Dexamethasone downregulates caveolin-1 causing muscle atrophy via inhibited insulin signaling

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Abstract

Glucocorticoids play a major role in the development of muscle atrophy in various medical conditions, such as cancer, burn injury, and sepsis, by inhibiting insulin signaling. In this study, we report a new pathway in which glucocorticoids reduce the levels of upstream insulin signaling components by downregulating the transcription of the gene encoding caveolin-1 (CAV1), a scaffolding protein present in the caveolar membrane. Treatment with the glucocorticoid dexamethasone (DEX) decreased CAV1 protein and *Cav1* mRNA expression, with a concomitant reduction in insulin receptor alpha (IR α) and IR substrate 1 (IRS1) levels in C2C12 myotubes. On the basis of the results of promoter analysis using deletion mutants and site-directed mutagenesis a negative glucocorticoid-response element in the regulatory region of the *Cav1* gene was identified, confirming that *Cav1* is a glucocorticoid-target gene. *Cav1* knockdown using siRNA decreased the protein levels of IR α and IRS1, and overexpression of *Cav1* prevented the DEX-induced decrease in IR α and IRS1 proteins, demonstrating a causal role of *Cav1* in the inhibition of insulin signaling. Moreover, injection of adenovirus expressing *Cav1* into the gastrocnemius muscle of mice prevented DEX-induced atrophy. These results indicate that CAV1 is a critical regulator of muscle homeostasis, linking glucocorticoid signaling to the insulin signaling pathway, thereby providing a novel target for the prevention of glucocorticoid-induced muscle atrophy.

Key Words

- caveolin-1
- glucocorticoid
- insulin signaling
- muscle atrophy
- C2C12 myotube

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Introduction

Glucocorticoids have diverse and tissue-specific effects on cellular metabolism. During metabolic stress, such as starvation, glucocorticoids promote degradation and suppress synthesis of muscle proteins in the skeletal muscle, thereby providing amino acids for glucose production in the liver (Kuo *et al.* 2013). Many pathological conditions, such as cancer, severe injury, sepsis, and long-term steroid therapy, cause persistent elevation of glucocorticoid levels, resulting in the development of muscle atrophy,

in which a loss of muscle mass leads to muscle weakness. Thus, muscle atrophy is considered to be a prognostic factor for underlying diseases (Schakman *et al.* 2013).

Glucocorticoids cause muscle atrophy by upregulating the expression of two muscle-specific ubiquitin ligases, MuRF1 and MAFbx/Atrogin1 (Glass 2010). MuRF1 ubiquitinates troponin I, myosin heavy chain, and myosin binding protein C, and thus induces their proteasomal degradation (Clarke *et al.* 2007, Polge *et al.* 2011). In contrast,

MAFbx/Atrogin1 suppresses protein synthesis through the ubiquitination and degradation of eIF3f and MyoD, key factors regulating translation and transcription in muscle respectively (Attaix & Baracos 2010). Results described in recent reports have indicated that glucocorticoids regulate MuRF1 and MAFbx/Atrogin1 through *KLF15*, a transcription factor involved in muscle catabolism via cooperation with FoxO1 (Attaix & Baracos 2010).

Glucocorticoids also suppress muscle protein synthesis through inhibition of insulin/insulin-like growth factor 1 (IGF1) signaling. Glucocorticoid-induced REDD1 inhibits mTOR by stabilizing the TSC1–TSC2 complex, resulting in decreased phosphorylation of both eIF4E-binding protein 1 and ribosomal protein S6 kinase 1, two critical factors regulating translation in the ribosome (Attaix & Baracos 2010). In addition, glucocorticoids inhibit PI3K/AKT signaling through the reduction in the levels of insulin receptor substrate 1 (IRS1) protein, possibly by accelerating IRS1 degradation (Koh *et al.* 2013). Although the molecular mechanism regulating the glucocorticoid-mediated reduction in the insulin receptor alpha (IR α) and IRS1 levels is not fully understood, crosstalk between glucocorticoid and insulin/IGF1 signaling could play a crucial role in the maintenance of muscle mass.

Caveolin (CAV) is the major protein component of caveolae, which are invaginations of the plasma membrane found in most cell types. CAV functions as a scaffolding protein within the caveolae, where it regulates signaling pathways through its interaction with diverse membrane proteins, such as G-proteins or receptors (Shimizu *et al.* 2011). There are three CAV isoforms expressed in muscle (Kim *et al.* 2009). CAV3 is expressed predominantly in muscle, where it forms a homo-oligomer in the T tubule system and plays a role in muscle development and sarcolemma repair under mechanical stress. Mutations in the *CAV3* gene cause several degenerative muscle diseases in humans, including limb-girdle muscular dystrophy, rippling muscle disease, and distal myopathy (Koh *et al.* 2013). CAV1 is expressed in the muscle and lung, and forms a homo-oligomer or a hetero-oligomer with CAV2 (Hansen & Nichols 2010). *Cav1*-deficient mice have impaired nitric oxide and calcium signaling, resulting in dilated cardiomyopathy and thickening of the alveolar septa in the lung. In humans, *CAV1* mutations cause generalized lipodystrophy, insulin resistance, and hypertriglyceridemia (Gazzerro *et al.* 2010). Moreover, we have demonstrated that the level of CAV1 in skeletal muscle is related to insulin sensitivity *in vitro* and *in vivo* (Oh *et al.* 2008), indicating that CAV1 may regulate insulin signaling. Notably, *Cav1*-deficient mice have a lean phenotype

(Razani *et al.* 2002). Furthermore, CAV1 expression is dependent on androgens and exercise (Baehr *et al.* 2011, Oh *et al.* 2011). These data from *Cav*-deficient mice indicate that *Cav1* may play a key role in glucocorticoid regulation of the insulin pathway, thereby contributing to muscle atrophy.

In this study, we evaluated the role of CAV isoforms in a glucocorticoid-induced muscle atrophy model, and found that dexamethasone (DEX) treatment decreased *Cav1*, but not *Cav2* and *Cav3*, expression. Downregulation of *Cav1* suppressed expression of IR α and IRS1, thereby increasing MAFbx/Atrogin1 and MuRF1 expression. Conversely, overexpression of *Cav1* prevented DEX-induced muscle atrophy *in vitro* and *in vivo*. Our results indicate that *Cav1* mediates glucocorticoid-induced suppression of insulin signaling.

Methods and materials

Cell culture

C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. To induce myoblast fusion and differentiation, cells were grown to about 80% confluency, and the medium was replaced with DMEM containing 2% FBS. The medium was changed every 2 days. After 4 days, the C2C12 myotubes were treated with various doses of DEX (100 nM, 1 μ M, 10 μ M, and 100 μ M) for the indicated times to induce myotube atrophy. To confirm the nuclear translocation of p-GR after DEX treatment, cells were incubated with anti-pGR antibody (for phosphorylation at S²¹¹) and FITC-conjugated secondary antibody. The fluorescently stained cells were photographed using a LSM501META confocal microscope (Zeiss, Oberkochen, Germany). The cells were used at low passage (under ten passages) for all experiments to maintain their differentiation potential (Menconi *et al.* 2008).

Measurement of myotube diameter

After differentiation, C2C12 myotubes were stained using a modified PAS staining kit (Merck). C2C12 myotubes were treated for 5 min with periodic acid, followed by washing with PBS for 3 min. After washing with PBS, Schiff's reagent was added to the cells for 15 min at room temperature. After another wash with PBS, nuclei were stained for 2 min with Gill III solution. C2C12 myotubes were mounted in Neo-Mount solution and photographed under a phase-contrast microscope at 100 $\times$ magnification.

The diameters of 50 myotubes were measured in at least ten random fields using ImageJ Software (National Institutes of Health, Frederick, MD, USA). The results are expressed as percentages of the diameter of the control myotubes.

Promoter analysis

The *Cav1* promoter region (GenBank accession number NC_000072.6) spanning positions –2000 to +300 was amplified from genomic DNA using PCR (Roche Molecular Biochemicals). Four deletion mutants of the *Cav1* promoter, spanning from positions –2000, –1037, –500, and –100 to +300, were generated using PCR and cloned into pGL2-Basic (Promega) for the luciferase assays. Site-directed mutagenesis was performed following the manufacturer's instructions (Stratagene, La Jolla, CA, USA). All constructs were verified by DNA sequencing (Applied Biosystems 3730xl DNA Analyzer, Life Technologies). To define the transcriptional upstream region of the *Cav1* gene, we used FASTA data from the NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NC_000072.5). Putative glucocorticoid response element (GRE) sequences were identified in this region (positions –2000 to +300) of the *Cav1* gene using <http://www.cbil.upenn.edu/cgibin>, http://alggen.lsi.upc.es/cgibin/promo_v3, and <http://www.gene-regulation.com/cgi-bin>.

Transfection and luciferase assays

C2C12 cells (2×10^4) were seeded into a 12-well plate and cultured for 24 h. The cells were then transfected with reporter constructs using Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer's instructions. After reaching confluence, the cells were cultured in differentiation medium for 4 days and then treated with 1 μ M DEX for 24 h. After preparation of the myotube lysates, luciferase activity was assayed using a dual-luciferase reporter assay system (Promega) and normalized to *Renilla* luciferase activity. The results are expressed as the mean $\pm$ S.D. of at least five independent experiments.

Western blot analysis

We prepared lysates with RIPA buffer and performed SDS-PAGE as described previously (Menconi *et al.* 2008). An equal amount of protein was transferred to nitrocellulose membrane and probed with the following antibodies: MAFbx/Atrogin1 (ECM Bioscience, Versailles, KY, USA), MuRF1 (GeneTax, Irvine, CA, USA), CAV1, CAV2, CAV3

(BD, Franklin Lakes, NJ, USA), glucocorticoid receptor (GR), lamin, MyoD (Santa Cruz Biotechnology), COX1, EIF3F (Abcam, Cambridge, MA, USA), p-GR (phosphorylated at S²¹¹) (Cell Signaling Technology, Danvers, MA, USA), α -tubulin (Sigma–Aldrich), IR α (Santa Cruz Biotechnology), and IRS1 (Cell Signaling Technology). Secondary antibodies were added for 1 h at room temperature. The antibody–antigen complexes were detected using an ECL Kit (Pierce, Rockford, IL, USA). The signal intensity was determined using an LAS-3000 image reader (Fujifilm, Tokyo, Japan). To inhibit proteolysis, C2C12 myotubes were pretreated with MG132 (20 μ M) for 1 h before DEX treatment.

Real-time quantitative RT-PCR

Total RNA was isolated from C2C12 myotubes using the TRI reagent (Invitrogen; Menconi *et al.* 2008), and the expression levels of *MAFbx/Atrogin1*, *MuRF1*, *Cav1*, and *Gapdh* mRNA were assessed by real-time RT-PCR using an iCycler RT-PCR instrument (Bio-Rad). For each sample, 25 ng total RNA were subjected to RT-PCR (in duplicate) according to the protocol provided by the manufacturer. The following specific primers were used: *MAFbx/Atrogin1*, 5'-CTCTGTACCATGCC-GTTCCT-3', (forward) and 5'-GGCTGCTGAACAGATTCT-CC-3' (reverse); *MuRF1*, 5'-TGTCTGGAGGTCGTTCCG-3' (forward) and 5'-TGCCGGTCCATGATCACTT-3' (reverse); *Cav1*, 5'-ACG ATGTCTGGGGGCAAATAC-3' (forward) and 5'-TCATATCTCTTTCTGCGTGC-3' (reverse); and *Gapdh*, 5'-CCACCCATGGCAAATTCATGGCA-3' (forward) and 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (reverse).

siRNA knockdown of *Cav1*

siRNA specific for *Cav1* was purchased from Santa Cruz Biotechnology. siRNA (10 nM) was transfected into C2C12 myotubes for 10 min using the G-fectin transfection protocol (Genolution Pharmaceuticals, Seoul, Korea).

Animals

Male C57BL/6 mice (8 months old) were purchased from the Osong Aging Animal Laboratory (Cheongju, Chungcheong bukdo, Korea). Mice were given *ad libitum* access to food and water and were maintained on a 12 h light:12 h darkness cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Seoul National University College of Medicine. The animals were randomly assigned to four groups ($n=5$ for each group). The control group received saline, the DEX

group received 5 mg/kg of DEX every other day by i.p. injection for 12 days. The DEX-AdGFP and DEX-AdCav1 groups received adenovirus expressing GFP or CAV1 (3×10^{11} p.f.u.), respectively, in 10 μ l of saline by daily injection into the gastrocnemius muscle at eight sites for 3 days. The animals were anesthetized using isoflurane before adenovirus administration. After the animals were killed by cervical dislocation, the gastrocnemius muscle was obtained for western blot analysis and morphological evaluation.

Preparation of adenovirus

The adenoviral vectors and infective recombinant adenovirus expressing *Cav1* used in this study have been described previously (Oh *et al.* 2008). In brief, infective recombinant adenovirus was made using the AdEasy system. *Cav1* cDNA was inserted into the pAdTRACK-CMV plasmid (Invitrogen) and transfected into *Escherichia coli* BJ5183 with the adenovirus containing the pAdEasy-1 plasmid by electroporation. Recombinant viruses were selected with kanamycin. The virus stocks were amplified in HEK293 cells on 15 cm plates and purified using BD Adeno-X Purification Kits (Clontech).

Measurement of muscle fiber cross-sectional area

Muscle sections (10 μ m) were obtained and mounted on glass slides. The sections were fixed for 2 min with acetone and stained with hematoxylin and eosin (H&E). Images of muscle fiber cross-sectional area (CSA) were visualized at 200 $\times$ magnification using a light microscope and captured with a microscope camera (Zeiss). At least 200 fibers per muscle were randomly chosen and analyzed using ImageJ.

Statistical analyses

All data are presented as mean $\pm$ s.d. Differences between two variables were assessed using the unpaired Student's *t*-test or two-way ANOVA. A difference was considered significant if the *P* value was <0.05 . All statistical calculations were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

DEX negatively regulates *Cav1* expression

To examine the role of caveolins in glucocorticoid-induced muscle atrophy, we used DEX-treated C2C12

myotubes as an *in vitro* model (Menconi *et al.* 2007, Schakman *et al.* 2008). C2C12 myotubes were treated with DEX at several concentrations (100 nM, 1 μ M, 10 μ M, and 100 μ M) for various periods. Results of western blot analyses indicated that DEX treatment induced maximal GR expression at a concentration of 100 nM (Fig. 1A), and promoted the translocation of the GR into the nucleus (Fig. 1B). Protein levels of MAFbx/Atrogin1 and MuRF1, two markers of muscle atrophy, were increased in a dose- and time-dependent manner, and peaked following treatment with DEX for 24 h at 1 μ M (1.5- and 5.2-fold respectively; Fig. 1A). DEX-induced changes in protein abundance were confirmed by real-time PCR. Transcript levels of *MAFbx/Atrogin1* (2.6-fold, $P < 0.05$) and *MuRF1* (6.8-fold, $P < 0.001$) were increased by exposure to DEX (Fig. 1C). In contrast, protein levels of the muscle differentiation markers MyoD and EIF3F, as well as insulin signaling molecules IR α and IRS1, were decreased in a dose- and time-dependent manner by DEX treatment (Fig. 1A). Furthermore, DEX treatment (1 μ M, 24 h) reduced the mean diameter of myotubes by 20% in comparison with the control cells (Fig. 1D).

Under the same experimental conditions, we found that the level of CAV1 protein in C2C12 myotubes was decreased in a dose-dependent manner by DEX (0.55-fold, 1 μ M for 24 h), whereas the levels of CAV2 and CAV3 were not altered (Fig. 1A and Supplementary Fig. 1, see section on supplementary data given at the end of this article). DEX regulates the transcription of many genes in a positive or negative way. To test whether DEX regulates *Cav1* expression at the transcriptional level, we performed real-time PCR and found that *Cav1* mRNA abundance was significantly decreased in myotubes treated with DEX (Fig. 1C). Moreover, MG132 had no effect on CAV1 protein abundance after DEX treatment (results not shown), indicating that CAV1 was downregulated by DEX through transcriptional suppression, and not through Atrogin1- or MuRF1-mediated proteolytic degradation.

To further confirm the transcriptional regulation of *Cav1* by DEX, we generated promoter deletion constructs based on a sequence analysis that revealed approximately 30 putative GREs within a 2-kbp region upstream of the transcriptional start site of *Cav1*. The deletion constructs were fused with a luciferase reporter and transfected into the C2C12 myotubes. The reporter assay revealed that DEX caused a 50% reduction in luciferase activity in comparison with the control cells, but DEX failed to exhibit a suppressive effect after deletion of a region spanning from positions -500 to -100 of the *Cav1* gene (Fig. 2A). To determine the specific GRE responsible for the

Figure 1

Dexamethasone suppresses caveolin-1 expression at the transcriptional level in C2C12 myotubes. (A) Effect of DEX treatment on the expression of caveolins. C2C12 myotubes were treated with DEX (100 nM, 1 μ M, 10 μ M, and 100 μ M) at various time points. Cell lysates were analyzed by western blotting using antibodies against glucocorticoid receptor (GR), glucocorticoid receptor phosphorylated at S²¹¹ (p-GR), MAFbx/Atrogin1, MyoD, MuRF1, EIF3F, insulin receptor alpha (IR α), IRS1, caveolin-1 (CAV1), caveolin-2 (CAV2), and caveolin-3 (CAV3). (B) Nuclear translocation of p-GR following DEX treatment (1 μ M, 24 h) was monitored by confocal microscopy and western blot analysis. Cox1 and lamin were used as markers

for the cytosolic (Cyto) and nuclear (Nucl) fractions respectively. (C) Total RNA from C2C12 myotubes treated with 1 μ M of DEX for 24 h was analyzed by quantitative RT-PCR for *MAFbx/Atrogin1*, *MuRF1*, and *Cav1* ($n=3$). The data are given as mean $\pm$ s.d. ** $P<0.01$. (D) Myotubes treated with DEX (1 μ M, 24 h) were stained with PAS and photographed. The diameter of the myotubes from randomly selected fields was measured using ImageJ. The diameters are expressed as percentages of the diameter of the control myotubes ($n=50$). Results are expressed as mean $\pm$ s.d. Scale bars: 100 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-14-0490>.

negative regulation of *Cav1* gene, we generated six GRE mutants located in this region, and found that the site-specific mutation (CAGAG to CACAG) of a GRE located at position -387 produced mutants that failed to respond to DEX (Fig. 2B and Supplementary Fig. 2, see section on supplementary data given at the end of this article). These results indicate that DEX suppresses *Cav1* expression in C2C12 myotubes through a negative regulatory GRE.

Cav1 mediates DEX-induced suppression of insulin signaling

CAV1 is a scaffolding protein that recruits components of various signaling pathways to the caveolar membrane, thereby activating or repressing signaling (Boscher & Nabi 2012). To test whether CAV1 is involved in the regulation of signaling pathways required for muscle growth or protein degradation, we first examined the effect of *Cav1* knockdown on insulin signaling. C2C12 myotubes were treated with *Cav1*-specific siRNA for 24 h, and the protein levels of IR α and IRS1 were evaluated. *Cav1* siRNA

treatment resulted in a 40% reduction in CAV1 protein expression in comparison with control-siRNA-treated myotubes. In cells treated with *Cav1*-specific siRNA, protein levels of IR α and IRS1 were significantly decreased by up to 90 and 80%, respectively, in comparison with the control siRNA-treated myotubes (Fig. 3A and B).

Reduced insulin signaling decreases AKT activity, and thereby leads to a marked increase in nuclear FoxO. As increased nuclear FoxO promotes E3 ligase expression (Zhao *et al.* 2009), we next examined E3 ligase protein levels in *Cav1* siRNA-treated myotubes. Results of western blot analyses indicated that protein levels of MAFbx/Atrogin1 and MuRF1 were significantly increased by three- and tenfold respectively (Fig. 3A and B). Moreover, *Cav1*-knockdown caused a 20% reduction in the diameter of myotubes in comparison with those treated with the control siRNA (Fig. 3C and D). These results indicate that CAV1 mediates DEX-induced suppression of insulin signaling, thereby increasing E3 ligase expression.

To confirm the causal relationship between CAV1 expression and insulin signaling, we tested whether CAV1

Figure 2

Identification of a negative glucocorticoid-responsive element in the mouse caveolin-1 promoter. (A) Luciferase reporter activity of deletion constructs of the mouse *Cav1* promoter. C2C12 myotubes transfected with deletion constructs were treated with 1 μ M of DEX for 24 h. Cell lysates were assayed for luciferase activity. (B) The effect of site-specific mutagenesis of GREs (CAGAG to CACAG) on reporter activity. Luciferase activity is expressed relative to that for vehicle-treated myotubes ($n=5$). Data are expressed as mean $\pm$ s.d.

overexpression could suppress E3 ligase expression through upregulation of insulin signaling. To this end, adenoviruses expressing *Cav1* (*AdCav1*) and *Gfp* (*AdGfp*) were generated and infected into the cultured C2C12 myotubes. Western blot analysis showed that *AdCav1* increased the protein level of CAV1 1.7-fold in comparison with *AdGFP*-infected myotubes. Under the same experimental conditions, protein levels of IR α and IRS1 were elevated three- and twofold respectively. Concomitantly, protein levels of MAFbx/Atrogin1 and MuRF1 were decreased by 30 and 70% respectively (Fig. 4A and B). Moreover, when these cells were treated with DEX, *Cav1*-overexpression prevented more than 80% of the DEX-induced reduction in IR α and IRS1 expression, as well as 25–60% of the DEX-induced increase in MAFbx/Atrogin1 and MuRF1 expression (Fig. 4A and B). In addition, when the diameters of the myotubes were compared, *Cav1* overexpression prevented up to 20% of the DEX-induced reduction in mean diameter (Fig. 4C and D). These results obtained from the siRNA and adenoviral overexpression systems indicate that CAV1 is a key regulator of DEX-induced insulin signaling.

***Cav1* overexpression restores muscle loss in DEX-treated mice**

We next sought to extend these results from a cellular model to an *in vivo* model of DEX-induced muscle atrophy by assessing whether *Cav1* overexpression could restore muscle loss induced by DEX treatment. C57BL/6 mice received intraperitoneal injections of DEX (5 mg/kg body weight) every other day for 12 days, and were then treated with a daily injection of *AdCav1* or *AdGFP* virus to the gastrocnemius muscle in eight places for 3 days.

DEX treatment and adenovirus injection did not significantly affect the body weight of the mice during the treatment period (Fig. 5A). *AdCav1* injection restored the weight of the gastrocnemius muscle and the ratio of gastrocnemius to body weight after DEX treatment, but these results were not statistically significant (Fig. 5B). Thus, to support these findings at the tissue level, we compared the size of myofibers in the gastrocnemius muscle by measuring the CSA after H&E staining (Fig. 5C). In the gastrocnemius muscle, DEX treatment decreased the number of myofibers with a larger CSA in comparison with that of saline-treated mice. In contrast, *AdCav1* injection into the gastrocnemius muscle of DEX-treated mice increased the number of myofibers with a larger CSA in comparison with that of mice that received injections of virus encoding *AdGFP* (Fig. 5D), indicating that *Cav1* overexpression reversed the DEX-induced myofiber size reduction *in vivo*.

To confirm the role of CAV1 in DEX-induced muscle atrophy at the molecular level, we evaluated the levels of various proteins involved in insulin signaling in the gastrocnemius muscle. Results of western blot analysis indicated that DEX treatment decreased IR α and IRS1 protein levels, resulting in increased expression of E3 ubiquitin ligases (MAFbx/Atrogin1 and MuRF1) and decreased expression of MyoD and EIF3F in the gastrocnemius muscle. *Cav1* overexpression induced by *AdCav1* injection reversed the effects of DEX on the abundance of these insulin-related proteins. It is of note that *Cav1* overexpression had no effect on expression of *Cav2* or *Cav3* in the gastrocnemius muscle (Fig. 5E and F). Collectively, these results confirmed that DEX induced muscle atrophy through downregulation of CAV1.

Discussion

Skeletal muscle mass is maintained through a balance of protein synthesis and degradation regulated by a number of signaling pathways, including the insulin/IGF, myostatin, and NF- κ B pathways (Bonaldo & Sandri 2013). The insulin/IGF-PI3K-AKT pathway is a muscle regulation

Figure 3

Caveolin-1 knockdown decreases C2C12 myotube diameter through downregulation of IR α and IRS1. (A) Western blot analyses of C2C12 myotubes treated with Cav1 siRNA or scramble (SC) siRNA using antibodies against IR α , IRS1, MAFbx/Atrogin1, and MuRF1. (B) Relative band intensities from three independent experiments were estimated by densitometric quantification. Data are presented as mean $\pm$ s.d. ** P < 0.01.

(C) Representative photographs of C2C12 myotubes treated with Cav1 siRNA or scramble siRNA. Scale bars: 100 μ m. (D) Cells were stained with PAS and photographed. The diameter of myotubes from randomly selected fields was measured using Image J. Data are expressed as percentages of the diameter of the control myotubes (n = 50). Results are presented as mean $\pm$ s.d. * P < 0.05; SC, scramble siRNA.

pathways that promotes protein synthesis and suppresses protein degradation, resulting in an increase in the size of myofibrils (Glass 2010). The results presented here indicated that DEX treatment suppresses Cav1 expression in muscle cells, causing a decrease in IR α and IRS1 and an increase in MAFbx/Atrogin1 and MuRF1 protein expression. Conversely, overexpression of Cav1 in mouse muscle prevented DEX-induced muscle loss. These results indicate that glucocorticoids effectively inhibit the anabolic effects of insulin through negative regulation of upstream components of the signaling pathway. Moreover, the results from the Cav1 siRNA and overexpression assays indicate that regulation of insulin signaling by glucocorticoids is a crucial point of control in the maintenance of muscle homeostasis, especially under conditions of catabolic stress. Thus, downregulation of Cav1 gene expression is a new mechanism by which glucocorticoids inhibit the insulin signaling pathway.

Cav1 is abundantly expressed in adipocytes, endothelial cells, type 1 pneumocytes, and smooth muscle cells, and it is the main structural component of caveolae, which are plasma membrane invaginations that function as vesicular transporters for macromolecules and a variety of pathogens by transcytosis or endocytosis (Cohen *et al.* 2004). In addition, CAV1 functions as a scaffolding protein that recruits numerous signaling molecules to caveolae, thereby activating or inhibiting signaling pathways, depending on the cell-type and signaling molecules (Hansen & Nichols 2010). Thus, CAV1 is responsible for the pathogenesis of various diseases, including lipodystrophy, atherosclerosis, diabetes, pulmonary fibrosis, and cancer. However, the role of CAV1 in muscle is relatively unstudied. Although the mechanisms of signal-dependent regulation in the caveolae remain unclear, our results indicate that IR α and IRS1 are positively regulated by CAV1 in muscle cells. Consistent with these results,

Figure 4

Caveolin-1 overexpression prevents the dexamethasone-induced decrease in myotube diameter. (A) Western blot analysis of C2C12 myotubes infected with adenovirus expressing *Cav1* (*AdCav1*, 3×10^{11} plaque-forming units) or GFP (*AdGFP*, 3×10^{11} plaque-forming units) was performed using the indicated antibodies. Myotubes were cultured for 24 h in the presence or absence of DEX (1 μ M). (B) Relative band intensities of the western blots from three independent experiments were estimated by densitometric

quantification. Data are presented as mean $\pm$ s.d. ** $P < 0.01$ and * $P < 0.05$. (C) Representative photographs of C2C12 myotubes infected with *AdCav1* or *AdGFP*. Scale bars: 100 μ m. (D) Myotubes were stained with PAS and photographed. The diameter of the myotubes from randomly selected fields was measured using ImageJ. Data are expressed as percentages of the diameter of the control cells ($n = 50$). Results are expressed as mean $\pm$ s.d. * $P < 0.05$.

an increase in *Cav1* expression in skeletal muscle improves insulin sensitivity (Cohen *et al.* 2004, Oh *et al.* 2008). Conversely, *Cav1*-null mice displayed skeletal muscle abnormalities due to tubular aggregate formation (Schubert *et al.* 2007) and exercise intolerance (Razani *et al.* 2001), demonstrating that insulin/IGF signaling depends on the level of *Cav1* expression in muscle.

Cav1-null mice were viable and fertile, but displayed various phenotypes, including abnormal regulation of lipid metabolism and impaired nitric oxide and calcium signaling, which led to aberrant regulation of myogenic tone in the cardiovascular system (Drab *et al.* 2001) and impaired liver regeneration after injury (Fernandez *et al.* 2006). On the basis of these observations, homozygotic or heterozygotic mutations in the *CAV1* gene have been identified in patients with congenital generalized lipodystrophy type 3 (CGL3) (Kim *et al.* 2008), partial lipodystrophy, congenital cataracts, neurodegeneration syndrome (LCCNS) (Cao *et al.* 2008), and primary pulmonary hypertension 3 (PPH3) (Austin *et al.* 2012). Comparing the clinical features of these syndromes with those of patients who received long-term glucocorticoid

therapy indicates that, in addition to muscle wasting and insulin resistance, clinical manifestations such as cataracts, progressive loss of adipose tissue, and hypertension might be caused by glucocorticoid-induced downregulation of *Cav1* expression. Moreover, as a nongenomic effect of glucocorticoid, DEX treatment induced rapid phosphorylation of CAV1, which is required for activation of the PI3K–AKT pathway in the glucocorticoid-mediated growth arrest of A549 cells (Matthews *et al.* 2008). Therefore, these observations clearly support our findings that CAV1 is a key downstream effector of glucocorticoids.

CAV3, a muscle-specific isoform, plays a role in muscle development and physiology by forming a complex with dystrophin and glycoprotein, thereby stabilizing the sarcolemma. Thus, CAV3 deficiency due to mutations in the gene causes the degeneration of myofibrils, leading to skeletal muscle diseases (Gazzerro *et al.* 2010). Results of functional analyses of CAV3-null mice indicated that CAV3 is an enhancer of insulin signaling (Oshikawa *et al.* 2004). In addition, CAV3 inhibits myostatin signaling through its interaction with ALK5, resulting in increased myofiber size (Ohsawa *et al.* 2004). These findings indicate

Figure 5

Caveolin-1 overexpression restores dexamethasone-induced muscle atrophy in mice. C57BL/6 mice were treated every other day with injections of saline (CON) or dexamethasone (DEX; 5 mg/kg) for 12 days. Subsequently, received daily injections of *AdCav1* (DEX-*AdCav1*) or *AdGFP* virus (DEX-*AdGFP*) into the gastrocnemius muscle at eight sites for 3 days. Mice were killed on the 16th day. (A) Total body weight. (B) Gastrocnemius mass and ratio of weight of gastrocnemius (Ga) to total body weight, $n=5$ for each group. (C) Representative photographs of *AdCav1* or *AdGFP*-injected

gastrocnemius muscles from DEX-treated mice. The sections were stained with hematoxylin and eosin (H&E). Scale bars: 200 μ m. (D) The distribution of muscle fibers with different cross-sectional area (CSA). Two hundred fibers per gastrocnemius muscle were randomly chosen and the CSA was measured using a microscope. (E) Western blot analysis of gastrocnemius muscle using the indicated antibodies. (F) Relative band intensities from five mice were estimated by densitometric quantification. Data are presented as mean $\pm$ s.d. * $P<0.01$.

that CAV3 could be a plausible target for glucocorticoids. However, contrary to expectations, our results indicated that CAV3 protein in mouse skeletal muscle was increased, although the increase was not statistically significant rather than decreased in response to DEX treatment. Interestingly, overexpression of *Cav3* also induces muscle fiber degeneration by downregulating dystrophin (Galbiati *et al.* 1999). Taken together, these results indicate that both increased *Cav3* expression and decreased *Cav1* expression are likely to contribute to DEX-mediated muscle atrophy.

The transcriptional regulation of specific genes by glucocorticoids is dependent on specific GREs. It is well established that GREs have both positive and negative regulatory functions (Dostert & Heinzel 2004). Positive GREs activate transcription of related genes, while negative GREs (nGREs) inhibit transcription. Results described in reports indicated that DEX induced *Cav1* expression at both the mRNA and protein levels in endothelial cells from thoracic aorta and lung artery (Igarashi *et al.* 2013). Although CAV1 induction by DEX was glucocorticoid-receptor-dependent, specific GREs responsible for CAV1 induction in the endothelial cells were not identified. In contrast, our results indicated that DEX suppresses *Cav1* expression, and that a nGRE in the *Cav1* gene located 387 bp upstream of the promoter region is responsible for DEX-induced suppression of *Cav1* gene expression by site-directed mutagenesis. Results of gene expression analyses using microarrays indicated that a number of genes are downregulated by glucocorticoids (van Batenburg *et al.* 2010). However, nGREs have been identified only in the promoter region of a limited number of genes, because nGREs do not have a highly conserved sequence. Moreover, nGREs exhibit an inhibitory effect on the transcription of target genes in a cell-type-specific manner (Turney & Kovacs 2001), indicating that unidentified transcription factors such as coactivators or corepressors may be involved in the regulation of DEX–glucocorticoid receptor–GRE interaction. This idea is supported by the fact that DEX and corticosterone induced different muscle wasting responses in cultured myotubes. Moreover, the effects of RU38486, an antagonist of glucocorticoid receptor, depended on the muscle cell line used (Menconi *et al.* 2008). Thus, further studies are required to elucidate the physiological and molecular mechanisms of tissue-specific suppression by nGREs.

In summary, our results indicated that *Cav1* is negatively regulated by DEX through GR targeting of nGREs in the promoter region of the gene, thereby mediating DEX-induced muscle atrophy through the inhibition of insulin

signaling. Thus, these results define a new pathway linking glucocorticoid signaling and the insulin pathway in the induction of muscle atrophy, and provide a useful target that could be exploited to prevent muscle atrophy.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0490>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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