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Inhaled salbutamol induces leanness in well-trained healthy females but not males during a period of endurance training: a randomized controlled trial

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Take-home message
Prolonged treatment with inhaled salbutamol induces leanness only in female athletes and concurrently impairs aerobic and strength related training outcomes in both sexes. Athletes with uncontrolled asthma should avoid overreliance on inhaled salbutamol.
Abstract

Introduction: Many athletes use short-acting inhaled beta2-agonists multiple times weekly during training sessions to prevent exercise-induced bronchoconstriction, but it is unclear if treatment impairs training outcomes. Herein, we investigated performance adaptations in well-trained females and males training with prior inhalation of salbutamol. Methods: Nineteen females and 21 males with maximal oxygen uptake ($\text{VO}_{2\text{max}}$) of 50.5±3.3 and 57.9±4.9 mL/min/kg participated in this double-blinded, placebo-controlled, parallel-group study. We randomized participants to placebo or salbutamol inhalation (1600 µg/training day) for 6 weeks of combined endurance (1x/week) and high-intensity interval-training (2x/week). We assessed participants’ body composition, $\text{VO}_{2\text{max}}$, and muscle contractile function, and collected vastus lateralis muscle biopsies. Results: Salbutamol induced a sex-specific loss of whole-body fat mass (sex×treatment: $p=0.048$) where only salbutamol-treated females had a fat mass reduction compared to placebo (−0.8 kg at 6 weeks; 95%CI: −0.5 to −1.6; $p=0.039$). Furthermore, salbutamol-treated females exhibited a repartitioning effect, lowering fat mass while gaining lean mass ($p=0.011$), which was not apparent for males ($p=0.303$). Salbutamol negatively impacted $\text{VO}_{2\text{max}}$ in both sexes (treatment main-effect: $p=0.014$) due to a blunted increase in $\text{VO}_{2\text{max}}$ during the initial 4 weeks of the intervention. Quadriceps contractile strength was impaired in salbutamol-treated females (−39 Nm; 95%CI: -61 to -17; $p=0.002$) compared to placebo at 6 weeks. Muscle electron-transport-chain complex I-V abundance increased with salbutamol (treatment main-effect: $p=0.035$) while content of SERCAI, beta2-adrenoceptor, and desmin remained unchanged. Conclusion: Inhaled salbutamol appears to be an effective repartitioning agent in females but may impair aerobic and strength related training outcomes.
Introduction

Many athletes use short-acting inhaled beta2-agonists multiple times weekly during training sessions to prevent exercise-induced bronchoconstriction – a condition characterized by airway narrowing in conjunction with exercise [1-7]. This is particularly true for athletes engaged in sports with high ventilatory loads, training volumes, and aerobic demands [1-3, 7, 8]. However, it remains unclear whether inhaled beta2-agonists affect training outcomes when used in conjunction with training.

Short-acting beta2-agonist salbutamol is the most common drug used by elite athletes to counter exercise-induced bronchoconstriction. While the World Anti-Doping Agency (WADA) restricts use of salbutamol and other beta2-agonists in- and out-of-competition, athletes are permitted to inhale salbutamol at a daily maximum dose of 1600 µg not to exceed 600 µg in any 8-h period [9]. The dosing limit is in place to prevent misuse at supratherapeutic inhaled doses and via systemic routes, as salbutamol can impose performance-enhancing effects. Early findings by Martineau et al. [10] in the 1990s showed that daily ingestion of oral salbutamol for 2-3 weeks effectively increased muscle strength by 10-27%. And several studies have since underpinned the acute ergogenic effects of oral salbutamol on sprint performance and muscle strength as well as chronic effects in promoting muscle hypertrophy and power development [11-15] – independent of concurrent resistance training programs [12, 16]. Although systemic effects are not as apparent at therapeutic inhaled doses, a significant fraction of the inhaled dose reaches the systemic circulation [17-19] and could therefore influence the training response. For example, 0.4-0.6 mg inhaled salbutamol (equivalent to 2-3 puffs of a standard 0.2 mg salbutamol inhaler device) is sufficiently high to induce systemic effects, as reflected by increased heart rate, ventilation, and metabolic rate [20-22].

A few studies have shown that daily inhalation of beta2-agonist leads to adaptive changes during periods of exercise training in moderately trained males. Terbutaline, a short-acting beta2-agonist like salbutamol, attenuated aerobic exercise outcomes when used daily at inhaled doses (8 puffs of 0.5 mg) during 4 weeks of endurance-based training [23]. While the 4-week training period augmented maximum oxygen uptake (VO_{2max}) and incremental exercise capacity by 5 and 12% in the placebo group, the group randomized to terbutaline experienced no significant changes in either parameter. The same pattern was apparent for a range of exercise-responsive proteins involved in oxidative muscle metabolism [23]. Using a similar dosing regimen, terbutaline was also demonstrated to lower VO_{2max} and muscle oxidative capacity during 4 weeks of resistance training [24]. And while inhaled salbutamol at doses of
1.6 mg did not affect performance outcomes when administered daily for 5 weeks during a period of strength and power training program 3 times weekly, daily inhalation of long-acting beta2-agonists formoterol and salmeterol for 5 weeks was shown to enhance sprint performance [25].

Although the above studies indicate that daily inhalation of beta2-agonists can affect adaptations during periods with training, the treatment was administered on a daily basis and not in conjunction with training [23, 24, 26] as would be normal practice for an athlete with exercise-induced bronchoconstriction [7]. Furthermore, the studies with inhaled salbutamol and terbutaline utilized high doses and were conducted with moderately trained males only [23, 24, 26]. Potential sex-specific effects of beta2-agonists are important to consider — especially because Lee et al. showed that females have a higher anabolic response to oral formoterol than males [27]. Thus, there is a need for studies illuminating sex-specific effects of inhaled beta2-agonists when used in conjunction with training by endurance-trained individuals. Such studies will not only help guide anti-doping regulations but also practices if treatment is associated with detrimental effects. Indeed, recent studies highlight that chronic use of inhaled beta2-agonists may impose detrimental effects on aerobic exercise outcomes, including in VO2max and incremental exercise capacity [23, 28].

Herein, we investigated sex-specific effects of 6 weeks of aerobic exercise training with or without inhalation of salbutamol on training days on body composition, VO2max, and muscle function in well-trained males and females. Furthermore, we examined putative mechanisms in skeletal muscle. We hypothesized that females would respond to a greater extent than males in terms of lean mass gain and muscle adaptations with inhaled salbutamol, while adaptations to aerobic exercise training would be blunted with salbutamol treatment in both sexes.
Methods

Study design and ethics approval

The study was a randomized, double-blinded, placebo-controlled, parallel group study. Participants completed experimental trial days at baseline, and following 2 weeks, 4 weeks, and 6 weeks of training with concurrent inhalation of salbutamol or placebo (Figure 1A). The study was registered in Clinicaltrials.gov (Trial identifier: NCT03902106). The study was conducted in accordance with the 2013 Declaration of Helsinki and was approved by the regional ethics committee of Copenhagen (H-18007889). All participants were informed about possible risks involved and gave their oral and written consent before inclusion.

Participants and eligibility criteria

All trials were conducted at the Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark between April 2019 and December 2020. Inclusion criteria were age 18-45 years, body mass index <26 kg/m², and VO₂max >50 and >55 mL O₂/min/kg bw for females and males, respectively. Exclusion criteria were chronic use of beta₂-agonist or allergy towards beta₂-agonist, serious adverse effects to beta₂-agonist, chronic disease deemed by the study responsible medical doctor to interfere with any part of the study (such as asthma or exercise-induced bronchoconstriction), smoking, chronic use of prescription medicine (excluding contraceptives) deemed by the study responsible medical doctor to interfere with any part of the study, or pregnancy (for females).

Assessment of eligibility criteria

We assessed eligibility criteria during a medical examination with electrocardiography (ECG-2150, Nihon Kohden, Rosbach, Germany), blood pressure measurements, (M7 Intellisense, OMRON, Kyoto, Japan) and lung function (EasyOne® Air, NDD Medical Technologies, Massachusetts, US). Participants also completed a ramp test on a bicycle ergometer (Monark LC6, Monark, Vansbro, Sweden) for determination of VO₂max by indirect calorimetry (Oxycon Pro; CareFusion, San Diego, California, USA). The ramp test was preceded by 3×4-min bouts at 75, 100, and 125 W and 100, 150, and 200 W for females and males, respectively. Participants performed lung function testing of forced expiratory volume over 1 s (FEV₁) 5, 10, 15, and 20 min after conclusion of the ramp test to exclude exercise-induced bronchoconstriction (defined as a >10% decrease in FEV₁ from pre-test values). After
the last FEV$_1$ measurement, participants inhaled 800 µg salbutamol (onset of action ≈5 min [29]) and FEV$_1$ was assessed once more 10 min after inhalation to test for reversibility defined as a 12% and 200 mL increase in FEV$_1$ according to ERS guidelines [30].

Randomization and blinding

Upon inclusion, participants were allocated into two groups receiving either salbutamol or placebo during training days. Randomization was performed by minimization for sex, VO$_{2\text{max}}$, and lean body mass to ensure group homogeneity at baseline for the main outcome measures [31, 32]. Allocation was performed by personnel not involved in enrolment or conducting experimental trials. Salbutamol and placebo were administered in identical looking inhalers and the intervention was identical for both treatments, and thus allocation was concealed to assessors and participants throughout the whole intervention.

Outcome measures and sample size

The main response outcome measure was change in body composition (i.e., fat and lean mass) measured by dual energy X-ray absorptiometry and VO$_{2\text{max}}$ during a ramp test to exhaustion on a bike ergometer. Other outcome measures were changes in muscle function. All measurements were conducted on experimental trial days at baseline and after 2 weeks, 4 weeks, and 6 weeks.

Sample size was determined for the main outcome measure in GPower 3.1.9.3 with an α-level of 0.05 and β-level of 0.8 for a linear mixed model repeated measures design which resulted in a required sample size of 18 participants per treatment group with an equal distribution of males and females. Effect size and standard deviation (SD) were estimated from a previous study [33].

Training and study drugs

The intervention period consisted of 6 weeks of exercise training (3 sessions per week) consisting of a high-intensity interval training (HIT; 2 sessions per week) and endurance exercise (1 session per week) on an indoor spinning bike (Body Bike indoor cycle, W014). Intensity was monitored by heart rate sensors (Polar Team Pro, Polar Denmark) and an instructor supervised all training sessions.
HIT sessions consisted of 5 minutes warmup at 65% and 5 minutes at 75% of heart rate max (HR$_{\text{max}}$) followed by intervals of 4 minutes >85% of HR$_{\text{max}}$ interspersed by 2 minutes active recovery. The participants were encouraged to achieve the highest possible HR during each interval. Strong verbal encouragement was given during the training sessions. During the first week, sessions consisted of four repetitions and increased by one repetition every second week, resulting in six repetitions during the last two weeks.

Endurance training sessions consisted of a 20 minute warmup at 60-75% HR$_{\text{max}}$, followed by 3 × 20 min at 70, 75, and 80% HR$_{\text{max}}$, respectively, 20 min at 75% HR$_{\text{max}}$ and finally 40 min at 85% HR$_{\text{max}}$ for a total exercise duration of 3 hours (Figure 1C).

At each training session, participants were administered either salbutamol (Ventoline®, GlaxoSmithKline, Brentford, UK) or placebo (lactose monohydrate). Salbutamol and placebo inhalers were delivered by the regional pharmacy of Copenhagen, Denmark. During the intervention, participants were instructed to maintain their physical activity levels and not to make major changes in their nutrition regimens and training outside of the intervention. In addition, participants were asked not to donate blood during the intervention. Participants were asked to report their physical activity levels and any potential illness during the intervention at the post intervention trial. On trial days, participants kept a detailed nutritional log and replicated nutritional intake to minimize inter-day variation in study outcome measurements.

At training sessions, the administered dose of salbutamol was 800 µg on HIT training days and 1600 µg on endurance training days. These doses were chosen to be within those maximally allowed by WADA at the time of the study conception [34], and to reflect the dose that could conceivably be used by athletes in the duration of the respective training sessions. To further simulate a dosing regimen during the endurance training sessions, salbutamol was administered before the warmup (200 µg), after the warmup (400 µg), and immediately after the training session (200 µg). In addition, 400 µg was administered in the evening after the training session for a total dose of 1600 µg in 16 hours. All inhalations were supervised during the training sessions, and via video monitoring (e.g., Skype/FaceTime) for the evening inhalations.

**Experimental trial days**

At all experimental trial days, we measured body composition using dual-energy X-ray absorptiometry (DXA; Lunar DPX-IQ, Version 4.7 E, Lunar Corporation, Madison, WI, US) using the mean of two separate scans, followed by participants completing a standardized
warm-up at 3×4 min at 30, 50, and 70% of VO\textsubscript{2max}, respectively, as calculated from the screening ramp test.

After the warm-up, participant’s contractile properties of the quadriceps muscle were measured using a maximal voluntary contraction setup (MVC) with electrical percutaneous muscle stimulations delivered on top of the plateau of each MVC as well as 1 s before and after each bout. Participants completed three MVCs of 3-4 s duration interspersed by 60 s recovery.

Then, participants completed a bike ergometer ramp to task failure for determination of VO\textsubscript{2max} and incremental exercise performance, starting from 125 and 150 W for females and males, respectively, with workload continuously increasing every second for an average increase of 25 and 30 W/min for females and males, respectively, until task failure which was defined as a drop below 70 rpm despite strong verbal encouragement for >3 s. After the ramp test, participants rested for 10 min before completing a constant power output test at 110% incremental exercise performance from the prior ramp test to exhaustion (as suggested by Poole & Jones [35] for more accurate VO\textsubscript{2max} assessment). In addition, we collected a muscle biopsy from the left vastus lateralis muscle under local anaesthesia at the baseline and 6 weeks experimental trial day.

VO\textsubscript{2max} was determined as the highest mean value recorded in any consecutive 30-s period during either the ramp test or constant power output test at 110% incremental exercise performance. Incremental exercise performance was determined as the highest power output attained during the ramp test.

All trials were carried out at the same time of day for each participant, to minimize the influence of circadian hormones. Participants were instructed to standardize meal and fluid intake on the day of each experimental trial, and to refrain from alcohol, caffeine, and exercise 24 h before testing. A flowchart depicting the experimental trials is shown in Figure 1B.

**Experimental procedures**

Detailed descriptions of experimental procedures are available in supplementary material (S1).

**Statistics**

Statistical analysis was performed in SPSS version 27.0 (IBM, Armonk, USA). Data were tested for normality using the Shapiro-Wilk test and Q-Q plots. Normally distributed data are presented as mean ± 95% confidence intervals (CI) unless stated otherwise. To determine
within-sex changes with the intervention we employed a 2-factor mixed linear model on delta change from baseline from each sex separately with treatment (salbutamol/placebo) and time (2, 4, and 6 wks) as fixed factors and participant as a random factor. To determine between-sex changes with the intervention we employed a 3-factor mixed linear model on delta change from baseline with treatment (salbutamol/placebo), time (2, 4, and 6 wks), and sex (male/female) as fixed factors and participant as a random factor. For Western blot and gel electrophoresis data, we employed a mixed linear model as described above but on absolute pre and post 6-week values. To estimate between-treatment differences in body composition repartitioning for each sex, we performed a multivariate mixed linear model with treatment (salbutamol/placebo) and composition (lean/fat) as fixed factors.
Results

Participants

The experimental trials were conducted from March 2019 to December 2020. In total, 40 healthy well-trained participants (19 females and 21 males) completed the study distributed to either a salbutamol or placebo group (Table 1). None of the participants presented with post-exercise reductions in FEV$_1$, airflow obstruction, or a positive bronchodilator reversibility test after exercise (Table 1). Outside of the intervention, participants were engaged mainly in running, cycling, and cross-fit with a weekly training volume of 3-7 h. Mean compliance with the training protocol was 99.6% and compliance with the inhalation regimen was 100%. During the intervention, six participants in the salbutamol group reported side effects (light-headedness; n=1, insomnia; n=1, tachycardia; n=2, tiredness; n=2). Upon completion of the last trial visit, 7 participants in the salbutamol group thought they had received salbutamol (32%), 8 thought they had received placebo (36%), and 7 participants did not know (32%).

Table 1: Baseline characteristics of study participants.

<table>
<thead>
<tr>
<th></th>
<th>Male (n = 21)</th>
<th>Female (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salbutamol</td>
<td>Placebo</td>
</tr>
<tr>
<td></td>
<td>(n = 12)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>26 ± 5</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>182 ± 4</td>
<td>182 ± 5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>77.8 ± 10.1</td>
<td>76.6 ± 7.9</td>
</tr>
<tr>
<td>Whole body lean mass (kg)</td>
<td>62.7 ± 6.8</td>
<td>63.6 ± 7.9</td>
</tr>
<tr>
<td>Whole body fat mass (kg)</td>
<td>12.2 ± 3.8</td>
<td>10.1 ± 2.6</td>
</tr>
<tr>
<td>Whole body fat percentage (%)</td>
<td>16.1 ± 3.5</td>
<td>13.8 ± 3.7</td>
</tr>
<tr>
<td>VO$_{2\text{max}}$ (mL/min)</td>
<td>4589 ± 602</td>
<td>4592 ± 658</td>
</tr>
<tr>
<td>VO$_{2\text{max}}$ (mL/min/kg)</td>
<td>59.2 ± 5.6</td>
<td>59.9 ± 6.1</td>
</tr>
<tr>
<td>Incremental exercise performance (W)</td>
<td>378 ± 48</td>
<td>375 ± 28</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>5.8 ± 0.7</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>FEV$_1$ (L)</td>
<td>4.8 ± 0.6</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>FEV$_1$/FVC</td>
<td>0.83 ± 0.05</td>
<td>0.81 ± 0.08</td>
</tr>
<tr>
<td>Change in FEV$_1$ after exercise (%)</td>
<td>-3.9 ± 3.6</td>
<td>-1.2 ± 5.9</td>
</tr>
<tr>
<td>Change in FEV$_1$ after beta$_2$-agonist (%)</td>
<td>0.5 ± 3.0</td>
<td>3.9 ± 4.3</td>
</tr>
</tbody>
</table>

VO$_{2\text{max}}$: maximal oxygen uptake. FVC: forced vital capacity. FEV$_1$: forced expiratory volume in 1 second. Incremental exercise performance is the highest power output achieved during the ramp test to exhaustion. Values are means ± SD.
**Body composition**

Salbutamol exhibited a sex-specific effect on whole-body fat mass (sex × treatment: \( p = 0.048 \)), for which females lost more fat than males. Specifically, salbutamol-treated females had lost 0.6 kg (95%CI: 0.0 to 1.3; \( p = 0.054 \)) and 0.8 kg (95%CI: 0.5 to 1.6; \( p = 0.039 \)) after 4 and 6 weeks of treatment compared to placebo-treated females, while no treatment differences were apparent in males (Figure 2A). The sex-specific effect of salbutamol on fat mass was mainly apparent as a reduction in fat mass of the upper extremities (Figure 2B+C), where the salbutamol-treated females lost 0.1 kg (95%CI: 0.0 to 0.2; \( p = 0.020 \)) and 0.1 kg (95%CI: 0.0 to 0.2; \( p = 0.014 \)) of fat mass after 4 and 6 weeks of treatment, respectively, compared to placebo.

Visceral adipose tissue increased in a sex-specific manner (sex × treatment: \( p = 0.003 \)), but in contrast to the whole-body changes this was related to an increase in salbutamol-treated males compared to placebo-treated males at weeks 2 and 6 (\( p = 0.047 \) and \( p = 0.017 \), respectively), while no changes were apparent in females (Figure 2D).

There was no apparent effect of salbutamol treatment on whole-body lean mass when analyzing pooled data (treatment main effect: \( p = 0.165 \)), or when analyzing data for each sex independently (treatment main effect, females: \( p = 0.714 \); males: \( p = 0.135 \)). However, in a multivariate mixed model, females treated with salbutamol exhibited a repartitioning effect, lowering fat mass while gaining lean mass, compared to placebo (\( p = 0.011 \)), whereas no such effect was apparent for males (\( p = 0.303 \); Figure 3).

**\( VO_{2\text{max}} \) and incremental exercise performance**

Salbutamol treatment had an overall negative effect on changes in \( VO_{2\text{max}} \) (treatment main effect: \( p = 0.014 \)), which was independent of sex (treatment × sex interaction: \( p = 0.964 \)) and mainly attributed to an initially blunted increase in \( VO_{2\text{max}} \) in salbutamol-treated participants (Figure 4A). After 6 weeks, increases in \( VO_{2\text{max}} \) were of similar magnitude between treatments in both males and females.

Salbutamol treatment had no effect on training induced increases in incremental exercise performance (Figure 4B).
**Muscle function**

Salbutamol exhibited an overall negative effect on changes in MVC (treatment main effect: $p < 0.001$) independent of sex (treatment × sex interaction: $p = 0.517$). However, the effect of salbutamol treatment on MVC was only significant within females, where the change in MVC was lower compared to placebo by 22 Nm (95% CI: -43 to -1; $p = 0.039$) and 39 Nm (95% CI: -61 to -17; $p = 0.002$) after 4 and 6 weeks of treatment, respectively (Figure 5A). The decrease in MVC was paralleled by an overall decrease in voluntary activation level (main effect of treatment: $p < 0.001$), which was not different between sexes (treatment × sex interaction: $p = 0.517$). After 6 weeks of treatment, change in voluntary activation levels was 6 percentage points (95% CI: 12 to 0; $p = 0.036$) lower in males, and 8 percentage points (95% CI: 11 to 4; $p < 0.001$) in females, compared to placebo, respectively.

Time to peak tension after percutaneous electrical stimulation was shortened by 21.5% in females treated with salbutamol compared to placebo (treatment main effect: $p = 0.016$), whereas there was no effect in males (treatment main effect: $p = 0.263$), but with no difference between sexes (treatment × sex interaction: $p = 0.254$). Time to half-relaxation was prolonged in a sex-specific manner (treatment × sex interaction: $p = 0.007$), as females treated with salbutamol exhibited a 34.6% prolonged half relaxation time (treatment main effect: $p = 0.045$) while this effect was not apparent in males (treatment main effect: $p = 0.530$).

**Muscle fiber type distribution**

Salbutamol had no effect on muscle fiber type distribution with the intervention (treatment × time interaction: $p = 0.820$), and the response was not influenced by sex (sex × treatment × time interaction: $p = 0.956$).

| Table 2. Muscle fiber type distribution before and after 6 weeks of exercise training with concurrent salbutamol inhalation in moderately trained males (n = 21) and females (n = 19). |
|---|---|---|---|---|---|---|
| | Male | | | Female | | |
| | Placebo (n=9) | Salbutamol (n=12) | | Placebo (n=9) | Salbutamol (n=9) | |
| | Pre | Post | Pre | Post | Pre | Post |
| MHCI (%) | 62.1±15 | 59.0±12.5 | 61.2±13.3 | 59.5±9 | 64.2±12 | 60.5±9.9 | 70.1±9.1 | 67.3±10.7 |
| MHCII (%) | 37.9±15 | 41.0±12.5 | 38.8±13.3 | 40.5±9 | 35.8±12 | 39.5±9.9 | 29.9±9.1 | 32.7±10.7 |

MHC: myosin heavy chain.
Muscle protein content

Training induced increases in electron transport chain complexes were not affected by salbutamol treatment except for complex II which increased more in males treated with salbutamol compared with placebo (treatment × time: $p = 0.044$) (Figure 6A). However, mean muscle complex I-V expression was increased with salbutamol treatment (treatment main effect: $p = 0.035$).

Muscle protein content of desmin and SERCAI was not affected by salbutamol treatment, whereas beta2-adrenergic receptor content tended to be downregulated in males (treatment × time: $p = 0.089$) but not in females (treatment × time: $p = 0.287$) with no between-sex difference (treatment × time × sex interaction: $p = 0.195$).
**Discussion**

The key takeaway from this study was that inhaled salbutamol had a sex-specific effect on body composition when used by well-trained individuals in conjunction with a period of aerobic training. Specifically, salbutamol lowered fat mass, while retaining or even increasing lean mass in females, but not in males. Another key finding was that inhaled salbutamol had a sex-independent detrimental effect on isometric strength of the quadriceps muscle.

Females in the salbutamol group had a progressive fat mass loss from 0.4 kg at week 2 reaching 1.2 kg at week 6 versus only 0.4 kg at week 6 in the placebo group. Fat mass loss around the arms and legs accounted for a combined 0.4 kg, meaning that the predominant fat loss occurred in the abdominal and thoracic region during the 6-week intervention. This occurred concomitantly with an increase in lean mass in 9 out of 10 female participants and highlights the efficacy of salbutamol as a repartitioning agent – even at therapeutic inhaled doses in well-trained lean females. On the other hand, no such effect was evident in males. While the mechanism underlying this sex-dependent difference is not apparently clear, Lee et al. did observe a greater effect of beta2-agonist on protein synthesis in females than males [27]. And although we, and others, have previously found beta2-agonists to induce leanness in males [15, 28, 33, 36-38], concomitant endurance training was shown to blunt this effect [23, 33]. This collectively suggests that beta2-agonists exert sex-specific effects on body composition and that such effects depend on habitual training patterns.

Several studies have demonstrated that prolonged beta2-agonist use increases muscle mass and strength [14, 39]. Therefore, we were surprised to find a marked decline in isometric muscle torque in the salbutamol-treated participants. This likely reflects other factors than muscle cross-sectional area since leg lean mass remained stable throughout the study (data not shown). Furthermore, we observed no indication of a muscle fiber-type shift or sarcomeric re-organization, as reflected by no changes in muscle fiber type composition or in protein abundance of SERCA1 and desmin. The degree of voluntary activation declined by a few percentage-points in the salbutamol-treated participants, mainly in females, which suggests that participants were not as able to voluntarily recruit as many motor units as before the intervention – hence explaining some of the decline in isometric muscle torque. Salbutamol treatment also revealed some other notable sex-specific changes in muscle function, including a shortened time to peak tension and prolonged relaxation time in females but not in males. This points to salbutamol inducing muscle adaptations pertaining to the on-and-off kinetics of Ca2+-binding to troponinC either by changes in the regulation and expression of troponin
isoforms or its interaction with tropomyosin. This is supported by the observation that daily treatment with terbutaline has been shown to lower the abundance of both the alpha and beta chains of tropomyosin during a period of endurance-based training in moderately trained males [23]. Nonetheless, our findings collectively indicate that aerobic training with concomitant salbutamol treatment can impose negative effects on muscle function.

Another notable effect was that the salbutamol-treated participants temporarily had a lower increase in VO\textsubscript{2max} than placebo-treated participants during the first 4 weeks of the training intervention after which the salbutamol-treated participants approximated the change in VO\textsubscript{2max} observed in the placebo-treated participants at week 6. Although it could be expected that mitochondrial content would also be compromised, as has been shown for prolonged treatment with formoterol [28], our data did not support such an effect. In fact, salbutamol seemed to increase mitochondrial content as determined by electron transport complex protein expression, where there was an overall increase with salbutamol treatment. This could suggest a compensatory mechanism of mitochondrial content, which may explain that incremental exercise performance was not impaired in parallel to VO\textsubscript{2max} in the male participants. Although we cannot readily explain the temporal attenuated training response in VO\textsubscript{2max} with salbutamol treatment, it is unlikely to be explained by desensitization of the beta\textsubscript{2}-adrenergic receptor from repeated salbutamol exposure as immunoblotting analysis showed no change in its abundance in muscle homogenates. Furthermore, the participants did not report any changes in exercise habits during the intervention. Consistent with a potential negative effect of inhaled beta\textsubscript{2}-agonist, daily treatment with inhaled terbutaline attenuated VO\textsubscript{2max} in moderately trained males subjected to 4 weeks of endurance or resistance training [23, 24], while daily therapeutic inhaled doses of formoterol for 6 weeks lowered VO\textsubscript{2max} in well-trained females and males [28]. Thus, it seems clear that prolonged daily treatment with inhaled beta\textsubscript{2}-agonists can negatively affect VO\textsubscript{2max}—likely in a dose-dependent manner and dependent on weekly usage. For example, in the present study we only administered salbutamol during training days, in contrast to Jessen \textit{et al.} 2023 and Hostrup \textit{et al.} 2017 [23], where beta\textsubscript{2}-agonist was also administered on non-training days.

Few studies have investigated sex-specific adaptations to prolonged inhalation of beta\textsubscript{2}-agonist. The observation that salbutamol may induce a sex-specific effect contrasts with a previous study where we administered inhaled formoterol to well-trained males and females and reported no sex-specific effects on exercise performance or body composition [28]. However, a key difference in the present study was that dosing occurred during training
sessions, whereas in the previous study participants inhaled formoterol in the morning and evenings outside of their own training. Considering that concurrent training affects the hypertrophic response to prolonged beta2-agonist treatment [33], it is possible that exercise may modulate the responsiveness to treatment between males and females.

Taken together, our results demonstrate that inhaled salbutamol may have several sex-specific effects when used in conjunction with aerobic training. Salbutamol had profound effects in females in terms of fat mass loss but also had negative effects on VO2max and muscle function. Thus, this study provides new insights into the potential interactions between inhaled salbutamol and endurance exercise, which is of particular relevance to athletes who use inhaled salbutamol as part of their normal training routine.

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Author contributions

MH conceived the study. MH, SJ, CW, MB, and DK collected the data. MH, SJ, CW, DK, and JB analyzed the data. MH and SJ wrote the first draft. All authors critically revised the manuscript and approved the final version of the manuscript.

Conflicts of interest

Authors have no conflicting interests.
**Figure legends**

Figure 1: Experimental overview. Study design (A), experimental trial days (B), and training days (C). DXA: Dual-Energy X-ray Absorptiometry; VO\textsubscript{2max}: maximal oxygen uptake; HR\textsubscript{max}: maximal heart rate.

Figure 2. Change in body composition during 6 weeks of exercise training with concurrent salbutamol inhalation in moderately trained males (n = 21; left panels) and females (n = 19; right panels). Whole-body fat mass (A), arm fat mass (B), leg fat mass (C), and visceral adipose tissue mass (D). All values are changes relative to baseline. Symbols are means and shaded areas are 95% confidence intervals. #Between-group difference (p < 0.05).

Figure 3. Relationship between change in fat mass and lean mass after 6 weeks of exercise training with concurrent salbutamol inhalation in moderately trained males (n = 21; left panels) and females (n = 19; right panels). Plots on outer edges of panels denote the distributions of data points on the respective axes and were created in R with base density function using Gaussian distribution. Symbols with bold border are means and error bars are 95% confidence intervals.

Figure 4. Changes in maximum oxygen uptake (VO\textsubscript{2max}; A) and incremental exercise performance (B) during 6 weeks of exercise training with concurrent salbutamol inhalation in moderately trained males (n = 21; left panels) and females (n = 19; right panels). All values are changes relative to baseline. Symbols are means and shaded areas are 95% confidence intervals. #Between-group difference (p < 0.05).

Figure 5: Change maximal voluntary contraction torque (A) and voluntary activation level (B) during 6 weeks of exercise training with concurrent salbutamol inhalation in moderately trained males (n = 21; left panels) and females (n = 19; right panels). All values are changes relative to baseline. Symbols are means and shaded areas are 95% confidence intervals. #Between-group difference (p < 0.05).

Figure 6: Change in muscle individual and mean muscle content of mitochondrial electron transport chain complexes (A), and SECAI, beta\textsubscript{2}-adrenoceptor, and desmin content (B) after 6 weeks of exercise training with concurrent salbutamol inhalation in moderately trained males (n = 21; left panels) and females (n = 19; right panels). Bars are means with 95% confidence intervals. B2AR: beta\textsubscript{2}-adrenoceptor. #Between-group difference (p < 0.05).
References


Figure 1

A) Study design

- Trial 1: Training with salbutamol
- Trial 2: Training with placebo
- Trial 3: Training with salbutamol
- Trial 4: Training with placebo

n=40

B) Experimental trial days

1. DXA scan
2. Vastus lateralis biopsy (baseline and 6 weeks)
3. Standardized warm-up
4. Maximal voluntary contraction force
5. VO₂max determination (ramp test)
6. Constant power output at 110% incremental exercise performance

C) Training

Endurance training (once weekly)
- Warmup 60-75% HRmax
- 20 min 70% HRmax
- 20 min 75% HRmax
- 20 min 80% HRmax
- 20 min 75% HRmax
- 20 min 80% HRmax
- 60 min 85% HRmax

HIIT (twice weekly)
- Warmup 65-75% HRmax
- 4 x 4 min all-out (345% HRmax)
Figure 2
Figure 3
Figure 4
Figure 5

A. Change in maximal voluntary contraction torque (Nm)

- Male:
  - Treatment, $p = 0.072$
  - Time, $p = 0.690$
  - Treatment × time, $p = 0.907$

- Female:
  - Treatment, $p = 0.011$
  - Time, $p = 0.044$
  - Treatment × time, $p = 0.018$

B. Change in voluntary activation (% points)

- Male:
  - Treatment, $p = 0.070$
  - Time, $p = 0.051$
  - Treatment × time, $p = 0.284$

- Female:
  - Treatment, $p < 0.001$
  - Time, $p = 0.179$
  - Treatment × time, $p = 0.205$
Figure 6
Experimental procedures

Flow chart

Figure 1. Participant flow diagram.

Dual-Energy X-ray absorptiometry

DXA scans were performed using a Lunar iDXA (GE Healthcare, Brøndby, Denmark) which was calibrated daily according to manufacturer’s instructions. Participants rested on the scanner bed in the supine position for 10 min prior to the scanning to minimize potential effects of body fluid shifts. Each scan was conducted in duplicate to minimize inter-scan variation and the mean of the two scans was used. The scanner was calibrated before each experimental trial day, using daily calibration procedures (Lunar “System Quality Assurance”).

Muscle biopsy

Muscle biopsies were collected under local anaesthesia (20 mg/mL Xylocain without epinephrine; AstraZeneca, Cambridge, UK) through a small incision in the skin over the vastus
lateralis. Biopsies were collected with a modified Bergström needle with suction. Upon collection, the muscle biopsy piece was rinsed in saline (9 mg/mL, Fresenius Kabi, Sweden), frozen liquid nitrogen, and stored at -80°C until analysis.

**Immunoblotting and SDS-page**

Protein contents were determined by Western blotting. Approximately 1 mg d.w. muscle tissue was homogenized for 1 min at 30 Hz on a shaking bead-mill (TissueLyser II, Qiagen, Valencia, CA, USA) in ice-cold lysis buffer containing: 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 2 mM Na$_3$VO$_4$, 10 mM NaF, 2 mM PMSF, 1 mM EDTA (pH 8), 1 mM EGTA (pH 8) 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 3 mM benzamidine. Samples were rotated end-over-end for 30 min at 4°C and centrifuged (18,320 × g) for 20 min at 4°C. The protein concentration of each sample was determined in triplicate with a BSA kit (Thermo Fisher Scientific, MA, US) and samples were created in duplicate 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$_2$O to achieve equal protein concentration. Equal amounts of protein were loaded in wells of pre-cast 4-15% gels (Bio-Rad Laboratories, CA, US) with all samples for each participant loaded on the same gel. Proteins were then separated according to their molecular weight by SDS-PAGE and semi-dry transferred to a PVDF membrane (Millipore A/S, Copenhagen, Denmark). Membranes were blocked for 15 min in either 2% skim milk or 3% BSA in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) before an overnight incubation in primary antibody at 4°C and a subsequent incubation in horseradish peroxidase conjugated secondary antibody at room temperature for 1 h. Bands were visualized with ECL (Millipore) and recorded with a digital camera (ChemiDoc MP Imaging System, Bio-Rad Laboratories). Bands were quantified using Image Lab version 6.0 (Bio-Rad Laboratories) and determined as the total band intensity adjusted for background intensity. Primary antibodies used were: β2-adrenoceptor: EPR707(N) (#ab182136, Abcam, Cambridge, UK); SERCA1: VE121G9 (#MA3-912, ThermoFisher Scientific, Waltham, MA, USA); SIRT3: D22A3 (#5490, Cell Signaling Technology, Herlev, Denmark); Desmin: D33 (#M076029-2, Dako, Glostrup, Denmark). Secondary antibodies used were: β2-adrenoceptor: Goat Anti-Rabbit (1:5000; #4010-05, SouthernBiotech, Birmingham, AL, USA); SERCA1: Goat Anti-Mouse (1:5000; #P0447, Dako, Glostrup, Denmark); SIRT3:
Goat Anti-Rabbit (1:5000; #4010-05, SouthernBiotech, Birmingham, AL, USA); Desmin: Goat Anti-Mouse (1:5000; #P0447, Dako, Glostrup, Denmark).

Fiber type determination with gel electrophoresis

Muscle fiber type content was determined by myosin heavy chain (MHC) isoform separation. We used the sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) method. The immunoblotting sample preparations were diluted in heavy sample buffer (1:1 100% glycerol to Laemmlı buffer) to reach a protein concentration of 0.3 mg/l. Equal amounts of protein were loaded in wells of self-casted gels (separation gel: 30% glycerol, 8% acrylamide, 200 mM Tris-HCl with pH 8.80, 100 mM glycine, 0.4% SDS, 0.1% APS, 0.5% TCE, 0.05% TEMED; stacking gel: 30% glycerol, 4% acrylamide, 70 mM Tris-HCl with pH 6.80, 4 mM EDTA, 0.4% SDS, 0.1% APS, 0.05% TEMED). A lower running buffer (50 mM Tris, 75 mM glycine, 0.05% SDS) and a top running buffer (300 mM Tris, 75 mM glycine, 0.3% SDS) with 1 mM DTT added just before start were used. Electrophoresis ran in ice boxes for 16 h at a constant voltage of 73 V followed by 24 h at a constant current (10 mA). Bands were visualized by 5 min of UV exposure and recorded with a digital camera (ChemiDoc MP Imaging System, Bio-Rad Laboratories). Bands were quantified using Image Lab version 6.0 (Bio-Rad Laboratories) and determined as the total band intensity adjusted for background intensity. Results are expressed as the ratio of MHC type I to MHC type II.

Maximal voluntary contraction

During measurements of the contractile properties of the quadriceps muscle, participants were positioned on a table with their right leg fixed in a knee joint angle of 90° of flexion. The participants were sitting upright with thighs parallel to the floor and a 90° angle of flexion in the hips. Hands were positioned on handlebars on each side for further tension support. Isometric contraction force was recorded using a strain gauge (Tedea-Huntleigh) strapped around the right ankle just above the malleoli. Strain gauge signal was fed to an amplifier connected to a computer. Data were recorded at 1 kHz in LabChart 8 (ADInstruments). Before, during and immediately after each MVC, superimposed percutaneous electrical muscle stimulations were delivered to the vastus lateralis muscle and rectus femoris muscle by two self-adhesive electrodes (PALS Platinum 5 × 9 cm; Alexgaard Manufacturing, Lystrup, Denmark). Electrodes were placed on the skin 25% distal from spina iliaca anterior superior
and 25% proximal from patella covering m. vastus lateralis and m. rectus femoris. Muscle stimulations were produced by a constant current stimulator (Stimulator model DS7AH; Digitimer, Hertfordshire, UK) in rectangular pulses of 1 ms. All participants received a progressive familiarization to stimulation intensity at the screening. During the experimental trials an intensity of 999 mA was applied. To determine the degree of voluntary activation (VA) level, a single stimulation was delivered on top of the plateau of each MVC. A single stimulation was delivered 1 s following relaxation of each MVC to determine potentiated peak twitch force (T\textsubscript{Pot}). During MVC measurements, participants received verbal encouragement with no visual feedback.

The following parameters were determined: MVC (N): highest force during an MVC; T\textsubscript{Pot} (N): highest force during a potentiated single stimulation 1 s following relaxation from an MVC; half-relaxation time (HRT, ms): time from peak twitch force until force reached half of peak twitch force; and time-to-peak twitch force (TPT, ms): time from single stimulation until peak twitch force was reached.

Degree of VA level was calculated from the single twitches using the following equation 3:

\[ VA = \left(1 - \frac{T_S}{T_{Pot}}\right) \times 100 \]

where \(T_S\) is the superimposed twitch delivered on top of the plateau of the MVC and \(T_{Pot}\) is the potentiated twitch delivered following relaxation after an MVC. A correction was applied to the equation if the superimposed stimulation was delivered slightly before or after the peak MVC 4.

References