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Metabolism Clinical and Experimental 57 (Suppl 1) (2008) S32 – S38
Metabolism
Clinical and Experimental
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Modulation of peroxisome proliferator–activated receptor γ stability and transcriptional activity in adipocytes by resveratrol

Z. Elizabeth Floyd[a,*], Zhong Q. Wang[b], Gail Kilroy[a], William T. Cefalu[b]

[a]*Ubiquitin Biology Laboratory, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, USA*
[b]*Diabetes and Nutrition Laboratory, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, USA*

Abstract

The peroxisome proliferator–activated receptor (PPAR) γ is essential for the formation and function of adipocytes. It is also involved in regulating insulin sensitivity and is the functional target of the thiazolidinedione class of insulin-sensitizing drugs. Whereas thiazolidinediones activate PPARγ and decrease PPARγ protein levels, genetic models indicate that decreased expression of PPARγ is also associated with increased insulin sensitivity. In this study, we show that resveratrol modulates PPARγ protein levels in 3T3-L1 adipocytes via inhibition of PPARγ gene expression coupled with increased ubiquitin-proteasome–dependent degradation of PPARγ proteins. Resveratrol-mediated decreases in PPARγ expression are associated with repression of PPARγ transcriptional activity when assayed using a panel of PPARγ target genes in adipocytes. Finally, we demonstrate that resveratrol inhibits insulin-dependent changes in glucose uptake and glycogen levels and decreases insulin receptor substrate 1 and glucose transporter 4 protein levels, indicating that resveratrol represses insulin sensitivity in adipocytes. These results indicate that the resveratrol-mediated effects in adipocytes involve regulation of PPARγ expression and transcriptional activity along with decreased responsiveness to insulin.

1. Introduction

A remarkable range of health benefits is 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 (PPAR) γ transcriptional activity [3].

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

Activation of SIRT1 by resveratrol suggests a potential link between regulation of PPARγ activity, decreased PPARγ 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 diabetes mellitus states. In the current study, we show that resveratrol treatment in 3T3-L1 adipocytes represses the endogenous gene expression of transcriptional targets of PPARγ such as *aP2*, *Lpl*, and *Pepck*. In addition, resveratrol decreases *PPARg* gene expression while increasing targeting of PPARγ 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 insulin receptor substrate (IRS) 1 and glucose transporter (GLUT) 4. Thus, our data indicate that resveratrol may function as a nutritional regulator of PPARγ activity,

* Corresponding author. Pennington Biomedical Research Center, Baton Rouge, LA 70806, USA. Tel.: +1 225 763 2724; fax: +1 225 763 0273.
E-mail address: elizabeth.floyd@pbrc.edu (Z.E. Floyd).

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doi:10.1016/j.metabol.2008.04.006

expression, and stability while also decreasing insulin sensitivity in adipocytes.

2. Materials and methods

2.1. Cell culture

Murine 3T3-L1 preadipocytes were plated and grown to 2 days postconfluence in Dulbecco modified Eagle medium 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, and insulin as previously described [11]. The 3T3-L1 preadipocytes and fully differentiated adipocytes were maintained in a humidified chamber at 37°C.

2.2. Preparation of whole cell extracts

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

2.3. Thiazolidinedione and resveratrol treatment of 3T3-L1 adipocytes

Five micromoles per liter rosiglitazone (thiazolidinedione [TZD]) and 50 μmol/L resveratrol (Sigma-Aldrich, St Louis, MO) 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. Dimethyl sulfoxide (DMSO) was used as a solvent for both rosiglitazone and resveratrol.

2.4. Gel electrophoresis and immunoblotting

Proteins were separated in polyacrylamide (National Diagnostics, Atlanta, GA) gels containing sodium dodecyl sulfate (SDS) according to Laemmli [13] and transferred to nitrocellulose. After transfer, the membrane was blocked in 4% nonfat dry milk suspended in PBS (pH 7.4) with 0.1% Tween 20 for 1 hour at room temperature. The membranes were incubated with mouse monoclonal anti-PPARγ or antibodies against IRS-1, IRS-2, PI3K, AKT, phospho-AKT, PTP-1B, AMPKα1, AMPKα2, β-actin, anti–insulin receptor β subunit, GLUT1, and GLUT4 as indicated for 1 to 2 hours. After extensive washes with PBS (pH 7.4) with 0.1% Tween 20, the results were visualized with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (Pierce).

2.5. Real-time reverse transcriptase polymerase chain reaction

Total RNA was purified from cultured cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed via 2-step RT-PCR (High Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA) followed by PCR using TaqMan primer/probe pairs consisting of 2 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*). The 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 ± standard deviation.

2.6. Ubiquitin conjugation assay

The 3T3-L1 adipocytes were preincubated with 10 μmol/L MG132 and 1 μmol/L epoxomicin for 1 hour before adding 50 μmol/L resveratrol, 5.0 μmol/L 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 mmol/L *N*-ethylmaleimide, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L pepstatin, and 10 μmol/L leupeptin. Whole cell extracts were incubated with protein A–sepharose (RepliGen, Waltham, MA), and the unbound supernatant was collected for immunoprecipitation using a polyclonal anti-PPARγ followed by incubation with protein A–sepharose. The PPARγ-ubiquitin conjugates were detected by Western blotting using monoclonal anti-PPARγ and polyclonal anti-ubiquitin antibodies.

2.7. Determination of 2-[^{3}H] deoxyglucose uptake

Fully differentiated 3T3-L1 adipocytes at day 6 to 7 post-induction were incubated in the presence of resveratrol, rosiglitazone, or DMSO for 6 hours. Four hours before measuring glucose uptake, the cells were serum-deprived in Dulbecco modified Eagle medium containing 6.25 mmol/L glucose and 0.3% bovine serum albumin. At the end of each treatment, 2-[^{3}H] deoxyglucose uptake measurements were performed in triplicate; and the results were corrected for nonspecific uptake, which was measured in the presence of 5 μmol/L cytochalasin B [14]. The protein concentration of each lysate was determined using a BCA kit according to the manufacturer's instructions.

2.8. Glycogen content

The adipocytes were treated with rosiglitazone (5 μmol/L) or resveratrol (50 μmol/L) for 15 hours, with serum derivation during the final 2 hours of treatment. The cells were then incubated with 30 mmol/L glucose in the absence or presence of 100 nmol/L insulin. At the end of 2 hours, the cells were washed 3 times with cold PBS (pH 7.4); and whole cell extracts were harvested in 200 μL of 0.2 mol/L sodium

acetate (pH 4.8), followed by sonication. After removal of 50-μL aliquots for protein concentration determination, glycogen content was measured according to the method of Gomez-Lechon et al [15].

3. Results

3.1. Resveratrol treatment increases proteasome-dependent PPARγ degradation and decreases PPARγ gene expression

Overexpression of SIRT1 is associated with decreased PPARγ protein levels in 3T3-L1 adipocytes [3], suggesting a link between activation of SIRT1 via resveratrol and regulation of PPARγ protein levels. To determine the effect of resveratrol on PPARγ protein levels, we assayed the steady-state levels of PPARγ proteins in fully differentiated 3T3-L1 adipocytes. As shown in Fig. 1A, a 6-hour treatment with either rosiglitazone or resveratrol decreases the steady-state levels of PPARγ proteins in adipocytes. We and others previously demonstrated that activation of PPARγ is linked to the ubiquitin-proteasome–dependent degradation of PPARγ [12,16]. Therefore, we asked if resveratrol-mediated decreases in PPARγ protein levels were also associated with direct targeting of PPARγ for ubiquitin conjugation and degradation by the proteasome. As shown in Fig. 1A, a 1-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 PPARγ protein levels to those observed under control conditions in the presence of proteasome inhibition. The observed decreases in PPARγ protein levels are associated with increased targeting of PPARγ for ubiquitin-dependent degradation as shown by the increase in PPARγ-ubiquitin conjugate formation in the presence of resveratrol (Fig. 1B). The incomplete restoration of PPARγ 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 PPARγ protein levels. As shown in Fig. 1C, both resveratrol and rosiglitazone also decrease *Pparg* gene expression.

3.2. Resveratrol treatment represses expression of endogenous genes regulated by PPARγ activity

The parallel effects of resveratrol and rosiglitazone on PPARγ protein degradation and gene expression prompted us to ask if resveratrol affects PPARγ transcriptional activity.

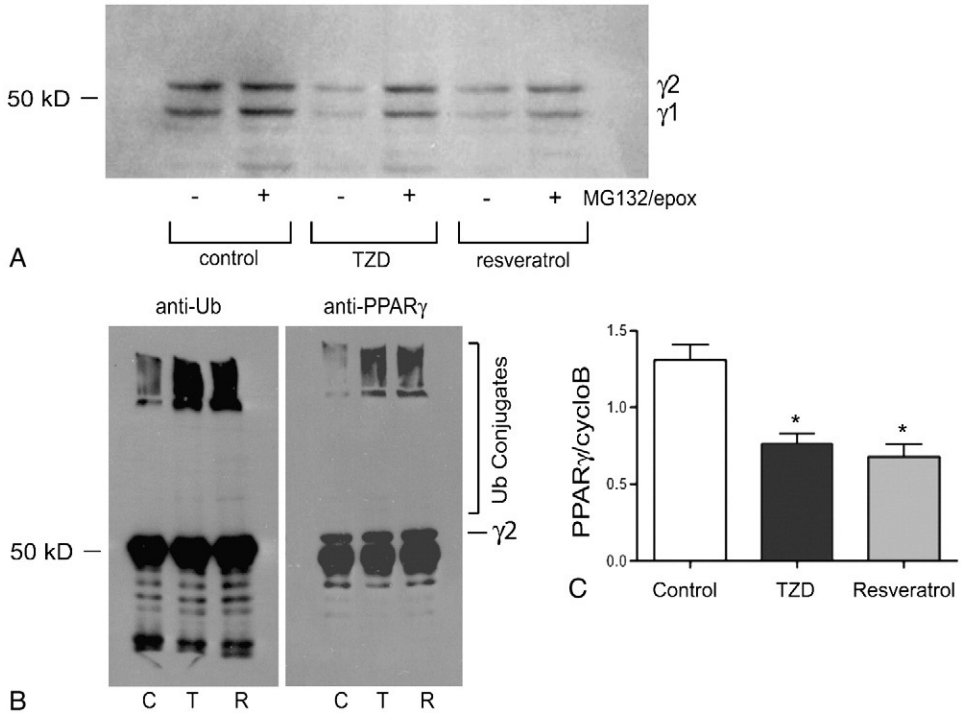

Fig. 1. Resveratrol decreases PPARγ gene and protein expression in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated for 6 hours under control (DMSO only) conditions or in the presence of rosiglitazone (5 μmol/L, TZD) or resveratrol (50 μmol/L). A, The adipocytes were pretreated for 1 hour in the presence (+) or absence (−) of MG132 (10 μmol/L) and epoxomicin (1 μmol/L) as indicated. Whole cell extracts were harvested and separated by SDS–polyacrylamide gel electrophoresis followed by Western blot analysis. B, The adipocytes were preincubated with 10 μmol/L MG132 and 1 μmol/L epoxomicin for 1 hour before adding 50 μmol/L resveratrol (R), 5.0 μmol/L 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-PPARγ antibody. The immunoprecipitated proteins were analyzed by Western blotting using anti-ubiquitin and anti-PPARγ antibodies as indicated. C, Total RNA was purified, and real-time RT-PCR was carried out using TaqMan chemistry (Applied Biosystems). The PPARγ expression is reported as the ratio of PPARγ expression to cyclophilin B gene expression. Each experiment was carried out in triplicate and reported as the average ± standard deviation. Statistical significance was determined using an unpaired Student *t* test. $^*P < .05$ (compared with control).

To determine if resveratrol affects PPARγ activity, we used real-time RT-PCR to assay expression of PPARγ target genes in fully differentiated adipocytes rather than a luciferase-based assay of PPARγ transactivation. Previous studies have shown that resveratrol activates PPARγ 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] has 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 because 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 PPARγ dependent: lipoprotein lipase (*Lpl*) [22,23], the fatty acid binding protein (*aP2*) [24], and the cytosolic phosphoenol-pyruvate carboxykinase (*Pepck*) [25-27]. We assayed gene expression under control conditions or in the presence of resveratrol or rosiglitazone (TZD) for 6 or 15 hours. As shown in Fig. 2A, a 6-hour treatment with resveratrol represses *aP2* and *Lpl* expression without affecting *Pepck* expression. Rosiglitazone (TZD) activation of PPARγ corresponds to increased expression of *aP2* and *Pepck* in both cases with no increase in *Lpl* expression. However, a 15-hour treatment (Fig. 2B) with resveratrol shows repression of all 3 PPARγ target genes, whereas rosiglitazone treatment continues to be associated with increased expression of *aP2* and *Pepck*.

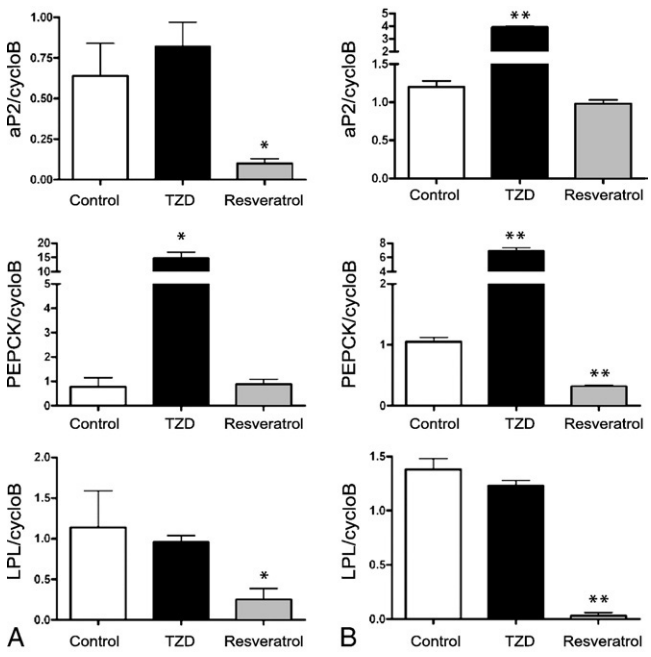

Fig. 2. Resveratrol regulates PPARγ transcriptional activity. Fully differentiated 3T3-L1 adipocytes were incubated for (A) 6 hours or (B) 15 hours under control (DMSO) conditions or in the presence of rosiglitazone (5 μmol/L) or resveratrol (50 μmol/L). Total RNA was purified, and real-time-PCR was carried out 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 ± standard deviation. Statistical significance was determined using an unpaired Student *t* test. **P* < .05 and ***P* < .005 (compared with control).

3.3. Resveratrol decreases insulin-dependent glucose uptake in adipocytes

Modulation of PPARγ 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 Fig. 3A demonstrate that resveratrol decreases insulin-stimulated glucose uptake in adipocytes when compared with 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-treated, or resveratrol-treated conditions in the absence or presence of insulin. As shown in Fig. 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 that 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 μmol/L has been shown to increase SIRT1 activity 3- to 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 (Fig. 3C). Insulin-stimulated glucose uptake is unaffected by treatment with resveratrol at 1 to 10 μmol/L, although basal levels of glucose uptake trend upward. Insulin-stimulated glucose uptake is inhibited in the presence of higher concentrations (30-100 μmol/L) of resveratrol.

3.4. Changes in insulin signaling pathway components in the presence of resveratrol

The resveratrol-mediated decreased glucose uptake suggests that resveratrol affects components of the insulin signaling pathway in adipocytes. As shown in Fig. 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

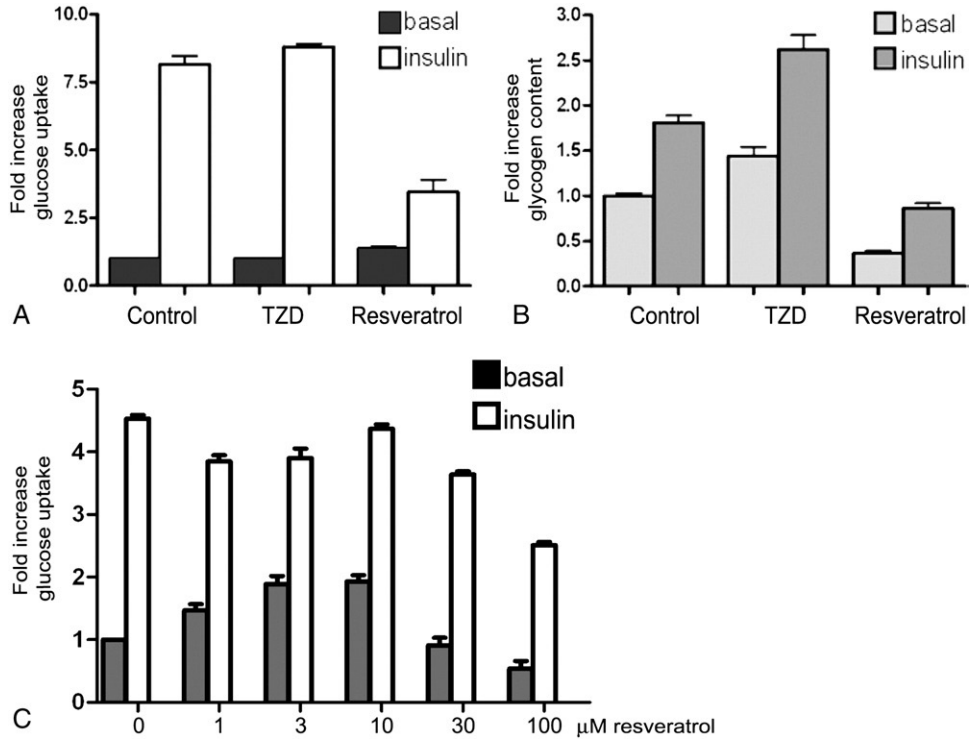

Fig. 3. Resveratrol decreases glucose uptake and glycogen content in adipocytes. Fully differentiated adipocytes were treated with DMSO (control), rosiglitazone (5 μmol/L), or resveratrol (50 μmol/L) for 15 hours and serum-deprived for 2 hours before adding vehicle control (10 mmol/L HCl) (■, basal) or 100 nmol/L insulin (□, insulin). Glucose uptake (A) and glycogen content (B) 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.

in insulin-stimulated AKT-P (Fig. 4A). In addition, total GLUT4 levels were decreased with resveratrol treatment (Fig. 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.

4. Discussion

Since PPARγ was identified as the functional receptor for the TZD class of insulin-sensitizing drugs [30], efforts to improve treatment of type 2 diabetes mellitus have included understanding the regulation of PPARγ in adipocytes. Although activation of PPARγ by the TZDs increases insulin sensitivity, studies of mice heterozygous for PPARγ show that reduced gene expression of wild-type PPARγ 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 PPARγ mutation (P465L) in leptin-deficient mice (*P465L/ob*) that results in insulin resistance in a setting of positive energy balance [31]. The wild-type PPARγ −/+ genetic model indicates that modulation of wild-type PPARγ expression could offer an alternative approach in the treatment of type 2 diabetes mellitus [32].

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

Resveratrol-mediated reductions in PPARγ gene expression and PPARγ 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]

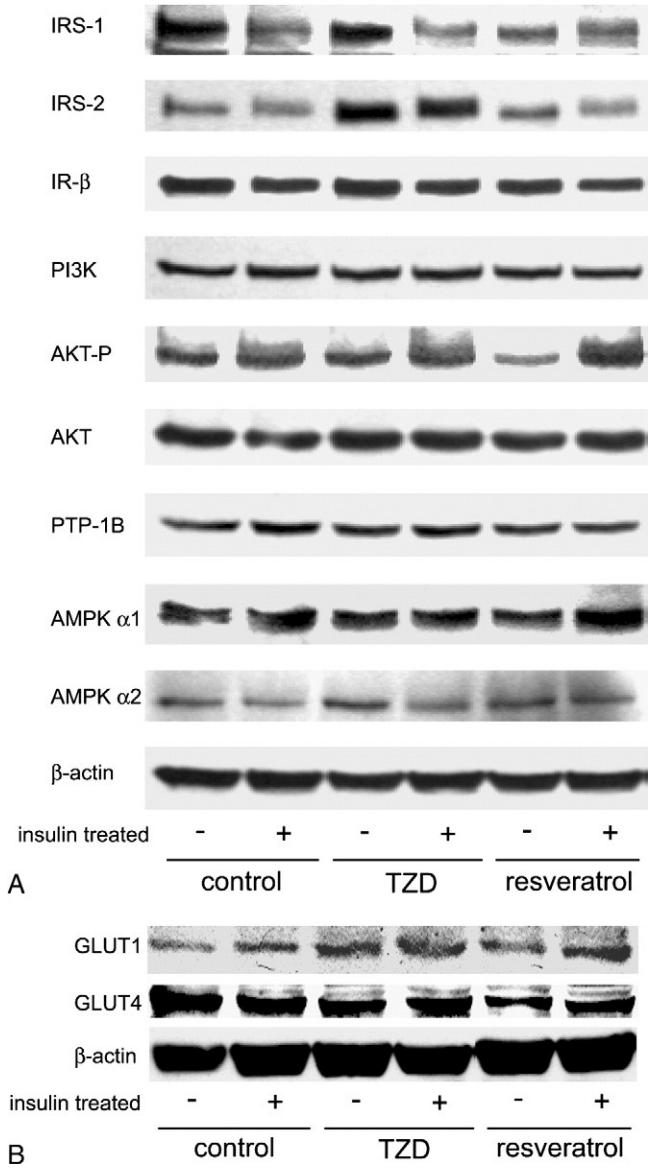

Fig. 4. Resveratrol-mediated changes in insulin signaling components in 3T3-L1 adipocytes. Fully differentiated adipocytes were incubated in the presence of 5 μmol/L rosiglitazone (TZD), 50 μmol/L resveratrol, or DMSO (control) for 14 hours in the absence or presence of 400 nmol/L insulin. Whole cell lysates were harvested and analyzed by SDS–polyacrylamide gel electrophoresis followed by detection using chemiluminence (Pierce). β-Actin is included as a loading control.

and reduction in PPARγ 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 that 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]. Although the current studies are not designed to determine if the resveratrol-mediated changes in insulin sensitivity are due to down-regulation of PPARγ activity and expression in adipocytes, our results are consistent with a recent finding from Liao et al [37] showing that direct attenuation of PPARγ expression decreases glucose uptake in 3T3-L1 adipocytes. Thus, resveratrol may serve as a pharmacological tool to explore the effects of reducing PPARγ protein and gene expression in adipocytes.

Acknowledgment/Conflict of Interest

This work is funded in part by the National Institutes of Health Grant P50AT002776-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS) and by the National Institute on Aging R03 AG025751 (to ZEF). The authors thank Dr Jeffrey Gimble for helpful discussions and critical reading of the manuscript.

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