



# Resveratrol prevents dexamethasone-induced expression of the muscle atrophy-related ubiquitin ligases atrogin-1 and MuRF1 in cultured myotubes through a SIRT1-dependent mechanism

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## ABSTRACT

Resveratrol (3,5,4'-trihydroxystilbene) has been ascribed multiple beneficial biological effects but the influence of resveratrol on glucocorticoid-induced muscle atrophy is not known. We examined the effects of resveratrol on dexamethasone-induced atrogin-1 and MuRF1 expression, FOXO1 acetylation, protein degradation and atrophy in cultured L6 myotubes. In addition, the role of the deacetylase SIRT1 in the effects of resveratrol was determined by transfecting myotubes with SIRT1 siRNA. The catabolic effects of dexamethasone were prevented by resveratrol and the protective effects of resveratrol on dexamethasone-induced atrogin-1 and MuRF1 expression were abolished in myotubes transfected with SIRT1 siRNA. Results suggest that resveratrol can prevent glucocorticoid-induced muscle wasting and that this effect is at least in part SIRT1-dependent.

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## 1. Introduction

Resveratrol (3,5,4'-trihydroxystilbene) is a natural polyphenol present in peanuts, pines, the skin of grapes, and red wine [1,2]. It has been ascribed multiple beneficial biological effects, including cardioprotection [3] and amelioration of some of the metabolic consequences of diabetes [4]. Different mechanisms by which resveratrol may exert beneficial effects have been described and include antioxidant effects [5,6], inhibition of NF-κB [7], and activation of AMP-activated protein kinase (AMPK) [8]. In other reports, resveratrol stimulated the activity of the histone deacetylase SIRT1 and recent studies suggest that this may be the most important mechanism of the metabolic effects of the drug [9–12].

Previous studies suggest that resveratrol may protect skeletal muscle from the influence of certain catabolic conditions, including diabetes [4], mechanical unloading [13], muscular dystrophy [14], and cancer [15]. Conflicting results have been reported, however, and in recent experiments, muscle wasting was not prevented, or was even worsened, by resveratrol [16]. In addition, the mechanisms by which resveratrol protects skeletal muscle from muscle wasting are unclear. In particular, the role of SIRT1 activation in

resveratrol-induced protection from muscle wasting is not well understood. This is important, because recent studies from our and other laboratories suggest that muscle wasting is associated with reduced expression and activity of histone deacetylases, including SIRT1 [17,18].

High levels of glucocorticoids result in increased expression of the muscle atrophy-related ubiquitin ligases atrogin-1 and MuRF1, increased ubiquitin–proteasome-dependent muscle proteolysis, and loss of muscle mass [19,20]. In addition, the catabolic effects of certain conditions, such as sepsis and severe injury, are at least in part mediated by glucocorticoids [19–22]. The effects of resveratrol on glucocorticoid-induced atrogin-1 and MuRF1 expression and muscle atrophy and the role of SIRT1 activation have not been reported. Here, we tested the hypothesis that resveratrol prevents dexamethasone-induced expression of atrogin-1 and MuRF1, protein degradation and atrophy in cultured myotubes and that the protective effects of resveratrol are SIRT1-dependent.

Previous studies suggest that atrogin-1 and MuRF1 expression is at least in part regulated by the transcription factor FOXO1 [23,24]. Other reports provided evidence that FOXO1 activity is increased by acetylation and can be inhibited by SIRT1 [25,26] although apparently contradictory results have also been reported [27], possibly reflecting differential regulation of FOXO1 activity by acetylation in different cell types. The regulation by glucocorticoids of FOXO1 acetylation in skeletal muscle and the effects of resveratrol are not known. In the present study, we therefore also tested

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the influence of dexamethasone and resveratrol on FOXO1 acetylation in cultured myotubes.

## 2. Materials and methods

### 2.1. Cell culture

L6 muscle cells, a rat skeletal muscle cell line (American Type Culture Collection, Manassas, VA), were maintained and cultured as described in detail recently [28]. Differentiated myotubes were treated for 24 h with 1  $\mu$ M dexamethasone (Sigma–Aldrich, St. Louis, MO), 100  $\mu$ M resveratrol (Sigma–Aldrich), or both drugs in combination. The concentrations of dexamethasone and resveratrol used here were based on previous studies [28–30]. Control myotubes were treated with solvent (0.1% ethanol).

### 2.2. Preparation of total cell lysates and nuclear extracts

Total cell lysates were prepared by harvesting the myotubes directly in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Nonidet P-40) containing Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN). After scraping the lysates into eppendorf tubes, the samples were briefly sonicated using a Sonic Dismembrator (Fisher Scientific, Model 100) followed by centrifugation at 14,000g for 10 min at 4 °C. Nuclear extracts were prepared using the NE-PER<sup>®</sup> Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Asheville, NC) according to the manufacturer's instructions. Concentrations of soluble proteins in the supernatants of the nuclear extracts and total cell lysates were determined by using the Bradford Protein Assay Kit (Thermo Fisher Scientific) with bovine serum albumin as standard. Nuclear extracts and cell lysates were stored at –80 °C until analyzed.

### 2.3. Real-time PCR

Messenger RNA levels for atrogen-1, MuRF1, and SIRT1 were determined by real-time PCR performed as described in detail recently [17,28,29,31]. The sequences of the forward, reverse, and double-labeled oligonucleotides for rat atrogen-1 and MuRF1 used here were reported recently [17,31]. SIRT1 mRNA levels were determined using the ABI Taqman Gene Expression Assay (Assay ID: RN01428093\_m1) from Applied Biosystems, Foster City, CA.

### 2.4. Western blotting

Western blotting was performed as described in detail recently [17,28] using the following antibodies: a rabbit polyclonal anti-mouse atrogen-1 antibody (1:1000; kindly supplied by Dr. Stewart Lecker, Harvard Medical School); a mouse polyclonal anti-rat MuRF1 antibody (1:1000; kindly supplied by Regeneron Pharmaceuticals, NY); a rabbit monoclonal anti-rat  $\alpha$ -tubulin antibody (1:5000; Sigma–Aldrich); a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). Levels of acetylated FOXO1 and PGC-1 $\alpha$  were determined by co-immunoprecipitation. Nuclear proteins (300  $\mu$ g) were pulled down with an anti-acetyl lysine antibody (Cell Signaling Technology, Danver, MA) followed by Western blotting using a rabbit polyclonal anti-rat FOXO1 antibody (Cell Signaling Technology) or a rabbit polyclonal anti-rat PGC-1 $\alpha$  antibody (Cell Signaling Technology). Immunoreactive protein bands were visualized by chemiluminescence using the Western Lighting Kit (Perkin Elmer Life Sciences Inc., Boston, MA) followed by exposure to X-ray blue film (Cole-Parmer, Vernon Hills, IL). Molecular weights of protein bands were determined by Dual Precision

molecular weight standards (BioRad, Hercules, CA). The protein bands were quantified by densitometry using ImageJ software (NIH, Frederick, MD).

### 2.5. Measurement of protein degradation and myotube diameter

Protein degradation rates were determined by measuring the release of trichloroacetic acid (TCA)-soluble radioactivity during 24 h from proteins prelabeled with [<sup>3</sup>H]-tyrosine as described [28,29]. For the measurement of myotube diameter, myotube cultures were photographed under a phase contrast microscope at 100 $\times$  magnification. The diameters were measured in a total of 60 myotubes from at least 10 random fields using ImageJ software (NIH) as described in detail [28,29]. The measurements were conducted in a “blinded” fashion on coded pictures with the investigator being unaware of the group (control, dexamethasone-, resveratrol-, or dexamethasone + resveratrol-treated myotubes) from which the cultures originated. Results were expressed as percent of control.

### 2.6. Cell transfections

L6 myotubes were transfected with SIRT1 siRNA or non-targeting (scrambled) siRNA (Santa Cruz Biotechnology) utilizing the transfection reagent Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY). The siRNA constructs were added to the culture medium at a concentration of 165 nM in combination with Lipofectamine according to the manufacturer's instructions. After 5 h, the medium was changed to fresh DMEM containing 2% FBS. After 48 h, myotubes were exposed for 24 h to 1  $\mu$ M dexamethasone, 100  $\mu$ M resveratrol, a combination of the drugs, or solvent alone (0.1% ethanol).

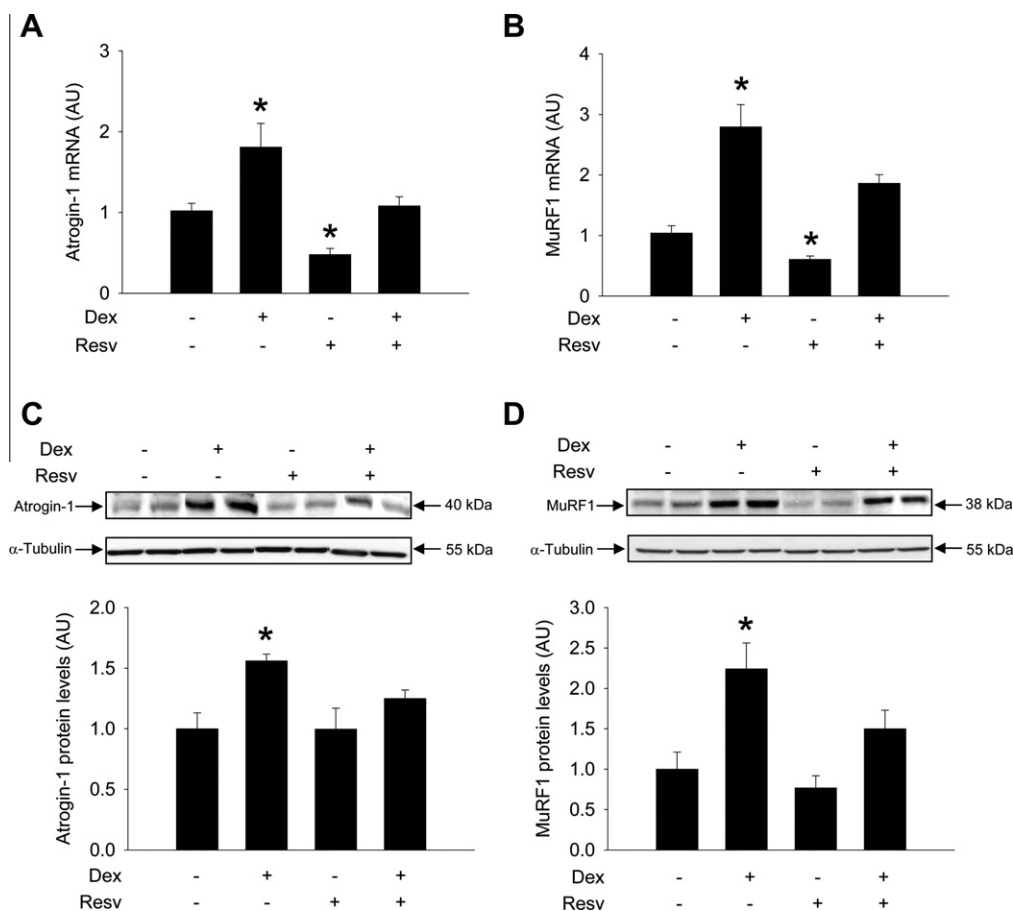
### 2.7. Statistics

Results are reported as means  $\pm$  SEM. Statistical analysis was performed by Student's *t*-test or ANOVA followed by Tukey's post hoc test as appropriate. *p* < 0.05 was considered statistically significant.

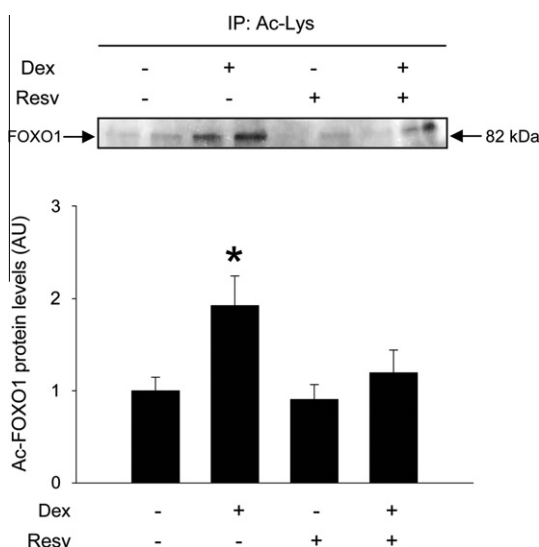
## 3. Results

Treatment of cultured L6 myotubes with 1  $\mu$ M dexamethasone for 24 h resulted in an approximately 2- to 3-fold increase in atrogen-1 and MuRF1 mRNA levels (Fig. 1A and B) and a 1.5- to 2-fold increase in corresponding protein levels (Fig. 1C and D). These effects of dexamethasone are similar to previous reports [28,29]. When dexamethasone-treated myotubes were exposed to 100  $\mu$ M resveratrol, atrogen-1 and MuRF1 mRNA and protein levels were not different from those in untreated control myotubes. Of note, resveratrol by itself reduced atrogen-1 and MuRF1 mRNA levels but did not influence the corresponding protein levels. Taken together, the results suggest that resveratrol inhibits dexamethasone-induced expression of atrogen-1 and MuRF1 in cultured myotubes.

Because previous studies suggest that atrogen-1 and MuRF1 expression is at least in part regulated by FOXO1 [23,24] and that FOXO1 activity may be stimulated by acetylation [25], we next tested whether FOXO1 acetylation is increased in dexamethasone-treated myotubes and can be reduced by resveratrol. When L6 myotubes were treated with dexamethasone, nuclear levels of acetylated FOXO1 increased, consistent with increased FOXO1 activity [25], and this effect of dexamethasone was blocked by resveratrol (Fig. 2).



**Fig. 1.** Resveratrol inhibits dexamethasone-induced expression of atrogin-1 and MuRF1 in cultured L6 myotubes. Myotubes were treated for 24 h with 1  $\mu$ M dexamethasone, 100  $\mu$ M resveratrol, both drugs in combination or solvent (0.1% ethanol) followed by measurement of mRNA levels for (A) atrogin-1 and (B) MuRF1 and protein levels for (C) atrogin-1 and (D) MuRF1. Results are means  $\pm$  SEM with  $n = 8$  per group. \* $p < 0.05$  vs all other groups by ANOVA.

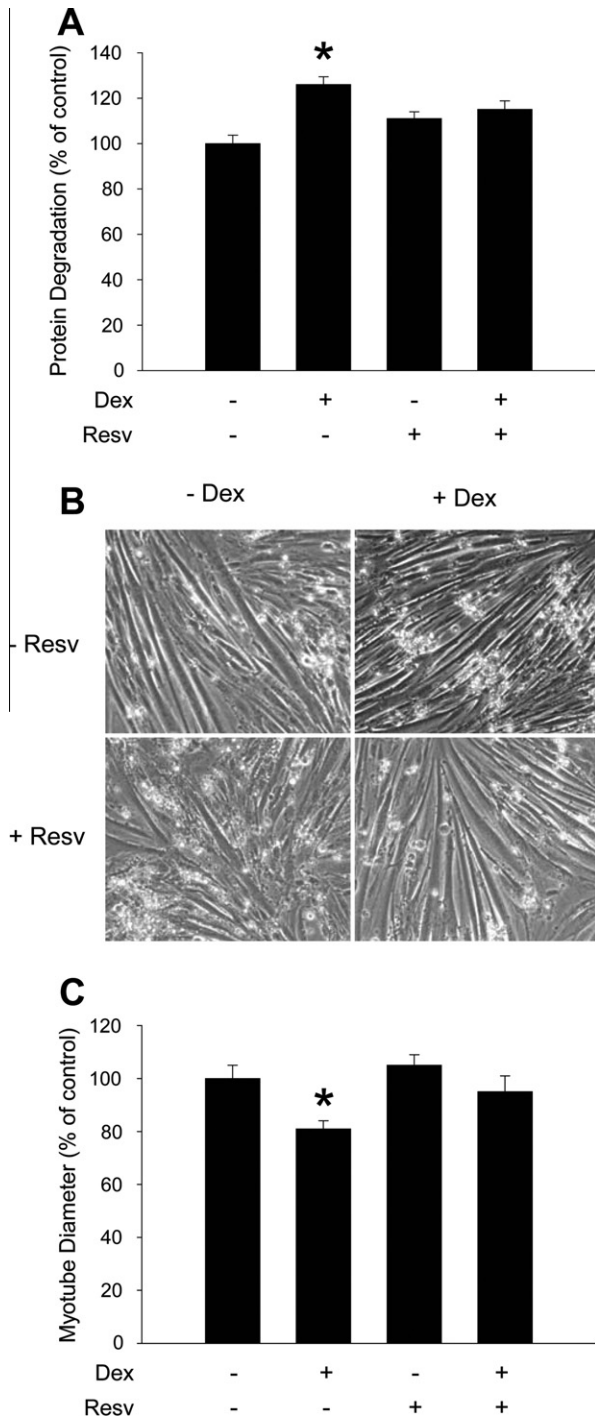


**Fig. 2.** Resveratrol blocks dexamethasone-induced acetylation of FOXO1 in cultured L6 myotubes. Myotubes were treated with dexamethasone and resveratrol as described in Fig. 1. Nuclear levels of acetylated FOXO1 were determined by co-immunoprecipitation as described in Section 2. Results are means  $\pm$  SEM with  $n = 6$  per group. \* $p < 0.05$  vs all other groups by ANOVA.

MuRF1 expression [23,24], we next examined whether resveratrol may prevent dexamethasone-induced increase in muscle protein degradation and myotube atrophy. Treatment of L6 myotubes with dexamethasone stimulated protein degradation by approximately 20% (Fig. 3A) and reduced myotube diameter by 20–30% (Fig. 3B and C), similar to previous reports [28,29]. Importantly, both the dexamethasone-induced increase in protein degradation and myotube atrophy were prevented by resveratrol.

Because studies suggest that activation of SIRT1 may be the most important mechanism of the metabolic effects of resveratrol [9–12], we next tested whether SIRT1 activation may be involved in the effects of resveratrol observed here. This was done by using two experimental approaches. First, we examined whether resveratrol activated SIRT1 in dexamethasone-treated myotubes by determining the acetylation status of the nuclear cofactor PGC-1 $\alpha$ , a well established marker of SIRT1 activity [8,32]. Treatment of the myotubes with resveratrol resulted in an approximately 50% decrease in acetylated PGC-1 $\alpha$  (Fig. 4A) suggesting that resveratrol activated SIRT1 under the present experimental conditions. Next, we transfected cultured myotubes with SIRT1 siRNA or non-targeting (scrambled) siRNA to test whether the effects of resveratrol on dexamethasone-induced atrogin-1 and MuRF1 expression would be reduced after knock-down of SIRT1 expression. Transfection of myotubes with SIRT1 siRNA reduced SIRT1 expression by approximately 40% (Fig. 4B). The protective effects of resveratrol on dexamethasone-induced atrogin-1 and MuRF1 expression were abolished in myotubes with downregulated expression of SIRT1 (Fig. 4C–F), suggesting that SIRT1 is involved in the effects of

Since glucocorticoid-induced loss of muscle mass is at least in part regulated by FOXO1-mediated upregulation of atrogin-1 and



**Fig. 3.** Resveratrol inhibits dexamethasone-induced protein degradation and atrophy in cultured L6 myotubes. Myotubes were treated with dexamethasone and resveratrol as described in Fig. 1. (A) Protein degradation, (B) myotube morphology, and (C) myotube diameter were determined as described in Section 2. Results in (A, B) are means  $\pm$  SEM with  $n = 8$  per group. \* $p < 0.05$  vs all other groups by ANOVA.

resveratrol in dexamethasone-treated myotubes. Of note, resveratrol alone did not influence atrogen-1 and MuRF1 mRNA levels in this experiment (see Fig. 4C–F) which differs from the results in resveratrol-treated, non-transfected myotubes (compare with Fig. 1A and B). Although we can not offer an explanation for these apparently contradictory results, taken together, the results in Figs. 1 and 4 support the concept that dexamethasone-induced

increase in atrogen-1 and MuRF1 expression is inhibited by resveratrol.

#### 4. Discussion

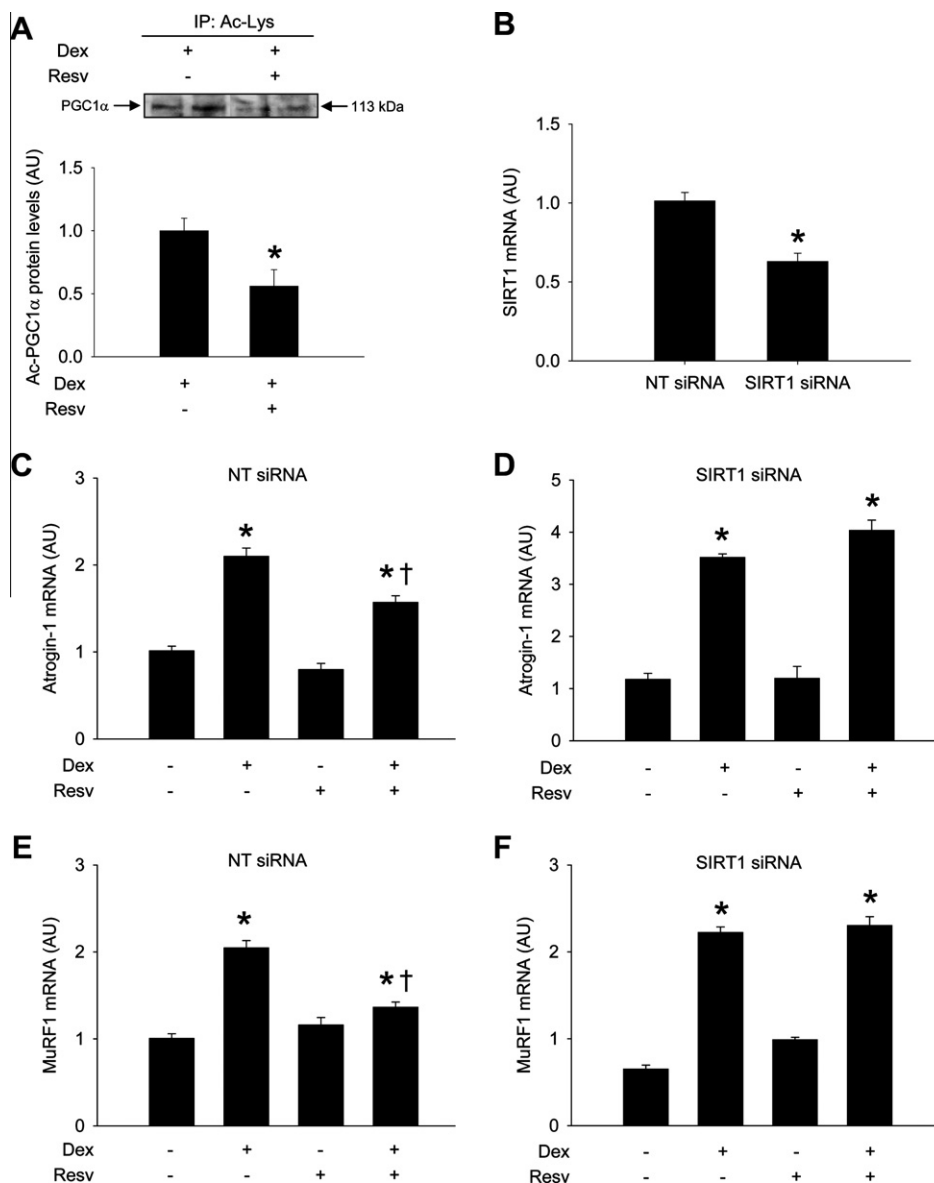
The present results suggest that resveratrol may inhibit glucocorticoid-induced muscle wasting and upregulation of atrogen-1 and MuRF1 and that these effects of resveratrol are at least in part regulated by SIRT1. The results have important clinical implications because muscle wasting is commonly seen as a significant side-effect in patients treated with corticosteroids [33,34] and in patients with Cushing's syndrome [35]. In addition, muscle wasting in several catabolic conditions, including sepsis and burn injury, is in part regulated by glucocorticoids [19–22]. Therefore, treatment that reduces glucocorticoid-mediated muscle wasting may benefit a large number of patients with different muscle wasting conditions. Other studies provided evidence of muscle sparing effects of resveratrol in additional conditions associated with muscle wasting, including diabetes [4], muscular dystrophy [14], muscle disuse [13], and cancer cachexia [15], further supporting the potential role of resveratrol in the prevention and treatment of muscle wasting. Of note, an apparently contradictory study in which resveratrol did not prevent muscle wasting in experimental cancer cachexia [16] may reflect insufficient doses of resveratrol used in that study (up to 25 mg/kg/day). Recent experiments suggest that a resveratrol dose of at least 200 mg/kg/day may be necessary to prevent cancer-induced cachexia [15].

Although the effects of resveratrol on glucocorticoid-induced muscle atrophy have not been reported previously, the influence of resveratrol on glucocorticoid-induced upregulation of uncoupling protein-3 (UCP3) in skeletal muscle was reported recently [30]. In that study, resveratrol prevented dexamethasone-induced increase in UCP3 expression in cultured muscle cells and evidence was found that this effect of resveratrol was SIRT1-dependent. Although the precise role of UCP3 in muscle wasting is not known at present, sepsis [36], endotoxemia [37], and cancer cachexia [38] are associated with increased UCP3 expression in skeletal muscle, suggesting that inhibited UCP3 expression may play a role in the muscle sparing effects of resveratrol.

The involvement of the histone deacetylase SIRT1 in the anti-catabolic effects of resveratrol is important because we and others found recently that hyperacetylation may be an important factor in muscle wasting [17,18,39–41]. Resveratrol-induced deacetylation of FOXO1, consistent with reduced FOXO1 activity [25,26], and deacetylation of PGC-1 $\alpha$ , consistent with increased PGC-1 $\alpha$  activity [8,32,42], as observed in the present study, are particularly important because atrogen-1 and MuRF1 expression is increased by FOXO1 activity [23,24] and reduced by PGC-1 $\alpha$  [43,44]. Thus, it is possible that the prevention by resveratrol of dexamethasone-induced atrogen-1 and MuRF1 expression observed here at least in part reflected reduced FOXO1 and increased PGC-1 $\alpha$  activities.

Although the present study and several previous reports suggest that activation of SIRT1 is involved in the muscle sparing effects of resveratrol [9–12], the results reported here do not rule out the possibility that some of the effects of resveratrol were SIRT1-independent. It should be noted that other potential mechanisms that may be involved in the anti-catabolic effects of resveratrol have been described. These mechanisms include antioxidant effects [5,6], inhibition of NF- $\kappa$ B [7] and iNOS activities [8], prevention of insulin resistance [4,45], and increased microvascular recruitment in skeletal muscle [46]. It is of course possible, and perhaps even likely, that several of these mechanisms are interconnected. For example, inhibition of NF- $\kappa$ B by resveratrol may reflect SIRT1-dependent deacetylation of NF- $\kappa$ B/p65 [47] and antioxidant effects may reflect improved PGC-1 $\alpha$ -regulated mitochondrial biogenesis [11,42].





**Fig. 4.** SIRT1 is involved in the protective effects of resveratrol in dexamethasone-treated L6 myotubes. (A) Nuclear levels of acetylated PGC-1 $\alpha$  were determined by co-immunoprecipitation in dexamethasone-treated myotubes in the absence or presence of resveratrol. (B) SIRT1 mRNA levels were determined in myotubes after transfection with non-targeting (NT) or SIRT1 siRNA. In other experiments, myotubes were treated with dexamethasone and resveratrol as described in Fig. 1 following transfection with non-targeting (NT) or SIRT1 siRNA and atrogin-1 (C, D) and MuRF1 (E and F) mRNA levels were determined by real-time PCR. Results are means  $\pm$  SEM with  $n = 6$  (A) or 8 per group. \* $p < 0.05$  by Student's  $t$ -test (A, B); \* $p < 0.05$  vs all other groups; † $p < 0.05$  vs Dex by ANOVA (C–F).

Interestingly, a recent study by Centeno-Baez et al. [8] suggests that mechanism(s) regulating the anti-catabolic effects of resveratrol may depend on the mediators involved in muscle wasting and that different effects of resveratrol may be regulated by different mechanisms. Thus, in that study [8], treatment of cultured L6 muscle cells with a mixture of TNF $\alpha$ , IFN $\gamma$ , and LPS resulted in increased iNOS expression and NO levels. These effects of the cytokine/LPS mixture were inhibited by resveratrol in a dose-dependent manner and because the effects of resveratrol were not blocked by different SIRT1 inhibitors, the authors concluded that the beneficial effects of resveratrol did not reflect SIRT1 activation. This conclusion was further supported by the inability of resveratrol to deacetylate PGC-1 $\alpha$  in untreated control cells (although the influence of resveratrol on the status of PGC-1 $\alpha$  acetylation in cytokine/LPS-treated muscle cells was not reported). In contrast, downregulation of AMPK with siRNA or treatment of the muscle cells with the AMPK

inhibitor Compound C blunted the ability of resveratrol to prevent the cytokine/LPS-induced iNOS expression and NO production and the authors concluded that resveratrol inhibition of iNOS in skeletal muscle involves AMPK but not SIRT1 [8]. Although these results suggest that resveratrol targets a different mechanism in muscle cells treated with cytokines and LPS [8] than in muscle cells treated with dexamethasone [present study and Ref. 30], the apparently contradictory results may also suggest that resveratrol inhibits the expression of atrogin-1, MuRF1, and UCP3 and the expression of iNOS through different mechanisms.

The present study is important because it provides novel information about the influence of resveratrol on glucocorticoid-induced muscle wasting. In addition, the results provide molecular evidence that the muscle-sparing effects of resveratrol are at least in part regulated by SIRT1 and reflect inhibited upregulation of the ubiquitin ligases atrogin-1 and MuRF1.

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