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Modulation of PPAR γ Stability and Transcriptional Activity in Adipocytes by Resveratrol

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Abstract

The peroxisome proliferator activated receptor gamma (PPAR γ) is essential for the formation and function of adipocytes. PPAR γ is also involved in regulating insulin sensitivity and is the functional target of the thiazolidinedione (TZDs) class of insulin sensitizing drugs. While TZDs activate PPAR γ and decrease PPAR γ protein levels, genetic models indicate decreased expression of PPAR γ is also associated with increased insulin sensitivity. In this study, we show that resveratrol modulates PPAR γ protein levels in 3T3-L1 adipocytes via inhibition of PPAR γ gene expression coupled with increased ubiquitin-proteasome dependent degradation of PPAR γ proteins. Resveratrol-mediated decreases in PPAR γ expression are associated with repression of PPAR γ transcriptional activity when assayed using a panel of PPAR γ target genes in adipocytes. Finally, we demonstrate that resveratrol inhibits insulin-dependent changes in glucose uptake and glycogen levels and decreases IRS-1 and GLUT4 protein levels, indicating resveratrol represses insulin sensitivity in adipocytes. These results indicate resveratrol-mediated effects in adipocytes involve regulation of PPAR γ expression and transcriptional activity along with decreased responsiveness to insulin.

Keywords

resveratrol; PPAR γ ; adipocyte; ubiquitin; proteasome

Introduction

A remarkable range of health benefits are ascribed to resveratrol, a bioactive plant polyphenol found in grapes, peanuts, and berries. Recent studies show that resveratrol treatment protects against diet-induced insulin resistance in rodents (1;2) and leads to decreased lipid accumulation in murine adipocytes (3). Resveratrol is a potent activator of SIRT1 (silencing information regulator-1) (4), a histone deacetylase that mediates the effects of resveratrol in mice (1). SIRT1 is also reported to inhibit the formation of adipocytes via repression of the peroxisome proliferator activated receptor gamma (PPAR γ) transcriptional activity (3).

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Regulation of PPAR γ activity in adipocytes provides a direct link between nutritional status, lipid metabolism, and adipocyte gene expression (5). Adipose PPAR γ is also required for the maintenance of insulin sensitivity (6) yet mice heterozygous for PPAR γ deficiency remain more insulin sensitive than wild-type mice when fed a high fat diet (7–9). The reduced PPAR γ gene expression in the PPAR γ $-/+$ mice correlates with decreased PPAR γ protein (10), suggesting modulation of PPAR γ activity and protein levels can play a role in regulating insulin sensitivity.

Activation of SIRT1 by resveratrol suggests a potential link between regulation of PPAR γ activity, decreased PPAR γ protein levels, and insulin sensitivity via resveratrol-mediated effects and points to a role for resveratrol in modulating insulin action associated with obesity and type 2 diabetic states. In the current study, we show that resveratrol treatment in 3T3-L1 adipocytes represses the endogenous gene expression of transcriptional targets of PPAR γ such as *aP2*, *Lpl* and *Pepck*. In addition, resveratrol decreases *PPARG* gene expression while increasing targeting of PPAR γ protein to the ubiquitin-proteasome system for degradation, a novel mechanism of resveratrol-mediated effects in adipocytes. We also show that resveratrol treatment in 3T3-L1 adipocytes reduces insulin sensitivity as measured by decreased insulin-dependent glucose uptake and glycogen content along with decreased protein content of IRS-1 and GLUT4. Thus, our data indicates resveratrol may function as a nutritional regulator of PPAR γ activity, expression and stability while also decreasing insulin sensitivity in adipocytes.

Materials and Methods

Cell Culture

Murine 3T3-L1 preadipocytes were plated and grown to 2 days post confluence in DMEM high glucose with 10% bovine serum and penicillin/streptomycin. The cells were induced to differentiate using a standard induction cocktail of 3-isobutyl-1-methylxanthine, dexamethasone, insulin (MDI) as previously described (11). The 3T3-L1 preadipocytes and fully differentiated adipocytes were maintained in a humidified chamber at 37°C.

Preparation of Whole Cell Extracts

Cell monolayers were rinsed with phosphate-buffered saline (PBS) and harvested in a non-denaturing buffer as previously described (12). Samples were extracted for 30 minutes on ice and centrifuged at $15,521 \times g$ at 4°C for 15 minutes. Supernatants containing whole cell extracts were analyzed for protein concentrations using a BCA kit (Pierce) according to the manufacturer's instructions.

TZD and Resveratrol Treatment of 3T3-L1 Adipocytes

5.0 μ M rosiglitazone (TZD) and 50 μ M resveratrol (Sigma-Aldrich) were added to fully differentiated 3T3-L1 adipocytes at the indicated times. Resveratrol was added in the indicated concentrations when glucose uptake was assayed in the presence of increasing concentrations of resveratrol. DMSO was used as a solvent for both rosiglitazone and resveratrol.

Gel Electrophoresis and Immunoblotting

Proteins were separated in polyacrylamide (National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (13) and transferred to nitrocellulose. Following transfer, the membrane was blocked in 4% non-fat dry milk suspended in phosphate buffered saline, pH 7.4 with 0.1% Tween 20 (PBS-T) for 1 hour at room temperature. The membranes were incubated with mouse monoclonal anti-PPAR γ or antibodies against IRS-1, IRS-2, PI3K, AKT, phospho-AKT, PTP-1B, AMPK α 1, AMPK α 2, β -actin, anti-insulin receptor β subunit, GLUT1, and GLUT4 as indicated for 1–2 hours. Following extensive washes with PBS-T, the

results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (Pierce).

Real-time RT-PCR

Total RNA was purified from cultured cells using TriReagent (Molecular Research Center) according to the manufacturer's instructions. Real-time RT-PCR was performed via two-step RT-PCR (High Capacity cDNA Archive Kit, Applied Biosystems) followed by PCR using TaqMan primer/probe pairs consisting of two sequence-specific PCR primers and a TaqMan assay-FAM™ dye-labeled MGB probe (Applied Biosystems, Taqman Gene Expression Assay) for each gene of interest. The genes of interest were fatty acid binding protein 4 (*aP2*), lipoprotein lipase (*Lpl*), and cytosolic phosphoenolpyruvate kinase (*Pepck*). PCR was performed using the 7900 Real-Time PCR system (Applied Biosystems) under universal cycling conditions. All results were normalized to a cyclophilin B expression control and reported as the mean or the fold change relative to baseline \pm standard deviation.

Ubiquitin Conjugation Assay

3T3-L1 adipocytes were preincubated with 10 μ M MG132 and 1 μ M epoxomicin for 1 hour prior to adding 50 μ M resveratrol, 5.0 μ M rosiglitazone or an equal volume of DMSO as a vehicle control. The cells were harvested after 30 minutes and lysed on ice in PBS containing 1% Triton X-100, 10 mM N-ethylmaleimide, 1 mM EDTA, 1 mM PMSF, 1 μ M pepstatin and 10 μ M leupeptin. Whole cell extracts were incubated with protein A-sepharose (RepliGen) and the unbound supernatant was collected for immunoprecipitation using a polyclonal anti-PPAR γ followed by incubation with protein A-sepharose. PPAR γ -ubiquitin conjugates were detected by western blotting using monoclonal anti-PPAR γ and polyclonal anti-ubiquitin antibodies.

Determination of 2-[³H] deoxyglucose Uptake

Fully differentiated 3T3-L1 adipocytes at day 6–7 post induction were incubated in the presence of resveratrol, rosiglitazone or DMSO for 6 hours. Four hours prior to measuring glucose uptake, the cells were serum-deprived in DMEM containing 6.25 mM glucose and 0.3% bovine serum albumin. At the end of each treatment, 2-[³H] deoxyglucose uptake measurements were performed in triplicate and the results were corrected for nonspecific uptake, which was measured in the presence of 5 μ M cytochalasin B (14). The protein concentration of each lysate was determined using a BCA kit according to the manufacturer's instructions.

Glycogen Content

The adipocytes were treated with rosiglitazone (5 μ M) or resveratrol (50 μ M) for 15 hours with serum derivation during the final 2 hours of treatment. The cells were then incubated with 30 mM glucose in the absence or presence of 100 nM insulin. At the end of 2 hours, the cells were washed three times with cold phosphate buffered saline (PBS), pH 7.4 and whole cell extracts were harvested in 200 μ l of 0.2 M sodium acetate, pH 4.8, followed by sonication. After removal of 50 μ l aliquots for protein concentration determination, glycogen content was measured according to the method of Gomez-lechon (15).

RESULTS

Resveratrol treatment increases proteasome-dependent PPAR γ degradation and decreases PPAR γ gene expression

Over-expression of SIRT1 is associated with decreased PPAR γ protein levels in 3T3-L1 adipocytes (3), suggesting a link between activation of SIRT1 via resveratrol and regulation

of PPAR γ protein levels. To determine the effect of resveratrol on PPAR γ protein levels, we assayed the steady-state levels of PPAR γ proteins in fully differentiated 3T3-L1 adipocytes. As shown in Figure 1A, a six hour treatment with either rosiglitazone or resveratrol decreases the steady-state levels of PPAR γ proteins in adipocytes. We and others previously demonstrated that activation of PPAR γ is linked to the ubiquitin-proteasome dependent degradation of PPAR γ (12;16). Therefore, we asked if resveratrol-mediated decreases in PPAR γ protein levels were also associated with direct targeting of PPAR γ for ubiquitin conjugation and degradation by the proteasome. As shown in Figure 1A, a one hour pretreatment of the adipocytes with the 20S proteasome inhibitors MG132 and epoxomicin partially reverses the effect of either rosiglitazone or resveratrol, but does not return PPAR γ protein levels to those observed under control conditions in the presence of proteasome inhibition. The observed decreases in PPAR γ protein levels are associated with increased targeting of PPAR γ for ubiquitin-dependent degradation as shown by the increase in PPAR γ -ubiquitin conjugate formation in the presence of resveratrol (Figure 1B). The incomplete restoration of PPAR γ protein levels in the presence of proteasome inhibition indicates that increased targeting for ubiquitin-proteasome dependent degradation is insufficient to account for the resveratrol-mediated changes in PPAR γ protein levels. As shown in Figure 1C, both resveratrol and rosiglitazone also decrease *Pparg* gene expression.

Resveratrol treatment represses expression of endogenous genes regulated by PPAR γ activity

The parallel effects of resveratrol and rosiglitazone on PPAR γ protein degradation and gene expression prompted us to ask if resveratrol affects PPAR γ transcriptional activity. To determine if resveratrol affects PPAR γ activity, we used real-time RT-PCR to assay expression of PPAR γ target genes in fully differentiated adipocytes rather than a luciferase-based assay of PPAR γ transactivation. Previous studies have shown that resveratrol activates PPAR γ in a variety of tissues (17–19), including macrophages (17) when measured using transactivation reporter assays. However, studies of other transcription factors such as Elk-1 and c-Fos (20) or control of T cell receptor β expression (21) have demonstrated discrepancies between luciferase-based transactivation assays and regulation of endogenous targets of transcription factor activity. These studies point to the pitfalls of measuring gene expression outside the usual chromatin structure as is the case with luciferase reporter assays. This consideration is particularly relevant since resveratrol action involves activation of SIRT1, a histone deacetylase. To circumvent this problem, we chose a small set of genes in adipocytes that are involved in lipid metabolism and insulin sensitivity and whose expression is well-described as PPAR γ dependent: lipoprotein lipase (*Lpl*) (22;23), the fatty acid binding protein (*aP2*) (24), and the cytosolic phosphoenolpyruvate carboxykinase (*Pepck*) (25–27). We assayed gene expression under control conditions or in the presence of resveratrol or rosiglitazone (TZD) for six or fifteen hours. As shown in Figure 2A, a six hour treatment with resveratrol represses *aP2* and *Lpl* expression without affecting *Pepck* expression. Rosiglitazone (TZD) activation of PPAR γ corresponds to increased expression of *aP2* and *Pepck* in both cases with no increase in *Lpl* expression. However, a fifteen hour treatment (Figure 2B) with resveratrol shows repression of all three PPAR γ target genes while rosiglitazone treatment continues to be associated with increased expression of *aP2* and *Pepck*.

Resveratrol decreases insulin-dependent glucose uptake in adipocytes

Modulation of PPAR γ activity and gene expression is associated with improved insulin sensitivity when assayed as plasma glucose and insulin levels (7–9). In addition, resveratrol treatment is associated with improved insulin sensitivity in murine models (1;2). To determine if resveratrol affects insulin sensitivity in adipocytes, we measured the effect of resveratrol on glucose transport. The results in Figure 3A demonstrate that resveratrol decreases insulin-stimulated glucose uptake in adipocytes when compared to control or TZD-treated conditions.

In addition to triglyceride synthesis, insulin-stimulated glucose uptake in adipocytes leads to an increase in glycogen content via stimulation of glycogen synthase activity (28;29). Therefore, we assayed the glycogen content of the 3T3-L1 adipocytes under control, rosiglitazone, or resveratrol-treated conditions in the absence or presence of insulin. As shown in Figure 3B, resveratrol treatment is associated with decreased glycogen content under basal and insulin stimulated conditions. This is consistent with the observed resveratrol-mediated decreases in glucose uptake, indicating resveratrol reduces insulin sensitivity in adipocytes.

In our experiments, we used resveratrol at a concentration previously shown to affect lipid accumulation in 3T3-L1 adipocytes (3). In addition, resveratrol at 50 μM has been shown to increase SIRT1 activity 3–4 fold (4). To determine if the observed resveratrol-mediated decreases in insulin sensitivity were dose-related, we carried out glucose uptake assays in the presence of increasing concentrations of resveratrol (Figure 3C). Insulin-stimulated glucose uptake is unaffected by treatment with resveratrol at 1–10 μM although basal levels of glucose uptake trend upward. Insulin-stimulated glucose uptake is inhibited in the presence of higher concentrations (30–100 μM) of resveratrol.

Changes in insulin signaling pathway components in the presence of resveratrol

The resveratrol-mediated decreased glucose uptake suggests resveratrol affects components of the insulin signaling pathway in adipocytes. As shown in Figure 4, we assessed the content of proteins involved in the insulin signaling pathway. Resveratrol treatment decreased the basal levels of IRS-1 and the phosphorylated form of protein kinase B (AKT-P), and resulted in a small increase in insulin-stimulated AKT-P (Figure 4A). In addition, total GLUT4 levels were decreased with resveratrol treatment (Figure 4B). The observed changes in insulin sensitivity in the presence of resveratrol are consistent with decreased IRS-1 and GLUT4 protein levels, although contrary to the slight increase in insulin-stimulated AKT phosphorylation.

DISCUSSION

Since PPAR γ was identified as the functional receptor for the thiazolidinedione (TZD) class of insulin sensitizing drugs (30), efforts to improve treatment of type 2 diabetes have included understanding the regulation of PPAR γ in adipocytes. Although activation of PPAR γ by the TZDs increases insulin sensitivity, studies of mice heterozygous for PPAR γ show that reduced gene expression of wild-type PPAR γ also improves insulin sensitivity (7;9). The improvement in insulin sensitivity was observed with aging (8) and may include resistance to changes in insulin sensitivity that accompany a high fat diet (7;8). This is in contrast to the effect of a dominant negative PPAR γ mutation (P465L) in leptin deficient mice (*P465L/ob*) that results in insulin resistance in a setting of positive energy balance (31). The wild-type PPAR γ $-/+$ genetic model indicates modulation of wild-type PPAR γ expression could offer an alternative approach in the treatment of type 2 diabetes (32).

The present studies demonstrate that PPAR γ transcriptional activity and protein levels are modulated in adipocytes by resveratrol, a bioactive plant polyphenol. The decreased levels of PPAR γ proteins in 3T3-L1 adipocytes in response to resveratrol are mediated by decreased *Pparg* gene expression coupled with increased ubiquitin-proteasome-dependent degradation of PPAR γ proteins, paralleling the effect of TZDs. Earlier evidence indicated PPAR γ is targeted for destruction via the ubiquitin-proteasome system under basal or activated conditions, (14; 16;33), supporting a model in which PPAR γ degradation serves to limit PPAR γ transcriptional activity. Therefore, downregulation of PPAR γ in the presence of resveratrol describes a novel mechanism of action for resveratrol that consistent with the overall scheme of limiting PPAR γ activity via ubiquitin-proteasome dependent degradation.

Resveratrol-mediated reductions in PPAR γ gene expression and PPAR γ proteins correlate with decreased cellular effects of insulin and insulin signaling proteins in adipocytes as assayed by decreases in protein content for IRS-1 and GLUT4 in addition to glucose uptake and glycogen content. Assessment of functional aspects of GLUT4 properties such as translocation to the plasma membrane was outside the scope of the current study. Although resveratrol treatment (2) and reduction in PPAR γ expression (7–9) are associated with generally improved insulin sensitivity in murine models, the observed effect of resveratrol in adipocytes is consistent with studies on longevity demonstrating calorie restriction is associated with inhibition of insulin signaling (34). In particular, selective loss of the insulin receptor in murine adipocytes (FIRCO mice) protects against developing age and obesity-related insulin resistance (35). The reduction in insulin sensitivity in adipocytes in response to resveratrol may mimic the overall insulin sensitizing effects of calorie restriction, where the predominant feature is a loss of fat mass as a result of decreased lipid storage and increased lipolysis in adipocytes (36). Clearly, any generalized improvement in insulin sensitivity coincident with decreased glucose uptake in adipocytes will involve the interaction of adipocytes with other tissues that are glucose responsive, such as skeletal muscle and the liver as well as independent effects of resveratrol on those tissues (1;2). While the current studies are not designed to determine if the resveratrol-mediated changes in insulin sensitivity are due to down-regulation of PPAR γ activity and expression in adipocytes, our results are consistent with a recent finding from Liao et al (37) showing direct attenuation of PPAR γ expression decreases glucose uptake in 3T3-L1 adipocytes. Thus, resveratrol may serve as a pharmacological tool to explore the effects of reducing PPAR γ protein and gene expression in adipocytes.

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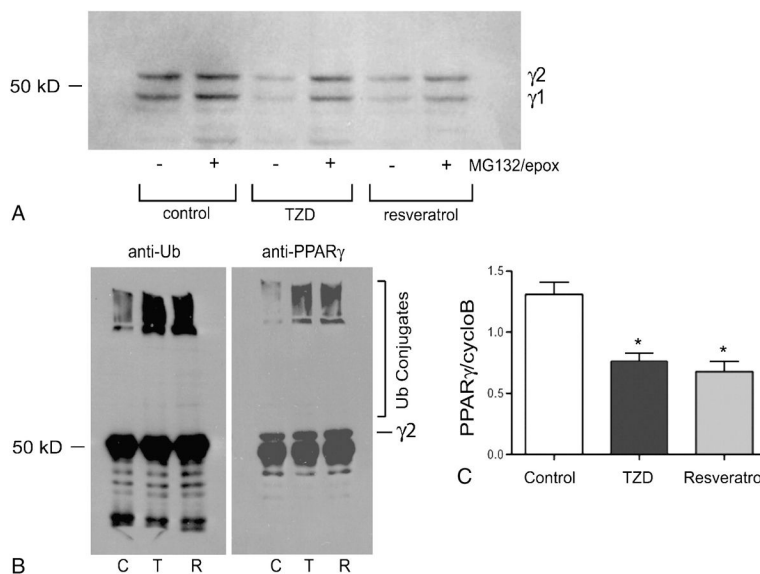


Figure 1. Resveratrol decreases PPAR γ gene and protein expression in 3T3-L1 adipocytes

Fully differentiated 3T3-L1 adipocytes were incubated for 6 hours under control (DMSO only) conditions or in the presence of rosiglitazone (5 μ M, TZD) or resveratrol (50 μ M). (A) The adipocytes were pretreated for 1 hour in the presence (+) or absence (-) of MG132 (10 μ M) and epoxomicin (1 μ M) as indicated. Whole cell extracts were harvested and separated by SDS-PAGE followed by western blot analysis. (B) The adipocytes were preincubated with 10 μ M MG132 and 1 μ M epoxomicin for 1 hour prior to adding 50 μ M resveratrol (R), 5.0 μ M rosiglitazone (T) or an equal volume of DMSO as a vehicle control (C). Whole cell extracts were harvested after 30 minutes and subjected to immunoprecipitation using a polyclonal anti-PPAR γ antibody. The immunoprecipitated proteins were analyzed by western blotting using anti-ubiquitin and anti-PPAR γ antibodies as indicated. (C) Total RNA was purified and real-time RT-PCR was carried using TaqMan chemistry (Applied Biosystems). PPAR γ expression is reported as the ratio of PPAR γ expression to cyclophilin B gene expression. Each experiment was carried out in triplicate and reported as the average \pm standard deviation. Statistical significance was determined using an unpaired Student's t test. *P<0.05 compared to control.

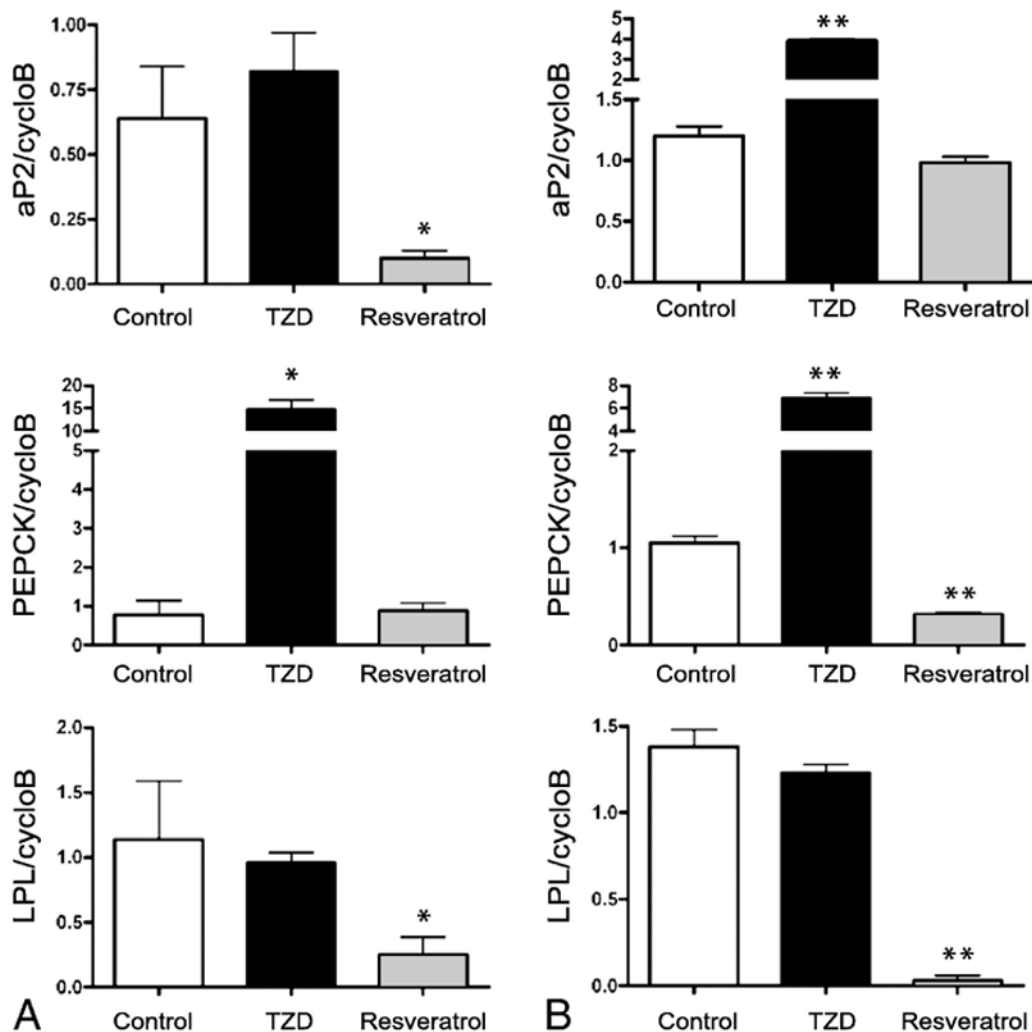


Figure 2. Resveratrol regulates PPAR γ transcriptional activity

Fully differentiated 3T3-L1 adipocytes were incubated for (A) 6 hours or (B) 15 hours under control (DMSO) conditions or in the presence of rosiglitazone (5 μ M) or resveratrol (50 μ M). Total RNA was purified and real-time-PCR was carried using TaqMan chemistry. The levels of *aP2*, *Pepck*, and *Lpl* were calculated as the ratio of the gene to cyclophilin B expression. Each experiment was carried out in triplicate and reported as the average \pm standard deviation. Statistical significance was determined using an unpaired Student's t test. *P<0.05, ** P<0.005 (compared to control)

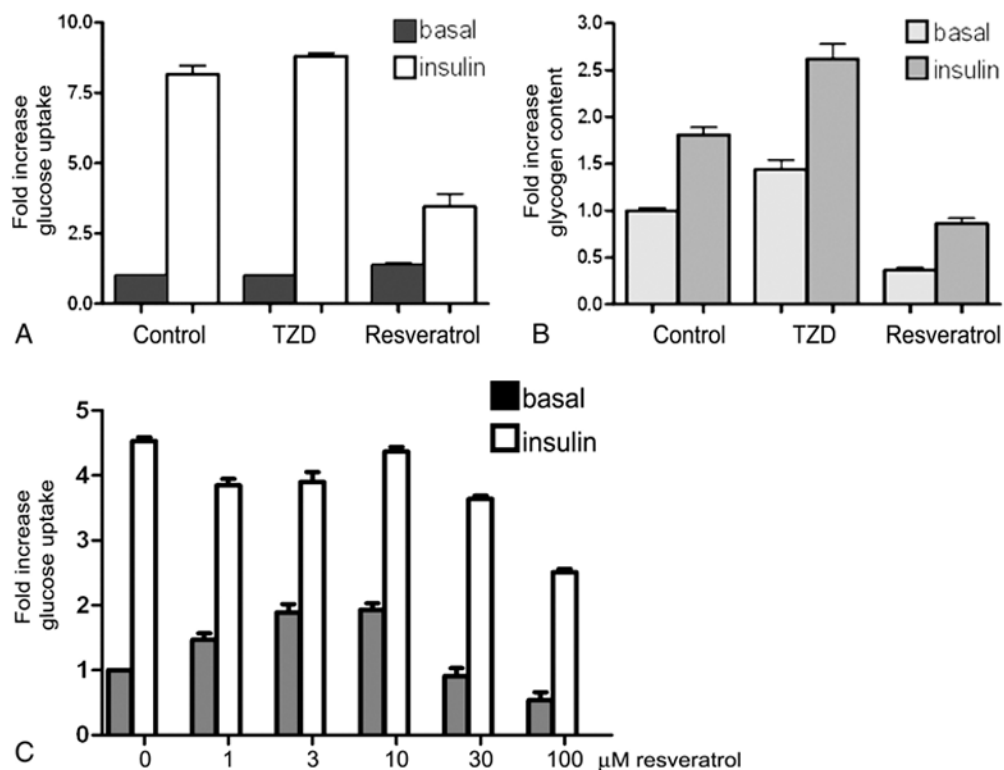


Figure 3. Resveratrol decreases glucose uptake and glycogen content in adipocytes

Fully differentiated adipocytes were treated with DMSO (control), rosiglitazone (5 μ M) or resveratrol (50 μ M) for 15 hours and serum-deprived for 2 hours prior to adding vehicle control (10 mM HCl) (■, basal) or 100 nM insulin (□, insulin). (A) Glucose uptake and (B) Glycogen content were measured as described in Materials and Methods. (C) Glucose uptake was measured in the 3T3-L1 adipocytes after treatment with the indicated concentration of resveratrol for 15 hours. Glucose uptake and glycogen content are reported as fold increase over basal control levels.

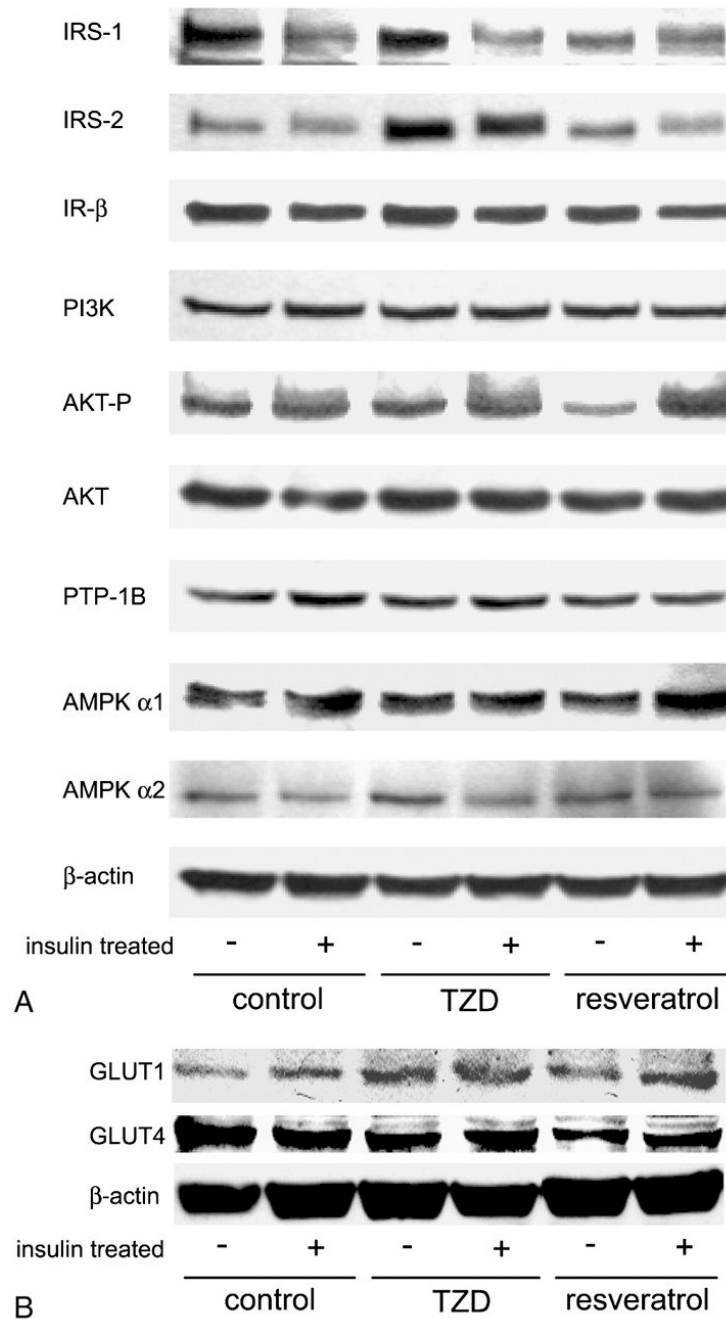


Figure 4. Resveratrol-mediated changes in insulin signaling components in 3T3-L1 adipocytes
Fully differentiated adipocytes were incubated in the presence of 5 μ M rosiglitazone (TZD), 50 μ M resveratrol or DMSO (control) for 14 hours in the absence or presence of 400 nM insulin. Whole cell lysates were harvested and analyzed by SDS-PAGE followed by detection using chemiluminescence (Pierce). β -actin is included as a loading control.