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Regulation of Akt-mTOR, ubiquitin-proteasome and autophagy-lysosome pathways in response to formoterol administration in rat skeletal muscle

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ABSTRACT

Administration of β_2-agonists triggers skeletal muscle anabolism and hypertrophy. We investigated the time course of the molecular events responsible for rat skeletal muscle hypertrophy in response to 1, 3 and 10 days of formoterol administration (i.p. 2000 μg/kg/day). A marked hypertrophy of rat *tibialis anterior* muscle culminated at day 10. Phosphorylation of Akt, ribosomal protein S6, 4E-BP1 and ERK1/2 was increased at day 3, but returned to control level at day 10. This could lead to a transient increase in protein translation and could explain previous studies that reported increase in protein synthesis following β_2-agonist administration. Formoterol administration was also associated with a significant reduction in *MAFbx/atrogin-1* mRNA level (day 3), suggesting that formoterol can also affect protein degradation of MAFbx/atrogin1 targeted substrates, including MyoD and eukaryotic initiation factor-3f (eIF3-f). Surprisingly, mRNA level of autophagy-related genes, *light chain 3 beta (LC3b)* and *gamma-aminobutyric acid receptor-associated protein-like 1 (Gabarapl1)*, as well as lysosomal hydrolases, *cathepsin B* and *cathepsin L*, was significantly and transiently increased after 1 and/or 3 days, suggesting that autophagosome formation would be increased in response to formoterol administration. However, this has to be relativized since the mRNA level of *Unc-51-like kinase1 (Ulk1)*, *BCL2/adenovirus E1B interacting protein3 (Bnip3)*, and *transcription factor EB (TFEB)*, as well as the protein content of Ulk1, Atg13, Atg5–Atg12 complex and p62/Sqstm1 remained unchanged or was even decreased in response to formoterol administration. These results demonstrate that the effects of formoterol are mediated, in part, through the activation of Akt-mTOR pathway and that other signaling pathways become more important in the regulation of skeletal muscle mass with chronic administration of β_2-agonists.

Abbreviations: Actb, actin beta; Atg, autophagy-related; Bnip3, BCL2/adenovirus E1B interacting protein 3; BSA, bovine serum albumin; COPD, chronic obstructive pulmonary disease; CREB, cAMP response element binding protein; EDL, *extensor digitorum longus*; eIF3-f, eukaryotic initiation factor 3 subunit f; Epac, exchange protein activated directly by cAMP; ERK, extracellular signal-regulated kinase; FoxO3, forkhead box O3; Gabarapl1, gamma-aminobutyric acid receptor-associated protein; GAS, *gastrocnemius*; Gαi, α, -subunit of the inhibitory G protein; Gαq, α, -subunit of the Gq protein; Gαs, α, -subunit of the stimulatory G protein; Hprt, hypoxanthine guanine phosphoribosyl transferase; IGF-1, insulin growth factor-1; IRS-1, insulin receptor substrate-1; LC3b, light chain 3 beta; MEF2, myocyte enhancer factor 2; mTOR, mammalian target of rapamycin; MuRF1, muscle RING finger-1; p62/Sqstm1, sequestosome 1; PE, phosphatidylethanolamine; PI3 K, phosphatidylinositol 3-kinase; PGC-1α, peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α; PKA, protein kinase A; Rap, ras-related protein; rpS6, ribosomal protein S6; SOL, *soleus*; TA, *tibialis anterior*; TBS, tris-buffered saline; TFEB, transcription factor EB; TSC2, tuberous sclerosis complex 2; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; Ulk1, unc-51-like kinase 1.

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1. Introduction

β_2-adrenoceptor-selective agonists (β_2-agonists) are derived from the chemical structure of adrenaline. They are traditionally used for the treatment of bronchospasm associated with asthma and the treatment of symptomatic patient with chronic obstructive pulmonary disease (COPD) to induce a bronchodilatation by relaxing airway smooth muscle (Cazzola et al., 1997). However, when experimentally used in animals, chronic administration of β_2-agonists is also known to trigger a gain in skeletal muscle mass. For instance, administration of formoterol (100 μg/kg/day), clenbuterol (2000 μg/kg/day) or fenoterol (2800 μg/kg/day) for 28 days increases skeletal muscle mass by about 20–30% (Gehrig et al., 2010; Koopman et al., 2010; Ryall et al., 2002). Even lower doses, such as 1 μg/kg/day of formoterol for 28 days (Ryall et al., 2006), as well as shorter duration treatment (2000 μg/kg/day of formoterol for 7 days), also triggers skeletal muscle hypertrophy (Busquets et al., 2004). β_2-agonist chronic administration can also attenuate skeletal muscle mass loss induced by different experimental settings including denervation (Zeman et al., 1987), hindlimb unloading (Yimlamai et al., 2005), and dexamethasone treatment (Agbenyega and Wareham, 1992), as well as in several muscle wasting conditions such as neuromuscular diseases and chronic diseases (see Joassard et al., 2013).

The hypertrophy and anti-atrophy effects of β_2-agonists can be explained by their actions on the balance between protein synthesis and protein degradation. Previous studies indicate that chronic administration of β_2-agonists not only increases muscle protein synthesis (Busquets et al., 2004; Emery et al., 1984; Koopman et al., 2010; MacLennan and Edwards, 1989; Navegantes et al., 2004), but also decreases muscle protein degradation (Busquets et al., 2004; Navegantes et al., 2000, 2001), thus leading to a net positive nitrogen balance. Activation of the Akt-mammalian target of rapamycin (mTOR) pathway stimulates protein translation in skeletal muscle (Bodine et al., 2001b; Rommel et al., 2001) and inhibits protein degradation via the inhibition of both ubiquitin-proteasome (Bodine et al., 2001a; Sandri et al., 2004; Stitt et al., 2004) and autophagy-lysosome pathways (Mammucari et al., 2007; Zhao et al., 2007). The activation of Akt-mTOR pathway and the subsequent inhibition of ubiquitin-proteasome pathway have been documented in response to β_2-agonist administration (Kline et al., 2007; Koopman et al., 2010; Sneddon et al., 2001), but the kinetic of this response in relation to the regulation of ubiquitin-proteasome and autophagy-lysosome pathways is currently unknown. This is of particular importance since prolonged β_2-adrenoceptor stimulation can lead to receptor desensitization and decrease β_2-adrenoceptor density (Beitzel et al., 2004; Rothwell et al., 1987; Ryall et al., 2002, 2004, 2006), thus leading to rapid dampening of receptor function and down-regulation of Akt-mTOR pathway. This also raises the question of the existence of other mechanisms that would be activated to sustain the hypertrophy. Finally, it is currently unknown whether β_2-adrenoceptor stimulation regulates autophagy-lysosome pathway in skeletal muscle.

The aim of this study was therefore to provide a detailed analysis of the molecular events and signaling pathways involved in the hypertrophy triggered by 1, 3 and 10 days of β_2-adrenoceptor stimulation by formoterol. Formoterol was used because unlike to the first generation of β_2-agonists like clenbuterol, the addition of a long carbon chain containing a second benzene ring confers a rapid onset and long duration of action (van Noord et al., 1996). In this study, we also examined the hypothesis that activation of Akt-mTOR signaling pathway in response to formoterol administration is temporally associated with the regulation of both ubiquitin-proteasome and autophagy-lysosome pathways.

2. Materials and methods

2.1. Ethical approval of the procedure

Experiments were approved by Jean Monnet University Animal Care and Use Committee, and were carried out in accordance with the European Community guidelines for the use of laboratory animals.

2.2. Animals

Thirty-four male Wistar rats (224 ± 19 g) were purchased from Charles River Laboratories (L'Arbresle, France). The animals were maintained under a constant 12 h light-dark cycle with food and water *ad libitum*. Rats were allocated into a control group ($n = 10$) or a formoterol-treated group (3 groups, $n = 6$–10/group). Formoterol-treated animals received daily intraperitoneal (i.p.) injections of 2000 μg/kg of formoterol (Sigma-Aldrich, l'Isle d'Abeau, France) in 0.9% endotoxin free NaCl for 1, 3 and 10 days. Control rats were injected with the saline solution for 10 days. Body weight and food intake were measured daily.

2.3. Tissue removal

Tissue removal was realized 24 h after the last formoterol injection. At the indicated time points, animals were anesthetized (i.p. injection of 90 mg/kg ketamine and 10 mg/kg xylazine). *Tibialis anterior* (TA) muscle was carefully dissected and trimmed of tendons and any non-muscle tissue and weighed. Central portions of right TA muscle were mounted in embedding medium and frozen in thawing isopentane and stored at −80 °C for subsequent analysis. The remaining right TA muscle and the entire left TA muscle were rapidly frozen in liquid nitrogen and stored at −80 °C for later analyses. *Soleus*, *extensor digitorum longus* (EDL), and *gastrocnemius* muscles were carefully removed and weighed. Rats were sacrificed as a consequence of heart excision, while deeply anesthetized.

2.4. Histomorphometric analysis

TA muscle transverse sections (12 μm) of rats treated with 2000 μg/kg of formoterol for 10 days were cut in a refrigerated (−20 °C) cryostat (Microm HM 560, Thermo Fisher Scientific, Brebières, France), and stained with hemalun-eosin-safran. Eight to 11 photographs covering the entire muscle section were used to determine the whole muscle cross-sectional area. Five fields consistently positioned across muscle sections were chosen. The cross-sectional area of 1062 ± 309 fibers per muscle was determined. Digital images of stained sections were captured using an upright light microscope (Eclipse E400, Nikon, Badhoevedorp, The Netherlands) connected to a digital camera (Coolpix 990, Nikon). Images were quantified using ImageJ analysis software.

2.5. RNA isolation, cDNA synthesis and real-time PCR

Total RNA was extracted from 20–30 mg of TA muscle using the RNeasy Fibrous tissue mini kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. cDNA was generated from 400 ng of RNA using iScript cDNA synthesis kit (Bio-Rad, Marnes-la-Coquette, France). The selected forward and reverse primer sequences are listed in Table 1. Real time PCR was performed in a 20 μl final volume and optimized concentrations for each primer using the SsoFast EvaGreen Super mix (Bio-Rad) and a CFX96 Real Time PCR Detection System, C1000 Thermal Cycler (Bio-Rad). Two reference genes, *actin beta* (*Actb*) and *hypoxanthine guanine phosphoribosyl transferase* (*Hprt*), were used to normalize the