Metabolomic Analysis Reveals Altered Skeletal Muscle Amino Acid and Fatty Acid Handling in Obese Humans

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Objective: Investigate the effects of obesity and high-fat diet (HFD) exposure on fatty acid oxidation and TCA cycle intermediates and amino acids in skeletal muscle to better characterize energy metabolism.

Methods: Plasma and skeletal muscle metabolomic profiles were measured from lean and obese males before and after a 5-day HFD in the 4 h postprandial condition.

Results: At both time points, plasma short-chain acylcarnitine species (SCAC) were higher in the obese subjects, while the amino acids glycine, histidine, methionine, and citrulline were lower in skeletal muscle of obese subjects. Skeletal muscle medium-chain acylcarnitines (MCAC) C6, C8, C10:2, C10:1, C10, and C12:1 increased in obese subjects, but decreased in lean subjects, from pre- to post-HFD. Plasma content of C10:1 was also decreased in the lean but increased in the obese subjects from pre- to post-HFD. CD36 increased from pre- to post-HFD in obese but not lean subjects.

Conclusions: Lower skeletal muscle amino acid content and accumulation of plasma SCAC in obese subjects could reflect increased anaplerosis for TCA cycle intermediates, while accumulation of MCAC suggests limitations in β-oxidation. These measures may be important markers of or contributors to dysregulated metabolism observed in skeletal muscle of obese humans.

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Introduction

Almost 70% of adults in the United States are overweight or obese (1), and obesity-related medical costs exceed $147 billion annually (2). As a consequence, investigators have focused on identifying factors that underlie the pathophysiology of obesity including insulin resistance and inflammation. Novel technologies, including metabolomics combined with functional and molecular-based genetic testing, have provided insight into the role of mitochondria in these processes. There are marked differences in substrate metabolism between normal-weight and obese cohorts, including fatty acid β-oxidation, glucose oxidation, amino acid catabolism, and the tricarboxylic acid (TCA) cycle flux (3-6); however, the mechanisms are complex and are just beginning to be explored.

Our previous studies in obese humans suggest that there is a decrement in the ability to oxidize fatty acids in skeletal muscle, particularly in the face of excess lipid load both in vivo and in vitro (7-9). While many have investigated obesity-related reductions in mitochondrial biogenesis and respiratory chain capacity (8,10,11), less is known about the dysregulation of skeletal muscle β-oxidation, intermediary metabolism of amino acids, and the TCA cycle in human obesity. Reduced β-oxidation and alterations in related metabolites have been observed in adipocytes (4,12) and plasma (3) in obese individuals, suggesting a global dysregulation of β-oxidation. However, very few studies have assessed the impact of nutritional challenge on metabolic intermediates in skeletal muscle, which may uncover additional deficiencies with obesity. Here, we hypothesize that an in-depth metabolomic analysis of plasma and skeletal muscle lipids and amino acids following a high-fat diet (HFD) would reveal dynamic markers of key regulatory metabolic intermediates and possible blocks in mitochondrial β-oxidation or TCA cycle function that characterize skeletal muscle of obese individuals (5,6,11). The unique biomarkers presented here suggest the novel hypothesis that
changes in plasma and skeletal muscle amino acid concentrations reflect a greater reliance on anaplerosis in cellular oxidative energy production in obese humans when challenged with a HFD.

Methods

Participants

Participants were lean (n = 6; BMI ≤24.9 kg/m²) and obese (n = 6; BMI ≥30 kg/m²) young Caucasian males (19-27 years) who were otherwise healthy, nonsmokers, and not taking medications known to alter metabolism. The present study represents a subset of a larger population already described (7). Subjects completed a health history prior to the study and were excluded if they had received prior medical diagnosis or treatment for metabolic diseases, or if they engaged in >60 min/week of planned physical activity. Participants maintained constant body mass (±2 kg) in the 6 months prior to the study. In accordance with the Declaration of Helsinki, the protocol was approved by the East Carolina University Policy and Review Committee on Human Research. Written informed consent was obtained prior to participation. Samples were obtained at the Human Performance Laboratory at East Carolina University.

Habitual dietary consumption was assessed by 3-day diet record with exclusion for subjects habitually consuming >40% kcal from fat. For screening measures, participants reported to the laboratory between 1 and 14 days prior to the start of the HFD. Following an overnight fast, a venous blood sample was drawn and body composition was measured using dual energy X-ray absorptiometry (DEXA, Hologic, Waltham, MA). Plasma was separated for subsequent analyses of glucose (YSI 2300 STAT Plus Glucose and Lactate Analyzer, YSI; Yellow Springs, OH) and insulin (Access Immunoassay System, Beckman Coulter; Fullerton, CA) and a homeostasis model assessment (HOMA-IR) value (fasting glucose [mmol/L] × fasting insulin [μU/mL]/22.1) was calculated (13).

Study design

We measured plasma and skeletal muscle content of amino acids, acylcarnitine species, and skeletal muscle content of TCA cycle intermediates 4 h after a single high-fat meal, which was chosen based on the standard time definition for postprandial testing (14), and after five consecutive days of a eucaloric HFD (pre- and post-HFD, respectively) to determine whether metabolic adaptation to a nutritional challenge is different in lean versus obese individuals. Participants reported to the laboratory between 0630 and 0800 h after a 12 h overnight fast on days 1 and 6. Participants were given a high-fat test meal designed to contain 65% fat, 15% protein, and 25% carbohydrate and comprise 35% of daily energy intake. Participants were instructed to refrain from eating or drinking anything except water before returning to the laboratory 4 h later. At this time a venous blood sample and vastus lateralis skeletal muscle biopsy were obtained and processed as described (7). Participants then consumed the prescribed HFD for 5 consecutive days (∼50% of the diet was provided). The diet was designed to contain 65% fat, 15% protein, and 25% carbohydrate. On experimental day 6, participants reported to the laboratory and repeated the assessments of day 1, including the consumption of the high-fat test meal 4 h prior to muscle biopsy. Adherence to the HFD was assessed by food diary, direct, and daily verbal communication with the subjects, and level of ketosis as determined by fasting plasma β-hydroxybutyrate levels as reported previously. Daily caloric requirements were estimated based on the Harris-Benedict equation (15). Also of note, and previously reported, there was no difference between the two groups regarding plasma nonesterified fatty acids before or after the 5 day HFD (7).

Biochemical analysis

Methods for sample preparation are as described previously (16). Measurement of free carnitine, acylcarnitines, and amino acids in plasma and muscle was completed by direct-injection electrospray tandem mass spectrometry (MS/MS), using a Micromass Quattro Micro LC-MS system (Waters-Micromass, Milford, MA) equipped with a model HTS-PAL 2777 auto sampler (Leap Technologies, Carrboro, NC), a model 1525 HPLC solvent delivery system (Agilent Technologies, Palo Alto, CA) and a data system running MassLynx 4.0 software (Waters Corporation, Milford, MA). Skeletal muscle organic acids, including lactate, succinate, fumarate, malate, citrate, and alpha-ketoglutarate (17), were quantified using methods described previously employing Trace Ultra GC coupled to a Trace DSQ MS operating under Excalibur 1.4 (Thermo Fisher Scientific, Austin, TX) (18).

mRNA content

Total RNA was isolated from ∼30 mg of frozen skeletal muscle tissue and quantitative PCR was performed and analyzed as described (7). Targeted gene expression was carried out pre- and postdiet in the postprandial state for citrate synthase (CS), carnitine palmitoyl transferase (CPT)-1, pyruvate dehydrogenase kinase (PDK) 4, fatty acid transporter CD36, uncoupling protein (UCP) 3, nuclear receptor coactivator (NCOA) 1, NCOA2, and transcription factors PPARα, PPARγ, PPARγ2, and PPARγ coactivator (PGC)-1α. These genes were selected as they are regulators of fatty acid oxidative metabolism and/or mitochondrial content and appear to be altered with obesity (19).

Statistical analyses

Independent t tests were used to determine differences between characteristics of lean and obese individuals in the pre-HFD condition. A one-tailed, paired t test was used to assess increases in fasting β-hydroxybutyrate levels. Repeated measures analyses of variance (ANOVA) were used to analyze metabolites between lean and obese subjects measured before and after the HFD, for BMI, diet, and interaction effects. All analyses were performed on raw or log transformed data where appropriate. Statistical significance was denoted at the P ≤0.05 level. Data are presented as the mean ± SEM. Statistical analyses were performed using SPSS Statistics software (IBM Corporation, Armonk, NY).

Results

Participant characteristics and HFD composition

Participant characteristics are shown in Table 1. By design, the obese individuals weighed more, had a higher BMI, and higher percent body fat than the lean individuals (P < 0.05). Obese individuals also had higher fasting insulin levels and higher HOMA-IR scores (P < 0.05). No differences were observed for circulating cholesterol or triglycerides. There were no differences in habitual macronutrient
consumption, or in HFD composition between groups. The high-fat meal comprised 34.4 ± 0.4% of daily kcal requirements and contained 63.7 ± 0.2% fat (18.8 ± 0.1% saturated, 56.2 ± 0.1% monounsaturated, 24.1 ± 0.1% polyunsaturated), 25.1 ± 0.1% carbohydrate, and 14.4 ± 0.1% protein. The HFD contained 62.8 ± 0.9% fat, 21.9 ± 0.9% carbohydrate, and 14.9 ± 0.3% protein. Fasting plasma β-hydroxybutyrate levels were increased from pre- to post-HFD (0.28 ± 0.08 and 0.42 ± 1.0 mmol/L, respectively; P < 0.05).

**Amino acid and TCA metabolites**

Skeletal muscle amino acid profiling revealed that several amino acids were lower in the obese individuals in both pre- and post-HFD conditions (glycine, histidine, methionine, citrulline; Figure 1A and B; P < 0.05). Proline also tended to be reduced in skeletal muscle with obesity (P = 0.07). For plasma measures, serine and histidine were reduced in the obese individuals (Figure 2A); while serine and glycine tended to be lower in the obese individuals (P < 0.07). Interestingly, plasma serine, asparagine, and glutamine all tended to show an effect of the HFD treatment, where both lean and obese groups tended to increase the content of these amino acids from pre- to post-HFD (P < 0.10). As shown in Figure 1C, skeletal muscle α-ketoglutarate increased in the lean individuals from pre- to post-HFD conditions, though decreased in the obese individuals across the same time points (P < 0.05).

**Acylcarnitine profile**

Skeletal muscle acylcarnitine profiling revealed differences between lean and obese individuals in response to the HFD. While the lean individuals exhibited decreases in C4/4i in response to the HFD, the obese individuals showed increased C4/4i from pre- to post-HFD (Figure 3B; P < 0.05). This same interaction effect, in which levels were decreased following the HFD in lean subjects but increased in the obese, was evident for skeletal muscle C6, C8, C10:2, C10:1, C10, and C12:1 measures (Figure 3B,C; P < 0.05). Lastly, both skeletal muscle C8 and C10:1 levels were consistently lower in obese compared with lean subjects at both time points (Figure 3C; P < 0.05). No differences in any of the long-chain acylcarnitine species were noted between or among subjects (Figure 3D).

As shown in Figure 4, there were no differences in plasma free carnitine or acetyl carnitine (C0, C2) between lean and obese subjects.

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**TABLE 1 Participant characteristics**

|                     | Lean (n = 6) | Obese (n = 6) |
|---------------------|-------------|---------------|
| Age (years)         | 21.1 ± 0.7  | 22.6 ± 1.1    |
| Weight (kg)         | 74.8 ± 2.4  | 128.0 ± 6.2a  |
| BMI (kg/m²)         | 22.3 ± 0.8  | 37.8 ± 1.6a   |
| Body fat (%)        | 15.9 ± 3.1  | 38.6 ± 3.0a   |
| Glucose (mmol/L)    | 4.7 ± 0.3   | 4.9 ± 0.2     |
| Insulin (µU/L)      | 0.9 ± 0.2   | 2.9 ± 0.7a    |
| Total cholesterol (mg/dL) | 162.5 ± 9.0 | 176.2 ± 20.4 |
| LDL (mg/dL)         | 87.0 ± 4.0  | 88.8 ± 22.0   |
| HDL (mg/dL)         | 53.0 ± 4.3  | 41.0 ± 3.7    |
| Triglycerides (mg/dL) | 112.3 ± 21.0 | 143.4 ± 43.2 |

Data are mean ± SEM.

*Significant difference from lean group (P < 0.05).
nor were there any effects of the HFD. Both plasma propionylcarnitine (C3) and isobutyrylcarnitine (C4/4i) were elevated in the obese compared with lean subjects (Figure 4B; $P < 0.05$). Unlike skeletal muscle measures, plasma C4/4i was increased in response to the HFD exposure in both lean and obese groups (Figure 4B; $P < 0.05$), while both C3 and C20 tended to increase in response to the HFD.
in both groups (Figure 4B,D; \( P = 0.09 \)). Similar to skeletal muscle measures, plasma medium-chain C10:1 was reduced in lean subjects in response to the HFD, but was increased in obese individuals from pre- to post-HFD (Figure 4C; \( P < 0.05 \)). Again, as with skeletal muscle tissue, there were no differences or changes in plasma long-chain acylcarnitines (Figure 4D).

mRNA content in skeletal muscle tissue
mRNA content of the transcriptional co-activators NCOA2 was reduced in the obese individuals at both time points (Figure 5; \( P < 0.05 \)) and PPAR\( \delta \) tended to be reduced (\( P = 0.08 \)). CD36 levels decreased as a result of the HFD in the lean individuals, but increased from pre- to post-HFD in the obese subjects (Figure 5; \( P < 0.05 \)).

Discussion
Altered skeletal muscle metabolism has been widely reported in obese and insulin resistant individuals (4-6,9,11,20). However, little is known about the cellular pathways involved, whether entire pathways or select enzymes are affected, or the energetic consequences in mitochondrial metabolism. Previously, we reported that consumption of a HFD induced accumulation of combined medium-chain acylcarnitine species (MCAC) in obese, but not lean skeletal muscle (7). Here we identified specific key acylcarnitine species and amino acids that were lower in the postprandial state in skeletal muscle and plasma of obese individuals. Likewise, content shifts in these metabolites were different in the obese, compared with lean subjects in response to the 5 day HFD. These results support our previous reports of reduced fatty acid oxidation in fasting skeletal muscle of obese versus age and gender matched lean individuals (6,9), and suggest that metabolic dysregulation involving specific amino acids, acylcarnitine species, and TCA cycle intermediates are indicative of important anaplerotic events. Conversely, these changes could be accounted for by altered amino acid transport, total amino acid body content, or dietary protein bioavailability in lean versus obese subjects. Overall, these results are the first to demonstrate a dysregulation of both fatty acid and amino acid metabolism in skeletal muscle in response to dietary lipid intake in obese humans.
A large body of literature supports derangements in amino acid concentrations, and especially branched chain amino acids (BCAA), in circulating plasma (21,22) as well as within skeletal muscle (23,24) and adipose tissues (12) in obese, insulin resistant humans. However, the role of amino acids in skeletal muscle mitochondrial metabolism, particularly in obese subjects following a dietary challenge, is still being explored. In contrast to other studies (4,24,25), we did not observe greater content of BCAA leucine/isoleucine in either skeletal muscle or plasma of our obese subjects. This may be due to the fact that most previous measures, in both animals and humans, have been taken in the overnight fasted state. In an attempt to preclude the effects of fasting induced increases in lipid oxidation, and the acute effects of a meal, we made our measures in the 4 h postprandial state (14). As demonstrated in previous reports (3,4,26,27), we observed increased plasma content of C3 (propionylcarnitine) and C4i (isobutyrylcarnitine) in the obese subjects and these metabolites correspond to the end products of BCAA catabolism (4,28,29). Interestingly, propionic acid and isobutyric acid are also known to be produced by gut flora (30), and increased content of these metabolites may indicate intrinsic difference in the gut microbiome between lean and obese individuals (31). Indeed, recent evidence links marked differences in bacterial populations between normal-weight and obese individuals that correspond to elevated C3 and C4/C4i (31,32).

Skeletal muscle content of amino acids did not appreciably change with the HFD exposure in either the lean or obese individuals. Nevertheless, we did observe lower content of several amino acids in obese compared with lean individuals in both pre- and post-HFD measures, including glycine, histidine, methionine, and citrulline. Though these amino acids are not directly related in metabolic pathways, many, if not all, of these amino acids ultimately feed into the TCA cycle in the setting of anaplerosis (33). Recent work in adipose tissue shows that amino acids are lower in skeletal muscle of obese individuals because they are being used to replenish TCA cycle intermediates and maintain oxidative metabolism, whereas lean individuals are anabolic in the postprandial state. Corroborating this hypothesis, increased plasma content of short-chain acylcarnitine species (SCAC) in the obese subjects could indicate amino acid anaplerosis, though more work is needed to confirm this.

While long-chain fatty acylcarnitine species (LCAC) concentration reflects transport and proximal β-oxidation efficiency (i.e., including and upstream of CPT-1), MCAC track more distal β-oxidation efficiency (i.e., downstream of CPT-1) (28,29), with sentinel species C6, C8, C10, and C10:1 typically used in evaluating MCAD activity and flux through the β-oxidation pathway (28). The SCAC are indicators of anaplerosis (4,28,29). Here, we found that several MCAC were lower in skeletal muscle of the obese compared with lean individuals at both time points and, perhaps more importantly, while many MCAC were decreased in lean individuals from pre- to post-HFD, these same MCAC were increased in obese subjects. Such MCAC variation in response to the HFD could indicate differences in MCAC flux through β-oxidation, where medium-chain acyl CoA dehydrogenase (MCAD) activity may be reduced. Alternatively, increased delivery of fatty acids to the mitochondria of the obese subjects could explain increases in MCAC from pre- to post-HFD. Despite our attempts to preclude immediate postprandial nutrient effects, it is also possible that there is increased substrate competition with glucose and/or amino acids downstream of β-oxidation that results in accumulation of SCAC and MCAC in the obese individuals. Nevertheless, our results point to distinct differences in fatty acid handling between lean and obese individuals as a result of a short-term HFD. We have previously reported accumulation of partially oxidized fatty acid metabolites in skeletal muscle from obese individuals (34), which would support the notion of reduced fatty acid flux through β-oxidation and/or the TCA cycle, rather than increased fatty acid influx. This interpretation is also supported by our observation of decreased glycine in both plasma and skeletal muscle of the obese versus lean individuals. Previous reports of low glycine in plasma of obese (22) and insulin resistant humans (4) remain largely unexplained, though it has been postulated that this could be the result of increased glycine incorporation into acylglycine compounds generated from surplus fatty acid oxidation intermediates. Alternatively, glycine is a known precursor of glutathione, and may be related to antioxidant response in the face of increased oxidative stress generated by incomplete β-oxidation (35).

Of the TCA cycle intermediates and substrates we measured, only α-ketoglutarate demonstrated differential responses to the HFD,
where content increased in lean but decreased in the obese subjects. We previously reported elevated pyruvate in skeletal muscle with obesity and suggested that this reflected a maximal rate of lipid oxidation both pre- and post-HFD in the obese subjects (7). However, in lean subjects, pyruvate increased from pre- to post-HFD, which corresponded to increases in mRNA content of β-oxidation associated genes (7). The pattern of α-ketoglutarate, reported here for the first time, follows the same pattern as pyruvate in obese subjects (i.e., reduced in response to the HFD, while lean individuals exhibit increases in α-ketoglutarate from pre- to post-HFD), while the other TCA intermediates do not change or differ significantly between groups or conditions. Both α-ketoglutarate and pyruvate are two key TCA metabolites that should increase concomitant with β-oxidation; α-ketoglutarate because of increased flux through the proximal TCA cycle and pyruvate secondary to inhibition of pyruvate dehydrogenase complex (PDC). For both pre- and post-HFD samples, we observed increased pyruvate in the skeletal muscle of obese, compared with lean individuals. Previous observations, by ourselves and others, show reduced lipid oxidation in skeletal muscle with obesity (6,9,20,36), though these measures were made under fasted or in vitro conditions. Few, if any, have investigated postprandial β-oxidation in lean and obese subjects. Those who have measured postprandial β-oxidation in individuals with type 2 diabetes, or a family history of type 2 diabetes, and have shown reduced β-oxidation in these individuals compared with nondiabetic subjects (20,37). Without measures of β-oxidation in our cohort, we cannot definitively state whether elevated skeletal muscle pyruvate is associated with an isolated increase in β-oxidation or reduced β-oxidation and a concomitant dysregulation of the PDC system. After the HFD, we observed increased α-ketoglutarate in the lean, but decreased α-ketoglutarate in the obese individuals. Reduced α-ketoglutarate may be the result of diminished proximal TCA substrate, secondary to a lower rate of β-oxidation in skeletal muscle from the obese subjects (specifically at the level of MCAD). Alternatively, this could be the result of increased distal TCA cycle flux in the form of anaplerotic metabolism and increased succinate dehydrogenase activity. However other TCA intermediates are not altered and prior literature documents lower succinate dehydrogenase activity in skeletal muscle of obese humans (38). Thus, our hypothesis, based on MCAC differences in obese skeletal muscle, is that reduced or incomplete β-oxidation is the primary reason for reduced α-ketoglutarate levels in skeletal muscle of the obese subjects after the HFD.

LCAC species were decreased in both the lean and obese individuals in response to the HFD. This suggests either inhibition of CPT-1 (i.e., less fatty acid influx) or that flux limitations occur at more distal steps of the pathway (i.e., downstream of LCAC metabolism). One alternative fate of skeletal muscle LCAC precursors, particularly in the face of β-oxidation flux limitations, is ectopic storage. Based on the qualitative observation of higher amounts of intramyocellular lipid storage in the muscle biopsies of obese subjects (38), it is possible that excess fatty acids are being stored within skeletal muscle tissue of the obese individuals, thus presenting with a reduction in LCAC from pre- to post-HFD. Our results showing an increase in mRNA content of CD36 fatty acid transporter in the obese individuals following the HFD, in contrast to a decrease in the lean subjects, suggest that obese individuals may have greater fatty acid uptake into the cell following the HFD. However, CD36 translocation to the cell membrane also plays an important role in its function (39). Thus, at present it is difficult to draw meaningful conclusions about CD36/fatty acid interactions in response to the HFD in either lean or obese individuals.

In conclusion, reduced skeletal muscle amino acid content and accumulation of SCAC in obese subjects could reflect increased anaplerosis for TCA cycle intermediates, while accumulation of MCAC suggests fatty acid flux limitations. Thus, these measures may be important metabolic markers or potential contributors to the disorder metabolism observed in skeletal muscle of obese humans. While the HFD exposure likely affects multiple pathways of whole body metabolism differently in lean versus obese individuals, there are clear differences in skeletal muscle composition of fatty acid and amino acid metabolites that warrant mechanistic investigation. Direct flux measurements of MCAC in primary myotubes derived from obese humans may help define their effects on disordered mitochondrial metabolism and persistent weight gain in obese individuals.

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