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Beta$_2$-adrenoceptor agonist salbutamol increases protein turnover rates and alters signalling in skeletal muscle after resistance exercise in young men

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Key points

- Animal models have shown that beta$_2$-adrenoceptor stimulation increases protein synthesis and attenuates breakdown processes in skeletal muscle. Thus, the beta$_2$-adrenoceptor is a potential target in the treatment of disuse-, disease- and age-related muscle atrophy.
- In the present study, we show that a few days of oral treatment with the commonly prescribed beta$_2$-adrenoceptor agonist, salbutamol, increased skeletal muscle protein synthesis and breakdown during the first 5 h after resistance exercise in young men.
- Salbutamol also counteracted a negative net protein balance in skeletal muscle after resistance exercise.
- Changes in protein turnover rates induced by salbutamol were associated with protein kinase A-signalling, activation of Akt2 and modulation of mRNA levels of growth-regulating proteins in skeletal muscle.
- These findings indicate that protein turnover rates can be augmented by beta$_2$-adrenoceptor agonist treatment during recovery from resistance exercise in humans.

Abstract The effect of beta$_2$-adrenoceptor stimulation on skeletal muscle protein turnover and intracellular signalling is insufficiently explored in humans, particularly in association with exercise. In a randomized, placebo-controlled, cross-over study investigating 12 trained men, the effects of beta$_2$-agonist (6×4 mg oral salbutamol) on protein turnover rates, intracellular signalling and mRNA response in skeletal muscle were investigated 0.5–5 h after quadriceps

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resistance exercise. Each trial was preceded by a 4-day lead-in treatment period. Leg protein turnover rates were assessed by infusion of [$^{13}C_6$]-phenylalanine and sampling of arterial and venous blood, as well as vastus lateralis muscle biopsies 0.5 and 5 h after exercise. Furthermore, myofibrillar fractional synthesis rate, intracellular signalling and mRNA response were measured in muscle biopsies. The mean (95% confidence interval) myofibrillar fractional synthesis rate was higher for salbutamol than placebo [0.079 (95% CI, 0.064 to 0.093) *vs.* 0.066 (95% CI, 0.056 to 0.075%) × h^{-1}] ($P < 0.05$). Mean net leg phenylalanine balance 0.5–5 h after exercise was higher for salbutamol than placebo [3.6 (95% CI, 1.0 to 6.2 nmol) × min^{-1} × 100 $g_{Leg\ Lean\ Mass}{}^{-1}$] ($P < 0.01$). Phosphorylation of Akt2, cAMP response element binding protein and PKA substrate 0.5 and 5 h after exercise, as well as phosphorylation of eEF2 5 h after exercise, was higher ($P < 0.05$) for salbutamol than placebo. Calpain-1, Forkhead box protein O1, myostatin and Smad3 mRNA content was higher ($P < 0.01$) for salbutamol than placebo 0.5 h after exercise, as well as Forkhead box protein O1 and myostatin mRNA content 5 h after exercise, whereas ActivinRIIB mRNA content was lower ($P < 0.01$) for salbutamol 5 h after exercise. These observations suggest that beta$_2$-agonist increases protein turnover rates in skeletal muscle after resistance exercise in humans, with concomitant cAMP/PKA and Akt2 signalling, as well as modulation of mRNA response of growth-regulating proteins.

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Introduction

Skeletal muscle encompasses ∼40% of body mass in lean individuals, making it the largest organ of the human body (Zurlo *et al.* 1990). Loss of muscle mass (muscle atrophy) can have critical consequences for contractile function and exercise capacity (Ryall *et al.* 2007) and may reduce quality of life and life expectancy (McLeod *et al.* 2016). During muscle atrophic conditions, muscle protein breakdown exceeds synthesis, resulting in a negative protein balance (Goldspink & Goldspink, 1977). Strategies that reduce muscle protein breakdown and/or increase synthesis are therefore an important area of research. Although resistance exercise training effectively promotes muscle hypertrophy and decelerates disuse- and age-related muscle atrophy and weakness (Macaluso & De Vito, 2004), pharmacological compounds may be used to augment the response to exercise (Kumar *et al.* 2009; Egan & Zierath, 2013).

Skeletal muscle beta$_2$-adrenoceptors are among potential therapeutic targets that have attracted interest in treatment of muscle atrophy and weakness (Lynch & Ryall, 2008; Joassard *et al.* 2013). Beta$_2$-adrenoceptors are the most predominant subtype of adrenoceptors in skeletal muscle (Williams *et al.* 1984; Jensen *et al.* 2002), where they serve a crucial role in the adrenergic fight-or-flight response (Emrick *et al.* 2010; Andersson *et al.* 2012; Hostrup *et al.* 2014*bb*). Furthermore, beta$_2$-adrenoceptors play a role in regulation of muscle protein turnover. Beta$_2$-adrenoceptor knockout mice display lower muscle mass than their wild-type peers (Hinkle *et al.* 2002) and beta$_2$-adrenoceptor stimulation with selective agonists (beta$_2$-agonists) increases muscle mass in mammals (Lynch & Ryall, 2008), including humans (Hostrup *et al.* 2015; Jessen *et al.* 2018). In rodents, beta$_2$-agonists have also been shown to accelerate muscle recovery from injury (Beitzel *et al.* 2004; Church *et al.* 2014) and to reverse muscle atrophy associated with ageing (Ryall *et al.* 2007), cancer cachexia (Busquets *et al.* 2004) and muscular dystrophies (Harcourt *et al.* 2007; Gehrig *et al.* 2010). Moreover, studies in humans have shown that a few weeks of beta$_2$-agonist treatment enhances muscle strength (Martineau *et al.* 1992; Hostrup *et al.* 2015, 2016) and preserves muscle function during disuse conditions when combined with resistance exercise (Caruso *et al.* 2004, 2005). Accordingly, beta$_2$-agonists have been proposed as pharmacotherapy to prevent muscle atrophy and loss of muscle function in muscle wasting conditions (Signorile *et al.* 1995; Lynch & Ryall, 2008; Atherton & Szewczyk, 2011; Joassard *et al.* 2013) and to augment muscle adaptations to exercise training (Caruso *et al.* 2004, 2005; Hostrup *et al.* 2018; Jessen *et al.* 2018).

Beta$_2$-agonists are widely used because of their application as first-line treatment of the broncho-constriction associated with asthma, exercise-induced bronchoconstriction (Price *et al.* 2014) and chronic obstructive pulmonary disease (Barnes, 2005). Although beta$_2$-agonists have been marketed for decades, information on the effect of these substances on skeletal muscle protein turnover in humans is lacking. Contra-dictory findings exist in that Robinson *et al.* (2010) found no effect of the non-selective beta-agonist isoproterenol on whole-body and muscle protein synthesis, whereas a recent study showed that 7 days of treatment with selective beta$_2$-agonist formoterol increased whole-body protein synthesis (Lee *et al.* 2015). In mice, however, Koopman *et al.* (2010) observed that beta$_2$-agonist only increased muscle protein synthesis after consecutive days of treatment and not after the first day. Thus, the therapeutic application of beta$_2$-agonists in humans may involve several days of treatment before a net positive protein balance incurs (Koopman *et al.* 2010; Atherton & Szewczyk, 2011). Nevertheless, despite the advances made, no studies have investigated the potential of consecutive days of beta$_2$-agonist treatment to improve muscle protein balance after exercise in humans.

In rodents, the muscle hypertrophic effect of beta$_2$-agonist is mediated by increased protein synthesis (Maltin *et al.* 1989; Hesketh *et al.* 1992) and/or reduced breakdown (Busquets *et al.* 2004; Yimlamai *et al.* 2005), resulting in an overall net positive protein balance. The mechanisms underlying the growth-promoting actions of beta$_2$-agonists, however, are not entirely clear, although they involve modulation of various signalling pathways and gene programs that regulate muscle protein synthesis and proteolysis in rodents (Spurlock *et al.* 2006; Pearen *et al.* 2009; Koopman *et al.* 2010). Beta$_2$-adrenergic signalling induces cAMP-dependent activation of protein kinase A (PKA), Epac and extracellular signal-regulated kinases 1/2 (Shi *et al.* 2007; Ohnuki *et al.* 2014), which have a wide range of downstream targets that regulate ribosomal translation processes and transcription of growth-modulating genes (Spurlock *et al.* 2006; Pearen *et al.* 2009), including cAMP response element binding protein (CREB) (Hinkle *et al.* 2002), Akt, mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) (Kline *et al.* 2007; Koopman *et al.* 2010). In addition, several regulators of protein synthesis may be modulated by beta$_2$-adrenergic signalling, including Akt-effector Forkhead box protein O1 (FoxO1), a regulator of the atrophy-related genes atrogin and MurF (Bodine & Baehr, 2014), and eEF2, a regulator of translational elongation. However, the myocellular signalling and mRNA response to beta$_2$-agonists in relation to muscle protein synthesis and breakdown after exercise remain unexplored in humans.

The present study aimed to investigate the effect of 5 days of beta$_2$-adrenoceptor stimulation with the selective beta$_2$-agonist salbutamol on protein turnover of skeletal muscle following resistance exercise in young men. Secondary purposes were to elucidate associated changes in intracellular signalling and mRNA content of selected canonical beta$_2$-adrenergic targets in skeletal muscle. We hypothesized that beta$_2$-agonist treatment would increase protein synthesis and reduce breakdown, resulting in an overall net positive protein balance compared to placebo during recovery from resistance exercise.

Methods

Human subjects and ethics

Thirteen healthy trained young men volunteered to participate in the present study. Before inclusion in the study, subjects underwent a medical examination where resting blood pressure, heart rate and ECG of the subjects were measured. Furthermore, body composition was measured by dual-energy X-ray absorptiometry (Lunar DPX-IQ, GE Healthcare, Chalfont St Giles, UK). Inclusion criteria were age 18–40 years and an active life-style, defined as more than 3 h of physical activity per week. Exclusion criteria were smoking, chronic disease, allergy towards medication and the use of beta$_2$-agonist or other prescription medication. Subjects were informed about risks and discomforts related to the different tests and procedures of the study. Each subject provided their written and oral informed consent prior to inclusion in the study. The study was approved by the Committee on Health Research Ethics of the Capital Region of Denmark (H-1-2012-119) and performed in accordance with the standards set by the *Declaration of Helsinki*. The study was registered in ClinicalTrials.gov (NCT02551276).

Of the 13 subjects who were screened, 12 were included in the study and completed it (Fig. 1). The characteristics of the 12 subjects who completed the study are presented in Table 1.

Study design

The study was designed as a randomized, double-blinded, placebo-controlled, cross-over study. During two identical trials, subjects received either oral salbutamol or placebo. Each trial was preceded by a 4-day lead-in period with oral salbutamol (4×4 mg $\times$ day^{-1}) or placebo treatment because animal studies have shown that the effect of beta$_2$-agonist on protein synthesis is evident after a few days of treatment (Koopman *et al.* 2010). The two trials were separated by 3–6 weeks to minimize potential confounding carry-over effects of salbutamol (Le Panse *et al.*, 2005). Prior to the first experimental trial, subjects met at the laboratory for two familiarizations to the resistance exercise protocol of the experimental trials.

Table 1. Subject characteristics (n = 12)

Age (years)	23.4 ± 3.8
Height (cm)	181.3 ± 5.6
Body mass (kg)	74.4 ± 9.2
Lean body mass (kg)	61.0 ± 5.4
Leg lean mass (kg)	17.8 ± 1.8

Values are the mean ± SD.

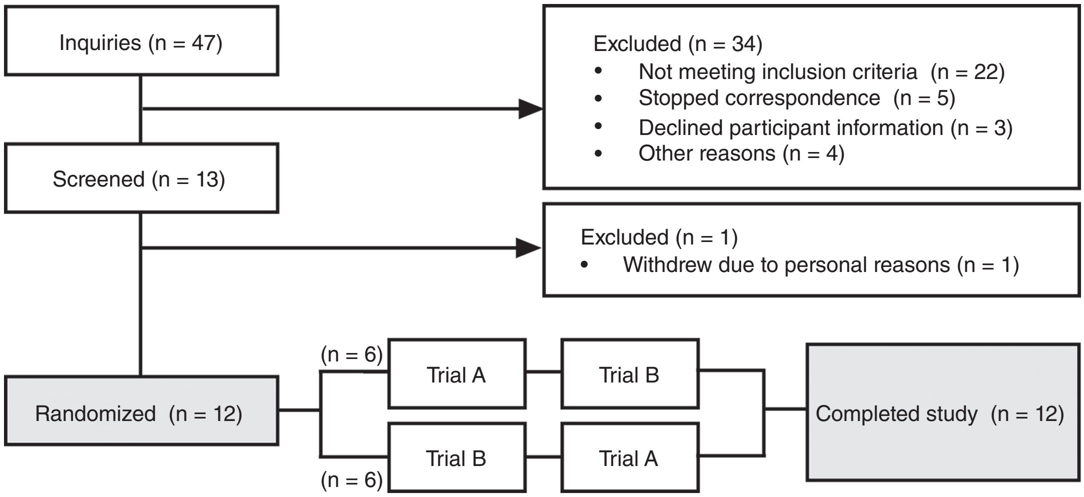

Figure 1. Flow diagram
Flow diagram providing details of the present study.

Experimental protocol

An overview of the experimental protocol is illustrated in Fig. 2. After the 4 days of lead-in treatment, subjects met in the morning after an overnight fast and received either oral salbutamol (6 × 4 mg) or placebo (same treatment as during lead-in) with a standardized light meal low on protein and fat consisting of white bread with jam (energy: 369 kcal; protein: 12 g; carbohydrate: 67 g; fat: 3 g) and 400 mL of water. Subjects then rested in a bed in the supine position and catheters were inserted: one in the dorsal hand vein for tracer infusion, one in the brachial artery and one in the femoral vein during local anaesthesia (lidocaine without epinephrine, Xylocaine; AstraZeneca, Cambridge, UK) for arterial and venous blood sampling. A primed, continuous infusion of stable amino acid isotope $[^{13}C_6]$-phenylalanine (L-phenylalanine, ring-13C6, 99%, CLM-1055-MPT; Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) was used for measurement of amino acid kinetics across the limb and incorporation of labelled phenylalanine into muscle. $[^{13}C_6]$-phenylalanine was dissolved in isotonic saline (0.9%) using a sterile procedure, filtered through disposable, sterile, non-pyrogenic filters with 0.2 μm pore size (Minisart; Sartorius Stedim Biotech, Aubagne, France) and kept at 5°C until infusion. The priming dose of 8 μmol × kg^{-1} lean body mass (LBM) labelled phenylalanine was dissolved in 20 mL saline and infused at once (1 min). The continuous infusion rate of labelled phenylalanine was 7 μmol × kg_{LBM}^{-1} × h^{-1} dissolved in saline and infused with a constant rate throughout the trial.

After 90 min of $[^{13}C_6]$-phenylalanine infusion (i.e. to reach tracer steady-state), subjects moved to a knee extensor resistance exercise model. Subjects then performed two sets of 10 repetition knee extensor exercise at an intensity corresponding to 50% of three repetition maximum, followed by eight sets of 12 repetitions of knee-extensor exercise at an intensity corresponding to 12 repetition maximum (75 ± 11 kg) (mean ± SD) with 2 min of recovery between each set. If subjects failed to perform 12 repetitions in a given set, load was decreased for the following set. The mean load performed during the final set was 69 ± 12 kg (mean ± SD). Intensity and recovery time were duplicated for each subject during the two trials. After exercise, subjects remained inactive in a supine position for 5 h. Biopsies were obtained from the vastus lateralis muscle 0.5 and 5 h after resistance exercise.

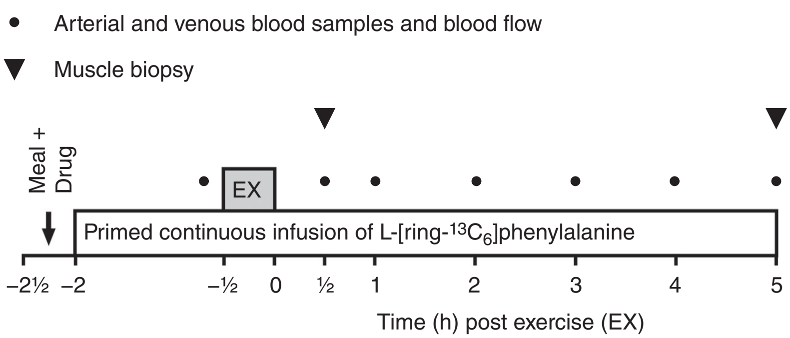

Figure 2. Overview of the experimental protocol
In a randomized placebo-controlled, double-blinded, cross-over design, the study participants conducted two experimental trials (salbutamol *vs.* placebo) that were separated by 3–6 weeks. Filled circles indicate when arterial and venous blood samples were drawn. Filled triangles indicate when muscle biopsies of the vastus lateralis were collected. EX, resistance exercise.

Brachial arterial and femoral venous blood samples were drawn in EDTA tubes (9 mL) prior to exercise as well as 0.5, 1, 2, 3, 4 and 5 h following exercise. Blood samples were kept at 5°C for 30 min before centrifugation at 5°C and 3200 *g* for 10 min, after which plasma was collected and stored at –80°C until analyses. Furthermore, 1 mL of arterial and venous blood were sampled in heparin tubes at same time points for determination of haematocrit using an ABL 800 flex (Radiometer, Copenhagen, Denmark). Prior to exercise as well as 0.5, 1, 2, 3, 4 and 5 h following exercise, femoral arterial blood flow was measured with ultrasound Doppler (Vivid E9; GE Healthcare, Brøndbyvester, Denmark) equipped with a linear probe operating at an imaging frequency of 8 MHz and Doppler frequency of 3.1 MHz, as described previously (Nyberg *et al.* 2014).

Subjects were asked to refrain from caffeine, nicotine and alcohol 24 h before each trial, as well as from exercise 48 h before each trial.

Study drugs

Salbutamol (Ventolin, 4 mg tablets; GlaxoSmithKline, London, UK) and identically looking placebo (lactose monohydrate/starch) were delivered by the hospital pharmacy of Copenhagen. Beta$_2$-adrenoceptors were stimulated with the highly selective beta$_2$-agonist salbutamol (Baker, 2010), which has a duration of action of 6–8 h and a plasma elimination half-life of 3–4 h (Rosen *et al.* 1986; Jacobson *et al.* 2015). Salbutamol concentrations peak systemically 1 h 30 min to 3 h after oral administration (Hostrup *et al.* 2014*a*). The dose administered during the lead-in period (16 mg $\times$ day^{-1}) was based on studies showing significant effect of daily treatment with oral salbutamol (16 mg $\times$ day^{-1}) on muscle strength (Martineau *et al.* 1992; Caruso *et al.* 2004). The increase in dose to 24 mg of oral salbutamol during the experimental day was because of potential desensitization of the beta$_2$-adrenoceptors during the lead-in treatment period. Drugs were administered in a double-blinded manner. Randomization was conducted in SPSS, version 24 (IBM Corp., Armonk, NY, USA) by personnel who did not take part in any of the experimental procedures or data analyses. To ensure a drug compliance of 100% during the 4-day lead-in period, subjects met at the laboratory in the morning or noon and ingested the study drugs during supervision. Eight of the 12 subjects experienced common side effects of salbutamol during the first 2 days of treatment, including tremors ($n = 7$) and palpitations ($n = 6$).

Dual-energy X-ray absorptiometry

Subjects laid in the scanner in supine position undressed for 20 min before the scan. To reduce variation, two scans at medium speed were performed in accordance with the manufacturer's guidelines. The scanner was calibrated before the scan using daily calibration procedures (Lunar 'System Quality Assurance'). All scans were conducted by the same hospital technician.

Muscle biopsies

Muscle biopsies were obtained from the vastus lateralis using a 4 mm Bergström biopsy needle (Stille, Stockholm, Sweden) with suction (Bergström, 1975). Before biopsies were sampled, two incisions were made in the skin at the belly of the vastus lateralis muscle during local anaesthesia (2 mL of lidocaine without epinephrine, Xylocaine 20 mg $\times$ mL^{-1}; Astra Zeneca). After sampling, the muscle biopsy was cleaned from visible blood, connective tissue and fat and immediately frozen in liquid nitrogen. Biopsies were stored in cryo tubes at –80°C until analyses.

Leg and muscle protein turnover rates

The influence of beta$_2$-agonist on muscle protein turnover and myofibrillar protein fractional synthesis rates (FSR) were measured 0.5 to 5 h during recovery from exercise by infusion of stable isotope-labelled phenylalanine, collection of arteriovenous blood samples and muscle biopsies from the vastus lateralis muscle, and measurement of femoral blood flow (Fig. 2).

Femoral arteriovenous plasma phenylalanine enrichment and concentration were measured using 400 μL of plasma with a known amount of [U-$^{13}C_9$]-phenylalanine added as internal standard. Samples were derivatized using *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide + 1% *tert*-butyl-dimethylchlorosilane (Regis Technologies, Morton Grove, IL, USA) and analysed on a triple-stage quadrupole-mass spectrometer via gas chromatography-tandem mass spectrometry (GC-MS/MS) (TSQ Quantum; Thermo Scientific, San Jose, CA, USA), as described previously (Holm *et al.* 2014).

Muscle specimens of ~20 mg wet weight were homogenized using a FastPrep 120A-230 homogenizer (Thermo Savant, Holbrook, NY, USA) in 1.5 mL of ice-cold Milli-Q saline water (Merck-Millipore, Burlington, MA, USA) and after a spin (5500 *g* for 10 min at 5°C), the supernatant containing the muscle free amino acids was transferred to a new vial. Muscle free phenylalanine enrichment was then measured by GC-MS/MS in the same way as described for the plasma phenylalanine enrichment. The pellet from the spin was added a Tris-buffer (pH 7.4, containing 2 mM EGTA chelating agent, 0.5% Triton-X100 and 0.25 M sucrose), homogenized once again, left 3 h at 5°C and spun (800 *g* for 20 min at 5°C) to pellet all structural proteins. Subsequently, myofibrillar proteins were dissolved in a 0.7 M KCl and 0.1 M $Na_4P_2O_7$-buffer and, after overnight incubation at 5°C and a subsequent spin (1600 *g* for 20 min at 5°C), the supernatant containing the myofibrillar proteins were transferred to other vials. The myofibrillar proteins were denatured by adding 2.3 volumes of ethanol. After 2 h of incubation at 5°C, vials were spun (1600 *g* for 20 min at 5°C) to pellet the myofibrillar proteins. After a wash with 70% ethanol, proteins were hydrolysed in 6 M HCl at 110°C overnight, and the ratio of $^{13}CO_2$ and $^{12}CO_2$ from *N*-acetyl *n*-propyl (NAP)-derivatized phenylalanine was analysed using a GC-combustion-isotope ratio mass spectrometer (Finnigan Delta Plus, Bremen, Germany), as described previously (Holm *et al.* 2014).

Calculations of phenylalanine kinetics across the leg and muscle, as well as muscle protein turnover parameters, were based on two- and three-pool modelling and myofibrillar FSR on a direct incorporation model (Wolfe & Chinkes, 2005; Smith *et al.* 2015). All calculations are based on phenylalanine enrichment as mole percent excess (MPE) or atomic percent excess, and phenylalanine concentration is the total concentration (i.e. unlabelled and labelled phenylalanine). Plasma flow, derived from blood flow and haematocrit, were used in the calculations. All phenylalanine kinetic values are expressed as nmol × min^{-1} × 100 g leg lean mass $(\text{LLM})^{-1}$. LLM was derived from the dual-energy X-ray absorptiometry scan. Models and calculations applied in the present study are in accordance with those described previously (Biolo *et al.* 1995; Wolfe & Chinkes, 2005; Smith *et al.* 2015):

Two- and three-pool models shared calculations.

$$\text{Leg plasma flow (LPF)} = \text{leg blood flow} \times (1 - \text{haematocrit})$$

$$\text{Delivery to the leg } (F_{\text{in}}) = C_{\text{A}} \times \text{LPF}$$

$$\text{Output from the leg } (F_{\text{out}}) = C_{\text{V}} \times \text{LPF}$$

$$\text{Leg net balance (leg NB)} = (C_{\text{A}} - C_{\text{V}}) \times \text{LPF}$$

where C_A and C_V are arterial and venous phenylalanine concentration, respectively.

Two-pool model calculations.

$$\text{Rate of disappearance in the leg (leg } R_{\text{d}}) = (C_{\text{A}} \times E_{\text{A}} - C_{\text{V}} \times E_{\text{V}}) \times \text{LPF}/E_{\text{A}}$$

$$\text{Rate of appearance in the leg (leg } R_{\text{a}}) = \text{leg } R_{\text{d}} - \text{leg NB}$$

where E_A and E_V are arterial and venous phenylalanine enrichment, respectively.

Three-pool model calculations.

$$\text{Muscle inward transport } (F_{\text{M,A}}) = ((C_{\text{V}} \times ((E_{\text{M}} - E_{\text{V}})/(E_{\text{A}} - E_{\text{M}}))) + C_{\text{A}}) \times \text{LPF}$$

$$\text{Muscle outward transport } (F_{\text{V,M}}) = ((C_{\text{V}} \times ((E_{\text{M}} - E_{\text{V}})/(E_{\text{A}} - E_{\text{M}}))) + C_{\text{V}}) \times \text{LPF}$$

$$\text{Arteriovenous shunting } (F_{\text{V,A}}) = F_{\text{in}} - F_{\text{M,A}}$$

$$\text{Muscle protein breakdown estimate } (F_{\text{M,O}}) = F_{\text{M,A}} \times (E_{\text{A}}/E_{\text{M}} - 1)$$

$$\text{Muscle protein synthesis estimate } (F_{\text{O,M}}) = F_{\text{M,O}} + \text{leg NB}$$

where E_M is muscle intracellular phenylalanine enrichment.

Direct incorporation model calculations for myofibrillar protein synthesis.

$$\text{Fractional synthesis rate (FSR)} = ((E_{\text{P2}} - E_{\text{P1}})/(E_{\text{precursor}} \times (T_2 - T_1))) \times 100$$

where E_{P1} and E_{P2} are the myofibrillar product enrichment at time point 0.5 and 5 h, respectively, $E_{\text{precursor}}$ is the muscle free phenylalanine enrichment (or venous or arterial plasma phenylalanine enrichment), and T_1 and T_2 are the specific time points at 0.5 and 5 h, respectively. Myofibrillar FSR is expressed as % × h^{-1}.

Protein phosphorylation in muscle homogenate lysates

Protein phosphorylation was determined by western blotting, as described previously (Thomassen *et al.* 2016). In short, ∼1.5 mg freeze dried muscle tissue was homogenized (Qiagen Tissuelyser II; Retsch GmbH, Haan, Germany) in a fresh batch of buffer containing (in mM): 10% glycerol, 20 Na-pyrophosphate, 150 NaCl, 50 Hepes (pH 7.5), 1% NP-40, 20 β-glycerophosphate, 2 Na_3VO_4, 10 NaF, 2 phenylmethylsulphonyl fluoride, 1 EDTA (pH 8), 1 EGTA (pH 8), 10 μg × mL^{-1} Aprotinin, 10 μg × mL^{-1} Leupeptin and 3 benzamidine. Samples were rotated end-over-end for 1 h at 4°C and centrifuged at 18,320 *g* for 20 min at 4°C to exclude non-dissolved structures and the supernatant (lysate) was used for further analyses. Total protein concentration in each sample was determined by a BSA standard kit (Thermo Fisher Scientific, Hvidovre, Denmark) and samples were mixed with 6 × Laemmli buffer (7 mL of 0.5 M Tris-base, 3 mL of glycerol, 0.93 g of dithiothreitol, 1 g of SDS and 1.2 mg of bromophenol blue) and ddH_20 to reach equal protein concentration before protein content was determined by western blotting.

An equal amount of total protein was loaded in each well of precast gels (Bio-Rad, Hercules, CA, USA). All samples from each subject were loaded on the same gel with a mixed human muscle standard lysate loaded in two different wells used for normalization. Analysis of phosphorylated proteins and corresponding total protein were performed

Table 2. Primary antibodies used for western blotting

Target protein	Manufacturer	Number	Molecular weight (kDa)
4E-BP1	Cell Signaling	9452	15–20
p-4E-BP1$_{Thr37/46}$	Cell Signaling	2855	15–20
Akt2	Cell Signaling	3063	60
p-Akt2$_{Ser473}$	Cell Signaling	9271	60
CREB	Cell Signaling	9197	43
p-CREB$_{Ser129/Ser133}$	Abcam	ab10564	37–43
eEF2	Abcam	ab130187	95
p-eEF2$_{Thr56}$	Cell Signaling	2331	95
mTOR	Cell Signaling	2972	289
p-mTOR$_{Ser2448}$	Cell Signaling	2971	289
p38MAPK	Cell Signaling	9212	37–43
p-p38MAPK$_{Thr180/Tyr182}$	Cell Signaling	9211	37–43
p70S6K	Cell Signaling	2708	70
p-p70S6K$_{Thr389}$	Cell Signaling	9234	70

Suppliers: Abcam, Cambridge, MA, USA; Cell Signaling, Beverly, MA, USA.

on separate gels. Proteins were separated according to their molecular weight by SDS page gel electrophoresis and semi-dry transferred to a polyvinylidene fluoride membrane (Bio-Rad). The membranes were blocked in either 2% skimmed milk or 3% BSA in Tris-buffered saline including 0.1% Tween-20 before overnight incubation in primary antibody at 4°C and a subsequent 1 h incubation in HRP conjugated secondary antibody at room temperature. The bands were visualized with ECL (Merck-Millipore) and recorded with a digital camera (ChemiDoc MP Imaging System; Bio-Rad). Densitometry quantification of the western blot band intensity was performed using Image Lab, version 4.0 (Bio-Rad) and determined as the total band intensity adjusted for background intensity. Primary antibodies used are presented in Table 2. Primary antibodies were optimized by use of mixed human muscle standard lysates. Two mixed study samples containing tissue from biopsies were used to ensure that the protein amount loaded would result in band signal intensities localized on the steep and linear part of a standard curve. Secondary antibodies used were HRP conjugated rabbit anti-sheep (P-0163), goat anti-mouse (P-0447; Dako, Glostrup, Denmark) and goat anti-rabbit IgM/IgG (4010-05; SouthernBiotech, Birmingham, AL, USA).

RNA isolation, reverse transcription, and real-time PCR

The method for RNA isolation, reverse transcription and real-time PCR has been described previously (Pilegaard *et al.* 2000; Brandt *et al.* 2016). Total RNA was isolated from ~5 mg wet weight muscle tissue using a modified guanidinium thiocyanate–phenol–chloroform extraction method from Chomczynski and Sacchi (1987) as described by Pilegaard *et al.* (2000), except for the use of a TissueLyser (TissueLyser II; Qiagen, Valencia, CA, USA) for homogenization. Superscript II RNase H- and Oligo dT (Invitrogen, Carlsbad, CA, USA) were used to reverse transcribe mRNA to cDNA (Pilegaard *et al.* 2000). Quantification of cDNA as a measure of mRNA content of a given gene was performed by real-time PCR using an ABI 7900 sequence-detection system (Applied Biosystems, Foster City, CA, USA). Probes and primers were either self-designed (Table 3) or pre-developed gene expression assays (ActivinRIIB Hs00609603_m1, calpain-1 Hs00559804_m1, Smad3 Hs00969210_m1) (Applied Biosystems). Self-designed probes and 5′-6-carboxyfluorescein (FAM)/3′-6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine labelled TaqMan probes were designed from human specific databases from ensemble (www.ensembl.org/homo_sapiens/info/index) using Primer Express, version 3.0 (Applied Biosystems) and were obtained from TAG Copenhagen (Copenhagen, Denmark).

Real-time PCR was performed in triplicates in a total reaction volume of 10 μL using Universal Mastermix with UNG (Applied Biosystems). The obtained cycle threshold values reflecting the initial content of the specific transcript in the samples were converted to a relative amount by using standard curves constructed from serial dilution of a pooled sample made from all samples. Target mRNA content was normalized to single-stranded DNA content in each sample determined by using OliGreen reagent (Molecular Probes, Leiden, The Netherlands) as described previously (Lundby *et al.* 2005).

Plasma concentrations of salbutamol

Plasma concentrations of salbutamol were measured by ultra high performance liquid chromatography (UPLC)-MS/MS using deuterated internal standard based

Table 3. Primers used for real-time PCR

Target gene	Sense primer	Antisense primer	TaqMan probe
Atrogin	5′-GATGTTACCCAAGGAAAGAGCAGTAT-3′	5′-ACGGATGGTCAGTGCCCTT-3′	5′-CCCTTCAGCTCTGCAAACACTGTCACAT-3′
FoxO1	5′-ACCGAACAGGATGATCTTGGA-3′	5′-CCATCTGCCGCAAAGATGGCCTCTA-3′	5′-CCATCTGCCGCAAAGATGGCCTCTA-3′
MurF	5′-GGAGCCACCTTCCTCTTGACT-3′	5′-CTCAAAGCCCTGCTCTGTCTTC-3′	5′-AACTCATCAAAAGCATTGTGGAAGCTTCCAA-3′
Myostatin	5′-ACCAGGAGAAGATGGGCTGAA-3′	5′-GTCAAGACCAAAATCCCTTCTGGA-3′	5′-CCGTTTTTAGAGGTCAAGGTAACAGACACACCA-3′
PGC-1α	5′-CAAGCCAAACCAACAACTTTATCTCT-3′	5′-CACACTTAAGGTGCGTTCAATAGTC-3′	5′-AGTCACCAAATGACCCCAAGGGTTCC-3′

on methods described previously (Jacobson *et al.* 2015). In brief, calibration samples were prepared using unlabelled salbutamol in drug free plasma over a concentration range of 2–200 ng × mL^{-1} and internal standard salbutamol-D3 (3-hydroxymethyl-D2, α-D1; Medical Isotopes, Inc., Pelham, NH, USA) was added to each plasma sample (200 μL) or calibration sample equivalent to 20 ng × mL^{-1}. Ammonia solution (200 μL, pH 9) was then added to each sample and vortex mixed before the addition of 1000 μL of HPLC grade ethyl acetate. This was vortex mixed for 1 min and then centrifuged at 15,000 *g* for 5 min. The organic supernatant was then transferred to a glass autosampler vial, from which the solvent was evaporated under nitrogen at 40°C. The residue was reconstituted using 100 μL of methanol and vortex mixed prior to analysis via UPLC-MS/MS consisting of a Waters Acquity® H-class UPLC system (Waters Corporation, Milford, MA, USA) with chromatography performed using an Astec® CHIROBIOTIC™ T2 chiral column (4.6 × 250 mm × 5 μm particles) (Sigma-Aldrich) coupled to a Waters Xevo® triple quadrupole mass spectrometer (Waters Corporation) with analyses undertaken using multiple reaction monitoring with conditions as described previously (Jacobson *et al.* 2015). Assay performance data were within acceptance criteria, with accuracy and precision (% relative SD; $n = 5$ at 5 ng × mL^{-1}) both less than 5% and calibration $r^2 > 0.9998$. Total salbutamol levels were calculated from the sum of individual enantiomers.

Statistical analysis

Statistical analyses were performed in SPSS, version 24 (IBM Corp., Armonk, US). Sample size was determined for the primary outcome measure (myofibrillar FSR) and was estimated from the effect of $beta_2$-agonist treatment on protein synthesis in animals (Koopman *et al.* 2010) and between-subject SD from resistance exercise studies in humans (Kumar *et al.*, 2009). Data were tested for normality using the Shapiro–Wilks test and *Q-Q* plots. Variables that violated normality were log-transformed (i.e. phosphorylation-ratio and mRNA level data). To estimate differences between treatments, two-tailed linear mixed modelling was used with treatment as a fixed effect and a random effect for subjects. In addition, age and LBM were included in the model as time invariant covariates because they may confound the effect of $beta_2$-agonist (White & Leenen, 1994; Cheymol, 2000). Area under the phenylalanine leg net balance–time curve (AUC) was analysed using the trapezoidal rule with inclusion of baseline net balance as a covariate in the mixed model. For mRNA content, technical replicates were nested within the fixed effects (Acharya & Zhu, 2011). In the case of repeated measures, sampling point

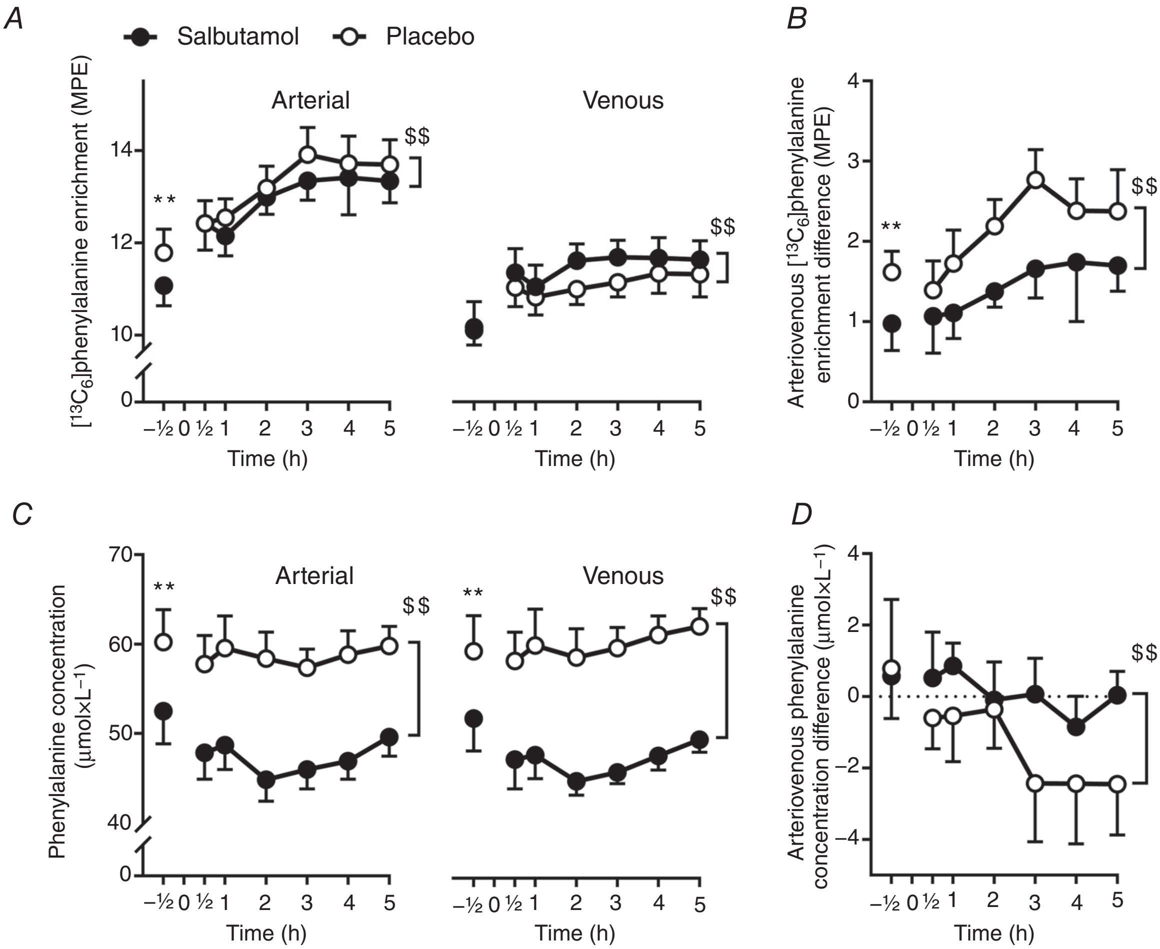

Figure 3. Circulating phenylalanine availability for salbutamol (black) and placebo (white) before (–0.5 h) and 0.5–5 h following resistance exercise

A, arterial and venous [^{13}C$_6$]-phenylalanine enrichment. *B*, arteriovenous difference in [^{13}C$_6$]-phenylalanine enrichment. *C*, femoral arterial and venous phenylalanine concentration. *D*, arteriovenous difference in phenylalanine concentration. Values are the mean ($n = 12$). Error bars represent upper or lower bound of the 95% CI. **Treatment difference ($P < 0.01$) at same point. $^{\$\$}$Overall treatment main effect ($P < 0.01$).

was included in the model as a fixed effect for a full factorial design. Within-sampling point P values were adjusted using the Bonferroni method. Data are presented as the mean with the 95% confidence interval (CI), unless otherwise stated, and exact P values (unless <0.01 or >0.50) to represent probability for treatment fixed effects.

Results

Plasma concentrations of salbutamol

Arterial plasma concentrations of salbutamol were 46.5 (95% CI, 39.1 to 53.8) and 52.0 (95% CI, 42.8 to 61.3) ng × mL^{-1} 0.5 and 5 h after exercise (2 h 30 min and 7 h after drug administration, respectively). No salbutamol was detected in the blood during the placebo trial.

Circulating phenylalanine availability

Average arterial plasma [^{13}C$_6$]-phenylalanine enrichment was lower ($P < 0.01$) for salbutamol than placebo, whereas femoral venous enrichment was higher ($P < 0.01$) for salbutamol than placebo (Fig. 3*A* and *B*). Average arterial and femoral venous plasma concentrations of phenylalanine were lower ($P < 0.01$) for salbutamol than placebo (Fig. 3*C* and *D*).

Leg phenylalanine kinetics based on the two-pool model

Average femoral arterial plasma flow was more than two-fold higher ($P < 0.01$) for salbutamol than placebo (Fig. 4*A*). No differences were observed in [^{13}C$_6$]-phenylalanine leg net balance between salbutamol

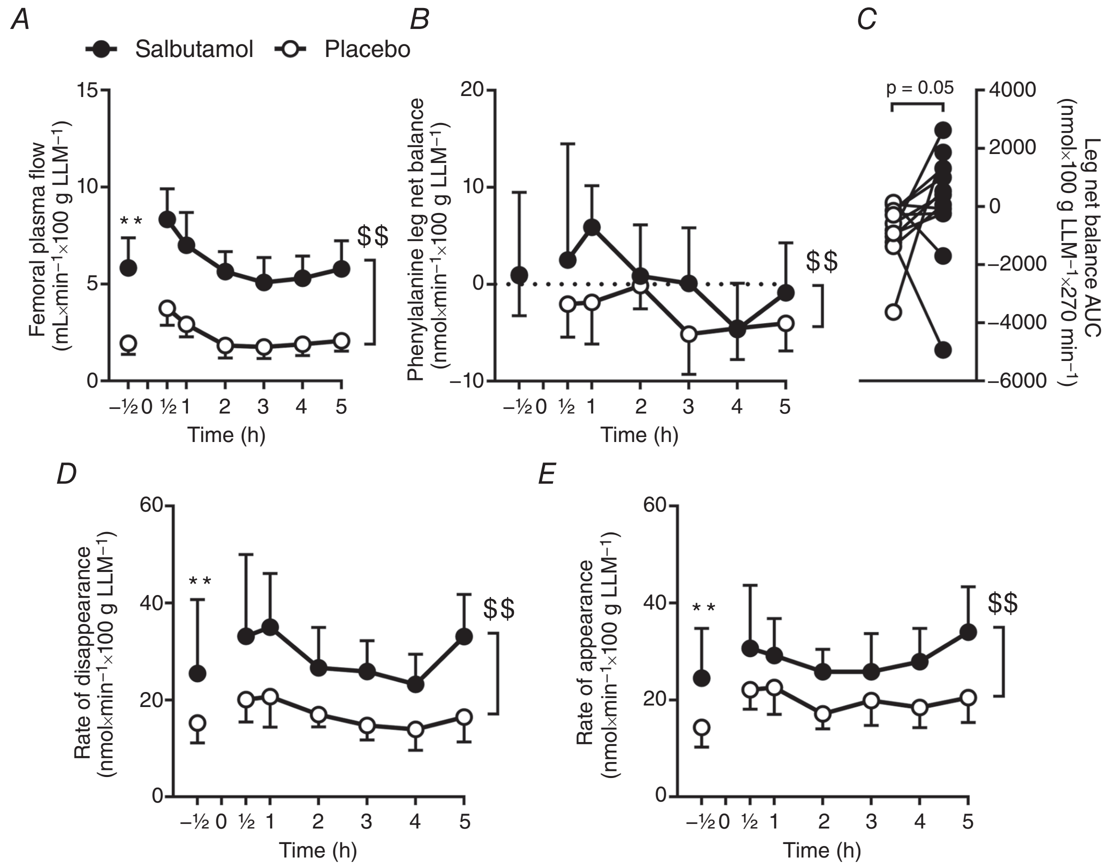

Figure 4. Leg phenylalanine kinetics based on the two-pool model for salbutamol (black) and placebo (white) before (–0.5 h) and 0.5–5 h following resistance exercise

A, femoral arterial plasma flow. *B*, leg net phenylalanine balance curve. *C*, area under the leg net phenylalanine balance–time curve. *C* and *D*, rate of disappearance. *E*, rate of appearance. Values are the mean (n = 12). Error bars represent upper or lower bound of the 95% CI.**Treatment difference (P < 0.01) at same point. $$Overall treatment main effect (P < 0.01).

and placebo before exercise (Fig. 4*B*). In the period 0.5–5 h after exercise, mean phenylalanine leg net balance was 0.6 (95% CI, −2.4 to 3.7) and –3.0 (95% CI, −0.7 to −5.2) nmol × min^{-1} × 100 g$_{\text{LLM}}$$^{-1}$ for salbutamol and placebo, respectively ($P < 0.01$) (Fig. 4*B*). Phenylalanine leg net balance AUC was higher ($P = 0.05$) for salbutamol than placebo (Fig. 4*C*). Average rate of disappearance and appearance of [$^{13}C_6$]-phenylalanine was higher ($P < 0.01$) for salbutamol than placebo (Fig. 4*D* and *E*).

Muscle protein synthesis rate and leg phenylalanine kinetics based on 3-pool model

Myofibrillar FSR was determined as the incorporation of tracer into myofibrillar proteins using the I.M. tracer enrichment (MPE) as precursor [salbutamol 0.5 h: 7.0 (95% CI, 6.4 to 7.6%) and 5 h: 7.6 (95% CI, 7.0 to 8.2%) and placebo 0.5 h: 7.6 (95% CI, 6.9 to 8.3%) and 5 h: 7.5 (95% CI, 6.8 to 8.2%)]. Myofibrillar FSR was 0.013 (95% CI, 0.001 to 0.025%) × h^{-1} higher ($P = 0.03$) for salbutamol than placebo (Fig. 5*A*). LBM was a significant negative confounder ($P = 0.03$) of the salbutamol-induced change in myofibrillar FSR, whereas age did not confound the response ($P > 0.50$). The effect of salbutamol on myofibrillar FSR was moderate (Cohen's $d = 0.78$).

Based on the three-pool model, salbutamol had higher estimates of protein synthesis ($F_{O,M}$) ($P < 0.01$) and breakdown ($F_{M,O}$) ($P < 0.01$) than placebo (Fig. 5*B* and *C*).

Inward and outward muscle transmembrane transport of phenylalanine was not significantly different between treatments 0.5 h after exercise, although it was higher ($P < 0.01$) for salbutamol than placebo 5 h after exercise (Table 4). Arteriovenous shunting was more than two-fold higher ($P < 0.01$) for salbutamol than placebo 0.5 and 5 h after exercise (Table 4).

Muscle signalling

PKA substrate intensity ($P = 0.01$) and phosphorylation of Akt2 ($P = 0.02$) and CREB ($P < 0.01$) were higher for

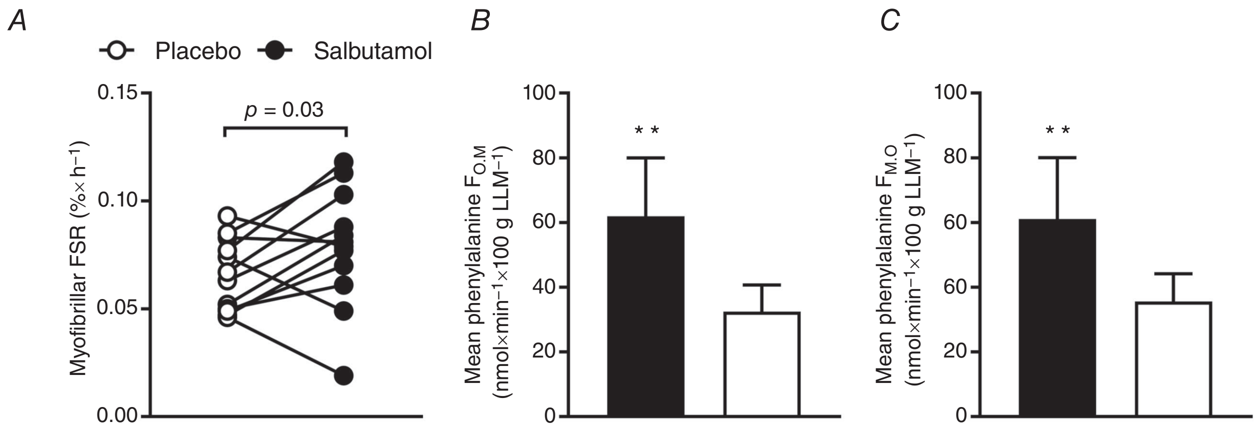

Figure 5. Muscle protein synthesis rate and leg phenylalanine kinetics based on the three-pool model for salbutamol (black) and placebo (white) 0.5–5 h following resistance exercise
A, myofibrillar FSR. *B*, average protein synthesis ($F_{O,M}$) (three-pool model). *C*, average protein breakdown ($F_{M,O}$) (three-pool model). Values are the mean (n = 12). Error bars represent upper or lower bound of the 95% CI. **Treatment difference ($P < 0.01$).

salbutamol than placebo 0.5 h after exercise, whereas no relevant differences were observed between the treatments in phosphorylation of 4E-BP1 ($P = 0.23$), eEF2 ($P = 0.17$), MAPK ($P > 0.50$), mTOR ($P = 0.17$) and p70S6K ($P = 0.39$) (Fig. 6*A*). PKA substrate intensity ($P < 0.01$) and phosphorylation of Akt2 ($P < 0.01$), CREB ($P < 0.01$) and eEF2 ($P = 0.01$) were higher for salbutamol than placebo 5 h following exercise, whereas phosphorylation of 4E-BP1 ($P = 0.38$), MAPK ($P > 0.50$), mTOR ($P > 0.50$) and p70S6K ($P = 0.13$) was not different between treatments (Fig. 6*B*).

mRNA content

The mRNA content of calpain-1 ($P < 0.01$), FoxO1 ($P < 0.01$), myostatin ($P < 0.01$) and Smad3 ($P < 0.01$) was higher for salbutamol than placebo 0.5 h after exercise, whereas no significant changes were induced by salbutamol in content of ActivinRIIB ($P = 0.25$), atrogin ($P > 0.50$), MuRF ($P > 0.50$) and PGC-1α ($P = 0.33$) compared to placebo (Fig. 7*A*). The mRNA content of ActivinRIIB was lower ($P < 0.01$) for salbutamol than placebo 5 h after exercise, whereas FoxO1 ($P < 0.01$) and myostatin ($P < 0.01$) mRNA content was higher for salbutamol than placebo (Fig. 7*B*). No treatment differences were observed in the mRNA content of atrogin ($P = 0.48$), calpain-1 ($P > 0.50$), PGC-1α ($P = 0.09$), MuRF ($P = 0.24$) and Smad3 ($P > 0.50$) 5 h after exercise (Fig. 7*B*).

Discussion

In the present study, we have reported the beta$_2$-adrenergically-induced changes in protein turnover rates and associated changes in intracellular signalling and mRNA content in skeletal muscle after resistance exercise in trained young men. The most important findings are that beta$_2$-adrenergic stimulation with the commonly prescribed selective beta$_2$-agonist, salbutamol, increased myofibrillar FSR and protein turnover rates, thus favouring an improved net protein balance in skeletal muscle following resistance exercise. Changes in protein turnover induced by salbutamol were associated with PKA-signalling, activation of Akt2 and modulation of mRNA response of growth-regulating proteins in skeletal muscle.

Although beta$_2$-agonists have been marketed for more than 50 years, the present study is first to show that a few days of beta$_2$-agonist treatment increases myofibrillar FSR and leg protein turnover rates, resulting in an improved leg net protein balance after resistance exercise in humans. The higher myofibrillar FSR induced by salbutamol was in agreement with our working hypothesis and consistent with reported observations in rodents (Maltin *et al.* 1989; Hesketh *et al.* 1992; Koopman *et al.* 2010). Contrary to our observations, isoproterenol was shown to have no effect on whole-body and muscle protein synthesis in young men (Robinson *et al.* 2010). The type of beta$_2$-agonist and dosing regimen applied may explain this discrepancy. In the present study, we administered salbutamol, which has superior selectivity for the beta$_2$-adrenoceptor than isoproterenol (Baker, 2010). Furthermore, we chose to administer salbutamol in supratherapeutic doses and daily for four days prior to the experiments because studies in rodents have shown that the stimulatory effect of beta$_2$-agonist on anabolism is dose-dependent and that the beta$_2$-agonist-induced increase in protein synthesis requires consecutive days of treatment (Koopman *et al.* 2010). Consistent with this, Lee *et al.* (2015) observed that 7 days of oral treatment

Table 4. Selected phenylalanine kinetics parameters based on the three-pool model

	Placebo		Salbutamol		Tests of fixed effects		
	0.5 h	5 h	0.5 h	5 h	Treatment	Time	Treatment by Time
Inward muscle transmembrane transport (PHE nmol × min^{-1} × 100 g LLM^{-1})	57 (40 to 73)	41 (32 to 49)	72 (39 to 105)	78 (62 to 94)**	<0.01	0.58	0.43
Outward muscle transmembrane transport (PHE nmol × min^{-1} × 100 g LLM^{-1})	59 (45 to 72)	45 (36 to 53)	69 (37 to 101)	79 (58 to 99)**	0.03	0.79	0.21
Arteriovenous shunting (PHE nmol × min^{-1} × 100 g LLM^{-1})	166 (107 to 224)	83 (54 to 112)	324 (249 to 399)**	209 (131 to 286)**	<0.01	<0.01	0.87

Values are the mean (95% CI) (n = 12). **Statistically significant difference ($P \leq 0.01$) compared to placebo at same time point.

with formoterol increased whole-body protein synthesis. In addition, we investigated the effect of beta$_2$-agonist on 5 h of protein turnover following resistance exercise and not during resting conditions as in the previous human studies (Robinson *et al.* 2010; Lee *et al.* 2015). Accordingly, the present study indicates that a few days of beta$_2$-agonist treatment increase protein turnover rates in the first 5 h period following resistance exercise, which is in agreement with the augmenting effect of daily salbutamol treatment on muscle adaptations to resistance training observed in previous studies (Caruso *et al.* 2004, 2005).

We observed that LBM confounded the effect of salbutamol on myofibrillar FSR, whereas no relevant confounding effect was observed for body mass (Pearson's r = –0.096, P = 0.32; data not shown). Distribution of drugs, including beta$_2$-agonists, is influenced by body composition (Cheymol, 2000), and LBM has been shown to be a superior predictor of the response to drugs than body mass (Morgan & Bray, 1994; Han *et al.* 2007). The influence of LBM on the response to salbutamol is probably related to the distribution kinetics of salbutamol, exhibiting extensive disposition in skeletal muscle (Jacobson *et al.* 2014). The beta-adrenoceptor cardiac response has also been shown to decline with age (White & Leenen, 1994), although we observed no relevant impact of subject age on the salbutamol-induced change in myofibrillar FSR. This may be explained by the relatively low heterogeneity in the present study population (range 19–32 years) and the different target tissue (cardiac *vs.* skeletal muscle). Nevertheless, the present observations suggest that LBM may be taken into consideration when investigating effects of beta$_2$-agonists. In this context, it has been speculated that the effect of beta$_2$-agonist on exercise performance and muscle excitation–contraction coupling depends on the training level of the subject (van Baak *et al.* 2004; Decorte *et al.* 2013).

Although studies in rodents have indicated that the hypertrophic effect of beta$_2$-agonist may involve both attenuation of muscle proteolytic processes (Busquets *et al.* 2004; Yimlamai *et al.* 2005) and an increase in protein synthesis (Maltin *et al.* 1989; Hesketh *et al.* 1992), we observed that salbutamol markedly increased leg protein turnover rates by almost doubling the rate of protein breakdown and synthesis during the 5 h period following exercise. Given that the resistance exercise undertaken was matched between the salbutamol and placebo trial, the higher rate of protein breakdown induced by salbutamol is related to factors other than the total work performed. Although a putative mechanism could be the pronounced increase in arterial femoral plasma flow (Biolo *et al.* 1997) induced by salbutamol, we observed no apparent association between femoral plasma flow and myofibrillar FSR (r = 0.11, P = 0.60) or leg net balance AUC (r = 0.05, P = 0.80). Furthermore, despite a higher femoral plasma flow and lower arterial plasma concentration of phenylalanine, the arteriovenous phenylalanine difference was more positive for salbutamol than placebo, in which there was a net release of phenylalanine in the 0.5–5 h following exercise. As such, our observations suggest that the greater protein turnover rates for salbutamol is related to myocellular mechanisms.

Despite the increase in rate of protein breakdown, salbutamol counteracted a net negative protein balance following resistance exercise, which was evident in the placebo condition. The negative protein balance observed for placebo is consistent with previous studies, where the balance is negative in the post-absorptive state following resistance exercise (Biolo *et al.* 1995; Phillips *et al.* 1997). In this context, it is important to emphasize that subjects in the present study consumed a standardized low-protein meal 2 h prior to the resistance exercise to provide some energy to be available for the exercise session, at the same time as being sufficiently low in protein to affect metabolism at the post-exercise measurements. Studies have shown that there is a graded response of muscle FSR to dietary protein or amino acid infusion (Bohé *et al.* 2003; Moore *et al.* 2009), and whether the effect of beta$_2$-agonist on protein turnover rates would have been different in completely fasting conditions or in conditions where subjects had consumed higher amounts of essential amino acids remains to be determined. Nonetheless, the beneficial effect of salbutamol in enhancing net balance to more positive levels than with placebo underpins the efficacy of beta$_2$-agonist in stimulating muscle anabolism.

We observed that salbutamol induced significant beta$_2$-adrenergic signalling in skeletal muscle 0.5 and

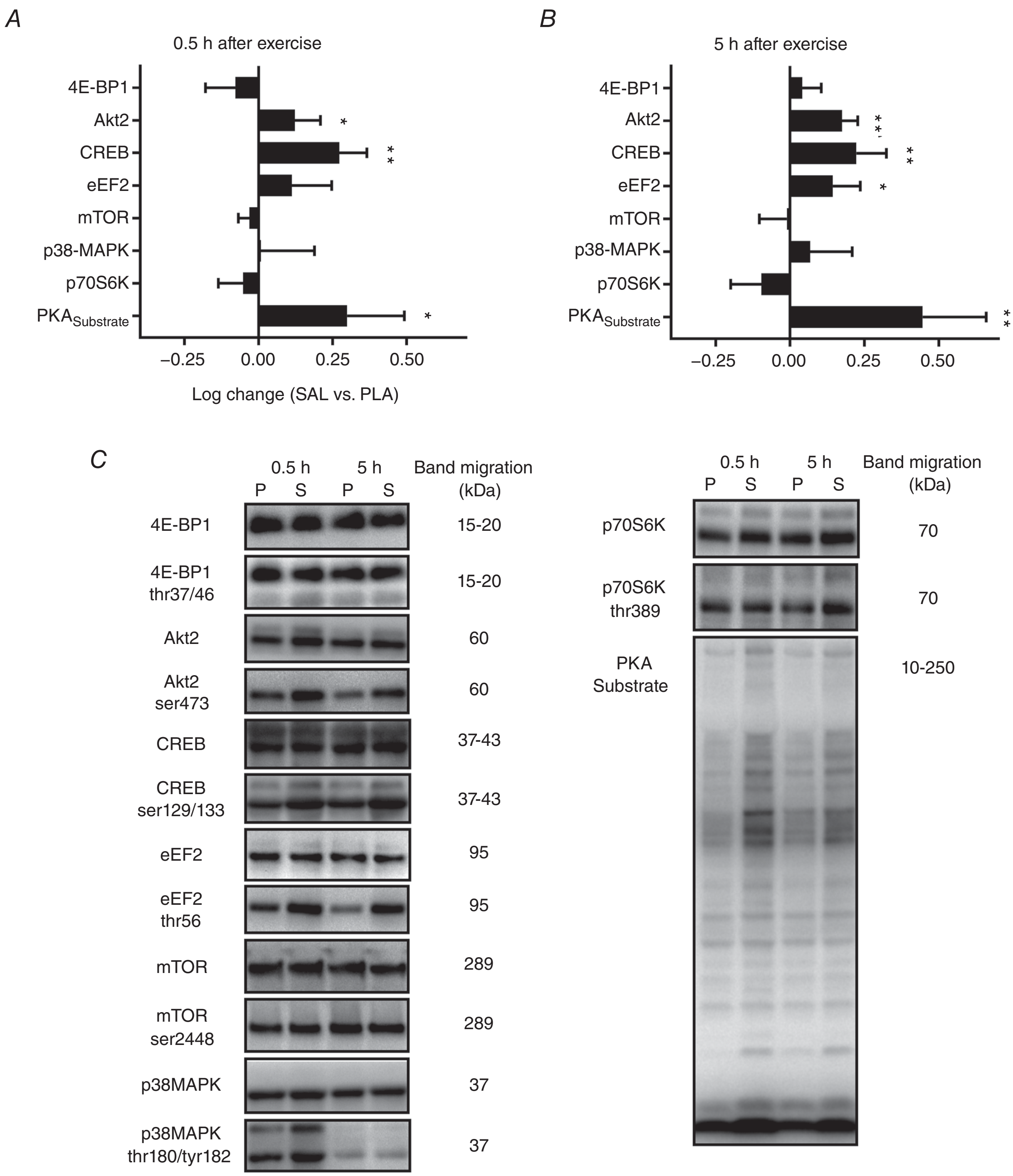

Figure 6. Phosphorylation-ratio induced by salbutamol (SAL) compared to placebo (PLA)
Phosphorylation-ratio induced by SAL compared to PLA in biopsies sampled from the vastus lateralis muscle 0.5 (*A*) and 5 h (*B*) after resistance exercise. Values are the mean log-change ($n = 12$). Error bars represent upper or lower bound of the 95% CI. *Treatment difference ($P < 0.05$). **Treatment difference ($P < 0.01$). *C*, representative blots for salbutamol (S) and placebo (P).

5 h following resistance exercise, as indicated by a higher phosphorylation of PKA substrates and downstream activation of cAMP/PKA-dependent target CREB. In rodents, the growth-promoting mechanisms of beta$_2$-adrenergic signalling involves Akt, mTOR and MAPK pathways (Kline *et al.* 2007; Sato *et al.* 2013), which are predominant in the regulation of translation initiation (Goodman, 2014) and cell proliferation and differentiation (Pearson *et al.* 2001). Despite the induced PKA-signalling and higher phosphorylation of Akt2 with salbutamol 0.5 and 5 h following exercise, we observed no changes in phosphorylation of mTOR^{Ser2448} and downstream effectors of translation initiation, p70S6K and 4E-BP1, or in phosphorylation of p38-MAPK. Although the latter observations may appear to be inconsistent with reports in rodents (Sato *et al.* 2013), beta$_2$-adrenergic signalling may be muscle fibre-type specific (Gonçalves *et al.* 2012) and some studies report no effect of beta$_2$-adrenergic stimulation on p38-MAPK phosphorylation (Kim *et al.* 2013). Reports in mice also indicate that beta$_2$-adrenergic stimulation does not phosphorylate mTOR^{Ser2448}, although it may phosphorylate mTOR^{Ser2481} (Sato *et al.* 2014), which could be a possible explanation of the observed increase in phosphorylation of Akt^{Ser473} (Copp *et al.* 2010). Indeed, mTOR^{Ser2448} may not be a target of Akt (Figueiredo *et al.* 2017). We also observed that salbutamol increased the phosphorylation of eEF2, which acts to reduce ribosomal elongation activity (Ryazanov *et al.* 1988). Although this may appear to be unexpected considering the higher protein synthesis rate and phosphorylation of Akt2 with salbutamol, studies have shown that cAMP-PKA-dependent signalling induces phosphorylation of eEF2 and inhibition of peptide elongation *in vitro* (Redpath & Proud, 1993).

Aside from the induced changes in signalling, we observed that salbutamol modulated mRNA levels of ActivinRIIB, calpain-1, FoxO1, myostatin and Smad3 following exercise. Most noteworthy was the upregulation of mRNA levels of the negative regulator of growth, myostatin. Although increased mRNA levels of myostatin may appear counterintuitive given the anabolic properties of beta$_2$-agonists, Abo *et al.* (2012) observed that hypertrophy induced by beta$_2$-agonist was associated with increased protein levels of myostatin in rats. Importantly, we also observed that salbutamol induced a downregulation of the mRNA level of the receptor target of myostatin, ActivinRIIB, 5 h after exercise. Thus, a potential upregulation of myostatin induced by beta$_2$-agonist may be counteracted by a concurrent downregulation of ActivinRIIB, which is consistent with that observed in rat tibialis anterior muscle following beta$_2$-agonist treatment (Pearen *et al.* 2009). In addition, we observed that salbutamol upregulated mRNA levels of Smad3 and FoxO1. Given that Smad3-null mice display loss of satellite cells and muscle atrophy (Ge *et al.* 2011), it may be speculated that a beta$_2$-agonist-induced upregulation of Smad3 plays a role in growth-promotion. FoxO1, a regulator of the atrophy-related genes atrogin and MuRF (Bodine & Baehr, 2014), is among the targets that are regulated by the ActivinRIIB-myostatin system and Akt signalling. However, despite significant Akt activation and upregulation of FoxO1 mRNA levels with salbutamol, we observed no changes in the mRNA level of atrogin and MuRF with salbutamol compared to placebo. Furthermore, although a potential

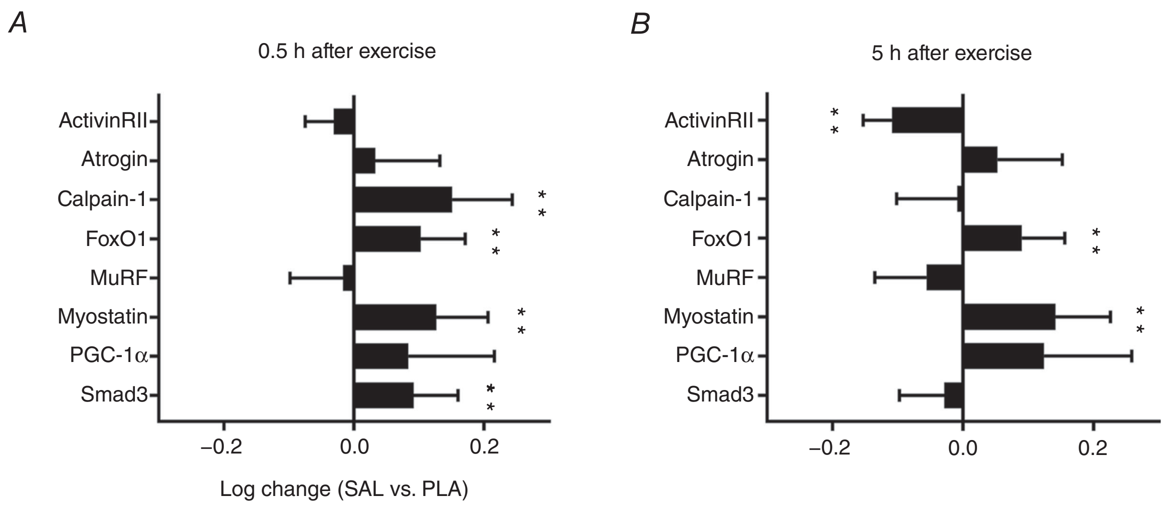

Figure 7. mRNA response induced by salbutamol (SAL) compared to placebo (PLA)
mRNA response induced by SAL compared to PLA in biopsies sampled from the vastus lateralis muscle 0.5 (*A*) and 5 h (*B*) after resistance exercise. Values are the mean log-change ($n = 12$). Error bars represent upper or lower bound of the 95% CI. **Treatment difference ($P < 0.01$).

effect of CREB activation is increased transcription of PGC-1α, we observed no effect of salbutamol on mRNA levels of PGC-1α compared to placebo. The latter observation is consistent with observations in rats, where beta$_2$-agonist treatment with clenbuterol did not necessarily affect PGC-1α mRNA levels (Kim *et al.* 2013; Shimamoto *et al.* 2017). We also observed that the calpain1 mRNA content was increased by salbutamol 0.5 h following exercise, which potentially may have contributed to Ca^{2+}-dependent proteolysis and thus the higher protein breakdown for salbutamol than placebo. The observation that salbutamol increased calpain mRNA levels is consistent with rodent studies, where beta$_2$-agonist treatment with formoterol increased calpain mRNA levels (Koopman *et al.* 2010).

The effect of beta$_2$-agonist on gene transcription and signalling possibly depends on timing of sampling and the biological samples (e.g. cells *vs.* tissue), as well as on type and dose of beta$_2$-agonist used (Baker, 2010; Wannenes *et al.* 2012). For example, although clenbuterol repressed mRNA levels of atrogin and MuRF in C2C12 muscle cell lines (Wannenes *et al.* 2012), no effect was found in rat soleus muscle after 3 days of treatment with clenbuterol (Gonçalves *et al.* 2012). Furthermore, unlike clenbuterol, salbutamol was shown to have no apparent effect on mRNA levels of atrogin and MuRF in C2C12 muscle cell lines (Wannenes *et al.* 2012). Based on the present study, along with studies in rodents, changes in muscle protein turnover induced by beta$_2$-agonists are possibly multifactorial, involving complex regulation of gene transcription and ribosomal translation (Pearen *et al.* 2009; Koopman *et al.* 2010).

In summary, the present observations show that selective activation of beta$_2$-adrenoceptors with salbutamol increases myofibrillar FSR and protein turnover rates in skeletal muscle following resistance exercise in trained young men. Furthermore, our observations indicate that LBM confounds the salbutamol-induced change in myofibrillar FSR. The effect of salbutamol in protein turnover rates was associated with pronounced PKA-signalling and phosphorylation of CREB and Akt2, as well as a concurrent mRNA response for growth-regulating genes, including ActivinRII, FoxO1 and myostatin.

Methodological considerations

We observed that the arterial phenylalanine enrichment rose from ∼12% to 13.5% MPE during the period of which the tracer measures were performed and therefore whether isotopic steady-state was achieved in the present study could be considered. Nonetheless, we observed a constant venous enrichment of ∼11.5% MPE and no difference in the I.M. enrichment at 0.5 and 5 h in recovery from exercise (0.079 ± 0.014 and 0.082 ± 0.013, respectively, $P = 0.32$) (mean ± SD), demonstrating that close to the actual site of protein turnover, tracer enrichments were not changing significantly. In clinical trials where homeostasis may be affected by drugs, exercise or other factors, minor fluctuations in circulating tracer enrichments may also be expected. In the present study, as well as in some other protocols (Rahbek *et al.* 2014; Mikkelsen *et al.* 2015), we applied a rather high tracer infusion rate (7 μmol × kg LBM^{-1} × h^{-1}) compared to 3.6 μmol × kg whole body weight^{-1} × h^{-1} used in other studies (Wilkinson *et al.* 2015; Wall *et al.* 2016). Our rationale for this infusion rate was to improve analytical sensitivity to allow detection of expectedly small intervention differences. However, with the precision of modern mass spectrometers, the relative high tracer infusion rate was probably not necessary and it is recommended to use a lower infusion rate to limit costs and reduce potential impact of the tracer on metabolism. It was shown, however, that the myofibrillar FSR was unaffected by flooding with 1665 mg phenylalanine (>10,000 μmol), increasing the blood (and most probably also intracellular) concentrations several-fold (Holm *et al.* 2014). For comparison, the infusion in the present study equalled a total amount of ∼500–600 mg phenylalanine over a 7 h time period, which probably did not affect phenylalanine metabolism and the muscle protein synthesis rate. In addition, in the present study, we used different tracer principles to investigate the effect of salbutamol *vs.* placebo on protein turnover rates. Although some variability was observed within the different estimates, consistency appeared across the findings when evaluated over the entire post-exercise period and, for the primary outcome measure (myofibrillar FSR determined by the direct incorporation method), we observed consistent findings with the three-pool tracer dilution estimate of protein synthesis rate ($F_{O,M}$) (Pearson's $r = 0.52$, $P = 0.009$). It should also be highlighted that the effect of beta$_2$-agonist *vs.* placebo on protein turnover rates observed in the present study was in a postprandial setting where the subjects also performed exercise. Therefore, any interpretation of the sole beta$_2$-adrenergic effect based on the present study should be made with caution because nutritional intake and exercise may confound the effect of beta$_2$-agonist.

Translational perspectives

The present study adds to animal studies showing that beta$_2$-agonist can alter protein turnover in skeletal muscle following resistance exercise in humans. The practical implications of beta$_2$-agonist-induced changes in protein turnover rates remain to be determined. Although studies in rodents have provided support of beta$_2$-agonists as treatment of muscle atrophy, concerns were raised because of concurrent adverse ventricular remodelling and collagen filtration (Gregorevic *et al.*

2005; Burniston *et al.* 2007). However, given the markedly lower relative doses prescribed to humans, such effects are possibly not a major concern. Furthermore, the most commonly used beta$_2$-agonists in humans, such as salbutamol and formoterol, have superior selectivity for the beta$_2$-adrenoceptor compared to clenbuterol and fenoterol (Baker, 2010), thus reducing or avoiding potential adverse activation of cardiac beta$_1$-adrenoceptors. Recent human studies also show that beta$_2$-agonists may hold some promise as anabolic agents with few minor side effects (Hostrup *et al.* 2015; Lee *et al.* 2015; Jessen *et al.* 2018). The beta$_2$-agonist-induced increase in protein turnover may also have implications for proteome signature remodelling of various components in skeletal muscle. Indeed, beta$_2$-agonist treatment has been shown to modulate proteome signature adaptations to endurance training in humans (Hostrup *et al.* 2018). Furthermore, given that remodelling and recycling of myocellular proteins are important adaptive processes with respect to stress and exercise (Camera *et al.* 2017), it may be that beta$_2$-agonists augment post-exercise recovery processes after resistance exercise. The observation that supratherapeutic oral doses of salbutamol increase protein turnover rates in association with resistance exercise provides support for the anti-doping regulatory restrictions regarding the supratherapeutic use of beta$_2$-agonists in competitive sport.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

MH designed the study and participated in the human experiments. LH and SR contributed to the conception of design and phenylalanine analyses. AK, JB, JE, MK, MN, SJ and VB participated in the human experiments. MT performed the immunoblotting. CMK and HP performed the RNA isolation, reverse transcription and real-time PCR. GAJ developed the salbutamol assay and performed the analysis of salbutamol in plasma. All authors contributed to the analysis and interpretation of data and the drafting of the manuscript. All authors approved the final version of the manuscript submitted for publication.

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