et al., 2003), and tremorogenesis (Guan et al., 2001; Lutes et al., 1988). Consistent with these reports, we observed a transient tremor following harmine administration to which the animals developed resistance after the first few doses (Lutes et al., 1988). No other side effects of harmine administration were observed in our studies. Clearly, given its CNS side effects, harmine itself is not a suitable candidate for drug development. However, it may eventually be possible to separate the CNS and metabolic effects of harmine through optimization of the chemical structure.

Several potential molecular targets for the central pharmacologic effects of harmine have been identified. These include MAO-A (Kim et al., 1997), 5-HT2A (Glennon et al., 2000), imidazoline receptors (I1 and I2 sites) (Husbands et al., 2001), and cyclin-dependent kinases (CDK1, 2, and 5) (Song et al., 2004). However, none of these appear to be the target for the metabolic actions of harmine in adipocytes. Highly specific ligands for these receptors did not share the ability to promote adipocyte differentiation or PPARγ expression (Figure S4 and data not shown). Furthermore, 6-methoxyharman, which promoted adipocyte differentiation and PPARγ expression, does not bind 5-HT2A receptors (Figure 4) (Glennon et al., 2000).

Nevertheless, given the fact that harmine is known to have CNS targets and the fact that CNS pathways regulate peripheral glucose and lipid metabolism, the possibility that CNS actions may contribute to the metabolic effects of harmine must be considered. Substrates of MAO-A include neurotransmitters such as serotonin, histamine, and catecholamines (Youdim et al., 2006). Catecholamines are known to stimulate gluconeogenesis in the liver; stimulate lipolysis and FFA release in adipose tissue; and increase glucose uptake in muscle, WAT, and BAT (Nonogaki, 2000). Chronic intracerebroventricular injection of histamine induces lipolysis and *UCP1* expression in BAT and *UCP3* expression in WAT (Masaki et al., 2001). 5-HT2A agonists have been reported to reduce food intake and increase oxygen consumption (Bovetto and Richard, 1995; Nonogaki, 2000). The imidazoline receptor I1 and I2 sites have been proposed to mediate

antihypertensive effects of α2-adrenergic agonists, stimulate sodium excretion in kidney, and modulate behavioral effects of morphine (Husbands et al., 2001). However, the overall metabolic effects of harmine are not consistent with activation of any of these CNS targets in vivo. For example, MAO inhibitors and selective serotonin reuptake inhibitors (SSRIs) have been widely used as antidepressants for decades, and there has been no report of an antidiabetic effect of these drugs (Ferguson, 2001; Youdim et al., 2006). Furthermore, although increased expression of *UCP1* and *UCP3* in WAT and higher oxygen consumption in harmine-treated mice could be explained by sympathetic tone in the autonomous nervous system, the lack of an effect of harmine on regulation of genes involved in gluconeogenesis in the liver, as well as the lack of evidence of increased lipolysis in adipose tissue (higher FFA levels), argues against this interpretation. We also did not observe a change in the activity of harmine-treated mice in energy-expenditure studies as might be expected with sympathetic activation. Nevertheless, identification of the molecular target of harmine's metabolic effects will be required in order to completely rule out these possibilities.

In conclusion, we have validated phenotypic screening of adipocytes as a promising approach to the discovery of antidiabetic small molecules. Furthermore, we have linked two biological activities with the antidiabetic actions of this compound: control of *PPARγ* mRNA expression and inhibition of Wnt signaling. Our results suggest that other small molecules that share these activities may be candidates for metabolic drugs. Moreover, given their distinct mechanism of action and side-effect profile, drugs acting through these pathways may represent a complementary approach to PPARγ ligands for the treatment of insulin resistance.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids

GW7845 was provided by T. Willson (GlaxoSmithKline). LG268 was provided by R. Heyman (Ligand Pharmaceuticals). Harmine hydrochloride and LiCl were purchased from Sigma (#H0625 and #L0505). RG14620 and PP1 were from BIOMOL International LP (#EI253-0005 and #EI275). Harmine hydrochloride and RG14620 were dissolved in water and DMSO, respectively. Insulin (#I0516), dexamethasone (#D2915), and 3-isobutyl-1-methylxanthine (IBMX, #I7018) were from Sigma. Actinomycin D was from Calbiochem. Purified Wnt-3a protein was from R&D Systems (#1324-WN). Plasmids encoding Gal4-DBD fusions of mPPARγ-LBD (pCMX-Gal4-mPPARγ-LBD), hRXRα-LBD (pCMX-hRXRα-LBD), reporter gene pMH-100-TK-luc, and pCMX-β-gal were described previously (Forman et al., 1995). A luciferase reporter under the control of the −5.4 kb aP2 promoter (Ross et al., 1990) (pGL3-aP2-Luc) was generated by cloning the promoter, amplified by primers 5′- ATTCCCAGCAGGAATCAGGTAGCTGGAG-3′ and 5′-ACAGGAGGGTGCTATGAG-3′, into the KpnI-NheI sites of pGL3-Basic (Promega).

Cell Culture

HEK293T cells were cultured in DMEM with 10% fetal bovine serum (FBS) with antibiotics at 37°C and 5% CO_2. 3T3-F442A and 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% calf serum. For differentiation of 3T3-F442A, cells were cultured in DMEM with 10% FBS and treated with insulin (5 μg/ml) at confluence. For 3T3-L1, cells were treated with dexamethasone (1 μM), IBMX (0.5 mM), and insulin (5 μg/ml) for 2 days after confluence, followed by treatment with insulin alone. Other chemicals and purified Wnt-3a were added as indicated in legends. For Wnt-3a-conditioned media, Wnt-3a-expressing L cells or control L cells (ATCC) were cultured in DMEM with 10% FBS, and medium was collected as described at http://www.atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=CRL-2647. Mouse primary hepatocytes were isolated from 2- to 3-month-old male C57BL/6 mice and cultured using the BioCoat Hepatocyte Differentiation Environment system (Becton Dickinson) as described in Pei et al. (2006). Bone marrow-derived macrophages were isolated as described elsewhere (Castrillo et al., 2003). Briefly, bone marrow isolated from femur and tibia was treated with red blood cell lysis buffer (Sigma R7757), washed with PBS, resuspended in DMEM supplemented with 20% FBS and 30% conditioned medium from L929 cells expressing macrophage colony-stimulating factor (M-CSF), and plated in six-well plates at density of 1×10^6 cells per well. Cells were used for experiments after a 5 day incubation. Retroviral overexpression of PPARγ2 was performed using pBabe puro-PPARγ2 and the packaging cell line Phoenix E as described elsewhere (Tontonoz et al., 1994). For generation of F442-luc20 cells, 3T3-F442A cells were cotransfected with pGL3-aP2-Luc (72 μg) and pSV2-bsr (1.2 μg) using Lipofectamine 2000. Blasticidin S-resistant colonies were isolated on day 11 and expanded. Luciferase activity during differentiation was determined, and clone #20 (F442-luc20) was selected based on the robustness and differentiation dependence of luciferase activity.

Cell-Based Chemical Screening

F442-luc20 cells (2000/well) were plated into 96-well plates in 180 μl DMEM with 10% FBS and antibiotics. Three days later, when cells were ~90% confluent, the libraries were transferred using a pin tool. On the next day, 20 μl medium containing insulin was added to the medium (final concentration 5 μg/ml). On day 5 after confluence, cells were lysed in 30 μl of Cell Culture Lysis Reagent (Promega), and luciferase activity was determined using the Luciferase Assay System (Promega). Small-molecule libraries were obtained from BIOMOL and used according to the manufacturer's recommendations. They included 72 ion channel inhibitors (#2805), 84 kinase/phosphatase inhibitors (#2831), 84 orphan ligands (#2825), 60 endocannabinoids (#2801), and 204 bioactive lipids (#2800). The compounds were dissolved in the manufacturer-recommended solvent and stored at −80°C in 384 wells.

RNA and Protein Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen). 0.5 μg of total RNA was reverse transcribed using MultiScribe (Applied Biosystems) and random hexamers according to the manufacturer's instructions. Real-time quantitative PCR (SYBR green) analysis was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Primer sequences are listed in Table S1. Expression was normalized to 36B4. For RNA from adipose tissue, homogenized TRIzol samples were centrifuged and the lipid layer was removed. After dissolving RNA into DEPC-treated water, phenol-chloroform extraction was performed twice followed by ethanol precipitation. Forty nanograms of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Protein preparation, SDS-PAGE, and western blotting were performed as previously described (Waki et al., 2003). Equal amounts of protein were loaded. Antiserum against murine PPARγ was a kind gift from B.M. Spiegelman (Dana-Farber Cancer Institute).

Luciferase Reporter Assays

For chimeric PPARγ-GAL4 assay, HEK293T cells (1×10^5 cells per well) were plated in 48-well plates in 250 μl of DMEM (2% FBS) without antibiotics. The next day, cells were cotransfected with pCMX-Gal-mPPARγ-LBD, pMH-100-TK-luc, and pCMX-β-gal using Lipofectamine 2000 (Invitrogen). One day later, chemicals were added to the

medium. After 18 hr incubation, cells were lysed in Cell Culture Lysis Reagent (Promega), and luciferase activity was determined using the Luciferase Assay System (Promega). Luciferase value was normalized to β-galactosidase activity. TOP-FLASH assays were performed as previously described (Korinek et al., 1997; Morin et al., 1997). HEK293T cells (5×10^6 cells) were plated in 10 cm plates in DMEM (10% FBS) without antibiotics. The next day, cells were cotransfected with plasmids for TOP-FLASH, LEF1, constitutively active mutant β-catenin (S37A), and renilla luciferase using Lipofectamine 2000. Eighteen hours later, cells were trypsinized, centrifuged, and resuspended in fresh DMEM with 10% FBS. Cells were replated in 24-well plates at 1:2 dilution and treated with either harmine (or derivatives), LiCl, or Wnt-3a-conditioned media for 24 hr. Firefly and renilla luciferase were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase value was normalized to renilla luciferase.

Animals

C57BLKS/J-Lepr$^{db/db}$ and C57BL/6 wild-type mice were purchased from The Jackson Laboratory. Nine-week-old male *db/db* mice were randomized into two groups according to glucose levels and body weight and treated with daily intraperitoneal (i.p.) injection of harmine (30 mg/kg) or saline. Harmine was dissolved in sterile saline (150 mM NaCl) at 3 mg/ml and stored at −20°C. Prior to use, the harmine solution was warmed in a 42°C water bath with agitation. For wild-type mice, 16-week-old C57BL/6 mice were treated with harmine (30 mg/kg, i.p.) twice per day. For troglitazone treatment, mice were fed chow containing troglitazone (0.4%). For blood analysis, *db/db* mice were fasted for 16 hr and blood was collected from the tail vein. Glucose levels were determined using an Accu-Chek Active glucometer (Roche). Free fatty acid levels were determined by the NEFA C test kit (Wako). Triglyceride levels were determined by the Serum Triglyceride Determination Kit (Sigma). Insulin levels were determined using an Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem Inc.). For glucose tolerance tests, mice were fasted for 16 hr, and glucose levels from tail-vein blood were determined before and 30, 60, 90, and 120 min after i.p. injection of glucose (2 g/kg). For insulin tolerance tests, random fed mice were injected i.p. with insulin (Humulin R, Eli Lilly) (2 U/kg). Glucose levels were determined before and 30, 60, 120, 180, and 210 min after injection. Animal studies were conducted in accordance with the Animal Research Committee of the University of California, Los Angeles.

Indirect Calorimetry

Oxygen consumption, heat production, and activity were measured over 3 days using the Oxymax Lab Animal Monitoring System (Columbus Instruments). Mice (five 10-week-old *db/db* males per group) were individually housed and allowed to acclimate in the experimental cages with free access to food and water. Animals were maintained on a 12 hr light/dark cycle and were treated with vehicle or harmine (30 mg/kg) for 17 days prior to the start of the experiment. During the experiment, mice were dosed daily with vehicle or harmine as described above. Data was analyzed using the OxymaxWin v3.32 software package. Statistical comparisons of repeated measurements were made using ANOVA. For ease of presentation, data are expressed as area under the curve (AUC) calculated for the light and the dark period over the 3 recording days; statistical comparison of these totals was made using Student's t test. All experiments were approved by the Institutional Animal Use and Care Committee of The Genomics Institute of the Novartis Research Foundation.

Histological Analysis of Adipose Tissue

White adipose tissue was fixed with paraformaldehyde, dehydrated, and embedded in paraffin, and 10 μm thick sections were subjected to hematoxylin-and-eosin staining. For immunohistochemistry, slides containing 5 μm sections were baked at 60°C for 30 min to melt the paraffin. The slides were deparaffinized with xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Antigen retrieval was performed by boiling the slides for 25 min at 95°C in 0.01 M sodium citrate buffer (pH 6.0). Purified rat anti-mouse Mac2 antibody (Cedarlane Laboratories, CL8942AP, clone M3/38) was applied at 1:5000 concentration overnight at 4°C. Slides were subsequently incubated with anti-rat biotinylated secondary antibody at 1:200 concentration (eBioscience, #13-4813-85). Histochemical reactions were performed using the VECTASTAIN ABC Kit (Vector Laboratories, #PK6100) and diaminobenzidine (DAB), and the slides were counterstained with hematoxylin.

Supplemental Data

Supplemental Data include ten figures and one table and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/5/5/357/DC1/.

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