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SIRT1 Improves Insulin Sensitivity under Insulin-Resistant Conditions by Repressing PTP1B

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SUMMARY

Insulin resistance is often characterized as the most critical factor contributing to the development of type 2 diabetes. SIRT1 has been reported to be involved in the processes of glucose metabolism and insulin secretion. However, whether SIRT1 is directly involved in insulin sensitivity is still largely unknown. Here we show that SIRT1 is downregulated in insulin-resistant cells and tissues and that knockdown or inhibition of SIRT1 induces insulin resistance. Furthermore, increased expression of SIRT1 improved insulin sensitivity, especially under insulin-resistant conditions. Similarly, resveratrol, a SIRT1 activator, enhanced insulin sensitivity in vitro in a SIRT1-dependent manner and attenuated high-fat-diet-induced insulin resistance in vivo at a dose of 2.5 mg/kg/day. Further studies demonstrated that the effect of SIRT1 on insulin resistance is mediated by repressing *PTP1B* transcription at the chromatin level. Taken together, the finding that SIRT1 improves insulin sensitivity has implications toward resolving insulin resistance and type 2 diabetes.

INTRODUCTION

Diabetes mellitus, the most common endocrine disorder, currently affects more than 170 million people worldwide and will prospectively affect more than 353 million by the year 2030 (Wild et al., 2004; Yach et al., 2006). Two forms of diabetes, type 1 and type 2, have been identified. Type 1 diabetes is primarily due to the autoimmune-mediated destruction of pancreatic islet β cells, resulting in absolute insulin deficiency. Type 2 diabetes, which more than 90% of diabetes patients suffer from, is characterized by the resistance of target tissues to insulin stimulation (Wild et al., 2004). The peptide hormone insulin lowers blood glucose levels by facilitating glucose uptake, mainly into skeletal muscle and fat tissue, and by inhibiting endogenous glucose production in the liver (Saltiel and Kahn, 2001). Insulin resistance occurs when a normal dose of the hormone is incapable of eliciting these metabolic responses (Saltiel, 2001). Development of insulin resistance is a multistep process with strong genetic and environmental influences, and the precise pathogenesis and the pathophysiological sequence resulting in insulin resistance are still largely unknown. Currently, alleviating insulin resistance is still one of the key avenues to treating type 2 diabetes (Mokdad et al., 2003).

SIRT1 is one of seven mammalian orthologs of the yeast protein silent information regulator 2 (Sir2), a conserved NAD-dependent protein deacetylase that plays a role in extension of life span and in chromatin remodeling associated with gene silencing (Blander and Guarente, 2004; Bordone et al., 2006; Longo and Kennedy, 2006; Rogina et al., 2002). SIRT1 has also been suggested to be involved in the processes of glucose homeostasis and insulin secretion. Rodgers et al. (2005) showed that SIRT1 controls hepatic glucose metabolism by interacting with and deacetylating PGC-1α, a key transcriptional coactivator that controls glucose metabolism in the liver at the level of gene transcription. Recently, SIRT1 was shown to be capable of upregulating insulin secretion in response to glucose stimulation in pancreatic β cells and thus improving glucose tolerance in mice (Moynihan et al., 2005). However, SIRT1 knockout mice exhibit lower blood glucose levels than wild-type mice (Bordone et al., 2006). Recently, elucidation of the role of SIRT1 in attenuating adipogenesis in 3T3-L1 adipocytes by repressing PPARγ (Picard et al., 2004) has provided a direction toward resolving obesity, as well as obesity-related metabolic diseases including insulin resistance and type 2 diabetes. Resveratrol, a plant-derived polyphenolic compound, has been receiving increasing attention as a potent activator of SIRT1 as evidenced by the findings that resveratrol can mimic caloric restriction and delay the aging process in a Sir2-dependent manner (Howitz et al., 2003). More recently, oral administration of resveratrol at a dose of 22.4 mg/kg/day was shown to improve insulin sensitivity and to slightly reduce the body weight of 1-year-old mice fed a high-fat diet (Baur et al., 2006). Additionally, resveratrol at a dose of 400 mg/kg/day has been demonstrated to prevent diet-induced obesity and alleviate obesity-related insulin resistance, and resveratrol-induced deacetylation of PGC-1α is mediated by SIRT1 with RNA

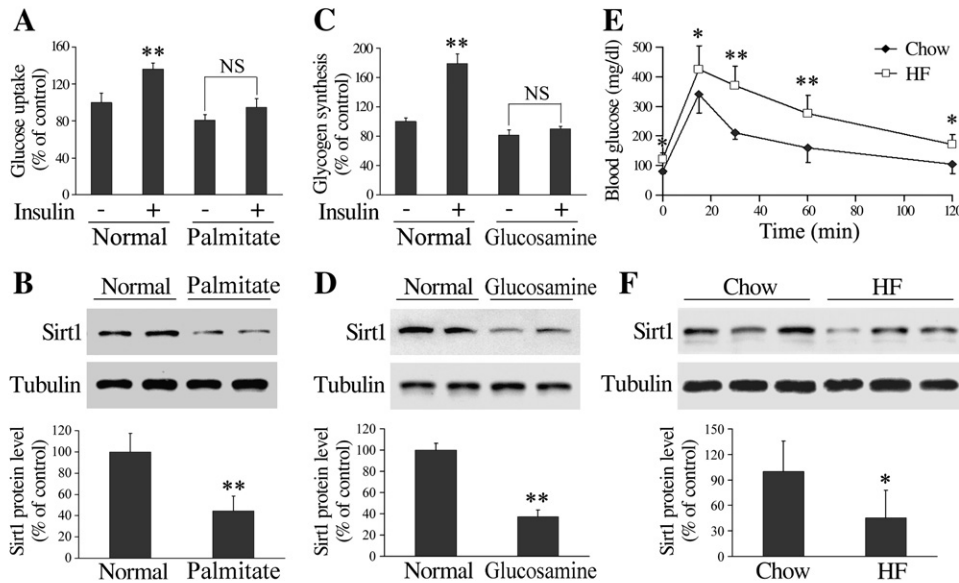

Figure 1. SIRT1 Protein Levels Decrease in Insulin-Resistant Cells and in Muscle of Mice with Impaired Glucose Tolerance

(A) Insulin resistance induced by palmitate in C2C12 myotubes was confirmed by glucose uptake assay. **p < 0.01 versus normal control without insulin treatment; NS, no significant difference. In this and all other figures, error bars represent SD.

(B) SIRT1 protein levels decreased in insulin-resistant C2C12 myotubes induced by palmitate when examined by western blot. **p < 0.01 versus normal control.

(C) Insulin resistance induced by glucosamine in HepG2 cells was confirmed by glycogen synthesis assay. **p < 0.01 versus normal control without insulin treatment; NS: no significant difference.

(D) SIRT1 protein levels decreased in insulin-resistant HepG2 cells induced by glucosamine. **p < 0.01 versus normal control.

(E) Glucose tolerance was impaired after 16 weeks on a high-fat (HF) diet. n = 8; *p < 0.05, **p < 0.01 versus chow-fed group at the same time point.

(F) SIRT1 protein levels decreased in the gastrocnemius muscle of mice from (E). *p < 0.05 versus chow-fed group. Quantification was performed with the results of at least three samples from different mice.

interference (RNAi) in C2C12 myotubes or *Sirt1*$^{-/-}$ murine embryonic fibroblast cells (Lagouge et al., 2006). However, whether SIRT1 has a direct effect on insulin resistance has yet to be elucidated, and whether resveratrol acts directly or indirectly through SIRT1 is still a subject of debate (Denu, 2005).

Protein tyrosine phosphatase (PTP) constitutes a family of phosphatases, including PTP1B, SHP1, SHP2, and LAR, which act to reverse tyrosine kinase action (Alonso et al., 2004; Tonks, 2006). As a negative regulator of the insulin signal transduction cascade, PTP1B has been shown to function as a key insulin receptor phosphatase (Moller, 2001). PTP1B-deficient mice are more sensitive to insulin, have improved glycemic control, and are more resistant to diet-induced obesity than wild-type mice (Elchebly et al., 1999). Diabetic mice treated intraperitoneally with PTP1B antisense oligonucleotides have lower PTP1B protein levels in liver, leading to decreases in fat, plasma insulin, and blood glucose levels (Zinker et al., 2002). These findings indicate that inhibition or downregulation of PTP1B is an effective strategy for improving insulin sensitivity.

In this study, we directly investigated the effect of SIRT1 on insulin sensitivity under normal versus insulin-resistant conditions. The role of resveratrol was studied both in vitro and in vivo. Our data reveal that SIRT1 and resveratrol improve insulin sensitivity by repressing *PTP1B* transcription at the chromatin level and that resveratrol is effective in vitro and in vivo at a much lower concentration than reported previously, in a SIRT1-dependent manner. Our findings suggest a potential therapeutic approach for preventing or treating insulin resistance and type 2 diabetes.

RESULTS

SIRT1 Protein Level Decreases under Insulin-Resistant Conditions

To investigate whether SIRT1 is involved in insulin resistance, we compared SIRT1 protein levels under normal and insulin-resistant conditions. As shown in Figure 1A, insulin-stimulated glucose uptake was blocked by palmitate in C2C12 myotubes. Similarly, glycogen synthesis was also blocked by glucosamine in HepG2 cells (Figure 1C). These results show that palmitate and glucosamine induce insulin resistance in C2C12 myotubes and HepG2 cells, respectively. As expected, SIRT1 protein levels decreased significantly under insulin-resistant conditions in both C2C12 myotubes and HepG2 cells (Figures 1B and 1D). To further investigate this decline in vivo, we measured SIRT1 protein levels in insulin-resistant mice. According to the results of a glucose tolerance test, 16 weeks of high-fat diet (HFD) feeding significantly impaired glucose tolerance (Figure 1E). As expected, SIRT1 protein levels were also decreased in the gastrocnemius muscle

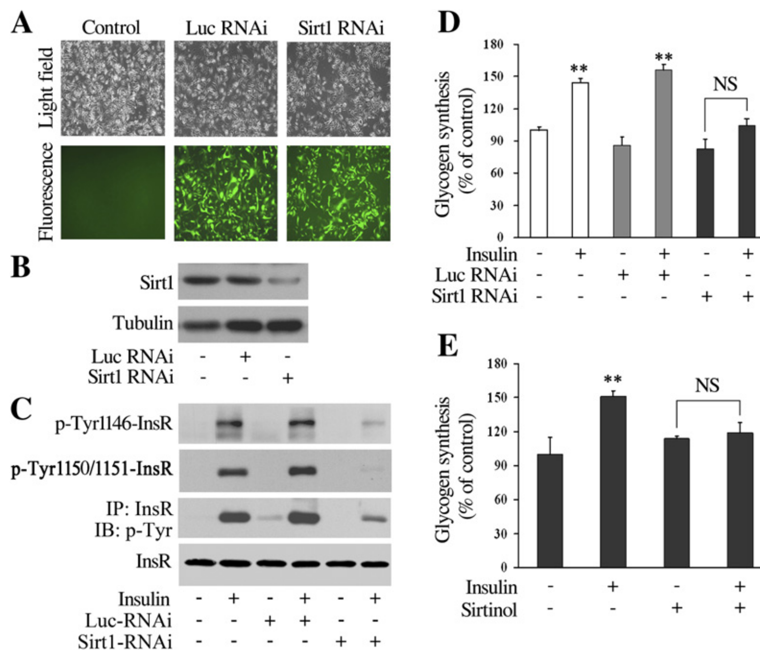

Figure 2. Knockdown or Inhibition of SIRT1 Induces Insulin Resistance

(A) HepG2 cells were infected with lentivirus expressing both EGFP and luciferase (Luc) RNAi or SIRT1 RNAi as evidenced by representative light-field or fluorescence photos. HepG2 cells without lentivirus infection were used as control.

(B) Lentivirus-mediated SIRT1 knockdown in HepG2 cells was confirmed by western blot. Luciferase RNAi was used as a control.

(C) Knockdown of SIRT1 impaired insulin receptor (InsR) phosphorylation stimulated by insulin in HepG2 cells.

(D and E) Knockdown of SIRT1 by RNAi (D) or inhibition of SIRT1 by sirtinol (E) blocked insulin-stimulated glycogen synthesis in HepG2 cells. **$p < 0.01$ versus respective control without insulin treatment; NS: no significant difference.

of mice fed a HFD as compared to the chow-fed group (Figure 1F). These results suggest that SIRT1 is involved in insulin resistance.

Knockdown or Inhibition of SIRT1 Induces Insulin Resistance

A reduction in SIRT1 protein levels under insulin-resistant conditions raises the possibility that knockdown or inhibition of SIRT1 might induce insulin resistance. To address this possibility, lentiviruses expressing both enhanced green fluorescent protein (EGFP) and SIRT1 RNAi or luciferase RNAi were packaged, and HepG2 cells were highly infected as evidenced by expression of EGFP (Figure 2A). As shown in Figure 2B, the endogenous SIRT1 protein level was effectively reduced by RNAi. At the same time, insulin-stimulated tyrosine phosphorylation of insulin receptor (InsR) was greatly impaired in the SIRT1 knockdown cells (Figure 2C). Similarly, insulin-stimulated glycogen synthesis was also impaired in the SIRT1 knockdown cells (Figure 2D). When HepG2 cells were treated with the SIRT1 inhibitor sirtinol (Grozinger et al., 2001), glycogen synthesis in response to insulin stimulation was also significantly impaired (Figure 2E). Taken together, these data show that SIRT1 is essential for insulin signaling and that knockdown or inhibition of SIRT1 is sufficient to induce insulin resistance.

Increased Expression of SIRT1 Improves Insulin Sensitivity

The reduction of SIRT1 protein levels under insulin-resistant conditions, as well as the induction of insulin resistance in SIRT1-downregulated cells, leads to a postulation that increased dosage of SIRT1 may attenuate insulin resistance. Thus, we increased SIRT1 expression in C2C12 myotubes by employing herpes simplex virus (HSV) infection. As shown in Figure 3D, SIRT1 protein levels increased remarkably in a dose-dependent manner in C2C12 myotubes infected with the recombinant HSV encoding *Sirt1*. We next determined the effect of increased SIRT1 dosage on glucose uptake and insulin signaling. As shown in Figure 3A, glucose uptake was significantly increased upon insulin stimulation in the presence or absence of HSV-SIRT1 virus infection, and no statistical difference was observed between helper virus-infected cells and HSV-SIRT1 virus-infected cells. Basal glucose uptake was not significantly affected by increased expression of SIRT1. However, under insulin-resistant conditions induced by palmitate, SIRT1 significantly enhanced insulin-stimulated glucose uptake and glycogen synthase activity upregulation (Figures 3B and 3C). Stimulation of C2C12 myotubes with insulin enhanced the phosphorylation of InsR at Tyr1146, as well as Akt at Thr308 and Ser473, PDK1 at Ser241, and GSK-3β at Ser9 (Figure 3D). Increased expression of SIRT1 under normal conditions did not affect insulin-stimulated phosphorylation of these downstream proteins (Figure 3D). When insulin resistance was induced in C2C12 myotubes by palmitate, the insulin-stimulated phosphorylation of these downstream proteins was impaired remarkably (Figure 3E). This impairment was significantly rescued by SIRT1, since the improvements in insulin-stimulated phosphorylation of the downstream proteins were observed in SIRT1-overexpressing cells (Figure 3E). (For quantitation of the results shown in Figure 3E, see Figure S1 in the Supplemental Data available with this article online.) Furthermore, SIRT1 improved the response to insulin under insulin-resistant conditions when cells were stimulated with different doses of insulin. The deacetylase activity of SIRT1 is required for its effect on improving insulin sensitivity, since the SIRT1-363Y mutant, which lacks deacetylase activity, failed to improve

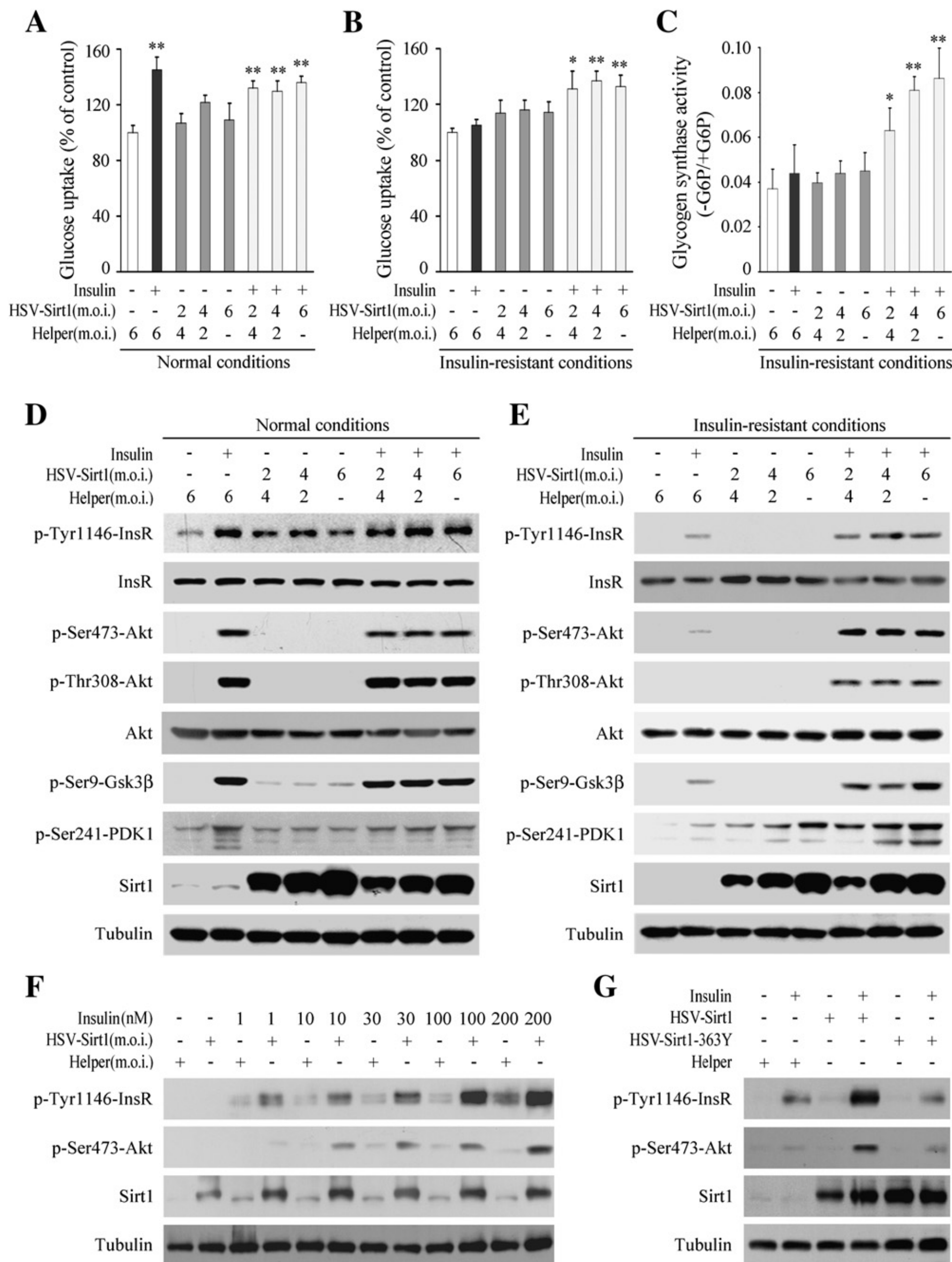

Figure 3. Increased Expression of SIRT1 Improves Insulin Sensitivity under Insulin-Resistant Conditions but Not under Normal Conditions

(A) Increased expression of SIRT1 with herpes simplex virus (HSV) had no significant effect on basal or insulin-stimulated glucose uptake in C2C12 myotubes under normal conditions. Helper virus used for recombinant HSV packaging was used as a control. **p < 0.01 versus cells only infected with helper virus and without insulin treatment. moi, multiplicity of infection.

(B and C) Increased expression of SIRT1 with HSV improved insulin-stimulated glucose uptake and glycogen synthase activity upregulation in C2C12 myotubes under insulin-resistant conditions induced by palmitate. *p < 0.05, **p < 0.01 versus cells only infected with helper virus and with insulin treatment. G6P, glucose-6-phosphate.

(D) Increased expression of SIRT1 had no significant effect on insulin signaling, including the phosphorylation of InsR, Akt, GSK-3β, and PDK1, in C2C12 myotubes under normal conditions. Protein levels were analyzed by western blot.

(E) Increased expression of SIRT1 significantly enhanced insulin signaling, including the phosphorylation of InsR, Akt, GSK-3β, and PDK1, in C2C12 myotubes under insulin-resistant conditions induced by palmitate.

(F) The response to different doses of insulin was improved by SIRT1 in C2C12 myotubes under insulin-resistant conditions induced by palmitate.

(G) The SIRT1-363Y mutant failed to improve insulin signaling in C2C12 myotubes under insulin-resistant conditions induced by palmitate.

insulin sensitivity (Figure 3G). These data clearly indicate that increased expression of SIRT1 improves glucose uptake and insulin signaling, especially under insulin-resistant conditions.

Resveratrol Improves Insulin Sensitivity In Vitro in a SIRT1-Dependent Manner

To further ascertain the positive effect of SIRT1 on insulin sensitivity, we applied the SIRT1 activator resveratrol (Howitz et al., 2003) at a wide range of concentrations. As shown in Figure 4A, glucose uptake in C2C12 myotubes stimulated by insulin was significantly enhanced by resveratrol under normal conditions. Correspondingly, resveratrol application also enhanced the insulin-evoked phosphorylation of InsR, Akt, PDK1, and GSK-3β under normal conditions (Figure 4C). Resveratrol enhanced insulin-stimulated glucose uptake under insulin-resistant conditions as well (Figure 4B). The effect of resveratrol on insulin sensitivity under insulin-resistant conditions was further confirmed by elevated phosphorylation of these downstream proteins in insulin signaling (Figure 4D). Interestingly, besides acting as a SIRT1 activator, resveratrol upregulated SIRT1 protein levels, especially under insulin-resistant conditions (Figure 4D).

To investigate whether resveratrol has a similar effect on other cells responding to insulin, primary cultured rat adipocytes and HepG2 cells were also examined. As we expected, the application of resveratrol improved insulin-stimulated glucose uptake significantly in primary rat adipocytes (Figure 4E). Notably, basal glucose uptake was also enhanced by resveratrol treatment. Similarly, resveratrol enhanced basal glycogen synthesis in HepG2 cells (Figure 4F). These data demonstrate that resveratrol can enhance insulin sensitivity in different types of insulin-responsive cells.

To address whether resveratrol functions in a SIRT1-dependent manner, we infected HepG2 cells with lentivirus expressing SIRT1 RNAi or luciferase RNAi as a control. Glycogen synthesis was stimulated significantly by all three tested concentrations of resveratrol in HepG2 cells expressing luciferase RNAi, but resveratrol-stimulated glycogen synthesis was markedly diminished in the SIRT1 knockdown cells (Figure 4F). These findings clearly indicate that resveratrol functions in a SIRT1-dependent manner.

Resveratrol Improves Insulin Sensitivity In Vivo

We next investigated whether resveratrol improves insulin sensitivity in vivo as well as in vitro. Mice fed a HFD exhibited impaired glucose tolerance, and resveratrol treatment significantly improved glucose tolerance in HFD-fed mice, with glucose disposal curves comparable to those of chow-fed mice (Figures 5A and 5B). The ability of insulin to decrease blood glucose concentration was also markedly reduced in HFD-fed mice compared with chow-fed mice, and resveratrol administration significantly improved the response to insulin in HFD-fed mice (Figures 5C and 5D). Furthermore, we found that there was a dramatic increase in fasting plasma insulin levels in HFD-fed mice in comparison to chow-fed mice, and resveratrol administration attenuated the increase in plasma insulin levels significantly (Figure 5E). In addition, the increased total cholesterol and low-density lipoprotein levels induced by HFD were partially restored by resveratrol administration (Figures 5F and 5G), while no significant changes were observed in the levels of triglycerides and high-density lipoprotein by resveratrol administration (data not shown). Collectively, these data demonstrate that resveratrol improves insulin sensitivity in HFD-fed mice via the findings that resveratrol-treated mice were better able to control plasma glucose levels using nearly normal levels of insulin.

The difference between our applied dose of resveratrol and the doses previously reported (Baur et al., 2006; Lagouge et al., 2006) drove us to investigate the exact levels of resveratrol in vivo. Plasma, liver, and muscle samples from HFD-fed mice receiving resveratrol at a dose of 2.5 mg/kg/day were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) as shown in Figure S2. The levels of resveratrol in the plasma, liver, and muscle samples from resveratrol-treated mice were 0.45 ± 0.39 nmol/l, 5.59 ± 3.45 nmol/kg, and 0.72 ± 0.50 nmol/kg, respectively (Figure 5H). The detected levels of resveratrol in vivo were quite close to the concentrations that we applied in vitro. These results demonstrated the resveratrol concentration range used in vitro in this study was reasonable.

In addition, we administered the SIRT1 inhibitor nicotinamide to mice fed a chow diet for 8 weeks. Treatment with nicotinamide significantly increased body weight, fat mass, energy intake, and fasting blood glucose levels in these mice (Figures 5I–5L). Although impaired glucose tolerance was not observed in nicotinamide-treated mice (data not shown), the increased fat mass and fasting blood glucose levels induced by nicotinamide provide further evidence for the role of SIRT1 in regulating insulin sensitivity.

SIRT1 and Resveratrol Improve Insulin Sensitivity by Repressing PTP1B

To examine the underlying mechanism responsible for SIRT1-mediated improvement of insulin sensitivity, we tested several pharmaceutical targets affecting insulin sensitivity. PTP1B, a well-known negative regulator of the insulin signaling pathway (Elchebly et al., 1999; Moller, 2001), was found to be an effective target downregulated by SIRT1 or resveratrol. Under normal conditions, PTP1B protein levels were decreased slightly by SIRT1 (Figure 6A, left panel), while under insulin-resistant conditions, PTP1B protein level decreased strikingly in the SIRT1-overexpressing cells (Figure 6A, right panel). The deacetylase activity of SIRT1 was required for its downregulatory effect on PTP1B protein levels (Figure 6B). Moreover, we found that *PTP1B* mRNA levels were decreased significantly by increased expression of SIRT1 under insulin-resistant conditions (Figure 6C). Resveratrol decreased PTP1B protein and mRNA levels remarkably under both normal and insulin-resistant conditions (Figures 6D and 6E). These results suggest that the effects of SIRT1 and resveratrol on insulin sensitivity might be due to PTP1B repression.

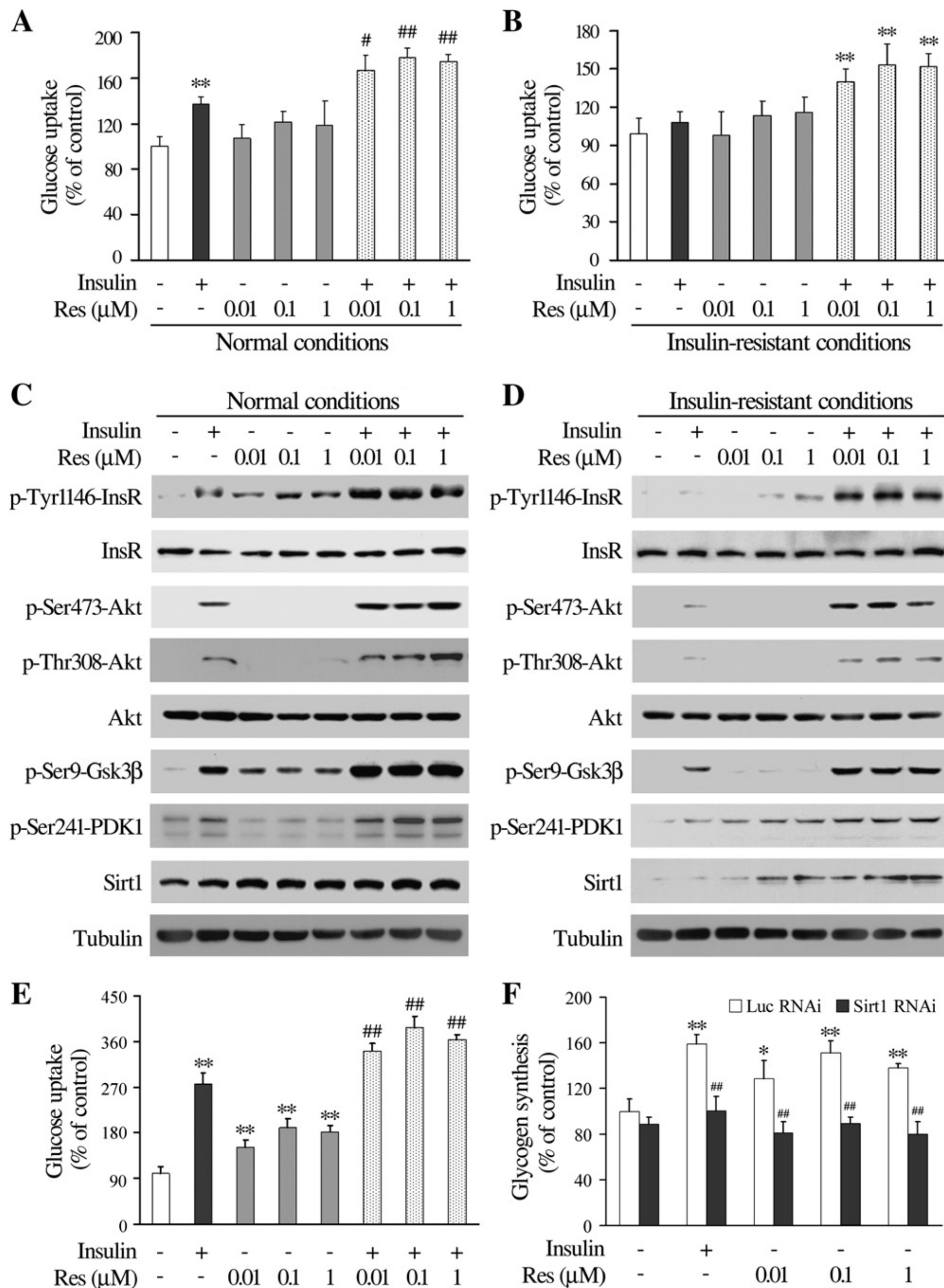

Figure 4. Resveratrol Enhances Insulin Sensitivity under Both Normal and Insulin-Resistant Conditions

(A and B) Resveratrol enhanced insulin-stimulated glucose uptake in C2C12 myotubes under both normal and insulin-resistant conditions. **p < 0.01 versus cells without any treatment; #p < 0.05, ##p < 0.01 versus insulin-treated cells in the absence of resveratrol.

(C and D) Resveratrol improved insulin signaling, including the phosphorylation of InsR, Akt, GSK-3β, and PDK1, in C2C12 myotubes under both normal and insulin-resistant conditions. Protein levels were analyzed by western blot. SIRT1 was upregulated by resveratrol under insulin-resistant conditions.

(E) Resveratrol enhanced glucose uptake in primary-cultured rat adipocytes. **p < 0.01 versus cells without any treatment; ##p < 0.01 versus insulin-treated cells in the absence of resveratrol.

(F) Resveratrol enhanced glycogen synthesis in HepG2 cells, and this effect required SIRT1. *p < 0.05, **p < 0.01 versus cells expressing luciferase (Luc) RNAi without insulin and resveratrol treatments; ##p < 0.01 versus cells expressing Luc RNAi treated with the same concentration of resveratrol.

To further confirm the above in vitro results, we explored the relationship between SIRT1 and PTP1B in vivo. A previous report indicated that SIRT1 protein levels are increased in the fasting state in mouse liver (Rodgers et al., 2005). Thus, we employed this strategy to upregulate SIRT1 protein level in vivo. As shown in Figure 6F,

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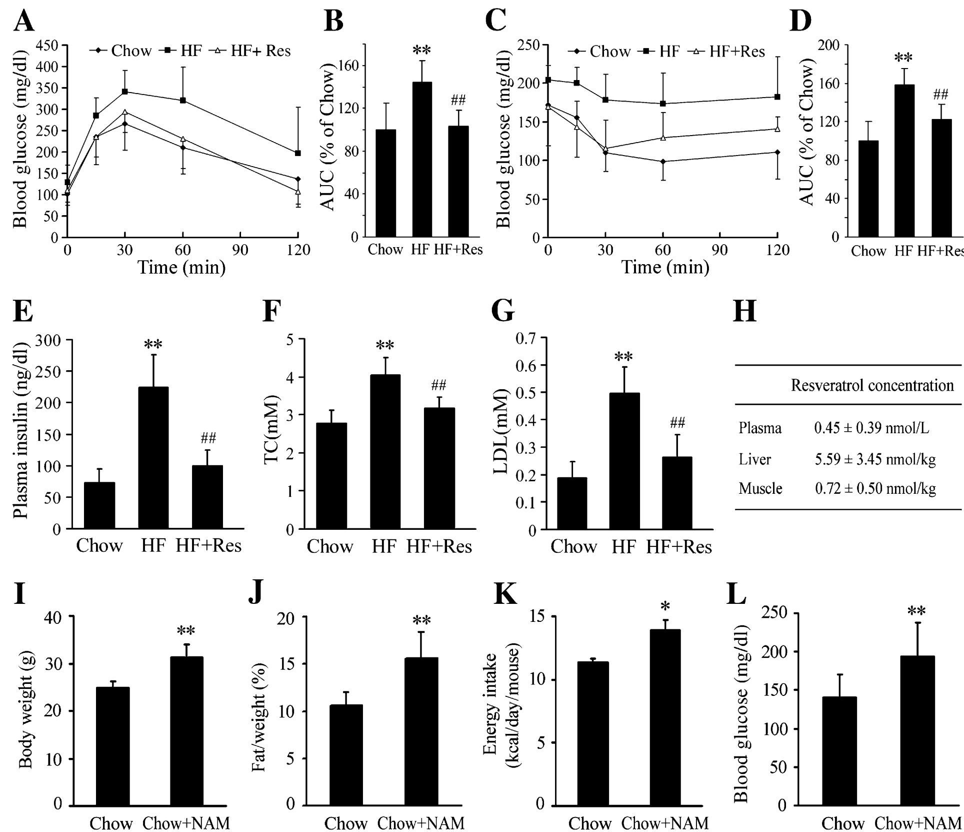

Figure 5. Resveratrol Ameliorates High-Fat-Diet-Induced Glucose Intolerance and Insulin Resistance, and Nicotinamide Increases Body Weight and Fasting Blood Glucose Levels in Chow-Fed Mice

(A) Resveratrol (Res) improved glucose tolerance in mice fed a high-fat (HF) diet as determined by glucose tolerance test (n = 8).

(B) Resveratrol significantly reduced the area under the curve (AUC) of the glucose tolerance test in (A). **p < 0.01 versus chow; ##p < 0.01 versus HF.

(C) Resveratrol improved insulin sensitivity in HF-fed mice as determined by insulin tolerance test (n = 8).

(D) Resveratrol significantly reduced the AUC of the insulin tolerance test in (C). **p < 0.01 versus chow; ##p < 0.01 versus HF.

(E–G) Plasma insulin (E), total cholesterol (TC, [F]), and low-density lipoprotein (LDL, [G]) in HF-fed mice was attenuated by resveratrol treatment. **p < 0.01 versus chow; ##p < 0.01 versus HF.

(H) Levels of resveratrol in plasma, liver, and muscle of resveratrol-treated mice analyzed by LC-MS/MS (n = 6). Resveratrol was extracted and measured after animals were administered resveratrol at 2.5 mg/kg/day for 6 weeks.

(I–L) Nicotinamide (NAM) significantly increased body weight (I), fat mass (J), energy intake (K), and fasting blood glucose levels (L) in chow-fed mice. n = 8; *p < 0.05, **p < 0.01 versus chow.

SIRT1 protein levels in liver were increased significantly by fasting and returned to normal chow-fed levels upon refeeding. At the same time, PTP1B protein levels were decreased remarkably in the fasting state and were restored by refeeding (Figures 6F and 6G). PTP1B protein levels were slightly but significantly increased in palmitate-treated C2C12 myotubes (Figure S3). Furthermore, SIRT1 protein levels were significantly decreased in gastrocnemius muscle of HFD-fed mice, and PTP1B levels increased simultaneously (Figures 6H and 6I). Resveratrol treatment to activate SIRT1 restored PTP1B to levels comparable to the chow-fed group. These data suggest that SIRT1, as well as resveratrol, may negatively regulate PTP1B in vivo.

To confirm that SIRT1 improves insulin sensitivity by repression of PTP1B, we next determined the effect of increased PTP1B expression on SIRT1-enhanced insulin sensitivity by upregulating expression of SIRT1 and PTP1B in C2C12 myotubes via infection with HSV encoding *Sirt1* and *PTP1B*, respectively. Western blots showed that the infected cells expressed PTP1B and/or SIRT1 strongly (Figure 7A). As expected, increased expression of SIRT1 improved insulin-stimulated InsR phosphorylation under insulin-resistant conditions, and this improvement was largely prevented by increased expression of PTP1B (Figure 7A). Similarly, the improved insulin-stimulated glucose uptake by SIRT1 was largely impaired by increased expression of PTP1B (Figure 7B). These data demonstrate that improvement of insulin sensitivity by SIRT1 is dependent on PTP1B repression. Furthermore, the PTP1B inhibitor vanadate reversed the effect of SIRT1 RNAi on insulin signaling (Figure 7C). Similarly, knockdown of PTP1B by RNAi also reversed the effect of SIRT1 RNAi on insulin signaling (Figures 7D and 7E).

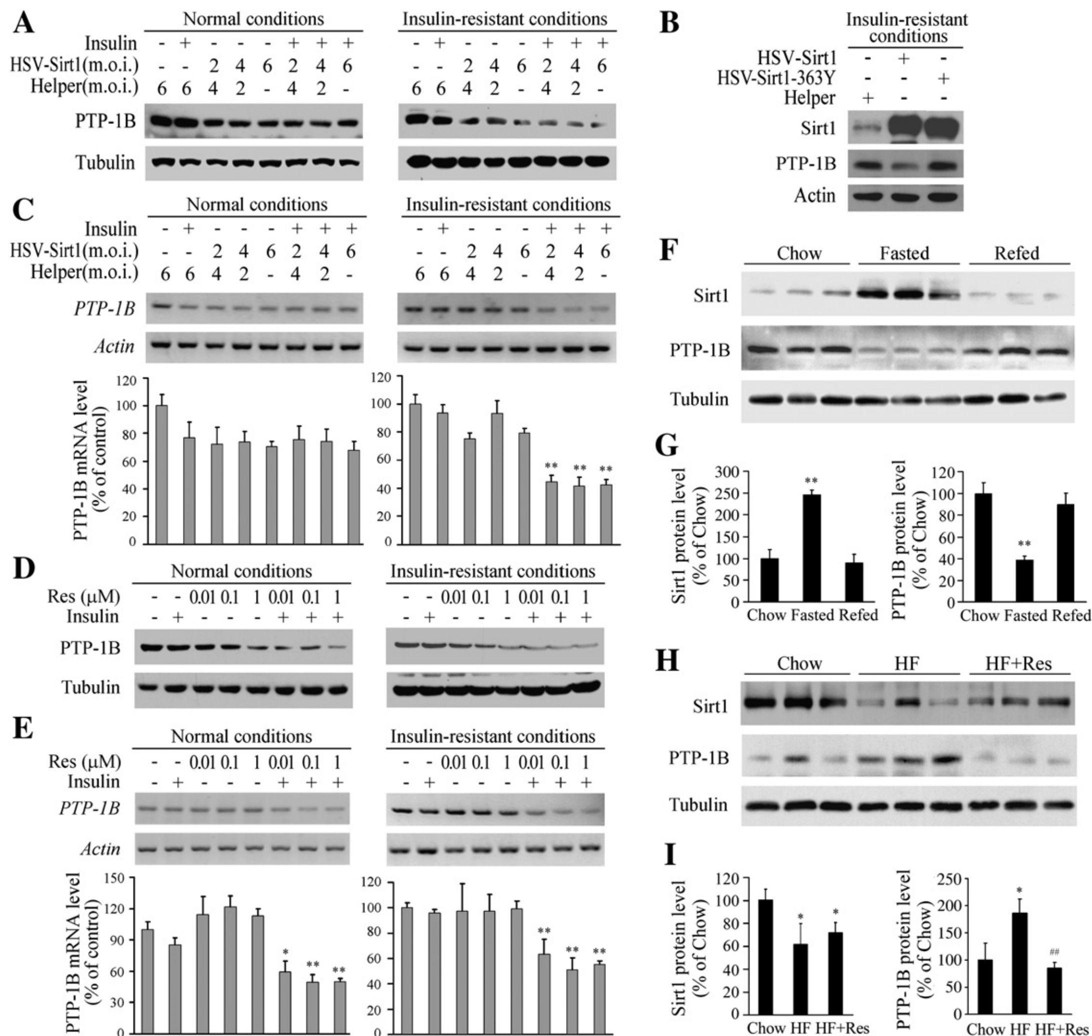

Figure 6. SIRT1 and Resveratrol Downregulate PTP1B at Both the Protein and the mRNA Level

(A) Increased expression of SIRT1 repressed PTP1B protein levels in insulin-treated C2C12 myotubes under insulin-resistant conditions induced by palmitate. Protein levels were analyzed by western blot. moi, multiplicity of infection.

(B) The SIRT1-363Y mutant failed to repress PTP1B protein levels in insulin-treated C2C12 myotubes under insulin-resistant conditions induced by palmitate.

(C) Increased expression of SIRT1 downregulated *PTP1B* mRNA levels in insulin-treated C2C12 myotubes under insulin-resistant conditions. PTP1B mRNA levels were analyzed by RT-PCR. $^{**}p < 0.01$ versus insulin-treated cells in the absence of resveratrol.

(D and E) Resveratrol (Res) treatment decreased PTP1B protein levels (D) and mRNA levels (E) in insulin-treated C2C12 myotubes. *PTP1B* mRNA levels were analyzed by RT-PCR. $^{*}p < 0.05$, $^{**}p < 0.01$ versus insulin-treated cells in the absence of resveratrol.

(F) SIRT1 protein levels were negatively correlated with PTP1B protein levels in vivo. Liver lysates from mice fed ad libitum with chow, fasted for 24 hr, or fasted for 24 hr and refed for 24 hr were analyzed by western blot (n = 6).

(G) Quantification of SIRT1 and PTP1B protein levels corresponding to (F). $^{**}p < 0.01$ versus chow or refed group.

(H) SIRT1 protein levels and activity were negatively correlated with PTP1B protein levels in gastrocnemius muscle of HF-fed mice with or without resveratrol treatment. Protein levels were analyzed by western blot.

(I) Quantification of PTP1B and SIRT1 protein levels corresponding to (H). $^{*}p < 0.05$ versus chow; $^{\#\#}p < 0.01$ versus HF.

These results further confirm that the effect of SIRT1 on insulin signaling is mediated by PTP1B.

To determine how PTP1B is regulated by SIRT1, the intracellular localization of SIRT1 and PTP1B was detected by immunofluorescence (Figure 7F). PTP1B and SIRT1 were not colocalized, suggesting that SIRT1 might regulate PTP1B at the transcriptional level. A series of reporter constructs containing different lengths of *PTP1B* promoter were applied, and SIRT1 failed to activate the *PTP1B* promoter (Figure 7H). Deacetylation of histone H3 by SIRT1 was observed in C2C12 myotubes (Figure 7G), providing the possibility that *PTP1B* transcription is regulated at the chromatin level, since deacetylation of histone usually leads to the inhibition of gene transcription. A chromatin immunoprecipitation (ChIP) assay showed that SIRT1 decreased acetylated histone H3

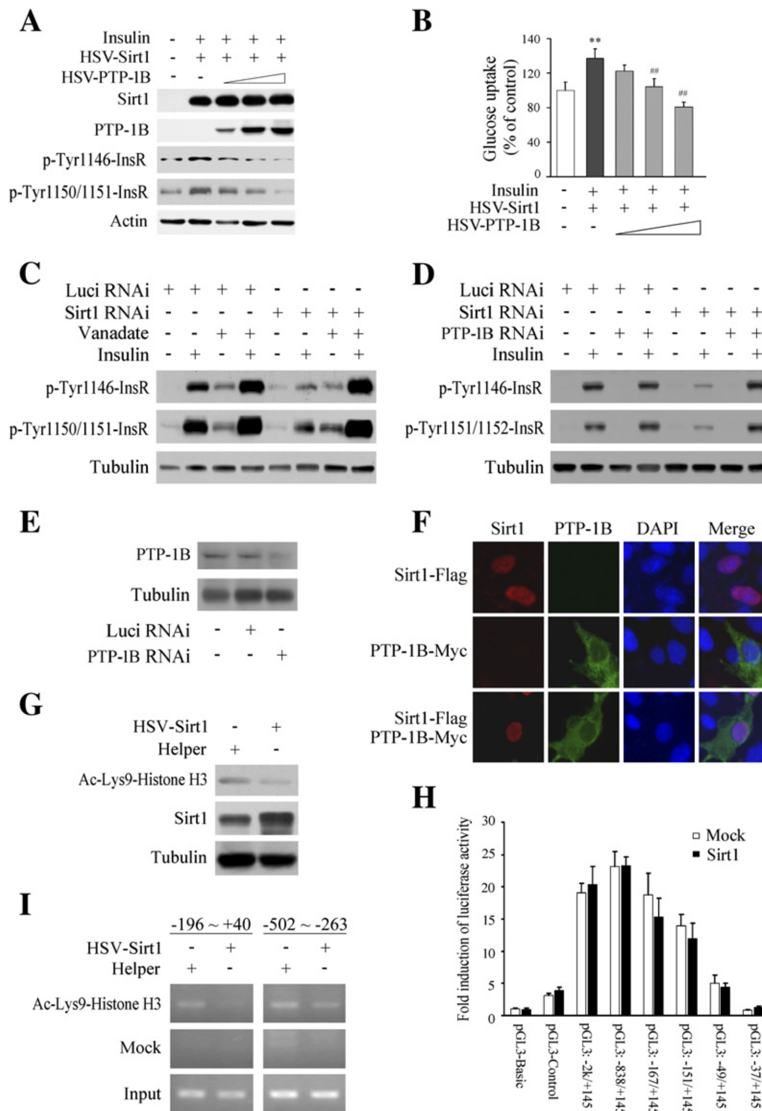

Figure 7. The Effect of SIRT1 on Insulin Signaling Is Mediated by PTP1B

(A) SIRT1's ability to improve insulin-stimulated InsR phosphorylation in C2C12 cells under insulin-resistant conditions induced by palmitate was impaired by increased PTP1B protein levels. Protein levels were analyzed by western blot.

(B) SIRT1's ability to improve insulin-stimulated glucose uptake in C2C12 cells under insulin-resistant conditions was impaired by increased PTP1B protein levels. **$p < 0.01$ versus control virus-infected cells; ##$p < 0.01$ versus only SIRT1-overexpressing cells.

(C) The PTP1B inhibitor vanadate reversed the effect of SIRT1 RNAi on insulin-stimulated tyrosine phosphorylation of InsR.

(D) PTP1B RNAi also reversed the effect of SIRT1 RNAi on insulin-stimulated phosphorylation of InsR.

(E) Lentivirus-mediated PTP1B RNAi in HepG2 cells was confirmed by western blot. Luciferase RNAi was used as a control.

(F) Intracellular localization of SIRT1 and PTP1B was detected by immunofluorescence. Nuclei were stained with DAPI.

(G) SIRT1 decreased the acetylation level of histone H3 in C2C12 cells under insulin-resistant conditions induced by palmitate.

(H) SIRT1 failed to activate the *PTP1B* promoter. A series of reporter constructs containing different lengths of *PTP1B* promoter were used for luciferase assay.

(I) Increased expression of SIRT1 decreased the binding of acetylated histone H3 to the *PTP1B* promoter region. ChIP assay was performed for two *PTP1B* genomic DNA fragments near the start site.

binding to the *PTP1B* promoter region (Figure 7I). These data demonstrate that SIRT1 represses *PTP1B* transcription at the chromatin level.

DISCUSSION

Recently, it has been demonstrated that resveratrol improves insulin sensitivity in a diet-induced obesity model (Baur et al., 2006; Lagouge et al., 2006); these studies raised the possibility that SIRT1 might be involved in regulation of insulin sensitivity. However, evidence for direct SIRT1 association with insulin sensitivity was still not available. In the present study, we provide some evidence that SIRT1 is directly involved in the regulation of insulin sensitivity. First, SIRT1 protein levels were markedly decreased in insulin-resistant cells (Figures 1B and 1D). Second, there was a reduction in SIRT1 protein levels in the gastrocnemius muscle of mice with impaired glucose tolerance compared to mice with normal glucose tolerance (Figure 1F). Such a decrease in epididymal fat tissue in a diet-induced obesity model was also reported recently in another study (Qiao and Shao, 2006). Third, knockdown or inhibition of SIRT1 impaired InsR phosphorylation and glycogen synthesis in response to insulin stimulation (Figures 2C–2E). However, in contrast to our findings, SIRT1 knockout mice exhibit better performance in maintaining systemic glucose homeostasis as compared to wild-type mice (Bordone et al., 2006). The precise mechanism responsible for such a difference observed in vivo and in vitro remains to be elucidated in the future. It is worth noting that only a small percentage of SIRT1 null mice are viable; most die during the immediate postnatal period. The viable mice exhibit growth retardation and defects in development of the eye, lung, pancreas, and heart, and both sexes are sterile (Cheng et al., 2003; McBurney et al., 2003). Therefore, it is reasonable to propose that the developmental defects in SIRT1-deficient mice may lead to abnormalities in physiological processes such as

glucose metabolism. Hence, heterozygous *Sirt1*$^{+/-}$ mice seem to be a useful model for studying the precise role of SIRT1 in glucose metabolism in vivo. Importantly, we also found that increased expression of SIRT1 can attenuate insulin resistance (Figures 3B, 3C, and 3E) and that this effect is dependent on its deacetylase activity (Figure 3G). It has been reported that pancreatic β cell-specific SIRT1-transgenic mice show improved glucose tolerance as a result of enhanced glucose-stimulated insulin secretion (Moynihan et al., 2005). Here, we showed that SIRT1 can directly improve insulin sensitivity by stimulating insulin signal transduction and that resveratrol improves HFD-induced insulin resistance and hyperinsulinemia. Therefore, other tissue-specific SIRT1-transgenic mice should be established in the future to investigate the in vivo function of SIRT1 in regulation of insulin sensitivity.

In addition to SIRT1, we found that resveratrol was also capable of improving insulin sensitivity in vitro. Moreover, resveratrol was observed to improve insulin sensitivity in mice with HFD-induced insulin resistance (Figures 5A–5E). This role of resveratrol in regulation of insulin sensitivity was consistent with recent reports (Baur et al., 2006; Lagouge et al., 2006). Notably, the dose of resveratrol applied in our study was 2.5 mg/kg/day, which was much lower than the doses of 22.4 or 400 mg/kg/day used in the above studies. To ascertain an effective concentration of resveratrol for improving insulin sensitivity, we utilized LC-MS/MS to determine resveratrol concentrations in animal tissues. The level of resveratrol in the plasma of resveratrol-treated mice was 0.11 ± 0.09 ng/ml (0.45 ± 0.39 nM), which was rather low compared to the 10–120 ng/ml concentration in resveratrol-treated mice administered a dose of 200 or 400 mg/kg/day (Lagouge et al., 2006). In addition to plasma, resveratrol was also detected in liver and muscle tissues, while it was difficult to detect in white adipose tissue (data not shown), suggesting that liver and muscle might be the major glucose-metabolizing tissues responding to resveratrol in vivo. Because several studies have shown that resveratrol is more effective in vivo than in cell culture models (Wenzel and Somoza, 2005), we selected several concentrations of resveratrol (0.01, 0.1, and 1.0 μM) close to in vivo concentrations to treat cultured cells. A previous study reported that resveratrol at concentrations of 25–50 μM administered over a short duration (30 min) improved phosphorylation of AMPK, leading to a potential increase in insulin sensitivity, but that resveratrol failed to improved phosphorylation of AMPK at 12.5 μM (Baur et al., 2006). Here, we also observed that phosphorylation of AMPK was induced by resveratrol at 50 μM, and, as expected, low concentrations of resveratrol from 0.01–1 μM used to improve insulin sensitivity in vitro failed to induce AMPK phosphorylation (data not shown). These results suggest that resveratrol may function at different concentration ranges with different mechanisms. When the resveratrol concentration is higher than 10 μM over a 24 hr duration, it produces toxic effects in differentiated C2C12 cells (data not shown). Consistent with this finding, a recent investigation has suggested that high concentrations of resveratrol (100 μM) over a 24 hr duration can induce cell apoptosis (Hambrock et al., 2007). Moreover, it has been found that high concentrations of resveratrol (100 μM) over a short treatment duration (10–30 min) inhibit insulin response through a SIRT1-independent pathway (Zhang, 2006). It seems that, at low concentrations, resveratrol exhibits biological functions in a SIRT1-dependent manner, whereas at high concentrations, it probably does so via a SIRT1-independent pathway. However, the effect of 50 μM resveratrol over a 24 hr duration has also been shown to be SIRT1 dependent (Lagouge et al., 2006). Therefore, the precise mechanisms for the functions of resveratrol remain to be elucidated. However, low effective concentrations of resveratrol in insulin sensitivity are of great therapeutic importance since lower concentrations mean greater biological safety and lower pharmaceutical cost.

SIRT1 is mainly linked to negative regulation of gene expression as a cofactor through protein deacetylation (Vaziri et al., 2001). In the present study, we found that SIRT1 can repress PTP1B at both the protein and the mRNA level. It is well demonstrated that decreased PTP1B results in better responses to insulin stimulation (Elchebly et al., 1999; Zinker et al., 2002). Moreover, increased expression of PTP1B counteracted SIRT1-mediated improved glucose uptake and InsR phosphorylation in response to insulin stimulation (Figures 7A and 7B). The reduction of PTP1B by SIRT1 should contribute, at least in part, to the enhancement of insulin sensitivity by SIRT1. Notably, PTP1B protein levels were decreased by increasing levels of SIRT1 in liver of fasted mice (Figure 6F). This finding raises the possibility that SIRT1 may also negatively regulate PTP1B in vivo, although this needs to be verified in the future. Consistent with our findings, a previous study also showed that PTP1B protein levels are decreased in liver of fasted mice (Gu et al., 2003); however, the mechanism involved was not fully elucidated. Our data suggest that the decrease in liver PTP1B protein levels in the fasting state is probably due to the repressive effect of SIRT1 on PTP1B. Although resveratrol has been reported to activate phosphatase SHP2 (Haider et al., 2005) as well as SIRT1, resveratrol also repressed PTP1B both in vitro and in vivo (Figures 6D, 6E, 6H, and 6I), indicating that resveratrol has a positive effect on insulin sensitivity. It has been demonstrated that SIRT1 can deacetylate histone H3 (Vaquero et al., 2004). Here, we observed similar results in C2C12 myotubes and demonstrated that SIRT1 represses *PTP1B* transcription at the chromatin level. In addition, caloric restriction is considered an effective dietary intervention for increasing insulin sensitivity (Barzilai et al., 1998; Fontana et al., 2004). SIRT1 protein levels are increased in calorie-restricted animals (Howitz et al., 2003). Thus, SIRT1-dependent repression of PTP1B might be involved in improved insulin sensitivity by caloric restriction.

In conclusion, by repressing PTP1B, SIRT1 improves insulin sensitivity under insulin-resistant conditions. Similarly, resveratrol, a potent activator of SIRT1, is also capable of enhancing insulin sensitivity both in vitro and in vivo

by repressing PTP1B. These findings suggest that drugs including resveratrol that enhance SIRT1 function and/or expression might provide a valuable new strategy for treating insulin resistance and type 2 diabetes.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Lentivirus and Herpes Simplex Virus

The H1 promoter was amplified by PCR from pSilencer 3.1-H1 hygro (Ambion) and inserted into XbaI and XhoI sites of the pLentiLox 3.7. The oligonucleotides and primers used in this study are described in the Supplemental Experimental Procedures. The oligonucleotides for RNAi were annealed and ligated into the BamHI and XhoI sites of pLentiLox 3.7 with the H1 promoter. Recombinant lentiviruses were produced in 293T cells by cotransfecting the construct containing luciferase RNAi, SIRT1 RNAi, or PTP1B RNAi with the packaging vectors Δ8.9 and VSVG and were subsequently concentrated and titered as described previously (Abbas-Terki et al., 2002). pBabepuro-hSIRT1 and pBabepuro-hSIRT1-363Y were a kind gift from W. Gu (Luo et al., 2001). The *Sirt1* and *Sirt1*-363Y genes were obtained from pBabepuro-hSIRT1 and pBabepuro-hSIRT1-363Y and subcloned into reconstructed pHSVPrPUC-myc vector at the BamHI site. The *PTP1B* cDNA was amplified by RT-PCR from mouse muscle tissue and subcloned into MluI and SalI sites in pHSVPrPUC-myc vector. The recombinant HSV-SIRT1 and HSV-PTP1B virus particles were prepared, amplified, and titered as described previously (Wang et al., 2005).

Cell Culture and Treatments

C2C12 myoblasts were maintained in DMEM with 10% FBS and differentiated in DMEM with 2% horse serum after reaching confluence. After 4 days, the C2C12 cells were differentiated into myotubes and treated with resveratrol or infected with recombinant HSV for 24 hr at the indicated concentrations or titers. Myotubes were then incubated for 18 hr in DMEM containing 2% BSA and 10% FBS in the absence or presence of 0.75 mM palmitate to induce insulin resistance. Subsequently, the treated cells were stimulated with 100 nM insulin for 20 min, and cells were then harvested for RT-PCR, western blot, or glucose uptake assay. HepG2 cells were maintained in DMEM with 25 mM glucose and 10% FBS. To induce insulin resistance, HepG2 cells were treated with 18 mM glucosamine for 18 hr in DMEM with 5 mM glucose. For lentivirus infection, HepG2 cells were treated with 6 μg/ml polybrene overnight and then infected with the indicated lentivirus for at least 3 days. After infection with lentivirus expressing SIRT1 RNAi, cells were treated with resveratrol at the indicated concentrations for 24 hr. To inhibit SIRT1, HepG2 cells were pretreated with 50 μM sirtinol (Sigma) for 12 hr. To inhibit PTP1B, cells were treated with 100 μM vanadate for 30 min. For western blot, cells were subsequently treated with 100 nM insulin for 20 min. Rat adipocytes were isolated from the epididymal fat pads of 150–170 g Sprague-Dawley rats (SLACCAS) as described previously (Wu et al., 2003). The prepared adipocytes were cultured in DMEM containing 2% insulin-free BSA (Sigma), 25 mM HEPES, and 0.5% FBS. Resveratrol was applied to rat adipocytes at the indicated concentrations for 10 hr, and the cells were then treated with 100 nM insulin for 20 min before glucose uptake assay.

Glucose Uptake Assay

Glucose uptake was determined using a method described previously (Jove et al., 2006). In brief, cells were incubated for 20 min at 37°C in the absence or presence of 100 nM insulin in Krebs-Ringer phosphate buffer (128 mM NaCl, 4.7 mM KCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$ [pH 7.4]). $[^3H]$2-deoxyglucose (Amersham) and 2-deoxyglucose were then added to a final concentration of 1 μCi/ml and 0.1 mM, respectively, and incubated for 5 min. After washing four times with ice-cold PBS, the cells were lysed with 0.5 M NaOH for 30 min. Radiolabeled glucose in lysates was measured in a high-flashpoint scintillation cocktail (PerkinElmer) using a liquid scintillation counter.

Glycogen Synthesis Assay and Measurement of Glycogen Synthase Activity

Glycogen synthesis was determined using a modified method described previously (Sakai et al., 2002). HepG2 cells cultured in 24-well plates were treated with 100 nM insulin and 1 μCi/ml $[^3H]$glucose in DMEM with 5 mM glucose for 3 hr and then harvested for glycogen synthesis assay. Cells in each well were lysed in 200 μl 30% KOH with 5 mg/ml glycogen at 60°C for 30 min. The cell lysates were collected, and glycogen was precipitated overnight at −20°C by adding 1 ml of ethanol. Glycogen was separated by centrifugation at 5000 × g for 10 min, and the pellets were washed twice with 75% ice-cold ethanol. Finally, the pellets were solubilized in 200 μl of 0.1 M HCl and measured by a liquid scintillation counter by adding 0.7 ml of high-flashpoint scintillation cocktail. Glycogen synthase activity was determined as described previously (Lazar et al., 1995) using UDP-$[^{14}C]$glucose purchased from PerkinElmer. C2C12 myotubes under palmitate-induced insulin-resistant conditions were used for glycogen synthase activity assay.

Western Blot and Immunoprecipitation

Protein samples were analyzed with antibodies against SIRT1 and PTP1B (Upstate); InsR, Tyr1146-phosphorylated InsR, Tyr1150/1151-phosphorylated InsR, phospho-Tyr, Akt, Ser308-phosphorylated Akt, Ser473-phosphorylated Akt, and Ser9-phosphorylated GSK-3β (Cell Signaling); and β-tubulin and actin (Sigma). The immune complexes were detected using a horseradish peroxidase-conjugated secondary antibody and visualized with a chemiluminescence reagent (Pierce). Each blot shown in the figures is representative of at least three experiments. Protein quantification was performed by Quantity One software (Bio-Rad), and the intensity values were normalized to tubulin. Immunoprecipitation of InsR antibody was performed according to the manufacturer's instructions.

RNA Isolation and RT-PCR

Total RNA was prepared from C2C12 cells by TRIzol reagent (Invitrogen). cDNA was then reverse transcribed and amplified by PCR. Quantification was carried out by Quantity One software (Bio-Rad), and the results were normalized to actin.

Animal Experiments

Animal experiments were performed as described in the Supplemental Experimental Procedures.

Luciferase Assay

Luciferase reporter constructs were a generous gift from N. Tonks (Fukada and Tonks, 2001). Luciferase assays were performed in HEK293T cells in 24-well plate cotransfected with SIRT1 plasmid (0.6 μg/well), luciferase reporter construct (0.1 μg/well), and pSV40-β-gal (0.1 μg/well) using Lipofectamine 2000 (Invitrogen). The transfected plasmids were balanced with empty vector. After transfection for 40 hr, cells were harvested and measured with a luciferase assay kit (Promega) and normalized to β-galactosidase activity.

Chromatin immunoprecipitation Assay

ChIP was carried out on C2C12 cells using a ChIP Assay Kit (Upstate Biotechnology).

Statistics

Data are expressed as mean ± SD of at least three independent experiments. Statistical significance was assessed by Student's t test. Differences were considered statistically significant at $p < 0.05$.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/6/4/307/DC1/.

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