

# Effect of protein overfeeding on energy expenditure measured in a metabolic chamber<sup>1–3</sup>

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## ABSTRACT

**Background:** Energy expenditure (EE) increases with overfeeding, but it is unclear how rapidly this is related to changes in body composition, increased body weight, or diet.

**Objective:** The objective was to quantify the effects of excess energy from fat or protein on energy expenditure of men and women living in a metabolic chamber.

**Design:** We conducted a randomized controlled trial in 25 participants who ate ~40% excess energy for 56 d from 5%, 15%, or 25% protein diets. Twenty-four-hour EE (24EE) and sleeping EE (SleepEE) were measured on days 1, 14, and 56 of overfeeding and on day 57 while consuming the baseline diet (usually day 57). Metabolic and molecular markers of muscle metabolism were measured in skeletal muscle biopsy specimens.

**Results:** In the low-protein diet group whose excess energy was fat, the 24EE and SleepEE did not increase during the first day of overfeeding. When extra energy contained protein, both 24EE and SleepEE increased in relation to protein intake ( $r = 0.50$ ,  $P = 0.02$ ). The 24EE over 8 wk in all 3 groups was correlated with protein intake ( $r = 0.60$ ,  $P = 0.004$ ) but not energy intake ( $r = 0.16$ ;  $P = 0.70$ ). SleepEE was unchanged by overfeeding in the low-protein diet group, and baseline surface area predicted increased 24EE in this group. Protein and fat oxidation were reciprocally related during overfeeding. Observed 24EE was higher than predicted on days 1 ( $P \leq 0.05$ ), 14 ( $P = 0.0001$ ), and 56 ( $P = 0.0007$ ). There was no relation between change in fat mass and change in EE.

**Conclusions:** Excess energy, as fat, does not acutely increase 24EE, which rises slowly as body weight increases. Excess energy as protein acutely stimulates 24EE and SleepEE. The strongest relation with change in 24EE was the change in energy expenditure in tissue other than muscle or fat-free mass. This trial was registered at clinicaltrials.gov as NCT00565149. *Am J Clin Nutr* 2015; 101:496–505.

**Keywords** adaptive thermogenesis, low-protein diet, sleeping energy expenditure, hormones, molecular markers

## INTRODUCTION

The concept that both over- and underfeeding leads to adaptations in metabolism has been a recurring theme for over 100 y and is still an unsettled question (1–3). Metabolic adaptations reflect changes in energy expenditure (EE),<sup>4</sup> which are not attributable to changes in body metabolic size, exercise levels, exposure to cold or heat, or pharmacogenic agents. Some in-

vestigators (1) but not all (2) hypothesize that a metabolic adaptation in response to overfeeding could explain why some individuals are resistant to weight gain.

On the basis of an analysis of published studies on overfeeding, Stock (3) showed that individuals eating either low- or high-protein diets gained less weight than anticipated from the excess energy ingested, leading to the concept of “metabolic inefficiency.” Indeed, dietary protein may play an important role in energy balance because it produces a larger stimulation of EE compared with carbohydrate and fat (4, 5). Metabolism of dietary protein is modified by energy intake. In a study comparing the effects of exercise and excess calories on nitrogen balance with a fixed-protein diet (6), excess energy intake favored nitrogen retention. The efficiency of protein metabolism may therefore play an important role in nutrient balance. Some studies suggest that individuals only adapt to high- or low-protein diets by dissipating food energy as “heat” when they consume a surfeit of a nutritionally imbalanced diet (7). Because energy intake affects protein metabolism, and conversely, dietary protein has been proposed to affect the storage of energy when overeating, we have examined the effect of different levels of dietary protein during positive energy balance by overfeeding 25 healthy men and women with diets that differed in protein content. In this article, we report the effects of these diets on 24-h EE (24EE) and substrate oxidation measured prospectively on 5 occasions in a metabolic chamber.

This analysis was designed to answer several questions. First, does overfeeding produce changes in EE that exceed what is predicted from the change in metabolic body size measured as

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<sup>4</sup>Abbreviations used: DXA, dual-energy X-ray absorptiometry; EE, energy expenditure; FFM, fat-free mass; FM, fat mass; HPD, high-protein diet; LPD, low-protein diet; NPD, normal-protein diet; REE, resting energy expenditure; SleepEE, sleeping energy expenditure; TDEE, total daily energy expenditure; 24EE, 24-h energy expenditure.

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fat-free body mass and fat mass? If so, does this occur in both sleeping and total EE? Finally, does the quantity of dietary protein intake affect the pattern of response?

## METHODS

### Participants

Twenty-five healthy men and women aged 18–35 y with a BMI (in kg/m<sup>2</sup>) of 19.7–29.6 and who led a sedentary lifestyle (<2 h of exercise per week) completed this study. Participants were randomly assigned to one of 3 different protein diets, consumed while living in the Pennington Biomedical Research Center (Baton Rouge, LA) for approximately 12 wk without leaving. All participants signed a consent form approved by the Pennington Biomedical Research Center Institutional Review Board. This trial was registered at clinicaltrials.gov as NCT00565149.

### Protocol

Many details of this study protocol were previously described (8) [the entire protocol is available in Bray et al. (8)]. Briefly, it was a randomized, parallel arm, inpatient study outlined in **Figure 1**. Participants were recruited between June 2005 and October 2007. As shown, the mean ( $\pm$ SD) energy required for weight stabilization during baseline was  $2412 \pm 444$  kcal/d (top left line). This estimate was significantly higher than the free-living EE measured by doubly labeled water ( $2176 \pm 441$  kcal/d;  $P = 0.01$ ; mean  $\pm$  SD). The baseline diet (left-hand box) can be contrasted with the dietary intake for each group during overfeeding (box in the central compartment). Participants were overfed by approximately 40% above the baseline weight maintenance energy requirement or  $950 \pm 167$  kcal/d for 56 d. The overfeeding diets contained 5% [low-protein diet (LPD)], where all excess energy was fat; 15% [normal-protein diet (NPD)]; or 25% [high-protein diet (HPD)] of energy from protein. These latter 2 diets had more protein than the baseline diet. During the final 24-h period

(day 57), the baseline diet was restored. Study personnel (except dietitians and kitchen staff) were blinded to the dietary assignment. Subjects were weighed daily and allowed to leave the metabolic unit only under supervision and with permission of the study director. Randomization was done by the study statistician or study dietitian by using the minimization allocation method with stratification for sex and BMI.

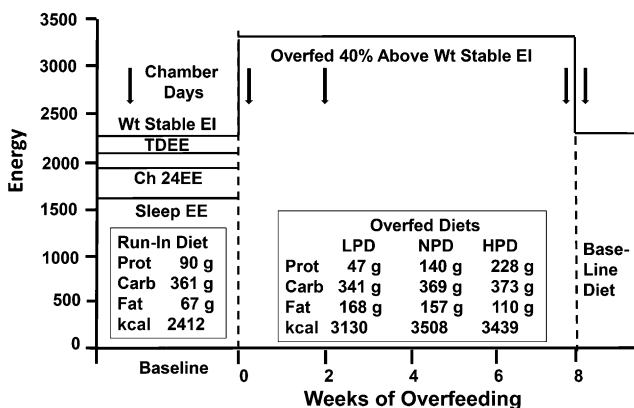
A baseline period of 13–25 d was used to establish energy requirements for weight maintenance and perform baseline tests (8). During this baseline period, subjects consumed an isocaloric diet (15% protein, 25% fat, and 60% carbohydrate). The calorie content of the baseline diet was initially estimated from measured resting EE (REE) multiplied by an activity factor of 1.3–1.4. Subjects consumed this diet for 2 d and then, on day 3, entered the metabolic chamber for assessment of 24EE and sleeping EE (SleepEE). When they exited the chamber on day 4, we started an assessment of EE on the metabolic ward by using a doubly labeled water study. This was also the first day of the weight stabilization period. Weight stability was defined as fluctuations in body weight <1 kg during a consecutive 10-d period without changes in energy intake. During weight stabilization, participants continued to consume the baseline diet, but the caloric prescription was determined from 24EE measured in the metabolic chamber (on day 3) multiplied by 1.15 to account for the increased EE from activity on the metabolic ward (9). Body weight was measured daily and weight stability evaluated each 5 d. If body weight was stable (i.e.,  $\pm 1$  kg), monitoring of body weight and activity was continued for an additional 5 d without any adjustment to energy intake. If body weight fluctuated more than 1 kg during a 5-d period, the caloric prescription was adjusted and the 10-d weight stabilization period was restarted (8). The energy intake that maintained body weight within  $\pm 1$  kg over 10 consecutive days was considered to represent the baseline energy requirement for weight maintenance (Figure 1).

### Study diets

All food was prepared by the metabolic kitchen and provided to the participants in a 5-d rotating menu over the entire inpatient stay. Overfeeding diets provided 40% of energy above the final caloric prescription determined at baseline to maintain weight on the metabolic ward. Menus were prepared in duplicate and food composites analyzed for nutrient composition (8). To ensure feeding compliance, all meals were consumed under supervision by inpatient personnel. There were 3 experimental diets with goals of 5%, 15%, and 25% energy from protein (Figure 1 and **Table 1**). When expressed per kilogram of weight, the 5% protein group consumed  $0.68 \pm 0.07$  g/kg per day, the NPD group consumed  $1.80 \pm 0.25$  g/kg per day, and the HPD group consumed  $3.01 \pm 0.30$  g/kg per day during the overfeeding period. Carbohydrate intake in grams per day was maintained throughout the overfeeding period with the balance of energy as fat. Carbohydrate provided 60% of calories at baseline and 42–43% in all 3 overfeeding diets. Fat, as a percentage of energy, was 49% in the LPD, 42% in the NPD, and 30% in the HPD.

### 24EE and substrate oxidation

Energy expenditure and substrate oxidation were measured for 24 h in a metabolic chamber stay at baseline; on days 1, 14, and



**FIGURE 1** Experimental protocol. This figure shows the baseline period followed by the 8 wk of overfeeding and the timing of the experimental procedures. The top horizontal lines represent energy intake at baseline, during overfeeding, and after energy intake was returned to baseline levels. The boxes inside the baseline and overfeeding periods summarize the macronutrient intakes. The 5 vertical arrows indicate the timing of the 24EE assessment in the metabolic chamber. Carb, carbohydrate; EE, energy expenditure; EI, energy intake; HPD, high-protein diet; LPD, low-protein diet; NPD, normal-protein diet; Prot, protein; TDEE, total daily energy expenditure; Wt, weight; 24EE, 24-h energy expenditure.

**TABLE 1**  
Diet composition<sup>1</sup>

Characteristic	Baseline	Percentage of protein in overfed diets		
		5%	15%	25%
Calories, kcal/d	2412 ± 444	3130 ± 364	3508 ± 684	3439 ± 649
Carbohydrate, g/d	361 ± 64	341 ± 37	369 ± 72	374 ± 79
Fat, g/d	67 ± 12	168 ± 21	157 ± 30	110 ± 18
Protein, g/d	90 ± 16	47 ± 4.7	140 ± 29	228 ± 48
Protein, g/kg BW per day	1.83 ± 0.98	0.68 ± 0.069	1.80 ± 0.25	3.01 ± 0.29
Nitrogen, g/d	22.1 ± 12.8	7.46 ± 0.75	22.3 ± 4.6	36.5 ± 7.7
Nitrogen, g/kg BW per day	0.29 ± 0.16	0.11 ± 0.011	0.29 ± 0.04	0.48 ± 0.047

<sup>1</sup>Values are means ± SDs. BW, body weight.

56 of overfeeding; and on day 57, the first day immediately after overfeeding when participants consumed the baseline diet. Subjects entered the metabolic chamber before breakfast at ~0800 on each morning and left the chamber at ~0715 the next day (10, 11). Diets were partitioned as 3 meals and 2 snacks provided at scheduled times. Lights were turned off at 2230, and the participants were awakened at 0630. All urine was collected for measurement of urinary nitrogen, creatinine, and catecholamine excretion rates. Energy expenditure was calculated by indirect calorimetry corrected for urinary nitrogen excretion and respiratory quotient based on the equation of Weir (12). We partitioned 24EE into various components as follows: 24EE represented the total EE for the 23 h 15 min extrapolated to 24 h, and SleepEE represented the EE between 0200 and 0500 extrapolated to 24 h when there was no physical activity, as indicated by the radar. Oxidation rates of carbohydrate, fat, and protein were calculated by using the equations from Jéquier et al. (13).

### Body composition

Body weight was measured each morning during the study. Body composition was measured by dual-energy X-ray absorptiometry (DXA) (Hologic QDR 4500; Hologic) at baseline and every 2 wk thereafter. The mass attributable to brain, skeletal muscle, adipose tissue, and bone was calculated according to the method of Hayes et al. (14) by using the baseline and 8-wk DXA scans and REE. The residual mass was the difference between the sum of these 4 components and the DXA weight at either baseline or 8 wk. The energy associated with the 4 tissue masses was calculated by multiplying organ mass by the energy coefficient in the article by Hayes et al. (14). The constant for energy related to residual mass,  $k_{RW}$ , was calculated according to Hayes et al. (14) by using baseline REE and 8-wk REE and, in contrast to the other 4 tissues, was different at baseline and 8 wk. Physical activity level was calculated as total daily energy expenditure (TDEE)/REE; measurement of TDEE and REE was previously described in detail (8).

### Clinical chemistry

A 24-h pooled urine sample collected during the chamber stays was used to measure concentrations of urinary nitrogen (ANTEK), creatinine (DXC 600 Pro; Beckman Coulter), and adrenaline and norepinephrine (HPLC; Bio-Rad). In fasting blood samples,

triiodothyronine, thyrotrophin-stimulating hormone, and thyroxine (Immulite 2000; Siemens) were measured.

### Muscle biopsy

Muscle biopsy specimens were obtained from the vastus lateralis following an overnight fast of at least 10 h by using the Bergstrom needle biopsy technique (15). Samples were collected at baseline and, after 2 and 8 wk of overfeeding, snap frozen in liquid nitrogen after trimming “debris” and stored at  $-80^{\circ}\text{C}$  until used.

Total messenger RNA was extracted from frozen muscle samples by using the miRNEasy Mini Kit (Qiagen), and complementary DNA was made by using the High Capacity cDNA Kit (Applied Biosystems). Detection of gene expression was performed by using TaqMan Gene Expression Assays-on-Demand (Applied Biosystems). Real-time polymerase chain reaction was carried out by using the 7900HT Fast Real-Time PCR system (Applied Biosystems), and expression levels were determined against a standard curve. Gene expression was adjusted to the expression of ribosomal protein large PO variant (*RPLPO*), a housekeeping gene commonly used in studies of human skeletal muscle genes.

Protein expression was measured from total protein extracts by using Western immunoblotting and adjusted to GAPDH expression (Abcam). Peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC1 $\alpha$ ) and uncoupling protein 3 (UCP3) antibodies were purchased from Abcam. The adenine translocator-1 (ANT1) antibody was purchased from Santa Cruz Biotechnology. The pT172-adenosine 5'-monophosphate kinase (AMPK) and total AMPK antibodies were purchased from Cell Signaling.

### Statistical analysis

Protein diets were randomly assigned, and there were no changes in protocol outcomes (8). The primary analysis was a post hoc evaluation by regression analysis of the protein intake on components of EE measured in the metabolic chamber and calculated from DXA measurements at baseline and 8 wk. Energy expenditure was evaluated as absolute values (unadjusted), as well as adjusted for age, sex, and baseline value. Residuals (difference between measured and predicted EE) were calculated from a linear regression of 24EE or SleepEE at baseline by using fat-free mass (FFM), fat mass (FM), sex, and age or DXA estimates of baseline tissue EE (14) as covariates. Effects of treatment on change from baseline on weekly average body weight, body composition, and EE were evaluated by using simple regression

analysis and again after adjusting for age, sex, and baseline covariates. Analysis of variance was used to compare treatment effects, and where significant differences existed, post hoc comparisons were done with Tukey's honestly significant difference test. Sex and age were included as a fixed effect and baseline energy requirements and baseline values for body weight, FFM, or baseline FM as covariates where appropriate. We set  $\alpha$  at  $P \leq 0.05$ . Analyses were done with the JMP-7 statistical package (SAS Institute).

## RESULTS

The baseline characteristics of the 25 normal-weight and overweight (BMI: 19.7–29.7) individuals (16 men, 9 women) who completed the study are shown in **Table 2**. There were no untoward effects from overfeeding. Representative patterns of EE over the 24 h spent in the metabolic chamber at baseline and on day 56 for each level of protein intake are shown in **Figure 2**. The increase in EE following each meal and the ~15% decline in EE during sleep are clearly evident. Also evident is that the 15% and 25% protein diets had higher nighttime levels of EE during overfeeding than the 5% protein group.

Changes from baseline to day 57 by diet group are shown for body weight (**Figure 3A**), urinary nitrogen (Figure 3B), 24EE (Figure 3C), and SleepEE (Figure 3D). Body weight increased steadily in all 3 diet groups, averaging  $8.7\% \pm 0.7\%$  in the HPD group and  $7.7\% \pm 0.7\%$  in the NPD group, which was significantly larger than in the LPD group ( $4.6\% \pm 0.8\%$ ,  $P < 0.05$ ). The increase in body weight was predicted by both intake of energy ( $r = 0.73$ ,  $P < 0.05$ ) and dietary protein ( $r = 0.77$ ,  $P < 0.01$ ) during overfeeding. In contrast, the increase in FM was predicted by energy intake ( $r = 0.52$ ,  $P < 0.05$ ) but not by protein intake, whereas the increase in FFM was strongly predicted by protein intake ( $r = 0.85$ ,  $P < 0.0001$ ) but less by energy intake ( $r = 0.65$ ,  $P < 0.01$ ).

Urinary nitrogen (Figure 3B) decreased within the first 24 h in the LPD group and rose in both the NPD and HPD groups. Nitrogen balance, calculated from the difference between nitrogen intake and urinary nitrogen excretion, rose from  $3.5 \pm 0.6$  g/d at baseline to  $6.4 \pm 2.3$  g/d in the NPD group and  $29.9 \pm 2.4$  g/d in the HPD compared with a fall to  $-0.07 \pm 2.5$  g/d in the LPD group despite their extra calorie load. Thus, there was no evidence of protein sparing by the high calorie intake. Urinary nitrogen excretion remained low in the LPD group until the final chamber measure (day 57), when it increased slightly because protein intake had been returned to the baseline level. On this final day (day 57), nitrogen balance became positive compared with baseline by  $t$  test in the LPD group ( $+8.2 \pm 1.5$  g/d,  $P = 0.0015$ ) but was not significantly different from baseline in the other 2 groups (NPD =  $1.1 \pm 1.5$ ,  $P = 0.22$ ; HPD =  $0.19 \pm 1.2$  g/d,  $P = 0.15$ ). Urinary nitrogen excretion in the NPD group remained near baseline levels throughout.

Protein balance calculated from protein oxidation and dietary protein intake was slightly positive at baseline in all groups, but there was no significant difference between them (LPD =  $21.0 \pm 6.9$  g/d, NPD =  $17.0 \pm 6.5$  g/d, and HPD =  $28.1 \pm 6.9$  g/d). On day 1 of overfeeding, protein balance in the LPD group dropped to  $0.01 \pm 15.9$  g/d compared with an increase in the NPD ( $39.7 \pm 15.0$  g/d) and HPD ( $130 \pm 15.9$  g/d) groups. At day 56, the protein balance was  $18.0 \pm 14.0$  g/d in the LPD group,  $52.3 \pm 13.2$  g/d in the NPD group, and  $101.6 \pm 15.0$  g/d in the HPD group. Protein oxidation measured in the chamber was inversely related to fat oxidation at days 1, 14, and 56 of overfeeding but not at baseline (**Figure 4**). Both fat balance and protein balance on day 1 predicted the 8-wk increase in body weight ( $r = 0.58$ ,  $P = 0.0021$ ) and body fat ( $r = 0.49$ ,  $P = 0.0008$ ), but only protein balance predicted the change in FFM ( $r = 0.66$ ,  $P = 0.0002$ ).

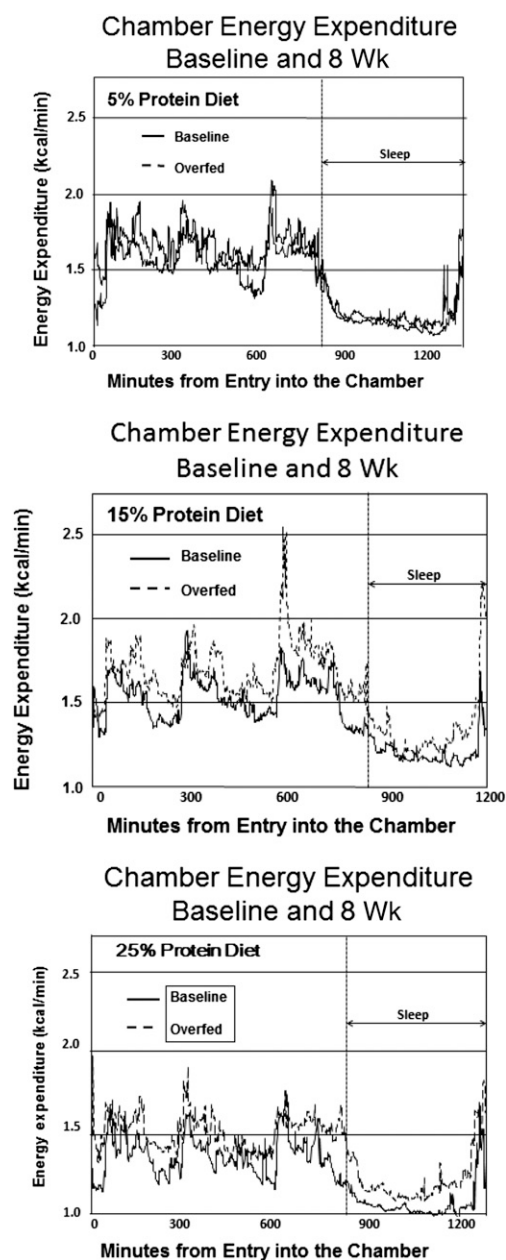
With the onset of overfeeding, changes in EE reflected diet composition, not energy intake (Figure 3C, D). In the LPD group, in which dietary protein decreased from 85 g/d to 47 g/d, all of the overfed energy was fat, and there was no increase in either 24EE

**TABLE 2**  
Baseline characteristics of the participants<sup>1</sup>

Characteristic	LPD	NPD	HPD	P value (ANOVA)	Overall
Sex, M/F, <i>n</i>	5/3	6/3	5/3		16/9
Ethnicity, W/O/B, <i>n</i>	4/1/3	0/1/8	5/0/3		9/1/14
Age, y	22.9 $\pm$ 2.7 <sup>2</sup>	22.9 $\pm$ 5.5	26.8 $\pm$ 2.0	0.09	24.1 $\pm$ 4.1
Weight, kg	69.1 $\pm$ 11.6	77.7 $\pm$ 13.0	76.1 $\pm$ 15.4	0.38	74.4 $\pm$ 13.4
Residual weight, kg	17.1 $\pm$ 0.66	18.8 $\pm$ 4.9	18.3 $\pm$ 4.2	0.71	18.1 $\pm$ 0.81
K <sub>RW</sub> , kcal/kg	41.8 $\pm$ 11.9	33.1 $\pm$ 12.4	33.0 $\pm$ 5.9	0.18	35.8 $\pm$ 10.9
BMI, kg/m <sup>2</sup>	24.4 $\pm$ 3.5	25.5 $\pm$ 3.0	25.6 $\pm$ 2.9	0.77	25.2 $\pm$ 3.0
Surface area, m <sup>2</sup>	1.81 $\pm$ 0.18	1.95 $\pm$ 0.22	1.91 $\pm$ 0.26	0.33	1.89 $\pm$ 0.22
EI from weight balance, kcal/d	2263 $\pm$ 250	2478 $\pm$ 512	2487 $\pm$ 545	0.58	2412 $\pm$ 444
EI from DLW, kcal/d	2233 $\pm$ 360	2176 $\pm$ 528	2090 $\pm$ 453	0.30	2176 $\pm$ 441
Urinary nitrogen, g/d	10.3 $\pm$ 2.9	12.0 $\pm$ 2.0	10.4 $\pm$ 4.0	0.51	10.9 $\pm$ 3.0
24-h energy expenditure, kcal/d	1935 $\pm$ 212	2079 $\pm$ 466	1953 $\pm$ 405	0.70	1993 $\pm$ 371
Sleeping energy expenditure, kcal/d	1555 $\pm$ 151	1657 $\pm$ 335	1557 $\pm$ 259	0.66	1592 $\pm$ 257
OF 40%, kcal/d	868 $\pm$ 123	1030 $\pm$ 178	952 $\pm$ 168	0.13	954 $\pm$ 167
Actual protein intake, %	6	15	26	—	—

<sup>1</sup>NPD > LPD by Tukey's honestly significant difference test. B, African American; DLW, doubly labeled water; DXA, dual-energy X-ray absorptiometry; EI, energy intake; HPD, high-protein diet; LPD, low-protein diet; NPD, normal-protein diet; O, other; OF, overfeeding; REE, resting energy expenditure; RW, residual weight; W, white; Wt, weight. RW = (DXA Wt) - (weight of muscle, adipose, brain, and bone). K<sub>RW</sub> = REE - [energy in (muscle + adipose + brain + bone)/RW].

<sup>2</sup>Mean  $\pm$  SD (all such values).



**FIGURE 2** Composite figure for one representative individual from each of the 3 diet groups showing the energy expenditure measured over 24 h at baseline (black line) and then again after 8 wk (dashed line) of overfeeding: (A) 5% protein diet, (B) 15% protein diet, and (C) 25% protein diet. The increased energy expenditure in the normal-protein (15%) and high-protein (25%) diets compared with the low-protein (5%) diet is evident.

or SleepEE [24EE = 20.3 (95% CI: -40.7, 75.5) kcal/d; SleepEE = -45.4 (95% CI: -122.7, 25.1) kcal/d]. In contrast, 24EE rose significantly during the first day of overfeeding in the 2 groups in which protein increased as part of the excess calories [HPD = 130 (95% CI: 80.2, 192.9) kcal/d; NPD = 75.5 (95% CI: 21.1, 123.8) kcal/d]. The changes in 24EE on day 1 were significantly correlated to dietary protein ( $r = 0.50$ ,  $P = 0.020$ ) but not dietary energy intake ( $r = 0.14$ ,  $P = 0.82$ ). By 14 d of overfeeding, there was a significant increase in 24EE in all 3 groups [LPD = 111.4 (95% CI: 25.8, 197.0) kcal/24 h; NPD = 111.4 (95% CI: 25.8, 197.1) kcal/24 h; HPD = 259.1 (95% CI: 179.0, 339.2) kcal/24 h],

which persisted at day 56. At both times, 24EE in the HPD group was significantly higher than in the LPD group ( $P < 0.05$ ). The increase in 24EE in the LPD group was significantly related to the baseline surface area but not FM, FFM, or body weight.

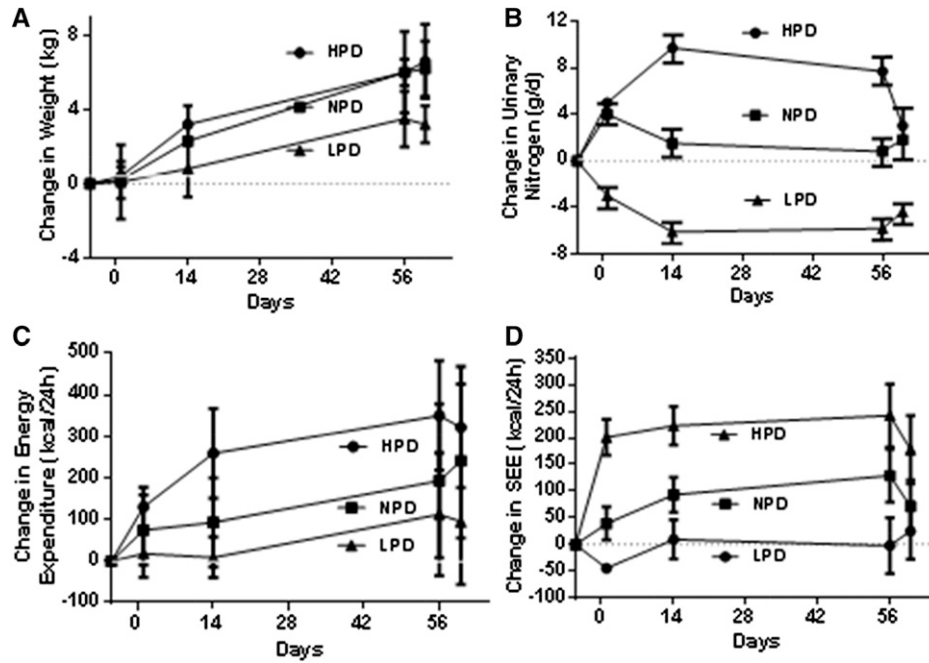
On day 57, when the diet was returned to baseline energy and protein levels, the 24EE ( $P < 0.01$ ) and SleepEE ( $P < 0.05$ ) remained significantly above baseline in the HPD group. The 24EE was also higher on day 57 in the NPD group ( $P < 0.01$ ), but the SleepEE was not. In the LPD group, neither measure of EE was increased on day 57 relative to baseline.

Because there were significant differences in the effects of diet during the first 24 h related to dietary composition, we also compared the changes in 24EE from day 1 to day 14 and from day 1 to day 56. There was a significant increase in 24EE from days 1–14 ( $92 \pm 21$  kcal/d;  $P < 0.001$ ) and days 1–56 ( $144 \pm 31$  kcal/d;  $P < 0.0001$ ), but the increase was not different between the 3 diet groups. SleepEE also increased from days 1–56 ( $64 \pm 28$  kcal/24 h;  $P < 0.05$ ), but the change from days 1–14 was borderline ( $40 \pm 20$  kcal/24 h;  $P = 0.062$ ).

Because EE is highly correlated with body size and body size increased differently during overfeeding between the 3 diets, changes in EE during overfeeding were adjusted for changes in body composition. The difference between the observed and predicted 24EE on day 1, day 14, and day 56 is shown in **Figure 5**. It is clear that on day 1 ( $P < 0.05$ ), day 14 ( $P < 0.0001$ ), and day 56 ( $P < 0.01$ ), the observed values for 24EE in most individuals were higher than the predicted 24EE based on age, sex, FFM, and FM. Similarly, observed SleepEE was higher than the predicted SleepEE on day 1 ( $P < 0.05$ ), day 14 ( $P < 0.01$ ), and day 56 ( $P < 0.05$ ). This adaptive thermogenesis was not different between the 3 diet groups ( $P = 0.6$ ).

The increase in FFM in both the NPD and HPD groups but not in the LPD group raises the question of where the extra nitrogen is deposited. To address this question, we used the DXA data to estimate the mass of brain, skeletal muscle, bone, adipose tissue, and residual mass at baseline and the change from baseline as described by Hayes et al. (14) and Heymsfield et al. (16) (**Table 3**). Skeletal mass was the largest component of body mass ( $30.9 \pm 8.06$  kg), with adipose tissue ( $21.2 \pm 7.33$  kg) and residual mass (liver, skin, viscera, etc.) ( $18.1 \pm 4.05$  kg) not far behind. During overfeeding, there were significant effects of diet on the change in tissue mass, which varied by diet group. Compared with baseline, the skeletal mass showed a graded increase in mass with protein intake. The LPD group had a significantly smaller increase than the normal- and high-protein groups, which were not statistically different from each other. Residual mass, brain mass, and bone mass did not change significantly during overfeeding. Adipose mass increased similarly in all 3 groups (**Table 3**).

Energy expended by these different tissue compartments can also be estimated at baseline and 8 wk by using the approach of Hayes et al. (14) (**Table 3**). The 24EE and SleepEE at baseline both had significant relationships with the residual mass ( $r = 0.85$ ,  $P < 0.0001$ ), skeletal mass ( $r = 0.88$ ,  $P < 0.0001$ ), bone mass ( $r = 0.68$ ,  $P < 0.001$ ), and brain mass ( $r = 0.55$ ,  $P < 0.01$ ) but not with adipose tissue mass. There were similar correlations at the end of overfeeding ( $r = 0.61$ ,  $P = 0.0014$  for residual mass;  $r = 0.89$ ,  $P < 0.0001$  for skeletal mass;  $r = 0.73$ ,  $P < 0.0001$  for bone;  $r = 0.65$ ,  $P = 0.0005$  for brain; and  $r = 0.11$ ,  $P = 0.059$  for



**FIGURE 3** Temporal changes in body weight (A; mean SD), urinary nitrogen (B; mean SE), 24EE (C; mean SD), and SEE (D; mean SD) at baseline and through the 8 wk of overfeeding and the final day that food intake was returned to the baseline level. The change data are expressed as means  $\pm$  SEs. The change in urinary nitrogen (B) was significantly greater in the HPD group than in the LPD group at all times from day 1 to day 57. The NPD group was lower than the HPD group at days 14 and 56 but not at day 1 or day 57. Weight gain was significantly greater for the HPD group and NPD group than for the LPD group ( $P < 0.01$ ). Urinary nitrogen excretion was significantly higher in the HPD group than in the NPD or LPD group at weeks 2 and 8 ( $P < 0.05$ ). Change in 24EE (C) and SEE (D) from baseline to week 8 was significantly greater in the HPD than in the LPD group ( $P < 0.05$ ). There were 8 participants in the LPD group, 9 in the NPD group, and 8 in the HPD group. HPD, high-protein diet; LDP, low-protein diet; NPD, normal-protein diet; SEE, sleeping energy expenditure; 24EE, 24-h energy expenditure.

adipose tissue). An increase in EE from baseline to day 56 occurred in the residual mass, skeletal muscle mass, and adipose tissue mass, but as seen in **Figure 6**, the increase was much larger in the residual mass. The increase in EE in the residual tissue during overfeeding accounts for nearly two-thirds of the increase in 24EE in the HPD group [residual weight EE;  $RWEE_{HPD} = 198 \pm 35$  (mean  $\pm$  SE) compared with  $24EE_{HPD} = 350 \pm 63$  kcal/d] and similarly in the NPD group [ $RWEE_{NPD} = 114 \pm 30$  (mean  $\pm$  SE) compared with  $24EE_{NPD} = 199 \pm 53$  kcal/d].

The residual mass includes the energy consumed by the liver and kidneys plus skin and intestine. The constant,  $k_{RM}$ , which relates the energy contribution from the residual tissue compartment to the other compartments, was significantly increased by protein and was differentially affected by diet. The change in  $k_{RM}$  from baseline to 8 wk, adjusted for age, sex, and baseline residual mass (RM), fell slightly in the LPD group ( $-2.42 \pm 2.06$ ) but rose in the NPD ( $4.80 \pm 1.95$ ) and HPD groups ( $10.0 \pm 2.21$ ;  $P = 0.01$ , HPD  $>$  LPD), suggesting differential influences of diet on the metabolism of the organs or tissues in the residual mass. The change in this energy constant ( $k_{RM}$ ) from baseline to 8 wk was strongly related to protein intake but not to energy intake during overfeeding, as shown in **Figure 7**.

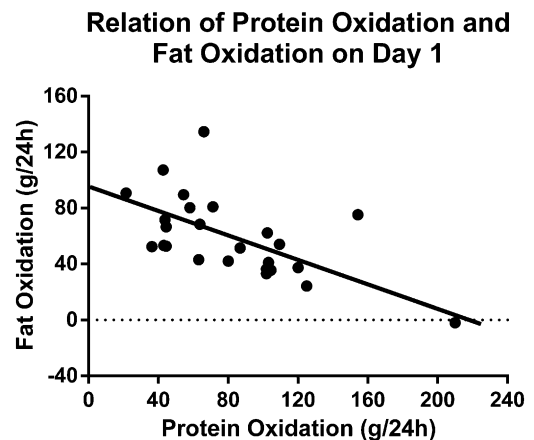
Physical activity level was the same at baseline in each group and did not change during overfeeding (baseline:  $1.46 \pm 0.05$ ; week 8:  $1.56 \pm 0.05$ ;  $P = 0.20$ ), suggesting that physical activity was not a major source of the increased EE.

We evaluated several potential molecular markers of muscle function by measuring expression of genes and proteins in skeletal muscle biopsy specimens (**Table 4**). There were some differences related to overfeeding but no significant effects of

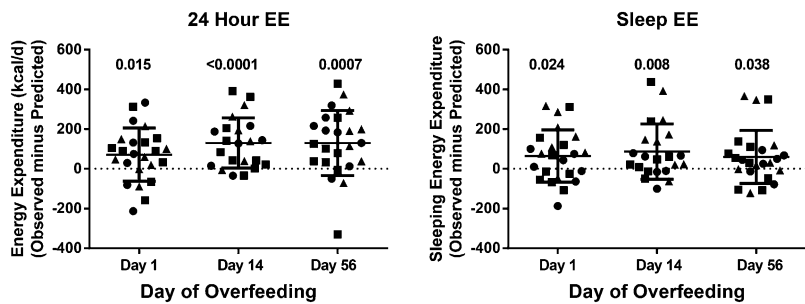
diet on any of these markers. Thyroid hormones and urinary catecholamines were also measured. The increase in triiodothyronine was significantly and negatively correlated to the change in FFM ( $r = -0.57$ ,  $P = 0.005$ ) but not FM or to 24EE. SleepEE expressed as absolute values or after adjusting for body composition. Baseline triiodothyronine had no significant relation to any of the measured changes.

## DISCUSSION

This study on the effect of different levels of dietary protein during overfeeding with participants residing in a metabolic



**FIGURE 4** Protein oxidation and fat oxidation on day 1 were significantly ( $P = 0.0017$ ) and inversely related ( $r = -0.59$ ).



**FIGURE 5** Observed minus predicted EE ( $\pm$ SD) (24-h EE and sleep EE) on days 1, 14, and 56. Each individual is identified by his or her diet group and each time. Circles = low-protein diet ( $n = 8$ ); squares = normal-protein diet ( $n = 9$ ); triangles = high-protein diet ( $n = 8$ ). EE, energy expenditure.

calorimeter on 5 occasions showed 1) that dietary protein had a significant effect on changes in both 24EE and SleepEE, 2) that excess caloric intake as fat did not actually increase 24EE or prevent the loss of protein with the lowest protein diet, 3) that initial changes in protein and fat balance predicted subsequent changes in fat and protein storage and not adaptive thermogenesis, and 4) that the effect of changing body composition on thermogenesis involved increased metabolism in organs or tissues other than muscle or adipose tissue—presumably the liver the kidney or supporting tissues.

All 3 groups were overfed by approximately 40% above estimated baseline energy requirements. With an HPD, the increase in 24EE was immediate and became highly significant during sleep on the first day. This is consistent with the findings of others (17–19). In the LPD group, however, 24EE did not increase, and actually SleepEE decreased in the first 24 h. We thus conclude that the level of dietary protein plays a major role in the stimulation of 24EE during overfeeding, confirming previous studies (20–22). In addition, this study suggests that protein may alter the rate of energy utilization in certain organs or tissues. The estimated mass of muscle and bone increased with overfeeding in the diet groups with protein content above baseline (HPD) and fell in the LPD group. Although the mass included in the “residual mass” did not change, the estimated contribution of residual mass to EE did. Energy consumed by the residual mass was also affected

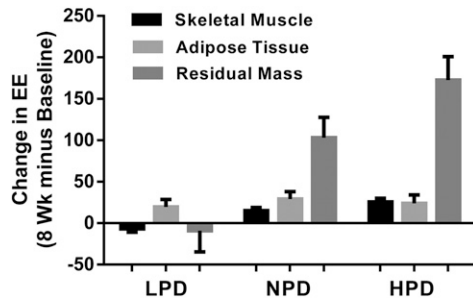
significantly by dietary protein, rising in the NPD and NPD but not in the LPD. The constant for EE by the residual mass from the DXA model (14) showed a highly significant positive relation to protein intake. This would be consistent with the idea that liver and/or kidney, which are significant sources of energy utilization in the residual mass, had increased their energy utilization to catabolize the excess protein.

Dietary fat represented a significant amount of energy in each group, ranging from 50.3%  $\pm$  0.5% of energy in the LPD group to 42.1%  $\pm$  0.2% in the NPD group and 30.2%  $\pm$  0.8% in the HPD group. Flatt (22), Flatt et al. (23), and Bennett et al. (24) showed that the addition of fat to the diet did not acutely increase EE within a few hours, over the entire day (24, 25), or over several days (26). In support of their observation, the LPD in which all the excess energy was fat did not increase 24EE or SleepEE. It is important to note that fat has been added as the primary component of overfeeding in several protocols (27–30), and these diets are often compared with carbohydrate supplementation (27–30). These studies show a similar weight gain, but carbohydrate has a larger effect on EE. In contrast to our overfeeding study, all of these previous studies had normal levels of protein (12–15%) that were reduced by one-third with overfeeding to around 8–12%. Therefore, the extra energy provided as fat or carbohydrate produced similar changes in fat storage relative to the other diet, but none had very high fat.

**TABLE 3**  
Mass and energy expenditure of the brain, skeletal mass, adipose tissue, bone, and residual mass at baseline and after 8 wk using the multicompartiment dual-energy X-ray absorptiometry model<sup>1</sup>

Variable	8 wk				<i>P</i> value, diet effect from baseline
	Baseline	LPD	NPD	HPD	
Brain, kg	1.53 $\pm$ 0.13	1.53 $\pm$ 0.13	1.54 $\pm$ 0.11	1.48 $\pm$ 0.12	0.18
Skeletal muscle, kg	30.9 $\pm$ 8.06	28.1 $\pm$ 7.1	34.2 $\pm$ 8.2	33.0 $\pm$ 10.8	0.0004 (HPD = NPD > LPD)
Bone, kg	2.56 $\pm$ 0.48	2.34 $\pm$ 0.38	2.73 $\pm$ 0.43	2.57 $\pm$ 0.58	0.34
Adipose tissue, kg	21.2 $\pm$ 7.33	21.1 $\pm$ 8.4	25.4 $\pm$ 9.4	27.2 $\pm$ 8.0	0.50
Residual mass, kg	18.1 $\pm$ 4.05	19.6 $\pm$ 10.4	19.5 $\pm$ 5.6	18.7 $\pm$ 4.55	0.64
Brain EE	367 $\pm$ 31.9	367 $\pm$ 31.4	369 $\pm$ 30.2	356 $\pm$ 29.0	0.18
Skeletal muscle EE	401 $\pm$ 103	365 $\pm$ 92.3	444 $\pm$ 105	429 $\pm$ 141	<0.0001 (HPD = NPD > LPD)
Bone EE	5.9 $\pm$ 1.09	5.39 $\pm$ 0.86	6.27 $\pm$ 1.00	5.94 $\pm$ 1.34	0.045
Adipose tissue EE	91.8 $\pm$ 47.2	106 $\pm$ 27.7	115 $\pm$ 42.4	122 $\pm$ 35.8	0.74
Residual mass EE	635 $\pm$ 206	685 $\pm$ 201	710 $\pm$ 151	785 $\pm$ 168	0.0006 (HPD = NPD > LPD)

<sup>1</sup>Values are means  $\pm$  SDs. Comparison was done with the fit model platform adjusting for age, sex, and baseline value. *P* value was calculated from model of change from baseline by diet, age, sex, and baseline value with contrasts based on Tukey’s honestly significant difference test. EE, energy expenditure; HPD, high-protein diet; LPD, low-protein diet; NPD, normal-protein diet.



**FIGURE 6** Mean ( $\pm$ SE) increase in EE in DXA compartments for skeletal muscle, adipose tissue, and residual mass during overfeeding where residual mass is the difference between DXA weight and the weights for the bone, brain, skeletal muscle, and adipose tissue. The change in EE in skeletal muscle was significantly higher in the HPD and NPD groups than in the LPD group ( $P < 0.0001$ ). The change in EE in the residual mass was significantly higher in the HPD and NPD groups than in the LPD group ( $P = 0.0002$ ). DXA, dual energy x-ray absorptiometry; EE, energy expenditure; HPD, high-protein diet; LPD, low-protein diet; NPD, normal-protein diet.

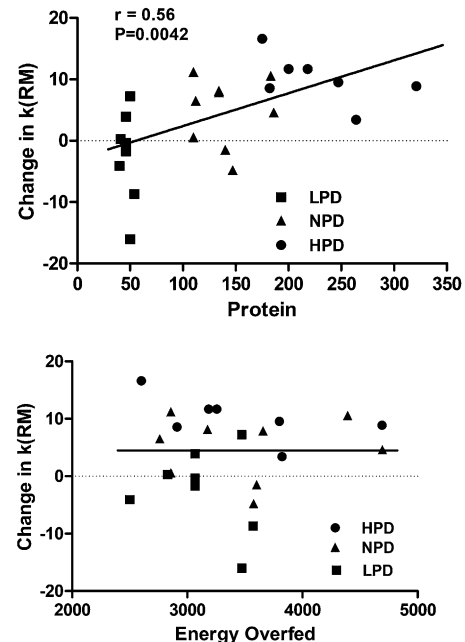
The level of protein in the LPD was 0.68 g/kg body weight per day or about 15% below the recommended level of 0.8 g/kg body weight. Protein sparing (i.e., slowing nitrogen loss by increasing caloric intake above maintenance levels) has been described for more than 75 y (31, 32) and was an expected observation in our study. However, the initial rapid loss of nitrogen with a persistent continued nitrogen excretion, even when fecal and skin nitrogen were not included, suggests that in the face of positive energy balance, the participants in the LPD group were unable to conserve nitrogen adequately to maintain balance, even in the presence of an extra 945 kcal/d.

Krebs (21) postulated 2 mechanisms for the effect of dietary protein during overfeeding. The first was related to the energy required for urea synthesis and the second for protein synthesis. The acute changes in urinary nitrogen observed in our experiments are entirely consistent with the first mechanism for disposal of protein and for the role of protein intake as a significant predictor of EE at baseline and during overfeeding. The effects of protein on EE were seen on the first day in total 24EE and particularly clearly with SleepEE. Most previous studies that have examined SleepEE during overfeeding also found it to be increased (26, 33–35), except Lammert et al. (28), who claimed that “poor sleep quality” might be a factor (36).

The increase in FFM with the 15% and 25% protein diets and the lack of a change with the 5% protein diet raise the question of where the nitrogen is deposited. Physical activity either in the respiration chamber (data not shown) or on the metabolic ward was low and not significantly different between diets and did not change during the course of the overfeeding, as shown by a low physical activity level (TDEE/REE). For nitrogen to accumulate as protein in “muscle” would be unexpected with the very low level of activity in these participants. This is supported by the absence of changes in the genes involved with protein synthesis (mTOR) and other genes associated with thermogenesis (*UCP3* and *ANT1*) and muscle metabolism (*AMPK1*, *PPARGC1A*, and *COX5a*). The marked and expected differences in protein turnover suggest that changes in enzymatic activity in the liver (or kidney) would be likely, but we do not have any

direct evidence for this. The turnover of extracellular matrix in fat (37–39) or in muscle in response to overfeeding (40) also might contribute to the increase in lean body mass.

There are differences of opinion about whether overfeeding induces changes in EE beyond that predicted by the changes in body metabolic size and, if so, how to interpret it (1, 2). Westerterp (2) concludes that intake-induced expenditure changes are largely explained by proportional changes in diet-induced EE, in activity-induced EE, and in maintenance expenditure as a function of changes in body weight and body composition and that there is little to support adaptive changes during overfeeding. In contrast, Dulloo et al. (1) note that in assessing adaptive thermogenesis as greater than predicted changes in EE by adjusting for FFM, it is assumed that the “composition of the fat-free mass remains constant.” As they note, FFM has several compartments with different metabolic rates. Our observation that overfeeding significantly increases the difference between measured and predicted EE on the first day of overfeeding when there is no measurable change in body composition, as well as days 14 and 56, when there are measurable changes, suggests that the correction for FFM does not provide an adequate understanding of the metabolic adaptations to overfeeding. The first requirement for interpreting changes in EE in relation to changes in body composition is to control protein intake because high protein intake can stimulate EE, even when FFM does not increase, as we observed during the first day of overfeeding. The second finding from our study relevant to assessing EE during overfeeding is that not all FFM is the same, as also suggested by Dulloo et al. (1). The fact that bone and brain contribute to EE, without any measurable change in mass during overfeeding, suggests that more sophisticated techniques to assess the contribution of tissues and organs or changes in organ sizes should be used in future studies. Much of what is referred to as



**FIGURE 7** Relation of change in energy constant for RM by protein intake and calorie intake, where RM is the difference between dual-energy X-ray absorptiometry weight and the weights for bone, brain, skeletal muscle and adipose tissue. HPD, high-protein diet; LPD, low-protein diet; NPD, normal-protein diet; RM, residual mass.



**TABLE 4**  
Baseline and change from baseline in metabolic and molecular markers<sup>1</sup>

Variable	Baseline value <sup>2</sup>	Change from baseline <sup>3</sup>			<i>P</i> value
		LPD	NPD	HPD	
T3	119 ± 19	10.9 ± 5.0	−6.4 ± 4.8	−0.53 ± 6.14	0.049
Thyroxine	7.12 ± 1.49	−0.17 ± 0.35	−0.64 ± 0.32	0.18 ± 0.37	0.39
Thyroid-stimulating hormone	1.88 ± 0.90	−0.63 ± 0.24	−0.31 ± 0.23	−0.13 ± 0.30	0.56
Reverse T3	0.21 ± 0.051	0.008 ± 0.011	−0.032 ± 0.14	−0.00 ± 0.017	0.85
Urinary epinephrine	20.0 ± 17.8	3.1 ± 9.3	1.29 ± 8.92	5.57 ± 11.4	0.92
Urinary norepinephrine	104 ± 53	50.9 ± 29.6	18.4 ± 28.3	28.5 ± 36.2	0.62
Gene expression data					
OGDH	1.39 ± 1.62	−0.006 ± 1.01	0.94 ± 0.89	0.21 ± 1.17	0.62
UCP-3	1.04 ± 0.43	−0.33 ± 0.27	0.12 ± 0.23	−0.041 ± 0.31	0.34
AMPKα1	1.38 ± 0.93	0.73 ± 1.13	1.14 ± 1.00	0.43 ± 1.31	0.86
ATP5b	1.09 ± 0.39	0.27 ± 0.23	0.69 ± 0.20	0.09 ± 0.26	0.90
COX5a	0.97 ± 0.22	0.47 ± 0.31	0.17 ± 0.27	0.11 ± 0.35	0.42
NDUFS8 CoI	1.13 ± 0.52	0.87 ± 0.44	0.54 ± 0.49	0.25 ± 0.64	0.79
SDH	1.19 ± 0.64	0.21 ± 0.53	0.61 ± 0.46	0.21 ± 0.61	0.83
Western blot data					
tAMPK	0.23 ± 0.12	−0.029 ± 0.067	−0.079 ± 0.075	−0.035 ± 0.088	0.87
pAMPK	0.29 ± 0.58	−0.016 ± 0.20	−0.15 ± 0.22	−0.039 ± 0.26	0.92
pAMPK/tAMPK	0.99 ± 1.34	0.12 ± 5.35	11 ± 5.94	−0.10 ± 7.0	0.50
ANT1	3.44 ± 1.38	0.22 ± 0.37	−0.066 ± 0.50	−0.44 ± 0.56	0.51
UCP-3	3.94 ± 4.32	−1.17 ± 1.12	−0.047 ± 1.15	−3.27 ± 1.53	0.33
PGC1α	6.80 ± 8.86	−3.0 ± 6.2	5.5 ± 4.6	−4.5 ± 5.5	0.49

<sup>1</sup>Fit model: T3\_BL is not related to change in 24EE, SEE, change in FFM, or change in FM or change in protein oxidation. Change in T3 (week 8 to baseline) was related to change in FFM but not change in FM, change in 24EE, change in SEE, or change in protein oxidation. EE needs to be adjusted for changes in body composition. AMPKα1, adenosine 5'-monophosphate kinase α1; ANT1, adenine translocator 1 or solute carrier 25 member 4; ATP5b, complex V-β subunit; COX5a, complex IV-subunit 5a; EE, energy expenditure; FFM, fat-free mass; FM, fat mass; NDUFS8, complex I-NADH dehydrogenase Fe-S protein 8; OGDH, α-ketoglutarate dehydrogenase; pAMPK, (p172T) adenosine 5'-monophosphate activated kinase; PGC1α, peroxisomal proliferator-activated receptor-γ coactivator 1α; SDH, complex II-succinate dehydrogenase, Fe-S containing subunit B; SEE, sleep EE; tAMPK, total adenosine 5'-monophosphate activated kinase; T3, triiodothyronine; T3\_BL, baseline triiodothyronine; UCP-3, uncoupling protein 3; 24EE, 24-h energy expenditure.

<sup>2</sup>Values are means ± SDs.

<sup>3</sup>Values are means ± SEs, adjusted for age and sex.

“adaptive” thermogenesis may thus be a reflection of changes in protein-induced thermogenesis occurring in tissues other than muscle. The fact that protein can differentially influence the EE of individual tissues implies that in addition to controlling protein, changes in metabolism in tissues whose mass does not measurably change also needs to be assessed. The findings in our study concur with those of Westerterp (2) that adaptive thermogenesis probably does not occur during overfeeding, except as a measurement artifact, which has also been suggested by others (1).

This study has several strengths and some limitations. It is a relatively long-term overfeeding study, and only a few have studies have made measurements in a metabolic chamber after overfeeding for 14 d or more (26–28, 33–35). Our participants lived in a metabolic ward during the overfeeding period, and the composition of the foods consumed was verified independently in food composites. Gold-standard techniques were used to assess changes in EE and body composition. One weakness of this study is the relatively small number of participants. A second is the unbalanced ethnic distribution of subjects between the 3 diet groups. A third is that there was no measure of the composition of the FFM in terms of water, glycogen, and collagen markers or measures of skeletal muscle efficiency to better elucidate the mechanisms underlying our observations. In addition, there were no measures of nitrogen lost in skin and stools.

Also, there are no studies measuring protein anabolism with stable isotopes, one of the mechanisms proposed by Krebs (21). Clearly, further studies are needed to understand the role of changes in the architecture of FFM with weight gain and the contributions of expanded lean mass to EE. In conclusion, these 5 respiration chamber measurements in volunteers consuming 40% additional calories for 56 d showed that calories supplemented as fat do not increase EE during the first 24 h of overfeeding and only slowly after that. When some of the excess energy was protein, there was a strong relation between protein intake and EE and no evidence for a role of adaptive thermogenesis in body weight regulation during overfeeding. Our findings concur with Westerterp (2) that adaptive thermogenesis probably does not occur during overfeeding in most individuals, although some individuals showed significant adaptive thermogenesis, corresponding to an increase in daily EE of 200–400 kcal/d, as has been suggested by others (1).

The authors' responsibilities were as follows—GAB, SRS, LdJ, and JR: designed the research; LdJ, JC, CB, SM, CKM, and SRS: conducted the research; CB: provided essential reagents or essential materials; GAB, LMR, and LdJ: analyzed data or performed statistical analysis and wrote the article; and GAB: had primary responsibility for final content. The authors declared no conflicts of interest with respect to this study.

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