Atomoxetine Prevents Dexamethasone-Induced Skeletal Muscle Atrophy in Mice

Sean R. Jesinkey, Midhun C. Korrapati, Kyle A. Rasbach, Craig C. Beeson, and Rick G. Schnellmann

Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, South Carolina (S.R.J., M.C.K., K.A.R., C.C.B., R.G.S.); and Ralph H. Johnson Veterans Administration Medical Center, Charleston, South Carolina (R.G.S.)

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ABSTRACT

Skeletal muscle atrophy remains a clinical problem in numerous pathologic conditions. β_2 -Adrenergic receptor agonists, such as formoterol, can induce mitochondrial biogenesis (MB) to prevent such atrophy. Additionally, atomoxetine, an FDA-approved norepinephrine reuptake inhibitor, was positive in a cellular assay for MB. We used a mouse model of dexamethasone-induced skeletal muscle atrophy to investigate the potential role of atomoxetine and formoterol to prevent muscle mass loss. Mice were administered dexamethasone once daily in the presence or absence of formoterol (0.3 mg/kg), atomoxetine (0.1 mg/kg), or sterile saline. Animals were euthanized at 8, 16, and 24 hours or 8 days later. Gastrocnemius muscle weights, changes in mRNA and protein expression of peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) isoforms, ATP synthase β , cytochrome *c* oxidase subunit I, NADH dehydrogenase (ubiquinone) 1 β subcomplex, 8, ND1, insulin-like growth factor 1 (IGF-1), myostatin,

muscle Ring-finger protein-1 (muscle atrophy), phosphorylated forkhead box protein O 3a (p-FoxO3a), Akt, mammalian target of rapamycin (mTOR), and ribosomal protein S6 (rp-S6; muscle hypertrophy) in naive and muscle-atrophied mice were measured. Atomoxetine increased p-mTOR 24 hours after treatment in naive mice, but did not change any other biomarkers. Formoterol robustly activated the PGC-1 α -4-IGF1-Akt-mTOR-rp-S6 pathway and increased p-FoxO3a as early as 8 hours and repressed myostatin at 16 hours. In contrast to what was observed with acute treatment, chronic treatment (7 days) with atomoxetine increased p-Akt and p-FoxO3a, and sustained PGC-1 α expression and skeletal muscle mass in dexamethasone-treated mice, in a manner comparable to formoterol. In conclusion, chronic treatment with a low dose of atomoxetine prevented dexamethasone-induced skeletal muscle wasting and supports a potential role in preventing muscle atrophy.

Introduction

Skeletal muscle is remarkably malleable, phenotypically adapting to functional demands. Exercise training is known to induce muscle hypertrophy and is characterized by growth of existing myofibrils (Fluck, 2006). Skeletal muscle atrophy is defined as a decrease in muscle mass that occurs when protein degradation exceeds protein synthesis (Fanzani et al., 2012). Glucocorticoids are well established inducers of catabolism, and numerous pathologic conditions characterized by muscle atrophy (cachexia, chronic kidney disease, metabolic acidosis,

sepsis, diabetes, etc.) are associated with increases in circulating glucocorticoids, suggesting a potential role for them in the development of atrophy (Slee, 2012; Schakman et al., 2013). Clinically, severe muscle atrophy, especially when it occurs concurrently with other chronic disease states, is associated with increased rates of morbidity and mortality (Metter et al., 2002; Pocock et al., 2008; He et al., 2013). Currently, no FDA-approved drugs are available to treat muscle atrophy, underscoring the importance of elucidating atrophy-associated signaling pathways and developing drugs to prevent skeletal muscle atrophy.

Recent reports describe an intricate network of signaling pathways that operate in muscle cells to regulate myofiber size and muscle performance (Bonaldo and Sandri, 2013). These different pathways cross-talk and modulate one another, simultaneously coordinating protein synthesis and degradation. Major pathways leading to atrophy are activation of myostatin, nuclear factor κ B, and the E3-ligase transcription factor forkhead box protein O 3a (FoxO3a), which accelerates protein degradation primarily through activation of muscle atrophy F-box/muscle-specific ubiquitin E3-ligases atrophy

S.R.J. and M.C.K. contributed equally to this work.

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ABBREVIATIONS: ADHD, attention deficit hyperactivity disorder; β_2 -AR, β_2 -adrenergic receptor; EX2, exon 2; FoxO3a, forkhead box protein O 3a; IGF-1, insulin-like growth factor 1; MB, mitochondrial biogenesis; mTOR, mammalian target of rapamycin; MuRF-1, muscle RING-finger protein-1; MYSTN, myostatin; NDUFB8, NADH dehydrogenase (ubiquinone) 1 β subcomplex, 8; NRI, norepinephrine reuptake inhibitor; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 α ; qPCR, quantitative real-time polymerase chain reaction; rp-S6, ribosomal protein S6.

gene-1, and muscle RING-finger protein-1 (MuRF-1) (McPherron et al., 1997; Bodine et al., 2001; Gomes et al., 2001; Lee, 2004; Sacheck et al., 2007; Bonaldo and Sandri, 2013). High-dose glucocorticoid treatment (e.g., dexamethasone) is a standard animal model of skeletal muscle atrophy. Dexamethasone is thought to induce atrophy, in part, by indirectly preventing the activation of FoxO3a and MuRF-1 (Qin et al., 2010; Bonaldo and Sandri, 2013; Schiaffino et al., 2013).

In contrast, skeletal muscle growth is regulated by the insulin-like growth factor 1 (IGF-1)-Akt-mammalian target of rapamycin (IGF-1-Akt-mTOR) pathway (Musaro et al., 2001; Adams, 2002a,b; Schiaffino et al., 2013). Akt stimulates protein synthesis by activating mTOR and its downstream

effector ribosomal protein S6 (rp-S6) (Adams, 2002b; Bonaldo and Sandri, 2013; Schiaffino et al., 2013). In addition, Akt can prevent muscle protein degradation by phosphorylating FoxO3a to prevent nuclear translocation and activation of MuRF-1 (Sandri et al., 2004; Senf et al., 2010).

In vivo and in vitro models have established a role for peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) in preventing muscle wasting (Bonaldo and Sandri, 2013). PGC-1 α is a transcriptional coactivator that regulates the transcription of genes that drive mitochondrial biogenesis (MB) (Wu et al., 1999). Recently, alternative splice variants of the PGC-1 α gene have been identified (Ruas et al., 2012). Each of the individual PGC-1 α isoforms regulates a discrete gene

Fig. 1. PGC-1 α isoform gene expression in skeletal muscle of naive mice after formoterol or atomoxetine treatment. Naive mice were given a single dose of either formoterol (0.3 mg/kg i.p.), atomoxetine (0.1 mg/kg i.p.), or sterile saline (veh i.p.) and euthanized at 8, 16, and 24 hours. Gastrocnemius muscle was excised from animals at each time point and RNA was isolated for qPCR analysis. Total PGC1 α (EX2), PGC1 α 1, and PGC1-1 α 4 at 0, 8, 16, and 24 hours after treatment with formoterol (A–C) or atomoxetine (D–F). Data were normalized to vehicle and represented as a relative fold-change. Data are expressed as means $\pm$ S.E. ($n = 5$). *Significantly different from untreated mice ($P \leq 0.05$).

program. For example, the induction of the *PGC-1 α 1* isoform promotes MB and regulates mitochondrial oxidative phosphorylation gene expression (Mootha et al., 2004; Schreiber et al., 2004). Interestingly, PGC-1 α 1 has also been reported as preventing muscle atrophy by negatively regulating expression of MuRF-1, preventing active nuclear forms of FoxO from binding to the MuRF-1 promoter region (Sandri et al., 2006; Brault et al., 2010; Qin et al., 2010). Treatment with high-dose dexamethasone has been reported to reduce total PGC-1 α protein expression and increase transcription of the MuRF-1 atrogene (Qin et al., 2010). In contrast, the PGC-1 α 4 isoform specifically activates IGF-1 expression and represses myostatin, which was shown to increase muscle mass, strength, and resistance to muscle wasting in a model of cancer cachexia (Ruas et al., 2012).

The benefits of therapeutic intervention with β_2 -adrenergic receptor (β_2 -AR) agonists in animal models of muscle atrophy have been previously established (Sato et al., 2011). β_2 -AR agonists, such as clenbuterol and formoterol, are considered pro-growth and anti-atrophic drugs (Kline et al., 2007). Formoterol induces skeletal muscle hypertrophy through activation of the Akt-mTOR-rp-S6 pathway and prevention of protein degradation (Joassard et al., 2013a). However, chronic administration of high doses of β_2 -AR agonists have been documented as causing adverse cardiovascular effects (Sato et al., 2011). Therefore, drugs that can prevent muscle atrophy in the absence of adverse cardiovascular effects are needed.

Atomoxetine (or tomoxetine) is an FDA-approved drug to treat attention deficit hyperactivity disorder (ADHD) by preventing the neuronal reuptake of norepinephrine (Bymaster et al., 2002). A recent screen to discover compounds that promote MB identified atomoxetine and β -adrenergic receptor agonists as potent inducers of MB (Peterson et al., 2013). Given the previously established links between MB-associated pathways and the prevention of muscle atrophy, we sought to evaluate the role atomoxetine in preventing dexamethasone-induced skeletal muscle atrophy and to identify associated signaling pathways. Efficacy and signaling mechanisms were compared with formoterol, which is well characterized and has previously been used in this model.

Materials and Methods

Animals and Treatments

Male C57BL/6 mice (The Jackson Laboratories, Bar Harbor ME), 6–8 weeks of age (25–30 g), were housed in temperature-controlled conditions under a light/dark photocycle with food and water supplied ad libitum.

Acute Treatment Details. Groups of naive mice were injected intraperitoneally with a single dose of sterile saline, 0.3 mg/kg of formoterol fumarate dihydrate (Sigma-Aldrich, St. Louis, MO) or 0.1 mg/kg atomoxetine (Tocris Bioscience, Bristol, UK). Animals were euthanized at 8, 16, and 24 hours after treatments.

Dexamethasone-Induced Muscle Atrophy Model. One group of mice was co-injected intraperitoneally with sterile saline daily for 7 days.

Fig. 2. IGF-1 and myostatin gene expression in skeletal muscle of naive mice after formoterol or atomoxetine treatment. Mice were treated as described in Fig. 1. Expression of IGF-1 and myostatin at 0, 8, 16, and 24 hours after treatment with formoterol (A and B) or atomoxetine (C and D). Data were normalized to vehicle and represented as a relative fold-change. Data are expressed as means $\pm$ S.E. ($n = 5$). *Significantly different from untreated mice ($P \leq 0.05$).

Three groups of mice were coinjected intraperitoneally with 25 mg/kg water-soluble dexamethasone (Sigma-Aldrich) followed by a second injection of sterile saline, 0.3 mg/kg of formoterol, or 0.1 mg/kg atomoxetine, respectively, daily for 7 days. Animals were euthanized on the 8th day.

Body weights were measured and gastrocnemius muscles were removed, weighed, and flash-frozen for mRNA and protein analysis. All animal and treatment protocols were in compliance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and were approved by our Institutional Animal Care and Use Committee.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from mouse gastrocnemius tissue samples using TRIzol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. cDNA was synthesized via reverse transcription using the iScript Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA) with 5 μ g of RNA. Quantitative real-time polymerase chain reaction (qPCR) analysis was performed with cDNA and carried out using 5 μ l of cDNA template combined with Brilliant II SYBR Green master mix (Stratagene/Agilent Technologies, La Jolla, CA) at a final concentration of 1 $\times$ and primers (Integrated DNA Technologies, Inc., Coralville, IA) at a concentration of 400 nM. mRNA expression of all genes was calculated using the $2^{-\Delta\Delta CT}$ method normalized to β -actin. Primer sequences are as follows:

Total PGC-1 α (EX2) (FW: 5'-TGA TGT GAA TGA CTT GGA TAC AGA CA-3', REV: 5'-GCT CAT TGT TGT ACT GGT TGG ATA TG-3'),
PGC-1 α 1 (FW: 5'-GGA CAT GTG CAG CCA AGA CTC T-3', REV: 5'-CAC TTC AAT CCA CCC AGA AAG CT-3'),
PGC-1 α 4 (FW: 5'-TCA CAC CAA ACC CAC AGA AA-3', REV: 5'-CTG GAA GAT ATG GCA CAT-3'),
Myostatin (FW: 5'-AGT GGA TCT AAA TGA GGG CAG T-3', REV: 5'-GTT TCC AGG CGC AGC TTA-3'),
IGF-1 (FW: 5'-TGC TCT TCA GTT CGT GTG-3', REV: 5'-ACA TCT CCA GTC TCC TCA G-3'),
 β -Actin (FW: 5'-GGG ATG TTT GCT CCA ACC AA-3', REV: 5'-GCG CTT TTG ACT CAG GAT TTA-3').

Mitochondrial DNA Content

qPCR was used to measure the relative quantity of mtDNA in mouse gastrocnemius tissue samples. After treatment, DNA was extracted from tissue using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and 5 ng of DNA was used for qPCR. ND1 (FW: 5'-TAG AAC GCA AAA TCT TAG GG-3', REV: 5'-TGC TAG TGT GAG TGA TAG GG-3') was used as the mitochondrial gene, and expression was normalized to nuclear-encoded β -actin expression.

Immunoblot Analysis

Mouse gastrocnemius skeletal muscle tissue was homogenized in 5 volumes of protein lysis buffer (1% Triton X-100, 150 mM NaCl,

Fig. 3. Acute effects of atomoxetine and formoterol on protein synthesis signaling mechanisms in skeletal muscle of naive mice. Mice were treated as described in Fig. 1. Representative immunoblots for markers of muscle protein homeostasis: total and phosphorylated forms of Akt-mTOR-rp-S6 axis and FoxO3a at 0, 8, and 24 hours after either formoterol (A) or atomoxetine (F). Densitometric analysis of FoxO3a (B), Akt (C), mTOR (D), and rp-S6 (E) $\pm$ formoterol; densitometric analysis of FoxO3a (G), Akt (H), mTOR (I), and rp-S6 (J) $\pm$ atomoxetine. Data were normalized to vehicle and represented as relative fold-change. Data are expressed as means $\pm$ S.E. ($n = 4$). *Significantly different from untreated mice ($P \leq 0.05$).

10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA; 2 mM sodium orthovanadate; 0.2 mM phenylmethylsulfonyl fluoride; 1 mM HEPES, pH 7.6; 1 μ g/ml leupeptin; and 1 μ g/ml aprotinin) using a Polytron homogenizer. The homogenate was stored on ice for 10 minutes and then centrifuged at 7500g for 5 minutes at 4°C. The supernatant was collected, and protein was measured with a bicinchoninic acid kit (Sigma-Aldrich) with bovine serum albumin as the standard. Proteins (50–75 μ g) were separated on 4–20% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked either in 5% dried milk or bovine serum albumin in 0.1% Tween 20 in 1× Tris-buffered saline and incubated with 1:1000 antibody dilutions of MuRF1 (ECM Biosciences, Versailles, KY); anti-PGC-1 α (EMD Millipore, Billerica, MA); anti-ATP synthase β , COX-1 (Abcam, Cambridge, MA); anti-NADH dehydrogenase (ubiquinone) 1 β subcomplex, 8 (NDUFB8; Invitrogen); total and phosphorylated anti-FoxO3a, Akt, mTOR, rp-S6 (Cell Signaling Technologies, Danvers, MA); and anti-glyceraldehyde 3-phosphate dehydrogenase (Fitzgerald, Acton, MA) overnight at 4°C. After incubation for 2 hours at room temperature with secondary antibodies (1:2000) conjugated with horseradish peroxidase, membranes were detected using chemiluminescence.

Data and Statistical Analysis

Data are expressed as means $\pm$ S.E. ($n = 4$ –5) for all experiments. Multiple comparisons of normally distributed data were analyzed by one-way analysis of variance, as appropriate, and group means were compared using the Student-Newman-Keuls post-hoc test. Single comparisons were analyzed with the Student's t test where appropriate. The criterion for statistical differences was $P \leq 0.05$ for all comparisons.

Results

Acute Treatment with Formoterol, but Not Atomoxetine, Differentially Modulates PGC-1 α Isoform Expression in Skeletal Muscle of Naive Mice. PGC-1 α 1 has been described as a “master regulator” of MB (Pearen et al., 2008, 2009; Wills et al., 2012). In contrast, PGC-1 α 4, a recently discovered PGC-1 α splice variant, induces a discrete gene program resulting in muscle hypertrophy but not MB (Ruas

et al., 2012). It is important to note that all variants of the PGC-1 α gene identified by Ruas et al. (2012) contain the exon 2 (EX2) region. Therefore, primer sequences that contain this region measure total PGC-1 α expression (all isoforms). Because identification of the specific PGC-1 α isoform induced by formoterol or atomoxetine has not been identified, we evaluated the effects of acute formoterol or atomoxetine treatment on PGC-1 α isoform mRNA in skeletal muscle of naive mice.

Formoterol caused a 12-fold induction of total PGC-1 α (EX2, representative of all PGC-1 α isoforms) at 8 hours post-treatment, which decreased and returned to baseline at 24 hours (Fig. 1A). PGC-1 α 1 gene expression was suppressed at 8 hours post-treatment with formoterol and returned to control levels by 24 hours (Fig. 1B). Formoterol induced PGC-1 α 4 gene expression at 8 hours after treatment (6-fold increase over vehicle) and returned to control levels by 24 hours (Fig. 1C). In contrast, atomoxetine had no effect on EX2 or PGC-1 α 1 and expression of PGC-1 α 4 was decreased 20–25% at 16 and 24 hours after treatment (Fig. 1, D–F).

Formoterol, but Not Atomoxetine, Treatment Acutely Increases IGF-1 Gene Expression and Suppresses Myostatin. PGC-1 α 4 regulates a discrete gene program responsible for inducing skeletal muscle hypertrophy via induction of IGF-1 and suppression of myostatin (Ruas et al., 2012). Formoterol increased IGF-1 gene expression 2-fold at 8 hours post-treatment, which returned to baseline at 24 hours (Fig. 2A). In addition, formoterol suppressed myostatin (MYSTN) gene expression 50% at 16 hours post-treatment (Fig. 2B). Atomoxetine did not alter IGF-1 or MYSTN (Fig. 2, C and D).

Atomoxetine Acutely Increases Phosphorylated mTOR Protein Expression. Formoterol induces skeletal muscle hypertrophy through the Akt-mTOR-rp-S6 pathway (Joassard et al., 2013a,b). In addition, formoterol prevents muscle atrophy via phosphorylation (p) of FoxO3a (Joassard et al., 2013a). Formoterol increased protein expression of p-FoxO3a, p-Akt, p-mTOR and p-rp-S6 8 hours after treatment (Fig. 3,

Fig. 4. Proposed pathways of formoterol action in the skeletal muscle of mice after acute treatment. Acute formoterol (Form) treatment leads to alternative splicing of the PGC-1 α gene resulting in higher PGC-1 α 4 expression. PGC-1 α 4 induces IGF-1 transcription and protein production, which acts via autocrine signaling to activate the IGF-1R. The IGF-1R initiates phosphorylation (P) of the Akt-mTOR-rp-S6 axis and hypertrophy. Inactivation of atrophy is mediated by Akt phosphorylation of FoxO3a and PGC-1 α 4 suppression of MYSTN gene expression. Alternatively, phosphorylation of Akt may result from β_2 -AR stimulation. Solid shapes represent proteins and helices within the nucleus represent genes, solid black arrows are pathways suggested by our data and supported by the literature. A dotted line represents a potential alternative mechanism and a “?” denotes that future experiments are warranted for full elucidation. All arrows represent activation, with the exception of the arrow from Akt to p-FoxO3a, where phosphorylation inactivates FoxO3a. Lines with blunted ends represent inhibition.

A–E). Atomoxetine increased p-mTOR protein expression 24 hours after treatment without altering rp-S6 phosphorylation (Fig. 3, F–J). These results reveal acute formoterol treatment activates hypertrophy pathways via PGC-1 α 4-mediated increases in IGF-1 expression and represses MYSTN expression downstream of p-FoxO3a, p-Akt, p-mTOR and p-S6 (Fig. 4). In contrast, atomoxetine had minimal or no effects on these pathways with the exception of a modest increase in p-mTOR at 24 hours.

Chronic Treatment with Formoterol and/or Atomoxetine Restores Mitochondrial Proteins, Increases Hypertrophy Markers, and Preserves Gastrocnemius Muscle Mass in Mice with Dexamethasone-Induced Muscle Atrophy. Using a previously described model of skeletal muscle atrophy (Wada et al., 2011), chronic treatment with dexamethasone resulted in a 17% reduction in gastrocnemius muscle mass, and low-dose formoterol or atomoxetine prevented this loss (Fig. 5A). After seven daily doses, neither

dexamethasone nor atomoxetine treatment had any effect on the expression of PGC-1 α 1 mRNA. However, formoterol increased PGC-1 α 1 mRNA content (Fig. 5B) but had no effect on mtDNA copy number, as measured by ND1 gene expression (Fig. 5C). Dexamethasone reduced PGC-1 α protein 1 week after treatment. In contrast, concomitant treatment of dexamethasone with either formoterol or atomoxetine sustained PGC-1 α protein (Fig. 5, D and E). ATP synthase β and mitochondrial cytochrome *c* oxidase subunit I (COX I) protein expression increased after treatment with formoterol in dexamethasone-treated mice, but there was no effect on NDUFB8 (Fig. 5D). Atomoxetine did not alter mitochondrial protein expression in dexamethasone-treated mice (Fig. 5E). These results reveal that prevention of PGC-1 α protein loss by formoterol leads to partial MB with an increase in mitochondrial proteins, whereas atomoxetine prevents PGC-1 α protein loss but does not induce MB.

Fig. 5. Chronic effects of atomoxetine and formoterol on skeletal muscle mass and mitochondrial proteins in dexamethasone-treated mice. Mice were coadministered a daily dose of 25 mg/kg water soluble-dexamethasone $\pm$ 0.3 mg/kg of formoterol/0.1 mg/kg of atomoxetine or sterile saline (i.p.) for 7 days. Appropriate saline controls were maintained throughout the experiment. Animals were euthanized after 8 days and gastrocnemius muscle was isolated from both right and the left hind limbs. (A) Normalized gastrocnemius muscle mass 8 days after treating dexamethasone-dosed mice with saline, formoterol, or atomoxetine. Gene expression analysis for PGC-1 α 1 (B) and ND1 (C). Representative immunoblots and respective densitometry for mitochondrial proteins: total PGC1 α 1, ATP synthase β , COX-1, and NDUFB8 8 days after either formoterol [BD 0.3 mg/kg; (D)] or atomoxetine [0.1 mg/kg; (E)]. Data were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as means $\pm$ S.E. ($n = 4-5$). *Significantly different from either saline-treated controls or all other groups of mice ($P \leq 0.05$). #Significantly different from dexamethasone-treated mice ($P \leq 0.05$).

Fig. 6. Chronic effects of atomoxetine and formoterol on hypertrophy and atrophy-associated proteins in skeletal muscle of dexamethasone-treated mice. Mice were treated as described in Fig. 5. qPCR analysis of gene expression for proteins of muscle hypertrophy, PGC-1 α 4 (A), IGF-1 (B), and atrophy, MYSTN (C). Data were normalized to vehicle and represented as a relative fold-change. Data are expressed as means $\pm$ S.E. ($n = 5$). *Significantly different from untreated mice ($P \leq 0.05$).

IGF-1 gene expression increased with formoterol in dexamethasone-treated mice despite a decrease in the PGC-1 α 4 isoform (Fig. 6, A and B). Atomoxetine had no effect on PGC-1 α 4, IGF-1, or MYSTN in dexamethasone-treated mice (Fig. 6, A–C). Formoterol and atomoxetine consistently increased phosphorylation of Akt in dexamethasone-treated mice (Fig. 7). However, only formoterol increased Akt and mTOR phosphorylation and, surprisingly, decreased p-rp-S6 (Fig. 7B). Atomoxetine markedly increased phosphorylation of FOXO3a but did not affect phosphorylation of mTOR or rp-S6 (Fig. 7). Formoterol and atomoxetine prevented the increase in protein expression of MuRF-1 in dexamethasone-treated mice (Fig. 8).

Discussion

Recently, alternative splice variants of PGC-1 α have been identified (Ruas et al., 2012), each regulating a discrete gene program. Specifically, induction of the PGC-1 α 1 isoform regulates MB, whereas PGC-1 α 4 induces muscle hypertrophy independent of MB (Ruas et al., 2012). A low dose of formoterol (0.3 mg/kg), a long acting β_2 -adrenergic receptor agonist, stimulated alternative splicing of the PGC-1 α gene, increasing PGC-1 α 4 expression and suppressing PGC-1 α 1. The induction of PGC-1 α 4 was associated with the suppression of myostatin and the activation of IGF-1–Akt–mTOR–rp-S6 pathway in acute naive animals. In unpublished studies, we observed that ICI 118,551, a highly selective β_2 -adrenergic receptor antagonist, blocked formoterol-induced increases in PGC-1 α 4 at 16 hours (data not shown). Thus, low-dose formoterol

promotes skeletal muscle hypertrophy and prevents protein turnover via the β_2 -adrenergic receptor.

The observed alterations in the phosphorylation status of FoxO3a, PGC-1 α 1 protein expression, and the activation of the Akt–mTOR–rp-S6 axis were similar to previously reported data (Pearen et al., 2009; Koopman et al., 2010; Qin et al., 2010). Prior to the discovery of the PGC-1 α 4 isoform, Pearen et al. (2008, 2009) reported an increase in total PGC-1 α gene expression approximately 8 hours post-treatment with formoterol and observed no change in myostatin in the tibialis anterior of naive C57BL/6 mice. In contrast, we found that upregulation of total PGC-1 α (EX2) gene expression observed at 8 hours was driven primarily by PGC-1 α 4 and not the PGC-1 α 1 isoform. PGC-1 α 4 is an inducer of IGF-1 (Ruas et al., 2012), which then functions to repress myostatin gene expression. All PGC-1 α splice variants contain the exon 2 region and this may explain the disparity between our data and that of (Pearen et al., 2009). Specifically, primers spanning EX2 were incorporated in their studies. Also, the divergence in findings may be attributable to model dissimilarities, including types of skeletal muscle tissue analyzed.

Recently, we reported that atomoxetine, an FDA-approved norepinephrine reuptake inhibitor (NRI) to treat ADHD, stimulates MB in a high throughput screening assay with renal proximal tubules cells (Peterson et al., 2013). Subsequent cheminformatic profiling of the β_2 -adrenergic receptor agonists and nisoxetine and atomoxetine led to the identification of four chemical moieties shared by atomoxetine and formoterol (Peterson et al., 2013), suggesting that atomoxetine and nisoxetine may

Fig. 7. Chronic effects of atomoxetine and formoterol on protein-synthesis signaling mechanisms in skeletal muscle of dexamethasone-treated mice. Mice were treated as described in Fig. 5. Representative immunoblots and densitometric analysis for markers of muscle protein homeostasis: total and phosphorylated forms of FoxO3a, Akt, mTOR, and ribosomal protein rp-S6 after respective treatments with either formoterol [0.3 mg/kg; (A and B)] or atomoxetine [0.1 mg/kg; (C and D)]. Data were normalized to vehicle and represented as a relative fold-change. Data are expressed as means $\pm$ S.E. ($n = 4-5$). *Significantly different from either saline-treated controls or all other groups of mice ($P \leq 0.05$).

act directly on the β_2 -adrenergic receptor. Other studies have shown that atomoxetine may activate β -adrenergic receptors indirectly by increasing norepinephrine (Springer et al., 1994; Mirbolooki et al., 2013). Despite chemical similarity to

formoterol, acute atomoxetine treatment in naive mice did not increase phosphorylated FoxO3a, activate the Akt-mTOR-rp-S6 axis, or induce skeletal muscle hypertrophy. Additionally, short-term exposure to atomoxetine did not appear to

Fig. 8. Chronic effects of atomoxetine and formoterol on muscle atrophy markers in skeletal muscle of dexamethasone-treated mice. Mice were treated as described in Fig. 5. Representative immunoblots and densitometric analysis for markers of skeletal muscle protein breakdown: MuRF-1 after respective treatments with either formoterol [Form; 0.3 mg/kg; (A)] or atomoxetine [0.1 mg/kg; (B)]. Data were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as means $\pm$ S.E. ($n = 4-5$). *Significantly different from saline-treated controls ($P \leq 0.05$). #Significantly different from dexamethasone-treated mice ($P \leq 0.05$).

Fig. 9. Proposed mechanisms preventing muscle atrophy associated with chronic treatment of atomoxetine or formoterol in skeletal muscle of dexamethasone-treated mice. Chronic formoterol (Form) (A) treatment sustained PGC-1 α gene and protein expression, leading to increased oxidative phosphorylation proteins. IGF-1 gene expression also increased at this time, but what drives its expression is uncertain (?). Formoterol sustained an increase in phosphorylation of the Akt-mTOR axis, indicating activation and a possible mechanism for protein synthesis. Atomoxetine (Atomox) (B) sustained p-Akt but could not activate the entire axis. In both treatment groups, concomitant inactivation of FoxO3a occurred, as depicted by increased phosphorylation with suppression of MuRF-1 gene expression, blocking a major mechanism for protein degradation. In addition, increased PGC-1 α observed with either treatment may block the transcription of MuRF-1 and represent an antiatrophic mechanism. Red lines shown are proposed mechanisms for protein degradation with dexamethasone treatment operating through the glucocorticoid receptor (GR). Refer to Fig. 4 for further description of the diagram.

modulate PGC-1 α 1/4 gene expression but did increase p-mTOR, as did formoterol. The interpretation of this finding is difficult given that there are numerous upstream modulators and

downstream effectors of mTOR activation. On the basis of these data, we surmise that atomoxetine is not associated with the direct activation of the β -adrenergic receptor and that downstream

signaling is elicited through an alternative pathway, perhaps related to its NRI pharmacology.

In an atrophy model, treatment with dexamethasone resulted in the activation of atrophic pathways, such as decreases in p-FoxO3a and increased MuRF-1 expression. Dexamethasone treatment also inhibited mTOR, thereby disrupting hypertrophic effects of the IGF-1–Akt–mTOR–rp-S6 axis downstream of IGF-1 (Wang et al., 2006). We observed no change in mTOR or rp-S6 in dexamethasone-treated animals, but we observed evidence of atrophic activation through FoxO3a and increased expression of MuRF-1. We hypothesize that skeletal muscle atrophy in our dexamethasone model was attributable to atrophic mechanisms and not the inactivation of the IGF-1–Akt–mTOR–rp-S6 axis.

As expected, treatment with dexamethasone suppressed PGC-1 α 1 expression at 8 days; however, concomitant treatment with both formoterol or atomoxetine maintained PGC-1 α 1 levels equal to that of controls. Interestingly, PGC-1 α 4 expression declined with formoterol treatment, confirming that formoterol can cause differential expression of these alternatively spliced variants. Despite sustained expression of PGC-1 α 1, MB was initiated but remained incomplete, as only some mitochondrial proteins increased with formoterol treatment.

It is important to note that p-Akt prevents the dephosphorylation of FoxO3a, limiting its entry into the nucleus and the subsequent induction of MuRF-1 (Sandri et al., 2006). Furthermore, transcriptional activity of nuclear FoxO3a can be inhibited by PGC-1 α 1 (Sandri et al., 2006). Our observations of increased p-FoxO3a and decreased MuRF-1 with atomoxetine or formoterol treatment provide evidence that atomoxetine and formoterol have antiatrophic properties. Our findings in the dexamethasone study are summarized for formoterol (Fig. 9A) and atomoxetine (Fig. 9B) in a diagram.

Because atomoxetine is an NRI (Bymaster et al., 2002) and norepinephrine has been demonstrated to modulate PGC-1 α 1 through the β_2 -adrenergic receptor (Wu et al., 1999), it is plausible that indirect β_2 -adrenergic receptor agonism by atomoxetine through norepinephrine may explain our results. However, norepinephrine is a less potent stimulator of the β_2 -adrenergic receptor than formoterol, and downstream signaling events may be more susceptible to internal regulation, a concept that would explain the lack of observable changes in naive mice.

In summary, we have identified formoterol as a potent inducer of skeletal muscle hypertrophy, which is associated with concomitant increases in PGC-1 α 4 and IGF-1, downregulation of myostatin, and activation of the Akt–mTOR–rp-S6 axis. Formoterol also prevented catabolism, as evidenced by a decrease in MuRF-1. In addition, we report that atomoxetine, used at a dose lower than that clinically approved for ADHD, was efficacious in the prevention of skeletal muscle atrophy in a model of dexamethasone-induced muscle atrophy. Furthermore, atomoxetine prevented muscle atrophy by sustaining PGC-1 α 1 expression, activating Akt, increasing p-FoxO3a, and suppressing MuRF-1. Additional experiments are warranted to define the full dose response of atomoxetine.

Although β_2 -adrenergic receptor agonists are potent catabolic agents, their potential clinical success for skeletal muscle atrophy is limited by their potential cardiac side effects (Carbo et al., 1997; Soppa et al., 2005; Ryall et al., 2006; Pearen et al., 2009). Atomoxetine did not stimulate skeletal muscle hypertrophy

in either of the models tested but was efficacious in preventing atrophy in the dexamethasone model. These characteristics suggest that atomoxetine has potential for therapeutic use in preventing skeletal muscle atrophy.

Authorship Contributions

Participated in research design: Jesinkey, Korrapati, Rasbach, Beeson, Schnellmann.

Conducted experiments: Jesinkey, Korrapati.

Performed data analysis: Jesinkey, Korrapati, Schnellmann.

Wrote or contributed to the writing of the manuscript: Jesinkey, Korrapati, Schnellmann.

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Address correspondence to: Dr. Rick G. Schnellmann, Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, 280 Calhoun St., MSC140, Charleston, SC 29425. E-mail: schnell@muscc.edu