Original article

MOLECULAR METABOLISM

Defects in muscle branched-chain amino acid oxidation contribute to impaired lipid metabolism

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ABSTRACT

Objective: Plasma levels of branched-chain amino acids (BCAA) are consistently elevated in obesity and type 2 diabetes (T2D) and can also prospectively predict T2D. However, the role of BCAA in the pathogenesis of insulin resistance and T2D remains unclear.

Methods: To identify pathways related to insulin resistance, we performed comprehensive gene expression and metabolomics analyses in skeletal muscle from 41 humans with normal glucose tolerance and 11 with T2D across a range of insulin sensitivity (S_I, 0.49 to 14.28). We studied both cultured cells and mice heterozygous for the BCAA enzyme methylmalonyl-CoA mutase (Mut) and assessed the effects of altered BCAA flux on lipid and glucose homeostasis.

Results: Our data demonstrate perturbed BCAA metabolism and fatty acid oxidation in muscle from insulin resistant humans. Experimental alterations in BCAA flux in cultured cells similarly modulate fatty acid oxidation. Mut heterozygosity in mice alters muscle lipid metabolism *in vivo*, resulting in increased muscle triglyceride accumulation, increased plasma glucose, hyperinsulinemia, and increased body weight after high-fat feeding.

Conclusions: Our data indicate that impaired muscle BCAA catabolism may contribute to the development of insulin resistance by perturbing both amino acid and fatty acid metabolism and suggest that targeting BCAA metabolism may hold promise for prevention or treatment of T2D.

Keywords Insulin sensitivity; BCAA; Fatty acid oxidation; TCA cycle

1. INTRODUCTION

The global epidemic of type 2 diabetes (T2D) impacts both individual and public health and highlights the urgent need for improved understanding of T2D pathophysiology to facilitate new approaches to prevention and therapy. A key feature of T2D risk is insulin resistance, a metabolic phenotype which can both precede and predict the development of diabetes decades later in high-risk individuals [1,2]. Emerging data underscore important links between energy metabolism and insulin resistance [3]. Potential contributors to insulin resistance include alterations in lipid oxidative metabolism leading to accumulation of acylcarnitines and other lipid intermediates [4,5], ceramides [6], reactive oxygen species [7], pro-inflammatory signals [8], and endoplasmic reticulum stress [9].

Branched-chain amino acids (BCAAs) have been identified as potential contributors to insulin resistance [10]. Plasma concentrations of amino acids, including BCAA, are increased in obesity [11] and in parallel with glycemia in patients with T2D [12]. Unbiased metabolomics approaches have confirmed the association between plasma amino acids and metabolic disorders [10,13–15]. Newgard and colleagues reported elevated plasma BCAAs in obese insulin resistant humans and demonstrated that increased BCAA intake contributes to insulin resistance in high fat diet-fed rodents [10]. Conversely, BCAA levels are reduced after bariatric surgery or weight loss, paralleling improve-

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Table 1 — Clinical characteristics of study subjects.

	IS	IR	T2D	p value
Subjects	n = 18	n = 22	n = 11	—
Age (years)	35.7 ± 2.3	39.4 ± 2.6	50.7 ± 3.9[c]	**0.005**
Females	10 (56%)	12 (55%)	7 (64%)	—
BMI (kg/m^2)	25.8 ± 1.0	27.9 ± 1.2	31.5 ± 2.7	0.067
Fasting Glucose (mmol/l)	4.89 ± 0.07	5.27 ± 0.09	7.25 ± 0.85[d]	**<0.0001**
Fasting Insulin (pmol/l)	28 ± 3	58 ± 7	137 ± 46[c]	**0.001**
HbA1c (%)	5.0 ± 0.1	5.1 ± 0.1	6.9 ± 0.4[d]	**<0.0001**
Triglycerides (mmol/l)	0.69 ± 0.09	1.07 ± 0.10[a]	1.16 ± 0.17[a]	**0.013**
Cholesterol (mmol/l)	4.42 ± 0.19	4.43 ± 0.14	4.05 ± 0.24	0.333
HDL-Cholesterol (mmol/l)	1.48 ± 0.08	1.23 ± 0.09	1.00 ± 0.07[c]	**0.004**
LDL-Cholesterol (mmol/l)	2.60 ± 0.24	2.94 ± 0.14	2.58 ± 0.27	0.356
S_I (IVGTT)	8.12 ± 0.56	2.81 ± 0.27[d]	1.69 ± 0.45[d]	**<0.0001**
Leucine	0.97 ± 0.05	1.02 ± 0.03	0.97 ± 0.04	0.560
Isoleucine	1.03 ± 0.06	1.14 ± 0.04	1.00 ± 0.05	0.135
Valine	0.98 ± 0.04	0.98 ± 0.05	1.00 ± 0.04	0.793

Subjects were metabolically characterized and insulin sensitivity (S_I) was determined by intravenous glucose tolerance test (IVGTT). Subjects without T2D were categorized as insulin sensitive (IS) or resistant (IR) based on S_I values above or below the median value of 4.78 for a larger population of normoglycemic individuals at the Joslin Diabetes Center. Relative quantitation of fasting plasma BCAA was performed; levels are relative to the median of the IS group. Data are mean ± SEM. One-Way ANOVA was applied to detect statistical significance between groups; statistical significant features were further analyzed by post-hoc Dunnet's test: [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.005$, [d]$p < 0.0001$ vs IS.
Bold indicates $p < 0.05$.

ments in insulin sensitivity [16,17], and changes in BCAA are correlated with improved insulin sensitivity after exercise training [18]. Elevations in branched-chain and aromatic amino acids can predict T2D [19] and are also associated with progression of insulin resistance in children in longitudinal cohort studies. Despite these intriguing associations, it remains incompletely understood whether elevations in BCAA in insulin resistant humans reflect chronically increased intake, altered gut microbiome [20], resistance to insulin's ability to suppress proteolysis, or alterations in their metabolism [21], possibly mediated in part by hypothalamic insulin resistance [22]. Furthermore, increases in BCAA may directly modulate insulin action or Tor-dependent nutrient signaling, as seen in cultured cells and animals [23,24].

Emerging data suggest that perturbations in metabolism of BCAA may not only promote increases in plasma BCAA levels but also contribute to insulin resistance [10,21,25]. BCAA are converted into their corresponding branched-chain α-ketoacids (BCKA) by the BCAA transaminase. BCKA are further catabolized within the mitochondrial matrix by the branched-chain α-ketoacid dehydrogenase (BCKDH) complex; a subsequent series of metabolic reactions yield acetyl-CoA (from isoleucine and leucine) and methylmalonyl-CoA (from valine and isoleucine). Methylmalonyl-CoA is then converted by methylmalonyl-CoA mutase (MUT) to succinyl-CoA, which can be incorporated into the tricarboxylic acid (TCA) cycle or enter complex II of the electron transport chain. Thus, altered BCAA metabolism may impact anaplerotic flux into the TCA. Indeed, Adams and colleagues demonstrated elevations in BCAA, but reductions in amino acid-derived carnitine species, in plasma from insulin resistant women and suggested that "anaplerotic stress" could contribute to incomplete fatty acid oxidation [12,26]. Interestingly, recent metabolomics studies revealed correlations between plasma levels of the three BCKA derived from BCAA and both insulin resistance and elevated fasting plasma glucose [27]. Thus, alterations in BCAA oxidative metabolism rather than BCAA concentrations *per se* may be more closely associated with insulin resistance. Using an integrative approach to analyze gene expression in parallel with metabolite profiling in skeletal muscle of insulin resistant humans, we now demonstrate that insulin resistance is associated with alterations in both BCAA and lipid metabolism. Moreover, experimental modulation of BCAA metabolism in cultured cells and in mice also perturbs lipid metabolism. Together these studies provide novel insights into the mechanisms underlying the association of BCAA with dysregulated metabolism in skeletal muscle.

2. METHODS

2.1. Study design

The aims of the observational human study were to test the hypothesis that muscle gene expression patterns would differ in individuals with established T2D in comparison to healthy controls. Participants with or without T2D were recruited based on family history of T2D (either or both parents with T2D). Results from these analyses were previously reported [28]. In secondary analyses, we aimed to identify genes and pathways correlating with insulin sensitivity and thus potentially pathogenic for T2D risk. We now report data from these analyses, which formed the basis of the hypotheses further tested in animal and cell culture models in the current report. The demographic and clinical characteristics of the participants are shown in Table 1. The Joslin Diabetes Center Institutional Review Board approved the human study; written informed consent was received from participants prior to inclusion in the study.

2.2. Human metabolic characterization and muscle biopsies

Written informed consent was obtained from participants. All subjects had normal coagulation, liver function, and no other major systemic illness. Individuals with and without established T2D were recruited for the study. Subjects with diabetes were treated with dietary measures and exercise, and were drug-naïve for diabetes medication. All participants without a history of diabetes underwent a 75 g glucose tolerance test to exclude impaired glucose tolerance or diabetes, analyzed according to World Health Organization criteria [29]. An intravenous glucose tolerance test was performed on a non-sequential day on all subjects without diabetes; data were analyzed using Minimal Model software (MINMOD) [30] for calculation of S_I. Subjects without diabetes were classified as insulin sensitive or insulin resistant on the basis of S_I values above or below median (4.79) for a larger population of normal glucose tolerant individuals studied at Joslin [31]. Since S_I values were not normally distributed over the subject population, values were log-transformed, and log S_I was chosen as the primary metabolic variable for correlation analyses. Fasting blood samples were obtained for insulin, glucose, cholesterol and liver enzymes. Following local anesthesia with 1% lidocaine, percutaneous biopsies of vastus lateralis muscle were performed, using a triport cannula and Bergstrom needle. Samples were blotted free of blood, connective tissue and visible fat, frozen in liquid nitrogen, and stored at −80 °C until further analysis.

2.3. RNA isolation and microarray analysis

Total RNA was isolated from frozen muscle after homogenization with a Polytron (Brinkmann Instruments) in TRIzol® (Invitrogen, Carlsbad, CA), using high-salt precipitation modification. RNA was purified using RNeasy® columns (Qiagen, Chatsworth, CA). An equal number of samples from all three groups were included at all steps to minimize risk of technical bias. Five μg of DNAse I-treated total RNA from each of these samples was used to generate double-stranded cDNA (SuperScript Choice, Invitrogen), followed by *in vitro* transcription (ENZO BioArray RNA labeling kit, Affymetrix, Santa Clara, CA). Five μg of

