Modulating energy metabolism: pathophysiological aspects and novel interventions

Straat, M.E.

CHAPTER 9

Stimulation of the beta-2-adrenergic receptor with salbutamol activates human brown adipose tissue

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Cell Rep Med, accepted.

SUMMARY

Brown adipose tissue (BAT) is activated by the beta-3-adrenergic receptor (ADRB3) in rodents, while the ADRB2 is dominantly present in human BAT biopsies and responsible for noradrenergic activation of human brown adipocytes. Here, we assessed whether ADRB2 agonism activates human BAT by performing a randomized double-blinded crossover trial in young and lean males to compare a single intravenous bolus of salbutamol without and with the ADRB1/2 antagonist propranolol. Salbutamol, compared to salbutamol with propranolol, increased glucose uptake by BAT as assessed by a dynamic 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) PET-CT scan, heart rate and whole-body energy expenditure, with the salbutamol-induced glucose uptake by BAT positively associated with the increase in energy expenditure. Notably, participants with high salbutamol-induced glucose uptake by BAT had a lower body fat mass, waist-hip ratio and serum LDL-cholesterol concentration. Our data show that the ADRB2 activates human BAT and warrant investigation of ADRB2 activation in long-term studies.

INTRODUCTION

Over the last decades, brown adipose tissue (BAT) has become an attractive target to stimulate energy dissipation to improve cardiometabolic health (1). BAT is a highly vascularized thermogenic organ mainly located in the deep neck region, along large blood vessels and in the supraclavicular area, and combusts triglyceride-derived fatty acids and glucose into heat (2-4). Naturally, the most potent activator of BAT is cold exposure, which increases sympathetic outflow towards beta-adrenergic receptors on BAT (5, 6).

In rodents, the beta-3-adrenergic receptor (ADRB3) is the main adrenergic receptor found on brown adipocytes and activation of the ADRB3 has been shown to effectively activate BAT and improve cardiometabolic outcomes in mice (7-9). In humans, however, the involvement of ADRB3 in BAT activation is less clear (10-17). The ADRB3 agonist mirabegron increases the uptake of the glucose analogue 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) by BAT, increases whole body lipolysis and increases resting energy expenditure. Nevertheless, this only occurs after administration of a supratherapeutic dose of 200 mg (10, 12, 13), which highly exceeds the therapeutic dose of 50 mg to treat hyperactive bladder in the clinic. In addition, at 200 mg cardiovascular side effects occur, such as an increase in heart rate and systolic blood pressure, raising the possibility that mirabegron cross-reacts with other beta-adrenergic receptors such as the ADRB1 and ADRB2 that are also present on the cardiovascular system and as such contribute to the increase in energy expenditure (18).

Indeed, we recently showed that the therapeutic dose of 50 mg mirabegron is ineffective to increase oxidative metabolism in BAT, and that the ADRB2 is in fact the dominant adrenergic receptor expressed in human BAT biopsies and brown adipocytes, while the expression of ADRB3 is negligible (12). Accordingly, evidence supporting the hypothesis that ADRB2 is responsible for stimulating thermogenesis in human BAT stems from our *in vitro* experiments in human brown adipocytes, where we demonstrated that: 1) stimulation with mirabegron did not increase oxygen consumption; 2) stimulation with the ADRB2 agonist formoterol increased oxygen consumption, which was inhibited when pre-exposed with a selective ADRB2 antagonist; and 3) knock-down of ADRB2, but not ADRB1 or ADRB3, reduced norepinephrine-stimulated oxygen consumption (12). Therefore, the aim of this study was to investigate the role of ADRB2 in activation of human BAT *in vivo*. To this end, as a proof of concept, we evaluated the acute effect of the specific ADRB2 agonist salbutamol on glucose uptake by BAT without and with the ADRB1/2 antagonist propranolol in healthy lean men.

METHODS

Study design

This study was a randomized double-blinded crossover trial, carried out at the Leiden University Medical Center (LUMC). The intervention consisted of a single intravenous bolus of salbutamol (250 µg) in combination with orally administered propranolol (80 mg) or placebo, in random order (**Figure 1A**). On both study days all participants underwent a dynamic [^{18}F]FDG Positron Emission Tomography and (low dose) Computed Tomography (PET-CT) scan. The study was approved by the Medical Ethical Committee of the LUMC and undertaken in accordance with the principles of the revised Declaration of Helsinki. Written informed consent was obtained from all participants prior to participation. The clinical trial is registered at the Netherlands Trial Register (NTR; NL9345), and at the European Union Drug Regulating Authorities Clinical Trials (EudraCT; 2020-004059-34).

Participants

In total, 10 healthy white Caucasian men were enrolled in this study, aged 19 to 35 years and body mass index between 19.2 and 26.5 kg/m^2. Eligibility for inclusion was assessed during a screening that consisted of anthropometry, electrocardiography, a questionnaire on medical history, and an overnight 10 h fasted blood sample. Exclusion criteria were the presence of any endocrine, cardiac, renal, of hepatic disease, a first-degree family member with sudden cardiac death, the use of medication known to influence glucose and/or lipid metabolism, the use of beta-adrenergic receptor agonists (*e.g.*, for asthma), any contra-indications for the use of salbutamol or propranolol, abuse of alcohol or other substances, smoking, participation in an intensive weight-loss program or vigorous exercise program during the last year before the start of the study, and/or clinically relevant abnormalities in clinical chemistry or electrocardiogram. After inclusion, participants were asked to adhere to several lifestyle rules prior to the study visits: no vigorous exercise 48 hours preceding the study days and no alcohol or drinks with caffeine 24 hours preceding the study visits. In addition, they were instructed to eat a standardized meal (prepared supermarket meal including pasta or noodles, ranging from 450-600 kcal) in the evening prior to the study visits, and not to eat or drink anything (with an exception for water) afterwards until completion of the study visits.

Randomization

After inclusion, participants (n=10) were randomized to determine whether they would receive salbutamol in combination with placebo on the first study day (n=5), or salbutamol in combination with propranolol on the first study day (n=5; **Figure 1A**). All measurements during the two study visits were identical (**Figure 1B**). Randomization was executed by the LUMC department of Clinical Pharmacology and Toxicology.

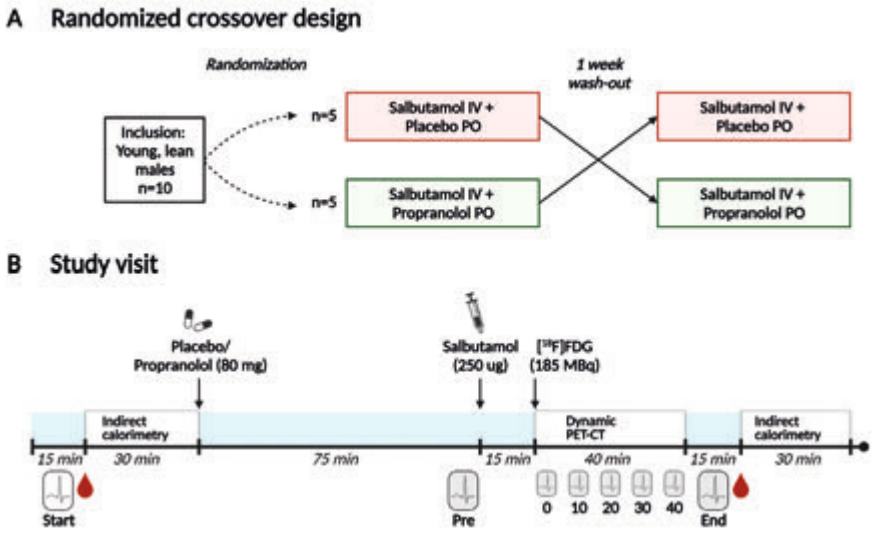

Figure 1. Study design and time line of study procedures.
A) This study had a randomized, double blinded, crossover design.
B) Both study visits started with the measurement of blood pressure and heart rate (indicated by the ECG icon). Thereafter, the first blood sample (indicated by blood drop icon) was drawn, followed by an indirect calorimetry measurement for 30 min. Then, participants received either placebo or propranolol (80 mg, in two capsules; per oral; PO), depending on the study visit. After 75 min, blood pressure and heart rate were measured again and a single bolus of salbutamol (250 µg, intravenous; IV) was injected over a continuous time course of 5 min. 15 min after initiation of the injection, a low dose computed tomography (CT) scan was performed, directly followed by injection of 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG; 185 MBq) and a dynamic positron emission tomography (PET) acquisition, during which heart rate was monitored. After termination of the scan, blood pressure and heart rate were measured, the final blood sample was drawn, and indirect calorimetry was performed for 30 min.

Anthropometric measurements

After arrival (9:00 AM), body weight (digital balance; E1200, August Sauter GmBH, Albstadt, Germany), height, and waist and hip circumference were obtained. Waist-hip-ratio (WHR) was calculated as: 'waist circumference'/'hip circumference'. Body composition (fat mass and fat percentage) was estimated using bioelectrical impedance analysis (InBody720, InBody CO., Ltd., CA, USA). In addition, heart rate and blood pressure were measured using a cuff connected to a digital blood pressure device (Model). In total, heart rate and blood pressure were measured at three time points (at the start of the study day, before administration of salbutamol and at the end of the study day). In addition, heart rate was measured at 5 time points (t=0, 10, 20, 30, 40 min) during the PET scan using a 3-lead ECG connected to a bedside patient monitor (Intellivue MP5, Philips Healthcare, Best, the Netherlands).

Indirect calorimetry

At the start and at the end of the study day, resting energy expenditure and substrate utilization were measured for 30 min with a metabolic cart (Vyntus™ CPX, Carefusion, Hochberg, Germany) equipped with a ventilated hood system that measures total carbon dioxide production (VCO_2) and oxygen consumption (VO_2) every 10 sec. Before each measurement, volume and gas calibrations were performed. The Weir formula was used to estimate energy expenditure (ignoring urinary nitrogen excretion): energy expenditure (kcal/day) = (3.941*VO_2 (L/min)) + (1.106*VCO_2 (L/min))*1440. The first 5 min of gas exchange data of every new recording was discarded, whereafter the most stable 5 min were selected for further analyses, as previously described (19).

Administration of medication

Directly after the first indirect calorimetry measurement, subjects received either placebo or 80 mg propranolol *per os*, divided over two capsules each, followed by 75 min of rest to reach peak plasma concentrations of propranolol. Afterwards, subjects were placed in supine position within the PET-CT scanner where salbutamol (250 µg) was administered via a single intravenous bolus (10 mL) into the antecubital vein over a time course of 5 min.

[18F]FDG PET-CT scan

Fifteen minutes after initiation of salbutamol administration, a low dose (30 mA, effective dose 0.7 mSv) CT scan of the cervicothoracic area centered on the supraclavicular region was performed. This was directly followed by the administration of a single bolus of [^{18}F]FDG tracer in a dosage of 185 MBq using an injection pump, after which the line was flushed with saline and the dynamic list-mode PET acquisition was started. The list-mode data were reconstructed into 32 time frames (1 x 30 sec, 12 x 10 sec, 8 x 30 sec, 6 x 90 sec and 5 x 300 sec). The time radioactivity curves for [^{18}F]FDG of metabolic tissues were analyzed using the Patlak linearization method (20), with the plasma input function taken from the aortic arch (21). The slope of the linear phase of the Patlak plot denotes the net influx rate (influx constant, Ki, in min^{-1}), which is the accumulated [^{18}F]FDG relative to the amount of [^{18}F]FDG that has been available in plasma. Ki was then multiplied by circulating glucose levels at the time of the PET image acquisition, and divided by the lumped constant, to calculate the net glucose uptake. Finally, this value was divided by tissue density and multiplied with 1,000 to obtain the net glucose uptake in the preferred unit: $nmol \cdot g^{-1} \cdot min^{-1}$. For adipose tissue, the lumped constant of 1.14 and tissue density 0.925 g/mL was used, for skeletal muscle, the lumped constant of 1.16 and tissue density 1.06 g/mL was used (22, 23). In summary, the following formula was used to estimate the net glucose uptake by metabolic tissues after intervention:

*Net glucose uptake (nmol/g/min) = ((Ki * circulating glucose)/ (Lumped constant))/ (Tissue density * 1000)*

PET-CT image data were analyzed using PMOD software (PMOD technologies LLC, Zürich, Switzerland). Regions of interest (ROIs) were drawn independently by two researchers (M.E.S, C.A.H) on the aortic arch for the plasma input function, four skeletal muscles (*e.g.*, m. sternocleidomastoid, m. trapezius, m. pectoralis major, m. deltoideus; all left and right), posterior cervical subcutaneous white adipose tissue (scWAT) and supraclavicular BAT (left and right). For skeletal muscles and supraclavicular BAT, values from left and right side were averaged.

Blood samples

At the start of the study visit, a catheter was inserted in the antecubital vein, for venous blood sampling and for administration of salbutamol and [^{18}F]FDG tracer. At two time points 10 h fasted blood samples were collected: at the start and at the end of the study visit. Blood was collected in Vacutainer® SST™ II Advance tubes. After a clotting time of at least 30 min, samples were centrifuged to obtain serum, which was aliquoted and stored at -80°C until batch-wise analyses. Commercially available enzymatic kits were used to measure serum concentrations of free fatty acids (FFA; Wako chemicals, Nuess, Germany), triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C; all Roche Diagnostics, Woerden, the Netherlands), glucose (Instruchemie, Delfzijl, the Netherlands), insulin and C-peptide (both Meso Scale Diagnostics, Rockville, Maryland, USA). Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald equation (24).

Statistical analyses

Statistical analyses were performed with SPSS® Statistics (version 25, IBM® Corporation, Armonk, NY, USA). Normal distribution of the data was tested using the Shapiro-Wilk test, visual histograms, and Q-Q plots. To assess the effect of treatment, and to compare the changes after treatment between the treatment regiments, general linear models with repeated measures and pairwise comparisons were used with two within-subject factors: treatment (salbutamol *vs.* salbutamol with propranolol) and timepoint (*e.g.*, before and after treatment). Not normally distributed data were log10 transformed (*e.g.*, energy expenditure, serum FFA, and serum insulin levels). To compare the glucose uptake by BAT and skeletal muscles between treatments, non-parametric Wilcoxon Signed Rank tests were used. To compare the glucose uptake by scWAT between treatments two-tailed paired Student's t-tests was used. Associations between parameters were tested using Pearson correlations (r) or nonparametric Spearman-rank correlations (rho). Baseline characteristics were compared between

non-responders and responders using Mann-Whitney U tests. Absolute changes in heart rate, blood pressure, expenditure, fat oxidation, carbohydrate oxidation, and serum markers were calculated as: 'end study visit'-'start study visit'. Changes in energy expenditure were calculated using the following formula: ('end study visit'-'start study visit')/ ('start study visit')*100%. The homeostasis model assessment-estimated insulin resistance (HOMA-IR) levels were calculated as: 'fasting glucose in mmol/L'*'fasting insulin in μU/mL'/22.5. A P-value of $P \leq 0.05$ was considered statistically significant. All data are presented as mean ± standard deviation. Figure 1 was created with BioRender.com. All other figures were prepared with Prism 9 for Windows (version 9.0.1, 2021, GraphPad Software, LLC, San Diego, California, USA).

RESULTS

Salbutamol increases heart rate and tends to increase energy expenditure

In total, 10 young (age: 24.4 ± 4.3 years) and lean (body mass index: 23.1 ± 2.3 kg/m^2) males were included in this study and participated in two experimental study visits as described above (see **Figure 1B**). A single intravenous bolus of salbutamol (250 μg) acutely increased heart rate (+16.9 ± 10.5 bpm, P=0.001), but not when combined with propranolol (-2.8 ± 8.9 bpm, P=0.35; interaction between treatments P<0.001; **Figure 2A**). Moreover, after salbutamol injection heart rate remained higher than after administration of salbutamol with propranolol until the end of the study visit ($P^{time*treatment}$<0.001, **Figure 2B**). Salbutamol did not affect systolic blood pressure (+2.4 ± 8.0 mmHg; P=0.38) or diastolic blood pressure (+3.2 ± 9.7 mmHg, P=0.35), whereas salbutamol combined with propranolol tended to decrease systolic blood pressure (-10.7 ± 16.5 mmHg, P=0.09) and decreased diastolic blood pressure (-7.0 ± 8.0 mmHg, P=0.03) (**Figure 2C**).

Next, we assessed the effect of salbutamol on energy expenditure and substrate utilization. For one male, the gas exchange measurement failed due to technical issues, leaving a total of 9 males for these analyses. Salbutamol tended to increase energy expenditure (+7.2%, +122 ± 168 kcal/day, P=0.06), whereas salbutamol with propranolol decreased energy expenditure (-9.4%, -192 ± 91 kcal/day, P<0.001), leading to a significantly different change in energy expenditure between the two treatment regimens (P=0.005; **Figure 2D**). Of note, the salbutamol-induced change in energy expenditure did not correlate with the increase in heart rate (rho: -0.59, P=0.10; not shown). Fat oxidation did not change after salbutamol (-0.004 ± 0.04 g/min, P=0.77), but decreased after salbutamol with propranolol (-0.06 ± 0.04 g/min, P=0.003; change between treatments P=0.03; **Figure 2E**). Carbohydrate oxidation did not change after

salbutamol (+0.03 ± 0.10 g/min, P=0.34), but increased after salbutamol with propranolol (+0.12 ± 0.09 g/min, P=0.009; change between treatments: P=0.19; **Figures 2F**).

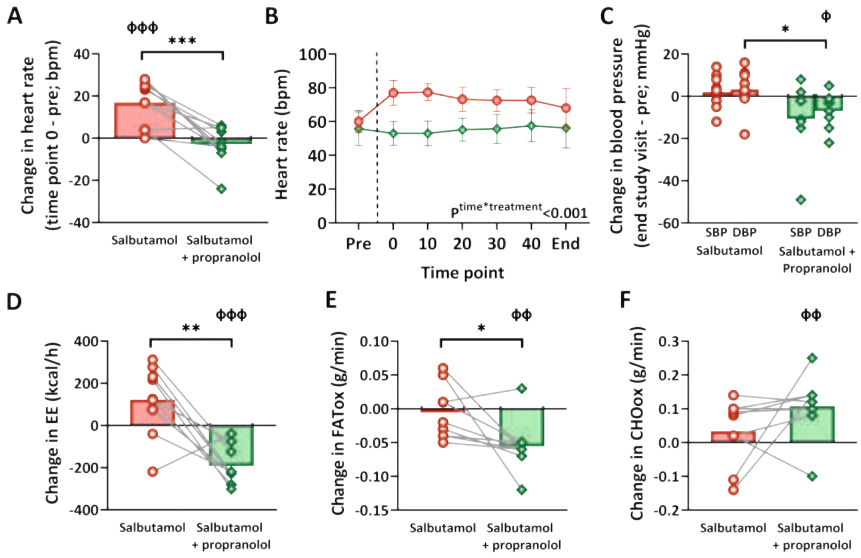

Figure 2. The effect of salbutamol vs. salbutamol with propranolol on heart rate, blood pressure, energy expenditure and nutrient oxidation rates.
The change in heart rate (A), systolic blood pressure (SBP) and diastolic blood pressure (DBP) (C), energy expenditure (EE) (D), fatty acid oxidation (FATox) (E), and carbohydrate oxidation (CHOox) (F) after salbutamol (n=10; red bars with circles) vs. salbutamol with propranolol (n=10; green bars with diamonds). For one male, EE measurement failed due to technical issues (n=9). General linear models with repeated measures and pairwise comparisons were used to test the effect of treatment and to compare the treatment regimens. Bars represent means, circles/diamonds represent individual values, and grey lines represent paired data. Before vs. after treatment: ΦP≤0.05, ΦΦP≤0.01, ΦΦΦP≤0.001. Salbutamol vs. salbutamol with propranolol: *P≤0.05, **P≤0.01, ***P≤0.001.
B) The effect of salbutamol (red circles) vs. salbutamol with propranolol (green diamonds) on heart rate over time. General linear model with repeated measures was used to test for an interaction between treatment regime and the effect of treatment over time. Bars represent means, error bars represent standard deviation.

Salbutamol increases net glucose uptake by brown adipose tissue, and this positively correlates to the change in energy expenditure

We next assessed the effect of the ADRB2 agonist salbutamol on net glucose uptake by supraclavicular BAT, as calculated from the [^{18}F]FDG influx rate. Salbutamol, compared to salbutamol with propranolol, increased the net glucose uptake by BAT (salbutamol *vs.* salbutamol with propranolol: 67.1 ± 87.0 nmol/g/min *vs.* 16.2 ± 5.2 nmol/g/min, P=0.03; **Figure 3A-C**). In contrast, after salbutamol, glucose uptake by skeletal muscle (9.5 ± 3.0

nmol/g/min *vs.* 12.6 ± 2.4 nmol/g/min, P=0.06) and scWAT (21.4 ± 3.7 nmol/g/min *vs.* 24.5 ± 3.4 nmol/g/min, P=0.06; **Figure 3A**) tended to be lower compared to after salbutamol with propranolol. Interestingly, we observed a physiologically plausible outlier with very high glucose uptake values by BAT (285.1 nmol/g/min, see identification number 10 in **Figure 4A** and **Supplementary figure 1**). After sensitivity analyses excluding the values of this participant, differences in glucose uptake by BAT after salbutamol vs. salbutamol with propranolol remained significant (salbutamol *vs.* salbutamol with propranolol: 36.7 ± 31.2 nmol/g/min *vs.* 14.9 ± 3.2 nmol/g/min, P=0.05; data not shown).

After salbutamol, glucose uptake by BAT was positively associated with the percentage change in energy expenditure (rho=0.73, P=0.03; **Figure 3D**, whereas the glucose uptake by skeletal muscle was negatively associated (rho=-0.83, P=0.008; **Figure 3E**). After salbutamol with propranolol, no significant correlation was found between the glucose uptake by BAT and energy expenditure (rho=-0.58, P=0.11; **Figure 3F**), and a tendency between the change in glucose uptake by skeletal muscle and energy expenditure (rho=-0.65, P=0.07; **Figure 3G**). In addition, during both treatment regimes, no significant correlations were found between the change in glucose uptake by scWAT and energy expenditure (salbutamol: rho=-0.16, P=0.68; salbutamol with propranolol: rho=-0.32, P=0.41; not shown).

High responders to salbutamol-induced glucose uptake by BAT have a more beneficial metabolic phenotype than low responders

A marked variability was observed between participants in the salbutamol-induced glucose uptake by BAT. Specifically, as is evident from **Figure 4A** and **Supplementary figure 1**, five participants showed glucose uptake by BAT above the median (ranging from 29.2 to 285.1 nmol/g/min; 'responders'), whereas the other five participants showed glucose uptake below the median (ranging from 11.1 to 17.6 nmol/g/min; 'non-responders'; P=0.008). Compared to non-responders, responders had a lower body fat mass (11.8 ± 1.3 % *vs.* 16.9 ± 2.3 %, P=0.008; **Figure 4B**), lower WHR (0.8 ± 0.02 *vs.* 0.9 ± 0.1, P=0.03; **Figure 4C**), and lower serum concentrations of total cholesterol (3.0 ± 0.7 mmol/L *vs.* 4.5 ± 0.5 mmol/L, P=0.008; **Figure 4D**) and LDL-cholesterol (1.6 ± 0.4 mmol/L *vs.* 2.7 ± 0.5 mmol/L, P=0.02; **Figure 4D**). No significant differences were observed in glucose, insulin or C-peptide levels between non-responders and responders (all P≥0.1, see supplementary table 1). Moreover, responders tended to have a higher heart rate at the start of the study visit (77.8 ± 10.0 bpm *vs.* 66.5 ± 8.3 bmp, P=0.06), without differences in baseline energy expenditure (1887 ± 384 kcal/day *vs.* 2056 ± 68 kcal/day, P=1.0). There was no significant difference between the groups in salbutamol-induced change in energy expenditure (+10.0 ± 12.1 % *vs.* +3.5 ± 3.8 %, P=0.35) or heart rate (+12.2 ± 13.2 bpm *vs.* +21.6 ± 4.4 bpm, P=0.55). A full overview of baseline characteristics between the two phenotypes can be found in **Supplementary table 1**.

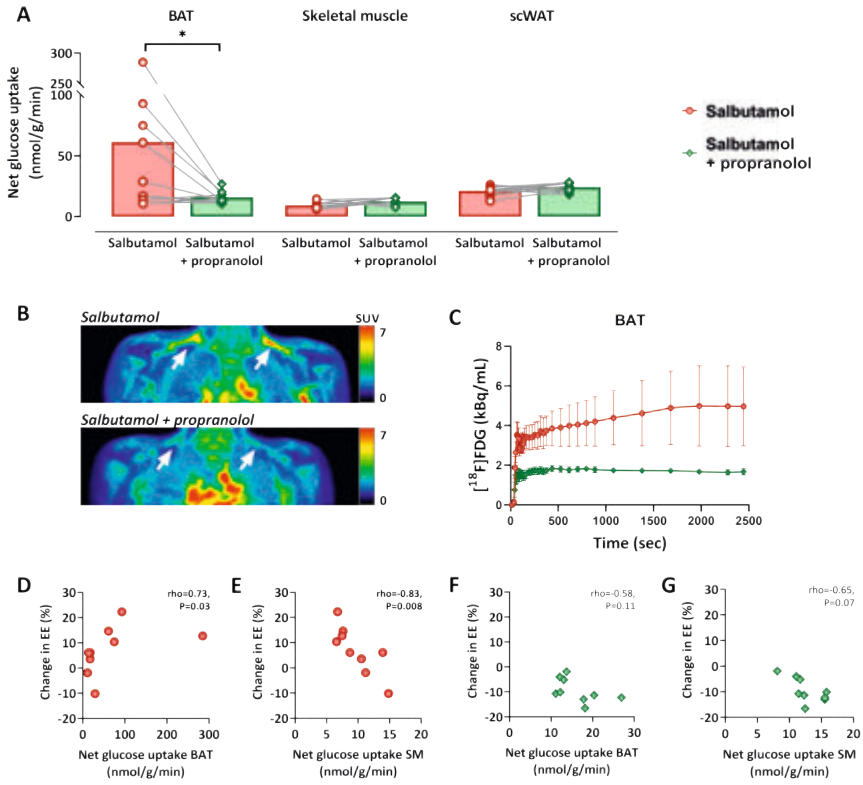

Figure 3. The effect of salbutamol vs. salbutamol with propranolol on glucose uptake by brown adipose tissue, skeletal muscle, and subcutaneous adipose tissue, and the association with the change in energy expenditure.

A) The glucose uptake by human brown adipose tissue (BAT), skeletal muscle (i.e., average of m. pectoralis, m. trapezius, m. deltoideus, and m. sternocleidomastoideus), and subcutaneous white adipose tissue (scWAT) after salbutamol vs. salbutamol with propranolol. A paired Student's t-test, or nonparametric equivalent, was used to compare the two treatment regimes. Bars represent means, dots/diamonds represent individual values, and grey lines represent paired data. *P≤0.05.

B) Positron emission tomography images of the supraclavicular area illustrating the [^{18}F] fluorodeoxyglucose [^{18}F]FDG uptake, expressed by body-weighted standardized uptake values (SUV), in response to salbutamol (top) and salbutamol with propranolol (bottom). The same representative participant is presented for both images. White arrows show supraclavicular BAT depots.

C) Time-activity curve showing the concentration of [^{18}F]FDG in BAT depots. Left and right, and all participants are averaged. Data represent mean with standard error of the mean.

D+E) Correlation plots between the change in energy expenditure (EE) (%) and the glucose uptake by human BAT after salbutamol (D) and skeletal muscle (SM) after salbutamol (E).

F+G) Correlation plots between the change in EE and the glucose uptake by human BAT after salbutamol with propranolol (F) and SM after salbutamol with propranolol (G).

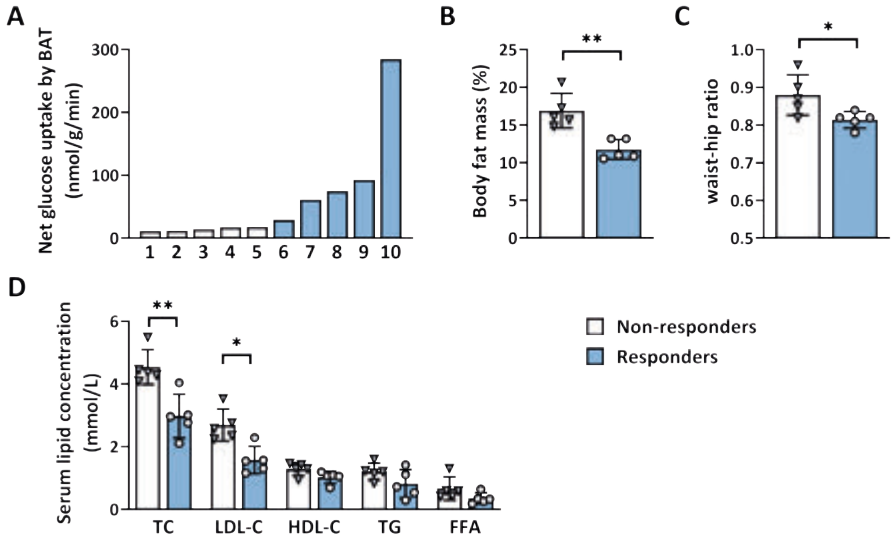

Figure 4. Differences in body composition and baseline serum lipid concentrations between non-responders and responders in terms of salbutamol-induced glucose uptake by brown adipose tissue. A) Waterfall plot showing the distribution in net glucose uptake by supraclavicular brown adipose tissue (BAT; left and right averaged). Each bar represents the individual value of a participant.
B+C+D) Differences in body fat mass percentage (B), waist-hip ratio (C), and baseline serum concentrations of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), and free fatty acids (FFA) (D) between participants who showed a high salbutamol-induced net glucose uptake by brown adipose tissue ('responders', blue bars with circles) vs. participants who showed low salbutamol-induced net glucose uptake by brown adipose tissue ('non-responders', white bars with triangles). Values illustrated in the figures were measured at baseline during the placebo-visit.
A paired Student's t-test, or nonparametric equivalent, was used to compare the two groups. Bars represent means, error bars represent standard deviation. *P≤0.05, **P≤0.01.

Salbutamol does not affect serum lipid or glucose concentrations

Lastly, we aimed to assess the acute effects of salbutamol on measures of lipoprotein and glucose metabolism. Salbutamol did not affect serum concentrations of triglycerides (Start *vs.* end study day: 1.0 ± 0.4 mmol/L *vs.* 1.0 ± 0.4 mmol/L, P=0.78), FFA (0.5 ± 0.3 mmol/L *vs.* 0.4 ± 0.1 mmol/L, P=0.21), total cholesterol (3.8 ± 1.0 mmol/L *vs.* 3.8 ± 0.9 mmol/L, P=0.74), HDL-cholesterol (1.2 ± 0.2 mmol/L *vs.* 1.2 ± 0.2 mmol/L, P=0.70), or LDL-cholesterol (2.1 ± 0.7 mmol/L *vs.* 2.2 ± 0.7 mmol/L, P=0.78; **Figure 5A-E**). Salbutamol with propranolol only decreased serum FFA levels (0.6 ± 0.3 mmol/L *vs.* 0.2 ± 0.1 mmol/L, P<0.001; **Figure 5B**). Moreover, salbutamol did not affect serum concentrations of glucose (5.5 ± 0.2 mmol/L *vs.* 5.6 ± 0.6 mmol/L, P=0.45), insulin (12.7 ± 4.3 μU/mL *vs.* 14.0 ± 7.3 μU/mL, P=0.75), or C-peptide (1.6 ± 0.4 ng/mL *vs.* 1.8 ± 0.6 ng/mL, P=0.08),

whereas salbutamol with propranolol decreased serum concentrations of glucose (5.5 ± 0.4 mmol/L *vs.* 5.2 ± 0.5 mmol/L, P=0.002), insulin (12.6 ± 7.1 µU/mL *vs.* 4.4 ± 2.5 µU/mL, P<0.001), and C-peptide (1.5 ± 0.6 ng/mL *vs.* 1.0 ± 0.4 ng/mL, P=0.001; **Figure 5F-H**). Importantly, the glucose uptake by BAT after salbutamol with propranolol was not associated with the decrease in glucose (rho=0.28, P=0.43) nor insulin (rho=0.45, P=0.19; not shown).

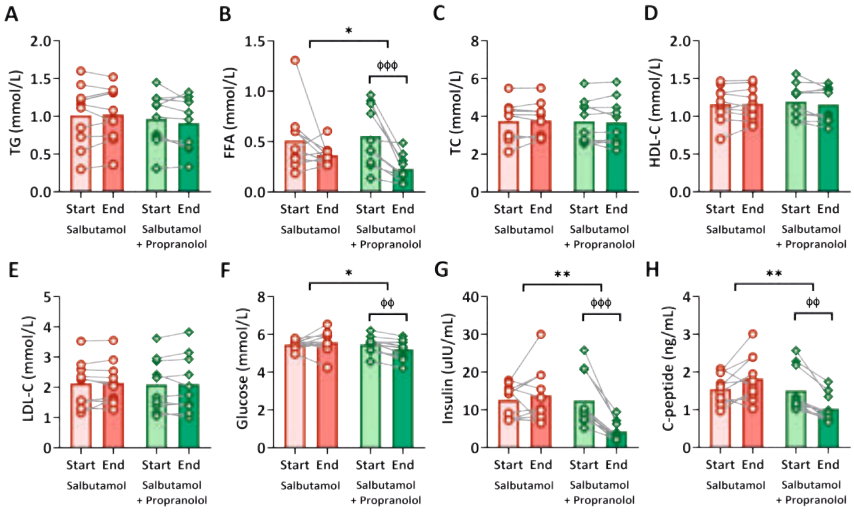

Figure 5. The effect of salbutamol vs. salbutamol with propranolol on serum concentrations of lipid and glucose metabolism.
The effect of salbutamol (red bars with circles) vs. salbutamol with propranolol (green bars with diamonds) on serum concentrations of triglycerides (TG; A), free fatty acids (FFA; B), total cholesterol (TC; C), high-density lipoprotein cholesterol (HDL-C; D), low-density lipoprotein cholesterol (LDL-C, E), glucose (F), insulin (G), and C-peptide (H). General linear models with repeated measures and pairwise comparisons were used to test the effect of treatment and to compare the treatment regimens. Bars represent means, dots/diamonds represent individual values, and grey lines represent the paired nature of the data. Start vs. end: ΦΦP≤0.01, ΦΦΦP≤0.001. Salbutamol vs. salbutamol with propranolol: *P≤0.05, **P≤0.01.

DISCUSSION

In this proof-of-concept study, we show that pharmacological stimulation of the ADRB2 acutely increases glucose uptake by human BAT *in vivo*. Specifically, we demonstrate that a single intravenous administration of the specific ADRB2 agonist salbutamol increased glucose uptake by BAT, which could be largely prevented when blocking the ADRB1/2 with propranolol. The salbutamol-induced uptake of glucose by BAT, but

not skeletal muscle, was positively associated with whole-body energy expenditure. Notably, participants with high salbutamol-induced glucose uptake by BAT had a more favorable metabolic phenotype (among other lower WHR and LDL-cholesterol levels) compared to participants with low glucose uptake by BAT. Together, our data underline the relevance of the ADRB2 for sympathetically induced glucose uptake by human BAT.

By revealing that stimulation of the ADRB2 increases glucose uptake by human BAT, we now provide *in vivo* evidence for our recent findings that the ADRB2 is the adrenergic receptor that activates human brown adipocytes *in vitro* (12). Moreover, since blocking the ADRB1/2 effectively inhibited the salbutamol-induced glucose uptake by BAT in our study, the ADRB3 apparently was not involved. Over the last decades, many research groups have focused on mimicking the sympathetic activation of BAT by targeting the ADRB3 (10, 14, 15, 25). Direct targeting the ADRB3 using CL 316,243, one of the most commonly used ADRB3 agonists, effectively activates BAT and improves cardiometabolic outcomes in mice (7, 9). However, pharmacological targeting of the ADRB3 by the use of mirabegron led to inconsistent results in humans (10, 12-17). In fact, mirabegron only activates human BAT oxidative metabolism at maximal allowable dose (*i.e.*, 200 mg), which also induces cardiovascular side effects (12, 13, 26), suggesting cross-activation with other beta-adrenergic receptors. Indeed, the human heart mainly expresses ADRB1 (80%) and ADRB2 (20%), with negligible expression of ADRB3 (27). In human BAT, in principle all three receptors are present (12, 28-31). In immortalized brown adipocytes originating from just a single donor, stimulation of the ADRB1, but not ADRB2 or ADRB3, increased uncoupling protein 1 (UCP1) expression and lipolysis (31). In contrast, we demonstrated using both immortalized and *in vitro* differentiated primary brown adipocytes from multiple donors from independent labs that ADRB2 agonism increases respiration, and knockdown of the ADRB2, but not ADRB1 and ADRB3, hampers norepinephrine-induced respiration (12). Taken together, although the involvement of the ADRB1 cannot be excluded, the ADRB2 seems to play the most important role in sympathetic activation of human BAT *in vivo*.

We show that ADRB2 stimulation with salbutamol tended to increase whole-body energy expenditure, and the change in energy expenditure correlated positively with the net glucose uptake by BAT. This is in line with previous studies that also demonstrated that intravenous administration of salbutamol with and without atenolol (*i.e.*, ADRB1 blocker) increased energy expenditure, although in those studies the tissues involved had not been explored (32-34). Besides BAT, skeletal muscles are also considered responsible for the increase in thermogenesis during cold exposure (35), and deeper and more centrally located skeletal muscles have been shown to contribute to the cold-induced glucose turnover (36). However, we here show that the change in energy expenditure

after salbutamol in fact negatively correlates with the net glucose uptake by skeletal muscle. Moreover, comparable to previous findings that acute ADRB2 stimulation does not affect insulin-stimulated glucose uptake by skeletal muscle (37), we did not find an increase in glucose uptake by skeletal muscle after acute salbutamol administration. Interestingly, long term (four weeks) daily inhalation of terbutaline (*i.e.*, an ADRB2 agonist) increases insulin-stimulated whole-body glucose disposal, without changes in GLUT4 in skeletal muscle or abdominal WAT (38). Although it has been shown that this may in part be due to muscle hypertrophy (38), we propose a potential role for BAT through increased glucose uptake coinciding with increased energy dissipation.

Previous studies demonstrated that continuous or repetitive intravenous administration of salbutamol with and without atenolol increases lipolysis and fat oxidation together with an increase in energy expenditure (32-34). In the present study, we did not observe a change in circulating lipids or in fat oxidation after a single bolus of salbutamol. Importantly, we performed indirect calorimetry and blood sampling approx. 70 min after salbutamol administration. Hence, we may have missed an acute effect of salbutamol on lipolysis and fat oxidation. Indeed, salbutamol with atenolol (*i.e.*, resulting in specific ADRB2 stimulation), increases fat oxidation only after 45 min of continuous intravenous administration (32). Furthermore, we used a single intravenous bolus of salbutamol, which may have not been sufficiently potent to induce a lipolytic effect in white adipose tissue. Interestingly, it has been reported previously that the salbutamol-induced increase in energy expenditure is independent of circulating fatty acid levels, as substrate utilization switches from fat to carbohydrate when peripheral lipolysis is blocked with acipimox (*i.e.*, a niacin derivative that inhibits adipose triglyceride lipase) (33). It is conceivable that the single bolus of salbutamol only transiently increases lipolysis, circulating fatty acid levels, and fat oxidation, and eventually increases energy expenditure due to an increase in both fat and glucose oxidation.

The finding that a single bolus of salbutamol activates human BAT and whole-body energy metabolism, is a promising new lead for the development of BAT targeted drugs to treat obesity-related cardiometabolic disorders. Nevertheless, although salbutamol is approved in the clinic for the treatment of respiratory diseases, it is unlikely that conventional tissue-unspecific ADRB2 agonism will be applied to treat cardiometabolic pathophysiology due to common cardiovascular side effects (*i.e.*, increase in heart rate, blood pressure and tremors). Moreover, pharmacological stimulation of the ADRB2 using salbutamol does not fully mimic the sympathetic cold-induced activation of BAT just yet, as during cold exposure heart rate decreases (12), possibly explained by cold-induced local norepinephrine release affecting tissue specific beta- as well as alpha-receptors. Nevertheless, it is conceivable that BAT-targeted drug delivery systems

may be developed. Such delivery systems have already been developed to target the liver, taking advantage of the exclusive expression of the asialoglycoprotein receptor on hepatocytes (39), and hepatocyte-targeted modulation of PCSK9 by Inclirisan has recently been approved by the FDA to reduce cardiovascular disease risk (40).

Interestingly, we found notable individual variability in the glucose uptake by BAT after salbutamol administration, with responders having a lower body fat mass percentage and lower WHR as well as lower total cholesterol and LDL-cholesterol levels compared to non-responders. This seems consistent with previous studies showing that [^{18}F]FDG uptake by cold exposed BAT (41, 42) or thermoneutral BAT (43, 44) is associated with a lower body fat mass percentage and/or less central adiposity. As an explanation, a higher body fat mass may influence the detectability of BAT using [^{18}F]FDG PET acquisitions, as human BAT is scattered between white adipocytes (45) and the proportion between classical BAT, beige/brite and white adipose tissue varies substantially between individuals (46, 47). Also, relatively high circulating triglyceride levels at baseline may interfere with glucose uptake by BAT. Under stimulated conditions, BAT increases oxidation of intracellular triglyceride-derived fatty acids, followed by replenishment of the intracellular lipid stores via the uptake of glucose and triglyceride-derives fatty acids from the circulation (48). A higher triglyceride-derived fatty acid flux towards the BAT will thus reduce its glucose uptake (49). However, in the current study baseline triglyceride levels were not significantly different. Alternatively, variations in responsiveness of the ADRB2, due to gene polymorphisms, receptor sensitivity, and/or receptor density could explain differences in salbutamol-induced glucose uptake by BAT and disparities in metabolic phenotype. Various polymorphisms in the ADRB2 gene haven been identified, of which the two most commonly studied gene polymorphisms have a frequency of approx. 35 to 50% in Europe (50). Indeed variations in polymorphisms of the ADRB2 gene can result in different thermogenic responses upon ADRB2 stimulation (34). In addition, loss-of-function mutations of the ADRB2 gene are related to higher body fat and circulating lipid variability (51-57), and low ADRB2 sensitivity and low ADRB2 density are linked to higher circulating triglycerides and LDL-cholesterol, respectively (58, 59). With respect to the current study, the latter would suggest that the responsiveness of the ADRB2 influences both the metabolic phenotype as the salbutamol-induced glucose uptake by BAT, which may be an important factor to consider when targeting BAT to improve cardiometabolic health.

This study is not without limitations. While we used [^{18}F]FDG as tracer for BAT activation, triglyceride-derived fatty acids are probably much more important as fuel for BAT oxidative metabolism (48). Nonetheless, a PET-compatible triglyceride tracer has not been described as yet. Alternative tracers include 14 (R,S)-[^{18}F]fluoro-6-thia-

heptadecanoic acid ([^{18}F]FTHA) or [^{11}C]acetate, which trace circulating fatty acid uptake by BAT and oxidative metabolism and perfusion of BAT, respectively. Using these tracers it has been reported that BAT glucose uptake can be uncoupled from BAT oxidative metabolism, as shown in individuals with obesity or type 2 diabetes whom have maintained BAT oxidative metabolism and fatty acid uptake, despite reduced glucose uptake (60). Hence, future work should examine how the salbutamol-induced changes in BAT glucose uptake reflect changes in BAT oxidative metabolism. In addition, only young, lean males were included in this study. As we already observe baseline differences between responders and non-responders considering body composition and circulating lipid levels, future studies should focus on metabolically compromised individuals and additionally include women.

In summary, we provide evidence that stimulation of the ADRB2 using salbutamol acutely increases the rate of glucose uptake by human BAT *in vivo*, which is suppressed after blocking the ADRB1/2, suggesting that this effect is not mediated by cross-reaction of salbutamol with the ADRB3. As such, these findings provide *in vivo* evidence for our recent findings that the ADRB2 is responsible for adrenergic stimulation of human brown adipocytes (12). Identification of ADRB2 as the most important receptor involved in the sympathetic activation of human BAT thermogenesis allows to maximize the therapeutic potential of targeting BAT to combat cardiometabolic diseases.

ACKNOWLEDGMENTS

We thank Trea Streefland (Department of Medicine, Div. of Endocrinology, LUMC) for her excellent technical assistance and Petra Dibbets-Schneider (Department of Radiology, Div. of Nuclear Medicine, LUMC) for her extended support concerning the PET-CT acquisitions.

FUNDING

This work was supported by a Fonds de Recherche du Québec-Santé (FRQS) Doctoral Training Award (to L.D.), the GSK Chair in Diabetes of Université de Sherbrooke (to D.P.B.), a FRQS J1 salary award (to D.P.B.), the Dutch Diabetes Foundation (2015.81.1808 to M.R.B.), an NWO-VENI grant (09150161910073 to M.R.B.), a Maria Zambrano fellowship by the Ministerio de Universidades y la Unión Europea – NextGenerationEU (RR_C_2021_04 to B.M.T.), and the Netherlands Cardiovascular Research Initiative: an initiative with support of the Dutch Heart Foundation (CVON2017 GENIUS-2 to P.C.N.R.).

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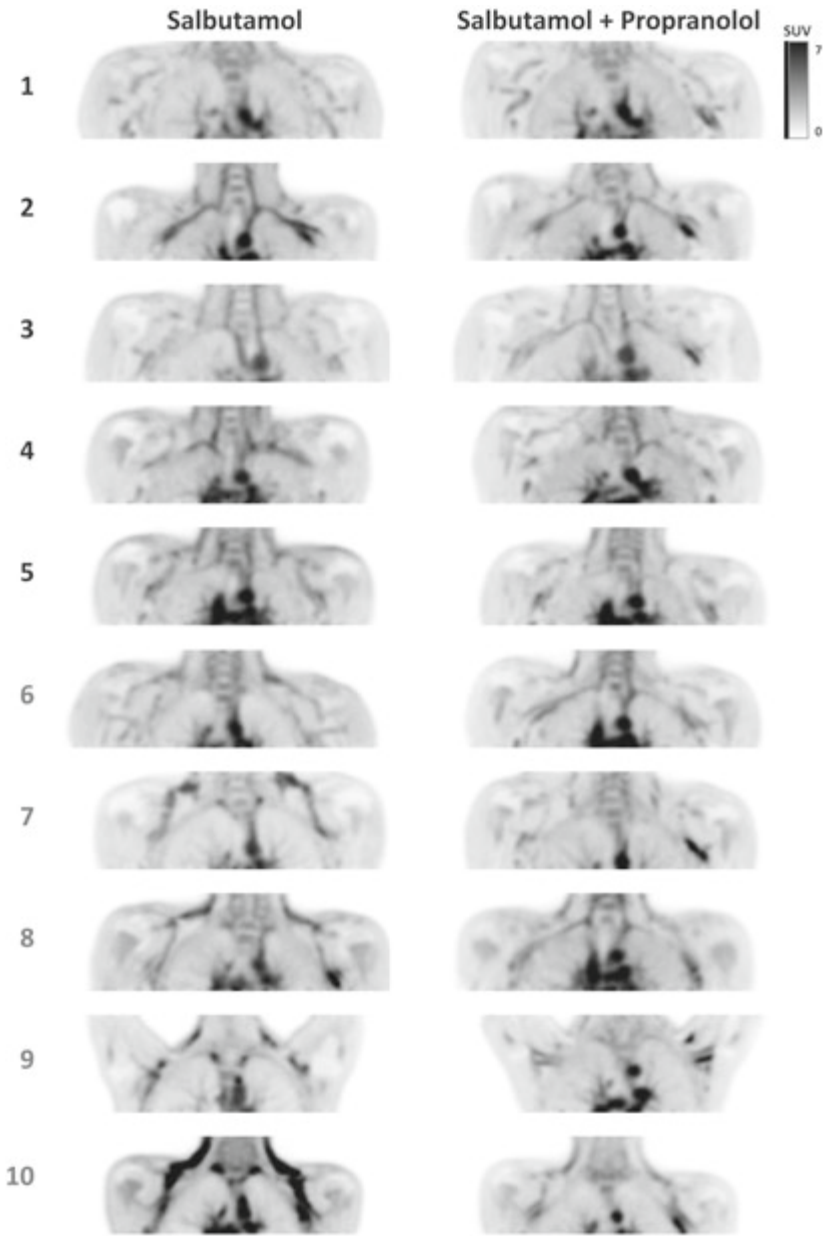
SUPPLEMENTAL DATA

Supplementary table 1. Differences in clinical characteristics, serum measurements, and the effect of salbutamol on metabolic parameters between non-responders and responders.

	Non-responders (n=5)	Responders (n=5)
Glucose uptake by BAT after salbutamol, nmol/g/min	14.4 ± 3.1	108.7 ± 101.3 **
Glucose uptake by BAT after salbutamol with propranolol, nmol/g/min	13.7 ± 2.8	18.4 ± 5.6
Clinical characteristics		
Age, years	25.6 ± 5.5	23.2 ± 2.7
Weight, kg	83.6 ± 8.3	76.1 ± 12.6
Body mass index	24.4 ± 1.2	21.9 ± 2.4 #
Fat mass, %	16.9 ± 2.3	11.8 ± 1.3 **
Waist circumference, cm	84.9 ± 6.3	74.3 ± 7.3 *
Hip circumference, cm	96.7 ± 7.5	91.0 ± 8.2
Waist-hip ratio	0.9 ± 0.1	0.8 ± 0.02 *
Systolic blood pressure (start), mmHg	125.3 ± 10.7	128.9 ± 8.5
Diastolic blood pressure (start), mmHg	79.9 ± 7.1	72.4 ± 2.9 #
Heart rate (start), bpm	66.5 ± 8.3	77.8 ± 10.0 #
Heart rate (pre), bpm	57.8 ± 3.7	62.4 ± 8.2
Heart rate (end), bpm	63.6 ± 8.3	72.4 ± 13.5
Baseline energy expenditure, kcal/h	2056 ± 68	1887 ± 384
Serum measurements		
Triglycerides, mmol/L	1.2 ± 0.3	0.8 ± 0.4
Free fatty acids, mmol/L	0.7 ± 0.4	0.4 ± 0.2 #
Total cholesterol, mmol/L	4.5 ± 0.5	3.0 ± 0.7 **
HDL-cholesterol, mmol/L	1.3 ± 0.2	1.0 ± 0.2 #
LDL-cholesterol, mmol/L	2.7 ± 0.5	1.6 ± 0.4 *
Glucose, mmol/L	5.5 ± 0.1	5.4 ± 0.3
Insulin, µU/mL	13.2 ± 5.3	12.3 ± 3.6
C-peptide, ng/mL	1.5 ± 0.5	1.6 ± 0.3
HOMA-IR	3.2 ± 1.3	2.9 ± 0.8
Effect salbutamol		
Glucose uptake by skeletal muscle, nmol/g/min	10.5 ± 2.3	8.6 ± 3.5
Glucose uptake by scWAT, nmol/g/min	22.2 ± 3.4	20.6 ± 4.3
Change in energy expenditure, %	+3.5 ± 3.8	+10.0 ± 12.1
Change in heart rate, bpm	+21.6 ± 4.4	+12.2 ± 13.2

Values are presented as mean ± standard deviation. Values are measured at baseline during the salbutamol + placebo-visit. Significance levels are obtained from independent-Samples Mann-Whitney U tests, comparing values from participants that showed a high salbutamol-induced glucose uptake by brown adipose tissue (BAT) ('responders') vs. participants that showed low salbutamol-induced glucose uptake by BAT ('non-responders'). #P<0.1, *P<0.05, **P<0.01.
HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; scWAT, subcutaneous white adipose tissue.



Supplementary figure 1. Tissue 2-[^{18}F]fluoro-2-deoxy-D-glucoseuptake in response to salbutamol and salbutamol with propranolol.
Positron emission tomography (PET) images after salbutamol are shown on the left and PET images after salbutamol with propranolol are shown on the right. Numbers of the scans coincide with the numbers in the waterfall plot of Figure 4A and are ordered from lowest (non-responders to salbutamol, black numbers) to highest (responders to salbutamol, blue numbers) glucose uptake by brown adipose tissue (BAT).
SUV, body-weighted standardized uptake value.