Effects of Dietary Carbohydrate Restriction with High Protein Intake on Protein Metabolism and the Somatotropic Axis

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Context: Alterations in dietary macronutrient intake can influence protein turnover.

Objective: The purpose of this study was to assess the influence of a low-carbohydrate/high-protein diet (LC/HP) on skeletal muscle protein synthesis and whole-body protein turnover, without the confounding influence of a negative energy balance.

Design: Nine-day dietary intervention was applied.

Setting: Subjects remained in the General Clinical Research Center throughout the 9-d study.

Participants: Eight young, healthy volunteers participated.

Intervention: Subjects ate a typical Western diet (60% carbohydrate, 30% fat, 10% protein) for 2 d, followed immediately by 7 d of an isocaloric LC/HP (5% carbohydrate, 60% fat, 35% protein).

Main Outcome Measures: Skeletal muscle fractional synthetic rate and whole-body proteinolysis (leucine rate of appearance in plasma [Ra]) were measured after an overnight fast before and after 2 and 7 d of LC/HP. We also measured plasma concentrations of insulin, GH, and IGF-I.

Results: Leucine Ra was increased (P = 0.03) after 2 and 7 d of LC/HP, and muscle fractional synthetic rate was approximately 2-fold higher (P < 0.01) after 7 d of LC/HP. Fat free mass was not altered by LC/HP. Average 24-h plasma insulin concentration was 50% lower (P < 0.001) after 2 and 7 d of LC/HP, whereas GH secretion and total plasma IGF-I concentrations were unchanged with LC/HP. However, plasma free IGF-I decreased by approximately 30% after 7 d of LC/HP (P = 0.002), whereas muscle IGF-I mRNA increased about 2-fold (P = 0.05).

Conclusions: Increasing dietary protein content during a 7-d carbohydrate restricted diet stimulated muscle protein synthesis and whole-body proteinolysis without a measurable change in fat free mass. (J Clin Endocrinol Metab 90: 5175–5181, 2005)

Maintaining lean tissue mass is critical for the preservation of functional capacity and normal metabolic function. Alterations in dietary macronutrient intake can greatly affect the balance between tissue protein synthesis and protein degradation (i.e., proteolysis) (1), which is the primary determinant for the maintenance of lean tissue. Specifically, dietary carbohydrate restriction has been found to increase nitrogen excretion, which is believed to be indicative of protein loss (2). On the other hand, high protein intake has been found to increase protein synthesis (3), suggesting that the ingestion of a high-protein diet during carbohydrate restriction may compensate for the protein loss associated with low-carbohydrate diets. However, despite the reemerging popularity of low-carbohydrate/high protein diets (LC/HP), little is understood about the effects of marked alterations in these macronutrients on protein metabolism.

Many of the metabolic effects of a low-carbohydrate diet are attributed to the chronic suppression of plasma insulin. Insulin is considered a potent anabolic hormone, primarily through its role in blunting proteolysis (4), but it has also been shown to aid in the stimulation of protein synthesis under certain conditions (5–7). Protein synthesis is responsive to growth factors, such as IGF-I, through distinct signal transduction pathways, including the protein kinase mammalian target of rapamycin (mTOR) signaling pathway (6). The mTOR pathway regulates protein synthesis through activation of downstream signaling intermediates. Phosphorylation (i.e., activation) of downstream targets of mTOR [e.g., eukaryotic initiation factor (eIF)4G, ribosomal protein S6] is associated with an enhanced rate of protein synthesis (8, 9). However, the effect of carbohydrate restriction combined with a high protein intake on the secretion of insulin, GH, and IGF-I and mTOR signaling is not clearly understood.

The primary purpose of this study was to determine the influence of dietary carbohydrate restriction, in combination with a high protein intake, on skeletal muscle protein synthesis and whole-body protein degradation. We also examined the influence of this diet on GH and insulin secretion, plasma levels of IGF-I, and skeletal muscle IGF-I mRNA to determine whether alterations in these anabolic factors were associated with diet-induced changes in protein metabolism. Finally, we evaluated the effect of the LC/HP on the phos-
phosphorylation state of key signaling proteins within the mTOR pathway (i.e. S6 and eIF4G).

Subjects and Methods

Experimental subjects

Eight healthy subjects (four men and four women) volunteered for participation in this study (age, 29 ± 4 yr; body mass index, 24.2 ± 1.1 kg/m²). Subjects had no evidence of metabolic or cardiovascular disease, were not taking any prescription medications, and were sedentary (i.e.< 1 h exercise/wk) before their involvement in the study. All women were premenopausal, and studies were performed within the first 2 wk of the follicular phase of their menstrual cycle. All subjects were fully informed of the possible risks associated with the study and signed an informed consent form approved by the University of Michigan Institutional Review Board.

General experimental design

Subjects were admitted to the General Clinical Research Center at the University of Michigan Hospital at 1200 h, 2 d before beginning the week-long experimental LC/HP, and they remained in the hospital until the completion of the 1-wk experimental diet (a total of 9 d). Body composition was assessed using dual-energy x-ray absorptiometry before and at the end of the experimental diet. Postabsorptive resting energy expenditure (REE) was measured during a 30-min screening visit and at 0630 h each morning of the study, using a metabolic cart (Delta-Trac; SensorMedics, Yorba Linda, CA). Oxygen consumption and carbon dioxide production were measured for at least 20 min while subjects were lying supine, awake but undisturbed in a dark, quiet room. Subjects ate a standardized Western diet for the first 2 d of their hospital stay and a LC/HP for the next 7 d (see Dietary intervention below for more detail). We assessed whole-body and skeletal muscle protein metabolism using tracer isotope techniques on three separate occasions during their hospital visit: 1) immediately before starting the LC/HP, 2) after 2 d on the diet, and 3) after 1 wk of the diet (see Measurement of protein kinetics for more detail). Plasma insulin concentrations were measured hourly throughout the day and every 2 h during the overnight period. Additionally, we measured plasma insulin concentration every 20 min for 2 h after each meal. Plasma GH concentrations were measured at 20-min intervals over a 24-h period starting at 0700 h before introducing the low carbohydrate diet, as well as on the second and seventh days of the diet.

Dietary intervention. After being admitted to the hospital, subjects ate a standardized diet for 2 d, consisting of 60% of total energy from carbohydrate, 30% fat, and 10% protein, and a daily caloric content estimated to maintain body weight [i.e. kcal = 1.3 × REE (10)]. This diet approximates the macronutrient content of a normal diet in the United States (11), and using this diet as a control allows us to best evaluate the metabolic consequences of a LC/HP. During the 1-wk LC/HP, subjects consumed a weight-maintaining (i.e. kcal = 1.3 × REE) diet consisting of 5% of total energy from carbohydrate, 60% fat, and 35% protein. Daily caloric content for both the standardized control diet and the LC/HP were 40 ± 2 kcal/kg fat free mass (28 ± 1 kcal/kg body weight). On most days, meals were served at 0800, 1300, and 1830 h. However, on days in which protein kinetics were measured, the meals were delayed until 1300, 1645, and 1945 h.

Measurement of protein kinetics. We measured skeletal muscle protein fractional synthetic rate (FSR) before the LC/HP and again after 7 d of the diet. Due to technical limitations, muscle FSR was determined in six subjects. Whole-body protein turnover was also measured immediately before the carbohydrate restricted diet, after 2 d of the diet, and again after 7 d of the diet. At 0600 h in the morning of each of these days, a Teflon catheter was inserted into the antecubital vein of one arm for isotope infusion. Plasma insulin concentration was measured every 20 min intervals over a 24-h period starting at 0700 h before introducing the low carbohydrate diet, as well as on the second and seventh days of the diet.

Analytical methods

Leucine rate of appearance (Ra). Whole-body protein turnover was determined at 0830 h and again at 1300 h for measurement of [1-3H]–leucine incorporation into skeletal muscle. Arterialized blood samples were taken at the following times: 0700 h in the morning of each day, 0800 h, 1300 h, and 1830 h (no. 1 and 2) and at 0700 h, 0800 h, and 1300 h (no. 3). The supernatant was transferred to a new tube and added to 3 ml hexane and 3 ml distilled water, mixed gently for 15 min on a platform shaker and centrifuged for 15 min at 3000 rpm at 5 C. The upper (hexane) layer was then discarded, and the lower (aqueous) layer was dried down overnight under vacuum. Samples were derivatized with t-butyldimethylsilyl by adding 100 µl of a 1:1 acetonitrile:N-methyl-N-tert(butyldimethylsilyl)tri-fluoroacetamide solution and incubated for 30 min at 70 C. Samples were analyzed via electron ionization using a gas chromatograph-mass spectrometer (Agilent 6890 gas chromatograph with 5973 mass selective detector; Agilent Technologies, Inc., Wilmington, DE), and the abundance measured at mass-to-charge ratios (m/z) of 200 (m/z = m/0) and 203 m/z (m+3) was measured with selective ion monitoring.

Skeletal muscle FSR. Mixed muscle rate of protein synthesis was determined by evaluating the incorporation of [3,3-2H]–leucine in muscle samples from the vastus lateralis, according to the methods of Patterson et al. (13), using plasma α-ketosocaproate (α-KIC) enrichment as the precursor pool (14). Briefly, 20–30 mg muscle was minced and homogenized on ice in a 3% trichloroacetic acid solution and centrifuged at 2500 rpm for 15 min. The resulting pellet then underwent four rinses in 2 ml normal saline at 1 min each, and the protein concentration of the supernatant was then determined by centrifugation at 12,0000 g for 20 min, and the protein concentration of the supernatant was measured. Samples were then diluted 1:2 with Laemmli sample buffer (containing 5% β-mercaptoethanol) (Bio-Rad Laboratories, Inc., Hercules, CA) and boiled for 5 min before gel electrophoresis. Samples (40 µg protein) were then separated using 4–8% PAGE, and proteins were subsequently transferred to a nitrocellulose membrane and incubated in 5% milk/Tris-buffered saline + tween-20 blocking buffer for 1 h at room temp. After rinsing (3 × 10 min in Tris-buffered saline + tween-20), blots were incubated in rabbit anti-eIF4G (BL896; Bethyl Laboratories, Inc., Montgomery, TX), rabbit anti-phospho-S6 ribosomal protein (Ser235/236) (no. 2211; Cell Signaling Technology, Inc., Beverly, MA), rabbit anti-β6 ribosomal protein (no. 2212; Cell Signaling Technology, Inc.), or rabbit anti-phospho-S6 ribosomal protein (Ser235/236) (no. 2211; Cell Signaling Technology, Inc.) overnight at 4 C. Blots were then analyzed using incubation with antirabbit IgG coupled to horseradish peroxidase (Cell Signaling Technology, Inc.) and developed using chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). Blots were then analyzed using a densitometer (Bio-Rad Laboratories, Inc.).
Skeletal muscle IGF-I mRNA expression. We used real-time RT-PCR to assess the mRNA expression of IGF-I in skeletal muscle samples. Total RNA was isolated from approximately 25 mg frozen muscle by using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was DNase-treated (DNA-free; Ambion, Inc., Austin, TX) for purity, and total RNA was determined spectrophotometrically at 260 nm. First-strand cDNA was generated from 2 μg RNA using Maloney-Murine Leukemia Virus reverse-transcriptase (Invitrogen).

 Primer pairs for each gene were designed using DNASTAR (Madison, WI) (Lasergene) computer software. Gene sequences were obtained from GenBank: IGF-I, NM_000618, forward primer 5′-TCATTTTGCCTCTGCTGTGGT-3′ and reverse primer 5′-CCCTGGGGGATTTTTGACGTCGTG-3′; 261-bp product. A BLAST search for each primer was conducted to confirm homologous binding to the target gene.

 RT-PCR was performed with use of a DNA engine Opticon continuous fluorescence detection system (MJ Research, Inc., Watertown, MA). Samples for each gene were run simultaneously and in duplicate to control for amplification efficiency. For mRNA quantification, a real-time PCR mix of 2× SYBR Green PCR Master Mix (QIAGEN, Valencia, CA), forward and reverse primers (3 μM/liter) and cDNA (12 ng) were run for 36 cycles in a total vol of 25 μl. After the final cycle, samples were subjected to melting curve analysis to ensure the detection of only one product. Additionally, all amplification products were separated by agarose gel electrophoresis to validate the presence and size of the appropriate product. To account for variations in RNA input amounts and transcription efficiency, 18S mRNA was determined, and results were normalized to these values. mRNA levels were quantified for each gene via determination of the critical threshold value as described by Schmittgen et al. (16). Finally, the IGF-I mRNA expression for each sample was calculated relative to the IGF-I mRNA expression measured in a control muscle sample. This control muscle sample was taken from another healthy subject who did not undergo the dietary intervention, and the mRNA expression in this sample was measured concurrently with the muscle samples from the subjects in this study.

Calculations

Leucine Ra. Because tracer:tracee ratios were achieved during steady-state conditions, leucine Ra was calculated using the steady-state Steele equation (17). The average tracer:tracee ratio for each set of three samples taken in 5-min intervals before and during the leucine infusion (e.g. 1050, 1055, and 1100 h) was used to calculate Ra.

Skeletal muscle protein FSR. FSR was calculated as the rate of [3H]-leucine tracer incorporated into muscle protein using the plasma α-KIC enrichment as the precursor pool (14) and the following equation: FSR (%/h⁻¹) = ([E₂ – E₁] × 100)/[E₀ × (t₁ – t₀)], where E₀ and E₁ represent muscle [3H]-leucine enrichments after 6 h and 1 h, respectively, E₀ represents the average plasma [3H]-α-KIC enrichment, and (t₁–t₀) represents the time between muscle biopsies (i.e. 5 h).

Phosphorylation of S6 and eIF4G. To obtain a more accurate representation of the change in phosphorylation of these proteins with our dietary treatment, we normalized the expression of phosphorylated S6 and eIF4G relative to the total amount of S6 and eIF4G, respectively. Therefore, the phosphorylation state of S6 and eIF4G was calculated by dividing the measured amount of phosphorylated S6 and eIF4G by the total amount of each protein.

Statistical analysis

Plasma GH concentration profiles were analyzed by Cluster (version 7), with t statistics of 2 and 2 × 1 configurations for GH increase and decrease, respectively. Baseline GH concentrations were calculated as the mean of the lowest four samples (5%) in each series. A one-way ANOVA for repeated measures with Tukey post hoc analysis was used to determine differences in body weight, fat mass, fat free mass, leucine Ra, skeletal muscle FSR, S6 and eIF4G phosphorylation, plasma concentrations of insulin, GH, IGF-I, and IGFBP-3, as well as muscle IGF-I mRNA. We performed a log transformation on the skeletal muscle IGF-I data to compensate for the asymmetric distribution of values and the heterogeneous variances. P ≤ 0.05 was considered statistically significant.

Results

Body weight and fat free mass

The 1-wk LC/HP did not affect fat free mass, which was 50.3 ± 3.6 kg before and 49.9 ± 3.6 after the diet. Percent body fat (28.7 ± 4.7% and 28.3 ± 4.9%) and total fat mass (20.2 ± 3.0 kg and 19.7 ± 3.2 kg) were also not different before vs. after the LC/HP. However, the diet slightly, yet significantly, reduced total body weight from 70.5 ± 3.7 kg to 69.5 ± 3.6 kg (P = 0.01).

Plasma hormone concentrations and muscle IGF-I mRNA

Carbohydrate restriction combined with high protein intake markedly suppressed daily plasma insulin concentration (Fig. 1A). The 24-h insulin concentration (i.e. area under the curve) was more than 50% lower (P < 0.001) during the second day of the LC/HP (14 ± 1 μU/liter·h) vs. before the diet (28 ± 2 μU/liter·h) and remained suppressed after 1 wk of the diet (12 ± 1 μU/liter·h). Not surprisingly, the differences in insulin secretion occurred exclusively after the meals, whereas there was no significant difference in post-absorptive plasma insulin concentration before (7.0 ± 0.7 μU/ml) compared with after 2 d (6.3 ± 1.1 μU/ml), and 7 d (6.4 ± 1.0 μU/ml) of the LC/HP. Mean 24-h GH concentrations, measured in 20-min intervals for 24 h, were unchanged as a result of the LC/HP (Fig. 1B and Table 1). Additionally, no changes in discrete parameters of GH pulsatility (pulse

FIG. 1. A, Plasma insulin concentration for 24 h before the LC/HP and during d 2 and 7 of the diet. B, Plasma GH concentration in 20-min increments for 24 h before the LC/HP and during d 2 and 7 of the diet. Data are presented as mean ± se. Area under the curve (AUC) for insulin before the diet was greater than AUC after d 2 and 7 of the diet.
frequency, pulse amplitude, baseline GH) were detected during the study (Table 1). Similarly, postabsorptive plasma total IGF-I was not altered during the dietary intervention (Table 1). In contrast, 7 d of carbohydrate restriction combined with high protein intake reduced (P = 0.002) free IGF-I and IGFBP-3 concentrations (Table 1), whereas skeletal muscle expression of IGF-I mRNA increased about 2-fold (P = 0.046) (Fig. 2).

**Whole-body proteolysis and skeletal muscle protein synthesis**

Postabsorptive leucine Ra increased approximately 20% (P = 0.03) after only 2 d of the LC/HP and remained elevated above basal levels after 1 wk of the diet (Fig. 3). In parallel with the increase in proteolysis, skeletal muscle protein FSR of the vastus lateralis was nearly 2-fold higher (P < 0.01) after 7 d of the LC/HP (i.e. before, 0.049 ± 0.004%/h; after, 0.094 ± 0.013%/h) (Fig. 4). Despite the marked increase in skeletal muscle FSR in response to the dietary intervention, we found the diet had no effect on total content of either ribosomal protein S6 (2.54 ± 0.51 vs. 2.75 ± 0.43 arbitrary units) or eIF4G (3.71 ± 0.96 vs. 4.92 ± 1.23 arbitrary units). Moreover, the phosphorylation of these proteins was also not affected after 1 wk of the LC/HP (Fig. 5).

**Discussion**

Maintaining lean tissue mass is essential for the preservation of metabolic and overall health. Preventing a decline in lean tissue mass requires the rate of protein synthesis to be the same or greater than the rate of protein degradation. It has been suggested that adequate dietary carbohydrate and protein are both required for optimal protein balance (7).
increased phosphorylation of these mTOR signaling proteins. Specifically, the binding of eIF4E to eIF4G forms a complex involved in the regulation of translation initiation and is associated with amino acid-induced stimulation of protein synthesis (8, 29). Additionally, our findings of an elevated FSR in the absence of activation of specific mTOR signaling intermediates may reflect an adaptive response to chronic high protein availability combined with a relatively low 24-h exposure to plasma insulin, which may stimulate protein synthesis through an as-yet-undetermined mechanism (30).

The increase in muscle protein synthesis with our LC/HP was accompanied by an increase in protein degradation. Insulin potently inhibits protein degradation (4, 26) and low plasma insulin concentration during fasting (31) or, in type 1 diabetics (32), is associated with an enhanced rate of whole-body proteolysis. Contrary to our findings, Bisschop et al. (2) reported no change in postabsorptive leucine Ra after 11 d of a low-carbohydrate diet, despite lower postprandial and postabsorptive insulin concentrations. It remains uncertain why a reduction in plasma insulin concentration did not correspond with an increased postabsorptive rate of proteolysis in their study. In the study by Bisschop et al. (2), nitrogen excretion was elevated after the carbohydrate-restricted diet, suggesting that overall protein degradation was greater than protein synthesis. One major difference between our study and that of Bisschop et al. (2) is that, in their study, they substituted dietary fat for carbohydrate, whereas protein content was not changed. Therefore, data from Bisschop et al. (2) suggest that dietary carbohydrate restriction, per se, does not affect postabsorptive proteolytic rate. Alternatively, our findings indicate that carbohydrate restriction in combination with a high protein intake can increase the postabsorptive rate of whole-body proteolysis. This is clinically relevant because recommendations for most low-carbohydrate diets suggest an increase in dietary protein content (33). Although it may seem counterintuitive, several studies have reported that increasing dietary protein content enhances the postabsorptive endogenous proteolysis (18, 34, 35). The mechanism(s) by which high protein intake may stimulate proteolytic rate is unknown but may be related to the increased rate of protein synthesis and the possible accretion of tissue protein found with high protein and/or high amino acid availability. Under our dietary conditions, the low 24-h plasma insulin concentration with carbohydrate restriction likely played an important role in increasing protein degradation.

Interestingly, the strict dietary carbohydrate restriction did not alter GH secretion. An energy deficit (i.e. fasting or a low-calorie diet) reduces hepatic IGF-I production with a resultant suppression in plasma IGF-I concentration (36). Consequently, because IGF-I is a potent inhibitor of GH secretion, plasma GH concentrations increase markedly in response to the reduction in IGF-I during energy deficit (36). In contrast, our strict carbohydrate restriction without an accompanying energy deficit led to a decrease in free IGF-I without a concomitant rise in plasma GH, suggesting that a different mechanism is responsible for regulating the soma-

![Fig. 5. Phosphorylation of ribosomal protein S6 (A) and eIF4G (B) in skeletal muscle before and after 7 d of the LC/HP. Data are presented as the amount of phosphorylated (phospho) protein divided by the amount of total protein. Representative blots are displayed for two subjects. Data are mean ± SE.](Image)
In conclusion, 1 wk of dietary carbohydrate restriction, in combination with an increase in protein intake, enhanced the rates of skeletal muscle protein synthesis and whole-body proteolysis. These alterations in protein kinetics were associated with a suppressed 24-h insulin exposure and an increase in muscle IGF-I expression but were independent of changes in plasma IGF-I and GH concentrations and activation of ribosomal protein S6 and eIF4G within skeletal muscle. Our results suggest that increasing dietary protein content during a carbohydrate restricted diet may be important for preventing or attenuating a net loss of body protein.

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