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ORIGINAL ARTICLE

Substrate metabolism during basal and hyperinsulinemic conditions in adolescents and young-adults with Barth syndrome

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Abstract
Background Barth syndrome (BTHS) is a rare X-linked disorder that is characterized by mitochondrial abnormalities, infantile or childhood onset of cardioskeletal myopathy, and high mortality rates. It is currently unknown if BTHS related mitochondrial dysfunction results in substrate metabolism abnormalities and thereby contributes to cardioskeletal myopathy in patients with BTHS.
Methods Adolescents and young adults with BTHS (n=5, 20±4 yrs) and age and activity matched healthy controls (n=5, 18±4 yrs) underwent an hyperinsulinemic-euglycemic clamp procedure with stable isotopically labeled tracers for measurement of lipolysis, fatty acid oxidation, glucose disposal, and whole-body proteolysis rates; dual energy x-ray absorptiometry for measurement of body composition and 2-D and strain echocardiography for measurement of left ventricular function.
Results Participants with BTHS had lower fat-free mass (FFM) (BTHS: 31.4±6.9 vs. Control: 46.7±5.3 kg, $p<0.005$), lower systolic function (strain, BTHS: -15.2±2.4 vs. Control: −19.0±2.4 %, $p<0.05$), greater insulin-stimulated glucose disposal rate per kg FFM (BTHS: 96.5±16.3 vs. Control: 67.4±17.6 μmol/kgFFM/min, $p<0.05$), lower basal (BTHS: 4.6±2.7 vs. Control: 11.9±4.4 μmol/kgFM/min, $p<0.05$) and hyperinsulinemic (BTHS: 1.6±0.4 vs. Control: 3.6±1.6 μmol/kgFM/min, $p<0.05$) lipolytic rate per kg fat mass (FM), and a trend towards higher basal leucine rate of appearance per kg FFM (BTHS: 271.4±69.3 vs. Control: 193.1±28.7 μmol/kgFFM/hr, $p=0.07$) compared to controls. Higher basal leucine rate of appearance per kg FFM (i.e.

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whole-body proteolytic rate) tended to be associated with lower left ventricular systolic strain (r=−0.57, p=0.09).
Conclusion Whole-body fatty acid, glucose and amino acid metabolism kinetics when expressed per unit of body composition are altered and appear to be related to cardioskeletal myopathy in humans with BTHS. Further studies examining myocardial substrate metabolism and whole-body substrate metabolism during increased energy demands (e.g., exercise) and their relationships to skeletal and cardiac function are recommended.

Introduction

Barth syndrome (BTHS) is a rare X-linked disorder caused by mutations in the *tafazzin (TAZ)* gene (G4.5) located on the Xq28.12 region of the chromosome resulting in infantile or childhood onset of cardioskeletal myopathy, growth delay, neutropenia, and high mortality rates (Barth et al 1983, 2004). *Tafazzin* is an acyltransferase involved in the reacylation of cardiolipin; an inner mitochondrial membrane protein important in oxidative phosphorylation and the overall molecular and crista membrane structure of the mitochondria (Schlame et al 2000). BTHS-specific defects in CL metabolism include low CL content (Schlame et al 2003), abnormal CL remodeling and altered fatty acid composition (Vreken et al 2000; Schlame et al 2002). Although total mitochondrial ATP production appears to be preserved (Xu et al 2005), it is unclear if CL-mediated mitochondrial abnormalities result in altered substrate metabolism and if these abnormalities contribute to cardioskeletal myopathy seen in patients with BTHS. Thus, the focus of this study was to examine the role of altered substrate metabolism in cardioskeletal myopathy in humans with BTHS.

In other states of chronic heart failure such as congenital and acquired heart failure, whole-body and myocardial substrate metabolism are altered and may contribute to/worsen the pathology of disease (Bergmann et al 2001; Davila-Roman et al 2002; de las Fuentes et al 2003; Norrelund et al 2006). In BTHS, musculoskeletal abnormalities such as decreased skeletal muscle oxidative function during exercise (Spencer et al 2011) and growth delay (Spencer et al 2006) suggests altered skeletal muscle fatty acid and protein metabolism. If fatty acids are not efficiently utilized for energy production, other substrates (i.e., glucose, amino acids) must be utilized to compensate for the energy deficit, possibly resulting in chronic amino acid utilization to meet energy needs. Chronic proteolysis would likely occur in skeletal and possibly cardiac muscle and may result in skeletal muscle weakness/atrophy and worsening left ventricular function. Overall, we hypothesized that BTHS-mediated mitochondrial dysfunction would result in impaired whole-body fatty acid oxidation and higher whole-body proteolysis/amino acid utilization, and would lead to skeletal muscle atrophy and worsening heart function. To test this hypothesis we compared whole-body fatty acid oxidation and proteolytic rates in participants with BTHS with those of healthy aged-matched controls under basal and hyperinsulinemic conditions. We also tested the relationship between substrate metabolism and left ventricular (LV) function and skeletal muscle mass. Phenotypic information about skeletal and myocardial substrate metabolism in BTHS and how they relate to body composition and left ventricular function will advance our understanding of the underlying pathogenesis for BTHS and may provide insights into potentially safe and effective treatments for BTHS.

Methods

Participants

Participants with BTHS (n=5) were recruited from the Barth Syndrome Registry located at the University of Florida (https://www.peds.ufl.edu/barthsyndromeregistry/). Any participant with a diagnosis of BTHS and a confirmed *TAZ* mutation was eligible for the study. Control participants (n=5) were recruited through Volunteers for Health at Washington University School of Medicine and from the local St. Louis community. Upon screening, all participants received a physical examination, including a medical history, fasting blood chemistry and complete blood cell count.

Participant #1 was not working nor in school. His hobbies included video games, riding his bike and stretching. He did not engage in regular physical activity other than riding his bike short distances for transportation needs. He was limited in walking distances over approximately 1–2 city blocks (> 4 min duration). Participant #2 was enrolled in high school and did not have a job. His hobbies included watching professional baseball, participation in occasional pick up baseball games, participation in after school activities and he was the manager for a high school sports team. He reported limitations in household chores and walking long distances (>2-3 city blocks). Participant #3 was working full-time for a family member in which physical activity such as lifting and unloading boxes was a requirement although not frequent. His hobbies included going out to dinner and a movie with friends. He did not engage in regular physical activity other than walking. He was able to walk moderate distances (>15 minutes) without having to take rests. Participant #4 was a high school senior and his hobbies included video games and watching movies. He did not participate in regular physical activity and was limited to

ambulating 1–2 city blocks without rest; he would require a wheelchair for distances longer than this. He reports his condition limited participation in sports but not social activities. Participant #5 was not in school nor employed at the time of the study. His hobbies included spending time with his girlfriend. His condition limited participation in sports but not social activities. He was able to walk moderate distances and perform household activities but needed to take short, frequent rests.

Participants with BTHS were taking cardiac and other medications/supplements at the time of testing. Participant #1 was taking atenolol (25 mg QD), losartan (25 mg QD), sertraline (100 mg QD), levocarnitine (5 ml TID), co-enzyme Q (7000 mg TID), and co-trimoxazole (800 mg QD). Participant #2 was taking furosemide (20 mg QD), digoxin (0.125 mg QD), enalapril (5 mg BID), and filgrastim (0.7 ml every other day). Participant #3 did not take any medications. Participant #4 was taking digoxin (0.5 mg QD) and ibuprofen (200 mg PRN). Participant #5 was taking digoxin (0.125 mg QD) and lisinopril (5 mg QD). Control participants were healthy age- matched males who were not taking any medications at the time of testing. BTHS and controls were physically inactive (not participating in any organized sport for at least 2 months, or regular exercise more than 1x/week). Informed written and verbal consent was obtained from all participants or the parents of the participants and the study was approved by the Human Research Protection Office at Washington University School of Medicine.

Body composition assessment

Whole-body fat mass (FM), fat-free mass (FFM (excluding bone)), and bone mineral density were quantified using a Hologic Discovery (version 12.4; Waltham, MA, USA) enhanced-array dual-energy X-ray absorptiometer (DXA).

Echocardiography

Conventional two-dimensional (2-D), pulsed-wave Doppler, and tissue Doppler echocardiography (General Electric Vivid E9; Waukesha, WI, USA) was performed on each participant. Left ventricular (LV) end-diastolic and end-systolic volumes were determined using the method of discs and LV ejection fraction was calculated. LV mass was determined by M-mode echocardiography according to recommendations of the American Society of Echocardiography (Lang et al 2005). Pulsed-wave Doppler mitral inflow velocities of early LV filling (E) and atrial filling (A) were obtained at the mitral leaflet tips in the apical 4 chamber view for measurements of the mitral E/A ratio, mitral deceleration and isovolumic relaxation time intervals. Tissue Doppler imaging (TDI) was performed in the apical 4 chamber view at the lateral and septal LV base; the averages of the peak systolic velocity (S_m) and peak early diastolic relaxation velocity (E_m) were derived for assessment of systolic and diastolic function, respectively. 2D speckle tracking echocardiographic-derived peak systolic strain was determined from the apical 4 chamber, 2 chamber and apical long axis views and calculated as the average of the basal, mid and apical segments.

Clinical metabolism studies

Background acetate study

To determine the between group acetate correction factor differences necessary to calculate plasma free fatty acid oxidation rate (Sidossis et al 1995), all participants completed an acetate infusion study one day prior to the substrate metabolism study at the Clinical Research Unit in the Washington University School of Medicine Institute of Clinical and Translational Sciences. The evening prior to study, participants were provided a standardized meal containing 12 kcal/kg body weight and 55 % carbohydrate, 30 % fat and 15 % protein at 1800 h. At 1900 h, participants ingested a carbohydrate beverage (80 gm carbohydrates, 12.2 gm fat, 17.6 gm protein, Ensure; Ross Laboratories, Columbus, OH, USA) to ensure adequate muscle and hepatic glycogen stores. Participants then fasted overnight and until completion of the study the following day. The following morning, a catheter was inserted into an antecubital vein (0700 h) and used to administer [1-^{13}C] acetate. At 0800 h, [1-^{13}C] acetate was infused at a constant rate (0.04 μmol•kg-^{1}•-min^{-1}) for 120 min. Serial breath samples were collected into evacuated exetainers at baseline, 1 hr and every 10 min during the final 30 min of the study. Whole body oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured using indirect calorimetry (ParvoMedics, Sandy, UT, USA) at 75 min of the study. Acetate recovery factor was not determined during hyperinsulinemia due to the added risk and burden to the participant of an additional insulin infusion.

Hyperinsulinemic-euglycemic clamp

Participants were fed the standardized meal and carbohydrate beverage the evening prior to the hyperinsulinemic-euglycemic clamp as described above. The following morning, at 0800 h, a catheter was inserted into an antecubital vein and used to administer stable isotope labeled tracers where primed-constant intravenous infusions of [6,6-$^{2}H_2$]-glucose (22.5 μmol/kg prime; 0.25 μmol•kg^{-1}•min^{-1} constant), [1-^{13}C] palmitate (0.04 μmol•kg-^{1}•min^{-1} constant) and [5'5'5' $^{2}H_3$]-leucine (0.06 μmol•kg-^{1}•min^{-1} constant) were initiated. A second catheter was inserted into a hand vein on the contralateral arm; the hand was heated (55°C)

using a thermostatically controlled box to obtain arterialized venous blood samples (Jensen and Heiling 1991). All tracers came from Cambridge Isotope Laboratories (Andover, MA, USA). After a baseline period (0–120 min), a one-stage hyperinsulinemic-euglycemic clamp was initiated. During the hyperinsulinemic stage of the clamp, a prime of 120 mU•$(m^2)^{-1}$•min^{-1}×5 min; followed by 60 mU•$(m^2)^{-1}$•min^{-1}×5 min was administered; then a constant 30 mU•$(m^2)^{-1}$•min^{-1} infusion of regular human insulin was administered intravenously and continued for 180 min (total study time: 300 min). Plasma glucose concentration was maintained at 5 mmol/L (90 mg/dL) by a variable-rate infusion of 20 % dextrose containing 1.5 % [6,6-$^2H_2$]-glucose. Blood samples were obtained every 10 min during the clamp to quantify plasma glucose concentrations and used to adjust the 20 % dextrose infusion rate. Blood and breath samples were obtained before starting the tracer infusions to quantify background ^2H and ^{13}C enrichments, every 10 min during the last 30 min of the basal period, and during hyperinsulinemia to quantify hormone levels, substrate levels, and substrate kinetics. Whole body oxygen consumption and CO_2 production rates were measured by indirect calorimetry into 240 min of the hyperinsulinemic clamp.

Sample analyses

Plasma glucose concentration was measured using an automated glucose analyzer (Yellow Springs Instruments Co, Yellow Springs, OH, USA). Plasma insulin levels were quantified using a chemiluminescent immunometric method (Immulite; Siemens, Los Angeles, CA, USA). The plasma insulin assay range is 2–300 μU/mL and the inter-assay coefficient of variation is 4 % in the low (10.5μU/mL) and high insulin concentration range (55.1μU/mL). Amino acid quantification was performed at St. Louis Children's Hospital via tandem mass spectrometric analysis.

The tracer-to-tracee ratios for plasma $^2H_2$-glucose, ^{13}C palmitate, and $^2H_3$-leucine were quantified using capillary gas chromatography–mass spectrometry (GC-MS; Agilent 6890 N gas chromatograph and Agilent 5973 N mass selective detector; Agilent, Palo Alto, CA, USA) as previously described (Mittendorfer et al 2001; Yarasheski et al 2003, 1998). For $^2H_2$-glucose enrichment quantification, plasma proteins were precipitated with cold acetone, lipids were extracted into hexane, and the aqueous phase was dried (Labconco, Kansas City, MO, USA). The heptafluorobutyric derivative of glucose was formed, and $^2H_2$-glucose enrichment was quantified using GC-electron ionization-MS and selective ion monitoring (mass/charge ratio [m/z] 519 and 521). For quantifying plasma leucine concentration, an internal standard ([U-$^{13}C_6$]-leucine) was added to the plasma before preparation. Plasma leucine was isolated using cation-exchange chromatography. Plasma leucine was converted to the heptafluorobutyric propyl ester derivative; $^2H_3$-leucine enrichment was quantified using GC-MS in negative-chemical ionization mode by monitoring ions at m/z 275 and 278 (Yarasheski et al 2004). Plasma 1-^{13}C palmitate enrichment was quantified after plasma proteins were precipitated with cold acetone, lipids were extracted into hexane, and the fatty acid methyl esters were produced via iodomethane and dichloromethane. Plasma 1-^{13}C palmitate enrichment was quantified using GC-EI-MS with selective ion monitoring (*m/z* 270 and 271). The GC-MS instrument response was calibrated using gravimetric standards of known isotope enrichment. Breath $^{13}CO_2$ enrichment was measured by isotope ratio mass spectrometry (IRMS; Finnigan Delta + XL, Bremen, Germany).

Calculations

Plasma glucose, palmitate and leucine rates of appearance (R_a) were calculated by dividing each tracer infusion rate by the average tracer-to-tracee (TTR) ratio obtained during the last 30 min of each stage (i.e., baseline, hyperinsulinemia) of the clamp as previously described (Matthews et al 1980; Yarasheski et al 1998). Glucose rate of disposal (R_d) was calculated as the sum of endogenous glucose R_a plus infused dextrose. Palmitate oxidation rate was determined by dividing breath $^{13}CO_2$ production ($^{13}CO_2$ TTR x VCO_2 production) by the plasma palmitate TTR. Carbohydrate oxidation rate was obtained using indirect calorimetry and calculations from Frayn (Frayn 1983). Kinetic rates were expressed per kilogram fat-free and fat mass where appropriate.

Statistics

Mean ± SD are presented in the tables and figures. Substrate kinetics were expressed in both absolute terms (μmol/min) and per unit of body mass (fat-free or fat mass, μmol/kg/min). Demographic and serum metabolite and hormone variables were compared between groups by independent t-tests. Differences in substrate kinetics between groups and conditions (i.e., baseline, hyperinsulinemic) were examined by a two-way analysis of variance (ANOVA, group x condition) with post-hoc testing using Tukey's honestly significant difference analysis. Relationships between outcome

Table 1 Demographic and metabolic variables

Variable	Control (n=5)	BTHS (n=5)
Age (yrs)	18±4 (15–25)	20±4 (16–24)
Height (cm)	176.6±7.5 (170.1-184.0)	170.0±18.5 (140.5-186.7)
Weight (kg)	65.0±10.6 (52.4-77.9)	51.8±8.5 (44.6-59.3)
BMI	20.9±3.3 (17.9-26.2)	18.2±3.4 (14.3-22.6)
FFM (kg)	46.7±5.3 (37.9-51.1)	31.4±6.9* (20.0-37.9)
Fat mass (kg)	8.4±6.4 (3.4-17.6)	13.2±5.8 (5.0-19.2)
Fat mass (%)	13.2±7.9 (7–26)	28.1±13.0 (14–49)
BMD (g/cm^2)	1.04±0.06 (0.94-1.09)	0.81±0.09* (0.72-0.95)
Fasting glucose (mg/dL)	87.4±7.9 (76.0-97.0)	80.8±13.8 (63.0-94.0)
Fasting insulin (μU/mL)	6.8±2.5 (3.8-10.4)	9.5±10.8 (2.0-28.4)
TG (mg/dL)	90.0±24.4 (66.0-124.0)	59.5±21.6 (36.0-88.0)
HDL (mg/dL)	36.0±5.1 (29.0-41.0)	35.8±4.2 (33.0-42.0)
LDL (mg/dL)	76.8±22.9 (59.0-109.0)	79.5±31.7 (32.0-95.0)
Chol (mg/dL)	130.8±28.5 (126.0-172.0)	127.3±36.3 (73.0-148.0)
Lactate (μmol/L)	890.2±150.0 (689.0-1049.0)	1544.4±1677.1 (413.0-4498.0)
FFA (μmol/L)	298.0±126.6 (182.5-505.0)	816.0±427.4** (485.0-1542.5)
IGF-1 (ng/mL)	221.5±118.5 (50.5-340.0)	150.6.6±33.1 (110.0-182.0)

BTHS: Barth syndrome, BMI: body mass index, FFM: fat free mass, BMD: bone mineral density, TG: triglyceride, HDL: high density lipoprotein, low density lipoprotein, Chol: total cholesterol, FFA: free fatty acid, IGF-1: insulin growth factor 1

*: $p<0.01$, **: $p<0.05$

variables were examined using univariate analysis (Pearson product correlation coefficient). Statistical significance was considered at $p<0.05$.

Results

Demographics, body composition, and serum metabolite and hormone levels

Participants were similar in age and height but participants with BTHS had significantly lower absolute fat-free mass (FFM), and bone mineral density, and tended to have lower % FFM ($p=0.07$) than controls (Table 1). There was a trend toward lower body weight ($p=0.06$) and higher absolute ($p=0.10$) and percentage of total fat ($p=0.06$) in participants with BTHS compared to controls (Table 1). Fasting plasma glucose, lactate, triglyceride and lipoprotein concentrations were similar between groups, however total plasma free fatty acid concentration was almost 3-fold higher in BTHS than controls (Table 1).

Echocardiography

Heart rate, left ventricular structure and diastolic function were not different between groups (Table 2). Systolic blood pressure was significantly lower in BTHS compared to controls. Systolic global strain and right wall velocity during systole were significantly lower in BTHS than controls and there was a trend toward lower LV wall velocity during systole in BTHS ($p=0.08$) (Table 2).

Metabolism studies

Energy expenditure

Due to the difference in body composition between groups, energy expenditure and substrate kinetics were also

Table 2 Echocardiographic Parameters

Variable	Control (n=5)	BTHS (n=5)
HR (bpm)	69±14 (58–93)	80±18 (61–102)
SBP (mm/Hg)	120±8 (108–128)	100±13* (80–112)
DBP (mm/Hg)	71±13 (51–80)	61±14 (48–78)
LV structure		
LVM (g)	143±12 (128–158)	138±33 (88–173)
EDV (mL)	96±11 (86–113)	76±18 (55–103)
ESV (mL)	40±7 (34–51)	37±16 (24–64)
LV systolic function		
EF (%)	58±3 (55–61)	52±9 (38–61)
LVOT TVI (cm/s)	19.5±3.1 (16.8-23.9)	15.7±1.1 (14.1-16.9)
LV S_m (cm/s)	11.2±1.9 (10.0-14.5)	9.0±1.6 (7.0-11.5)
Global strain (%)	-19.0±2.5 (−16.3- -21.3)	-15.2±2.4** (−12.4- -17.7)
LV diastolic function		
E wave (cm/s)	80±14 (66–90)	72±7 (62–77)
E/A ratio	1.7±0.6 (1.1-2.4)	1.8±1.0 (1.2-3.6)
E_m (cm/s)	16.6±2.8 (13.0-20.5)	15.3±0.7 (15.0-16.0)
E/E_m	4.4±0.5 (3.9-4.5)	3.7±0.5 (3.1-4.3)

BTHS: Barth syndrome, HR: heart rate, bpm: beats per minute, SBP: systolic blood pressure, DBP: diastolic blood pressure, mmHg: mL of mercury, LV: left ventricle, LVM: left ventricular mass measured by 2-D echocardiography, EDV: end diastolic volume, ESV: end systolic volume, EF: ejection fraction, LVET: left ventricular ejection time, S_m: systolic myocardial velocity averaged from septum and lateral wall, E_m: myocardial velocity during early diastole averaged from septum and lateral wall. *: $p<0.01$, **: $p<0.05$

normalized to FFM and FM where appropriate. Participants with BTHS had lower absolute resting energy expenditure during the basal and hyperinsulinemic periods compared to controls (Table 3). When expressed per FFM, resting energy expenditure was similar between groups during both conditions (Table 3).

Glucose kinetics

Plasma insulin concentrations during the basal (BTHS: 9.5±1.08 vs. Control: 6.8±2.5 μU/ml) and hyperinsulinemic (BTHS: 47.9±13.2 vs. Control: 36.7±10.1 μU/ml) conditions were not different between groups. Absolute (i.e. μmol/min) basal endogenous glucose R_a was significantly lower in BTHS compared to controls however when normalized to FFM, was similar between groups. Conversely, absolute endogenous glucose R_a during hyperinsulinemia was similar between groups but was significantly higher in BTHS compared to controls when normalized to FFM (Table 3). Absolute glucose R_a was not different between groups during basal or hyperinsulinemic conditions, but was significantly greater in BTHS than controls during

Table 3 Substrate metabolism

Variable	Control (n=5)	BTHS (n=5)
Basal		
Energy expenditure (kcal/day)	1746±139 (1732–1879)	1329±202* (1122–1585)
Energy expenditure (kcal/kgFFM/day)	37.9±6.6 (33.3-49.6)	43.5±3.8 (33.6-56.1)
Endogenous glucose R_a (μmol/min)	1053±129 (867–1194)	733±101* (631–880)
Endogenous glucose R_a (μmol/kgFFM/min)	22.7±3.0 (18.9-26.7)	24.0±4.5 (20.5-31.5)
Carbohydrate oxidation (g/day)	198.6±103.2 (68.0-328.0)	119.5±33.8 (84.6-159.3)
Carbohydrate oxidation (g/kgFFM/day)	4.2±2.4 (1.3-6.8)	3.7±0.6 (2.7-4.3)
Palmitate R_a (μmol/min)	84.0±46.5 (41.0-151.7)	62.4±16.9 (45.7-84.4)
Palmitate R_a (μmol/kgFFM/min)	11.9±4.4 (6.3-12.2)	4.6±2.7** (2.4-8.4)
Palmitate R_a (μmol/kgFFM/min)	1.8±0.9 (0.8-3.0)	2.0±0.3 (1.6-2.3)
FAOX (μmol/min)	10.8±6.0 (5.0-19.6)	9.8±1.0 (8.9-11.1)
FAOX (μmol/kgFFM/min)	0.23±0.11 (0.10-0.39)	0.31±0.08 (0.28-0.44)
Non-oxidative FA disposal (μmol/min)	70.2±38.9 (40.4-126.4)	50.6±15.1 (35.6-70.1)
Non-oxidative FA disposal (μmol/ kgFFM/min)	1.5±0.7 (0.7-2.5)	1.6±0.3 (1.3-1.8)
Fasting serum leucine (μM)	144.6±23.8 (121.7-177.0)	144.1±29.5 (126.5-190.2)
Leucine R_a (μmol/min)	9071±2008 (6522–11243)	8211±1436 (7236–10016)
Leucine R_a (μmol/kgFFM/min)	193.1±28.7 (172.1-227.8)	271.4±69.3 (195.7-361.8)
Acetate correction factor (%)	31±6 (27–41)	28±7 (20–37)
Hyperinsulinemia		
Energy expenditure (kcal/day)	1770±75 (1642–1834)	1424±212* (1130–1639)
Energy expenditure (kcal/kgFFM/day)	38.2±3.4 (35.2-43.3)	47.3±12.7 (32.0-65.4)
Carbohydrate oxidation (g/day)	302.7±53.7 (255.1-365.3)	284.1±110.1 (182.9-462.0)
Carbohydrate oxidation (g/kgFFM/day)	6.3±1.8 (4.6-9.2)	9.1±3.8 (5.0-14.8)
Endogenous glucose R_a (μmol/min)	248.4±117.3 (97.4-402.2)	300.9±220.6 (85.4-596.6)
Endogenous glucose R_a (μmol/kgFFM/min)	5.2±2.1 (2.1-7.9)	9.4±6.6** (2.5-19.6)
Palmitate R_a (μmol/min)	22.2±7.0 (17.6-33.8)	23.6±3.2 (21.5-25.2)
Palmitate R_a (μmol/kgFM/min)	3.6±1.4 (1.9-5.2)	1.6±0.4* (1.4-2.1)
Palmitate R_a (μmol/kgFFM/min)	0.5±0.1 (0.4-0.7)	0.8±0.4 (0.6-1.4)
FAOX (μmol/min)	7.0±1.0 (5.7-8.1)	7.4±1.6 (5.4-9.1)
FAOX (μmol/kgFFM/min)	0.15±0.02 (0.12-0.18)	0.25±0.13 (0.21-0.46)
Non-oxidative FA disposal (μmol/min)	**15**.2±6.2 (11.9-25.7)	16.2±2.3 (15.0-18.1)
Non-oxidative FA disposal (μmol/kgFFM /min)	0.32±0.10 (0.25-0.50)	0.54±0.24 (0.36-0.91)
Fasting serum leucine (μM)	90.5±35.1 (60.6-146.3)	61.3±7.5** (50.7-69.5)
Leucine R_a (μmol/min)	5908±1456 (4504–8383)	4959±563 (4236–5627)
Leucine R_a (μmol/ kgFFM /min)	125.8±21.8 (115.1-164.1)	167.9±58.0 (111.7-260.1)
Delta baseline→hyperinsulinemia (μmol/ kgFFM /min)	-67.2±25.5 (−53.2- -112.7)	-103.5±28.7 (−60.5- 136.6)
Delta baseline→hyperinsulinemia (%)	34±9 (31–49)	39±9 (31–48)

BTHS: Barth syndrome, R_a: rate of appearance, R_d: rate of disappearance, FAOX: fatty acid oxidation, μM: micromolar, FFM: fat free mass, FM: fat mass, *p<0.01, **p<0.05

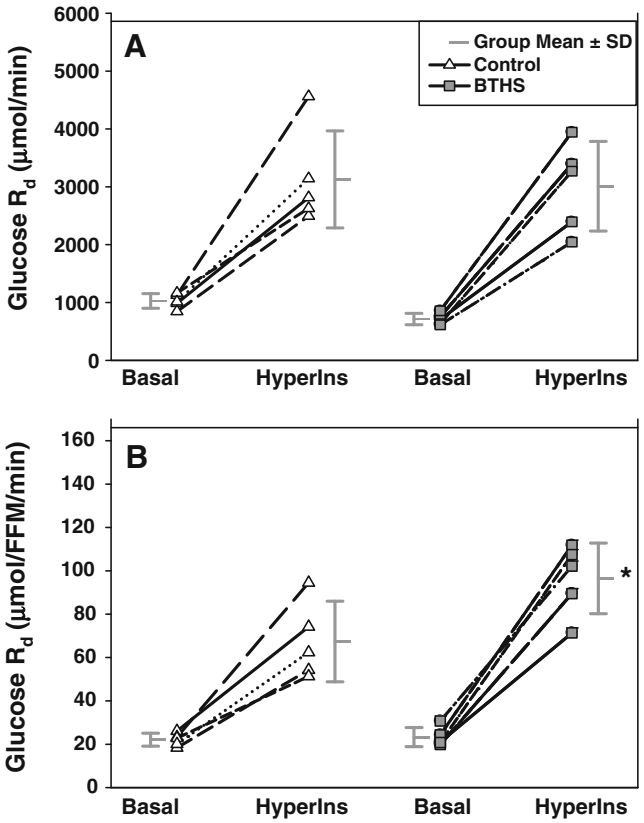

Fig. 1 Glucose rate of disappearance in BTHS and controls during the basal and hyperinsulinemic condition. BTHS: Barth syndrome, R_d: rate of disappearance, HyperIns: hyperinsulinemia, FFM: fat free mass. *: $p<0.05$

hyperinsulinemia when expressed per FFM (Fig. 1). Glucose R_d per FFM increased from baseline to hyperinsulinemia in both groups but increased to a greater degree in BTHS than controls (Fig. 1) and as a percent change (BTHS: 75±5 vs. Control: 66±7 %, $p<0.04$). Absolute and FFM normalized carbohydrate oxidation was not different between groups during the basal and hyperinsulinemic conditions.

Fatty acid kinetics

Absolute palmitate R_a (i.e., lipolytic rate) was similar between groups during the basal and hyperinsulinemia periods (Table 3, Fig. 2a). Palmitate R_a normalized to fat mass was significantly lower during basal and hyperinsulinemic conditions in BTHS compared to controls (Table 3, Fig. 2a) however the insulin-mediated suppression of whole-body lipolysis was similar between groups (BTHS: 90±12 vs. Control: 95±2 %) indicating a similar responsiveness of adipose tissue to insulin. Palmitate R_a normalized to FFM (as skeletal muscle is the most significant user of serum free fatty acids) was similar between groups during basal and hyperinsulinemic conditions. Basal and hyperinsulinemic palmitate oxidation rates were similar between groups when expressed per min or per FFM. Absolute non-oxidative palmitate R_d and when normalized to FFM was similar between groups during the basal and hyperinsulinemic conditions (Table 3). Acetate recovery/correction was not different between the groups under basal conditions (Table 3) and therefore was not used in the calculation of fatty acid oxidation rate.

Amino acid kinetics

Fasting (8 hr) serum phenylalanine and proline concentrations were higher in BTHS compared to controls (Fig. 3). Serum threonine concentrations tended to be higher ($p=0.09$) in BTHS compared to controls (Fig. 3). In addition, fasting serum arginine and ornithine were significantly lower in BTHS compared to controls (Fig. 3) and there was a trend toward lower citrulline ($p=0.08$) and glutamine ($p=0.15$) levels in BTHS compared to controls (Fig. 3). Fasting serum leucine concentration at baseline was similar between groups but was significantly lower in BTHS during hyperinsulinemia (Table 3). Absolute basal leucine R_a was similar between groups however when normalized to FFM tended to be greater ($p=0.09$) in BTHS than controls (Fig. 4). During

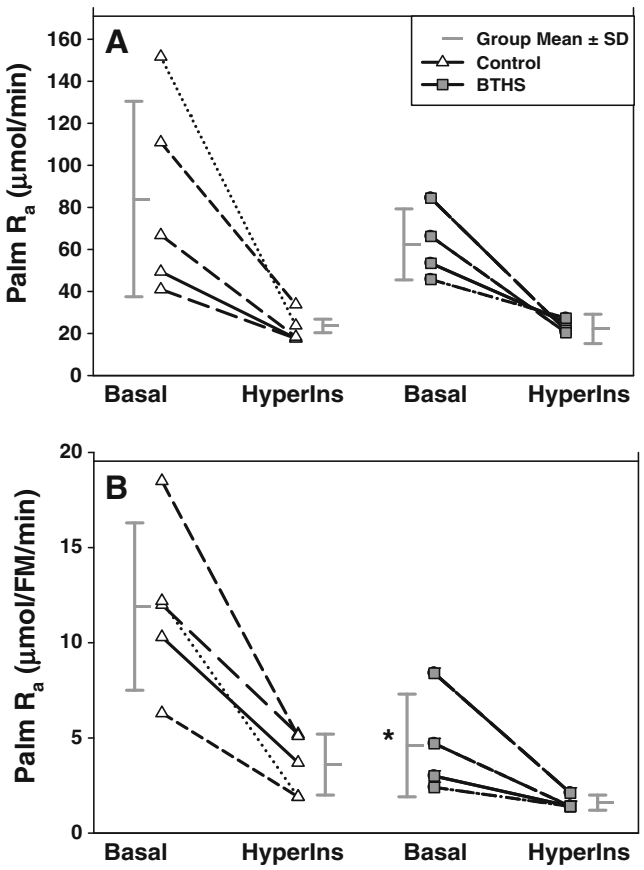

Fig. 2 Palmitate rate of appearance in BTHS and controls during the basal and hyperinsulinemic condition. BTHS: Barth syndrome, R_a: rate of appearance, HyperIns: hyperinsulinemia, FM: fat mass. *: $p<0.05$

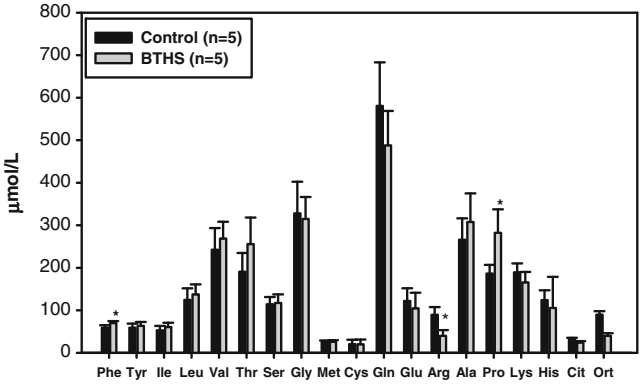

Fig. 3 Plasma Amino Acid Levels in BTHS and Control Participants. Phe: phenylalanine, Tyr: tyrosine, Ile: isoleucine, Leu: leucine, Val: valine, Thr: Threonine, Ser: serine, Gly: Glycine, Met: methionine, Cys: cysteine, Gln: glutamine, Glu: glutamate, Arg: arginine, Ala: alanine, Pro: proline, Lys: lysine, His: histidine, Cit: citrulline, Ort: ornithine. *: $p<0.05$

hyperinsulinemia, leucine R_a expressed absolutely and when expressed per kg FFM was similar between groups. Insulin-mediated suppression of proteolysis (i.e., leucine rate of appearance) expressed absolutely and normalized to FFM, significantly decreased in controls and tended to decrease

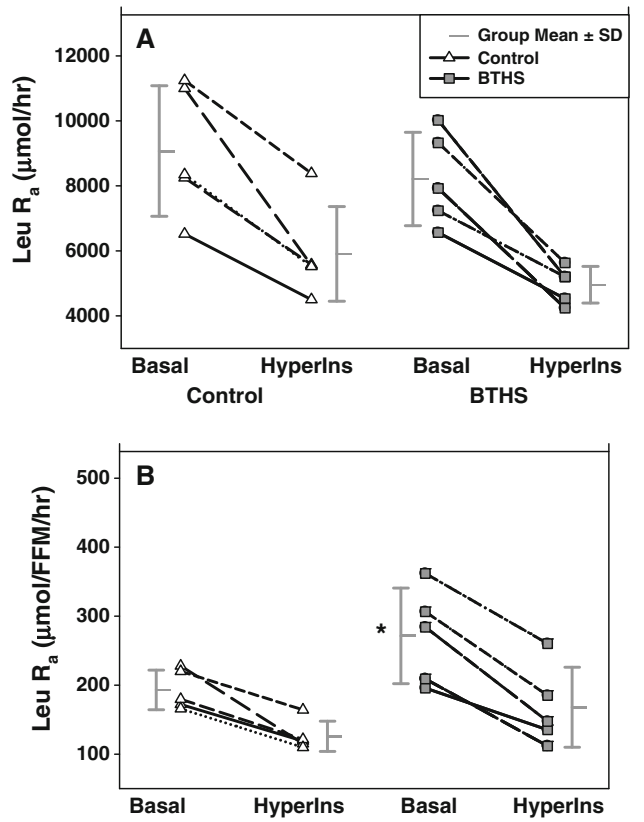

Fig. 4 Leucine rate of appearance in BTHS and controls during the basal and hyperinsulinemic condition. BTHS: Barth syndrome, R_a: rate of appearance, HyperIns: hyperinsulinemia, FFM: fat free mass. *: $p=0.09$

in BTHS ($p=0.17$); however the percent suppression between groups was similar (Table 3, Fig. 3).

Correlation with endpoints

Lower serum arginine levels tended to correlate with lower FFM ($r=0.62$, $p=0.06$) and there was a tendency for a significant association between higher baseline leucine R_a normalized for FFM and lower global strain ($r=-0.57$, $p=0.09$).

Discussion

This is the first report of substrate metabolism kinetics in humans with BTHS. Specifically, participants with BTHS demonstrated: 1) greater insulin-stimulated glucose disposal rate expressed per kg fat-free mass, 2) blunted insulin-mediated suppression of endogenous glucose production, 3) lower basal and insulin-induced suppression of lipolytic rates expressed per unit of fat mass, and 4) a trend toward a higher basal whole-body proteolytic rate expressed per unit of fat-free mass. Substrate kinetics expressed in absolute terms (i.e., µmol/min) were similar between groups except basal endogenous glucose production which was higher in BTHS. This study is also the first to report body composition abnormalities in BTHS, specifically lower fat-free mass (i.e., skeletal muscle) and a trend towards greater adiposity. Also, this study is the first to report lower absolute resting energy expenditure in BTHS; however, these differences disappeared when expressed per unit of fat-free mass. Higher whole-body proteolytic rate per unit of fat-free mass tended to be associated with lower left ventricular systolic function suggesting that amino acid metabolism alterations may be related to the etiology of BTHS-related heart failure. Overall, these findings suggest that alterations in substrate metabolism may be part of the BTHS phenotype and may mediate or contribute to clinical features (e.g., muscle atrophy, left ventricular dysfunction) of the disease; however larger studies are needed to confirm these findings.

Body composition

Participants with BTHS had lower fat-free mass and tended to have a greater total and percentage of fat mass compared to their peers. The cause of lower fat-free or skeletal muscle mass in BTHS is not clear. A sedentary lifestyle and/or a delayed growth pattern (Barth et al 2004) in BTHS may contribute. In addition, it is possible that the observed chronic elevation in basal whole-body proteolytic rate per unit of fat-free mass may also play a role. However, due to the inherent contrast in body composition between BTHS and controls, it was difficult to determine whether

differences in proteolytic rate were due to differences in group body composition or rather represent inherent physiologic abnormalities in BTHS. Therefore, for all analyses, we examined substrate kinetics in absolute terms (μmol/min) and per kg of the appropriate metabolically active tissue mass; fat mass for lipolytic rate, lean mass for glucose, leucine and energy metabolism. Normalization to fat free mass essentially compensates for individual differences in resting energy expenditure (Koutsari and Jensen 2006); the variable most related to substrate kinetics (esp. lipid metabolism) (Nielsen et al 2003).

Lipid kinetics

Fatty acid (palmitate) kinetics expressed both in absolute terms (μmol/min) and per kg fat free mass were not different between BTHS and controls. This suggests that *tafazzin* mutations are not associated with abnormalities in basal (i.e., resting) lipolysis (fatty acid release from adipose depots) and fatty acid utilization in BTHS, and that a higher lipolytic rate was not responsible for the elevated serum free fatty acid levels seen in BTHS participants. When expressed per kg fat mass, lipolytic rate was lower in BTHS compared to controls. One interpretation from this finding is that an inherent defect in adipose tissue lipolysis in BTHS exists; however, this interpretation is speculative as adipose tissue biopsies and in vitro analyses were not performed. Alternatively, higher serum free fatty acid levels in BTHS may have lowered the lipolytic rate by reducing the expression of or inhibiting adipose tissue hormone sensitive lipase (Burns et al 1978; Lei et al 2004). Also, a reduction in sympathetic nerve innervation/catecholamine concentration in BTHS, as frequently observed in adult and childhood obesity, may have also mediated the lower lipolytic response (Bougneres et al 1997; Enoksson et al 2000; Klein et al 1996); however sympathetic output in chronic heart failure is frequently elevated (Esler and Kaye 2000).

Fatty acid oxidation rate (absolute and when expressed per kg fat-free mass) was similar between groups during baseline and hyperinsulinemia; contrary to our hypothesis. This finding was surprising because mitochondrial myopathy is a hallmark feature in the drosophila model of BTHS (Xu et al 2006), and this would be predicted to affect fatty acid oxidation. The absence of fatty acid oxidation abnormalities in BTHS participants in the current study may be due to the fact that fatty acid oxidation kinetics were measured during bed rest and that a stimulus for increased mitochondrial respiration (e.g., low intensity activity/exercise) is needed to reveal deficits in fatty acid oxidation in this population. Thus, BTHS-mitochondrial function (and subsequently fatty acid oxidation) may be adequate during rest but during daily living and recreational activities (i.e., activities requiring increased energy production (Gunn et al 2004)), fatty acid oxidation may be inadequate and alternate substrate (e.g., amino acid) metabolism is required. Impaired skeletal muscle fatty acid oxidation during low level activities may also explain the elevated serum fatty acid levels in BTHS (i.e., inability to oxidize mobilized fatty acids). However, since this was not measured directly, this is also speculative. Interestingly, the percentage of the labeled acetate carbon recovered in the breath was similar between BTHS and controls indicating similar acetyl-coA flux through the mitochondrial tricarboxlyic cycle (Schrauwen and Hesselink 2008), and further suggesting preserved mitochondrial function in BTHS during rest. Future studies examining the relationship between myocardial fatty acid oxidation and whole-body fatty acid oxidation during increased energy demands (i.e., exercise) and left ventricular function are needed.

Glucose kinetics

Endogenous glucose production (i.e., hepatic) expressed in absolute terms, was significantly higher in BTHS during the basal period and was suppressed to a lesser extent during hyperinsulinemia (%) in BTHS compared to controls. However, absolute basal and insulin stimulated glucose disposal rate (Fig. 1) and carbohydrate oxidation rate (by indirect calorimetry, Table 3) were not different between groups suggesting similar absolute glucose utilization in BTHS and controls. Examining glucose utilization in absolute terms indicates that participants with BTHS were able to utilize glucose (in both conditions) to a similar extent as controls, even though BTHS had significantly less skeletal muscle mass; the predominant site for glucose disposal. Indeed, when expressed per kg fat free-mass (gold standard expression in substrate kinetic studies (Miyazaki et al 2002)), insulin-stimulated glucose disposal was higher in BTHS than controls (Fig. 1). However, without body composition matched controls, it is unclear whether differences in glucose metabolism in BTHS are due to contrasts in physiology or body composition or both.

Amino acid kinetics

Serum levels of several amino acids were significantly altered in BTHS participants compared to controls. Of note, amino acids that are associated with anaplerosis in the citric acid cycle (arginine, ornithine and citrulline) were significantly lower in BTHS suggesting that amino acids utilization for energy use may be occurring in BTHS to a greater extent than controls thus supporting the hypothesis of alternate substrate utilization in BTHS. This hypothesis is further supported by the finding of higher basal leucine rate of appearance per kg fat-free mass in BTHS; a surrogate measure of whole-body proteolysis (Waterlow 2006). Although

absolute leucine rate of appearance was not different between groups, a similar leucine rate of appearance was achieved with a significantly lower lean mass in BTHS. This is important as skeletal muscle represents the largest portion of lean mass and muscle is the largest storage site of amino acids (Waterlow 2006). Leucine oxidation rate was not measured and therefore the use of leucine (and other amino acids) for energy production is uncertain. However, taken in combination with the finding of lower levels of select amino acids involved in the citric acid cycle, the higher proteolysis rate is consistent with the hypothesis of the increased use of amino acids for energy needs in BTHS. This finding is also consistent with non-BTHS heart failure studies where whole-body and myocardial protein/amino acid metabolism were altered (Norrelund et al 2006).

The alternative substrate metabolism remodeling hypothesis remains speculative as free fatty acid oxidation rate in the current study was similar among groups during bed rest. However, if increased chronic skeletal and cardiac muscle protein breakdown is occurring to supplement energy needs in BTHS, this may contribute to low lean (i.e., muscle) mass and reduced heart function seen in this population; a condition termed "cardiac cachexia" (Anker and Sharma 2002). Indeed, in the current study, the higher basal leucine rate of appearance per unit of fat-free mass was associated with lower left ventricular function suggesting that increased amino acid/protein turnover may contribute to the cardioskeletal myopathy in BTHS.

Limitations. The sample size in the current study is small and trends in the data were likely a result of inadequate statistical power. However, BTHS is an extremely rare disease and the logistics and effort for BTHS participants to travel to St. Louis and undergo intensive metabolic measurement is considerable. Despite the small sample size, we were able to find several significant body composition and metabolic differences between groups. Although control participants were matched to BTHS for age, race, and sedentary lifestyle, inherent body composition differences existed which made interpretation of kinetic data challenging. Also, all participants had stable heart function and were generally healthy and therefore the results may represent only healthier boys and young men with BTHS. Although BTHS participants had low normal ejection fraction (i.e. systolic function), systolic strain was significantly lower than controls and it appears that systolic functional abnormalities are most observed during exercise (Spencer et al 2011); however, exercise stimulated heart function was not measured in the current study. Another limitation is that amino acid oxidation was not measured; therefore the contribution of amino acid oxidation for energy could not clearly be determined. In addition, fatty acid oxidation rates were not corrected for the loss of ^{13}C label in the citric acid cycle and therefore are underestimated in both groups. However, the basal acetate correction factor was similar and did not affect the comparison between groups. Lastly, the results of this study were shown in post-pubescent adolescents and young men and therefore cannot be imputed to pre-pubescent boys with BTHS; additional studies in pre-pubescent participants are warranted.

Conclusions

Absolute whole-body fatty acid, glucose and amino acid kinetics remain largely unaffected however when expressed as a function of body composition, they are altered in humans with BTHS during fasting and hyperinsulinemic conditions. The complete physiologic and functional consequences of altered substrate metabolism in BTHS are unclear but increased whole-body proteolytic rate normalized to fat-free mass appears to be related to left ventricular dysfunction. Further studies examining myocardial nutrient metabolism and whole-body nutrient metabolism during increased energy demands (e.g., exercise) and their relationships to skeletal and cardiac function are recommended.

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