Cellular mechanisms underlying temporal changes in skeletal muscle protein synthesis and breakdown during chronic β-adrenoceptor stimulation in mice

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Chronic stimulation of β-adrenoceptors with β-adrenoceptor agonists (β-agonists) can induce substantial skeletal muscle hypertrophy, but the mechanisms mediating this muscle growth have yet to be elucidated. We investigated whether chronic β-adrenoceptor stimulation in mice with the β-agonist formoterol alters the muscle anabolic response following β-adrenoceptor stimulation. Twelve-week-old C57BL/6 mice were treated for up to 28 days with a once-daily injection of either saline (control, $n = 9$) or formoterol ($100\,\mu g\,kg^{-1}$; $n = 9$). Rates of muscle protein synthesis were assessed at either 1, 7 or 28 days of treatment, 6 h after injection. Protein synthesis rates were higher in formoterol-treated mice at day 7 (∼1.5-fold, $P < 0.05$), but not at day 1 or 28. The increased muscle protein synthesis was associated with increased phosphorylation of S6K1 ($r = 0.49$, $P < 0.01$). Formoterol treatment acutely reduced maximal calpain activity by ∼25% ($P < 0.05$) but did not affect atrogin-1 protein levels and proteasome-mediated proteolytic activity, despite significantly enhanced phosphorylation of Akt ($P < 0.05$). Formoterol increased CREB phosphorylation by ∼30% ($P < 0.05$) and PPARγ coactivator-1α (PGC-1α) by 11-fold ($P < 0.05$) on day 1 only. These observations identify that formoterol treatment induces muscle anabolism, by reducing calpain activity and by enhancing protein synthesis via increased PI-3 kinase/Akt signalling.

Abbreviations AC, adenylate cyclase; FSR, fractional synthetic rate; MHC, myosin heavy chain; MPS, muscle protein synthesis; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PI3K, phosphoinositol 3-kinase; PKA, protein kinase A; SDH, succinate dehydrogenase; TA, tibialis anterior.

Introduction

The biological importance of β-adrenergic signalling has been well documented in the heart but only very recently have we begun to understand the importance of this signalling pathway in skeletal muscle (Lynch & Ryall, 2008). Chronic stimulation of β-adrenoceptor signalling with β-adrenoceptor agonists (β-agonists) has been shown to induce skeletal muscle growth and could represent an effective treatment for skeletal muscle wasting conditions. In addition to promoting muscle hypertrophy in healthy animals (Maltin *et al.* 1987; Ryall *et al.* 2006), treatment with β-agonists can prevent or reverse the muscle wasting and weakness associated with numerous conditions and pathologies, including sarcopaenia (age-related muscle wasting) (Ryall *et al.* 2007), cancer cachexia (Busquets

et al. 2004) and muscular dystrophies (Harcourt *et al.* 2007; Gehrig *et al.* 2010). Chronic treatment with formoterol, a highly selective (newer generation) β₂-agonist, increased muscle mass, fibre cross-sectional area (CSA) and maximal force producing capacity in wild-type and *mdx* dystrophic mice (Harcourt *et al.* 2007; Gehrig *et al.* 2010) and in young, adult and aged rats (Ryall *et al.* 2004, 2006, 2007). However, the therapeutic potential of β-agonists for conditions associated with muscle wasting and weakness has so far been compromised by deleterious side effects, including hypertrophy and dysfunction of the heart (Ryall *et al.* 2008*b*). It is therefore important to identify the mechanisms underlying β-adrenoceptor mediated skeletal muscle hypertrophy so that targets can be identified that modulate β-adrenergic signalling to enhance muscle mass and function without side-effects. Such an outcome would

have considerable clinical significance for the treatment of many muscle wasting disorders.

The hypertrophic response of skeletal muscle following chronic, high-dose β-agonist administration has been associated with an increase in muscle protein synthesis (MPS), a decrease in protein degradation, or a combination of both mechanisms (Ryall *et al.* 2008*a*). The data are equivocal as to the precise mechanisms that mediate β-agonist-induced skeletal muscle hypertrophy. Canonical β-agonist signalling has been well described and involves selective coupling to a heterotrimeric G-protein ($G\alpha\beta\gamma$) to initiate downstream signalling. Traditionally, β-agonist signalling is believed to occur via the stimulatory $G\alpha$ subunit ($G\alpha s$) coupling to adenylate cyclase (AC), with production of cyclic AMP (cAMP) and subsequent activation of protein kinase A (PKA) and phosphorylation of cAMP response element binding (CREB) protein (Lynch & Ryall, 2008). The activation of this pathway has been linked to the inhibition of Ca^{2+}-dependent proteolytic pathways, and possibly to increased mixed muscle and mitochondrial protein synthesis, via increases in transcription of genes encoding muscle regulatory factors such as MyoD and peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) (Pearen *et al.* 2008). The $G\beta\gamma$ subunits of the G-protein have also been suggested to play an active role in various cell signalling processes, which may have important roles in β-agonist-induced hypertrophy of skeletal muscle. Specifically, β-agonists initiate $G\beta\gamma$-mediated activation of the phosphoinositol 3-kinase (PI3K)–protein kinase B (Akt) signalling pathway, which has been implicated as a regulator of both MPS and ubiquitin-proteasome-dependent protein degradation (Kline *et al.* 2007).

It has been shown previously that chronic administration of most β-agonists in rats activates MPS significantly (Maltin *et al.* 1989) but MPS does not appear to be increased in rats after acute (oral) administration of the (older generation) β_2-agonist clenbuterol (Maltin *et al.* 1989), or infusion of isoproterenol, a non-specific β-agonist in humans (Robinson *et al.* 2010). In contrast, protein degradation is affected more rapidly (Maltin *et al.* 1987). Thus, the anabolic response to a single administration of a β-agonist might be different from that with chronic treatment because of differences in the activation of β-adrenoceptor signalling. To investigate whether there is a shift in the mechanism that primarily governs β-adrenergic signalling mediated muscle hypertrophy with chronic stimulation, muscle protein synthesis and degradation and the associated signalling events need to be measured at different times after β-agonist administration.

The aim of this study was to determine the pathways responsible for skeletal muscle hypertrophy after chronic β-adrenoceptor stimulation with administration of formoterol. We measured mitochondrial, myofibrillar and sarcoplasmic protein synthesis and degradation and associated signalling after 1, 7 and 28 days of β-adrenoceptor stimulation. We tested the hypothesis that changes in muscle protein degradation predominate in the initial stages of formoterol treatment which are followed by a shift to changes in MPS during the later stages of chronic β-adrenoceptor stimulation.

Methods

Animals

Twelve-week-old C57BL/6 mice ($n = 72$) were used in this study. All mice were obtained from the Animal Resources Centre (Canning Vale, Western Australia), and housed in the Biological Research Facility at The University of Melbourne under a 12 h light/dark cycle, with drinking water and standard chow provided *ad libitum*. All experiments were approved by the Animal Experimental Ethics Committee of The University of Melbourne and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council (NHMRC Australia).

Mice were allocated into either control (3 groups, $n = 12$ per group) or formoterol treated groups (3 groups, $n = 12$ per group). Treated animals received 100 μg kg^{-1} of formoterol (Sigma-Aldrich Co., Castle Hill, NSW, Australia) in saline once daily via intraperitoneal (I.P.) injections for up to 28 days. Control mice received an identical volume of saline only. All animals were weighed daily throughout the study. We have shown previously that 4 weeks of treatment with this dose of formoterol increases muscle size and strength in mice by 20–30% (Gehrig *et al.* 2010).

Experimental procedures

We have shown previously that the increase in muscle fibre CSA with chronic administration of the β-agonist fenoterol was associated with a reduction in oxidative enzyme activity in selected muscles of rats (Ryall *et al.* 2004). As such, we hypothesized that the acute and chronic anabolic response to formoterol administration would be different in mitochondrial and myofibrillar protein fractions in skeletal muscle. Therefore, protein synthesis rates in myofibrillar, mitochondrial and sarcoplasmic fractions and muscle protein breakdown rates were determined after 1, 7 or 28 days of formoterol treatment. MPS was measured 6 h after the injection of either formoterol or saline, with food withdrawn during the last 5 h. MPS was assessed in the gastrocnemius/soleus/plantaris muscle complex using the

flooding-dose method as described previously (Osowska *et al.* 2006). Briefly, 50 min before being killed, each mouse was injected subcutaneously with a flooding dose of L-[1-^{13}C]valine (50%, 300 μmol per 100 g; Cambridge Isotope laboratories, Andover, MA, USA). Three mice per group did not receive the tracer and served as controls to measure baseline free and protein-bound L-[1-^{13}C]valine enrichments. Thirty minutes after the tracer injection, mice were anaesthetized with sodium pentobarbitone (Nembutal, 60 mg kg^{-1}, Sigma-Aldrich) via I.P. injection. While anaesthetized deeply, the right and left gastrocnemius/soleus/plantaris muscles were dissected as a unit, weighed and rapidly frozen in liquid N$_2$ for later determination of free and protein-bound L-[1-^{13}C]valine enrichment. The tibialis anterior (TA) and quadriceps muscles were carefully dissected and trimmed of tendon and any adhering non-muscle tissue. The right TA was then mounted in embedding medium and frozen in thawing isopentane, while the other muscles were frozen directly in liquid N$_2$ and stored at $-80°$C for subsequent analyses. Mice were killed as a consequence of heart excision, while anaesthetized deeply.

Muscle analyses

Muscle protein synthesis. The two gastrocnemius/soleus/plantaris complexes ($\sim$350 mg) were used for isolation of myofibrillar, sarcoplasmic and mitochondrial proteins as described previously (Rooyackers *et al.* 1996; Guillet *et al.* 2004; Zangarelli *et al.* 2004). Briefly, muscle samples were homogenized in a 5% ice-cold buffer containing 0.25 M sucrose, 2 mM EDTA and 10 mM Tris-HCl (pH 7.4) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at low speed (600 *g*) and the pellet containing myofibrillar protein was collected. From the supernatant centrifuged at 7000 *g*, the sarcoplasmic protein fraction was isolated after centrifugation at 100,000 *g* for 60 min. The pellet of the 7000 *g* supernatant, containing mitochondria, was washed twice using an ice-cold buffer containing 100 mM KCl, 5 mM EGTA, 5 mM MgSO$_4$, 50 mM Tris-HCl (pH 7.4) and 1 mM ATP and spun at 7000 *g*. The final mitochondrial pellet was suspended in the sucrose/EDTA/Tris-HCl buffer for analysis (Guillet *et al.* 2004). The applied protein fractionation method separates subsarcolemmal mitochondria from myofibrillar and sarcoplasmic proteins (Rooyackers *et al.* 1996). The purity of the myofibrillar, mitochondrial and sarcoplasmic fractions was confirmed by Western blot analyses for myosin and COX-IV protein abundance and citrate synthase activity (data not shown). The myofibrillar protein fraction is enriched in contractile proteins, but also contains some intermyofibrillar mitochondria. Therefore, we refer to the three protein fractions as being enriched in myofibrillar, mitochondrial or sarcoplasmic protein.

Each protein fraction was hydrolysed using 6 M HCl (110°C for 24 h), and the constituent amino acids in the hydrolysate were purified by cation exchange chromatography (Dowex 50W 8X; Bio-Rad Laboratories, Hercules, CA, USA). Amino acids were eluted in 4 ml of 4 M NH$_4$OH and dried in a SpeedVac (Savant Instruments Inc., USA). Amino acids were derivatized as their *N*-acetyl-propyl and isotopic enrichments measured by gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS, mGas System, Fisons Instruments/VG Isotech, Middlewich, UK) (Osowska *et al.* 2006). Tissue fluid valine enrichments were determined using *t*-butyldimethylsilyl derivatives, as described previously (Osowska *et al.* 2006) and used as the precursor pool enrichment to calculate fractional synthesis rates (FSRs). Another set of three mice was used for the determination of basal isotopic abundance in skeletal muscle.

Calpain activity. An $\sim$15 mg portion of the TA muscle was homogenized in a Tris-based buffer containing EDTA, 0.1% β-mercaptoethanol and a protease inhibitor cocktail (Wei *et al.* 2005). Following centrifugation, supernatants were equalized to 1 mg protein per ml and calpain activity was determined using 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid labelled casein (BODIPY-FL-casein, Invitrogen) as a calpain substrate. Muscle extracts (50 μg) and the labelled casein were added to a micro-titre plate and incubated at 25°C for 60 min. The reaction was stopped by adding 25 μl of 100 mM EDTA, and fluorescence was read at 485 nm excitation and 530 nm emission wavelengths. Duplicate assays were performed under identical conditions, except that calcium was omitted and 100 mM EDTA was added to the assay. Calpain activity was calculated as the difference between activity measured in the presence of 10 mM calcium and the activity measured in the absence of calcium and the presence of 100 mM EDTA. The assay measured calcium-dependent degradation of casein (Wei *et al.* 2005).

Proteasome activity. The 20S proteasome activities were determined in quadriceps muscles *ex vivo* as described in detail elsewhere (Hobler *et al.* 1999). Briefly, muscles were homogenized and proteasomes isolated by sequential (ultra)centrifugation steps. Protein content (determined with Bio-Rad DC Protein Assay; Bio-Rad, Gladesville, NSW, Australia) of the isolated proteasome fractionations were equalized and the peptidase activities of the 20S proteasome determined

fluorometrically by measuring the hydrolysis of the fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC, Sapphire Bioscience, Redfern, NSW, Australia), t-butoxycarbonyl-Leu-Arg-Arg-7-amido-4-methylcoumarin (Boc-LRR-AMC, Sigma), and Z-Leu-Leu-Glu-amido-4-methylcoumarin (Z-LLE-AMC, Sapphire Bioscience). These substrates are hydrolysed preferentially by the chymotrypsin-, trypsin-like and the peptidyl glutamyl peptide hydrolase (PGPH) activities of the 20S proteasome, respectively (Minnaard *et al.* 2005). Peptidase activity was determined by measuring the accumulation of the fluorogenic cleavage product amido-4-methylcoumarin (AMC) using a luminescence spectrometer. The difference between arbitrary fluorescence units recorded with or without $40\,\mu\text{M}$ of the proteasome inhibitor MG132 (Sigma) in the reaction medium was corrected for the amount of protein in the reaction and the time course for the accumulation of AMC after hydrolysis of the substrate was analysed by linear regression to calculate peptidase activities, as described previously (Minnaard *et al.* 2005).

Western blot. Thirty milligram portions of the TA muscle were homogenized in ice-cold buffer ($10\,\text{mM}$ Tris-HCl (pH 7.4), $100\,\text{mM}$ NaCl, $1\,\text{mM}$ EDTA, $1\,\text{mM}$ EGTA, $1\,\text{mM}$ NaF, 1% Triton, 10% glycerol, 0.1% SDS, $20\,\text{mM}$ $Na_4P_2O_7$, $0.5\,\text{mM}$ Na_3VO_4, 0.5% sodium deoxycholate, $0.1\,\text{mM}$ PMSF and protease and phosphatase inhibitors, all from Sigma). Samples were centrifuged at $10,000\,g$ for 5 min at 4°C and the resulting supernatant analysed for total protein content (Bio-Rad DC Protein Assay), with BSA as the standard. Samples were normalized to $3\,\mu\text{g}\,\mu\text{l}^{-1}$ in homogenizing buffer, resolved in SDS-buffer and heated for 5 min at 95°C. Primary phospho-specific antibodies (anti-phospho Akt (S^{473}), anti-phospho S6K1 (T^{389}), anti-phospho CREB (S^{133}) and anti-Akt, anti-S6K1, anti-CREB and anti-calpastatin were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-atrogin-1 and anti-tubulin were purchased from ECM-Biosciences (Versailles, KY, USA) and Sigma, respectively.

Equal amounts of protein ($60\,\mu\text{g}$ per lane) were run on SDS-polyacrylamide gels and proteins were transferred to 0.45 mm PVDF. After Ponceau S staining and de-staining, membranes were blocked for 2 h at room temperature (RT) in Tris-buffered saline–Tween 20 (TBST) containing 5% BSA. Membranes were incubated overnight at 4°C in primary antibodies. The following day membranes were washed for $3 \times 5\,$min in TBST and then incubated for 1 h at RT in horseradish peroxidase-conjugated secondary antibodies (mouse anti-rabbit or goat anti-mouse immunoglobulins) diluted in TBST containing 5% BSA. After $3 \times 5\,$min washes in TBST, membranes were treated with enhanced chemiluminescence (ECL plus; GE Healthcare, Little Chalfont, UK). The signal was imaged using a ChemiDoc XRS machine (Bio-Rad) and blots were quantified using Quantity One software (Bio-Rad). Tubulin was used as the loading control.

Real-time RT-PCR. MuRF-1, atrogin-1, myostatin, calpastatin, MyoD, PGC-1α, Myf5, β-arrestin-2, β_2-adrenoceptor, BNIP3, LC3, myosin heavy chain (MHC) IIa, MHCIIb, MHCIIx, calpain-1, calpain-2 and calpain-3 mRNA expression were determined by real-time RT-PCR as described in detail elsewhere (Murphy *et al.* 2010). Total RNA was extracted from 10–20 mg of TA muscle using a commercially available kit, according to the manufacturer's instructions (PureLink RNA Mini Kit, Invitrogen, Carlsbad, CA, USA). RNA was transcribed into cDNA using the Invitrogen SuperScript™ VILO cDNA Synthesis Kit, and the resulting cDNA was stored at −20°C for subsequent analysis. Real-time PCR was performed using the Bio-Rad iCycler Thermal Cycler. Primer sequences are shown in Table 1. Measurements included a no-template control as well as an RT (reverse transcription) negative control. The content of single stranded DNA (ssDNA) in each sample was determined using the Quanti-iT OliGreen ssDNA Assay Kit (Molecular Probes/Invitrogen, Eugene, OR, USA), as described previously (Murphy *et al.* 2010). Gene expression was quantified by normalizing to the cDNA content of each sample and expressed as arbitrary units (AU).

Skeletal muscle histology. Serial sections were cut transversely through the TA muscles from mice killed after 28 days of treatment with formoterol or saline, using a refrigerated (−20°C) cryostat (CTI Cryostat, IEC, Needham Heights, MA, USA). Sections were reacted with caveolin-3 to assist in the determination of mean myofibre CSA and succinate dehydrogenase (SDH) to determine fibre oxidative capacity (Koopman *et al.* 2006). Digital images of stained sections were obtained using an upright microscope with camera (Axio Imager D1, Carl Zeiss MicroImaging GmbH, Jena, Germany), controlled by AxioVision AC software (AxioVision AC Rel. 4.7.1, Carl Zeiss). Images were quantified using AxioVision 4.7.1 software as described previously (Koopman *et al.* 2006).

Statistical analyses. All values are expressed as means ± S.E.M. unless stated otherwise. Saline- and formoterol-treated groups were compared using Student's unpaired t test or a two-way ANOVA (time, treatment), where appropriate. Bonferroni's *post hoc* test was used to determine significant differences between individual groups. The level of significance was set at $P < 0.05$ for all comparisons.

Table 1. Primer sequences used

Gene	GeneBank accession no.	Sense primer (5′–3′)	Antisense primer (5′–3′)
MuRF-1	NM_009066	AGGTGTCAGCGAAAAGCAGT	CCTCCTTTGTCCTCTTGCTG
Atrogin-1	AF_441120	GTTTTCAGCAGGCCAAGAAG	TTGCCAGAGAACACGCTATG
MyoD	NM_010866	AGTGAATGAGGCCTTCGAGA	GCATCTGAGTCGCCACTGTA
Myf5	NM_008656	AACCAGAGACTCCCCAAGGT	AGCTGGACACGGAGCTTTTA
BNIP3	NM_009760	GGGTTTTCCCCAAAGGAATA	TGACCACCCAAGGTAATGGT
LC3	NM_026160	CGGCTTCCTGTACATGGTTT	ATGTGGGTGCCTACGTTCTC
$β_2$-ADR	NM_007420	GAGCACAAAGCCCTCAAGAC	GTTGACGTAGCCCAACCAGT
β-Arrestin-2	NM_145429	AAGTCGAGCCCTAACTGCAA	GGTGAGGGTCACGAACACTT
Myostatin	NM_010834	GGTACAAGGTATACTGGAATCCGATC	CTTCACATCAATGCTCTGCCA
PGC-1$α$	NM_008904	CCGAGAATTCATGGAGCAAT	TTTCTGTGGGTTTGGTGTGA
MHCIIa	NM_001039545	GAACCCTCCCAAGTACGACA	TAAGGGTTGACGGTGACACA
MHCIIb	NM_030679	CTTCAACCACCACATGTTCG	GAGCTTCAACCTTGCCTTTG
MHCIIx	NM_010855	CCAGAGTCACCTTCCAGCTC	CTTCCCTTTGCTTTTGCTTG
Calpastatin	NM_009817	TTGATGATGCCTTGGATGAA	TCCCAGTTTCTCGCTATGCT
Calpain-1	NM_007600	ACATTTTACGAGGGCACCTG	CTCCCGGTTGTCATAGTCGT
Calpain-2	NM_009794	TGACAATTTTGTGCGGTGTT	GAAAAACTCAGCCACGAAGC
Calpain-3	NM_007601	ACGGGAATAAGCAACACCTG	AAACGAAGATGATGGGCTTG

Primers were designed using Invitrogen software from gene sequences obtained from GeneBank. Primer specificity was determined using a BLAST search.

Results

Formoterol treatment induces skeletal muscle hypertrophy

Daily injections with $100\ \mu g\ kg^{-1}$ formoterol for 28 days increased body mass in C57BL/6 mice (Fig. 1*A*, significant treatment main effect; $P < 0.05$). The higher body mass in formoterol treated animals was associated with greater TA muscle mass (Fig. 1*B*, significant treatment main effect; $P < 0.05$). The increase in muscle mass after 28 days of treatment was associated with a 15% increase in muscle fibre cross-sectional area ($P < 0.01$), without changes in muscle fibre oxidative enzyme activity ($P = 0.40$, Fig. 1*C–H*). Total muscle oxidative capacity (sum of all muscle fibre CSA × SDH density) was significantly higher (+27%) in the formoterol treated mice compared with saline treated controls ($P < 0.05$).

Formoterol treatment does not acutely increase muscle protein synthesis

MPS was measured 6 h after administration of $100\ \mu g\ kg^{-1}$ formoterol for 1, 7 and 28 days of treatment in skeletal muscle mitochondrial, myofibrillar and sarcoplasmic enriched protein fractions (Fig. 2) to identify changes in the muscle anabolic response to formoterol over time. A single I.P. injection of formoterol (day 1) did not acutely increase protein synthesis in any of the muscle protein fractions (Fig. 2*B–D*). After 7 days of treatment (i.e. 6 h after the 7th daily injection), MPS was elevated significantly in the formoterol treated mice compared with control mice in the mitochondrial (2-fold), myofibrillar (1.6-fold), and sarcoplasmic protein enriched fractions

(1.6-fold), as seen in Fig. 2*B–D* ($P < 0.05$). After 28 days of treatment (i.e. 6 h after the 28th daily injection), no significant differences in the rates of myofibrillar and sarcoplasmic protein synthesis were observed between treatments, although mitochondrial protein synthesis rates tended to be higher ($\sim$20%, $P = 0.10$) in the formoterol treated group compared with the saline treated controls.

Formoterol treatment reduces maximum calpain activity but does not affect ubiquitin-proteasome-dependent proteasome activity

In addition to measuring MPS we also assessed muscle protein degradation, specifically the Ca^{2+}-dependent (calpains) and ATP-dependent (proteasome) proteolytic activity *ex vivo*. Maximal calpain activity was reduced significantly with chronic administration of formoterol (Fig. 3*A*, treatment main effect, $P < 0.05$). In contrast, there was no acute or chronic effect of formoterol administration on ubiquitin-proteasome-dependent protein degradation. Maximal proteasome activities (i.e. trypsin-like, chymotrypsin-like and peptidyl glutamyl peptide hydrolase) were unchanged 6 h after formoterol administration on days 1, 7 and 28 of treatment (Fig. 3*B–D*).

Formoterol induces signalling through p70 S6K1 to increase muscle protein synthesis

To elucidate the signalling pathway regulating protein synthesis and breakdown with *β*-adrenoceptor stimulation, we performed Western blotting using

phospho-specific antibodies and real-time RT-PCR. Formoterol treatment induced significant increases in Akt phosphorylation (Fig. 4*A* and *B*, main treatment effect, $P < 0.05$), without altering S6K1 phosphorylation at days 1 and 28 (Fig. 4*C*). Although we could not detect significant treatment, time and interaction effects of formoterol treatment on S6K1 phosphorylation, we

observed significant correlations between the extent of S6K1 phosphorylation and the fractional synthetic rate of myofibrillar (Fig. 4*F*), mitochondrial ($r = 0.49$, $P < 0.01$) and sarcoplasmic protein ($r = 0.42$, $P < 0.01$). The increased Akt phosphorylation was associated with a reduction in atrogin-1 mRNA expression after 7 days of formoterol treatment, but there was no effect on atrogin-1
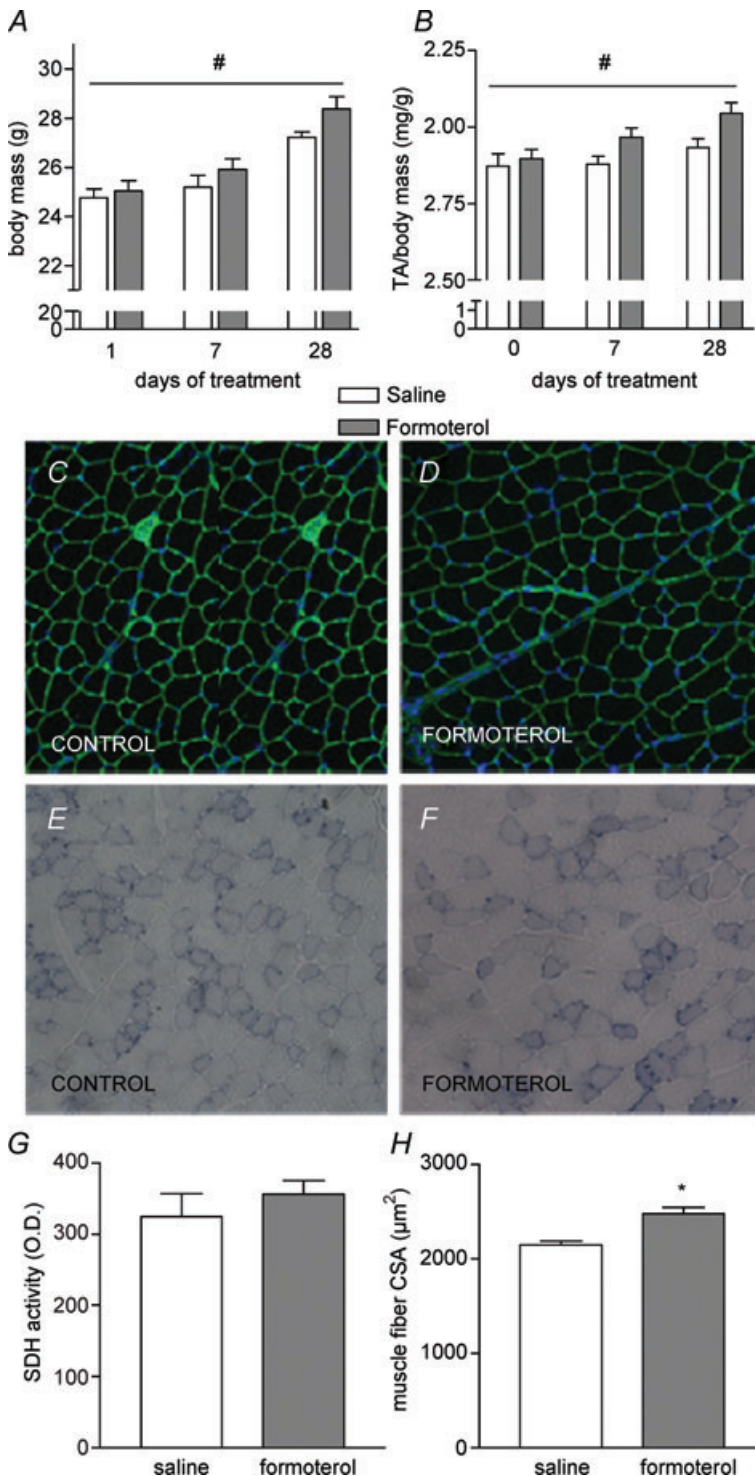
Figure 1. Effect of formoterol treatment on body and muscle mass, fibre cross-sectioned area and oxidative enzyme capacity

A and *B*, body mass (*A*), TA muscle mass to body mass rate (*B*) from C57BL/6 mice after 1, 7 or 28 days treatment with either saline or formoterol ($100 \, \mu g \, kg^{-1} \, day^{-1}$). *C–F*, muscle cross-sections from mice treated for 28 days reacted for laminin and DAPI (*C* and *D*) and SDH activity (*E* and *F*). *G* and *H*, the average muscle fibre SDH activity (*G*) and cross-sectional area (CSA, *H*) were quantified using Axiovision 4.7 software. TA, tibialis anterior; SDH, succinate dehydrogenase. Data are means ± s.e.m.; $n = 9$ per group. *$P < 0.05$ *versus* saline, #$P < 0.05$ treatment main effect.

protein expression (Fig. 4*D/E*). We did not observe any changes in MuRF-1 mRNA levels following formoterol administration (Table 2). Interestingly, mRNA levels of BNIP3 and LC3, genes involved in the regulation of autophagy, tended to be reduced at day 7 of treatment (Table 2), but there were no significant correlations between Akt or S6K1 phosphorylation status and BNIP and LC3 mRNA levels.

Chronic *β*-adrenoceptor stimulation alters the change in CREB phosphorylation after acute formoterol administration

In addition to studying the PI3 kinase/Akt/mTOR signalling pathway, we performed measurements of the proteins involved in *β*-adrenergic signalling. A single injection of formoterol increased the phosphorylation status of CREB by 30% ($P < 0.05$, Fig. 5*A* and *B*), but with repeated injections (i.e. after 7 and 28 days of treatment) CREB phosphorylation was not different from the saline control. MyoD mRNA expression was significantly higher (Fig. 5*C*, treatment main effect, $P < 0.05$) in the formoterol treated group compared with controls. Formoterol injections (acute and chronic) did not affect mRNA expression of the $β_2$-adrenoceptor, *β*-arrestin-2, myostatin, calpastatin (Fig. 5*D*), calpain-1, calpain-2, calpain-3, Myf-5, or myosin heavy chain

(MHC) IIa, IIb and IIx isoforms (Table 2). In contrast, muscle calpastatin protein levels were increased at day 7 in the formoterol treated mice compared with the saline treated controls (Fig. 5*E*, $P < 0.05$). No significant correlations were observed between calpastatin protein levels, calpain mRNA levels and the measured calpain activity (data not shown). Formoterol injections acutely increased PGC-1$α$ mRNA expression (Fig. 5*F*, $P < 0.01$), but following repeated injections (i.e. after 7 and 28 days of treatment) PGC-1$α$ mRNA was not different from the saline control (Fig. 5*F*). Formoterol treatment did not alter PGC-1$α$ protein levels (Fig. 5*G*).

Discussion

The importance of *β*-adrenergic signalling in skeletal muscle is only beginning to emerge, especially the potential therapeutic applications of modulating *β*-adrenoceptor mediated pathways that regulate muscle growth. In this study, we found that muscle protein synthesis was increased only after chronic (7 days) *β*-adrenoceptor stimulation with formoterol administration and that treatment induced muscle anabolism, by reducing calpain activity, and by enhancing muscle protein synthesis via increased PI-3 kinase/Akt signalling. This knowledge enhances our understanding of the pathways that maintain skeletal muscle homeostasis with implications for treating muscle wasting conditions.
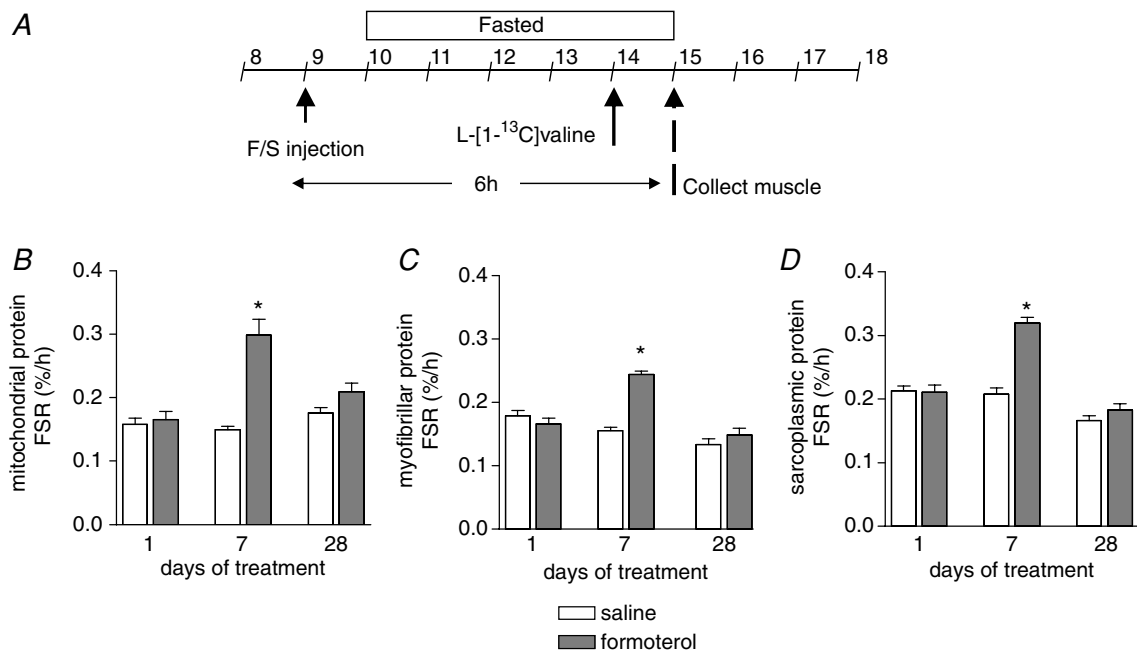

Figure 2. Effect of formoterol treatment on the acute protein synthetic response to *β*-agonist administration
A, muscle protein synthetic response was determined 6 h after a single injection of formoterol (100 $μ$g kg^{-1}) or saline at 1, 7 and 28 days of treatment by injecting a large flooding dose of L-[1-^{13}C]valine 50 min before muscle collection. *B–D*, protein synthetic rates were determined in mitochondrial (*B*), myofibrillar (*C*) and sarcoplasmic (*D*) protein enriched fractions. Data are means $±$ S.E.M.; $n = 9$. *$P < 0.05$ *versus* saline at same time point.

A single injection of formoterol did not increase the rates of mitochondrial, myofibrillar and sarcoplasmic protein synthesis within 6 h of administration (Fig. 2). Since it has been shown previously, at least for the β-agonist clenbuterol, that maximum muscle concentrations of the drug are attained 30–45 min after administration (Chang *et al.* 2009), and the elimination half-life from muscle is about 6 h (Chang *et al.* 2009), we expected that formoterol administration would acutely affect muscle protein synthesis. In fact, MPS in all measured protein fractions were elevated significantly 6 h after formoterol injection at day 7, but protein synthesis rates were not increased at day 28 (Fig. 2). These findings are consistent with data from studies performed in rats (Maltin *et al.* 1992) and humans (Robinson *et al.* 2010), using similar methods (i.e. injection of labelled phenylalanine) to measure MPS *in vivo*, showing that MPS rates are not increased immediately after β-agonist administration.

We observed increased phosphorylation of CREB, combined with increased mRNA levels of MyoD and PGC-1α, 6 h after the first injection of formoterol, indicating that stimulation of the β-adrenoceptor initially signals through Gαs coupling to AC, production of cAMP with subsequent activation of PKA, and phosphorylation of CREB protein. Interestingly, the increased PGC-1α mRNA expression in the formoterol treated mice was not associated with altered PGC-1α protein levels or mitochondrial FSR indicating that subsequent changes in protein levels (if any) occur after 6 h post-treatment. We did not observe any increase in CREB phosphorylation, PGC-1α mRNA or protein expression at days 7 and 28, suggesting that other signalling pathways become more important in the regulation of MPS with chronic β-adrenoceptor stimulation. By using rapamycin to inhibit mTOR activity, it has been established that PI-3 kinase/Akt signalling is implicated in β-agonist mediated hypertrophy (Kline *et al.* 2007). Our new findings reveal an increase in Akt phosphorylation after β-adrenoceptor stimulation with formoterol treatment and significant correlations between the extent of S6K1 phosphorylation and MPS in mitochondrial, myofibrillar and sarcoplasmic protein enriched fractions after formoterol treatment. These data indicate that the increase in overall protein synthesis
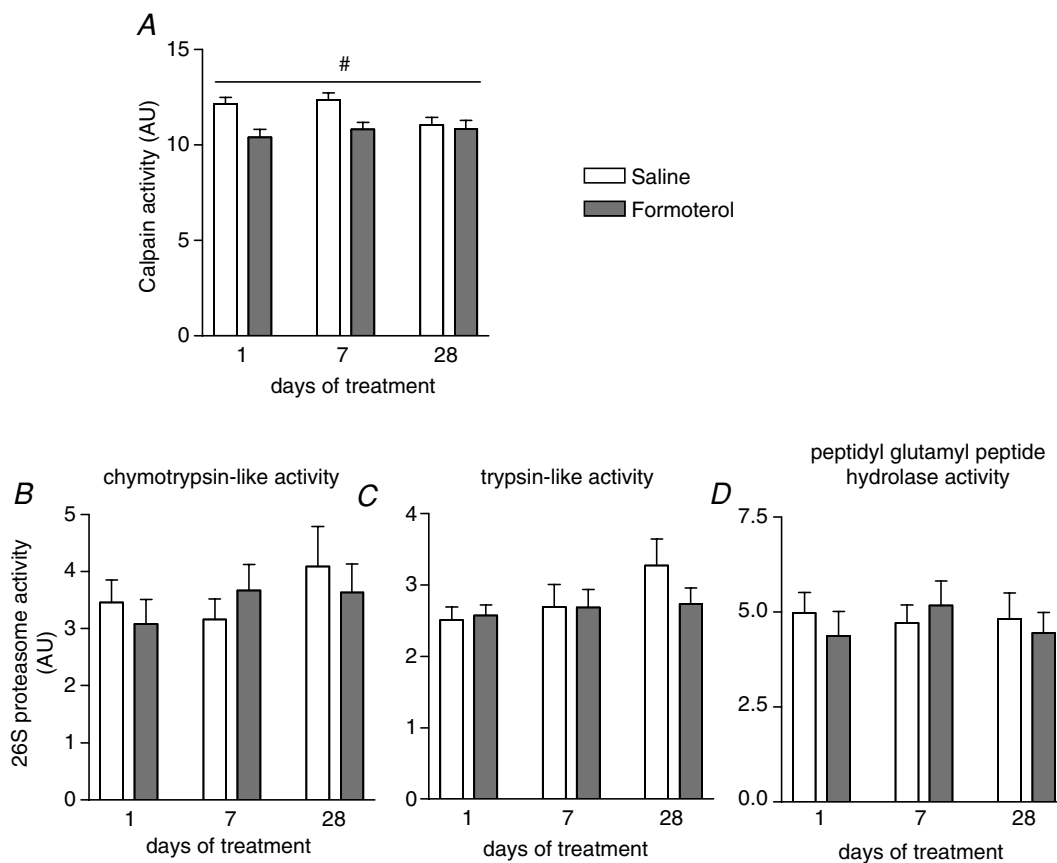

Figure 3. Effect of formoterol treatment on Ca^{2+}-dependent (A) and ubiquitin-proteasome dependent (B–D) protein degradation
Maximal calpain activity (*A*) and proteasome activity (*B–D*, chymotrypsin-like, trypsin-like and peptidyl glutamyl peptide hydrolase activities) were determined *ex vivo* as described in Methods. Data are means ± s.e.m.; $n = 9$. #$P < 0.05$ treatment main effect.
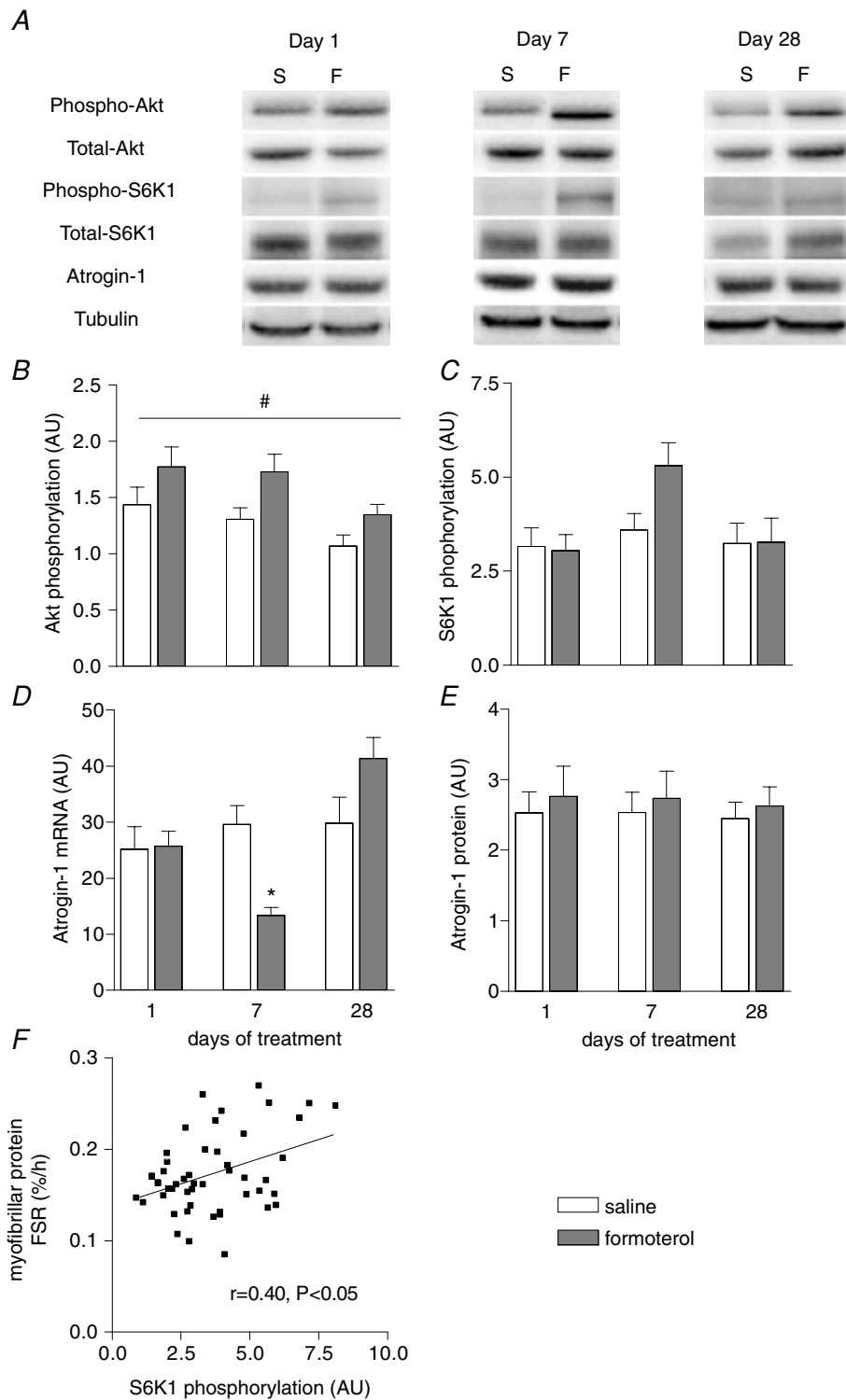
Figure 4. Representative blots (*A*) and group data for Akt (S^{457}) phosphorylation (*B*), S6K1 (T^{389}) phosphorylation (*C*), atrogin-1 mRNA (*D*) and protein (*E*) levels in TA muscles of mice after 1, 7 or 28 days of treatment with either saline or formoterol, and the plotted relationship between S6K1 phosphorylation and myofibrillar protein synthesis rates (*F*)
Data are means ± s.e.m.; $n = 9$. *$P < 0.05$ *versus* saline at same time point. #$P < 0.05$ treatment main effect.

Table 2. mRNA expression in mouse TA muscles 6 h after formoterol administration at day 1, 7 or 28 of treatment

Gene	Time (days)	Saline	Formoterol	*P*-values Time/treatment/interaction
MuRF-1	1	1.78 ± 0.11	1.80 ± 0.16	0.09/0.57/0.17
	7	2.19 ± 0.36	1.93 ± 0.22	
	28	1.33 ± 0.12	1.86 ± 0.16	
Myf5	1	1.27 ± 0.34	1.37 ± 0.13	<0.01/0.24/0.39
	7	1.39 ± 0.23	1.36 ± 0.14	
	28	0.52 ± 0.04	1.04 ± 0.17	
BNIP3	1	1.92 ± 0.29	1.92 ± 0.24	0.13/0.70/<0.05
	7	2.65 ± 0.24	1.81 ± 0.25‡	
	28	2.21 ± 0.20	2.79 ± 0.38	
LC3	1	1.58 ± 0.13	1.53 ± 0.14	0.11/0.14/0.07
	7	1.97 ± 0.12	1.38 ± 0.13	
	28	1.84 ± 0.13	1.92 ± 0.21	
Myostatin	1	10.24 ± 1.69	14.43 ± 1.32	0.05/0.74/<0.05
	7	20.32 ± 3.83	14.49 ± 1.82	
	28	11.98 ± 0.75	15.36 ± 1.76	
β_2-ADR	1	1.32 ± 0.21	0.83 ± 0.12	0.25/0.19/0.20
	7	1.56 ± 0.23	1.18 ± 0.28	
	28	1.28 ± 0.14	1.48 ± 0.20	
β-Arrestin-2	1	0.63 ± 0.11	0.71 ± 0.07	0.12/0.09/0.65
	7	0.62 ± 0.11	0.70 ± 0.09	
	28	0.39 ± 0.06	0.61 ± 0.07	
MHCIIa	1	26.70 ± 4.29	36.02 ± 3.51	<0.01/0.12/0.16
	7	49.90 ± 6.74	45.07 ± 4.06	
	28	56.68 ± 9.19	81.71 ± 12.33	
MHCIIb	1	5.50 ± 1.20	7.11 ± 1.05	<0.01/0.68/0.45
	7	8.80 ± 1.32	6.72 ± 0.43	
	28	14.50 ± 2.63	13.45 ± 1.65	
MHCIIx	1	3.51 ± 0.64	4.43 ± 0.51	0.10/0.16/0.54
	7	4.01 ± 0.54	3.96 ± 0.36	
	28	4.52 ± 1.09	5.93 ± 0.66	
Calpain-1	1	3.94 ± 0.42	3.65 ± 0.33	<0.05/0.77/0.15
	7	4.77 ± 0.33	4.22 ± 0.37	
	28	4.46 ± 0.60	5.64 ± 0.63	
Calpain-2	1	3.85 ± 0.33	4.13 ± 0.28	<0.01/0.57/0.44
	7	4.50 ± 0.29	4.90 ± 0.30	
	28	4.92 ± 0.60	6.26 ± 0.68	
Calpain-3	1	1.54 ± 0.15	1.30 ± 0.14	0.56/0.25/0.21
	7	1.51 ± 0.12	1.14 ± 0.11	
	28	1.41 ± 0.15	1.57 ± 0.22	

Data are in means ± S.E.M.; *n* = 9 per group. ‡*P* = 0.09 *versus* saline.

during formoterol treatment is driven mainly by signalling through the PI-3 kinase/Akt pathway.

In addition to our measurements of MPS, we examined potential changes in muscle protein breakdown by assessing maximal Ca^{2+}-dependent (calpains) and ubiquitin-proteasome-dependent proteolytic activity *ex vivo*. Calpain activity was acutely and chronically reduced after formoterol administration (Fig. 3*A*), consistent with data from *in vitro* studies in rats that showed reduced Ca^{2+}-dependent protein degradation with clenbuterol (Navegantes *et al.* 2001). We found that the reduction in calpain activity was not associated with changes in calpain-1–3 and calpastatin mRNA expression with formoterol treatment, indicating that β-adrenergic signalling does not control the transcription of these factors. These observations are consistent with previous studies showing that mRNA expression of calpain-1, -2 or -3 and calpastatin are either increased (Parr *et al.* 1992) or not changed (Busquets *et al.* 2004) after β-agonist administration. Calpastatin protein levels were increased on day 7 of formoterol treatment suggesting that part of the reduction in calpain activity with formoterol treatment was due to increased expression of calpastatin protein. Further work is needed to investigate the interaction
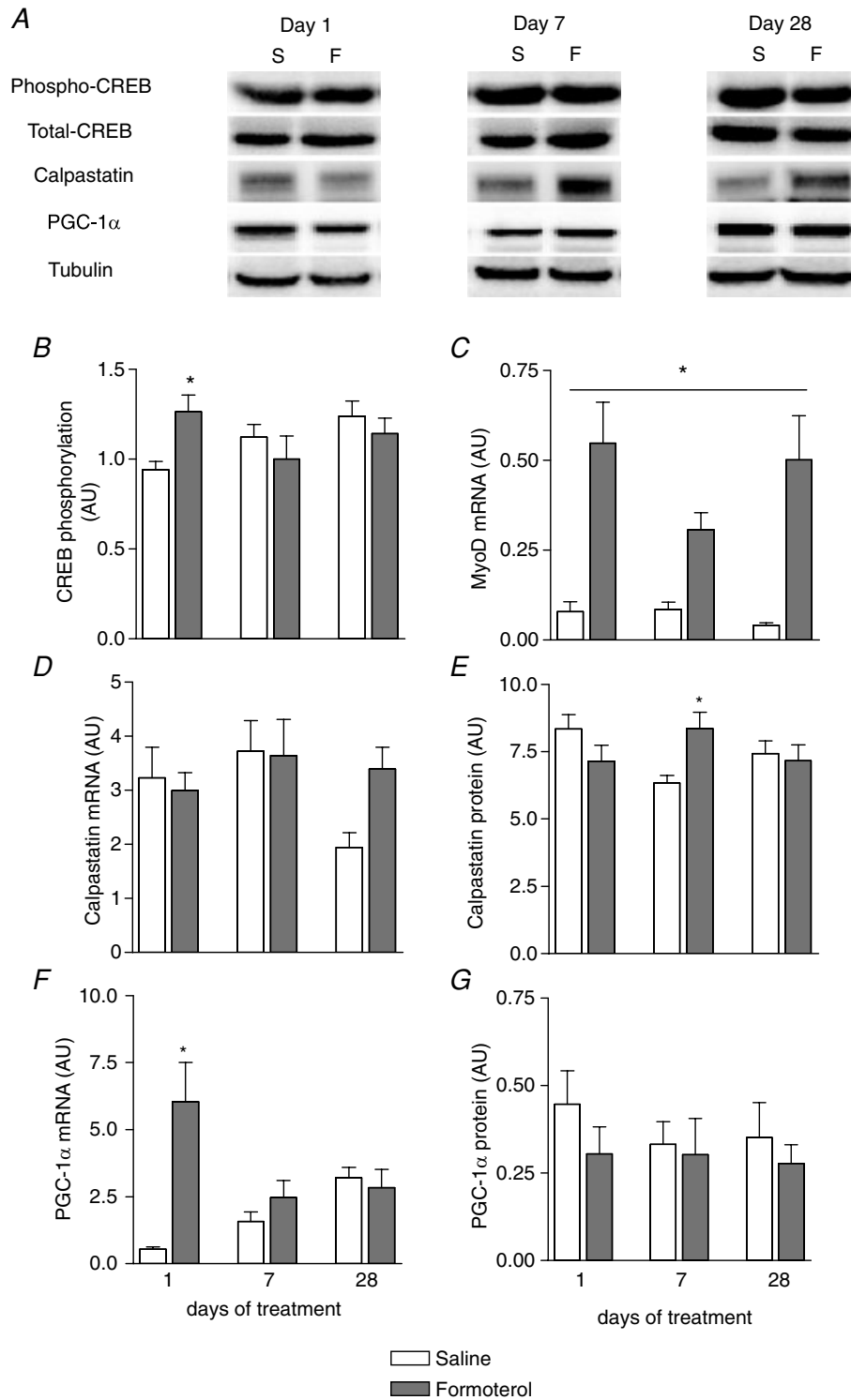
Figure 5. Representative blots (*A*) and group data for CREB (S[133]) phosphorylation (*B*), MyoD (*C*) and calpastatin (*D*) mRNA expression, calpastatin protein levels (*E*), PGC-1α mRNA expression (*F*), and PGC-1α protein levels (*G*) in TA muscle of mice after 1, 7 or 28 days of treatment with either saline or formoterol
Data are means ± s.e.m.; $n = 9$. *$P < 0.05$ *versus* saline at same time point. #$P < 0.05$ treatment main effect.

between β-adrenergic signalling and calpain activity and the regulation of calpain activity by calpastatin in response to β_2-agonist administration.

We did not observe any acute or chronic effects of formoterol treatment on proteasome-dependent protein breakdown, which is different from that reported by Busquets *et al.* (2004), who found reduced proteasome-dependent protein breakdown after chronic formoterol treatment in control and cachectic mice, without substantial changes in mRNA expression in proteasome subunits (Busquets *et al.* 2004). Although we observed a reduction in atrogin-1 mRNA expression with formoterol administration on day 7 (but not on days 1 and 28), consistent with the increased Akt signalling, altered mRNA expression did not translate to changes in atrogin-1 protein expression. In addition, MuRF-1 mRNA expression was not affected by formoterol treatment. Our results for MuRF-1 mRNA and atrogin-1 mRNA and protein expression data are consistent with our observation that proteasome activity is unchanged with acute and chronic formoterol treatment in C57BL/6 mice.

Taken together, our data show that acute stimulation of the β-adrenergic signalling pathway can reduce muscle protein breakdown, while prolonged stimulation promotes protein synthesis, findings that highlight how modulating the β-adrenergic signalling pathway is an appropriate therapeutic target for skeletal muscle wasting and weakness due to its dual roles in the mechanisms regulating protein synthesis and degradation. This may be particularly important for muscle wasting conditions that result from activation of Ca^{2+}-dependent protein breakdown, as occurs during the later stages of sepsis (Voisin *et al.* 1996), or for wasting disorders that result from reduced (basal) MPS, such as immobilization (de Boer *et al.* 2007). It has been suggested that a reduced anabolic response (e.g. S6K1 phosphorylation and MPS) to food intake, insulin and exercise in the elderly plays a key role in the aetiology of sarcopaenia (Cuthbertson *et al.* 2005; Rasmussen *et al.* 2006; Kumar *et al.* 2009) and as such, our findings that acute and chronic formoterol treatment effectively stimulates PI3-kinase/Akt signalling supports the contention that stimulation of the β-adrenergic signalling pathway has therapeutic potential for age-related muscle wasting and weakness.

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Author contributions

Acknowledgements