PORTLAND PRESS

Research Article

Excessive glucocorticoid-induced muscle MuRF1 overexpression is independent of Akt/FoXO1 pathway

Xiao Juan Wang, Jing Jing Xiao, Lei Liu, Hong Chao Jiao and Hai Lin

Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, Department of Animal Science, Shandong Agricultural University, 61 Daizong Street, Taian City, Shandong Province 271018, China

Correspondence: Hai Lin (hailin@sdau.edu.cn)

OPEN ACCESS

The ubiquitin-proteasome system (UPS)-dependent proteolysis plays a major role in the muscle catabolic action of glucocorticoids (GCs). Atrogin-1 and muscle-specific RING finger protein 1 (MuRF1), two E3 ubiquitin ligases, are uniquely expressed in muscle. It has been previously demonstrated that GC treatment induced MuRF1 and atrogin-1 overexpression. However, it is yet unclear whether the higher pharmacological dose of GCs induced muscle protein catabolism through MuRF1 and atrogin-1. In the present study, the role of atrogin-1 and MuRF1 in C2C12 cells protein metabolism during excessive dexamethasone (DEX) was studied. The involvement of Akt/forkhead box O1 (FoXO1) signaling pathway and the cross-talk between anabolic regulator mammalian target of rapamycin (mTOR) and catabolic regulator FoXO1 were investigated. High concentration of DEX increased MuRF1 protein level in a time-dependent fashion ($P<0.05$), while had no detectable effect on atrogin-1 protein ($P>0.05$). FoXO1/3a (Thr24/32) phosphorylation was enhanced ($P<0.05$), mTOR phosphorylation was suppressed ($P<0.05$), while Akt protein expression was not affected ($P>0.05$) by DEX. RU486 treatment inhibited the DEX-induced increase of FoXO1/3a phosphorylation ($P<0.05$) and MuRF1 protein; LY294002 (LY) did not restore the stimulative effect of DEX on the FoXO1/3a phosphorylation ($P>0.05$), but inhibited the activation of MuRF1 protein induced by DEX ($P<0.05$); rapamycin (RAPA) inhibited the stimulative effect of DEX on the FoXO1/3a phosphorylation and MuRF1 protein ($P<0.05$).

Introduction

Skeletal muscle is an essential component of the body. The maintenance of skeletal muscle mass is of paramount importance for systemic energy homeostasis [1]. The muscle mass usually results from a dynamic balance between protein synthesis and catabolism [2]. When the body is suffering from severe stress, excessive glucocorticoids (GCs) in circulation cause muscular loss by impeding protein synthesis and enhancing protein breakdown [3-5]. GCs, as the final effectors of the hypothalamic–pituitary–adrenal axis, participate in the maintenance of whole-body homeostasis. Many pathological conditions, including muscle atrophy, result from an increase in circulating GC levels [6].

The increased muscular loss during stress is mainly caused by activation of proteolytic systems [7]. Amongst these proteolytic systems, ubiquitin-proteasome system (UPS) is considered to play a major role in the catabolic action of GCs [8]. The stimulation of UPS is mediated through the increased expression of several atrogenes, such as atrogin-1 and muscle-specific RING finger protein 1 (MuRF1), which are two E3 ubiquitin ligases involved in the targetting of protein to be degraded by the proteasome machinery [8]. MuRF1 and atrogin-1 are uniquely expressed in muscle [9,10]. Muscle wasting is associated with an increase in circulating GC levels [11]. Dexamethasone (DEX), a synthetic GC that is specific for the GC

1

receptor (GR) and delayed plasma clearance [12], is widely used in studies of muscle atrophy in C2C12 cells. In the previous study, it has been demonstrated that DEX treatment (0.05–10 μmol/l) induced MuRF1 and atrogin-1 overexpression [13-15]. However, it is yet unclear whether the higher pharmacological dose of DEX induced catabolic effect on skeletal muscle through MuRF1 and atrogin-1.

The forkhead transcriptional factor subfamily forkhead box O1 (FoxO1) plays essential roles in the transcriptional cascades that are responsible for metabolism in the muscle, liver, brain, pancreas, and adipose tissues [16-19]. Both the translocation and activity of FoXO1 are required for up-regulation of atrogin-1 and MuRF1. FoxO1 is an important downstream target of the PI3K/Akt pathways [20]. Activated Akt phosphorylates FoXO1, leading to its nuclear exclusion and proteasomal degradation [21,22]. We thus hypothesize that Akt/FoXO1 signaling pathway is required in the ubiquitin ligase expression and in the dysregulation of muscular protein metabolism induced by excessive GCs.

In this study, we investigated whether excessive GCs enhanced proteolysis through the MuRF1 and atrogin-1, and the involvement of Akt/FoXO1 signaling pathway was also evaluated. According to previous report [23] and our recent studies [24,25], a hyperglucocorticoid milieu (100 μmol/l, DEX) in C2C12 cells suppressed protein synthesis and enhanced proteolysis. In the present study, 100 μmol/l DEX was employed to induce a hyperglucocorticoid milieu.

Materials and methods
Cell culture and *in vitro* treatments
Murine C2C12 cells (CCTCC, Wuhan, China) were cultured in DMEM (HyClone, Logan, UT, U.S.A.) supplemented with 10% FBS (HyClone, Logan, UT, U.S.A.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Solarbio, Shanghai, China) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. When cells were 70% confluent, the proliferation medium was replaced with DMEM containing 2% horse serum (Gibco, U.S.A.) for inducing myogenic differentiation. After 84 h, the cultures were shifted to serum-free DMEM for 12 h [25].

Trial 1: DEX and its time-dependent effect
After a 12-h incubation in serum-free medium, cells were exposed to DMEM-LM (Thermo, Fremont, CA, U.S.A.) with or without 100 μmol/l DEX [23-25] and collected after 3, 8, 12, and 24 h, for the analysis of mRNA expression by RT-PCR and protein expression by immunoblotting.

Trial 2: DEX and epoxomicin (proteasome inhibitor) treatment
After a 12-h incubation in serum-free medium, cells were exposed to one of the four following treatments: basal serum-free medium (Control); basal medium with 100 μmol/l DEX for 8 h (DEX); basal medium with 1.5 μmol/l epoxomicin (EPOX) for 5 h; basal medium with 1.5 μmol/l EPOX for 5 h prior to addition of 100 μmol/l DEX for 8 h (DEX + EPOX). Following this, all cells were collected and subjected to the further analysis.

Trial 3: DEX and RU486 (GR inhibitor) treatment
After a 12-h incubation in serum-free medium, cells were exposed to one of the four following treatments: basal serum-free medium (Control); basal medium with 100 μmol/l DEX for 8 h (DEX); basal medium with 10 μmol/l RU486 for 5 h (RU486); basal medium with 10 μmol/l RU486 for 5 h prior to addition of 100 μmol/l DEX for 8 h (DEX+RU486). Following this, all cells were collected and subjected to the further analysis.

Trial 4: DEX and LY294002 (PI3K inhibitor) treatment
After a 12-h incubation in serum-free medium, cells were exposed to one of the four following treatments: basal serum-free medium (Control); basal medium with 100 μmol/l DEX for 8 h (DEX); basal medium with 10 μmol/l LY294002 for 5 h (LY); basal medium with 10 μmol/l LY294002 for 5 h prior to addition of 100 μmol/l DEX for 8 h (DEX+LY). Following this, all cells were collected and subjected to the further analysis.

Trial 5: DEX and rapamycin (mammalian target of rapamycin inhibitor) treatment
After a 12-h incubation in serum-free medium, cells were exposed to one of the four following treatments: basal serum-free medium (Control); basal medium with 100 μmol/l DEX for 8 h (DEX); basal medium with 25 μmol/l rapamycin (RAPA) for 5 h; basal medium with 25 μmol/l RAPA for 5 h prior to addition of 100 μmol/l DEX for 8 h (DEX+RAPA). Following this, all cells were collected and subjected to the further analysis.

Protein preparation and Western blot analysis

Cell samples were homogenized on ice in radioimmunoprecipitation lysis buffer (50 mmol/l Tris/HCl at pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mmol/l sodium orthovanadate, 1 mmol/l sodium fluoride) and centrifuged at 12000 g for 5 min at 4°C. Protein concentration was determined using the BCA assay kit (Beyotime, Jiangsu, China) according to the manufacturer's protocol. After boiling at 100°C for 5 min, the protein extracts were electrophoresed in 7.5–10% SDS polyacrylamide gels (Bio-Rad, Richmond, CA) according to the procedure described by Laemmli [26]. The separated proteins were then transferred on to a nitrocellulose membrane in Tris-glycine buffer containing 20% methanol at 4°C. The membranes were blocked and then probed with primary antibodies including anti-MuRF1, anti-atrogin-1, anti-phospho-FoxO1/3a (Thr24/32) (Abcam, Cambridge, U.K.), anti-FoxO1, anti-p-mTOR (mammalian target of RAPA) (Ser^{2448}), anti-mTOR (Cell Signaling Technology, Beverly, U.S.A.), anti-p-Akt (Ser^{473}), and anti-Akt (Beyotime, Jiangsu, China). Protein detection was performed using goat anti-rabbit IgG (H + L)-HRP conjugated secondary antibody (Bio–Rad, Richmond, CA) or HRP-labeled goat anti-mouse IgG (H + L) secondary antibody (Beyotime, Jiangsu, China) with ECL using Western blot detection reagents (Beyotime, Jiangsu, China). β-actin was used as a loading control (Beyotime, Jiangsu, China). Western blots were developed and quantified using BioSpectrum 810 with VisionWorksLS 7.1 software (UVP LLC, Upland, CA).

RNA preparation and analysis

Total RNA extraction and real-time PCR were performed as described previously [25]. Primers were designed to span an intron to avoid genomic DNA contamination. Standard curves were generated using pooled cDNA from the samples that were assayed and to calculate the efficiency of the real-time PCR primers. The primers were as follows: Atrogin-1: forward, GCTGGATTGGAAGAAGAT and reverse, GAGAATGTGGCAGTGTTTG; MuRF1 forward, CTGGAGGTCGTTTCCGTTGC and reverse, TCGGGTGGCTGCCTTTCTGC; FoXO1: forward, GAGTGGATGGTGAAGAGCGT and reverse, GGGACAGATTGTGGCGAAT; FoXO3: forward, CCCTAACCCAGCAGAGACTGT and reverse, GGAAACAAACACAAGACGACACT; β-actin: forward, ACCACACCTTCTACAATGAG and reverse, ACGACCAGAGGCATACAG. Primer against β-actin was used as internal controls. The comparative CT method ($2^{-\Delta\Delta CT}$) was used to quantitate mRNA expression, according to Livak and Schmittgen method [27]. All samples were included in the same assay for one gene to avoid interassay variability.

Statistical methods

The data are presented as the mean ± S.E.M. All the data were subjected to one-way ANOVA analysis performed with the Statistical Analysis Systems statistical software package (Version 8e, SAS Institute, Cary, NC, U.S.A.) to test the main effect of treatments. The homogeneity of variances amongst treatments was confirmed using Bartlett's test (SAS Institute). When the primary effect of treatment was significant, differences between means were assessed by Duncan's multiple range analysis. Means were considered significantly different at $P<0.05$.

Results

The time-dependent effect of DEX treatment

The effect of high concentration of DEX on the UPS was investigated. Atrogin-1 mRNA level was significantly increased at 3 h ($P<0.05$, Figure 1A), decreased at 8 h ($P<0.05$, Figure 1A, 2A) and 12 h ($P<0.05$, Figure 1A), and not changed at 24 h of DEX ($P>0.05$, Figure 1A), whereas atrogin-1 protein level was not significantly changed by DEX at 3 h ($P>0.05$, Figure 1C) and 8 h ($P>0.05$, Figure 1C and 2C). DEX treatment significantly up-regulated the mRNA level of MuRF1 at 3 h ($P<0.05$, Figure 1B), 8 h ($P<0.05$, Figure 1B and 2A), 12 h ($P<0.05$, Figure 1B), and 24 h ($P<0.05$, Figure 1B), and up-regulated the protein level of MuRF1 at 8 h ($P<0.05$, Figure 1D and 2C) but not at 3 h ($P>0.05$, Figure 1D).

The ubiquitin ligase MuRF1 was dramatically regulated by DEX treatment at 8 h; therefore, we then detected the DEX effect at 8 h. The upstream pathway of atrogin-1 and MuRF1, Akt/FoXO1, was then measured. DEX treatment increased the FoXO1 mRNA and decreased FoXO3 mRNA ($P<0.05$, Figure 2A), while increased FoXO1/3a (Thr24/32) phosphorylation ($P<0.05$, Figure 2B). Akt protein was not affected by DEX ($P>0.05$, Figure 2D). As a crucial protein synthesis regulator, mTOR phosphorylation was suppressed by DEX ($P<0.05$, Figure 2D).

Effect of proteasome inhibition with EPOX on DEX-induced effects

To further confirm the role of ubiquitin ligases in UPS pathway during GC treatment, EPOX was used to inhibit proteasome in C2C12 cells. MuRF1 protein expression was suppressed by DEX + EPOX compared with DEX treatment
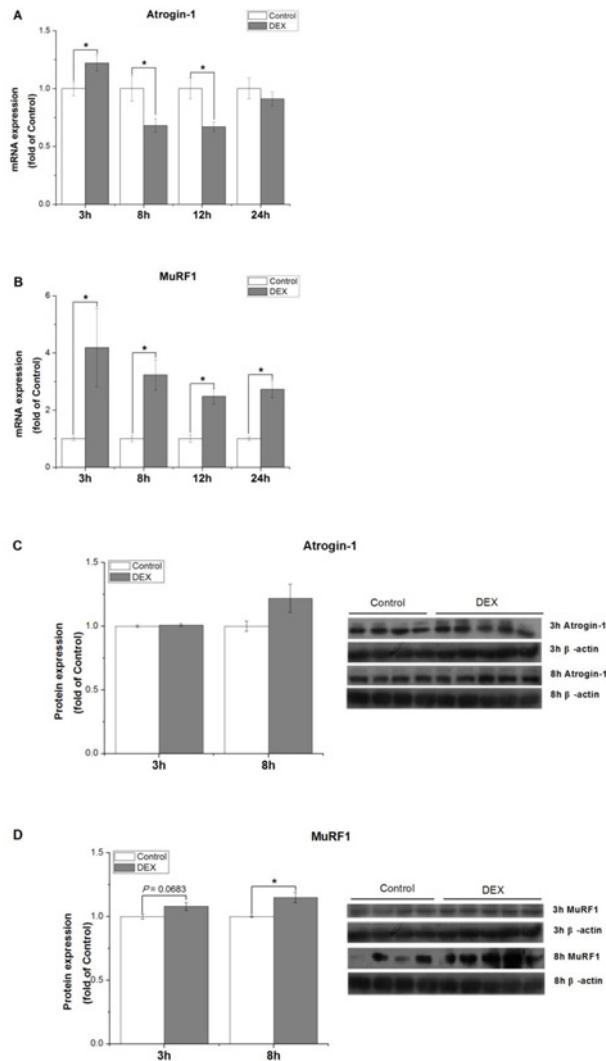
Figure 1. The time-dependent effect of DEX treatment
The effect of DEX treatment (100 μM for 3, 8, 12, and 24 h) on atrogin-1 (**A**, **C**) and MuRF1 (**B**, **D**) expressions in C2C12 cells. Results are presented as the mean $\pm$ S.E.M. ($n \geq 4$); control and DEX treatments were compared at each time point, and means with * are significantly different ($P < 0.05$).

alone ($P < 0.05$, Figure 3A). However, DEX and EPOX had no influence on the atrogin-1 protein expression ($P > 0.05$, Figure 3B).

Effect of GR blockage with RU486 on DEX-induced effects

To further explore the mechanism underlying GC effect on UPS pathway, RU486 was used to inhibit GR in C2C12 cells. DEX-induced increase of FoXO1/3a phosphorylation was significantly inhibited by DEX+RU486 treatment ($P < 0.05$, Figure 4A), and DEX-induced increase of MuRF1 protein was partially attenuated (-20.5%) by DEX+RU486 (Figure 4B). However, DEX and RU486 had no influence on the atrogin-1 protein expression ($P > 0.05$, Figure 4B).

Effect of PI3K blockage with LY294002 on DEX-induced effects

To further explore the effect of PI3K/Akt on UPS pathway, LY294002 was used to inhibit PI3K in C2C12 cells. DEX+LY294002 did not restore the stimulative effect of DEX on the FoXO1/3a phosphorylation ($P > 0.05$, Figure 5A), but inhibited the activation of MuRF1 protein induced by DEX ($P < 0.05$, Figure 5B).
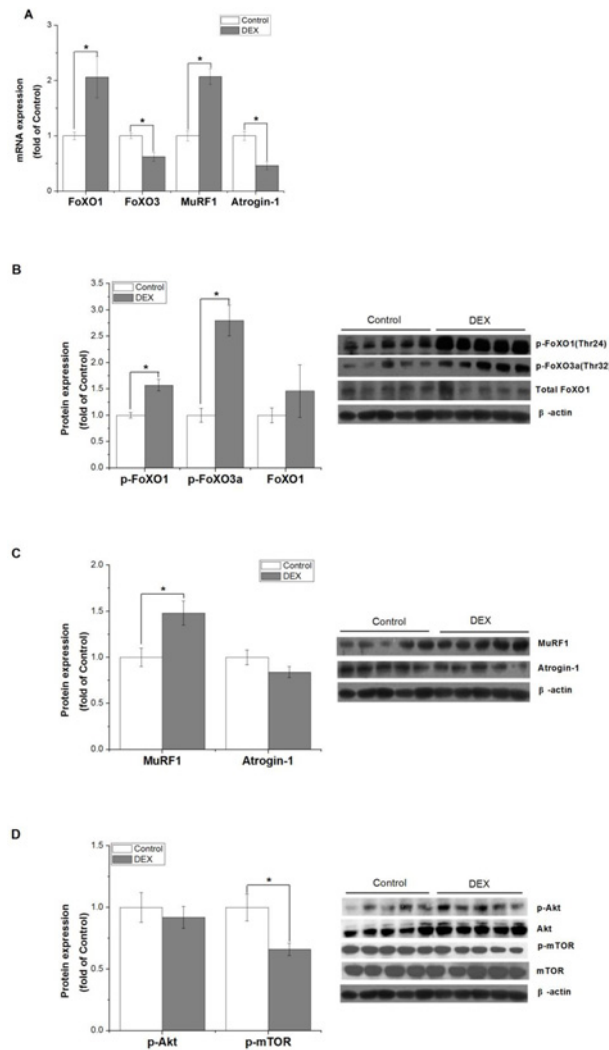
Figure 2. Dex effects on protein degradation regulators and protein synthesis regulators

The effect of DEX treatment (100 μM for 8 h) on mRNA expressions of FoXO1, FoXO3, MuRF1, and atrogin-1 (**A**), protein expressions of p-FoXO1/3a (**B**), MuRF1, atrogin-1 (**C**), p-mTOR and p-Akt (**D**) in C2C12 cells. Results are presented as the mean ± S.E.M. ($n \geq 5$); control and DEX treatments were compared for each gene, and means with * are significantly different ($P < 0.05$).

Effect of mTOR blockage with RAPA on DEX-induced effects

To further explore the effect of mTOR on UPS pathway, RAPA was used to inhibit mTOR in C2C12 cells. DEX + RAPA inhibited the stimulative effect of DEX on the FoXO1 phosphorylation and MuRF1 protein ($P < 0.05$, Figure 6A,B). However, DEX and RAPA had no detectable effect on atrogin-1 protein level ($P > 0.05$, Figure 6B).

Discussion

The previous report [23] and our recent studies [24,25] have demonstrated that excessive GCs (100 μmol/l DEX) suppressed muscle protein synthesis and enhanced proteolysis. On the basis of above studies, the role of ubiquitin ligases in protein metabolism during excessive GC exposure was investigated in the present study. In line with our previous study, excessive GCs stimulated UPS through MuRF1, but not atrogin-1. We novelly find that other pathway that is independent of Akt/FoXO1 pathway may account for the excessive GC-induced MuRF1 activation. The cross-talk between anabolic regulator and catabolic regulator is also noteworthy. Proposed model of excessive GC action on protein metabolism in C2C12 cells is shown in Figure 7.
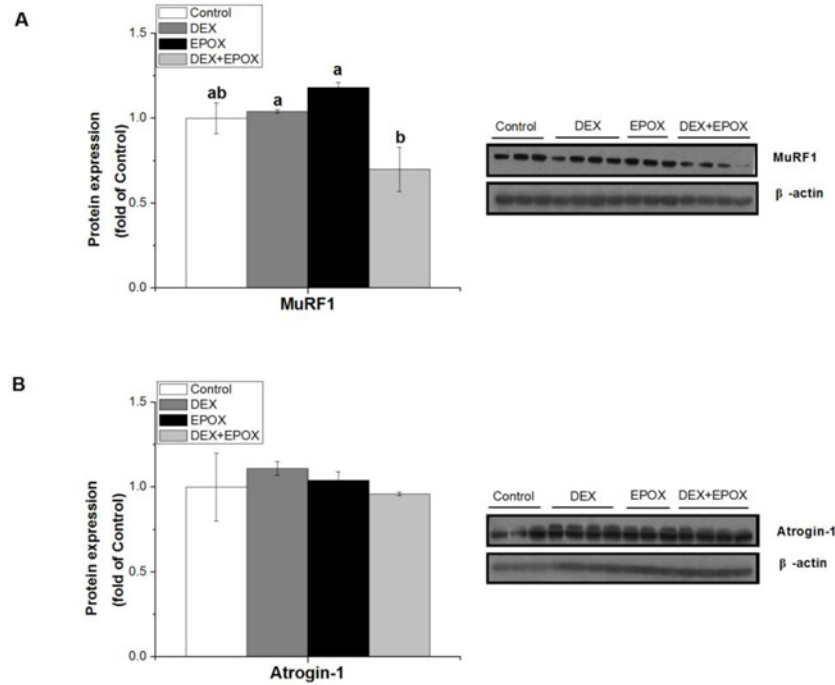
Figure 3. Effect of proteasome inhibition with EPOX on DEX-induced effects

The effect of DEX (100 μM for 8 h) and EPOX (1.5 μM for 5 h) on protein expressions of MuRF1 (**A**) and atrogin-1 (**B**) in C2C12 cells. Results are presented as the mean ± S.E.M. ($n \geq 3$); four groups were compared for each gene, and means without a common letter are significantly different ($P < 0.05$).
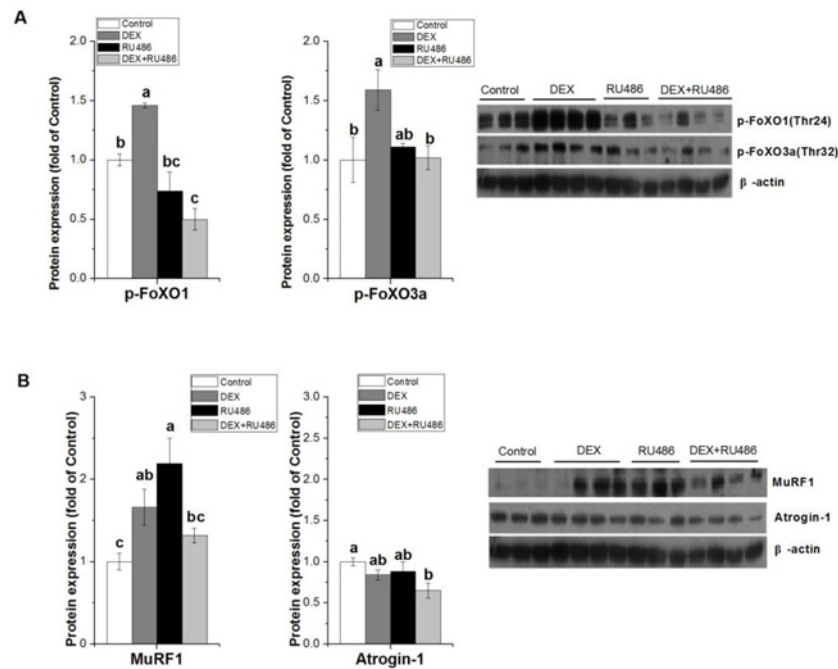

Figure 4. Effect of GR blockage with RU486 on DEX-induced effects

The effect of DEX (100 μM for 8 h) and RU486 (10 μM for 5 h) on protein expressions of p-FoXO1/3a (**A**), MuRF1 and atrogin-1 (**B**) in C2C12 cells. Results are presented as the mean ± S.E.M. ($n \geq 3$); four groups were compared for each gene, and means without a common letter are significantly different ($P < 0.05$).
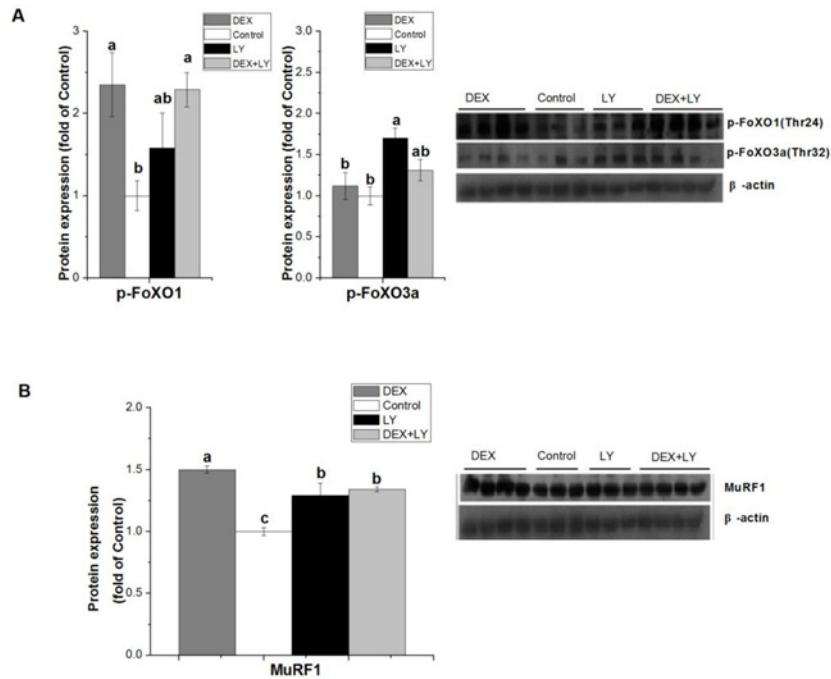
Figure 5. Effect of PI3K blockage with LY294002 on DEX-induced effects

The effect of DEX (100 μM for 8 h) and LY294002 (10 μM for 5 h) on protein expressions of p-FoXO1/3a (**A**) and MuRF1 (**B**) in C2C12 cells. Results are presented as the mean ± S.E.M. ($n \geq 3$); four groups were compared for each gene, and means without a common letter are significantly different ($P < 0.05$).
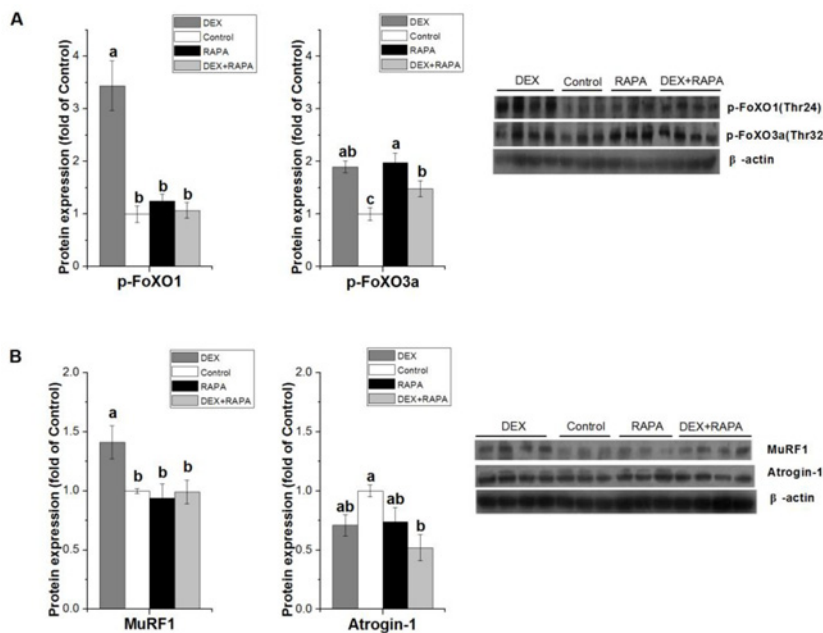

Figure 6. Effect of mTOR blockage with RAPA on DEX-induced effects

The effect of DEX (100 μM for 8 h) and RAPA (25 μM for 5 h) on protein expressions of p-FoXO1/3a (**A**), MuRF1 and atrogin-1 (**B**) in C2C12 cells. Results are presented as the mean ± S.E.M. ($n \geq 3$); four groups were compared for each gene, and means without a common letter are significantly different ($P < 0.05$).
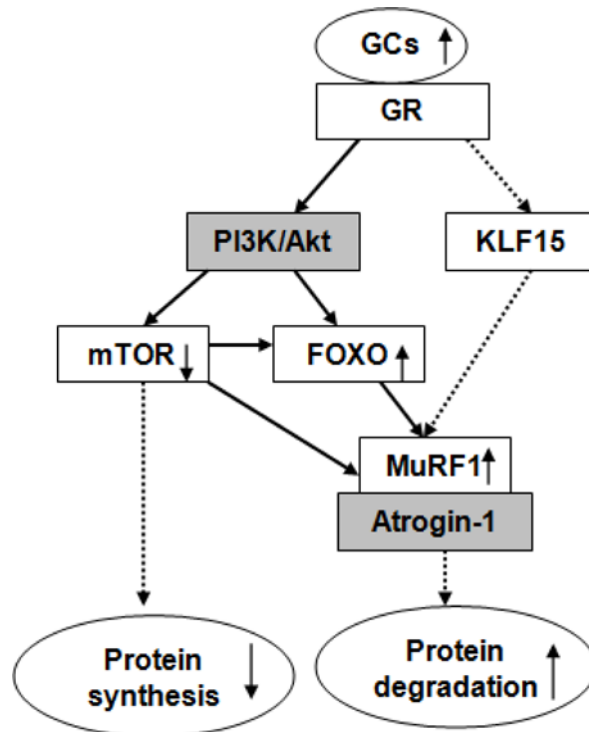
Figure 7. Proposed model of excessive GC action on protein synthesis and protein degradation in C2C12 cells
(↑ increase; ↓ decrease; gray blocks represent unaffected proteins; solid arrows represent findings in this study and dashed arrows represent findings reported previously [23-25,38,39]). Excessive GCs stimulated protein degradation through MuRF1, but not atrogin-1. Other pathway that is independent of Akt/FoXO1 pathway may account for the excessive GC-induced MuRF1 activation. There is a cross-talk between anabolic regulator mTOR and catabolic regulator FoXO1-MuRF1.

The stimulation by excessive GCs of the UPS is mediated through MuRF1, but not atrogin-1

The catabolic effect and muscle atrophy caused by GCs have been well studied in mammals [28] and in chickens [29]. Muscle mass loss occurs when the degradation rate is higher than the synthesis rate. Amongst three proteolytic systems (lysosomal, proteasomal, and calpain dependent), UPS is considered to play a major role in the catabolic action of GCs [8]. We thus assessed two ubiquitin ligases, atrogin-1 and MuRF1, in the present study. At the early stage of DEX exposure (3 h), the increased mRNA levels of atrogin-1 and MuRF1 in the present study were in agreement with previous study that also used 100 μmol/l DEX for 4 h [30]. At 8–24 h, we found that MuRF1, but not atrogin-1, was increased by DEX at both transcriptional and translational levels; the results are in line with our recent study [24], but contrary to many other studies where atrogin-1 was up-regulated by DEX with lower dose [13-15,30], indicating a dose- and time-dependent manner of GC effect. The role of MuRF1 is further confirmed, as we find MuRF1 expression was suppressed by DEX+proteasome inhibitor compared with DEX. However, DEX and proteasome inhibitor had no influence on the atrogin-1.

Adrenal GCs produce their actions via a signal pathway involving the ubiquitously expressed GR. Upon binding GCs, GR translocates into the nucleus and binds to the GC response element (GRE) in the promoters of target genes. It has been reported that the promoter regions of MuRF1 [31], but not atrogin-1 [32], contain functional GREs. Consistently, in the present study, MuRF1 expression was significantly elevated during DEX treatment, and was partially attenuated after GR inhibition, while atrogin-1 was not significantly increased by DEX. The results suggest the stimulation by GCs of the UPS is mediated mainly through MuRF1, but not atrogin-1. In other studies using DEX with lower dose [13-15,30], however, atrogin-1 expression was also increased by DEX. We thus speculate the lower concentrations of GCs up-regulate MuRF1 and atrogin-1 through GR and non-GR-dependent mechanism respectively, whereas the higher concentrations of GCs only up-regulate MuRF1 through GR-dependent mechanism.

Other pathway that is independent of Akt/FoXO1 pathway may account for the excessive GC-induced MuRF1 overexpression

Several studies have demonstrated that muscle-specific ubiquitin ligase MuRF1 is the target of FoxO1, and FoxO1 inhibition prevents MuRF1 activation [32,33]. The proximal promoter of the FoxO1 gene contains multiple functional GREs and FoxO1 gene expression is regulated by binding of the GREs [34]. The reduced phosphorylation is an important mechanism of FoxO1 activation [35]. We thus investigated whether FoxO1/3a phosphorylation is associated with MuRF1 expression during high dose of DEX treatment. In line with previous study, FoxO1 mRNA was up-regulated by DEX [34,36]. The phosphorylation of FoXO1/3a was increased by DEX, suggesting that GCs induced the inactivation of FoxO1. Furthermore, the increases of FoXO1/3a phosphorylation by DEX were inhibited after RU486 treatment, indicating that GC effect is a GR-driven mechanism. The suppression of FoxO1 pathway by DEX was in line with the previous studies [24,28]. However, the up-regulated MuRF1 mRNA and protein by DEX indicated the activated muscle atrophy-related genes, consistent with previous results [28,34,36]. These results implied that FoxO1 is not a major factor contributing to the GC-induced MuRF1 overexpression.

FoxO1 is an important downstream target of the PI3K/Akt pathways [20,37]. FoxO1 phosphorylation by Akt leads to exclusion of FoxO1 from the nucleus and the phosphophorylated FoxO1 is ubiquitinated in the cytoplasm followed by degradation [22]. In the present results, unexpectedly, Akt expression was not affected by DEX; protein levels of phospho-FoxO1/3a were not affected when adding PI3K/Akt inhibitor compared with DEX treatment alone, suggesting PI3K/Akt is not necessary in GC-induced FoxO1 phosphorylation. Although the involvement of Akt/FoXO1 transcription factors is well known in the gene regulation of MuRF1 under the presence of excessive GCs [32,33], a recently discovered transcription factor that promotes skeletal muscle atrophy via transcriptional regulation of atrogenes in GC treatment has been reported. According to the study of Shimizu et al. [38], the up-regulation of MuRF1 in response to DEX might be mediated through an Akt/FoXO-independent pathway, such as Kruppe-like factor 15 (KLF15) transcription factor – overexpression of KLF15 causes myotube atrophy in an Akt-independent manner [39]; KLF15 co-operating with FoxO significantly enhanced the promoter activity of atrogenes [38]. All these results implicate that Akt and FoxO1 were not essential in the regulation of MuRF1 by GCs. It is likely that an Akt/FoXO1-independent pathway, such as KLF15, up-regulates the expression of ubiquitin ligases MuRF1 after DEX treatment. The present result is noteworthy, and is in fact contrary to the known effects of DEX on Akt and FoxO1 expression [15]. One possible explanation is the difference in DEX dose.

Cross-talk between mTOR and FoXO1-MuRF1 pathways

Excessive GCs cause muscular loss by impeding protein synthesis and enhancing protein breakdown [5]. The mTOR pathway plays a major role in the regulation of skeletal muscle fiber size [40-42]. In the present study, mTOR phosphorylation was depressed by DEX, consistent with our recent study in C2C12 cell, which indicated that DEX repressed protein synthesis rate through the involvement of mTOR pathways [24,25]. Moreover, DEX enhanced myostatin-dependent muscle proteolysis in our recent study [24], and increased the expression of atrophy-related gene MuRF1 in the present study as well as our recent report [24]. All these results imply that GCs facilitate proteolysis meanwhile inhibiting protein synthesis and further exacerbating the loss of protein and muscle mass.

Given GCs facilitated muscle proteolysis meanwhile blunting muscle protein synthesis, we speculate there are associations between atrophy-related genes and anabolic regulators. We thus investigated whether MuRF1 is affected by mTOR signaling, using mTOR inhibitor RAPA. Much of the evidence suggests that there is a cross-talk between mTOR and FoXO1-MuRF1 pathways. mTOR signaling could be inhibited directly by FoxO1 [43], whereas there is a disagreement on the involvement of mTOR in proteolysis and muscle atrophy. mTOR is considered to be a master regulator of autophagy, and the mTOR inhibitor RAPA induces autophagy in many cell systems [44]; the mTOR pathway is necessary for mediating mRNA changes of atrogenes during hypertrophy [45]; mTORC2 is required for signaling to Akt-FoXO [46]; inhibition of Rictor causes translocation of FoXO3 to the nucleus and induced autophagy [47]. Inversely, many studies reported FoXO1 activation is not affected by mTOR signaling – RAPA was unable to inhibit the phosphorylation of FoXO1 [48] and FoxO1 translocation [45]. In the current study, we found that DEX stimulated MuRF1 meanwhile suppressing mTOR. Moreover, DEX-induced MuRF1 overexpression was restored when pretreated with RAPA. These results suggest that GCs exert both a catabolic and an anti-anabolic action. When further suppressing anabolic pathway, the enhanced catabolism was attenuated, demonstrating a systemic compensation mechanism.

In conclusion, the present study indicates that excessive GCs increased UPS-dependent protein proteolysis via stimulating MuRF1, but not atrogin-1. GCs induced MuRF1 overexpression independent of Akt/FoXO1 pathway.

Competing interests
The authors declare that there are no competing interests associated with the manuscript.

Author contribution
H.L. designed the research. X.J.W. and J.J.X. conducted the research. L.L. analyzed data. H.C.J. prepared figures. X.J.W. wrote the paper. All authors read and approved the final manuscript.

Abbreviations
Akt, also known as Protein Kinase B; DEX, dexamethasone; DMEM, Dulbecco 's modified eagle medium; EPOX, epoxomicin; FoxO1, forkhead box O1; GC, glucocorticoid; GR, GC receptor; GRE, GC response element; HRP, horse radish peroxidase; KLF15, Kruppe-like factor 15; LY, LY294002; mTOR, mammalian target of rapamycin; MuRF1, muscle-specific RING finger protein 1; RAPA, rapamycin; RT-PCR, reverse transcription-polymerase chain reaction; UPS, ubiquitin-proteasome system.

References
1 Matthews, D.E. (1999) Proteins and amino acids. In *Modern Nutrition in Health and Diseases*, 9th edn, (Shils, M.E., Olson, J.A., Shike, M. and Ross, A.C., eds), pp. 11–48, Williams & Wilkins, Baltimore, MD

2 Hoffman, E.P. and Nader, G.A. (2004) Balancing muscle hypertrophy and atrophy. *Nat. Med.* **10**, 584–585

3 Goldberg, A.L., Tischler, M., DeMartino, G. and Griffin, G. (1980) Hormonal regulation of protein degradation and synthesis in skeletal muscle. *Fed. Proc.* **39**, 31–36

4 Löfberg, E., Gutierrez, A., Wernerman, J., Anderstam, B., Mitch, W.E., Price, S.R. et al. (2002) Effects of high doses of glucocorticoids on free amino acids, ribosomes and protein turnover in human muscle. *Eur. J. Clin. Invest.* **32**, 345–353

5 Tomas, F.M., Murray, A.J. and Jones, L.M. (1984) Interactive effects of insulin and corticosterone on myofibrillar protein turnover in rats as determined by N tau-methylhistidine excretion. *Biochem. J.* **220**, 469–479

6 Munck, A., Guyre, P.M. and Holbrook, N.J. (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr. Rev.* **5**, 25–44

7 Ventadour, S. and Attaix, D. (2006) Mechanisms of skeletal muscle atrophy. *Curr. Opin. Rheumatol.* **18**, 631–635

8 Schakman, O., Gilson, H. and Thissen, J.P. (2008) Mechanisms of glucocorticoid-induced myopathy. *J. Endocrinol.* **197**, 1–10

9 Bodine, S.C., Latres, E., Baumhueter, S., Lai, V.K., Nunez, L., Clarke, B.A. et al. (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704–1708

10 Gomes, M.D., Lecker, S.H., Jagoe, R.T., Navon, A. and Goldberg, A.L. (2001) Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14440–14445

11 Schakman, O., Gilson, H., Kalista, S. and Thissen, J.P. (2009) Mechanisms of muscle atrophy induced by glucocorticoids. *Horm. Res.* **72**, 36–41

12 Foucaud, L., Niot, I., Kanda, T. and Besnard, P. (1998) Indirect dexamethasone down-regulation of the liver fatty acid-binding protein expression in rat liver. *Biochim. Biophys. Acta* **1391**, 204–212

13 Krawiec, B.J., Nystrom, G.J., Frost, R.A., Jefferson, L.S. and Lang, C.H. (2007) AMP-activated protein kinase agonists increase mRNA content of the muscle-specific ubiquitin ligases MAFbx and MuRF1 in C2C12 cells. *Am. J. Physiol. Endocrinol. Metab.* **292**, E1555–E1567

14 Menconi, M., Gonnella, P., Petkova, V., Lecker, S. and Hasselgren, P.O. (2008) Dexamethasone and corticosterone induce similar, but not identical, muscle wasting responses in cultured L6 and C2C12 myotubes. *J. Cell. Biochem.* **105**, 353–364

15 Zhao, W., Qin, W., Pan, J., Wu, Y., Bauman, W.A. and Cardozo, C. (2009) Dependence of dexamethasone-induced Akt/FOXO1 signaling, upregulation of MAFbx, and protein catabolism upon the glucocorticoid receptor. *Biochem. Biophys. Res. Commun.* **378**, 668–672

16 Kamei, Y., Miura, S., Suzuki, M., Kai, Y., Mizukami, J., Taniguchi, T. et al. (2004) Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated type I (slow twitch/red muscle) fiber genes, and impaired glycemic control. *J. Biol. Chem.* **279**, 41114–41123

17 Kim, M.S., Pak, Y.K., Jang, P.G., Namkoong, C., Choi, Y.S., Won, J.C. et al. (2006) Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. *Nat. Neurosci.* **9**, 901–906

18 Kim, J.J., Li, P., Huntley, J., Chang, J.P., Arden, K.C. and Olefsky, J.M. (2009) FoxO1 haploinsufficiency protects against high-fat diet-induced insulin resistance with enhanced peroxisome proliferator-activated receptor gamma activation in adipose tissue. *Diabetes* **58**, 1275–1282

19 Nakae, J., Biggs, W.H., Kitamura, T., Cavenee, W.K., Wright, C.V., Arden, K.C. et al. (2002) Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat. Genet.* **32**, 245–253

20 Tang, E.D., Nunez, G., Barr, F.G. and Guan, K.L. (1999) Negative regulation of the forkhead transcription factor FKHR by Akt. *J. Biol. Chem.* **274**, 16741–16746

21 Aoki, M., Jiang, H. and Vogt, P.K. (2004) Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 13613–13617

22 Matsuzaki, H., Daitoku, H., Hatta, M., Tanaka, K. and Fukamizu, A. (2003) Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11285–11290

23 Desler, M.M., Jones, S.J., Smith, C.W. and Woods, T. L. (1996) Effects of dexamethasone and anabolic agents on proliferation and protein synthesis and degradation in C2C12 myogenic cells. *J. Anim. Sci.* **74**, 1265–1273

24 Wang, R.X., Jiao, H.C., Zhao, J.P., Wang, X.J. and Lin, H. (2016) Glucocorticoids enhance muscle proteolysis through a myostatin-dependent pathway at the early stage. *PLoS ONE* **11**, 0156225

25 Wang, X.J., Yang, X., Wang, R.X., Jiao, H.C., Zhao, J.P., Song, Z.G. et al. (2016) Leucine alleviates dexamethasone-induced suppression of muscle protein synthesis via synergy involvement of mTOR and AMPK pathways. *Biosci. Rep.* **36**, e00346

26 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685

27 Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 (−Delta Delta C (T)) method. *Methods* **25**, 402–408

28 Umeki, D., Ohnuki, Y., Mototani, Y., Shiozawa, K., Suita, K., Fujita, T. et al. (2015) Protective effects of clenbuterol against dexamethasone-induced masseter muscle atrophy and myosin heavy chain transition. *PLoS ONE* **10**, e0128263

29 Dong, H., Lin, H., Jiao, H.C., Song, Z.G., Zhao, J.P. and Jiang, K.J. (2007) Altered development and protein metabolism in skeletal muscle of broiler chichens (Gallus gallus domesticus) by corticosterone. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **147**, A189–A195

30 Nishimura, M., Mikura, M., Hirasaka, K., Okumura, Y., Nikawa, T., Kawano, Y. et al. (2008) Effects of dimethyl sulphoxide and dexamethasone on mRNA expression of myogenesis- and muscle proteolytic system-related genes in mouse myoblastic C2C12 cells. *J. Biochem.* **144**, 717–724

31 Waddell, D.S., Baehr, L.M., van den Brandt, J., Johnsen, S.A., Reichardt, H.M., Furlow, J.D. et al. (2008) The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. *Am. J. Physiol. Endocrinol. Metab.* **295**, 785–797

32 Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A. et al. (2004) Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* **117**, 399–412

33 Stitt, T.N., Drujan, D., Clarke, B.A., Panaro, F., Timofeyva, Y., Kline, W.O. et al. (2004) The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol. Cell* **14**, 395–403

34 Qin, W., Pan, J., Qin, Y., Lee, D.N., Bauman, W.A. and Cardozo, C. (2014) Identification of functional glucocorticoid response elements in the mouse FoxO1 promoter. *Biochem. Biophys. Res. Commun.* **450**, 979–983

35 Van Der Heide, L.P., Hoekman, M.F. and Smidt, M.P. (2004) The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. *Biochem. J.* **380**, 297–309

36 Castillero, E., Alamdari, N., Aversa, Z., Gurav, A. and Hasselgren, P.O. (2013) PPARβ/δ regulates glucocorticoid- and sepsis-induced FoxO1 activation and muscle wasting. *PLoS ONE* **8**, e59726

37 White, J.P., Gao, S., Puppa, M.J., Sato, S., Welle, S.L. and Carson, J.A. (2013) Testosterone regulation of Akt/mTORC1/FoxO3a signaling in skeletal muscle. *Mol. Cell. Endocrinol.* **365**, 174–186

38 Shimizu, N., Yoshikawa, N., Ito, N., Maruyama, T., Suzuki, Y., Takeda, S. et al. (2011) Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. *Cell Metab.* **13**, 170–182

39 Schakman, O., Kalista, S., Barbé, C., Loumaye, A. and Thissen, J.P. (2013) Glucocorticoid-induced skeletal muscle atrophy. *Int. J. Biochem. Cell Biol.* **45**, 2163–2172

40 Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R. et al. (2001) Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy *in vivo*. *Nat. Cell Biol.* **3**, 1014–1019

41 Liu, X., Powlas, J., Shi, Y., Oleksijew, A.X., Shoemaker, A.R., De Jong, R. et al. (2004) Rapamycin inhibits Akt-mediated oncogenic transformation and tumor growth. *Anticancer Res.* **24**, 2697–2704

42 Shah, O.J., Anthony, J.C., Kimball, S.R. and Jefferson, L.S. (2000) 4E-BP1 and S6K1: translational integration sites for nutritional and hormonal information in muscle. *Am. J. Physiol. Endocrinol. Metab.* **279**, 715–729

43 Southgate, R.J., Neill, B., Prelovsek, O., El-Osta, A., Kamei, Y., Miura, S. et al. (2007) FOXO1 regulates the expression of 4E-BP1 and inhibits mTOR signaling in mammalian skeletal muscle. *J. Biol. Chem.* **282**, 21176–21186

44 Lum, J.J., DeBerardinis, R.J. and Thompson, C.B. (2005) Autophagy in metazoans: cell survival in the land of plenty. *Nat. Rev. Mol. Cell Biol.* **6**, 439–448

45 Latres, E., Amini, A.R., Amini, A.A., Griffiths, J., Martin, F.J., Wei, Y. et al. (2005) Insulin-like growth factor-1 (IGF-1) inversely regulates atrophy-induced genes via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway. *J. Biol. Chem.* **280**, 2737–2744

46 Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J. et al. (2006) Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev. Cell* **11**, 859–871

47 Mammucari, C., Schiaffino, S. and Sandri, M. (2008) Downstream of Akt: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle. *Autophagy* **4**, 524–526

48 Gu, Y., Lindner, J., Kumar, A., Yuan, W. and Magnuson, M.A. (2011) Rictor/mTORC2 is essential for maintaining a balance between β-cell proliferation and cell size. *Diabetes* **60**, 827–837