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# Effects of 3 diets with various calcium contents on 24-h energy expenditure, fat oxidation, and adipose tissue message RNA expression of lipid metabolism-related proteins<sup>1-3</sup>

Niels Boon, Gabby BJ Hul, Nathalie Viguerie, Audrey Sicard, Dominique Langin, and Wim HM Saris

## ABSTRACT

**Background:** Evidence from molecular and animal research and epidemiologic investigations indicates that calcium intake may be inversely related to body weight, possibly through alterations in 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] metabolism.

**Objective:** We tested whether energy and substrate metabolism and adipose tissue enzyme messenger RNA (mRNA) expression can be altered by dietary calcium intake in healthy, nonobese, human volunteers consuming an isocaloric diet.

**Design:** Twelve healthy men [age: 28 ± 2 y; body mass index (BMI; in kg/m<sup>2</sup>): 25.2 ± 0.6] received 3 isocaloric diets [high calcium (1259 ± 9 mg/d), high dairy (high/high); high calcium (1259 ± 9 mg/d), low dairy (high/low); and low calcium (349 ± 8 mg/d), low dairy (low/low)] in a randomized crossover design. At the end of the 7-d dietary periods, 24-h energy expenditure and substrate metabolism were measured, and fat biopsy specimens were obtained to determine mRNA expression in genes involved in the lipolytic and lipogenic pathways.

**Results:** The 24-h energy expenditure was 11.8 ± 0.3, 11.6 ± 0.3, and 11.7 ± 0.3 MJ/24 h in the high/high, high/low, and low/low conditions, respectively. Fat oxidation in these conditions was 108 ± 7, 105 ± 9, and 100 ± 6 g/24 h. These differences were not statistically significant. mRNA concentrations of *UCP2*, *FAS*, *GPDH2*, *HSL*, and *PPARG* did not differ significantly. Serum 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations changed from 175 ± 16 to 138 ± 15, 181 ± 23 to 159 ± 19, and 164 ± 13 to 198 ± 19 pmol/L in the high/high, high/low, and low/low conditions, respectively, and was significantly different between the high/high and low/low conditions (*P* < 0.05).

**Conclusion:** Altering the dietary calcium content for 7 d does not influence substrate metabolism, energy metabolism, or gene expression in proteins related to fat metabolism, despite significant changes in 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations. *Am J Clin Nutr* 2005;82:1244-52.

**KEY WORDS** Dietary calcium, energy expenditure, adipose tissue message RNA expression, body weight regulation, substrate metabolism, 1,25-dihydroxyvitamin D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>

## INTRODUCTION

The prevalence of obesity has increased markedly during the past 2 decades, making obesity an important risk factor for the development of type 2 diabetes, various types of cancer, and

cardiovascular complications. In recent years, an inverse relation between dietary calcium and body mass index (BMI; in kg/m<sup>2</sup>) was repeatedly observed (1-8). Some intervention studies also showed that dietary calcium may have weight-lowering effects, and, in addition, an even stronger effect was observed with dairy sources of calcium (9-12).

However, the mechanism is still unclear. A hypothesis to explain this relation was provided by Zemel et al (13). With an increased concentration of 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] in cultures of human adipocytes and in transgenic mice, acute increases of the intracellular calcium concentration were observed (14, 15). This increased concentration of Ca<sup>2+</sup> in the adipocytes lowered lipolysis and stimulated lipogenesis. Lowering the intake of dietary calcium leads to an increase in the serum concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> within a few days (16). In this way, a low intake of dietary calcium may lead to a higher body weight by changing the balance of lipolysis and lipogenesis in adipose tissue through an increase in serum 1,25(OH)<sub>2</sub>D<sub>3</sub> (1).

Serum 1,25(OH)<sub>2</sub>D<sub>3</sub> is an important regulator of a large number of genes (17). Of the genes regulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> are genes that are either related to fat breakdown and storage [*HSL* (hormone sensitive lipase), *GPDH2* (glycerol phosphate dehydrogenase), and *FAS* (fatty acid synthase)] or adipocyte differentiation [*PPARG* (peroxisome-proliferator activated receptor  $\gamma$ )] (18-22). Furthermore, 1,25(OH)<sub>2</sub>D<sub>3</sub> decreases the messenger RNA (mRNA) expression of *UCP2* (23), which is correlated with basal metabolic rate (18). If changes in serum 1,25(OH)<sub>2</sub>D<sub>3</sub> are paralleled by changes in the expression of these genes, this may provide more insight on how calcium intake affects body weight through changes in lipogenesis, lipolysis, and energy expenditure (EE).

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The first well-controlled experimental trial to look at the effects of calcium intake on energy and fat metabolism in weight-stable subjects was published by Melanson et al (9), who observed a positive correlation between 24-h and sleeping fat oxidation and calcium intake, but they did not correct for protein intake. Furthermore, in most of the epidemiologic and dietary intervention studies performed in humans mentioned here, the investigators did not correct for protein intake (3, 4, 9, 24–26), which has been shown to have weight-lowering effects (27–31).

These data prompted us to investigate the effect of 3 isocaloric diets with a fixed macronutrient composition and different concentrations of calcium on 24-h EE and 24-h fat oxidation in vivo in humans. We hypothesized that a higher calcium intake would increase these indicators and that an additive effect may be observed of low-fat dairy. To study potential effects on a molecular level, mRNA expression of different genes related to fat metabolism (ie, *UCP2*, *FAS*, *GPDH2*, *HSL*, and *PPARG*) in adipose tissue was investigated as well.

## SUBJECTS AND METHODS

### Experimental subjects

Twelve untrained (did not perform any regular physical activity  $\geq 3$  h/wk), healthy men were recruited for participation in this study through advertisements in local newspapers. This study was approved by the local ethical committee of the University of Maastricht and the Academic Hospital of Maastricht. After the subjects had received a written and oral explanation of the procedures to be followed in this project, their informed consent was obtained. Furthermore, the habitual energy, micronutrient, and macronutrient intakes of the subjects was assessed by using 3-d food intake diaries, before the start of the experiment. Subjects were provided with a digital kitchen balance to allow them to make a more accurate estimation of their habitual food intake, and the macronutrient and micronutrient compositions of these habitual diets was calculated from these food records with the use of the Dutch food-composition table (32). Habitual physical activity of the subjects was also determined to assure that none of the subjects spent  $>3$  h/wk in heavy physical exercise. Finally, before the start of the first dietary intervention period, body composition and maximal aerobic capacity were determined.

### Maximal exercise test

At least 1 wk before the first stay in the respiration chamber, maximum oxygen uptake ( $\dot{V}O_{2\max}$ ), maximum workload ( $W_{\max}$ ), and maximal heart rate ( $Hf_{\max}$ ) were measured on an electronically braked cycle ergometer (Lode Excalibur, Groningen, Netherlands) during an incremental exhaustive exercise test. During this test, oxygen uptake was measured by using indirect calorimetry (Oxycon- $\beta$ ; Mijnhardt, Mannheim, Germany), and heart rate was measured with a heart rate monitor (Polar Sporttester, Polar Electro, Kempele, Finland). Exercise was started at 100 W for 5 min, and the power output was increased by 50 W every 2.5 min consequently until subjects reached a heart rate of 160. Thereafter, workload was increased by 25 W. We instructed our subjects to continue until exhaustion. To calculate the maximum power output, we used the following formula:

$$W_{\max} = W_{\text{out}} + (t/150) \times \partial W \quad (1)$$

in which  $W_{\text{out}}$  is the highest workload completed by the subject,  $t$  is the time (in sec) performed on the last workload, and  $\partial W$  is the final uncompleted load increment. The maximal power output was the workload at which subjects had to exercise for 45 min during their 3 consecutive stays in the respiration chamber.

### Body-composition and anthropometric measurements

To assure an accurate description, body composition was determined by using the 3-component model with water by Siri (33). This method combines determination of total body water with deuterium dilution and estimation of whole-body density by underwater weighing. The exact details of this method are described in detail elsewhere (34).

### Experimental design

All 12 subjects underwent three 7-d dietary interventions in a randomized, crossover design: a high-calcium, high-dairy (high/high) diet; a high-calcium, low-dairy (high/low) diet; and a low-calcium, low-dairy (low/low) control diet. Each diet was followed by a washout period of 7–14 d. The subjects consumed these diets at home during the first 5.5 days of the intervention periods and during the last 36 h of each dietary intervention, when the subjects were studied in the respiration chamber.

Blood samples were obtained in the fasted state on the first day of each dietary intervention period ( $t = 0$  d) and on the evening the subjects entered the respiration chamber in the postprandial state ( $t = 5.5$  d). The final blood sample of each period was obtained in the fasted state when subjects left the respiration chamber ( $t = 7$  d). Fat biopsies were obtained in the fasted state at baseline and on the 3 occasions that the subjects left the respiration chamber at the end of the 3 dietary intervention periods.

### Diets

During the 5.5-d periods preceding their 36-h stay in the respiration chamber, subjects were provided with 3 different diets for consumption at home. All food items were consumed as breakfast, lunch, dinner, and 3 snacks. From the dinner on day 6 onward, all food was consumed in the respiration chamber. During these experiments in the respiration chamber, subjects received breakfast, lunch, and dinner at 0730, 1230, and 1800 and snacks at 1000, 1500, and 2030. These diets were composed so that their macronutrient and micronutrient compositions resembled the average consumption in the Netherlands. However, we choose to use a relatively high-protein content to ensure that the protein balance was equally positive in all experimental conditions, because a high-protein diet may affect body weight (29). The macronutrient and micronutrient compositions of these diets were calculated with the use of the Dutch food-composition table (32). The energy content of the diets during the first 5.5 d of each dietary intervention was calculated by using the Harris and Benedict equation (35) for men, multiplying the outcome of this calculation by 1.6 to adjust for daily physical activity.

The characteristics of the experimental isocaloric diets were as follows. The high-calcium, high-dairy (high/high) diet provided 50% carbohydrate, 20% protein, and 30% fat. Low-fat dairy was used as the main source of protein. The calcium content of this diet was 1200–1300 mg/d. The high-calcium, low-dairy (high/low) diet used nondairy sources of protein (animal and vegetable protein) to match the contribution to the total energy intake (EI) of protein, fat, and carbohydrate with that of the high/high diet.

The calcium content of this diet was increased to the same amount as that of the high/high diet (ie, 1200–1300 mg/d) by supplementing with calcium carbonate (CalciChew; Christiaens BV, Breda, Netherlands) (ie, a nondairy source of calcium). The low-calcium, low-dairy (low/low) diet was similar to the high/low diet but without the calcium carbonate supplementation. The calcium content of this diet was 300–400 mg/d.

Subjects received written and oral instructions every time when the diets were handed out and a meal-to-meal description of all food items that were to be consumed. All food items were weighed and packed at the research facility and were handed out to the subjects on days 1 and 4 of the dietary intervention periods. Subjects were instructed to write down all deviations from the diets and to report all problems immediately to the researcher. Furthermore, compliance to the diets was also checked with interviews; subjects were also instructed to return all the items they did not consume on the evening they reported to the laboratory for their 36-h stay in the respiration chamber. These returned food items were not included in the calculation of the total dietary intake during the experimental periods. In general, no serious deviations from the diets were reported; that is, these deviations did not affect the macronutrient composition and calcium content of the diets.

Because it has been shown that deviations from energy balance (ie, the absolute difference between EI and EE) can significantly alter substrate metabolism (36), it was assured that the subjects were in energy balance (here defined as a maximal difference between EI and EE of <0.5 MJ/24 h) during the measurements of 24-h EE and fat oxidation. To achieve this, at 0700, sleeping metabolic rate (SMR; defined as the lowest mean EE during 3 consecutive hours between 2300 and 0700) during the first night was calculated as previously described by Schrauwen et al (37) and multiplied with a physical activity index (PAI) of 1.7, which is an activity level that was observed previously in studies with a comparable exercise regimen (38). Furthermore, at 1600 and 2000, EE was calculated again to adjust EI.

### Indirect calorimetry

A respiration chamber was used to accurately determine EE and substrate oxidation (39). This respiration chamber is a 14-m<sup>3</sup> room equipped with a bed, chair, radio, telephone, intercom, a computer with Internet connection, sink, and toilet. Furthermore, the air in the respiration chamber is ventilated at a rate of 70–80 L/min. This ventilation rate is measured with a dry-gas meter (Schlumberger, type G6, The Hague Netherlands). The concentrations of oxygen and carbon dioxide are measured by using paramagnetic oxygen analyzers (type Magnos G6; Hartmann & Braun, Frankfurt, Germany; type OA 184A; Servomex East Sussex, United Kingdom) and infrared carbon dioxide analyzers (type Uras 3G; Hartmann & Braun). During each 15-min period, 6 samples of outgoing air for each chamber, one sample of fresh air, zero gas, and calibration gas are measured. The gas samples to be measured are selected by a computer, which also stores and processes the data.

### Calculations

The 24-h EE was calculated from  $\dot{V}O_2$  and  $\dot{V}CO_2$  values obtained in the period from 0700 until 0700 the next day with the use of the Weir formula (40). With the data on urinary nitrogen losses (described in “Biochemical methods”), these  $\dot{V}O_2$  and  $\dot{V}CO_2$

values were used to calculate carbohydrate, fat, and protein oxidation with the following equations of Brouwer (41):

$$\text{Protein oxidation (g/d)} = 6.25 \times N \quad (2)$$

Fat oxidation (g/d) =

$$(1.718 \times \dot{V}O_2) - (1.718 \times \dot{V}CO_2) - (0.315 \times P) \quad (3)$$

Carbohydrate oxidation (g/d) =

$$(4.17 \times \dot{V}CO_2) - (2.965 \times \dot{V}O_2) - (0.390 \times P) \quad (4)$$

where  $N$  is the total nitrogen excreted in urine (in g/d),  $\dot{V}O_2$  is the oxygen consumption (in L/d),  $\dot{V}CO_2$  is the carbon dioxide production (in L/d), and  $P$  is protein oxidation (in g/d). Consequently, substrate balances were calculated by subtracting the calculated values of macronutrient oxidation from the calculated intake of these macronutrients during the 24-h stay in the respiration chamber.

At daytime, subjects were instructed not to perform any strenuous exercise apart from the 45 min of moderate intensity [50% of maximal workload ( $W_{\max}$ )] cycling exercise at 1100. Furthermore, they were told not to sleep at daytime. They were asked to go to bed at  $\approx$ 2300 throughout all 3 respiration chamber experiments, and they were woken up at 0700.

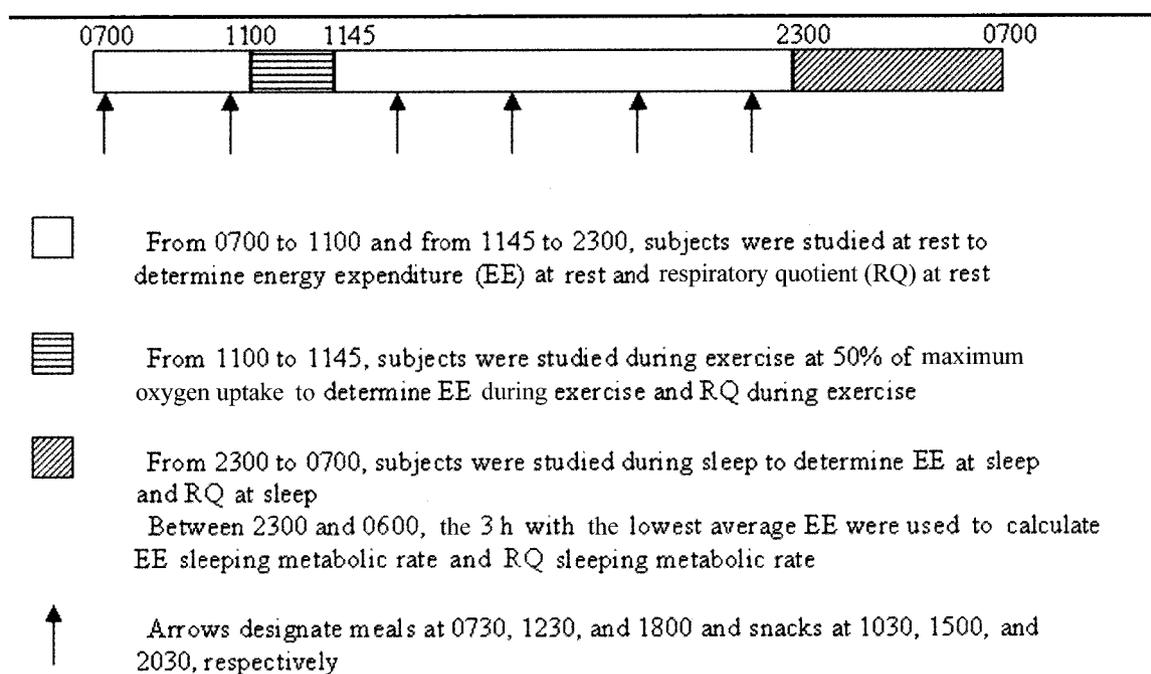
EE and substrate oxidation were calculated. The 4 individual periods included SMR [defined as the lowest mean EE during 3 consecutive hours between 2300 and 0700; respiratory quotient (RQ) was also determined during this period], EE and substrate oxidation during sleep ( $EE_{\text{sleep}}$  and  $RQ_{\text{sleep}}$ , defined as the average EE and RQ values from 2300 until 0700), resting EE and RQ ( $EE_{\text{rest}}$  and  $RQ_{\text{rest}}$ , defined as the average EE from 0700 until 2300, except for the 45 min of exercise), and EE and RQ during exercise ( $EE_{\text{exercise}}$  and  $RQ_{\text{exercise}}$ , defined as the average EE and RQ during exercise at 50%  $W_{\max}$ ).

Finally, the PAI was also calculated for the 3 different experiments in every subject. This was done by dividing the 24-h EE by the SMR during the second night of the respiration chamber measurements. For a schematic representation of these measurement periods, see **Figure 1**.

### Biochemical methods

After blood samples were drawn, they were centrifuged at  $4000 \times g$  for 10 min at 4 °C; aliquots of EDTA plasma and serum were frozen in liquid nitrogen and stored at –80 °C until further analysis. Total plasma free fatty acid (FFA), glucose, free glycerol, and  $Ca^{2+}$  concentrations in the blood samples were measured by using standard enzymatic techniques automated on Cobas FARA and MIRA centrifugal analyzers at 340 nm (for FFA: FFA-C test kit, Wako Chemicals, Neuss, Germany; for glucose: Roche Unikit III, Hoffman-LaRoche Inc, Basel, Switzerland; for free glycerol: Roche Molecular Biochemicals, Mannheim, Germany; for  $Ca^{2+}$ : Calcium CP, ABX Pentra, Montpellier, France). Plasma insulin was measured by using a specific double antibody radioimmunoassay for human insulin (Kabi Pharmacia, Uppsala, Sweden), and 1,25(OH)<sub>2</sub>D<sub>3</sub> was measured by using a monoclonal antibody radioimmunoassay (ImmunoDiagnostic Systems Ltd, Boldon, United Kingdom).

To allow us to calculate nitrogen excretion, 24-h urine was collected while the subjects were in the respiration chamber from the second voiding on the first morning until the first voiding on



**FIGURE 1.** Schematic representation of different periods of measurements of energy and substrate metabolism and timing of meals and snacks in the respiration chamber.

the second morning in containers with 10 mL of a 4 mol  $\text{H}_2\text{SO}_4$ /L solution to prevent nitrogen losses through evaporation. Volume of the 24-h urine was measured, and samples of the 24-h urine were stored at  $-20^\circ\text{C}$  until later analysis for nitrogen content.

#### Quantification of messenger RNAs in biopsies of human adipose tissue by using reverse transcriptase–quantitative polymerase chain reaction

After fat biopsies were obtained by using a percutaneous needle attached to a vacuum syringe, blood and connective tissue were washed off with a sterile 0.9% NaCl solution. Fat tissue was weighed and homogenized in 1 mL of a 1% solution of  $\beta$ -mercaptoethanol in RLT buffer (RNeasy Mini kit; Qiagen, Courtaboeuf, France) per 1000 mg fat tissue, frozen immediately in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until further analysis. Total RNA was extracted from adipose tissue biopsies by using the RNeasy Mini kit (Qiagen). First-strand complementary DNA (cDNA) was first synthesized from 200 ng total RNA in the presence of 100 U Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France) by using random hexamer primers (Promega, Charbonnières, France). Real-time quantitative polymerase chain reactions (qPCRs) were performed on a GeneAmp 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). cDNA (10 ng) was used as a template for real-time PCR. The thermal cycler limits for the real-time PCR were 2 min at  $50^\circ\text{C}$  followed by 40 cycles with 10 s at  $95^\circ\text{C}$  and 1 min at  $60^\circ\text{C}$ . For each primer pair, a standard curve was obtained by using serial dilutions of human adipose tissue cDNA before mRNA quantitation. For *HSL* a set of primers (Genset; Prologis France, Paris, France) was designed by using the software Primer Express 1.5 (Applied Biosystems) and used at a final concentration of 900 nmol/L with SYBR-Green–based chemistry. A dissociation curve was generated at the end of the PCR cycles to verify that a single gene product was amplified. For *UCP2*, *FAS*,

*GPDH2*, and *PPARG* the Taqman approach was used. Both primers and TaqMan probes were obtained from Applied Biosystems. The probes were labeled with a reporter dye (FAM) on the 5' end. The probe for 18S ribosomal RNA was labeled with the reporter dyes VIC and TAMRA (Applied Biosystems) on the 5' end and the 3' end, respectively. For Taqman assays, because of the high specificity of the method, a check for nonspecific product formation with dissociation curve is not needed. We used 18S rRNA as control to normalize gene expression by using the Ribosomal RNA Control TaqMan Assay kit (Applied Biosystems). All reactions were performed in duplicate.

#### Statistical analysis

Statistical power analysis by using the variance data from previously published, similar research, in which a SEM of the 24-h EE of 0.4 MJ and a SEM of the 24-h RQ of 0.01 were observed (42), indicated that a minimum of 12 subjects was required to detect a 0.02 decrease in RQ values and a 10% increase in EE between the high/high and low/low diets at a significance level of 95%. This would represent a difference in fat oxidation of  $\approx 20$  g/d and a difference in EE of  $\approx 1200$  kJ/d at an EE level of 12 MJ/d. These differences in RQ values and EE were based on the results of Melanson et al (9) who found even stronger effects of acute calcium intake on 24-h fat oxidation in a less well-controlled study.

Possible significant differences in substrate and energy metabolism induced by the 3 different diets were calculated by using a 2-factor analysis of variance (ANOVA), and a Scheffé test was used for post hoc analysis. Differences over time for the serum  $1,25(\text{OH})_2\text{D}_3$  concentration were tested for significance with an ANOVA repeated measures analysis. When a significant group  $\times$  time interaction was observed, a Scheffé post hoc analysis was used to determine the differences among the 3 dietary conditions on specific time points. Statistical difference was set at  $P < 0.05$ .

**TABLE 1**  
Physical characteristics of the male subjects<sup>1</sup>

	Value
Age (y)	28 ± 2 (20–40)
Height (m)	1.78 ± 0.02 (1.65–1.92)
Weight (kg)	80.0 ± 0.6 (65.5–95.9)
BMI (kg/m <sup>2</sup> )	25.2 ± 0.6 (21.2–27.9)
Body fat (%)	22.1 ± 1.6 (9.0–31.9)
W <sub>max</sub> (W)	271 ± 9 (233–336)
VO <sub>2</sub> max (L/min)	3.5 ± 0.1 (3.0–4.5)
Hf <sub>max</sub> (bpm)	193 ± 3 (175–203)
1,25(OH) <sub>2</sub> D <sub>3</sub> (pmol/L)	193 ± 27 (119–447)

<sup>1</sup> All values are  $\bar{x} \pm \text{SEM}$ ; range in parentheses;  $n = 12$ . W<sub>max</sub>, maximal workload; VO<sub>2</sub>max, maximal oxygen consumption; Hf<sub>max</sub>, maximal heart rate; bpm, beats per minute; 1,25(OH)<sub>2</sub>D<sub>3</sub>, baseline serum concentration of 1,25-dihydroxyvitamin D<sub>3</sub>.

All data are presented as means ± SEMs. SPSS for WINDOWS version 12.0.1 (Chicago, IL) was used for the statistical analysis.

## RESULTS

### Subject characteristics

The average age of the subjects was 28 ± 8 y. The average BMI was 25.2 ± 0.6 (Table 1).

### Habitual dietary intake

The subjects had an average energy consumption of 12.6 ± 1.0 MJ/d, which was consumed as 14.8 ± 0.9% protein, 45.9 ± 2.8% from carbohydrate, and 35.7 ± 2.2% from fat. The habitual daily calcium intake of these subjects was 1027 ± 82 mg, and the dietary fiber intake of the subjects was 21.2 ± 1.9 g/d (Table 2).

### Experimental diets

All subjects completed the 3 different dietary regimens without any adverse effects of the diets, except for 1 subject who reported after his first 5.5-d dietary run-in (high/low diet) that he had not consumed all of the food items provided, because he felt he was overeating. Because the macronutrient composition and calcium content of the diet he had consumed were in line with the experimental diets, the data from this experiment were included in the final analysis. His EI during the 2 following dietary intervention periods was adjusted according to his EE during the first

respiration chamber experiment. The average EI during the 7 days of the dietary interventions was 12.3 ± 0.3, 12.0 ± 0.6, and 12.3 ± 0.3 MJ/d for the high/high, high/low, and low/low diets, respectively. The experimental diets only differed in calcium concentration ( $P < 0.0001$ , low/low compared with high/high and high/low). All of the experimental diets provided significantly more dietary fiber and carbohydrates ( $P < 0.0001$ ) and significantly less fat ( $P < 0.01$ ) than the subjects' habitual diets (Table 2). The high-calcium diets provided significantly more calcium than the habitual diet ( $P < 0.01$ ), and the low-calcium diet provided significantly less calcium ( $P < 0.0001$ ). The content of energy and protein was not significantly different among any of the diets (Table 2). The dietary tool that was used in the present investigation does not allow for an accurate determination of vitamin D intake, but the average intake was  $\approx 16 \pm 8$  IU/d during the 3 experimental diets, whereas the habitual intake was  $28 \pm 8$  IU/d. This difference between the experimental and habitual diets was not statistically significant. To test whether the order in which the diets were given had an effect on the measurements of energy and substrate metabolism, all analyses were also performed with order of diet as a factor. No effects of order of the dietary intervention were observed.

### Metabolic measurements

In all experiments, energy balance was achieved [(EI – EE) < 0.5 MJ]. On average, the absolute deviation from energy balance was 0.2 ± 0.0 MJ/d (high/high), 0.3 ± 0.1 MJ/d (high/low), and 0.2 ± 0.1 MJ/d (low/low) during the 24-h measurements in the respiration chamber. The total 24-h EE was 11.8 ± 0.3, 11.6 ± 0.3, and 11.7 ± 0.3 MJ/24 h in the high/high, high/low, and low/low conditions, respectively. These differences were not statistically different. The EE during the other periods (EE<sub>SMR</sub>, EE<sub>sleep</sub>, EE<sub>rest</sub>, and EE<sub>exercise</sub>) and the PAI were not statistically different either (Table 3).

The average 24-h RQ was 0.85 ± 0.01, 0.85 ± 0.01, and 0.86 ± 0.01, and the average 24-h fat oxidation in the 3 conditions was 108 ± 7, 105 ± 9, and 100 ± 6 g/d in the high/high, high/low, and low/low conditions, respectively (Figure 2). These differences were not statistically different. The average RQ during SMR in the 3 different dietary conditions was not statistically different either (0.82 ± 0.01, 0.81 ± 0.02, and 0.82 ± 0.01, respectively). No significant differences in RQ were observed during the other periods (sleep, rest, or exercise) (Table 3).

**TABLE 2**

Energy intake, macronutrient composition, and content of calcium and dietary fiber in the habitual and experimental diets during the 7-d intervention<sup>1</sup>

	Diet			
	Habitual	High/high	High/low	Low/low
Energy intake (MJ/d)	12.6 ± 1.0	12.3 ± 0.3	12.0 ± 0.6	12.3 ± 0.3
Protein (% of energy)	14.8 ± 0.9	19.9 ± 0.1	19.7 ± 0.1	19.8 ± 0.1
Carbohydrate (% of energy)	45.9 ± 2.8 <sup>2</sup>	50.0 ± 0.1	49.9 ± 0.1	49.9 ± 0.1
Fat (% of energy)	35.7 ± 2.2 <sup>3</sup>	29.9 ± 0.1	30.2 ± 0.1	30.1 ± 0.1
Calcium (mg/d)	1027 ± 82	1259 ± 9 <sup>4,5</sup>	1259 ± 9 <sup>4,5</sup>	349 ± 8 <sup>6</sup>
Dietary fiber (g/d)	21.2 ± 1.9 <sup>2</sup>	34.7 ± 1.5	35.4 ± 1.1	31.2 ± 1.0

<sup>1</sup> All values are  $\bar{x} \pm \text{SEM}$ ;  $n = 12$ . Differences between the 4 diets were tested with a 2-factor ANOVA; a Scheffé test was used for post hoc analysis.

<sup>2,3</sup> Significantly different from all experimental diets: <sup>2</sup>  $P < 0.0001$ , <sup>3</sup>  $P < 0.01$ .

<sup>4,6</sup> Significantly different from the habitual diet: <sup>4</sup>  $P < 0.01$ , <sup>6</sup>  $P < 0.0001$ .

<sup>5</sup> Significantly different from the low/low diet,  $P < 0.0001$ .

**TABLE 3**  
Different components of energy expenditure (EE)<sup>1</sup>

	Diet		
	High/high	High/low	Low/low
EE (kJ/min)			
24-h EE	8.19 ± 0.21	8.04 ± 0.20	8.11 ± 0.20
EE <sub>sleep</sub>	5.58 ± 0.16	5.54 ± 0.13	5.55 ± 0.16
EE <sub>rest</sub>	8.04 ± 0.22	7.86 ± 0.21	7.86 ± 0.19
EE <sub>exercise</sub>	40.24 ± 1.36	38.45 ± 1.47	39.39 ± 1.39
PAI	1.60 ± 0.02	1.59 ± 0.03	1.61 ± 0.04
RQ			
24-h RQ	0.85 ± 0.01	0.85 ± 0.01	0.86 ± 0.01
RQ <sub>sleep</sub>	0.81 ± 0.01	0.82 ± 0.01	0.83 ± 0.01
RQ <sub>rest</sub>	0.87 ± 0.01	0.87 ± 0.01	0.87 ± 0.01
RQ <sub>exercise</sub>	0.90 ± 0.01	0.90 ± 0.01	0.91 ± 0.01
Absolute deviation from energy balance (MJ/24 h)			
Energy intake (MJ)	11.7 ± 0.3	11.6 ± 0.3	11.6 ± 0.3
EE (MJ)	11.8 ± 0.3	11.6 ± 0.3	11.7 ± 0.3
Absolute deviation <sup>2</sup>	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
Substrate balances (g/24 h)			
Protein	47.5 ± 4.6	40.3 ± 4.4	38.7 ± 5.0
Fat	-16.1 ± 6.0	-10.3 ± 7.6	-8.4 ± 5.2
Carbohydrate	11.6 ± 15.2	29.6 ± 19.6	8.6 ± 14.1

<sup>1</sup> All values are  $\bar{x} \pm \text{SEM}$ ;  $n = 12$ . Differences between the 3 diets were tested with a 2-factor ANOVA; a Scheffé test was used for post hoc analysis. None of the differences were statistically significant. PAI, physical activity index; RQ, respiratory quotient.

<sup>2</sup> Energy intake minus EE.

Because of the relatively high intake of dietary protein, a positive 24-h protein balance ( $47.5 \pm 4.6$ ,  $40.3 \pm 4.4$ , and  $38.7 \pm 5.0$  g in the high/high, high/low, and low/low conditions, respectively) was observed in all 3 experimental conditions. The 24-h fat balance was  $-16.1 \pm 6.0$ ,  $-10.3 \pm 7.6$ , and  $-8.4 \pm 5.2$  g in the high/high, high/low, and low/low conditions, respectively, and 24-h carbohydrate balance was  $11.6 \pm 15.2$ ,  $29.6 \pm 19.6$ , and  $8.6 \pm 14.1$  g in the high/high, high/low, and low/low conditions, respectively. No significant differences in substrate balance were observed among the 3 diets (Table 3).

No differences among the 3 diets were observed in the concentrations of plasma calcium, FFA, glucose, insulin, or glycerol. All values were within the normal range. The serum

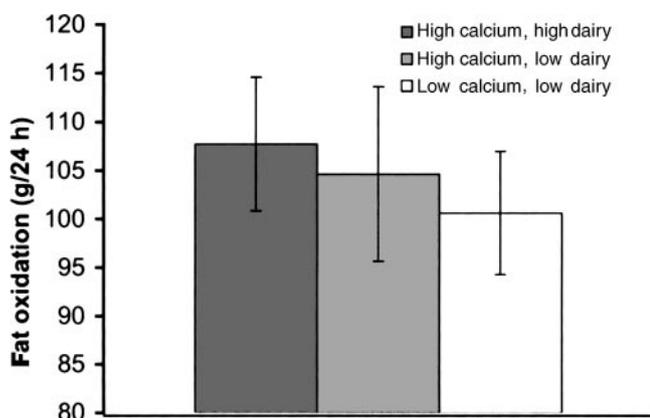
1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations at  $t = 0$  were not significantly different among the 3 interventions. The serum concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> decreased from  $175 \pm 16$  to  $138 \pm 15$  pmol/L during the high/high condition, decreased from  $181 \pm 23$  to  $159 \pm 19$  pmol/L in the high/low condition, and increased from  $164 \pm 13$  to  $198 \pm 19$  pmol/L in the low/low condition. A significant group  $\times$  time interaction was observed ( $P < 0.05$ ). The serum concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> was significantly different between the high/high and low/low conditions at  $t = 7$  ( $P < 0.05$ ). Furthermore, the change in serum 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration was  $-39 \pm 13$ ,  $-22 \pm 27$ , and  $33 \pm 15$  pmol/L for the high/high, high/low, and low/low conditions, respectively. The difference in change was significantly different between the high/high and low/low conditions ( $P < 0.05$ ). No differences were observed among the high/low and the other 2 conditions (Figure 3).

The mRNA concentrations in adipose tissue were determined for 10 subjects only, because for 2 subjects, no complete sets of biopsies could be obtained because of their relatively low percentage of body fat. No differences were observed in the mRNA concentration of *UCP2*, *FAS*, *GPDH2*, *HSL*, or *PPARG* among the 3 diets (Figure 4).

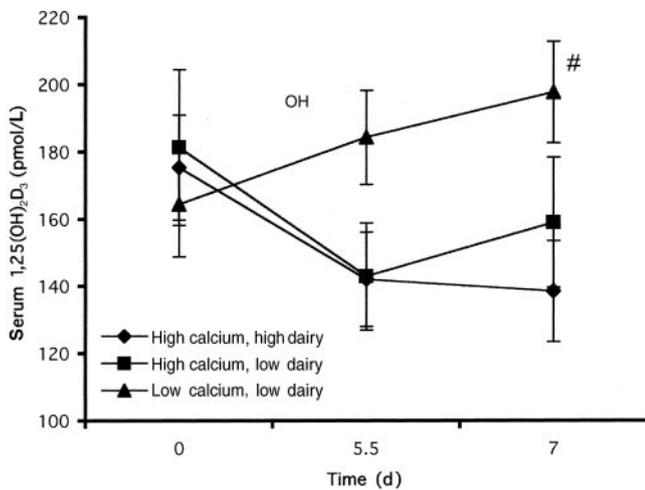
## DISCUSSION

In this carefully controlled dietary intervention study, no differences in energy or substrate metabolism were observed among 3 diets with varying contents of calcium and dairy protein, despite significant changes in the concentration of serum 1,25(OH)<sub>2</sub>D<sub>3</sub>. We did not see any differences in gene expression of 5 genes involved in the lipogenic and lipolytic pathways.

Because some epidemiologic investigations do show the long-term effects of an altered Ca<sup>2+</sup> intake, it may be argued that the



**FIGURE 2.** Mean ( $\pm \text{SEM}$ ) 24-h fat oxidation calculated from the indirect calorimetry values. The data were corrected for protein oxidation, which was calculated from 24-h nitrogen excretion in urine.  $n = 12$ . Differences between the 3 diets were tested with a 2-factor ANOVA; a Scheffé test was used for post hoc analysis. None of the differences were statistically significant.



**FIGURE 3.** Mean ( $\pm$ SEM) serum concentrations of 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] during the 7-d intervention period.  $n = 12$ . Differences over time between the 3 diets were tested for significance with a repeated-measures ANOVA. A Scheffé post hoc was used to determine differences between the 3 dietary conditions on different time points. A significant group  $\times$  time interaction was observed ( $P < 0.05$ ). <sup>#</sup>Significantly different from the low-calcium, low-dairy diet,  $P < 0.05$ .

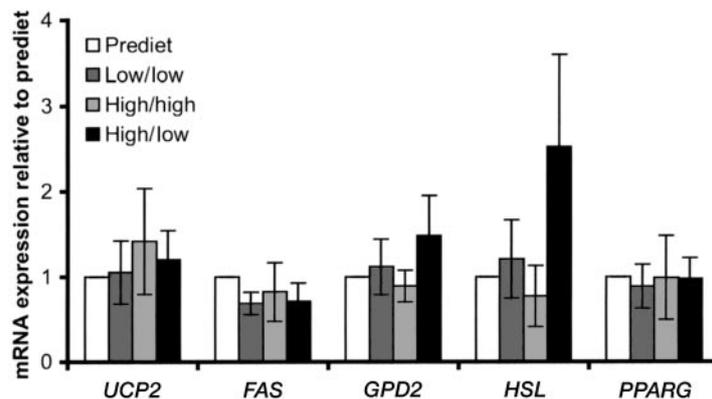
duration of the intervention period was not long enough to induce the necessary adaptations of the 1,25(OH)<sub>2</sub>D<sub>3</sub> metabolism. However, we observed significant differences among the 3 diets for this hormone within 7 d, which is in line with previous research (43). This hormone was shown to have immediate effects on lipolysis (22, 23, 44, 45), so the significant changes in 1,25(OH)<sub>2</sub>D<sub>3</sub> that were observed in this study should have induced the metabolic changes that we hypothesized. Nonetheless, other adaptations to a diet of high calcium and dairy may also occur that take longer than 7 d. The short duration of this intervention trial did not allow us to measure the effects of such longer-term adaptations.

It may also be argued that we studied the effects of calcium depletion rather than the effects of calcium supplementation because of the already high and possibly optimal calcium intake of our subjects. But this is contradicted by our data, because both the high/high and low/low conditions induced a significant change

from the baseline values of the serum 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration in these subjects with a habitual intake of high calcium. Along with the results from 2 other recent investigations in which no relation between 1,25(OH)<sub>2</sub>D<sub>3</sub> and body weight was observed (46, 47), our results indicate that the 1,25(OH)<sub>2</sub>D<sub>3</sub> metabolism may not play a significant role in the relation of calcium with body weight, which is not in line with previous studies that investigated the relation among calcium intake, 1,25(OH)<sub>2</sub>D<sub>3</sub>, and substrate and energy metabolism (4, 10, 48–50).

However, part of this research that shows significant effects of altering calcium intake on body weight was performed in rodents, and the results may not carry to the human situation. The high-calcium diet induced a 1.2-fold increase in calcium intake, whereas increases of up to 2-fold were observed in the animal studies (11, 12). Furthermore, the results of the various experiments performed in cell cultures are not directly applicable to whole-body human metabolism because the intracellular calcium concentrations in these experiments (14, 22, 23, 45, 51, 52) were manipulated directly by calcium channel and vitamin D receptor agonists and not through an altered dietary intake of calcium. Therefore, they do not reflect the normal physiologic situation. Thus, it seems that the results from these cell culture and animal studies are not directly applicable to the human in vivo situation.

An explanation for the discrepancy with the observational research and our results may be that because of the larger statistical power in epidemiologic investigations, the differences in body weight and body composition caused by subtle differences in energy and substrate metabolism that are expected from changes in calcium intake are more likely to be detected (3, 4, 25, 26, 53–58). Recently, Jacobsen et al (59) did not observe any differences in substrate and energy metabolism either, using a comparable study design. From a post hoc power analysis, it was calculated that to reach significant differences in EE with the variance and differences observed between groups in the present investigation, we should include at least 43 subjects. This shows that 7-d changes in dietary calcium content may not have a robust effect on either EE or fat oxidation, which is in line with recent work from Shapses et al (60). However, if the observed differences would sustain for a prolonged period, they may contribute to a significant effect on body weight. For example, if the effects



**FIGURE 4.** Mean ( $\pm$ SEM) changes in messenger RNA (mRNA) expression of adipose tissue *UCP2* (uncoupling protein 2), *FAS* (fatty acid synthase), *HSL* (hormone sensitive lipase), *PPARG* (peroxisome-proliferator activated receptor  $\gamma$ ), and *GPDH2* (glycerol phosphate dehydrogenase) genes relative to the prediet value, which was set at 1.00.  $n = 10$ . Differences between the 3 diets were tested with a 2-factor ANOVA; a Scheffé test was used for post hoc analysis. None of the differences were statistically significant.

on fat oxidation observed in this project would last for 1 y, they may account theoretically for a difference in body weight of  $\approx 3.0$  kg (assuming an additional fat oxidation of 8 g/d and with the assumption that an additional 38000 kJ needs to be expended to lose 1 kg body fat). An impairment of fat oxidation is suggested as an important prerequisite to become obese (61). This may be the link with the observed correlations between calcium intake and obesity in the epidemiologic studies.

Nonetheless, data from 2 interventional studies indicate a possible role for calcium in body weight regulation. In one human intervention trial, an additive effect of increasing dietary calcium intake on weight loss was observed (50). However, the endpoint of that experiment was body weight and not substrate metabolism. Furthermore, the subjects were obese, and it was previously shown that obese subjects show a larger increase in serum  $1,25(\text{OH})_2\text{D}_3$  in response to a low-calcium diet (16). In another human intervention study, Melanson et al (9) observed a relation between acute  $\text{Ca}^{2+}$  intake and fat oxidation in normal weight subjects who consumed an energy-balanced diet. The correlation between acute  $\text{Ca}^{2+}$  intake and fat oxidation was rather weak with  $r^2$  ranging from 0.32 to 0.38 ( $P = 0.03\text{--}0.07$ ), and food intake was not as well controlled as in the present randomized clinical trial. Furthermore, in the interventional study by Melanson et al (9) and also some other previously published observational studies (3, 4, 24–26), an increased protein intake that is related to an increase in calcium intake may have confounded the outcome measures. Although those 2 intervention studies may point at an effect of calcium intake on energy and substrate metabolism, differences in methods and subjects make it difficult to compare them with the present study.

An alternative mechanism that may be responsible for the observed epidemiologic relation between dairy and calcium intake and body weight is a reduced fat absorption from the gut induced by an increased calcium intake. In a recent investigation Jacobsen et al (59) observed that increasing the dietary calcium intake from 675 to 1850 mg/d induced no differences in energy or substrate metabolism but a rather large increase of the fecal fat excretion of 8.2 g/d (from 6.0 to 14.2 g/d). Papakonstantinou et al (62) also showed in rats that increasing the dietary calcium intake decreased the energy that was available from the diet from 94% to 90%. Although the magnitude of these results is not in line with previous research showing more modest effects of dietary calcium on fat absorption (63, 64), it is clear that fat absorption may be an important mechanism by which calcium intake can influence body composition.

Summarizing the results from this investigation, we have shown that a 7-d dietary intervention with either a high- or a low-calcium diet induces significant changes in the concentration of serum  $1,25(\text{OH})_2\text{D}_3$ . However, these changes were not accompanied by differences in energy and substrate metabolism or alterations in the mRNA concentration of 5 genes involved in fat and substrate metabolism, which is in contrast with previous data. Possible explanations for this discrepancy are first that we have successfully corrected for the confounding effects of protein intake. Second, the power of the present investigation may be too small to detect differences in energy and substrate metabolism induced by different concentrations of dietary calcium.

Finally, the results from other investigations (59, 63–65) also seem to indicate that the relation between intake of dairy and calcium and body weight that was observed in epidemiologic

reports may not be mediated by  $1,25(\text{OH})_2\text{D}_3$  but by other mechanisms, for example, the decreased fat absorption that is related to an increased calcium intake. This mechanism indeed warrants further investigation. 

NB was the principal investigator, GBJH provided practical assistance during the experiment, AS performed the mRNA analyses, NV assisted in the mRNA analyses and in writing the manuscript, DL supervised the mRNA analyses and assisted in writing the manuscript, WHMS supervised the study and the writing of the manuscript. None of the authors had a financial conflict related to this work.

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