Hostrup Morten (Orcid ID: 0000-0002-6201-2483)

Jacobson Glenn (Orcid ID: 0000-0002-3409-8769)

Thevis Mario (Orcid ID: 0000-0002-1535-6451)

Title Page

Beta$_2$-adrenergic ligand racemic formoterol exhibits enantioselective disposition in blood and skeletal muscle of humans, and elicits myocellular protein kinase A-signalling at therapeutic inhaled doses

Running Title

Enantioselective disposition of racemic formoterol in blood and muscle

Morten Hostrup[1]*, Christian K. Narkowicz[2], Sajad Habib[1], David S. Nichols[3], Glenn A. Jacobson[2]

[1]Section of Integrative Physiology, Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, [2]Discipline of Pharmacy, School of Medicine, University of Tasmania, Hobart, Australia, [3]Central Science Laboratory, University of Tasmania, Hobart, Australia

Corresponding Author
Morten Hostrup
Section of Integrative Physiology
Department of Nutrition, Exercise and Sports
University of Copenhagen
August Krogh Building 2nd floor
Universitetsparken 13
DK-2100 Copenhagen
Denmark
E-mail: mhostrup@nexs.ku.dk
Tel: +45 2447 4785
Fax: +45 3531 2179
ORCID ID: 0000-0002-6201-2483

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/dta.2580

Abstract

While studies have demonstrated substantial differences in beta$_2$-adrenergic agonist enantiomer pharmacology, enantioselective disposition of long-acting beta$_2$-adrenergic ligand racemic (*rac*)-formoterol in blood is unexplored after inhaled therapy given analytical challenges. Furthermore, information on enantioselective disposition and partitioning of beta$_2$-adrenergic agonist in skeletal muscle is absent despite its promising data on muscle anabolism in humans. Using a sensitive UPLC-MS/MS (ultra-high performance liquid chromatography-mass spectrometry) assay, we determined disposition of (*R,R*)-formoterol and (*S,S*)-formoterol in plasma and skeletal muscle samples from 11 non-asthmatic men who had inhaled *rac*-formoterol at therapeutic doses (2×27 µg). Mean (SD) concentrations of (*R,R*)- and (*S,S*)-formoterol in plasma and in muscle biopsies of the *vastus lateralis* 1 h after inhalation of formoterol were 31 (15) and 45 (18) pg×mL^{-1} for (*R,R*)-formoterol and (*S,S*)-formoterol, respectively, in plasma, and 0.56 (0.32) and 0.51 (0.29) pg×mg$_{\text{wet wt}}^{-1}$, respectively, in muscle. Formoterol exhibited different enantioselective disposition in plasma and muscle (p<0.0001). In plasma, mean log (*R,R*):(*S,S*)-formoterol ratio was lower than 0 [–0.17(0.07), p<0.0001], whereas in muscle, mean log (*R,R*):(*S,S*)-formoterol ratio was slightly higher than 0 [0.04(0.07), p<0.05]. Log (*R,R*):(*S,S*)-formoterol ratio in muscle was related to muscle fibre-type composition. Furthermore, formoterol induced an approximately two-fold increase in muscle p-PKA$^{\text{Ser/thr}}$ phosphorylation (p<0.01), indicating a substantial beta$_2$-adrenergic response. Collectively, these findings suggest that formoterol exhibits modest enantioselective disposition in plasma after inhaled therapy in humans, which appear related to a greater (*R,R*)-enantiomer disposition in skeletal muscle that may be dependent on fibre-type composition.

Keywords: beta-adrenoceptor, beta2-adrenoceptor, beta-2, arformoterol, LABA

Introduction

Inhaled beta$_2$-adrenergic agonists (beta$_2$-agonists) are commonly prescribed drugs, which is attributed to the high prevalence of asthma, exercise-induced bronchoconstriction and chronic obstructive pulmonary disease (COPD)[1,2]. While the primary application of inhaled beta$_2$-agonists is to induce bronchial smooth muscle relaxation, a large proportion of the drug enters the systemic circulation and distributes throughout the body[3,4]. Skeletal muscle, the largest tissue of the human body in non-obese individuals[5], has a high density of beta-adrenoceptors of which the beta$_2$-subtype accounts for approximately 90%[6,7]. Stimulation of muscle beta$_2$-adrenoceptors with selective agonists modulates myocellular excitation-contraction coupling and metabolism[8,9], which appear mediated by cAMP/protein kinase A (PKA)-dependent signalling[10-12]. Furthermore, beta$_2$-agonists stimulate muscle protein turnover and growth[13-16]. Given their anabolic and lipolytic properties, beta$_2$-agonists have attracted interest as potential treatment in muscle wasting disorders and obesity[17,18].

Beta$_2$-agonists are mainly sold as racemic (*rac*-) 1:1 mixtures, consisting of an *R*- and *S*-enantiomer with different pharmacodynamics and pharmacokinetics[3,19-21]. It is generally accepted that the (*R*)-enantiomer is responsible for the pharmacological activity, whereas the (*S*)-enantiomer is generally considered pharmacodynamically inert[22]. The therapeutic application of beta$_2$-agonists may therefore be superior for (*R*)-enantiopure formulations. There is a reasonable body of *in vitro* and *in vivo* work investigating enantioselective effects of beta$_2$-agonist *R*- and *S*-enantiomers in smooth muscle tissue of animals and humans mainly for respiratory research[23], as well as interest as an anabolic and lipolytic agent[17,18,24]. However, despite a substantial body of literature on beta$_2$-adrenergic effects in skeletal muscle[14,25], it is unexplored to what extent beta$_2$-agonists distribute in skeletal muscle of humans and whether

any disposition is enantioselective. A handful of studies in animals have demonstrated enantioselective disposition of beta$_2$-agonist clenbuterol[26-28] and salbutamol enantiomers in muscle tissue of rodents [29]. Given the enantioselective disposition of beta$_2$-agonists observed in blood and urine from humans[3,20,21], it seems plausible that skeletal muscles are major enantioselective deposits for beta$_2$-agonists.

Formoterol, *N*-(2-hydroxy-5-((*S*)-1-hydroxy-2-(((*S*)-1-(4-methoxyphenyl)propan-2-yl)amino) ethyl) phenyl) formamide, is a long-acting beta$_2$-agonist commonly prescribed in asthma as a *rac*- 1:1 mixture of (*R,R*)-formoterol and (*S,S*)-formoterol (Fig.1). There is little data on enantiomer levels of formoterol in humans, which may be related to analytic challenges associated with the microgram inhaled doses of formoterol administered clinically[30]. Median plasma concentrations of enantiomers following inhaled dosing with *rac*-formoterol were less than 15 pg×mL^{-1} following a 24 µg dose[31] and there have been further studies reporting formoterol enantiomers in urine present at sub-nanogram concentrations[20,32]. Last, in spite of the promising data on formoterol in relation to energy expenditure[17,33] and muscle protein turnover observed in clinical trials[13], no study has determined enantiomer disposition and partitioning of formoterol in human skeletal muscle as well as the resultant canonical beta$_2$-adrenergic PKA-signalling response.

The purpose of the present study was to determine enantioselective disposition of (*R,R*)- and (*S,S*)-formoterol in plasma and skeletal muscle of non-asthmatic men after inhaled therapy using UPLC-MS/MS (ultra-high performance liquid chromatography-mass spectrometry). Secondary purposes were to examine the muscle:plasma partition coefficient of each individual enantiomer, the relation between enantioselective disposition in muscle and muscle fibre-type

composition, and the beta$_2$-adrenergic PKA-signalling response to *rac*-formoterol in human skeletal muscle.

Materials and methods

Volunteer study

Stored venous plasma and *vastus lateralis* muscle biopsies sampled from 11 recreationally active men during a previous study[34] were used for the present study. Subjects were 21–43 years, 167–185 cm height and had a tissue mass of 72–103 kg (17–31% body fat). Subjects had no history of asthma and were beta$_2$-agonist naive. Subjects were informed about potential risks and discomforts related to the study, and each subject gave written and oral informed consent prior to inclusion. The study was approved by the Committee on Health Research Ethics of the Capital Region of Denmark and performed in accordance with the Helsinki declaration.

Sample collection

The experimental protocol has been described in Kalsen *et al.* (2016)[34]. Briefly, subjects met in morning after an overnight fast and inhaled two doses of 27 µg formoterol (Oxis Turbohaler®, AstraZeneca, Cambridge, UK), with the two doses being separated by 45 min. Approximately 1 h after the second dose, a venous blood sample (4 mL) and a muscle biopsy were collected for determination of enantioselective disposition of *rac*-formoterol. The blood sample was drawn in a gel-free lithium heparin tube (BD vacutainer, NJ, US) from a catheter in the medial ante cubital vein and stood in room temperature for 15 min before being spun at 4000 rpm for 15 min after which plasma was collected and stored at –80 °C until analysis. Muscle biopsies were sampled from the *vastus lateralis* using a 4-mm Bergström needle with

suction[35]. Prior to sampling, an incision was made through the skin and fascia at the belly of the *vastus lateralis* muscle during local anesthesia with lidocaine (2 mL lidocaine without epinephrine, Xylocaine® 20 mg×mL^{-1}, AstraZeneca, Cambridge, UK). Muscle biopsy specimens were snap-frozen in liquid nitrogen and stored at –80 °C until analysis.

Analysis of (R,R)- and (S,S)-formoterol enantiomer levels in plasma and muscle

Plasma and muscle biopsy samples were couriered from University of Copenhagen, Denmark to University of Tasmania, Australia under dry ice temperature-controlled conditions, where the chemical analyses were undertaken.

Enantioselective formoterol analyses follow on from our previous beta$_2$-agonist work[29,36] with the assay optimised for formoterol. Analyses were undertaken using UPLC-MS/MS (ultra-high performance liquid chromatography-mass spectrometry) consisting of a Waters Acquity® H-class UPLC system coupled to a Waters Xevo® triple quadrupole mass spectrometer; Waters Corporation, Milford, MA) with chromatography performed using an Astec® CHIROBIOTIC™ T2 chiral column (4.6×250 mm×5 μm particles) (Sigma-Aldrich, St. Louis, MI, US).

Pieces of muscle tissue (20 to 200 mg$_{\text{wet wt}}$) were weighed with an electron scale. Water (deionised, 500 μL), ammonia solution (pH ~10, 3.2 μL×mg^{-1} tissue mass) and 10 μL internal standard solution (*rac*-formoterol-D6; Toronto Research Chemicals, Toronto, Canada) equivalent to 10 ng were added. The muscle tissue was homogenised in this mixture using a mechanical rotor type tissue homogeniser (Tissue-Tearor, Biospec Products USA) until no intact muscle tissue was evident. Homogenate was adjusted to a pH of 8.5. Ethyl acetate (1 mL) was added to the homogenate and mixed using the homogeniser for 1 min. The tube was

then centrifuged at 15,000 *g* for 15 min. The separated ethyl acetate fraction was transferred into a glass vial. The tissue homogenate was extracted a second time with ethyl acetate in a similar fashion and the ethyl acetate fraction was combined with the first extract. Samples for analysis were prepared from this extract as described below.

Calibration samples were prepared by spiking drug free human plasma and bovine skeletal muscle samples with unlabelled *rac*-formoterol (formoterol fumarate dihydrate; Carbosynth, Compton, UK) over the calibration ranges of 0-20 and 0-10 ng×mL^{-1} for plasma and muscle, respectively. Internal standard (*rac*-formoterol-D6; Toronto Research Chemicals, Toronto, Canada) equivalent to 10 ng was added to each calibration and study sample aliquot (400 µL), then dilute ammonia solution (150 µL) was added to each and vortex mixed before the addition of 850 µL of HPLC grade ethyl acetate. This was vortex mixed for one min and then centrifuged at 15,000 *g* for five min. The organic supernatant was then collected, and the ethyl acetate extraction repeated and the two residues combined; solvent was then evaporated under nitrogen at 40 °C and reconstituted using 80 µL of methanol and vortex mixed prior to analysis via UPLC-MS/MS.

Analyses were undertaken using multiple reaction monitoring (MRM) in positive electrospray ionisation mode. The UPLC was operated with a mobile phase consisting of 100% methanol with 0.2% acetic acid and 0.025% ammonium hydroxide. Elution was isocratic for 30 min. The flow rate was 0.8 mL×min^{-1} and the column was held at room temperature. Injection volume was 50 µL. Electrospray ionisation was performed with a capillary voltage of 2.76 KV, a cone voltage of 30 V and individual collision energies for each MRM transition, as described below. The desolvation temperature was 450 °C, nebulising gas was nitrogen at 950 L×h^{-1} and cone gas was nitrogen at 50 L×h^{-1}. MRM transition monitored for formoterol was (*m/z*) 345 to

149, (collision energy 19 V), and MRM transition monitored for formoterol-D6 was (*m/z*) 351 to 155, (collision energy 19 V). Dwell time per channel was 36 ms.

Confirmation of enantiomer elution order was undertaken by analysis of (*R,R*)-formoterol standard (TLC Pharmaceutical Standards, Ontario, Canada). The "drop perpendicular" method of peak integration was used where a vertical line from the valley of the peaks is dropped to the horizontal baseline. The method detection limit (MDL) was determined by signal-to-noise ratio (S/N=3) extrapolated from the 0.2 ng×mL^{-1} calibration standard, accuracy (n=4) was estimated at the 1 and 2 ng×mL^{-1} for plasma and muscle, respectively, and precision estimated across the calibration range (0.2-20 and 0.1-10 ng×mL^{-1} for plasma and muscle, respectively; each n=4). For muscle:plasma partition coefficient (K_m) determinations, ng×mL^{-1} (plasma) was considered equivalent to ng×g^{-1} (muscle).

Immunoblotting and SDS-page

Western blotting was performed as previously described[25]. In brief, muscle pieces were homogenized (Qiagen Tissuelyser II, Retsch GmbH, Haan, Germany) in a fresh batch of buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 2 mM Na3VO4, 10 mM NaF, 2 mM PMSF, 1 mM EDTA (pH 8), 1 mM EGTA (pH 8), 10 µg×mL^{-1} Aprotinin, 10 µg×mL^{-1} Leupeptin and 3 mM 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 0.5 M Tris-base, 3 mL glycerol, 0.93 g DTT, 1 g SDS and 1.2 mg

bromophenol blue) and ddH_2O to achieve equal protein concentration. Equal amount of protein was loaded in each well of pre-casted gels (Bio-Rad Laboratories, US). Samples from same subject were loaded next to each other on the same gel with a mixed human muscle standard lysate loaded in two different wells used for normalization. Proteins were separated according to their molecular weight by SDS page gel electrophoresis and semi-dry transferred to a PVDF membrane (Bio-Rad Laboratories, US). The membranes were blocked in either 2% skimmed milk or 3% BSA in Tris-buffered Saline including 0.1% Tween-20 (TBST) before an overnight incubation in primary antibody at 4 °C and a subsequent 1 h incubation in horseradish-peroxidase (HRP) conjugated secondary antibody at room temperature. The bands were visualised with ECL (Millipore) and recorded with a digital camera (ChemiDoc MP Imaging System, Bio-Rad Laboratories, US). Densitometry quantification of the western blot band intensity was performed using Image Lab version 4.0 (Bio-Rad Laboratories, US) and determined as the total band intensity adjusted for background intensity. The primary antibodies used were phospho-(Ser/Thr) PKA substrate (#9621, Cell signalling, MA, US), SERCAI (MA3-912, Thermo Fischer Scientific, MA, US) and SERCAII (SC-8095, Santa Cruz Biotechnology, TX, US). Secondary antibodies used were HRP conjugated rabbit anti-sheep (P-0163, DAKO, Glostrup, Denmark), goat anti-mouse (P-0447, DAKO, Glostrup, Denmark) and goat anti-rabbit IgM/IgG (4010-05 Southern Biotech, AL, US).

Myosin heavy chain composition in muscle homogenate

Myosin heavy chain (MHC) composition of the *vastus lateralis* muscle was determined from homogenate using gel electrophoresis as described previously[37]. Muscle homogenate prepared as described for Western blotting was diluted with 6×Laemmli sample-buffer (7 mL 0.5 M Tris-base, 3 mL glycerol, 0.93 g DTT, 1 g SDS and 1.2 mg bromophenol blue) and 100%

glycerol (50/50). A total of 1 µg of protein was separated on 8% self-cast stain free gels (49:1 acrylmid : bis-acrylmid, 30% glycerol, 200 mM Tris-base, 0.4% SDS, 0.1% APS and 0.1 M glycine) containing 0.5% 2,2,2 Trichloroethanol[38] for 16 h at 140 V on ice. MHC protein bands were visualized by ultraviolet activation of the stain free gel (ChemiDoc MP Imaging System) and were quantified densitometrically using imaging software (Image Lab v. 4.0, Bio-Rad Laboratories, Hercules, CA, US).

Statistics

Statistical analyses were performed in SPSS version 25 (IBM, Armonk, NY, US). Data were tested for normality using the Shapiro-Wilks test and Q-Q plots. Variables that violated normality were log-transformed. To estimate enantioselective disposition in plasma and muscle, one-sample t-test was used. To estimate tissue differences in enantioselective disposition between plasma and muscle, two-tailed linear mixed modelling was used with tissue as a fixed effect. To estimate association between subjects' muscle fibre type composition and muscle enantioselective disposition of formoterol, principal component analysis (PCA) with varimax rotation and multiple linear regression analysis were used. A comparison of muscle:plasma partitioning between enantiomers was assessed by paired student's t-test. Data are presented as mean and standard deviations (SD), unless otherwise stated, and exact *p*-values (unless lower than 0.001) to represent probability.

Results

Enantioselective UPLC-MS/MS assay performance

All matrices met acceptance criteria for accuracy (<15% deviation), precision (<15%RSD), recovery >20% in plasma and muscle, and estimate of linearity (r^2>0.999). For plasma, the MDL was 7 pg×mL^{-1} for both (*R,R*)- and (*S,S*)-formoterol. The MDL in muscle was variable and determined by biopsy mass; therefore based on a mean biopsy wet mass of 83 mg, MDL was estimated to be equivalent to 0.09 pg×mg^{-1} (but would be lower for larger biopsy mass and vice versa). All tissue samples had measurable levels of formoterol enantiomers with signal to noise ratio >10. Precision (%RSD) for plasma (*R,R*)- and (*S,S*)-formoterol was 12.2 and 8.9%, respectively; precison for muscle (*R,R*)- and (*S,S*)-formoterol was 11.6 and 10.9%, respectively. Accuracy deviation (%) for plasma (*R,R*)- and (*S,S*)-formoterol was 5.0 and 1.8%, respectively; accuracy deviation for muscle (*R,R*)- and (*S,S*)-formoterol was 3.2 and 4.9%, respectively. Example chromatograms of a muscle and plasma sample are shown in Figure 2.

(R,R)- and (S,S)-formoterol enantiomer disposition in plasma and skeletal muscle

Enantiomer levels of (*R,R*)- and (*S,S*)-formoterol in plasma and in muscle biopsies of the *vastus lateralis* 1 h after inhalation of formoterol are presented in figure 3. Mean(SD) plasma concentrations were 31(15) and 45(18) pg×mL^{-1} for (*R,R*)-formoterol and (*S,S*)-formoterol, respectively, and mean(SD) muscle concentrations were 0.56(0.32) and 0.51(0.29) pg×mg$_{wet\ wt}^{-1}$ for (*R,R*)-formoterol and (*S,S*)-formoterol, respectively (Fig. 3A-B). Formoterol exhibited different enantioselective disposition in plasma and muscle ($p < 0.0001$)(Fig. 3C). In plasma, mean log (*R,R*):(*S,S*)-formoterol ratio was lower than 0 [–0.17(0.07), $p < 0.0001$], whereas in muscle, mean log (*R,R*):(*S,S*)-formoterol ratio was slightly higher than 0 [0.04(0.07), $p = 0.046$] (Fig. 3C). The mean(SD) muscle:plasma partition coefficient (K_m) was 14.3(6.7) for *rac*-

formoterol, 18.9(9.7) for (*R*,*R*)-formoterol and 11.4(5.0) for (*S*,*S*)-formoterol indicating partitioning of both enantiomers into muscle with (*R*,*R*)-formoterol partitioning to a greater extent than (*S*,*S*)-formoterol ($p = 0.0012$).

Muscle fibre-type composition and enantioselective disposition

Principal component analysis revealed that muscle (*R*,*R*):(*S*,*S*)-formoterol ratio loaded together with muscle SERCAI content and MHCII distribution on the first principal component (Fig. 4A). There was a significant correlation [$r^2_{adj} = 0.56$, $p = 0.015$] between muscle log(*R*,*R*):(*S*,*S*)-formoterol ratio and principal component scores of muscle SERCAI+II content and MHCI+II distribution in a multiple linear regression (fig. 4B).

Muscle PKA-signalling

Inhalation of formoterol induced significant PKA-signalling in human *vastus lateralis* muscle 1 h after administration as indicated by an approximately two-fold greater p-PKA$^{Ser/thr}$ phosphorylation for formoterol than placebo-control ($p = 0.002$)(Fig. 5).

Discussion

Herein we have described the enantioselective disposition of (*R*,*R*)- and (*S*,*S*)-formoterol in plasma and skeletal muscle in non-asthmatic men who inhaled repetitive therapeutic doses of *rac*-formoterol (2×27 µg). In addition, we investigated the PKA-signalling response to formoterol in human skeletal muscle. The new findings of the study are 1) that formoterol exhibits modest, but divergent enantioselective disposition in plasma and muscle after inhaled administration, 2) that formoterol partitions into muscle with (*R*,*R*)-formoterol partitioning to

a greater extent than (*S*,*S*)-formoterol, 3) that the enantioselective disposition in muscle is associated with muscle fibre-type composition, and 4) that inhalation of formoterol in therapeutic doses elicits a significant beta$_2$-adrenergic PKA-signalling response in skeletal muscle.

To the best of our knowledge, the present study is first to investigate formoterol enantiomer levels in skeletal muscle of humans following inhalation therapy. In the past, determination of individual formoterol enantiomers in biological fluids has been limited by assay performance in studies that employed HPLC[30]. We successfully developed a sensitive and specific UPLC-MS/MS assay with a MDL of around 7 pg×mL^{-1}, equivalent to around 3 pg on column, sufficient to measure formoterol enantiomers in human plasma and muscle tissue. Accordingly, all plasma and muscle samples collected in the present study had measurable levels of each formoterol enantiomer. In our laboratory, we have found formoterol enantiomers are more difficult to measure than other beta$_2$-agonists, as there is a more significant trade-off between mobile phase composition for sensitivity and selectivity compared to for example salbutamol enantiomers.

We observed that *rac*-formoterol exhibited a modest enantioselective disposition in plasma and muscle with relatively lower (*R*,*R*)-formoterol than (*S*,*S*)-formoterol in plasma and higher (*R*,*R*)-formoterol than (*S*,*S*)-formoterol disposition in muscle 1 h after repetitive inhalation of *rac*-formoterol. Other beta$_2$-agonists, such as *rac*-salbutamol, have been shown to exhibit extensive enantioselective disposition in the circulation, where the plasma S-enantiomer resides in higher concentrations than the active R-enantiomer. This is consistently observed in a number of salbutamol pharmacokinetic parameters[3,39]. Although the degree of

enantioselective disposition in plasma is much lower for *rac*-formoterol than that observed for salbutamol, every subject had higher plasma concentrations of the (*S,S*)-enantiomer than the (*R,R*)-enantiomer in the present study.

The higher concentration of (*S,S*)-formoterol in plasma may be related to several factors. Firstly, the present study indicates that skeletal muscle exhibits some degree of enantioselective partitioning in favour of the (*R,R*)-enantiomer. Although the degree of enantioselective disposition in muscle was small, skeletal muscle encompasses approximately 40% of body mass in non-obese individuals[5], meaning that even a minor difference between enantiomers in muscle could have a large effect on circulating levels of (*R,R*)- and (*S,S*)-formoterol. Secondly, studies have demonstrated enantioselective differences in the urine excretion of unchanged formoterol. Oral and inhaled administration of *rac*-formoterol has been reported to result in higher levels of unchanged (*S,S*)-formoterol in urine[20,40]. Last, enantioselective metabolism of formoterol via the glucuronidation pathway may be a factor, as total levels of (*R,R*)-formoterol (unchanged drug and glucuronide metabolite) has been shown to predominate in urine, at least following oral dosing[40].

Our observation of a slightly higher disposition of the (*R,R*)-enantiomer of formoterol in skeletal muscle is consistent with the enantioselective disposition observed for salbutamol in rodents[29]. However, in contrast to the modest degree for formoterol, salbutamol exhibits more extensive enantioselective disposition in rat skeletal muscle, where the *R*-enantiomer of salbutamol resides in much higher concentrations than the *S*-enantiomer. Collectively, these observations suggest that distribution kinetics of beta$_2$-agonists are enantioselective with a preference of the *R*-enantiomers in muscle tissue and *S*-enantiomers in the circulation. It should

also be noted that compared to *rac*-salbutamol, the muscle:plasma partition coefficient (K_m) for *rac*-formoterol in the present study was greater than that previously reported in a rodent model for oral dosing of salbutamol (14.3 versus 3.8)[29], acknowledging that the route of administration (oral) and sampling (4 h) were also different. This greater partitioning could be a pharmacokinetic factor for consideration of formoterol as a preferential therapeutic for muscle wasting and obesity[13,17,33] or doping misuse[34] compared to the short acting salbutamol.

While animal models have indicated that slow-twitch dominated muscles (e.g. *soleus*) respond differently to beta$_2$-agonists than fast-twitch dominated (e.g. *extensor digitorum longus*)[18], it is unknown whether muscle fibre-type specific differences exist in the disposition of beta$_2$-agonist enantiomers. Given human skeletal muscle predominantly has a mixed fibre-type composition as compared to rodents, we performed an exploratory analysis of the association between muscle fibre-type composition and enantioselective disposition of (*R*,*R*)- and (*S*,*S*)-formoterol in muscle. Muscle fibres can be classified according to MHC isoform distribution and SERCA isoforms[41,42], in which slow-twitch fibres predominantly express MHCI and SERCAII, whereas fast-twitch fibres express MHCII and SERCAI. We observed that muscle (*R*,*R*):(*S*,*S*)-formoterol ratio loaded together with MHCII and SERCAI in a principal components analysis. This suggests that the enantioselective disposition of (*R*,*R*):(*S*,*S*)-formoterol may be fibre type-specific in humans, with a higher relative disposition of (*R*,*R*)-formoterol in fast-twitch fibres as compared to slow-twitch fibres.

The main canonical signalling pathway associated with the beta$_2$-adrenoceptor is the cAMP/PKA-dependent[11,12]. The repetitive inhaled therapeutic dose of formoterol administered in the present study was sufficient to induce significant beta$_2$-adrenergic cAMP/PKA signalling

in skeletal muscle 1 h after inhalation. As such, formoterol induced a two-fold greater $PKA^{Ser/Thr}$ phosphorylation in the *vastus lateralis* muscle. This observation underpins the efficacy of formoterol in activating $beta_2$-adrenergic signalling pathways in skeletal muscle, even after inhalation in therapeutic doses. The formoterol-induced PKA-signalling in skeletal muscle likely explains the apparent effects of formoterol on protein turnover and substrate utilization observed in previous studies[13,17,33,34].

An important consideration of the present study was that muscle concentrations of formoterol enantiomers were determined 1 h after inhalation of two repetitive doses of *rac*-formoterol, consistent with time to reach maximum plasma levels (t_{max})[20]. Given the observation that the pharmacokinetics of individual formoterol enantiomers in plasma differ[20,31], it is likely that (*R,R*)- and (*S,S*)-formoterol concentrations also exhibit a different time-course in muscle. For instance, it may be that (*R,R*)- and (*S,S*)-formoterol have differential partitioning, distribution and clearance kinetics in skeletal muscle. Such assessment, however, would require repeated muscle biopsy sampling for several hours because of the relatively long half-life of formoterol[20,31]. In addition, substantial inter-variability exists in the pharmacokinetics of $beta_2$-agonists, which is also reflected by the spread of the formoterol enantiomer concentrations observed in plasma and muscle in the present study.

In summary, the present study shows that *rac*-formoterol exhibits modest, but significant and divergent enantioselective disposition in the circulation and skeletal muscle of humans. Despite the therapeutic inhaled dose used, formoterol partitioned in muscle to an extent that was sufficient to induce a significant PKA-signaling response. Furthermore, our data indicate that the disposition of the (*R,R*)-enantiomer in human skeletal muscle may be fiber-type specific. The application of $beta_2$-agonists in treatment of muscle wasting and for weight-loss has been

suggested by several authors[17,18], and formoterol has been shown to effectively increase energy expenditure and to induce protein anabolism in clinical trials[13,17]. Given that the activity of formoterol resides with the (*R,R*)-enantiomer, being 1,000 times more potent than the (*S,S*)-enantiomer[19], our observations of greater partitioning of (*R,R*)-formoterol in muscle provide a rationale for chiral switch enantiopure (*R,R*)-formoterol rather than racemic mixture in future clinical trials for the purpose of increasing muscle metabolic rate and anabolism. For doping control purposes relating to beta$_2$-agonists, it may also be that more emphasis should be on analysis of the *R*-enantiomers. Indeed, a significant number of enantiopure beta$_2$-agonist products are available on the market.

Acknowledgements

We thank Anders Kalsen, Johan Onslev, Martin Thomassen and Michael Kreiberg for their excellent assistance with the human experiments. This research was supported by the Danish Ministry of Culture (Science Council).

Competing interests

Morten Hostrup and Glenn A. Jacobson have received funding by the World Anti-Doping Agency for research in beta$_2$-agonists with respect to urine thresholds. Morten Hostrup and Glenn A. Jacobson have provided independent expert scientific reports in doping cases related to beta$_2$-agonists.

Authorship contributions

Participated in research design: Hostrup and Jacobson.

Conducted experiments: Hostrup, Habib, Narkowicz

Contributed new reagents or analytic tools: Nichols, Narkowicz, Jacobson

Performed data analysis: Hostrup, Habib, Nichols, Jacobson

Wrote or contributed to the writing of the manuscript: Hostrup, Habib, Narkowicz, Nichols, Jacobson

All authors approved the final version of the manuscript.

References

1. Price OJ, Hull JH, Backer V, Hostrup M, Ansley L. The impact of exercise-induced bronchoconstriction on athletic performance: a systematic review. *Sports Med.* 2014;44(12):1749-1761.
2. Rottenkolber M, Voogd E, van Dijk L, et al. Time trends of period prevalence rates of patients with inhaled long-acting beta-2-agonists-containing prescriptions: a European comparative database study. *PLoS One.* 2015;10(2):e0117628.
3. Ward JK, Dow J, Dallow N, Eynott P, Milleri S, Ventresca GP. Enantiomeric disposition of inhaled, intravenous and oral racemic-salbutamol in man--no evidence of enantioselective lung metabolism. *Br J Clin Pharmacol.* 2000;49(1):15-22.
4. Dyreborg A, Krogh N, Backer V, Rzeppa S, Hemmersbach P, Hostrup M. Pharmacokinetics of Oral and Inhaled Terbutaline after Exercise in Trained Men. *Front Pharmacol.* 2016;7:150.
5. Zurlo F, Larson K, Bogardus C, Ravussin E. Skeletal muscle metabolism is a major determinant of resting energy expenditure. *J Clin Invest.* 1990;86(5):1423-1427.
6. Williams RS, Caron MG, Daniel K. Skeletal muscle beta-adrenergic receptors: variations due to fiber type and training. *Am J Physiol.* 1984;246(2 Pt 1):E160-167.
7. Jensen J, Brennesvik EO, Bergersen H, Oseland H, Jebens E, Brors O. Quantitative determination of cell surface beta-adrenoceptors in different rat skeletal muscles. *Pflugers Arch.* 2002;444(1-2):213-219.
8. Hostrup M, Kalsen A, Ortenblad N, et al. beta2-adrenergic stimulation enhances Ca2+ release and contractile properties of skeletal muscles, and counteracts exercise-induced reductions in Na+-K+-ATPase Vmax in trained men. *J Physiol.* 2014;592(Pt 24):5445-5459.
9. Kalsen A, Hostrup M, Karlsson S, Hemmersbach P, Bangsbo J, Backer V. Effect of inhaled terbutaline on substrate utilization and 300-kcal time trial performance. *J Appl Physiol (1985).* 2014;117(10):1180-1187.
10. Lynch GS, Ryall JG. Role of beta-adrenoceptor signaling in skeletal muscle: implications for muscle wasting and disease. *Physiol Rev.* 2008;88(2):729-767.
11. Ohnuki Y, Umeki D, Mototani Y, et al. Role of cyclic AMP sensor Epac1 in masseter muscle hypertrophy and myosin heavy chain transition induced by beta2-adrenoceptor stimulation. *J Physiol.* 2014;592(24):5461-5475.
12. Shi Y, Wu Z, Cui N, et al. PKA phosphorylation of SUR2B subunit underscores vascular KATP channel activation by beta-adrenergic receptors. *Am J Physiol Regul Integr Comp Physiol.* 2007;293(3):R1205-1214.
13. Lee P, Birzniece V, Umpleby AM, Poljak A, Ho KK. Formoterol, a highly beta2-selective agonist, induces gender-dimorphic whole body leucine metabolism in humans. *Metabolism.* 2015;64(4):506-512.
14. Hostrup M, Reitelseder S, Jessen S, et al. Beta2 -adrenoceptor agonist salbutamol increases protein turnover rates and alters signalling in skeletal muscle after resistance exercise in young men. *J Physiol.* 2018;596(17):4121-4139.
15. Hostrup M, Kalsen A, Onslev J, et al. Mechanisms underlying enhancements in muscle force and power output during maximal cycle ergometer exercise induced by chronic β2-adrenergic stimulation in men. *J Appl Physiol* 2015;119(5):475-486.
16. Jessen S, Onslev J, Lemminger A, Backer V, Bangsbo J, Hostrup M. Hypertrophic effect of inhaled beta2 -agonist with and without concurrent exercise training: A randomized controlled trial. *Scand J Med Sci Sports.* 2018;28(10):2114-2122.

17. Lee P, Day RO, Greenfield JR, Ho KK. Formoterol, a highly beta2-selective agonist, increases energy expenditure and fat utilisation in men. *Int J Obes (Lond).* 2013;37(4):593-597.
18. Joassard OR, Durieux AC, Freyssenet DG. beta2-Adrenergic agonists and the treatment of skeletal muscle wasting disorders. *Int J Biochem Cell Biol.* 2013;45(10):2309-2321.
19. Trofast J, Osterberg K, Kallstrom BL, Waldeck B. Steric aspects of agonism and antagonism at beta-adrenoceptors: synthesis of and pharmacological experiments with the enantiomers of formoterol and their diastereomers. *Chirality.* 1991;3(6):443-450.
20. Lecaillon JB, Kaiser G, Palmisano M, Morgan J, Della Cioppa G. Pharmacokinetics and tolerability of formoterol in healthy volunteers after a single high dose of Foradil dry powder inhalation via Aerolizer. *Eur J Clin Pharmacol.* 1999;55(2):131-138.
21. Jacobson GA, Fawcett JP. Beta2-Agonist Doping Control and Optical Isomer Challenges. *Sports Med.* 2016;46(12):1787-1795.
22. Fozard JR, Buescher H. Comparison of the anti-bronchoconstrictor activities of inhaled formoterol, its (R,R)- and (S,S)-enantiomers and salmeterol in the rhesus monkey. *Pulm Pharmacol Ther.* 2001;14(4):289-295.
23. Jacobson GA, Raidal S, Hostrup M, et al. Long-Acting beta2-Agonists in Asthma: Enantioselective Safety Studies are Needed. *Drug Saf.* 2018;41(5):441-449.
24. Marchant-Forde JN, Lay DC, Jr., Marchant-Forde RM, McMunn KA, Richert BT. The effects of R-salbutamol on growth, carcass measures, and health of finishing pigs. *J Anim Sci.* 2012;90(11):4081-4089.
25. Hostrup M, Onslev J, Jacobson GA, Wilson R, Bangsbo J. Chronic beta2 -adrenoceptor agonist treatment alters muscle proteome and functional adaptations induced by high intensity training in young men. *J Physiol.* 2018;596(2):231-252.
26. Smith DJ. Total radioactive residues and clenbuterol residues in edible tissues, and the stereochemical composition of clenbuterol in livers of broilers after exposure to three levels of dietary [14C]clenbuterol HCl and three preslaughter withdrawal periods. *J Anim Sci.* 1998;76(12):3043-3053.
27. von Deutsch DA, Abukhalaf IK, Wineski LE, et al. Beta-agonist-induced alterations in organ weights and protein content: comparison of racemic clenbuterol and its enantiomers. *Chirality.* 2000;12(8):637-648.
28. Hirosawa I, Ishikawa M, Ogino M, et al. Enantioselective disposition of clenbuterol in rats. *Biopharm Drug Dispos.* 2014;35(4):207-217.
29. Jacobson GA, Yee KC, Premilovac D, Rattigan S. Enantioselective disposition of (R/S)-albuterol in skeletal and cardiac muscle. *Drug Test Anal.* 2014;6(6):563-567.
30. van den Berg BT, Portier EJ, van den Berg M, Braat MC, van Boxtel CJ. First high-performance liquid chromatography assay of formoterol concentrations in the low-picogram-per-milliliter range. *Ther Drug Monit.* 1994;16(2):196-199.
31. Kharidia J, Fogarty CM, Laforce CF, et al. A pharmacokinetic/pharmacodynamic study comparing arformoterol tartrate inhalation solution and racemic formoterol dry powder inhaler in subjects with chronic obstructive pulmonary disease. *Pulm Pharmacol Ther.* 2008;21(4):657-662.
32. Butter JJ, vandenBerg BTJ, Portier EJG, Kaiser G, vanBoxtel CJ. Determination by HPLC with electrochemical detection of formoterol RR and SS enantiomers in urine. *J Liq Chromatogr R T.* 1996;19(6):993-1005.
33. Onslev J, Jacobson G, Narkowicz C, et al. Beta2-adrenergic stimulation increases energy expenditure at rest, but not during submaximal exercise in active overweight men. *Eur J Appl Physiol.* 2017;117(9):1907-1915.
34. Kalsen A, Hostrup M, Backer V, Bangsbo J. Effect of formoterol, a long-acting β2-adrenergic agonist, on muscle strength and power output, metabolism, and fatigue

during maximal sprinting in men. *Am J Physiol Reg Integ Comp Physiol.* 2016;310(11):R1312-R1321.
35. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest.* 1975;35(7):609-616.
36. Jacobson GA, Hostrup M, Narkowicz CK, Nichols DS, Haydn Walters E. Enantioselective disposition of (R)-salmeterol and (S)-salmeterol in urine following inhaled dosing and application to doping control. *Drug Test Anal.* 2017;9(8):1262-1266.
37. Albers PH, Pedersen AJ, Birk JB, et al. Human muscle fiber type-specific insulin signaling: impact of obesity and type 2 diabetes. *Diabetes.* 2015;64(2):485-497.
38. Ladner CL, Yang J, Turner RJ, Edwards RA. Visible fluorescent detection of proteins in polyacrylamide gels without staining. *Anal Biochem.* 2004;326(1):13-20.
39. Jacobson GA, Yee KC, Wood-Baker R, Walters EH. SULT 1A3 single-nucleotide polymorphism and the single dose pharmacokinetics of inhaled salbutamol enantiomers: are some athletes at risk of higher urine levels? *Drug Test Anal.* 2015;7(2):109-113.
40. Zhang M, Fawcett JP, Shaw JP. Stereoselective urinary excretion of formoterol and its glucuronide conjugate in human. *Br J Clin Pharmacol.* 2002;54(3):246-250.
41. Harridge SD, Bottinelli R, Canepari M, et al. Whole-muscle and single-fibre contractile properties and myosin heavy chain isoforms in humans. *Pflugers Arch.* 1996;432(5):913-920.
42. Lamboley CR, Murphy RM, McKenna MJ, Lamb GD. Sarcoplasmic reticulum Ca2+ uptake and leak properties, and SERCA isoform expression, in type I and type II fibres of human skeletal muscle. *J Physiol.* 2014;592(6):1381-1395.

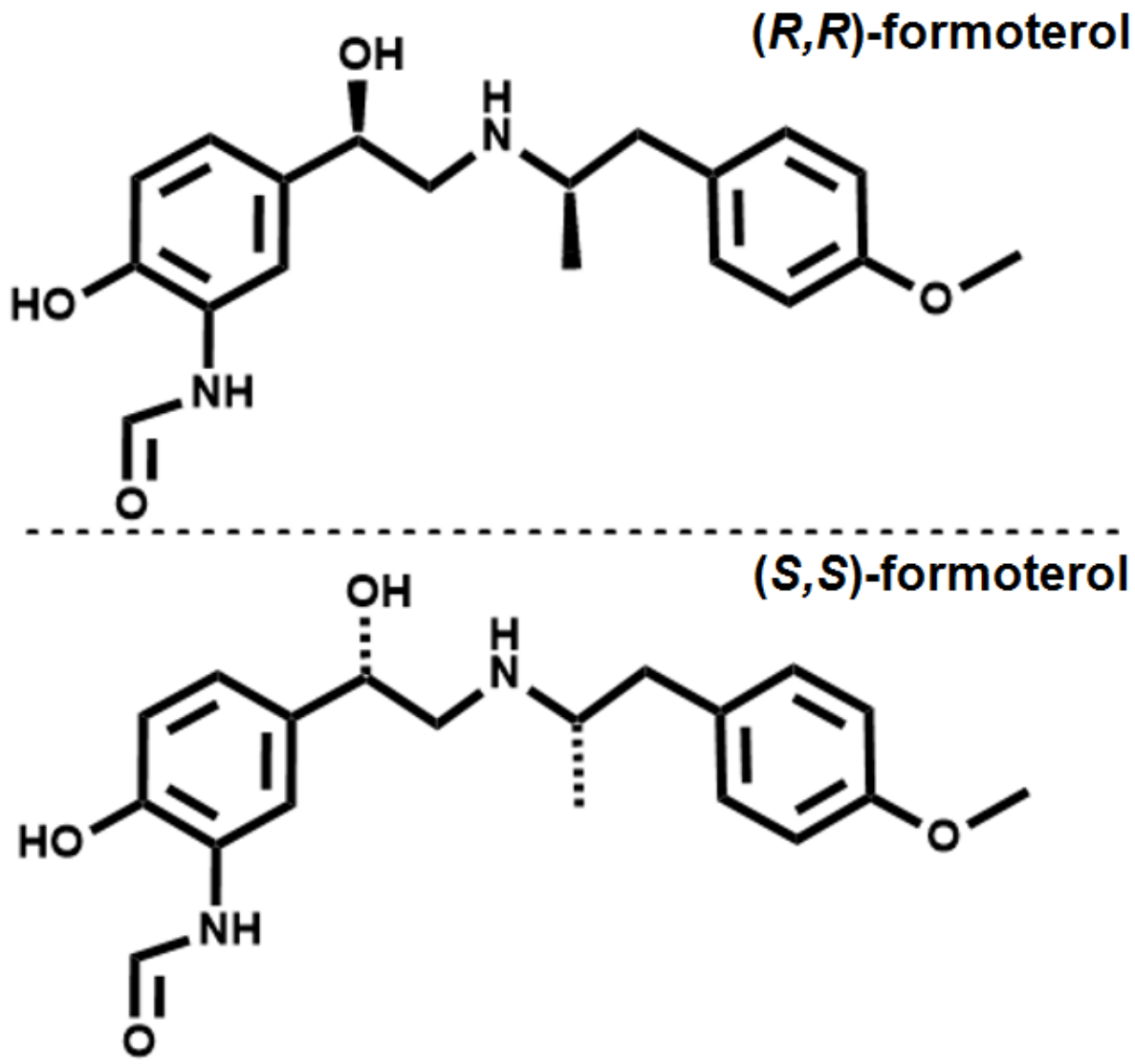

Fig. 1. Molecular structure of (*R*,*R*)-formoterol (upper panel) and (*S*,*S*)-formoterol (lower panel).

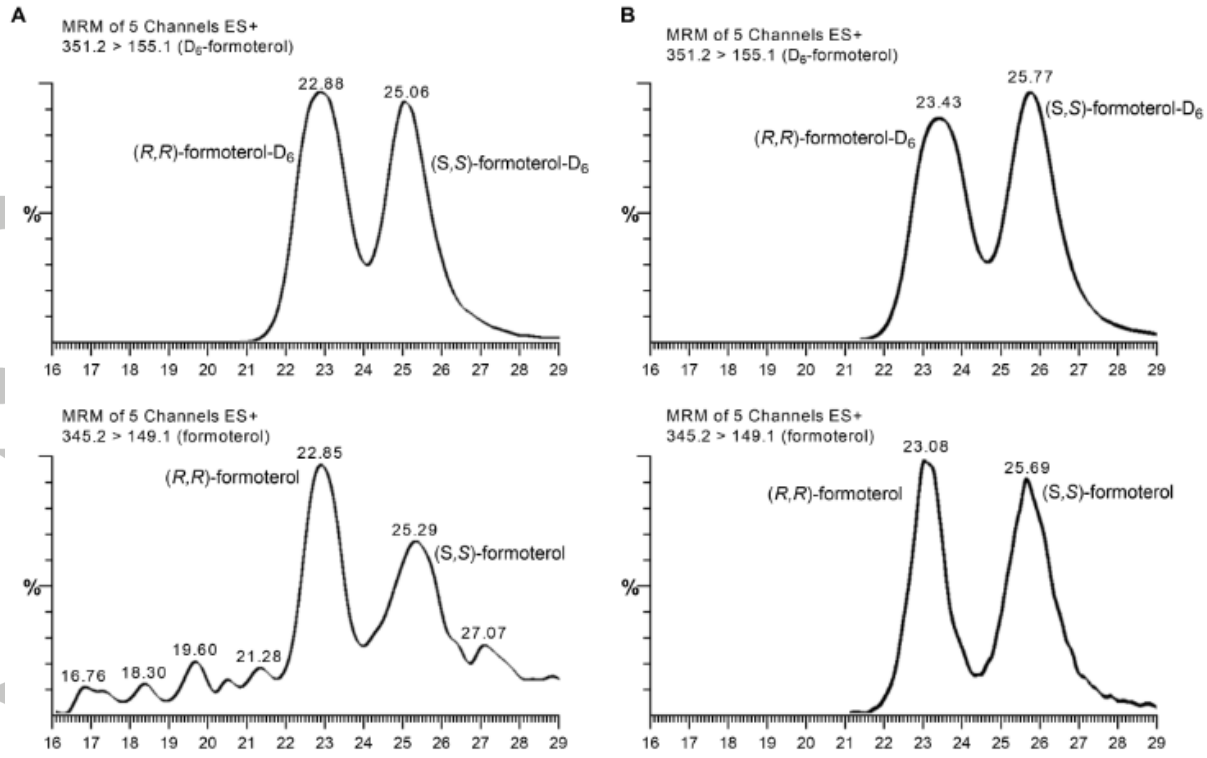

Fig. 2. Example chromatogram from a subject showing low level formoterol enantiomers in a muscle sample extract equivalent to 0.18 and 0.12 pg×mg^{-1} biopsy wet weight for (*R*,*R*)-formoterol and (*S*,*S*)-formoterol, respectively (**A**), and a corresponding plasma sample (**B**).

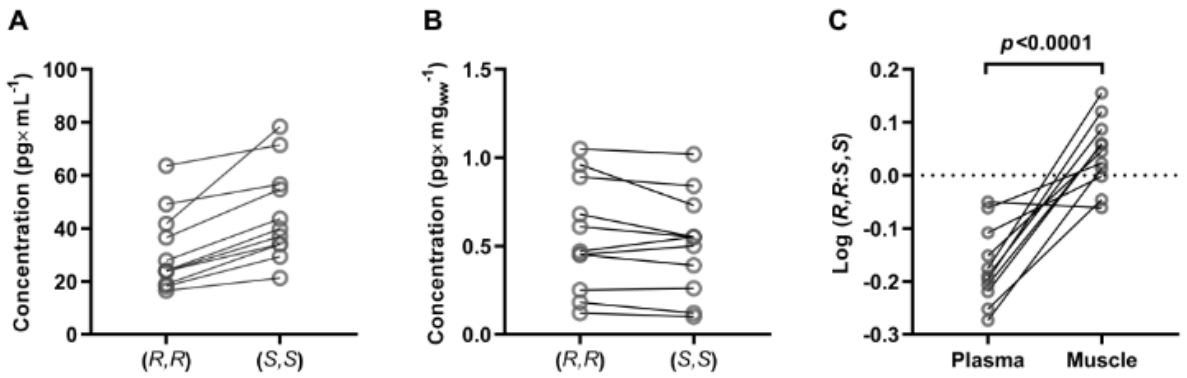

Fig. 3. Concentrations of (*R*,*R*)-formoterol and (*S*,*S*)-formoterol in plasma (**A**) and human *vastus lateralis* muscle (**B**), and enantioselective disposition [log(*R*,*R*):(*S*,*S*)-formoterol] in plasma and muscle (**C**). Individual values (n=11).

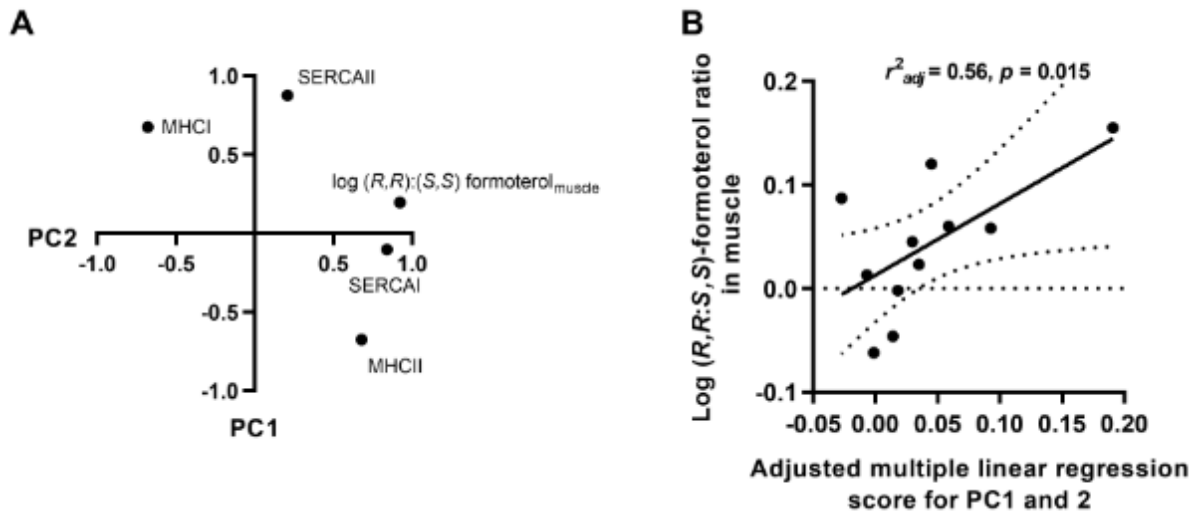

Fig. 4. Principal component analysis (**A**) and multiple linear regression analysis (**B**).

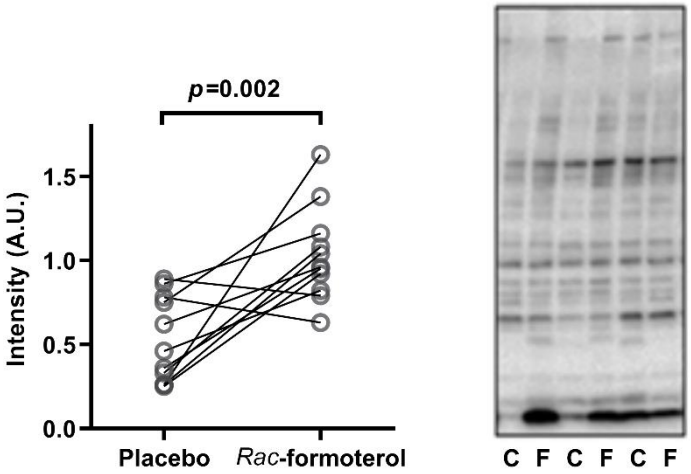

Fig. 5. Left panel shows individual values for p-PKA$^{Ser/thr}$ in human *vastus lateralis* muscle in subjects (n=11) who inhaled either placebo or formoterol (2×27 µg). Right panel shows representative blots for three subjects (C: placebo sample, F: formoterol sample).

Graphical Abstract

Information on enantioselective disposition and partitioning of beta$_2$-adrenergic agonists in skeletal muscle is absent despite their apparent clinical effects in human muscle. Herein we show that *rac*-formoterol exhibits modest, but significant and divergent enantioselective disposition in the circulation and skeletal muscle of humans. Despite the therapeutic inhaled dose used, formoterol partitioned in muscle to an extent that was sufficient to induce a significant PKA-signaling response.