

# Chronic $\beta_2$ -adrenoceptor agonist treatment alters muscle proteome and functional adaptations induced by high intensity training in young men

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## Key points

- While several studies have investigated the effects of exercise training in human skeletal muscle and the chronic effect of  $\beta_2$ -agonist treatment in rodent muscle, their effects on muscle proteome signature with related functional measures in humans are still incompletely understood.
- Herein we show that daily  $\beta_2$ -agonist treatment attenuates training-induced enhancements in exercise performance and maximal oxygen consumption, and alters muscle proteome signature and phenotype in trained young men.
- Daily  $\beta_2$ -agonist treatment abolished several of the training-induced enhancements in muscle oxidative capacity and caused a repression of muscle metabolic pathways; furthermore,  $\beta_2$ -agonist treatment induced a slow-to-fast twitch muscle phenotype transition.
- The present study indicates that chronic  $\beta_2$ -agonist treatment confounds the positive effect of high intensity training on exercise performance and oxidative capacity, which is of interest for the large proportion of persons using inhaled  $\beta_2$ -agonists on a daily basis, including athletes.

**Abstract** Although the effects of training have been studied for decades, data on muscle proteome signature remodelling induced by high intensity training in relation to functional changes in humans remains incomplete. Likewise,  $\beta_2$ -agonists are frequently used to counteract exercise-induced bronchoconstriction, but the effects  $\beta_2$ -agonist treatment on muscle remodelling and adaptations to training are unknown. In a placebo-controlled parallel study, we randomly assigned 21 trained men to 4 weeks of high intensity training with (HIT+ $\beta_2$ A) or without (HIT) daily inhalation of  $\beta_2$ -agonist (terbutaline, 4 mg dose<sup>-1</sup>). Of 486 proteins identified by mass-spectrometry proteomics of muscle biopsies sampled before and after the intervention, 32 and 85 were changing (false discovery rate (FDR)  $\leq 5\%$ ) with the intervention in HIT and HIT+ $\beta_2$ A, respectively. Proteome signature changes were different in HIT and HIT+ $\beta_2$ A ( $P = 0.005$ ), wherein  $\beta_2$ -agonist caused a repression of 25 proteins in HIT+ $\beta_2$ A compared to HIT, and an upregulation of 7 proteins compared to HIT.  $\beta_2$ -Agonist repressed or even downregulated training-induced enrichment of pathways related to oxidative phosphorylation and glycogen metabolism, but upregulated pathways related to histone trimethylation and the nucleosome. Muscle contractile phenotype changed differently in HIT and HIT+ $\beta_2$ A ( $P \leq 0.001$ ), with a fast-to-slow twitch transition in HIT and a slow-to-fast twitch transition in HIT+ $\beta_2$ A.  $\beta_2$ -Agonist attenuated training-induced enhancements in maximal oxygen consumption ( $P \leq 0.01$ ) and exercise performance (6.1 vs. 11.6%,  $P \leq 0.05$ ) in HIT+ $\beta_2$ A compared to HIT. These findings indicate that daily  $\beta_2$ -agonist treatment attenuates the beneficial effects of high intensity training

on exercise performance and oxidative capacity, and causes remodelling of muscle proteome signature towards a fast-twitch phenotype.

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**Abbreviations** ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CSA, cross-sectional area; FDR, false discovery rate; HIT, high intensity training + placebo group; HIT+ $\beta_2$ A, high intensity training +  $\beta_2$ -agonist group; MHC, myosin heavy chain; PKA, protein kinase A; TCA, tricarboxylic acid cycle;  $\dot{V}_{O_{2max}}$ , maximal oxygen consumption.

## Introduction

Exercise is essential for maintaining physical function and health, and is positively related to quality-of-life and life expectancy (Westerterp, 2001; Dhana *et al.* 2016). Thus, exercise is considered one of the best non-pharmacological strategies to prevent and even reverse several pathological conditions (Goodyear, 2008). In addition, exercise training is crucial for optimal performance in the vast majority of sport disciplines. High intensity training is widely practised because of its superior efficacy in improving physiological measures compared to low- and moderate intensity exercise (Milanovic *et al.* 2015). Only a few weeks of high intensity training improves cardiovascular fitness and induces several beneficial adaptations in skeletal muscle (Laursen & Jenkins, 2002; Hostrup & Bangsbo, 2017). The adaptations induced by high intensity training are related to major cellular perturbations in skeletal muscle that activate a variety of signalling pathways and gene programmes (Arany *et al.* 2008; Canto *et al.* 2009; Powers *et al.* 2010; Hoffman *et al.* 2015; Brandt *et al.* 2016). However, while considerable progress has been made in understanding myocellular adaptations induced by training (Egan *et al.* 2011; Petriz *et al.* 2012; Hawley *et al.* 2014; Murton *et al.* 2014; Padrão *et al.* 2016; Powers *et al.* 2016; Sollanek *et al.* 2017), only few studies have investigated the effect of high intensity interval training on muscle proteome signature changes (Holloway *et al.* 2009; Egan *et al.* 2011; Hussey *et al.* 2013) and integrated such changes with adaptations in muscle phenotype, contractile function and exercise performance in humans (Petriz *et al.* 2012; Padrão *et al.* 2016). Integrated approaches are needed to provide a global perspective on the relation between muscle remodelling and functional changes induced by training from a basic physiological standpoint and for future therapeutic applications.

A large proportion of the population experiences respiratory complications during physically demanding activities (Price *et al.* 2014). A common cause of this problem is asthma and exercise-induced bronchoconstriction, with a prevalence of around 10% in Western countries (Cruz, 2007; Kainu *et al.* 2013). Inhaled  $\beta_2$ -adrenoceptor agonists ( $\beta_2$ -agonists) are used as first-line treatment of the bronchoconstriction associated with

asthma and exercise-induced bronchoconstriction, and are as such among the most commonly prescribed medications worldwide (Rottenkolber *et al.* 2015). While the main application of inhaled  $\beta_2$ -agonists is to induce bronchodilatation, a large proportion of the drug enters the systemic circulation and distributes to all organs (Jacobson *et al.* 2014; Dyreborg *et al.* 2016). The largest organ of the body, skeletal muscle, contains a high density of  $\beta_2$ -adrenoceptors (Williams *et al.* 1984; Jensen *et al.* 2002) that serve an important role in the adrenergic fight-or-flight response (Jensen *et al.* 2008; Emrick *et al.* 2010; Andersson *et al.* 2012; Hostrup *et al.* 2014b). Thus, acute inhalation of  $\beta_2$ -agonist has been shown to affect ion handling and energy production of skeletal muscle in trained young men (Hostrup *et al.* 2014b; 2016; Kalsen *et al.* 2016a,b). Furthermore, when administered chronically,  $\beta_2$ -agonists induce hypertrophy and alter metabolic and contractile properties of skeletal muscle (Martineau *et al.* 1992; Dodd *et al.* 1996; Rajab *et al.* 2000; Hostrup *et al.* 2015). Therefore,  $\beta_2$ -agonists have been proposed as therapeutic agents to combat lifestyle-related diseases, muscle dysfunction and age-related muscle atrophy (Lynch & Ryall, 2008; Joassard *et al.* 2013). However, despite their widespread use, it is unknown what effect daily inhalation of  $\beta_2$ -agonist has on training-induced adaptations in skeletal muscle and functional capacity in humans. This is important, since inhaled  $\beta_2$ -agonists are often used in conjunction with physical activity in persons with asthma and exercise-induced bronchoconstriction (Arie, 2012; Price *et al.* 2014). For instance, in some sports, as many as 50% of athletes use inhaled  $\beta_2$ -agonists in conjunction with training and competition (Parsons & Mastronarde, 2005). A better understanding of these  $\beta_2$ -agonist mediated effects on muscle proteome and phenotype could also lead to improved treatment modalities in a diverse range of diseases such as muscle wasting and obesity.

Thus, the purpose of the present study was to investigate proteome signature and phenotype changes of skeletal muscle induced by high intensity training with and without concomitant daily treatment with  $\beta_2$ -agonist in therapeutic doses, integrating muscle remodelling with relevant functional measures related to maximal

**Table 1. Subject characteristics**

	HIT ( <i>n</i> = 9)	HIT+ $\beta_2$ A ( <i>n</i> = 12)	Between-group difference ( <i>P</i> value)
Age (years)	24.7 ( $\pm$ 3.0)	23.5 ( $\pm$ 1.7)	0.47
Height (cm)	184 ( $\pm$ 3)	185 ( $\pm$ 2)	0.85
Weight (kg)	78.4 ( $\pm$ 6.7)	77.6 ( $\pm$ 5.5)	0.86
Lean body mass (kg)	61.5 ( $\pm$ 4.4)	60.6 ( $\pm$ 2.9)	0.72
$\dot{V}_{O_2\max}$ (mL min <sup>-1</sup> )	3932 ( $\pm$ 233)	3945 ( $\pm$ 329)	0.95
MHCI (%)	49.4 ( $\pm$ 9.6)	49.2 ( $\pm$ 6.8)	0.97
MHCIIa (%)	44.2 ( $\pm$ 9.4)	45.8 ( $\pm$ 5.9)	0.74
MHCIIx (%)	6.5 ( $\pm$ 4.2)	5.0 ( $\pm$ 2.4)	0.46

$\dot{V}_{O_2\max}$ , maximal oxygen consumption. MHC, myosin heavy chain. Values are means ( $\pm$ 95% CI).

oxygen consumption ( $\dot{V}_{O_2\max}$ ), exercise performance and muscle contractile properties in trained young men. We hypothesized that daily  $\beta_2$ -agonist treatment would alter training-induced modulation of muscle proteome signature and cause a shift towards a fast-twitch muscle phenotype.

## Methods

### Human subjects and ethics

Twenty-four healthy trained men were initially screened of which 21 were included in the study. Prior to inclusion, subjects received oral and written information about the aims and contents of the study as well as possible risks involved, including side effects associated with the study drug (terbutaline). Each subject gave his oral and written informed consent. A physician screened each subject for unknown cardiopulmonary disease with lung and heart auscultation and electrocardiography. Inclusion criteria were males, aged 18–36 years, informed consent, a weekly training volume of 2–5 h, a  $\dot{V}_{O_2\max}$  between 40 and 60 mL min kg<sup>-1</sup>, and a lean mass index between 14 and 22 kg m<sup>-2</sup>. Exclusion criteria were smoking, allergy towards study drug, and chronic disease. Subjects were recreationally active, engaging in team sports, running, biking and/or light resistance training. Subject characteristics are presented in Table 1. Subjects were told to refrain from competitive events for the entire study and not to change their daily physical activities and nutritional habits, which were recorded. The study was conducted in accordance with the standards set by the 2013 version of the *Declaration of Helsinki* and was approved by the regional research ethics committee of Copenhagen, Denmark (H-4-2014-002). The study was registered in ClinicalTrials.gov (NCT02557581).

### Study design

The study was designed as a block-randomized controlled parallel study with two groups: Either 4 weeks of high intensity training and daily inhalation of placebo (HIT, *n* = 9) or 4 weeks of high intensity training and daily inhalation of terbutaline (HIT+ $\beta_2$ A, *n* = 12). Upon inclusion, subjects were randomly allocated in the two groups, stratified for  $\dot{V}_{O_2\max}$  and lean body mass. In HIT, subjects received a placebo inhalator (Turbohaler, AstraZeneca, Cambridge, UK) and in HIT+ $\beta_2$ A subjects received an inhalator of the selective  $\beta_2$ -agonist terbutaline (Bricanyl Turbohaler 0.5 mg dose<sup>-1</sup>, AstraZeneca, Cambridge, UK). Subjects were instructed to inhale eight doses once daily for 28  $\pm$  1 days.

### Study drugs

Terbutaline is a commonly prescribed short-acting  $\beta_2$ -agonist in Northern Europe, which has a high selectivity for the  $\beta_2$ -adrenoceptor (Baker, 2010) and a half-life of  $\sim$ 4 h (Krogh *et al.* 2017). The dosage of inhaled terbutaline administered (8  $\times$  0.5 mg) in the present study is higher than that normally prescribed to asthmatics (Bricanyl Turbohaler; product information, www.astrazeneca.com), but equivalent to the daily upper limit for inhaled salbutamol in competitive sports (2017 list of prohibited substances; www.wada-ama.org). The rationale for the dosage of 4 mg was to ensure an adequate systemic response, while staying within the current upper therapeutic limit in competitive sports. Systemic concentrations of terbutaline after inhalation of 4 mg have been shown to reach their peak within 0.5–1 h with concentrations of  $\sim$ 5–8 ng mL<sup>-1</sup> (Dyrborg *et al.* 2016; Kreiberg *et al.* 2017). Such concentrations are equivalent or even higher than those observed after oral administration of 10 mg terbutaline (Elers *et al.* 2012; Dyrborg *et al.* 2016). To ensure a drug compliance of 100%, inhalations were monitored by study staff on a daily basis via online video tools (FaceTime/Skype). Because  $\beta_2$ -agonists may affect energy turnover and ion handling in skeletal muscle (Kalsen *et al.* 2014; Hostrup *et al.* 2014a,b; Kalsen *et al.* 2016b), subjects inhaled their daily dose after exercise sessions on training days. During non-training days, subjects inhaled their daily dose in the time frame of 09.00–18.00. The duration of treatment was based on a previous study showing that 4 weeks of treatment with terbutaline leads to adaptations in skeletal muscle of humans (Hostrup *et al.* 2015). Both subjects and investigators were blinded to treatment. Terbutaline Turbohalers were delivered by the regional pharmacy of Copenhagen, Denmark. Placebo Turbohalers were kindly delivered by AstraZeneca. Randomization was conducted in SPSS (IBM, Armonk, NY, US) by staff who did not take part in any of the experimental procedures or data analysis.

## Experimental protocol

Before the start of the intervention, subjects attended two trials at the laboratory separated by 2 days. At the first trial, subjects' thigh lean mass was determined by Dual-energy X-ray absorptiometry (DXA) (Lunar iDXA, GE Healthcare, Belgium). Subjects were placed in the scanner in supine position undressed. All DXA scans were performed with 10 min of supine rest before scanning to allow distribution of body fluids. To reduce intra-day variation, two scans were performed. After the scans, subjects rested in a supine position for 20 min and resting metabolic rate was determined by indirect calorimetry breath-by-breath with a gas analyser system (Oxycon Pro, CareFusion, San Diego, CA, USA) for 15 min. Subjects then performed a standardized warm-up on a bike ergometer at 100 W for 10 min (Monark LC4, Monark Exercise AB, Vansbro, Sweden). After warm-up, subjects' contractile properties of the quadriceps muscle were assessed by maximal voluntary contraction (MVC). After MVC, subjects'  $\dot{V}_{O_{2,max}}$  and performance were determined during incremental cycling to exhaustion on the bike ergometer starting with 4 min of cycling at 100 W, in which substrate utilization was determined by indirect calorimetry as described by Jeukendrup & Wallis (2005), followed by 4 min at 150 W and 200 W, after which workload increased by 30 W every minute until exhaustion.

At the second trial, thigh lean mass and resting metabolic rate were determined similarly to the first trial to minimize day-to-day variation. Afterwards, subjects warmed up on the bike ergometer for 4 min, followed by cycling at a workload corresponding to 60% of subjects'  $\dot{V}_{O_{2,max}}$  for determination of substrate utilization at same relative intensity of  $\dot{V}_{O_{2,max}}$ . After the exercise, subjects' contractile properties of the quadriceps muscle were measured as performed during the first trial to minimize day-to-day variation. Subjects then rested for 30 min and had a muscle biopsy taken from the vastus lateralis of the right thigh using a Bergström needle with suction (Bergström, 1975). Prior to biopsy sampling, an incision (3 mm) was made through the skin and fascia at the vastus lateralis belly under local anaesthesia (2 mL lidocaine without epinephrine, 20 mg mL<sup>-1</sup> Xylocain, AstraZeneca).

After the second trial, subjects received either placebo or terbutaline according to their group.

Upon conclusion of the 4 week intervention, subjects attended two laboratory trials similar to those conducted before the intervention. To ensure complete washout of terbutaline (half-life ~4 h) (Krogh *et al.* 2017), post-testing was performed 2–3 days after last inhalation.

Subjects were told to abstain from caffeine, strenuous exercise and alcohol 48 h before each laboratory trial. To minimize variation, subjects ingested a standardized meal and fluid 1.5 h before the laboratory trials.

## Training intervention

During the 4 week intervention, subjects performed a high intensity-training programme of 3 sessions per week on indoor spinning bikes. Each training session was supervised by an instructor and consisted of a standardized 10 min warm-up followed by three blocks of exercise at ~85% of maximum heart rate for 10 min with 30 s of all-out sprinting at the end of each 10 min block. Repeated all-out sprints of 30 s were chosen, as studies have shown that this type of training effectively induces adaptations in skeletal muscle (Hostrup & Bangsbo, 2017). During each training session, subjects wore heart rate monitors that fed subjects' heart rate to the instructor's iPad (iPad 4, Apple Inc., CA, USA) using Polar Team app (Polar Electro Denmark, Holte, Denmark), thus ensuring that subjects kept their target heart rate. Training adherence was 100% for all subjects, apart from one subject who missed one training session during the 4 week intervention.

## Experimental procedures

**Contractile properties of the quadriceps muscle.** Subjects' contractile properties of the quadriceps muscle were determined during MVC with percutaneous electrical muscle stimulation during and immediately after each contraction as described previously (Hostrup *et al.* 2015). Subjects were familiarized with two 3–4 s submaximal isometric muscle contractions at 40 and 70 % of MVC and with constant current electrostimulation, (Digitimer, model DS7AH, Welwyn Garden City, UK) at 100, 400, 700 and 999 mA with 400 V for 200  $\mu$ s, before the first MVC. The electrostimulation used could activate 42.6 ( $\pm$ 3.3)% of subjects' peak MVC. Each MVC consisted of a 3–4 s maximal contraction with verbal encouragement. A potentiated twitch was applied when subjects reached their apparent peak MVC, as well as 1 s following relaxation. Peak twitch torque, time-to-peak twitch torque, half-relaxation time and voluntary activation level were calculated as previously described (Bachasson *et al.* 2013). Rate of force development was calculated as the average slope on the moment–time curve at the time interval 0–200 ms relative to the onset of muscle contraction during MVC (Aagaard *et al.* 2002). Onset of muscle contraction was defined as the time point at which the moment curve increased to values above 2.5% of the magnitude of the moment achieved during MVC.

For each variable, a grand mean was calculated from the average of the two highest values from both trials. The distance from the base of patella to the middle of the Velcro strap on the ankle was registered and used to calculate torque in newton metres (N m).

**Muscle biopsies.** Sampled biopsies were divided in two pieces of which the first piece (20–30 mg) was mounted

on an embedded medium (OCT Compound Tissue-Tek; Sakura Finetek, Zoeterwoude, The Netherlands), frozen in isopentane cooled to the freezing point in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until analysis for fibre type distribution, capillaries and fibre cross-sectional area by immunohistochemical analysis. The other biopsy piece (50–100 mg) was washed in ice-cold saline to reduce blood contamination, then dried and frozen in liquid nitrogen, and stored in cryo tubes for later proteomic, enzymatic and Western blot analyses. Prior to these analyses, each biopsy was freeze-dried and dissected free from apparent non-muscle tissue. The dissected muscle tissue was then divided in three pieces for proteomics ( $\approx 10$  mg dry weight), Western blotting ( $\approx 1.5$  mg dry weight) and enzyme activity assays ( $\approx 2$  mg dry weight). Because of inadequate muscle tissue yield from one subject in HIT, only proteomic analysis was performed for this subject.

**Mass spectrometry proteomics.** Proteins were extracted from muscle biopsies using two-step sequential extraction as described previously (Wilson *et al.* 2010; Nuez-Ortin *et al.* 2016, 2017). Proteins were extracted in progressively denaturing buffers (150 mM NaCl, 50 mM Tris, pH 8.0 followed by 7 M urea, 2 M thiourea, 50 mM Tris, pH 8.0). Protein concentrations were estimated by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), and 100  $\mu\text{g}$  of protein sample was reduced using 10 mM DTT (overnight at  $4^{\circ}\text{C}$ ), alkylated using 50 mM iodoacetamide (2 h at ambient temperature in the dark) then trypsin digested by co-precipitation (Wilson *et al.* 2010). Briefly, 1  $\mu\text{g}$  trypsin was spotted onto the wall of each sample Eppendorf tube then flushed into the sample with 1 mL 100% methanol chilled to  $-20^{\circ}\text{C}$ . Samples were kept at  $-20^{\circ}\text{C}$  overnight to allow co-precipitation of proteins with trypsin, then centrifuged in a benchtop microcentrifuge (5 min at 12,000 g) and pellets allowed to air-dry for 10 min. After addition of 100 mM ammonium bicarbonate, samples were then incubated for 5 h at  $37^{\circ}\text{C}$  with the further addition of 1  $\mu\text{g}$  trypsin after 3 h. Samples were acidified by the addition of 1% formic acid and non-digested protein removed by centrifugation in a benchtop microcentrifuge (5 min at 12,000 g). 1  $\mu\text{g}$  aliquots of each peptide sample were analysed using an LTQ-Orbitrap and Ultimate 3000 nano RSLC system (Thermo Fisher Scientific, MA, USA) using published methods for separation and data-dependent acquisition (Wilson *et al.* 2016).

Sequential extraction has been shown to be an effective approach to sample fractionation in complex and challenging tissues rich in structural proteins, such as cartilage (Wilson *et al.* 2010), whole fish larvae (Nuez-Ortin *et al.* 2016) and in white muscle (Nuez-Ortin *et al.* 2017) and skeletal muscle (Barbé *et al.* 2017). Prior to processing the complete set of samples, a sub-

set of six tissue biopsies was used to evaluate the extraction method for human skeletal muscle proteomics. Comparison of the extracted proteins indicated clear separation of the contractile proteins (e.g. actin, myosin and troponin) from readily soluble cytosolic proteins (e.g. glutathione S-transferases, 14-3-3 proteins and carbonic anhydrases) demonstrating the value of this approach to reduce sample complexity at the protein level.

**Protein identification and data processing.** Data files were imported into MaxQuant version 1.5.1.2 (<http://maxquant.org/>), where sequential extracts were defined as fractions of the same biological sample, and MS/MS spectra were searched against the Swiss-Prot Human reference proteome database (downloaded 18/03/2016; 201,015 entries) using the Andromeda search engine. Default settings for protein identification by LTQ-Orbitrap MS/MS and label-free quantitation (LFQ) included a maximum of two missed cleavages, mass error tolerances of 20 ppm then 4.5 ppm for initial and main peptide searches, respectively, 0.5 Da tolerance for fragment ions, variable methionine oxidation and fixed cysteine carbamidomethylation. A false discovery rate of 1% was used for both peptide-spectrum matching and protein identification. The LFQ protein intensity values were extracted from the MaxQuant *proteinGroups.txt* file into Perseus software (<http://perseus-framework.org/>), filtered to remove proteins identified by reverse database matching and on the basis of modified peptides only and then  $\log_2$ -transformed. Proteins detected in fewer than twelve biological samples were also excluded and remaining missing values were imputed using low-abundance LFQ intensity values drawn from a normal distribution, according to default values in Perseus. In total, 9932 unique peptides were identified matching 783 proteins. Prior to statistical comparative analysis, protein data were filtered for potential non-skeletal muscle contamination using the Human Protein Atlas (Uhlén *et al.* 2015; Thul *et al.* 2017). In accordance with the Human Protein Atlas (June 2017), 297 were determined not to be expressed at a protein level in skeletal muscle and were not included in further statistical analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005480.

**Immunohistochemistry and imaging.** Muscle fibre type distribution, capillaries and fibre cross-sectional area were determined by immunohistochemistry and confocal imaging as described previously (Nyberg *et al.* 2016). Briefly, the embedded muscle samples were cut in transverse sections of 8  $\mu\text{m}$  in a cryostat. Sections were fixed for 2 min in phosphate buffered saline (PBS, pH 7.2,

Gibco 70013-016, Life Technologies Denmark, Nærum, Denmark) containing 2% formaldehyde and washed in a 1:10 wash buffer (Dako S3006, Glostrup, Denmark), and blocked for 10 min in PBS containing 1% BSA for immunohistochemical staining. Antibodies were diluted in antibody diluent (Dako S0809). First, capillaries and myofibre type IIA were visualized using biotinylated *Ulex europaeus* agglutinin I lectin (1:100; VECTB-1065, VWR, Bie and Berntsen, Herlev, Denmark) and a monoclonal antibody (1:200; SC-71, Hybridoma Bank, Iowa City, IA, USA), respectively. Second, myofibre borders were visualized using an antibody against laminin (1:500; Dako Z0097) together with myosin heavy chain (1:1000; Sigma-Aldrich Denmark M8421, Brøndby, Denmark) added for distinction of myofibre type I. Specific secondary antibodies (order listed: Streptavidin/FITC, (1:200; DAKO F0422), Alexa-555 donkey anti-mouse (1:1000; Invitrogen A31570, Life Technologies Denmark), Alexa-350 goat anti-rabbit (1:1000; Invitrogen P10994) and Alexa-488 donkey anti-mouse (1:1000; Invitrogen A21202)) were applied to each primary antibody. Three individual muscle fibre types were identified as type I (green), type IIA (red), and type IIX (unstained/black). Visualization was performed on a computer screen using a light microscope (Carl Zeiss, Germany), and all morphometric analysis were performed using a digital analysis program (ImageJ, NIH ImageJ). Two or more separate sections of a cross-section were used for analysis, and the cross-sectional area was assessed by manually drawing the perimeter around each selected section. The number of muscle fibres and capillaries within each section was counted, and capillary supply was subsequently expressed as capillaries per fibre (capillary-to-fibre ratio) and capillary density (capillaries  $\text{mm}^{-2}$ ). Mean fibre area was assessed by manual drawing of the perimeter of each muscle fibre. All analyses were carried out manually by the same blinded investigator.

**Immunoblotting and SDS-PAGE.** Protein expression by Western blotting were determined as previously described (Thomassen *et al.* 2010). Protein concentration of each sample was determined with a BSA kit (Thermo Fisher Scientific, MA, USA). Samples were mixed with  $6 \times$  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 double distilled  $\text{H}_2\text{O}$  to reach equal protein concentrations. Equal amounts of protein was loaded in each well of pre-cast gels (Bio-Rad). 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. Bands were visualized with ECL (Millipore, MA, USA) and recorded with a digital camera (ChemiDoc MP Imaging System, Bio-Rad). Densitometry quantification of band intensity was done using Image Lab version 4.0 (Bio-Rad) and determined as the total

band intensity adjusted for background intensity. Primary antibodies used were citrate synthase (no. ab96600, Abcam, MA, US), GLUT4 (no. PA1-1065, Thermo Fisher Scientific), glycogen synthase (GS) (no. 3893, Cell Signalling Technology, MA, USA), OXPHOS (no. ab110411, Abcam, MA, USA) and phosphofructokinase (Sc-166722, Santa Cruz Biotechnology, Inc., Dallas, USA). The secondary antibodies used were HRP conjugated rabbit anti-sheep (P-0163), goat anti-mouse (P-0447, DAKO, Denmark) and goat anti-rabbit IgM/IgG (4010-05 Southern Biotech, AL, USA).

**Enzymatic activity.** Maximal enzyme activity of citrate synthase, hexokinase, phosphofructokinase, and lactate dehydrogenase was quantified in muscle homogenates using fluorometric methods (Fluoroscan Ascent, Thermo Fisher Scientific) as described by Lowry & Passonneau (1972).

## Statistics

Statistical analyses were performed in SPSS version 24. Sample size was based on previous studies of terbutaline (Hostrup *et al.* 2015; Dyreborg *et al.* 2016). Data were tested for normality using the Shapiro Wilks test and  $q$ - $q$  plots. Data were normally distributed and magnitudes of outcome statistics are presented as means ( $\pm 95\%$  confidence interval) and  $P$  values to represent probability. To estimate within- and between-group changes with the intervention, two-tailed linear mixed modelling was used with group and trial included as fixed factors and a random factor for subjects for a full factorial design. Age was included as a time-invariant covariate in the mixed model, since age has been shown to influence the response to  $\beta_2$ -agonists (White *et al.* 1994). For univariate correlation analyses, the Pearson product-moment correlation coefficient was used. For the proteomic data, a series of mixed models were performed on the  $\log_2$  expression for each protein to estimate protein-specific within- and between-group changes with the intervention. The false discovery rate (FDR) method as described by Storey & Tibshirani (2003) was used to control for multiple testing. Differently expressed proteins (FDR  $\leq 5\%$ ) were subjected to functional annotation enrichment analysis (David Bioinformatics resources 6.8, NIH, USA) based on gene ontology (GO) terms biological processes (GO:BP) and cellular components (GO:CC) as well as KEGG terms using an EASE score threshold of 0.1 and the Benjamini-Hochberg procedure to adjust  $P$  values. Linear discriminant analysis-principal component analysis with varimax rotation was used to estimate between-group separation in the muscle proteome, only including the first eight principal components in the discriminant analysis because of the sample size of nine in HIT.

**Table 2. Upregulated proteins in HIT (n = 9)**

Name	Description	Log <sub>2</sub> -change	q value
GYS1	Glycogen [starch] synthase, muscle	0.46	≤ 0.01
FUNDC2	FUN14 domain-containing protein 2	0.91	≤ 0.05
NDUFA2	NADH dehydrogenase [ubiquinone] 1 $\alpha$ subcomplex subunit 2	0.38	≤ 0.05
COX6C	Cytochrome c oxidase subunit 6C	0.63	≤ 0.05
S100A13	Protein S100-A13	1.15	≤ 0.05
DNAJA2	DnaJ homolog subfamily A member 2	1.00	≤ 0.05
NDUFS6	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	0.54	≤ 0.05
NDUFB6	NADH dehydrogenase [ubiquinone] 1 $\beta$ subcomplex subunit 6	0.51	≤ 0.05
UQCRH	Cytochrome b-c1 complex subunit 6, mitochondrial	0.36	≤ 0.05
MRPL12	39S ribosomal protein L12, mitochondrial	1.04	≤ 0.05
NDUFB3	NADH dehydrogenase [ubiquinone] 1 $\beta$ subcomplex subunit 3	0.40	≤ 0.05
PHB	Prohibitin	0.29	≤ 0.05
B2M	B-2-microglobulin;B-2-microglobulin form pI 5.3	0.97	≤ 0.05
ATP5H	ATP synthase subunit d, mitochondrial	0.28	≤ 0.05
SSBP1	Single-stranded DNA-binding protein, mitochondrial	0.22	≤ 0.05
MYOZ2	Myozenin-2	0.48	≤ 0.05
NDUFV2	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	0.28	≤ 0.05
STOML2	Stomatin-like protein 2, mitochondrial	0.43	≤ 0.05
ATP5J	ATP synthase-coupling factor 6, mitochondrial	0.35	≤ 0.05
COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	0.24	≤ 0.05
PLIN2	Perilipin-2	0.39	≤ 0.05
NDUFB8	NADH dehydrogenase [ubiquinone] 1 $\beta$ subcomplex subunit 8, mitochondrial	0.55	≤ 0.05
SDHA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	0.14	≤ 0.05
CHCHD3	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial	0.30	≤ 0.05
AGL	Glycogen debranching enzyme;4- $\alpha$ -glucanotransferase;Amylo- $\alpha$ -1,6-glucosidase	0.23	≤ 0.05
IMMT	Mitochondrial inner membrane protein	0.20	≤ 0.05

## Results

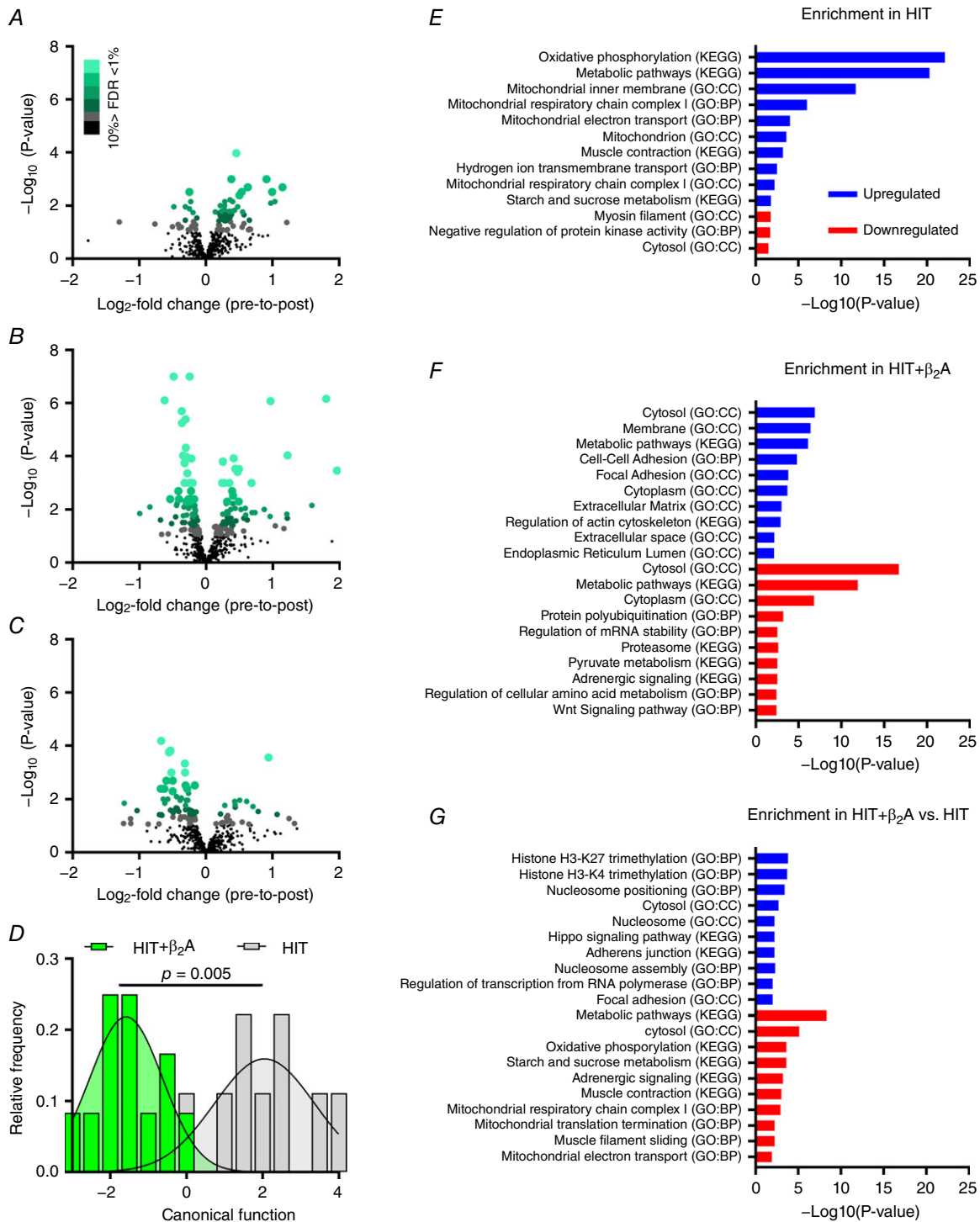
### Muscle proteome remodelling

Of the 486 proteins identified as human skeletal muscle proteins, training upregulated expression of 26 proteins (Table 2) and downregulated expression of 6 proteins (Table 3) in HIT (FDR ≤ 5%)(Fig. 1A), whereas concomitant daily inhalation of  $\beta_2$ -agonist upregulated expression of 34 proteins (Table 4) and downregulated expression of 51 proteins (Table 5) in HIT+ $\beta_2$ A (Fig. 1B). Inhalation of  $\beta_2$ -agonist caused a repression of 25 proteins in HIT+ $\beta_2$ A compared to HIT (group by trial interaction), and an upregulation of 7 proteins compared to HIT (Table 6; Fig. 1C). Proteome signature changes induced by the intervention in HIT and HIT+ $\beta_2$ A were markedly different as indicated by a clear separation of the two groups (Wilks' Lambda: 0.228,  $P = 0.005$ ) in a principal component-discriminant analysis that explained 70% of total proteome variance (Fig. 1D).

**Table 3. Downregulated proteins in HIT (n = 9)**

Name	Description	Log <sub>2</sub> -change	q value
PARK7	Protein DJ-1	-0.25	≤ 0.05
MYBPC1	Myosin-binding protein C, slow-type	-0.23	≤ 0.05
MYH1	Myosin-1	-0.48	≤ 0.05
PNPO	Pyridoxine-5-phosphate oxidase	-0.31	≤ 0.05
FKBP3	Peptidyl-prolyl cis-trans isomerase FKBP3	-0.25	≤ 0.05
HSPB1	Heat shock protein $\beta$ -1	-0.19	≤ 0.05

Functional annotation enrichment analysis of differently expressed proteins (FDR ≤ 5%) based on gene ontology (GO) terms biological processes (GO:BP), cellular components (GO:CC) and KEGG



**Figure 1. Proteome signature remodelling in HIT and HIT+ $\beta_2$ A**

Effect of 4 weeks of high intensity training with (HIT+ $\beta_2$ A,  $n = 12$ ) and without (HIT,  $n = 9$ ) daily inhalation of terbutaline ( $4 \text{ mg day}^{-1}$ ) on proteome signature of the vastus lateralis muscle in trained men. A–C, volcano plots of within-group changes in skeletal muscle proteins (486 proteins) in HIT (A) and HIT+ $\beta_2$ A (B), as well as between-group interaction (HIT+ $\beta_2$ A – HIT) (C). Green scale indicates % false discovery rate (FDR). D, Gaussian fit of frequency distribution of discriminant canonical function score of within-group changes in proteome signature using principal component-discriminant analysis (PCA-DA) of the eight first principal components that explained 70% of total proteome variance. E–G, enrichment analysis of the 10 most up- (blue) and downregulated (red) pathways (GO: biological processes, GO: cellular component, and KEGG pathways) in HIT (E) and HIT+ $\beta_2$ A (F) as well as between-group (HIT+ $\beta_2$ A – HIT) (G).



**Table 4. Upregulated proteins in HIT+ $\beta_2$ A ( $n = 12$ )**

Name	Description	Log <sub>2</sub> -change	q value
S100A13	Protein S100-A13	1.80	≤ 0.001
ACTG1	Actin, cytoplasmic 2;Actin, cytoplasmic, N-terminally processed	0.97	≤ 0.001
B2M	B-2-microglobulin;B-2-microglobulin form pl 5.3	1.22	≤ 0.01
PADI2	Protein-arginine deiminase type-2	0.41	≤ 0.01
SDHA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	0.25	≤ 0.01
AHNAK	Neuroblast differentiation-associated protein AHNAK	0.44	≤ 0.01
PCBP1	Poly(rC)-binding protein 1	0.49	≤ 0.01
EEF1A1P5;EEF1A1	Putative elongation factor 1- $\alpha$ -like 3;Elongation factor 1- $\alpha$ 1	1.96	≤ 0.01
HK1	Hexokinase-1	0.47	≤ 0.01
YWHAZ	14-3-3 protein zeta/delta	0.27	≤ 0.01
ACSL1	Long-chain-fatty-acid-CoA ligase 1	0.35	≤ 0.01
PACSLN3	Protein kinase C and casein kinase substrate in neurons protein 3	0.24	≤ 0.01
S100A6	Protein S100-A6	0.68	≤ 0.01
DES	Desmin	0.39	≤ 0.05
ACTR3	Actin-related protein 3	0.40	≤ 0.05
RPL5	60S ribosomal protein L5	0.31	≤ 0.05
STOML2	Stomatin-like protein 2, mitochondrial	0.49	≤ 0.05
HSPA5	78 kDa glucose-regulated protein	0.30	≤ 0.05
MAPT	Microtubule-associated protein tau	0.40	≤ 0.05
ANP32A	Acidic leucine-rich nuclear phosphoprotein 32 family member A	1.58	≤ 0.05
RPSA	40S ribosomal protein SA	0.43	≤ 0.05
PDIA6	Protein disulfide-isomerase A6	0.59	≤ 0.05
HNRNPK	Heterogeneous nuclear ribonucleoprotein K	0.37	≤ 0.05
MYL9	Myosin regulatory light polypeptide 9	0.87	≤ 0.05
PPP1R14B	Protein phosphatase 1 regulatory subunit 14B	0.52	≤ 0.05
TOM1	Target of Myb protein 1	0.31	≤ 0.05
HADHA	Trifunctional enzyme subunit $\alpha$ , mitochondrial	0.32	≤ 0.05
NDUFC2	NADH dehydrogenase [ubiquinone] 1 subunit C2	0.73	≤ 0.05
TIMM50	Mitochondrial import inner membrane translocase subunit TIM50	0.69	≤ 0.05
NDUFS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	0.33	≤ 0.05
ACTC1	Actin, $\alpha$ cardiac muscle 1	1.21	≤ 0.05
PFN1	Profilin-1	0.25	≤ 0.05
MAOB	Amine oxidase [flavin-containing] B	0.59	≤ 0.05
MYL6	Myosin light polypeptide 6	0.96	≤ 0.05

pathways revealed different enrichments induced by the intervention in HIT and HIT+ $\beta_2$ A (Fig. 1E–G). In HIT, the most dominantly upregulated pathways were related to the mitochondria, in particular the oxidative phosphorylation, as well as metabolic pathways, including glycogen metabolism (Fig. 1E). In contrast, HIT+ $\beta_2$ A predominantly had an upregulation of pathways related to the cytosol, metabolism and cell-to-cell adhesion (Fig. 1F). HIT had a minor downregulation of pathways related to myosin filaments, protein kinases and the cytosol (Fig. 1E), whereas HIT+ $\beta_2$ A had several down-regulated pathways, including those related to the cytosol and metabolism, including pyruvate and amino acid metabolism (Fig. 1F). Between-group analysis revealed that daily inhalation of  $\beta_2$ -agonist downregulated pathways related to glycogen metabolism and oxidative phosphorylation in HIT+ $\beta_2$ A compared to HIT, but

upregulated pathways related to histone trimethylation and the nucleosome (Fig. 1G).

### $\beta_2$ -Agonist blunts training-induced enhancements in $\dot{V}_{O_{2max}}$ and exercise performance

A key observation was that inhalation of  $\beta_2$ -agonist completely blunted ( $P \leq 0.01$ ) training-induced improvement in  $\dot{V}_{O_{2max}}$  and reduced ( $P \leq 0.05$ ) enhancement in exercise performance during incremental cycling to exhaustion (Fig. 2B). While training effectively augmented  $\dot{V}_{O_{2max}}$  in HIT ( $P \leq 0.01$ ), HIT+ $\beta_2$ A showed no relevant changes in  $\dot{V}_{O_{2max}}$  with the intervention (Fig. 2B). Furthermore, HIT had a twofold greater enhancement in exercise performance compared to HIT+ $\beta_2$ A (11.6 vs. 6.1%,  $P \leq 0.05$ ) (Fig. 2B). Between-group changes in  $\dot{V}_{O_{2max}}$  were still evident

**Table 5. Downregulated proteins in HIT+ $\beta_2$ A ( $n = 12$ )**

Name	Description	Log <sub>2</sub> -change	q value
HSPB1	Heat shock protein $\beta$ -1	-0.49	$\leq 0.001$
ALDH9A1	4-trimethylaminobutyraldehyde dehydrogenase	-0.25	$\leq 0.001$
GYG1	Glycogenin-1	-0.62	$\leq 0.001$
CFL2	Cofilin-2	-0.36	$\leq 0.001$
FHL1	Four and a half LIM domains protein 1	-0.31	$\leq 0.001$
PTGR2	Prostaglandin reductase 2	-0.36	$\leq 0.001$
CRYAB	A-crystallin B chain	-0.30	$\leq 0.01$
HSPA2	Heat shock-related 70 kDa protein 2	-0.35	$\leq 0.01$
GSTM2	Glutathione S-transferase Mu 2	-0.30	$\leq 0.01$
ST13	Hsc70-interacting protein	-0.22	$\leq 0.01$
CMBL	Carboxymethylenebutenolidase homolog	-0.32	$\leq 0.01$
HSPB7	Heat shock protein $\beta$ -7	-0.33	$\leq 0.01$
DNPEP	Aspartyl aminopeptidase	-0.28	$\leq 0.01$
ACO1	Cytoplasmic aconitate hydratase	-0.25	$\leq 0.01$
AGL	Glycogen debranching enzyme	-0.32	$\leq 0.01$
MACROD1	O-acetyl-ADP-ribose deacetylase MACROD1	-0.20	$\leq 0.01$
PSMA6	Proteasome subunit $\alpha$ type-6	-0.23	$\leq 0.01$
PSMB3	Proteasome subunit $\beta$ type-3	-0.32	$\leq 0.01$
NDRG2	Protein NDRG2	-0.23	$\leq 0.01$
MDH1	Malate dehydrogenase, cytoplasmic	-0.23	$\leq 0.01$
CUTC	Copper homeostasis protein cutC homolog	-0.42	$\leq 0.05$
PDLIM5	PDZ and LIM domain protein 5	-0.23	$\leq 0.05$
CAB39	Calcium-binding protein 39	-0.26	$\leq 0.05$
NDUFB7	NADH dehydrogenase [ubiquinone] 1 $\beta$ subcomplex subunit 7	-0.54	$\leq 0.05$
PREP	Prolyl endopeptidase	-0.24	$\leq 0.05$
TPM3	Tropomyosin $\alpha$ -3 chain	-0.41	$\leq 0.05$
WDR1	WD repeat-containing protein 1	-0.18	$\leq 0.05$
LMCD1	LIM and cysteine-rich domains protein 1	-0.29	$\leq 0.05$
PSMA5	Proteasome subunit $\alpha$ type-5	-0.24	$\leq 0.05$
TNNC1	Troponin C, slow skeletal and cardiac muscles	-0.30	$\leq 0.05$
MAPRE2	Microtubule-associated protein RP/EB family member 2	-0.44	$\leq 0.05$
YWHAG	14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed	-0.20	$\leq 0.05$
TXLNB	B-taxilin	-0.24	$\leq 0.05$
PYGM	Glycogen phosphorylase, muscle form	-0.31	$\leq 0.05$
LDB3	LIM domain-binding protein 3	-0.17	$\leq 0.05$
TPM2	Tropomyosin $\beta$ chain	-0.44	$\leq 0.05$
AMPD1	AMP deaminase 1	-0.33	$\leq 0.05$
LMNB2	Lamin-B2	-0.84	$\leq 0.05$
ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	-0.27	$\leq 0.05$
PRDX1	Peroxiredoxin-1	-0.17	$\leq 0.05$
CRYZ	Quinone oxidoreductase	-0.26	$\leq 0.05$
TPT1	Translationally-controlled tumor protein	-0.17	$\leq 0.05$
PHPT1	14 kDa phosphohistidine phosphatase	-0.16	$\leq 0.05$
PFN2	Profilin-2	-0.99	$\leq 0.05$
MYOM2	Myomesin-2	-0.21	$\leq 0.05$
NPEPPS	Puromycin-sensitive aminopeptidase	-0.17	$\leq 0.05$
ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	-0.29	$\leq 0.05$
PCBD2	Pterin-4- $\alpha$ -carbinolamine dehydratase 2	-0.34	$\leq 0.05$
LDHA	L-lactate dehydrogenase A chain	-0.19	$\leq 0.05$
NT5C1A	Cytosolic 5-nucleotidase 1A	-0.46	$\leq 0.05$
PARK7	Protein DJ-1	-0.15	$\leq 0.05$

**Table 6. Between-group interaction (HIT+ $\beta_2$ A – HIT)**

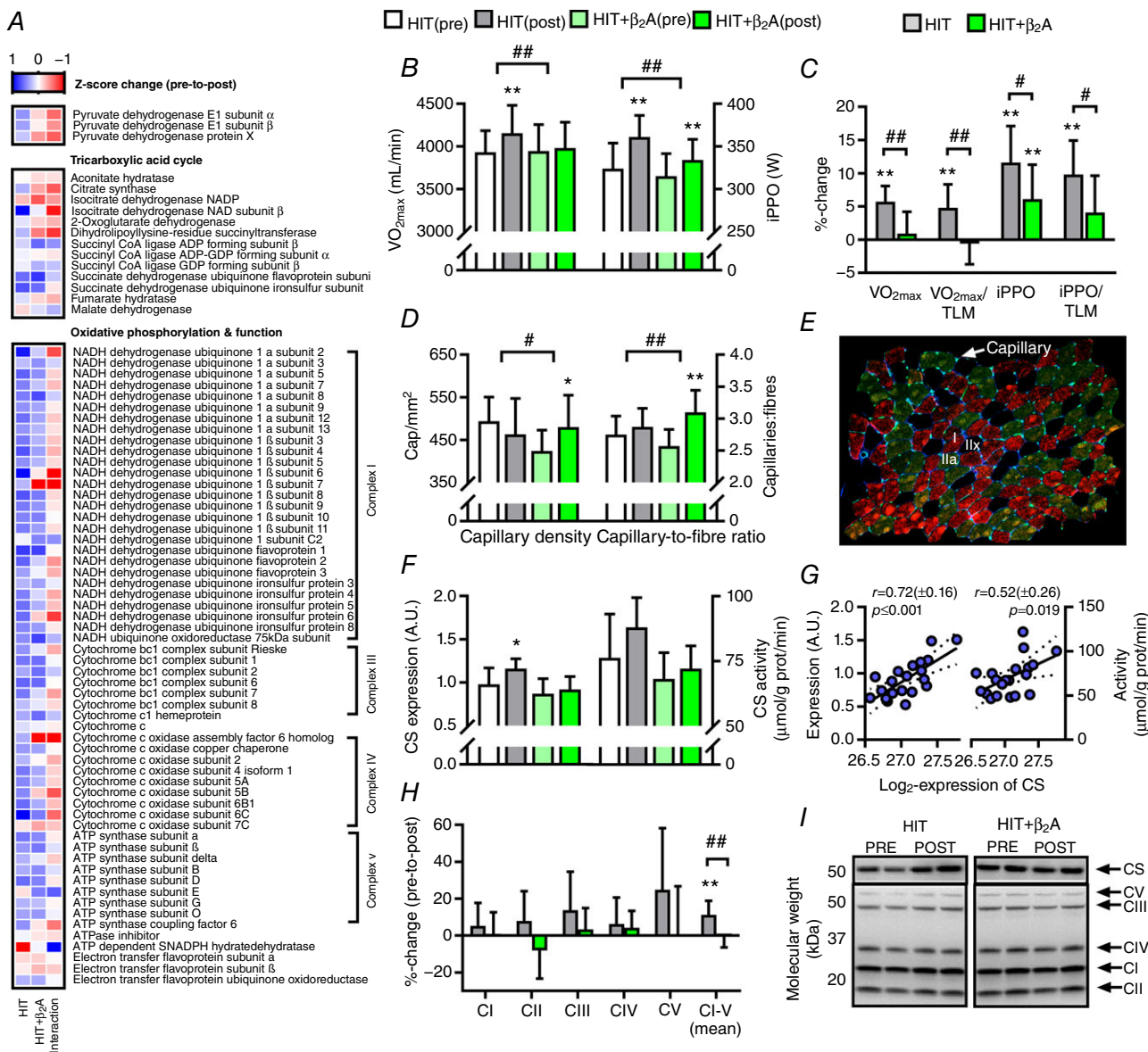
Name	Description	Log <sub>2</sub> -change	q value
GYG1	Glycogenin-1	-0.67	≤ 0.01
HSPB7	Heat shock protein $\beta$ -7	-0.53	≤ 0.01
AGL	Glycogen debranching enzyme	-0.55	≤ 0.01
ACTG1	Actin, cytoplasmic 2;Actin, cytoplasmic 2, N-terminally processed	0.94	≤ 0.01
FHL1	Four and a half LIM domains protein 1	-0.31	≤ 0.01
CFL2	Cofilin-2	-0.31	≤ 0.01
GYS1	Glycogen [starch] synthase, muscle	-0.52	≤ 0.01
ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	-0.59	≤ 0.05
TNNC1	Troponin C, slow skeletal and cardiac muscles	-0.50	≤ 0.05
CRYAB	A-crystallin B chain	-0.31	≤ 0.05
ALDH9A1	4-trimethylaminobutyraldehyde dehydrogenase	-0.17	≤ 0.05
ACO1	Cytoplasmic aconitate hydratase	-0.28	≤ 0.05
NDUFS6	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	-0.68	≤ 0.05
TPM3	Tropomyosin $\alpha$ -3 chain	-0.62	≤ 0.05
PYGM	Glycogen phosphorylase, muscle form	-0.50	≤ 0.05
LDB3	LIM domain-binding protein 3	-0.26	≤ 0.05
MRPS36	28S ribosomal protein S36, mitochondrial	-0.44	≤ 0.05
PSMB3	Proteasome subunit $\beta$ type-3	-0.37	≤ 0.05
NDUFB6	NADH dehydrogenase [ubiquinone] 1 $\beta$ subcomplex subunit 6	-0.57	≤ 0.05
NDUFB7	NADH dehydrogenase [ubiquinone] 1 $\beta$ subcomplex subunit 7	-0.64	≤ 0.05
HSPB1	Heat shock protein $\beta$ -1	-0.29	≤ 0.05
HIST1H1C;HIST1H1E HIST1H1D	Histone H1.2;Histone H1.4;Histone H1.3	0.51	≤ 0.05
LMCD1	LIM and cysteine-rich domains protein 1	-0.39	≤ 0.05
SSBP1	Single-stranded DNA-binding protein, mitochondrial	-0.27	≤ 0.05
ACTN1	A-actinin-1	0.61	≤ 0.05
AHNAK	Neuroblast differentiation-associated protein AHNAK	0.43	≤ 0.05
MRPL12	39S ribosomal protein L12, mitochondrial	-1.23	≤ 0.05
TPM2	Tropomyosin $\beta$ chain	-0.62	≤ 0.05
HK1	Hexokinase-1	0.44	≤ 0.05
YWHAZ	14-3-3 protein zeta/delta	0.28	≤ 0.05
NDUFA2	NADH dehydrogenase [ubiquinone] 1 $\alpha$ subcomplex subunit 2	-0.30	≤ 0.05
KPNB1	Importin subunit $\beta$ -1	0.71	≤ 0.05

after adjusting for adaptations in resting metabolic rate and thigh lean mass (Fig. 2C), which indicates that the increase in  $\dot{V}_{O_2\max}$  observed in HIT and the attenuating effect of  $\beta_2$ -agonist observed in HIT+ $\beta_2$ A were attributed to factors other than different adaptations in resting metabolic rate and hypertrophy of the exercising muscles. For training-induced enhancement in exercise performance, the between-group difference was also evident after adjusting for thigh lean mass (Fig. 2C). On the other hand, when adjusting for change in  $\dot{V}_{O_2\max}$ , no differences were observed in training-induced enhancement in exercise performance between the groups, thus indicating that the blunting effect of  $\beta_2$ -agonist on training-induced enhancement in performance in HIT+ $\beta_2$ A was related to the attenuating effect of  $\beta_2$ -agonist on improvement in  $\dot{V}_{O_2\max}$ .

Muscle capillary density has been shown to be related to  $\dot{V}_{O_2\max}$  and associated with endurance performance (Saltin *et al.* 1977; Coyle *et al.* 1991). Nonetheless, we

observed opposite adaptations in capillarization of the vastus lateralis muscle considering that observed for  $\dot{V}_{O_2\max}$  and exercise performance. Inhalation of  $\beta_2$ -agonist increased capillary density by 13% ( $P \leq 0.05$ ) and capillary-to-fibre ratio by 21% ( $P \leq 0.01$ ) in HIT+ $\beta_2$ A, whereas HIT showed no relevant changes with the intervention (Fig. 2D). Thus, the increased  $\dot{V}_{O_2\max}$  in HIT and the blunted effect in HIT+ $\beta_2$ A might be related to an inhibitory effect of  $\beta_2$ -agonist on adaptations of the heart or mitochondrial oxidative capacity of the exercised muscles (Holloszy & Coyle, 1984).

From proteomic analysis, we detected and grouped 13 proteins related to the tricarboxylic acid (TCA) cycle and 58 proteins related to oxidative phosphorylation of the mitochondria (Fig. 2A). For the TCA cycle, only minor changes were observed, in which succinate dehydrogenase flavoprotein subunit increased in both groups (Tables 2 and 4), whereas aconitate hydratase and malate dehydrogenase decreased in HIT+ $\beta_2$ A (Table 5)



**Figure 2. Maximal oxygen consumption, capillary density and muscle oxidative enzymes in HIT and HIT+ $\beta_2$ A**

Effect of 4 weeks of high intensity training with (HIT+ $\beta_2$ A,  $n = 12$ ) and without (HIT,  $n = 9$ ) daily inhalation of terbutaline (4 mg day $^{-1}$ ) on outcomes related to exercise performance and oxidative capacity in trained men. *A*, heat map of mean Z-score changes of the proteome related to tricarboxylate acid cycle and oxidative phosphorylation in HIT and HIT+ $\beta_2$ A, as well as between-group interaction (HIT+ $\beta_2$ A – HIT). *B*, mean ( $\pm 95\%$  CI) maximal oxygen consumption ( $\dot{V}O_{2\max}$ ; left y-axis) and incremental peak power output (iPPO; right y-axis). *C*, mean ( $\pm 95\%$  CI) relative change in  $\dot{V}O_{2\max}$  and iPPO when unadjusted and adjusted for thigh lean mass (TLM). *D*, mean ( $\pm 95\%$  CI) capillary density (left y-axis) and capillary-to-fibre ratio (right y-axis). *E*, representative image of immunohistochemical analysis; myosin heavy chain I (red), IIa (green/brown), IIx (black), laminin (blue), capillary (light green). *F*, mean ( $\pm 95\%$  CI) expression (left y-axis) and maximal activity (right y-axis) of citrate synthase (CS). *G*, bivariate correlation between intensity ( $\log_2$ -expression) of CS as determined by proteomics (y-axis) versus expression (left y-axis) and maximal activity (right y-axis) of CS as determined by Western blotting and fluorometrically, respectively. *H*, mean ( $\pm 95\%$  CI) relative change in abundance of OXPHOS complexes I–V (CI–V) determined by Western blotting. *I*, representative blots of CS and OXPHOS CI–V. #Between-group interaction ( $P \leq 0.05$ ). ##Between-group interaction ( $P \leq 0.01$ ). \*Within-group difference from pre ( $P \leq 0.05$ ). \*\*Within-group difference from pre ( $P \leq 0.01$ ).

with a between-group interaction for aconitate hydratase (Table 6). While proteomic analysis revealed no significant changes in citrate synthase, Western blot analysis showed a minor increase in citrate synthase content in HIT with a tendency towards a higher maximal enzymatic activity (Fig. 2F), but values were not statistically different from HIT+ $\beta_2$ A. There was a good correlation between the proteomic measurements of citrate synthase with that of Western blotting and enzymatic activity (Fig. 2G). In the oxidative phosphorylation, pronounced modulation was observed with the intervention. In HIT, abundance of a variety of the NADH dehydrogenase subcomplexes and ATP synthase subunits increased with the intervention (Table 2), whereas HIT+ $\beta_2$ A only had a few significantly upregulated proteins (Table 4) and even a blunted training-induced increase in abundance of four NADH dehydrogenase subcomplexes (Table 6). Consistent with this finding, Western blotting revealed an upregulation of overall abundance of OXPHOS complexes I–V in HIT ( $P \leq 0.01$ ), whereas this effect was attenuated ( $P \leq 0.01$ ) in HIT+ $\beta_2$ A (Fig. 2H).

### $\beta_2$ -Agonist alters contractile phenotype

Some clearly distinct adaptations in muscle contractile properties and phenotype were observed between the groups. As shown in Fig. 3A and B, muscle proteome signature changed differently in HIT and HIT+ $\beta_2$ A as indicated by a between-group separation (Wilks' Lambda: 0.127,  $P \leq 0.001$ ) (Fig. 3B) in a principal component-discriminant analysis that explained 80% of the variance for the contractile proteins listed in Fig. 3A. These between-group differences were primarily driven by opposing adaptations in myosin light chains, troponin C and tropomyosin  $\alpha$  and  $\beta$  chains (Table 6), all of which may affect contractile properties and myofibrillar  $\text{Ca}^{2+}$  sensitivity (Greaser *et al.* 1988; Lamboley *et al.* 2014). Muscle fibre phenotype has historically been categorized according to MHC isoforms (Lutz *et al.* 1979; Harridge *et al.* 1996). Expression of myosin 1, the most highly expressed myosin isoform in type IIx fibres (Murgia *et al.* 2015), decreased with the intervention in HIT ( $\log_2$ -fold change:  $-0.48$ ,  $P \leq 0.05$ ) (Fig. 3A and Table 2). Consistent with this observation, immunohistochemical analysis of the muscle biopsies revealed significant between-group changes ( $P \leq 0.05$ ) with the intervention, in which HIT had a reduction in distribution of MHCIIx ( $P \leq 0.05$ ) (Fig. 3C), whereas HIT+ $\beta_2$ A had an increase in distribution of MHCIIa by 4.1% ( $P \leq 0.05$ ) (Fig. 3C). There was a high degree of consistency between myosin expression data determined by proteomics and MHC isoform distribution determined immunohistochemically (Fig. 3D). Aside from muscle contractile phenotype changes, HIT and HIT+ $\beta_2$ A had some different adaptations in sarcomere architecture and

cell-to-cell adhesion. Compared to HIT, HIT+ $\beta_2$ A had an upregulation of the actin- and anchoring-related proteins actin and  $\alpha$ -actinin-1 (Table 6).

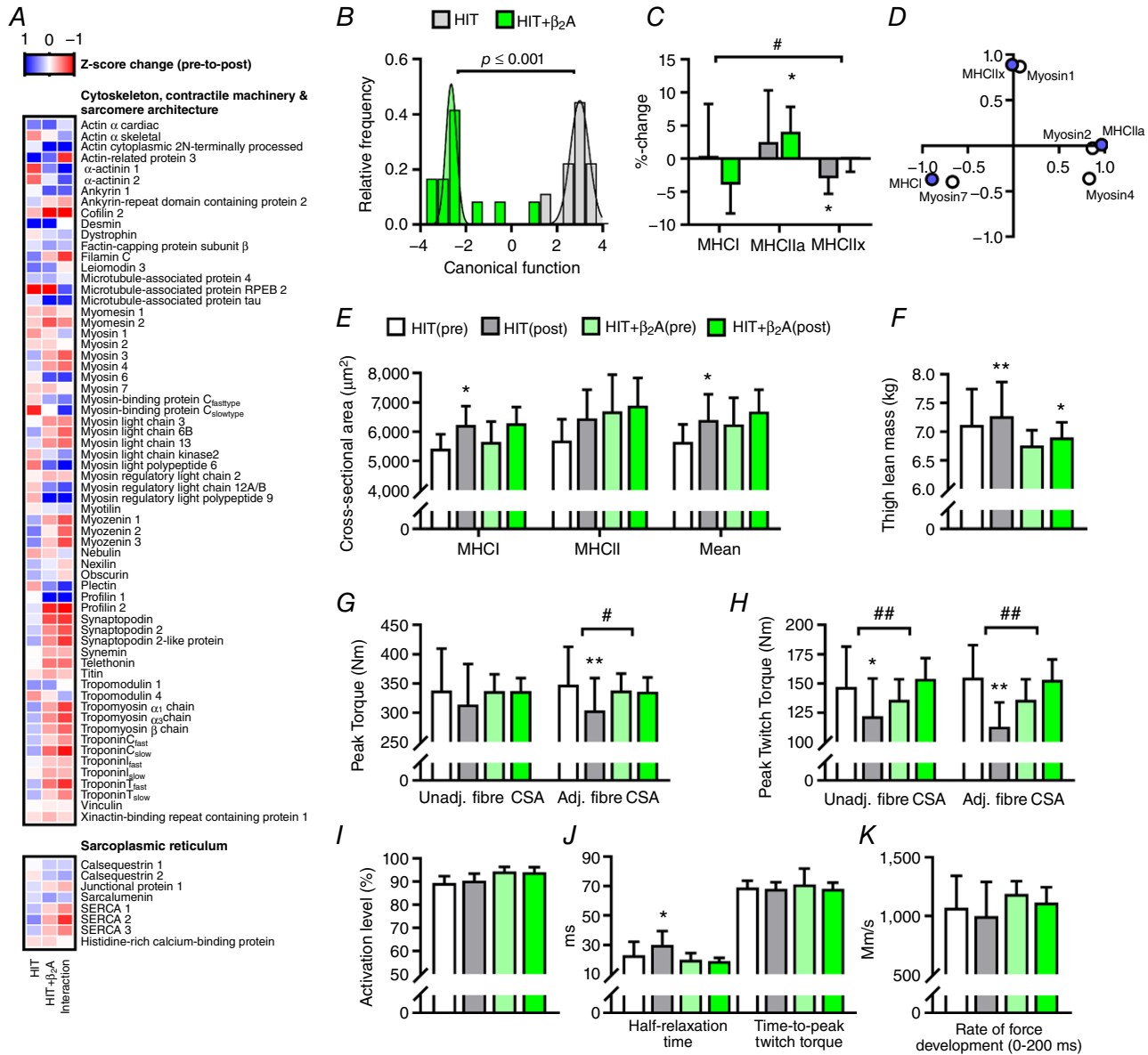
Although a common effect of  $\beta_2$ -agonist treatment is muscle growth (Lynch & Ryall, 2008; Joassard *et al.* 2013; Hostrup *et al.* 2015), we observed a similar degree of thigh muscle hypertrophy in both groups with the intervention (Fig. 3F). At the muscle fibre level, however, histochemical analysis revealed that only HIT had an increase in mean cross-sectional area (CSA) of muscle fibres ( $P \leq 0.05$ ), albeit not statistically different from HIT+ $\beta_2$ A (Fig. 3E). Despite the induced hypertrophy of the thigh, maximal isometric muscle strength (peak torque) of the quadriceps did not change in either HIT or HIT+ $\beta_2$ A (Fig. 3G). In fact, when adjusting for adaptations in muscle fibre CSA, peak torque declined by 19% with the intervention in HIT ( $P \leq 0.01$ ), whereas no change was observed in HIT+ $\beta_2$ A (Fig. 3G). Likewise, peak twitch torque declined by 17% ( $P \leq 0.05$ ) and 27% ( $P \leq 0.01$ ) in HIT when unadjusted and adjusted for muscle fibre CSA, respectively, whereas non-significant increases were observed in HIT+ $\beta_2$ A (Fig. 3H). Furthermore, muscle half-relaxation time was prolonged by 8 ( $\pm 6$ ) ms with the intervention in HIT ( $P \leq 0.05$ ), but not differently from HIT+ $\beta_2$ A which showed no relevant change with the intervention (Fig. 3J). Degree of voluntary activation level (Fig. 3I), time-to-peak twitch torque (Fig. 3J) and rate of force development (Fig. 3K) of the quadriceps did not change in either group with the intervention.

### $\beta_2$ -Agonist represses metabolic pathways

Various myocellular pathways regulate substrate choice during exercise, where glycogenolysis and glycolysis are predominant metabolic pathways for glucose metabolism and  $\beta$ -oxidation for lipid metabolism. Though only minor changes were observed in proteins related to lipid metabolism, HIT and HIT+ $\beta_2$ A had markedly diverse adaptations in the muscle proteome related to glycogenesis and glycogenolysis (Fig. 4A). Notably, in HIT+ $\beta_2$ A, inhalation of  $\beta_2$ -agonist attenuated the training-induced upregulation of glycogenin 1, glycogen debranching enzyme, glycogen synthase and glycogen phosphorylase observed in HIT (Table 6). HIT+ $\beta_2$ A even showed a marked downregulation of glycogen debranching enzyme, glycogen phosphorylase and glycogenin 1 (Fig. 4A, Table 5). Likewise, glycogen synthase, the rate-limiting enzyme in glycogenesis, which was the most significantly upregulated protein of the proteome in HIT (Fig. 4A, Table 2), did not change in HIT+ $\beta_2$ A. For further confirmation of this observation, Western blotting of glycogen synthase revealed a 25% increase in glycogen synthase abundance in HIT ( $P \leq 0.01$ ), which was blunted ( $P \leq 0.01$ ) in HIT+ $\beta_2$ A (Fig. 4D). In the glycolytic pathway, some minor changes were observed. Abundance

of hexokinase 1 increased with the intervention in HIT+ $\beta_2$ A compared to HIT (Tables 4 and 6) with a concurrent minor repression of lactate dehydrogenase A chain (Table 5). Partly in accordance with these observations, enzymatic analysis showed that maximal

activity of hexokinase increased ( $P \leq 0.01$ ) (Fig. 4B) and lactate dehydrogenase decreased in HIT+ $\beta_2$ A ( $P \leq 0.05$ ) (Fig. 4C). In contrast, HIT showed a minor increase in abundance and maximal activity of the glycolytic rate-limiting enzyme, phosphofructokinase ( $P \leq 0.05$ )



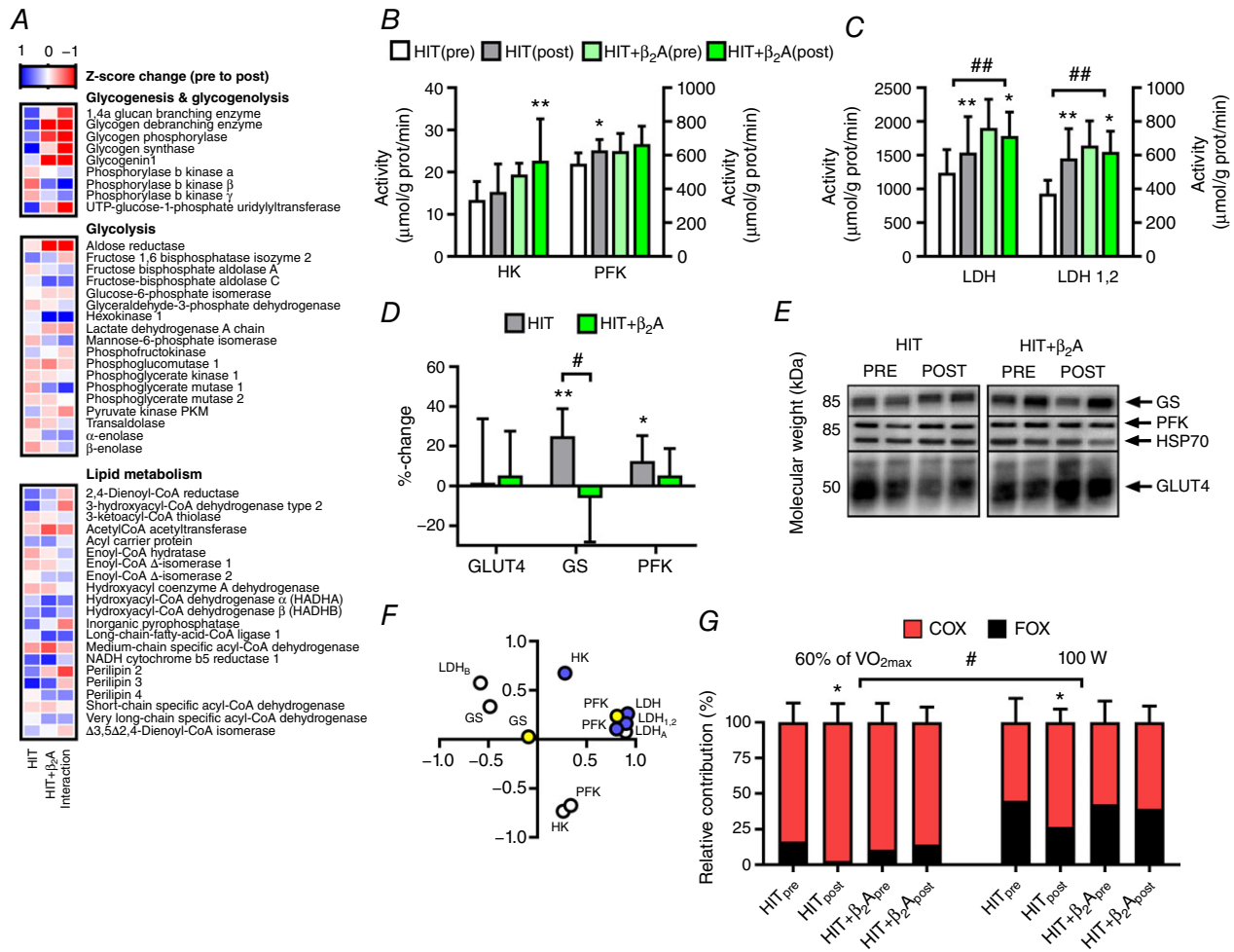
**Figure 3. Muscle contractile phenotype and function in HIT and HIT+ $\beta_2$ A**

Effect of 4 weeks of high intensity training with (HIT+ $\beta_2$ A,  $n = 12$ ) and without (HIT,  $n = 9$ ) daily inhalation of terbutaline ( $4 \text{ mg day}^{-1}$ ) on outcomes related to contractile phenotype and function in trained men. **A**, heat map of mean Z-score changes of the proteome related to the cytoskeleton and contractile phenotype in HIT and HIT+ $\beta_2$ A, as well as between-group interaction (HIT+ $\beta_2$ A – HIT). **B**, Gaussian fit of frequency distribution of discriminant canonical function score of within-group changes in the proteins listed in **A** using a principal component-discriminant analysis (PCA-DA) of the eight first principal components that explained 80% of the variance. **C**, mean ( $\pm 95\%$  CI) change in myosin heavy chain (MHC) isoform distribution. **D**, PCA of intensity ( $\log_2$ -expression) of myosin isoforms determined by proteomics (open circles) and MHC isoform distribution determined immunohistochemically (purple circles). **E–K**, mean ( $\pm 95\%$  CI) values for muscle mass and contractile properties. CSA, cross-sectional area. #Between-group interaction ( $P \leq 0.05$ ). ##Between-group interaction ( $P \leq 0.01$ ). \*Within-group difference from pre ( $P \leq 0.05$ ). \*\*Within-group difference from pre ( $P \leq 0.01$ ).

(Fig. 4B and D), and a marked increase in lactate dehydrogenase activity ( $P \leq 0.01$ ) (Fig. 4C). In general, principal component analysis revealed a good association between the different assays used to determine changes in glycogen synthase, hexokinase, phosphofructokinase and lactate dehydrogenase (Fig. 4F).

The major sources of substrate for ATP production during endurance exercise are glucose and free fatty acids, whereas the contribution of amino acids is negligible (Jeukendrup & Wallis, 2005). As a measure for changes

in substrate choice during exercise, we determined subjects' glucose and fat oxidation by indirect calorimetry during steady state cycling both at same absolute intensity at 100 W and at an intensity corresponding to 60% of  $\dot{V}O_{2max}$  before and after the intervention. While HIT showed an increase in the relative contribution of glucose oxidation by 15.9 ( $\pm 7.0$ ) percentage-points and a corresponding decline in fat oxidation with the intervention at both intensities ( $P \leq 0.01$ ), concomitant daily inhalation of  $\beta_2$ -agonist blunted ( $P \leq 0.05$ ) this



**Figure 4. Substrate choice during exercise and muscle metabolic enzymes in HIT and HIT+ $\beta_2$ A**  
 Effect of 4 weeks of high intensity training with (HIT+ $\beta_2$ A,  $n = 12$ ) and without (HIT,  $n = 9$ ) daily inhalation of terbutaline (4 mg day<sup>-1</sup>) on outcomes related to metabolism in trained men. A, heat map of mean Z-score changes of the proteome related to glycogenesis, glycolysis and lipid metabolism with the intervention in HIT and HIT+ $\beta_2$ A, as well as between-group interaction (HIT+ $\beta_2$ A – HIT). B and C, mean ( $\pm 95\%$  CI) maximal activity for hexokinase (HK), phosphofructokinase (PFK) and lactate dehydrogenase (LDH). D, mean ( $\pm 95\%$  CI) relative change in abundance of GLUT4, glycogen synthase (GS) and PFK determined by Western blotting. E, representative blots of GLUT4, GS and PFK. F, principal component analysis of intensity ( $\log_2$ -expression) of GS, HK, LDHA, LDHB and PFK determined by proteomics (open circles), GS and PFK expression determined by Western blotting (yellow circles), and HK, LDH and PFK activity determined fluorometrically (purple circles). G, mean ( $\pm 95\%$  CI) relative contribution of carbohydrate (COX; red bars) and fat oxidation (FOX; black bars) during cycling at 60% of maximal oxygen consumption ( $\dot{V}O_{2max}$ ; left side) and at 100 W (right side). #Between-group interaction ( $P \leq 0.05$ ). ##Between-group interaction ( $P \leq 0.01$ ). \*Within-group difference from pre ( $P \leq 0.05$ ). \*\*Within-group difference from pre ( $P \leq 0.01$ ).

training-induced change in substrate choice in HIT+ $\beta_2$ A (Fig. 4G).

## Discussion

Herein we have described adaptations in skeletal muscle proteome signature and phenotype induced by high intensity training with and without concomitant daily inhalation of  $\beta_2$ -agonist, as well as associated physiological adaptations in exercise performance,  $\dot{V}_{O_{2max}}$ , muscle contractile properties and substrate utilization during exercise. The most important findings were that daily  $\beta_2$ -agonist treatment altered training-induced changes in muscle proteome signature and phenotype and attenuated enhancements in  $\dot{V}_{O_{2max}}$  and exercise performance.

While several studies have investigated the effect of high intensity interval training in human skeletal muscle (Laursen & Jenkins, 2002; Padrão *et al.* 2016; Hostrup & Bangsbo, 2017) and the chronic effect of  $\beta_2$ -agonist treatment in rodent muscle (Pearen *et al.* 2009; Koopman *et al.* 2010), the present study is the first to describe changes in muscle proteome signature and phenotype with related functional measures after an intervention with high intensity interval training and  $\beta_2$ -agonist treatment in humans. Consistent with previous studies (Laursen & Jenkins, 2002; Milanovic *et al.* 2015), we observed that high intensity interval training effectively enhanced  $\dot{V}_{O_{2max}}$  and exercise performance in trained young men. More surprisingly, however, we observed that daily inhalation of  $\beta_2$ -agonist, terbutaline, completely blunted the training-induced increase in  $\dot{V}_{O_{2max}}$  and reduced enhancement in exercise performance. Although a few studies in rodents have shown that systemic chronic treatment with  $\beta_2$ -agonist clenbuterol blunts the effect of endurance training on exercise capacity (Ingalls *et al.* 1996; Duncan *et al.* 2000), the present study is the first to show such effect of  $\beta_2$ -agonists in humans. Furthermore, the present study is the first to show that daily  $\beta_2$ -agonist treatment attenuates the enhancing effect of endurance training on  $\dot{V}_{O_{2max}}$ . It is noteworthy that inhalation of  $\beta_2$ -agonist, at close to therapeutic doses, is capable of blunting the enhancing effects of high intensity interval training on  $\dot{V}_{O_{2max}}$  and exercise performance. This is possibly related to the relatively high systemic bioavailability of inhaled  $\beta_2$ -agonists (Dyrborg *et al.* 2016), partitioning into muscle (Jacobson *et al.* 2014) and inducing a continuous adrenergic fight-or-flight response (Emrick *et al.* 2010; Andersson *et al.* 2012; Hostrup *et al.* 2014b). Accordingly, high inhalation of terbutaline has been shown to induce significant  $\beta_2$ -adrenergic stimulation of skeletal muscle in humans (Hostrup *et al.* 2014b; 2016; Kalsen *et al.* 2016a,b). The dosage of inhaled terbutaline (4 mg) administered in the present study has also been shown to result in systemic concentrations that

exceed those observed after oral administration of 10 mg terbutaline (Elers *et al.* 2012; Dyrborg *et al.* 2016).

Our observations indicate that the attenuating effect of  $\beta_2$ -agonist on training-induced enhancement in exercise performance and  $\dot{V}_{O_{2max}}$ , at least in part, may be related to remodelling of muscle proteome signature and phenotype. Indeed, enhancements in exercise performance and  $\dot{V}_{O_{2max}}$  observed in HIT were complemented by pronounced upregulation of mitochondrial proteins and metabolic pathways in skeletal muscle, in particular related to oxidative phosphorylation and glycogen metabolism, being consistent with reports in humans and rodents (Holloway *et al.* 2009; Egan *et al.* 2011; Sollanek *et al.* 2017). In contrast, however, we observed that daily inhalation of  $\beta_2$ -agonist attenuated training-induced upregulation of NADH dehydrogenase subcomplexes of the oxidative phosphorylation and even caused a repression of carbohydrate metabolic pathways, including downregulation of glycogen debranching enzyme, glycogen phosphorylase and glycogenin 1. The repressing effect of  $\beta_2$ -agonist treatment on oxidative capacity and carbohydrate metabolism is in agreement with observations in rodents (Torgan *et al.* 1995), in which chronic  $\beta_2$ -agonist treatment has been shown to compromise mitochondrial function and pyruvate oxidation capacity (Hoshino *et al.* 2012). Given that a high capacity for oxidative metabolism and glycogenolysis is essential for energy and ion homeostasis of skeletal muscle and thus performance during intense exercise (Allen *et al.* 2008; Ørtenblad *et al.* 2013; Hostrup & Bangsbo, 2017), the attenuating effect of  $\beta_2$ -agonist on training-induced upregulation of oxidative and glycogenolytic proteins may explain why training-induced enhancement in exercise performance was reduced in HIT+ $\beta_2$ A. Furthermore, it may be speculated that  $\beta_2$ -agonist treatment compromised glycogen storage in HIT+ $\beta_2$ A compared to HIT in the present study, since glycogenin 1 was repressed by  $\beta_2$ -agonist treatment and that training-induced upregulation of glycogen synthase was blunted in HIT+ $\beta_2$ A. Accordingly, expression and activity of glycogenin are proportional to glycogen content in human skeletal muscle (Shearer *et al.* 2000, 2005) and glycogen synthase is a key enzyme in muscle glycogenesis (Bouskila *et al.* 2010). Thus, future studies should elucidate whether  $\beta_2$ -agonist-induced proteome changes in glycogen metabolism are associated with alterations in resting glycogen content and rate of glycogenolysis during exercise.

Aside from the effect of  $\beta_2$ -agonist treatment on training-induced adaptations in oxidative and metabolic pathways, we observed that terbutaline also modulated adaptations in muscle contractile phenotype and function. While the high intensity interval training regime undertaken induced a shift in muscle contractile phenotype from type IIx towards type IIa, concomitant daily inhalation of  $\beta_2$ -agonist induced a muscle phenotype



transition from slow- towards a fast-twitch phenotype. Although slow-to-fast-twitch muscle phenotype transition is a common phenomenon after chronic  $\beta_2$ -agonist treatment in rodents (Dodd *et al.* 1996; Zhang *et al.* 1996; Jones *et al.* 2004; Sirvent *et al.* 2014), this has not been described previously in humans. The contractile slow-to-fast twitch phenotype changes induced by chronic  $\beta_2$ -agonist treatment have been shown to be associated with enhancements in maximal force and acceleration of relaxation time of skeletal muscle in both rodents and humans (Martineau *et al.* 1992; Dodd *et al.* 1996; Zhang *et al.* 1996; Hostrup *et al.* 2015, 2016). In contrast to these findings, however,  $\beta_2$ -agonist treatment did not increase muscle force and peak twitch force or accelerate relaxation time of the quadriceps in HIT+ $\beta_2$ A. This discrepancy is possibly related to the fact that  $\beta_2$ -agonist treatment did not induce muscle hypertrophy or changes in expression of sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) isoforms in HIT+ $\beta_2$ A compared to HIT in the present study. As such, muscle hypertrophy has been shown to be the main mechanism by which chronic  $\beta_2$ -agonist treatment enhances peak twitch and tetanic muscle force (Dodd *et al.* 1996; Hostrup *et al.* 2015). Furthermore, studies suggest that the accelerating effect of chronic  $\beta_2$ -agonist treatment on muscle relaxation time is attributed to upregulation of SERCAI (Zhang *et al.* 1996; Hostrup *et al.* 2015) and concurrent downregulation of SERCAII and SERCA-regulatory subunit phospholamban (Zhang *et al.* 1996). In HIT, on the other hand, peak twitch force declined and relaxation time was prolonged with the intervention, thus indicating that changes in contractile phenotype were associated with some of the adaptations in quadriceps contractile properties. Given that muscle contractile properties are highly dependent on muscle fibre contractile phenotype (Harridge *et al.* 1996), the phenotype changes in HIT and HIT+ $\beta_2$ A may explain why HIT had a slower muscle relaxation time and a decline in peak twitch torque after the intervention.

The observation that  $\beta_2$ -agonist treatment alters training-induced remodelling of proteome signature and phenotype in relation to oxidative capacity, as well as metabolic and contractile properties of skeletal muscle, coincides with the acute and temporal changes in gene transcription profile observed in rodent skeletal muscle upon  $\beta_2$ -adrenergic stimulation (Spurlock *et al.* 2006; Pearen *et al.* 2009). Acute  $\beta_2$ -adrenoceptor activation has profound effects on global gene expression in rodent skeletal muscle, where  $\beta_2$ -agonists formoterol and clenbuterol have been shown to enrich a variety of pathways, including those related to metabolism, cell-to-cell communication and transcriptional regulation (Spurlock *et al.* 2006; Pearen *et al.* 2009). Apart from the proteome adaptations in metabolism, we also observed that  $\beta_2$ -agonist treatment induced an upregulation of proteome pathways related to trans-

criptional regulation compared to training alone, including histone trimethylation and the nucleosome. Because  $\beta_2$ -adrenergic stimulation induces an adrenergic fight-or-flight response in skeletal muscle (Emrick *et al.* 2010; Andersson *et al.* 2012), nucleosome adaptations to chronic  $\beta_2$ -agonist treatment seem to be a logical consequence of the continuous adrenergic myocellular stress and signalling (Pearen *et al.* 2009). Indeed, non-selective  $\beta$ -agonist isoproterenol has been shown to induce expression of histones in cells (Lim & Juhn, 2016), and Spurlock *et al.* (2006) observed that chronic  $\beta_2$ -agonist treatment with clenbuterol upregulated transcriptional and translational initiator genes in rodent muscle. In addition, we observed that  $\beta_2$ -agonist treatment upregulated proteome pathways related to adherens junction, cell-to-cell communication and cytoskeleton, inducing expression of actin and  $\alpha$ -actinin 1 compared to training alone. It could be speculated that such upregulations may have impacted between-fibre force transfer positively in HIT+ $\beta_2$ A (Hughes *et al.* 2016; Lambert *et al.* 2016; Nelson *et al.* 2016). Thus, a potential modulation of sarcomere architecture and cell-to-cell adhesion induced by  $\beta_2$ -agonist treatment may explain why HIT+ $\beta_2$ A had no reduction in peak twitch torque and mass-specific peak torque compared to HIT.

While the acute and chronic response to  $\beta_2$ -adrenoceptor activation may have some similarities with exercise training (Spurlock *et al.* 2006; Pearen *et al.* 2009; Hostrup *et al.* 2015), the present study, as well as studies in rodents (Ingalls *et al.* 1996; Lynch *et al.* 1996; Duncan *et al.* 2000; Mounier *et al.* 2007), indicate that chronic  $\beta_2$ -agonist treatment confounds the effect of exercise training. For instance, although a common feature of chronic  $\beta_2$ -agonist treatment is muscle hypertrophy in rodents and humans (Lynch & Ryall, 2008; Joassard *et al.* 2013; Hostrup *et al.* 2015), we observed no apparent induction of hypertrophy by  $\beta_2$ -agonist treatment compared to training alone. This observation is consistent with observations in rodents, in which exercise training blunts or reduces the hypertrophic effect of chronic clenbuterol treatment (Duncan *et al.* 2000; Mounier *et al.* 2007). The mechanisms underlying the attenuating effect of endurance training on the hypertrophic response to  $\beta_2$ -agonist treatment remain to be elucidated, but may be related to the fact that endurance training interferes with the growth-promoting signalling (Coffey & Hawley, 2017) induced upon  $\beta_2$ -adrenergic stimulation (Koopman *et al.* 2010).

In summary, the present study showed that daily inhalation of  $\beta_2$ -agonist alters adaptations to 4 weeks of high intensity endurance training in recreationally active young men, both in terms of proteome signature remodelling and phenotype changes of skeletal muscle, but also in relation to functional adaptations in exercise performance,  $\dot{V}_{\text{O}_{2\text{max}}}$ , muscle contractile properties and

substrate utilization during exercise. The most notable observation was that  $\beta_2$ -agonist treatment attenuated training-induced enhancements in exercise performance and  $\dot{V}_{O_{2\max}}$ , as well as blunted adaptations in oxidative phosphorylation and glycogen metabolism of skeletal muscle. Furthermore, that  $\beta_2$ -agonist treatment induced a slow-to-fast twitch muscle fibre type transition. Future studies should elucidate the molecular mechanisms underlying the confounding effect of  $\beta_2$ -agonist treatment on adaptations to endurance training.

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## Additional information

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005480.

## Competing interests

The authors have no competing interests.

## Author contributions

M.H. designed the study, participated in data collection, analysis and interpretation, and drafted the manuscript. J.O. conducted the human experiments and participated in data analysis and interpretation, as well as in drafting of the manuscript. G.A.J. and R.W. conducted the muscle proteomic analysis and assisted in the bioinformatics analysis and interpretation, as well as in revising the manuscript. J.B. participated in designing the study and in the analysis and interpretation data, as well as in drafting of the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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### Translational perspective

This study tested the hypothesis that daily inhalation of  $\beta_2$ -agonist alters adaptations in muscle proteome signature incurred from high intensity training. We observed that the  $\beta_2$ -agonist terbutaline blunted training-induced upregulation of proteome pathways related to oxidative phosphorylation and glycogen metabolism, while concurrently inducing a slow-to-fast twitch muscle phenotype transition. Notably, terbutaline also attenuated training-induced enhancements in  $\dot{V}_{O_{2max}}$  and exercise performance. These findings are of interest to the large proportion of persons using inhaled  $\beta_2$ -agonists on a daily basis, including athletes, and indicate that athletes who have a high use of  $\beta_2$ -agonist may benefit from lessening their reliance on  $\beta_2$ -agonist inhalers. This is also consistent with clinical guidelines, where regular reliance on  $\beta_2$ -agonists is indicative of poor asthma control. Furthermore, the present study provides a fingerprint of some of the muscle proteome and phenotype changes induced by high intensity training with and without  $\beta_2$ -agonist treatment, which may be used for future therapeutic applications. Indeed, it has long been proposed that  $\beta_2$ -agonists have potential therapeutic use in the treatment of muscle atrophic conditions (Lynch & Ryall, 2008; Joassard *et al.* 2013), despite their potential cardiomyotoxic effects when administered in high doses. Accordingly, chronic  $\beta_2$ -agonist treatment may repress oxidative capacity (Léger *et al.* 2011) and induce collagen infiltration and necrosis in rodent cardiac muscle (Duncan *et al.* 2000; Burniston *et al.* 2002, 2005, 2006; Gregorevic *et al.* 2005). Thus, the attenuating effect of daily  $\beta_2$ -agonist treatment on training-induced enhancements in exercise performance and  $\dot{V}_{O_{2max}}$  may involve modulating effects in both skeletal and cardiac muscle.