

Calcium and Dairy Products Inhibit Weight and Fat Regain during Ad Libitum Consumption Following Energy Restriction in Ap2-Agouti Transgenic Mice^{1,2}

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ABSTRACT We demonstrated previously that dietary calcium suppression of calcitriol reduces adipocyte Ca^{2+} , suppresses lipogenesis, and increases lipid utilization during energy restriction. Notably, dairy calcium sources exert markedly greater effects. To determine the effects of dietary calcium and dairy products on energy partitioning during subsequent refeeding, we induced obesity in ap2-agouti transgenic mice with a high-fat/high-sucrose diet, then restricted energy intake from a high-calcium (1.3%) diet for 6 wk to induce fat loss, and then provided free access to a low-calcium (0.4%) diet or to high-calcium (1.3%) diets that utilized either calcium-fortified foods or dairy products (milk or yogurt) for 6 wk. Refeeding the low-calcium diet caused the regain of all weight and fat, whereas all high-calcium diets reduced fat gain by 55% ($P < 0.01$). All high-calcium diets stimulated adipose tissue uncoupling protein (UCP)2 and skeletal muscle UCP3 expression ($P < 0.001$) and slightly increased core temperature ($P = 0.136$), but only the dairy-based diets elicited a marked (>10 -fold, $P < 0.001$) increase in skeletal muscle peroxisome proliferator-activated receptor- α expression. All 3 high-calcium diets produced significant increases in lipolysis, decreases in fatty acid synthase expression and activity, and reduced fat regain ($P < 0.03$), but the 2 dairy-containing high-calcium diets exerted significantly greater effects on regain ($P < 0.01$). Thus, high-Ca diets elicit a shift in energy partitioning and reduction of weight gain during refeeding, with dairy Ca sources exerting markedly greater effects. J. Nutr. 134: 3054–3060, 2004.

KEY WORDS: • obesity • intracellular calcium • dairy

Although obesity results from chronic energy imbalance, the energy status of an individual influences the partitioning of ingested food energy between oxidation and storage (1–3). Because energy balance in humans is a dynamic state, often characterized by substantial fluctuations in energy intake and expenditure, individual responses to such changes in energy and nutrient status may have important long-term consequences with respect to the retention of body fat. Long-term weight maintenance after successful weight loss may be considered an example of such an adaptation, and is clearly a greater challenge than successful short-term weight loss (4).

Previous data from this laboratory demonstrated that adipocyte intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)⁴ plays a key role in disorders associated with obesity (5–9). Our understanding of the effects of $[\text{Ca}^{2+}]_i$ derives from studies of the mechanism of

action *agouti*, an obesity gene expressed in rodent and human adipocytes (5,9). We demonstrated that recombinant *agouti* protein stimulates the expression and activity of fatty acid synthase (FAS) (10,11), and inhibits lipolysis in both human and murine adipocytes (12,13). We also demonstrated that *agouti* is expressed in human pancreas, and stimulates both Ca^{2+} -signaling and insulin release in human pancreatic islets, thereby contributing to hyperinsulinemia (14,15). This coordinated regulation of lipid metabolism by *agouti* appears to be responsible for the accumulation of triglyceride in adipose tissue. Further, these effects of *agouti* can be mimicked by stimulation of Ca^{2+} influx with KCl and blocked by $[\text{Ca}^{2+}]_i$ channel antagonists (16,17), indicating that strategies directed at reducing adipocyte intracellular $[\text{Ca}^{2+}]_i$ would also be expected to reduce triglyceride accumulation in adipose tissue. Recent data from our laboratory demonstrated that calcitropic hormones, such as 1,25-(OH)₂-cholecalciferol, cause a significant, sustained increase in $[\text{Ca}^{2+}]_i$ in primary cultured human adipocytes and a corresponding inhibition of lipolysis (18). Consistent with this, we also reported that suppression of 1,25-(OH)₂-cholecalciferol by feeding high-calcium diets inhibited $[\text{Ca}^{2+}]_i$ influx and subsequently attenuated diet-induced adiposity by simultaneously stimulating lipolysis and inhibiting lipogenesis (19,20), suggesting that dietary calcium may not only attenuate the diet-induced development of adiposity but also promote weight loss in established obesity. We also demonstrated that dairy sources of calcium exert substan-

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⁴ Abbreviations used: ACE, angiotensin-converting enzyme; AM, acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} ; CLA, conjugated linoleic acid; CPT, carnitine palmitoyltransferase; DTT, dithiothreitol; eIF, eukaryotic initiation factor; FAS, fatty acid synthase; FBS, fetal bovine serum; mTOR, mammalian target of rapamycin; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator-activated receptor; UCP, mitochondrial uncoupling protein.

tially greater antiobesity effects than calcium carbonate (19,20). Compared with calcium carbonate, the use of dairy products as a calcium source induced a significantly greater attenuation of diet-induced obesity and acceleration of weight and fat loss during energy restriction in both mice and humans (9,19–21). Thus, dairy products appear to contain additional bioactive compounds that provide an attenuation of adiposity beyond that found with calcium (5).

We have now used an experimental model to further investigate the effect of $[Ca^{2+}]_i$ in regulating lipid metabolism in aP2 transgenic mice under conditions of varying nutrient status: ad libitum consumption, energy restriction, and refeeding. The purpose of this study was to investigate the effect of dietary calcium content and sources in regulating lipid metabolism and energy partitioning in response to energy repletion after deprivation.

MATERIALS AND METHODS

Mice and diets. This study was divided into 3 phases. Phase I was designed to induce weight gain and fat accretion, Phase II to induce weight and fat loss, and Phase III to determine the role of calcium and dairy products in attenuating weight and fat gain during refeeding. We utilized aP2-*agouti* transgenic mice as a model of diet-induced obesity, as previously described (19,20). These mice are useful models for diet-induced obesity in a genetically susceptible human population in that they are not obese when they consume a standard AIN-93G diet, but develop mild-to-moderate obesity when fed high-sucrose and/or high-fat diets (19–21).

Phase I. aP2 transgenic mice (6 wk old) were fed the basal low-calcium/high-sucrose/high-fat diet during the 6-wk obesity induction period. At 6 wk of age, aP2-*agouti* transgenic mice ($n = 60$) from our colony began consuming a modified AIN 93G diet (22) with suboptimal calcium (calcium carbonate, 0.4%), sucrose as the sole carbohydrate source and providing 64% of energy; fat increased, to 25% of energy with lard and protein (casein-purified high nitrogen), provided 11% of energy. Mice were studied for 6 wk, during which food intake and spillage were measured daily and body weight, fasting blood glucose, food consumption, and core temperature were assessed weekly. At the end of Phase I, 5 representative mice were killed as described below to collect blood, fat pads, liver, and muscle.

Phase II. At the end of Phase I, the 55 mice remaining were fed a high-calcium diet with energy intake limited to 70% of that found during ad libitum consumption. The high-calcium diet used in this phase consisted of the basal diet with the addition of sufficient calcium-fortified cereal to bring the calcium content of the diet to 1.3%, with the macronutrients adjustments as described previously (19,20). Mice were then studied for 6 wk, during which food intake and spillage were measured daily and body weight, fasting blood glucose, food consumption, and core temperature were assessed weekly. At the end of Phase II, 5 mice were killed as described below to collect blood, fat pads, liver and muscle.

Phase III. At the end of the Phase II, the 50 mice remaining were further randomized into 5 groups that consumed the following diets ad libitum: 1) basal diet: modified AIN 93G diet with suboptimal calcium (0.4%), as described in Phase I; 2) high-Ca cereal: the high-Ca-(1.3%) cereal diet described in Phase II; 3) milk plus high-Ca-cereal: a high calcium cereal plus nonfat milk diet with 1.2% calcium derived from calcium-fortified cereal and an additional 0.1% calcium content derived from nonfat dry milk, and 20% protein replaced by nonfat dry milk; 4) yogurt: a high-calcium (1.3%) diet in which sufficient spray-dried nonfat yogurt was added to the basal diet to increase calcium to 1.3%, with 100% protein replaced by nonfat dry yogurt; and 5) cereal control: the basal diet with a calcium-free control cereal (identical to the calcium-fortified cereal used in diet 3, but without the calcium fortification) with a final calcium concentration of 0.4% and macronutrients adjusted to match the other diets. Ten mice/group were studied for 6 wk, during which food intake and spillage were measured daily and body weight, fasting blood glucose, food consumption, and core temperature were assessed weekly. At the conclusion of the study, all mice were killed under isoflurane anes-

thesia and blood collected via cardiac puncture; fat pads, soleus, and gastrocnemius muscles were immediately excised, weighed, and used for further study, as described below.

This study was approved by the Institutional Care and Use Committee of The University of Tennessee.

Core temperature. Core temperature was used as an indirect metabolic index to determine the effect of the diets on thermogenesis. Temperature was measured via a thermocouple (Columbus Instruments) weekly. The probe was inserted a constant distance (1.8 cm) into the rectum of each mouse. After stabilization (10 s), the temperature was recorded every 5 s for 30 s. All core temperature measurements were performed between 0800 and 0900 h.

Adipocyte $[Ca^{2+}]_i$ measurement. Adipose tissue was first washed several times with HBSS, minced into small pieces, and digested with 0.8 g/L type I collagenase in a shaking water bath at 37°C for 30 min. Adipocytes were then filtered through sterile 500- μ m nylon mesh and cultured in DMEM supplemented with 1% fetal bovine serum (FBS). Cells were cultured in suspension and maintained in a thin layer at the top of culture media for 2 h for cell recovery. $[Ca^{2+}]_i$ in isolated mouse adipocytes was measured by using a fura-2 dual wavelength fluorescence imaging system. Before $[Ca^{2+}]_i$ measurement, adipocytes were preincubated in serum-free medium for 2 h and rinsed with HBSS containing the following components (in mmol/L): NaCl, 138; $CaCl_2$, 1.8; $MgSO_4$, 0.8; NaH_2PO_4 , 0.9; $NaHCO_3$, 4; glucose, 5; glutamine, 6; HEPES, 20; and bovine serum albumin, 1%. Adipocytes were loaded with fura-2 acetoxymethyl ester (AM) (10 μ mol/L) in the same buffer in dark for 1 h at 37°C. Adipocytes were rinsed with HBSS 3 times to remove extracellular dye and then postincubated at room temperature for an additional 30 min to permit complete hydrolysis of cytoplasmic fura-2 AM. A thin layer of adipocytes was plated in 35-mm dishes with glass coverslips (P35G-0-14-C, MatTek). The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu 4915 CCD camera. Fluorescent images were captured alternatively at excitation wavelength of 340 and 380 nm with an emission wavelength of 520 nm. $[Ca^{2+}]_i$ was calculated by using a ratio equation as described previously (19).

Fatty acid synthase (FAS) activity assay. FAS activity was determined spectrophotometrically in crude cytosolic extracts of mouse adipose tissue. Mouse abdominal fat pads were homogenized in 250 mmol/L sucrose solution containing 1 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), and 100 μ mol/L phenylmethylsulfonyl fluoride (PMSF), pH 7.4. The homogenate was centrifuged at $18,500 \times g$ for 1 h, and the infranatant was used for measuring oxidation rate of NADPH. The final FAS activity was normalized to DNA, which was measured by CyQUANT cell proliferation assay kit according to the manufacturer's instruction (Packard Instrument).

Lipolysis assay. Perirenal adipose tissue was immediately dissected and incubated for 4 h. Glycerol released into the culture medium was used to measure lipolysis, as described previously (4). Glycerol was measured using a 1-step enzymatic fluorometric method (19). The final lipolysis data were normalized to DNA, as described above.

Total RNA extraction. A total cellular RNA isolation kit (Ambion) was used to extract total RNA from mouse abdominal adipose tissue and soleus muscle according to the manufacturer's instruction.

Quantitative real-time PCR. Mouse abdominal adipose tissue FAS, peroxisome proliferator-activated receptor (PPAR) γ , mitochondrial uncoupling protein (UCP)2 mRNA, and mouse soleus muscle UCP3 and PPAR α mRNA were measured quantitatively using a Smart Cycler Real Time PCR System (Cepheid) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems). The primers and probes for mouse genes were as follows. FAS: forward primer: 5'-CCCAGAGGCTTGTGCTGACT-3', reverse primer: 5'-CGAATGTGCTTGGCTTGGT-3', probe: 5'-CCGATCTGGAATCCGCACCG-3'; PPAR α : forward primer: 5'-CGATACTGTCCTCCTTGATGA-3', reverse primer: 5'-CGCGTGTGATAAAGCCATTG-3', probe: 5'-ACGCGATCAGCATCCCGTCTTTG-3'; PPAR γ forward primer: 5'-GCCTATGAGCACTTCACAAGAAATT-3', reverse primer: 5'-TGCGAGTGGTCTTCCATCAC-3', probe: 5'-TCTGCCCCACCAACTTCGGAATCAG-3'; UCP2 forward primer: 5'-GCGTTCTGGGTACCATCCTAAC-3', reverse primer: 5'-

GCGACCAGCCCATTGTAGA-3', probe: 5'-GCCTATGAGCACTTCACAAGAAATT-3'; UCP3 forward primer: 5'-CCTCTACGACTCTGTCAAGCAGTTCTAC-3', reverse primer: 5'-CAGCCTGCCAGAATCCTGAT-3', probe: 5'-CGCTGGA-TGGTCCGCTCCCT-3'.

Pooled mouse abdominal adipose tissue total RNA was diluted serially in the range of 1.5625–25 ng and used to establish a standard curve of the desired gene; total RNAs for unknown samples were also diluted in this range. Reactions of quantitative RT-PCR for standards and unknown samples were also performed according to the instructions of Smart Cycler System (Cepheid) and TaqMan Real Time PCR Core Kit (Applied Biosystems). The mRNA quantitation for each sample was further normalized using the corresponding 18s or cyclophilin quantitation, with 18S forward primer: 5'-AGTCCCTGCCCTTTGTA-CACA-3', reverse primer: 5'-GATCCGAGGGCCTCACTAAAC-3', probe: 5'-CGCCCGTTCGCTACTACCGATTGG-3'; cyclophilin forward primer: 5'-GGTGGAGAGACCAAGACAGA-3', reverse primer: 5'-GCCGGAGTCGACAATGATG-3', probe: 5'-AGCCG-GGACAAGCCACTGAAGGAT-3'.

Carnitine palmitoyltransferase (CPT) assay. Liver samples were first homogenized in 10 volumes (wt:vol) of 0.25 mol/L sucrose: 0.2 mmol/L EDTA (pH 7.5) in a glass tissue grinder to isolate hepatocyte mitochondria. The tissue homogenates were then centrifuged for 12 min at $750 \times g$ to collect the supernatant, which was further centrifuged for 12 min at $6700 \times g$. These centrifugations were repeated twice and the pellet was then suspended in 4 mL of mitochondrial suspension buffer (70 mmol/L sucrose, 220 mmol/L mannitol, 2 mmol/L HEPES buffer, 1 mmol/L EDTA, pH 7.4). CPT activity was determined by measuring the initial rates of total CoASH formation from palmitoyl CoA by mitochondria individually with l (-)-carnitine and d (+)-carnitine using a spectrophotometric method. Final data were normalized to cytosolic protein, which was assayed by the method of Bradford (23).

Statistical analysis. All data were evaluated for normality of distribution and equality of variance before statistical analysis. Variables with skewed distribution were ln-transformed. Outcomes are shown as means \pm SD and were evaluated for statistical significance by 1-way ANOVA; significantly different group means were then separated by the least significant difference (LSD) test using SPSS. Difference were considered significant at $P \leq 0.05$.

RESULTS

Although food consumption did not differ among the 5 groups of mice during the refeeding after energy restriction (food intake/mouse basal group: 3.5 ± 0.1 g/d, high-Ca cereal group: 3.7 ± 0.1 g/d, high-Ca cereal plus dry milk: 3.8 ± 0.1 g/d, yogurt group: 3.9 ± 0.2 g/d, and cereal control group: 3.7 ± 0.1 g/d, $P > 0.05$), there was a striking effect of diet on weight gain ($P < 0.05$) (Fig. 1A) and fat pad weight (Fig. 1B) ($P < 0.01$). Mice refed the 2 low-calcium diets exhibited 27% weight gains and 487% increases in fat pad mass. In contrast, mice refed high-calcium diets exhibited only ~50% of this regain. Further, the dairy-based, high-calcium diets were more effective in preventing fat gain than the high-calcium cereal diet; the high-dairy product diets (yogurt or cereal + milk diets) prevented 85% of the fat gain, whereas the high-calcium cereal diet prevented 55% of the fat gain.

Lipolysis was decreased 20% ($P < 0.05$) in mice that consumed the 70% energy-restricted, high-calcium cereal diet compared with those that consumed the high-sucrose, high-fat, low-calcium diet ad libitum (data not shown). Upon refeeding after energy restriction, the mice refed the milk-based, high-calcium cereal diet exhibited significantly greater lipolysis than mice refed low-calcium diets ($P < 0.03$). Adipose tissue FAS activity and expression responded to dietary manipulations in an inverse fashion to lipolysis responses. Energy restriction in mice fed the high-calcium cereal diet decreased FAS activity and expression by 91 and 99%, respec-

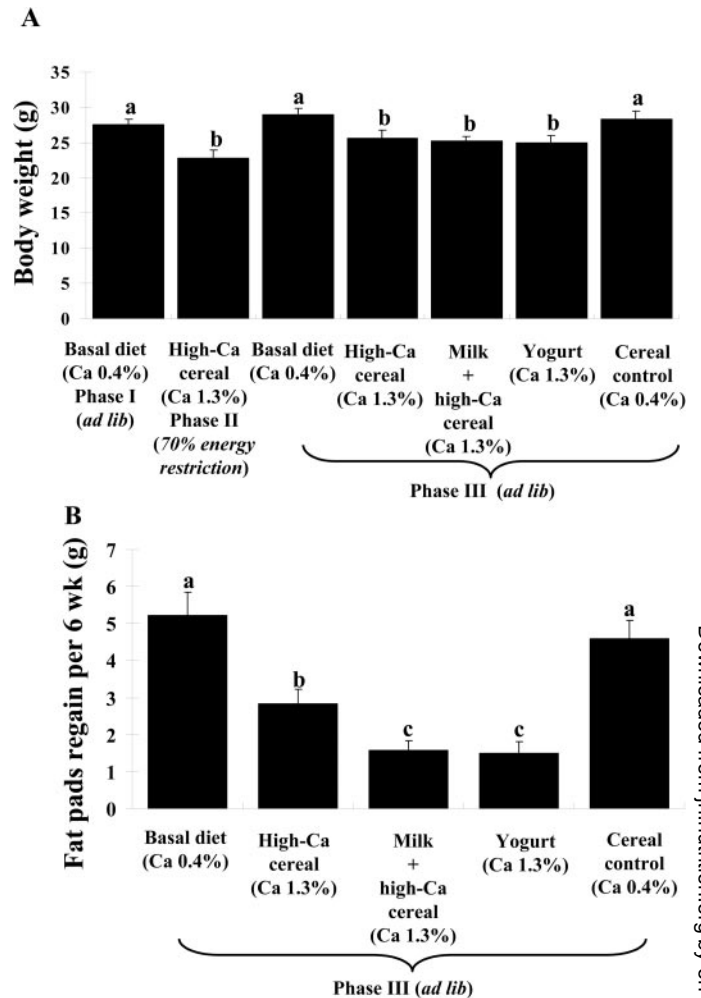


FIGURE 1 Effect of dietary calcium and dairy on body weight (A) and fat mass regain (B) in aP2 transgenic mice. Values are means \pm SEM ($n = 5$ for Phase I and Phase II, $n = 8$ for Phase III). Means without a common letter differ, $P < 0.05$.

tively (data not shown). Upon refeeding, the low-calcium diets restored FAS activity (Fig. 2B) and expression (Fig. 2C) to the basal level (cereal control diet) or higher (low-calcium basal diet, $P < 0.03$). In contrast, the groups refed high-calcium diets exhibited markedly lower adipose FAS activity and expression. Furthermore, mice refed the dairy-based, high-calcium diets had lower FAS expression than those fed the cereal-based, high-calcium diet ($P < 0.001$).

Energy restriction for 6 wk in aP2 mice fed a high-calcium diet resulted in a 79% decrease in adipocyte $[Ca^{2+}]_i$ (380 ± 43 vs. 79 ± 10 mol/L). Upon refeeding, the low-calcium diets induced 4- to 5-fold ($P < 0.001$) increases in adipocyte $[Ca^{2+}]_i$ (Fig. 3), whereas there was no increase in $[Ca^{2+}]_i$ among the mice refed the high-calcium diets, suggesting that diet-induced dysregulation of adipocyte $[Ca^{2+}]_i$ is associated with diet-induced adiposity in aP2-*agouti* transgenic mice.

To determine whether shifts in liver fat oxidation potentially contributed to changes in adiposity, we also examined liver CPT activity (Fig. 4). Refeeding aP2-transgenic mice the low-calcium diets slightly increased liver CPT activity (44 and 56%), whereas the high-calcium diets increased CPT activity by 150% ($P < 0.05$). However, the source of calcium did not affect liver CPT activity ($P = 0.213$).

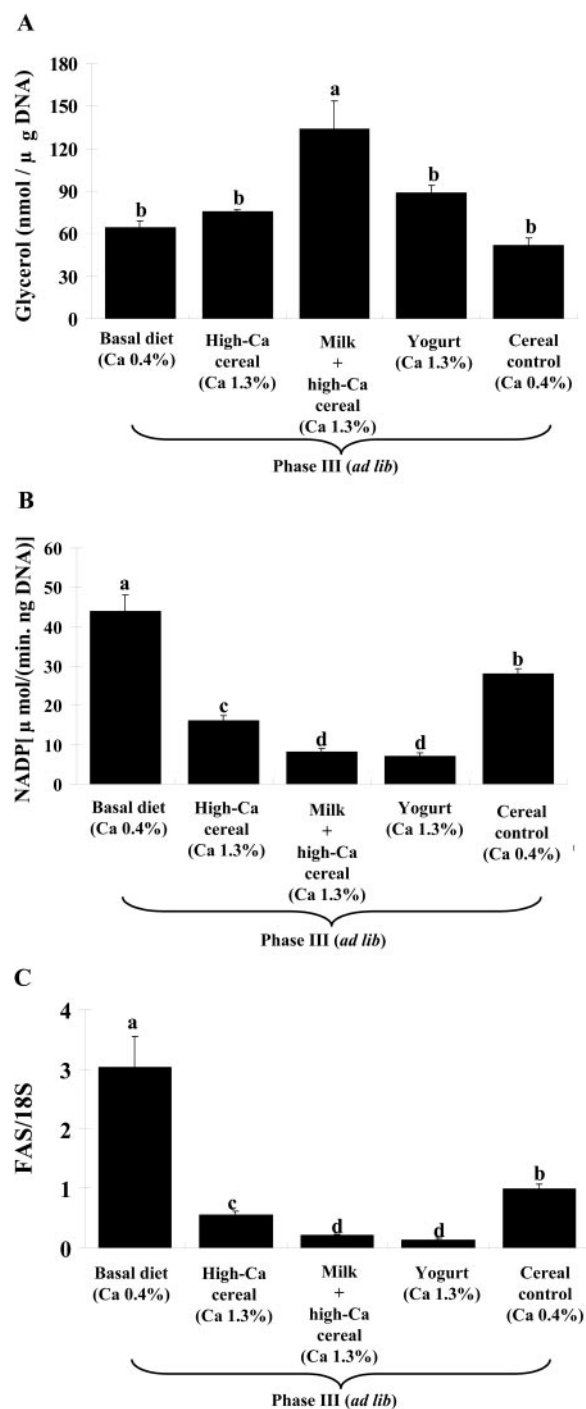


FIGURE 2 Effect of dietary calcium and dairy refeeding on adipose basal lipolysis (A), FAS activity (B) and FAS expression (C) in aP2 transgenic mice. Values are means \pm SEM, $n = 8$. Means without a common letter differ, $P < 0.05$.

The shift in energy metabolism with refeeding was further confirmed by dietary-induced increase in UCP2 expression in adipose tissue (Fig. 5) and UCP3 (Fig. 6) expression in skeletal muscle. Upon refeeding, the high-calcium diets induced 8- to 19-fold increases in UCP2 expression in white adipose tissue and 6- to 8-fold increases in UCP3 expression in soleus muscle, compared with the low-calcium diets ($P < 0.001$). Furthermore, the dairy-based, high-calcium diets had greater effects ($P < 0.05$) on adipose tissue UCP2 expression than the

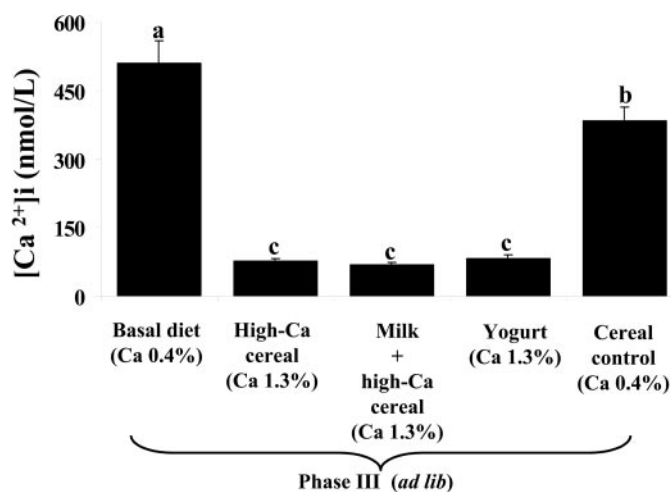


FIGURE 3 Effect of dietary calcium and dairy refeeding on adipocyte intracellular calcium in aP2 transgenic mice. Values are means \pm SEM ($n = 8$). Means without a common letter differ, $P < 0.05$.

cereal-based, high-calcium diet, although a comparable difference between calcium sources was not found for skeletal muscle UCP3 expression.

We also found that dietary calcium slightly increased core temperature ($P = 0.136$) (data not shown). This increase, coupled with the lack of difference in food intake during refeeding, suggests a shift in efficiency of energy metabolism from energy storage to thermogenesis.

This shift in energy metabolism was also evident in studies of PPAR α , a transcriptional factor that regulates cellular lipid catabolism. Refeeding the dairy-based, high-calcium diets markedly increased (>10-fold) skeletal muscle PPAR α compared with the low-calcium diets (Fig. 7, $P < 0.001$), whereas the cereal-based, high-calcium diet had less of an effect on PPAR α expression. Mice refed high-calcium diets exhibited significantly lower adipocyte PPAR γ expression than those refed the low-calcium diets ($P < 0.03$) (Fig. 8), suggesting that dietary calcium may also inhibit fat mass regain by suppressing adipogenesis.

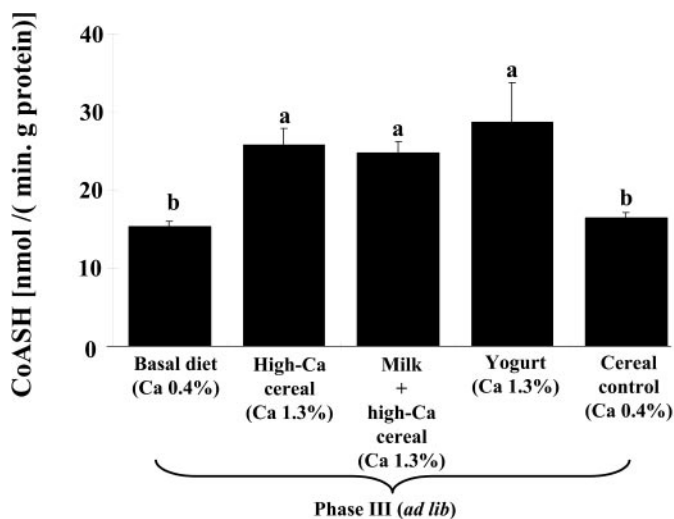


FIGURE 4 Effect of dietary calcium and dairy refeeding on liver CPT activity in aP2 transgenic mice. Values are means \pm SEM, $n = 8$. Means without a common letter differ, $P < 0.05$.

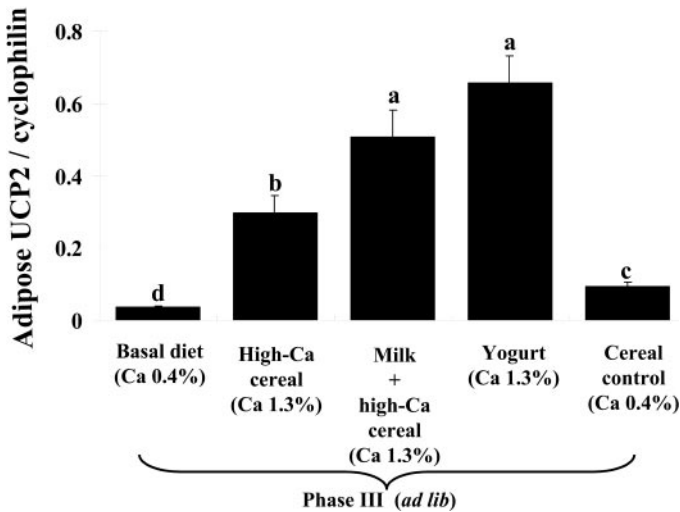


FIGURE 5 Effect of dietary calcium and dairy refeeding on adipose UCP2 expression in aP2 transgenic mice. Values are means \pm SEM, $n = 8$. Means without a common letter differ, $P < 0.05$.

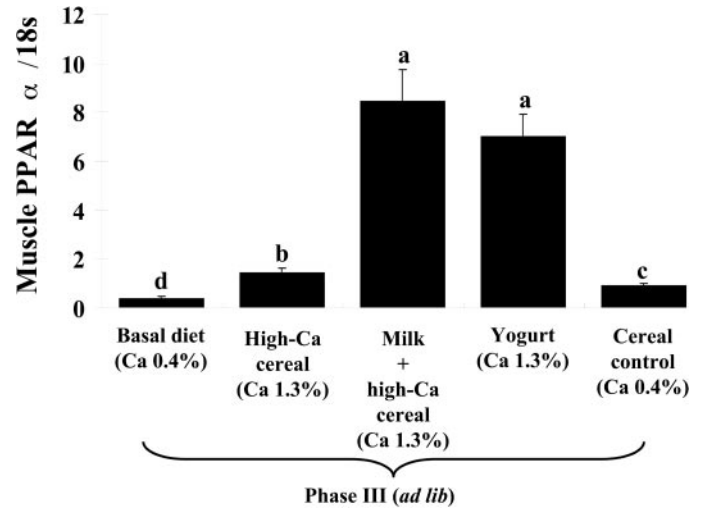


FIGURE 7 Effect of dietary calcium and dairy refeeding on PPAR α expression in aP2 transgenic mice. Values are means \pm SEM, $n = 8$. Means without a common letter differ, $P < 0.05$.

DISCUSSION

Obesity is a complex genetic trait, with multiple genes interacting to confer relative resistance or susceptibility to positive energy balance. Similarly, specific micro- or macronutrients, dietary patterns, or both may modulate the same metabolic pathways affected by these genetic factors, thereby altering nutrient and energy partitioning. This concept is supported by emerging evidence indicating that dietary calcium and dairy products may play a role in the modulation of adiposity independent of energy restriction. However, the effect of dietary calcium and dairy products on prevention of fat and weight regain is not yet clear.

The present study demonstrates that dietary calcium exerts marked effects in the regulation of weight and fat regain in aP2-agouti transgenic mice because the high-calcium diets inhibited weight and fat regain, whereas the low-calcium diets promoted fat and weight regain. Notably, dairy sources of calcium appear to exert greater effects than elemental calcium.

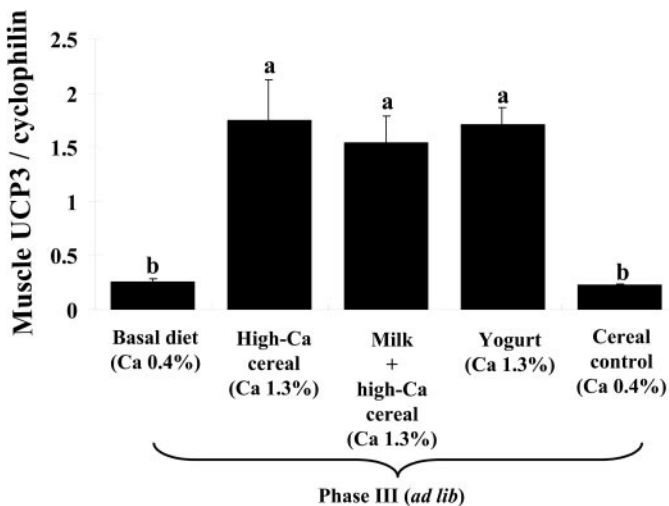


FIGURE 6 Effect of dietary calcium and dairy refeeding on adipose UCP3 expression in aP2 transgenic mice. Values are means \pm SEM, $n = 8$. Means without a common letter differ, $P < 0.05$.

Although the diets did not affect energy intake when the mice consumed feed ad libitum after the period of energy restriction, those refed the low-calcium diets showed a rapid, pronounced regain of weight and fat that exceeded that lost during energy restriction. In contrast, the mice refed the high-calcium diets regained only 50% of the weight lost during the energy-restriction period, suggesting that dietary calcium inhibits weight and fat regain by decreasing efficiency of energy utilization during rebound food ingestion.

We also observed that dietary calcium levels are inversely associated with $[Ca^{2+}]_i$ levels in white adipocyte tissue, with mice refed low-calcium diets exhibiting high adipocyte $[Ca^{2+}]_i$, whereas those refed high calcium exhibited low adipocyte $[Ca^{2+}]_i$. Adipocyte $[Ca^{2+}]_i$ was determined to regulate human and murine adipocyte metabolism, as recently reviewed (5). Accordingly, manipulation of $[Ca^{2+}]_i$ is an attractive and logical approach for development of therapeutic interventions in obesity. $1,25-(OH)_2D_3$ was shown previously

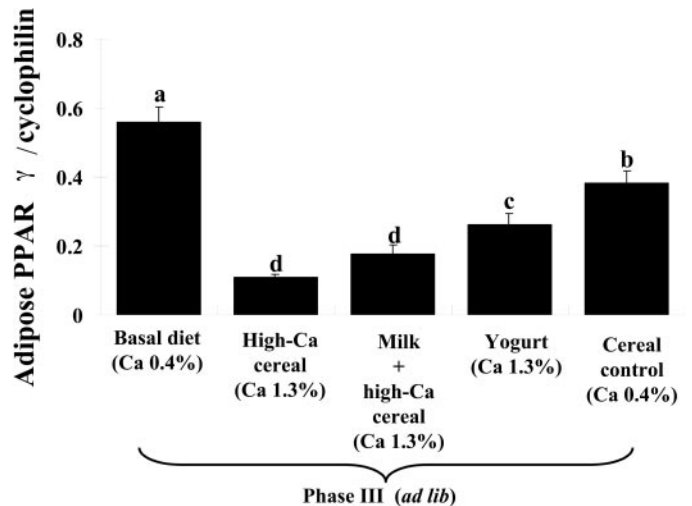


FIGURE 8 Effect of dietary calcium and dairy refeeding on PPAR γ expression in aP2 transgenic mice. Values are means \pm SEM, $n = 8$. Means without a common letter differ, $P < 0.05$.

to stimulate $[Ca^{2+}]_i$ in multiple cell types, including vascular smooth muscle cells, pancreatic β cells, and adipocytes (15,17,18,20). We demonstrated that 1,25-(OH)₂-cholecalciferol also acts on human adipocytes to cause a rapid sustained increase in $[Ca^{2+}]_i$ and a coordinated activation of FAS and inhibition of lipolysis (5,18,20). Increased 1,25-(OH)₂-cholecalciferol was also observed in obese humans, further suggesting that it may affect human energy metabolism (20). Consistent with this concept, we demonstrated recently that dietary calcium not only attenuates diet-induced obesity but also accelerates weight loss and fat mass reduction secondary to energy restriction in established obesity in both mice (19) and humans (9). In the present study, antagonism of $[Ca^{2+}]_i$ was again achieved through a dietary intervention by increasing dietary calcium. We proposed that suppression of 1,25-(OH)₂-D₃ dietary calcium would reduce adipocyte $[Ca^{2+}]_i$, thereby regulating lipid metabolism in response to different energy status. Indeed, the present study extended our previous observations by demonstrating that dietary calcium not only attenuates diet-induced obesity and accelerates weight loss and fat mass reduction in energy-restricted and obese mice, but also inhibits weight and fat mass regain during refeeding secondary to energy restriction.

Notably, increasing dietary calcium not only modulates energy partitioning by inhibiting lipogenesis and stimulating lipolysis, but also stimulates UCP2 and UCP3 expression and tends to increase core temperature, an indirect metabolic index associated with thermogenesis during refeeding after energy restriction. This may further contribute to the observed attenuation of weight and fat pad regain during refeeding. This concept is supported by our previous observation that 1,25-(OH)₂-cholecalciferol inhibits UCP2 expression in human adipocytes, indicating that its suppression by increasing dietary calcium may stimulate UCP2-induced thermogenesis and fat oxidation, thereby resulting in decreased energy efficiency.

The function of UCPs is not restricted to thermoregulation because a growing body of evidence links UCP expression to fatty acid metabolism and fatty acid flux in various tissues (24,25). Previous studies demonstrated that FFA increase UCP2 and UCP3 expression in adipocytes, pancreatic β cells, and myocytes (26–29). These data strongly support a regulation of UCP2 and UCP3 expression by fatty acids and also indicate that UCP activity could be related to fatty acid oxidation. In fact, UCP2 and UCP3 may function as FFA transporters, thereby increasing fatty acid utilization (30,31).

The regulation of UCP transcription is attributable to a group of ligand-regulated transcription factors such as peroxisome proliferation-activated factors, which can be activated upon binding fatty acid ligand (32). Because PPARs are also involved in the regulation of adipocyte differentiation and lipid oxidation (32), it is possible that they are the key regulators that coordinate the activation of lipid metabolism and oxidation in adipocytes. In fact, a PPAR response element was identified in the UCP promoter region (33,34), and PPAR α activation in mice is sufficient to induce liver UCP-2 expression (35). Consistent with this, our data showed that increases in UCP2 and UCP3 expression induced by a high-calcium diet were accompanied by marked increases in PPAR α expression during refeeding, indicating that high dietary calcium may stimulate UCPs via a PPAR α -dependent mechanism. Consistent with the effect of dietary calcium on stimulating thermogenesis and energy partitioning via upregulation of UCPs, we also observed that high-calcium diets significantly increased hepatic activity of CPT during refeeding, suggesting that dietary calcium may stimulate a diversion of hepatic fatty acid metabolism toward the oxidation pathway when refeeding

occurs ad libitum. These data suggest that dietary calcium reduces lipid accumulation via energy repartitioning and dissipation, resulting in less regain of fat when refeeding occurs ad libitum.

We also found that refeeding high calcium diets significantly decreased PPAR γ expression in white adipose tissue. Unlike PPAR α , which is highly expressed in liver and skeletal muscle and modulates fatty acid oxidation, PPAR γ is restricted mainly to white and brown adipose tissue and regulates adipogenesis (36). Accordingly, the present data suggest the possibility that dietary calcium may also inhibit adiposity by decreasing adipogenesis during food readministration.

It is also noteworthy that although dairy and elemental calcium exerted qualitatively comparable effects, the magnitude of these effects was significantly different. Although all 3 high-calcium diets similarly affected inhibition of body weight regain during refeeding, the inclusion of dairy products resulted in significantly less regain of fat and weight compared with mice fed elemental calcium. Consistent with this, calcium derived from dairy products exerted a markedly greater inhibitory effect on lipogenesis and greater stimulatory effect of lipolysis compared with the nondairy calcium source. Previous data demonstrated that the increase in dietary calcium from 1.2 to 1.3% by the addition of a small amount of nonfat dry milk doubled the rate of fat loss (unpublished data), and data from present study are consistent with these observations. Thus, utilizing a dairy source of calcium maximizes the antiobesity effects of calcium.

Additional factors in dairy products responsible for this effect in addition to calcium were recently discussed. Several investigators reported that conjugated linoleic acid (CLA), a component in dairy product and meat is an effective regulator of body fat accumulation and retention (37). However, the dairy diet utilized in this study included nonfat dry milk and fat-free yogurt, which do not contain CLA; thus, no effects can be attributed to CLA. Other components of dairy products that may contribute to these antiobesity effects include BCAA and small bioactive peptides. Our preliminary data in mice isolated most of the additional bioactivity of dairy products to the whey fraction (unpublished data), which contains a high proportion of BCAA (leucine, isoleucine, and valine). BCAAs, especially leucine, play a specific metabolic role in the regulation of energy metabolism and muscle protein synthesis (38,39). In skeletal muscle, leucine stimulates protein synthesis and inhibits protein catabolism through multiple independent mechanisms (40,41). Leucine stimulates protein synthesis through the mammalian target of rapamycin (mTOR) pathways, 70-kDa ribosomal protein S6 kinase activity, and enhances eukaryotic initiation factor (eIF)4E-binding protein phosphorylation and the association of eIF4E with eIF4G (42,43). Accordingly, dairy products provide sufficient BCAAs to be able to maintain the high concentration of intracellular leucine required by these signaling pathways. Milk proteins were also reported to contain substantial angiotensin-converting enzyme (ACE) activity (44,45). Furthermore, recent data demonstrated that adipocytes have an autocrine/paracrine rennin-angiotensin system and that adipocyte lipogenesis is regulated partially by angiotensin II (46). Moreover, inhibition of the renin-angiotensin system mildly attenuates obesity in rodents, and clinical observations in hypertensive patients treated with ACE inhibitors support this concept (21). In addition, our recent data demonstrated that dairy-derived ACE inhibitors augment the antiobesity effects of calcium in aP2 transgenic mice.

In summary, high-calcium diets exert potent effects in weight maintenance during refeeding after energy restriction

in $\alpha 2$ -agouti transgenic mice. High-calcium diets suppressed adipocyte $[Ca^{2+}]_i$, stimulated lipolysis, and inhibited lipogenesis and a corresponding increase in core temperature. Consequently, dietary calcium facilitates the reduction of fat tissue mass and body weight in refeeding by modulating energy metabolism, which serves to reduce energy storage and increase β -oxidation. This concept that calcium modulation of adiposity is consistent with the observations obtained epidemiologically in the NHANES III data set (20), the Quebec Family Study (47), CARDIA (48), and a recent clinical trial (9). Moreover, a dairy source of calcium exerts greater effects on preventing regain than supplementary calcium carbonate.

LITERATURE CITED

- Bray, G. A. (2003) Risks of obesity. *Endocrinol. Metab. Clin. North Am.* 32: 787-804.
- Yang, M. U., Presta, E. & Bjorntorp, P. (1990) Refeeding after fasting in rats: effects of duration of starvation and refeeding on food efficiency in diet-induced obesity. *Am. J. Clin. Nutr.* 51: 970-978.
- Walks, D., Lavau, M., Presta, E., Yang, M. U. & Bjorntorp, P. (1983) Refeeding after fasting in the rat: effects of dietary-induced obesity on energy balance regulation. *Am. J. Clin. Nutr.* 37: 387-395.
- Byrne, S., Cooper, Z. & Fairburn, C. (2003) Weight maintenance and relapse in obesity: a qualitative study. *Int. J. Obes. Relat. Metab. Disord.* 27: 955-962.
- Zemel, M. B. (2003) Role of dietary calcium and dairy products in modulating adiposity. *Lipids* 38: 139-146.
- Zemel, M. B. (1998) Nutritional and endocrine modulation of intracellular calcium: implications in obesity, insulin resistance and hypertension. *Mol. Cell. Biochem.* 188: 129-136.
- Zemel, M. B. (2004) Role of calcium and dairy products in energy partitioning and weight management. *Am. J. Clin. Nutr.* 79: 907S-912S.
- Shi, H., Halvorsen, Y. D., Ellis, P. N., Wilkison, W. O. & Zemel, M. B. (2000) Role of intracellular calcium in human adipocyte differentiation. *Physiol. Genomics* 3: 75-82.
- Zemel, M. B., Thompson, W., Milstead, A., Morris, K. & Campbell, P. (2004) Calcium and dairy accelerate weight and fat loss during energy restriction in obese adults. *Obes. Res.* 12: 582-590.
- Claycombe, K. J., Wang, Y., Jones, B. H., Kim, S., Wilkison, W. O., Zemel, M. B., Chun, J. & Moustaid-Moussa, N. (2000) Transcriptional regulation of the adipocyte fatty acid synthase gene by agouti: interaction with insulin. *Physiol. Genomics* 3: 157-162.
- Xue, B. & Zemel, M. B. (2000) Relationship between human adipose tissue agouti and fatty acid synthase (FAS). *J. Nutr.* 130: 2478-2481.
- Xue, B., Moustaid, N., Wilkison, W. O. & Zemel, M. B. (1998) The agouti gene product inhibits lipolysis in human adipocytes via a Ca^{2+} -dependent mechanism. *FASEB J.* 12: 1391-1396.
- Xue, B., Greenberg, A. G., Kraemer, F. B. & Zemel, M. B. (2001) Mechanism of intracellular calcium ($[Ca^{2+}]_i$) inhibition of lipolysis in human adipocytes. *FASEB J.* 15: 2527-2529.
- Xue, B. Z., Wilkison, W. O., Mynatt, R. L., Moustaid, N., Goldman, M. & Zemel, M. B. (1999) The agouti gene product stimulates pancreatic $[beta]$ -cell Ca^{2+} signaling and insulin release. *Physiol. Genomics* 1: 11-19.
- Shi, H., Moustaid-Moussa, N., Wilkison, W. O. & Zemel, M. B. (1999) Role of the sulfonylurea receptor in regulating human adipocyte metabolism. *FASEB J.* 13: 1833-1838.
- Kim, J. H., Kiefer, L. L., Woychik, R. P., Wilkison, W. O., Truesdale, A., Ittoop, O., Willard, D., Nichols, J. & Zemel, M. B. (1997) Agouti regulation of intracellular calcium: role of melanocortin receptors. *Am. J. Physiol.* 272: E379-E384.
- Kim, J. H., Mynatt, R. L., Moore, J. W., Woychik, R. P., Moustaid, N. & Zemel, M. B. (1996) The effects of calcium channel blockade on agouti-induced obesity. *FASEB J.* 10: 1646-1652.
- Shi, H., Norman, A. W., Okamura, W. H., Sen, A. & Zemel, M. B. (2001) $1\alpha,25$ -Dihydroxyvitamin D₃ modulates human adipocyte metabolism via non-genomic action. *FASEB J.* 15: 2751-2753.
- Shi, H., Dirienzo, D. & Zemel, M. B. (2001) Effects of dietary calcium on adipocyte lipid metabolism and body weight regulation in energy-restricted $\alpha 2$ -agouti transgenic mice. *FASEB J.* 15: 291-293.
- Zemel, M. B., Shi, H., Greer, B., Dirienzo, D. & Zemel, P. C. (2000) Regulation of adiposity by dietary calcium. *FASEB J.* 14: 1132-1138.
- Zemel, M. B. (2003) Mechanisms of dairy modulation of adiposity. *J. Nutr.* 133: 252S-256S.
- Reeves, P. G. (1997) Components of the AIN-93 diets as improvements in the AIN-76A diet. *J. Nutr.* 127: 838S-841S.
- Bradford, M. M. (1976) A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254.
- Millet, L. V., Vidal, H., Andreelli, F., Larrouy, D., Riou, J. P., Ricquier, D., Laville, M. & Langin, D. (1997) Increased uncoupling protein-2 and -3 mRNA expression during fasting in obese and lean humans. *J. Clin. Investig.* 100: 2665-2670.
- Carlsson, C., Borg, L. A. & Welsh, N. (1999) Sodium palmitate induces partial mitochondrial uncoupling and reactive oxygen species in rat pancreatic islets in vitro. *Endocrinology* 140: 3422-3428.
- Wang, S., Subramaniam, A., Cawthorne, M. A. & Clapham, J. C. (2003) Increased fatty acid oxidation in transgenic mice overexpressing UCP3 in skeletal muscle. *Diabetes Obes. Metab.* 5: 295-301.
- Wang, M. Y., Shimabukuro, M., Lee, Y., Trinh, K. Y., Chen, J. L., Newgard, C. B. & Unger, R. H. (1999) Adenovirus-mediated overexpression of uncoupling protein-2 in pancreatic islets of Zucker diabetic rats increases oxidative activity and improves beta-cell function. *Diabetes* 48: 1020-1025.
- Liu, R., Sun, C. H., Fu, R. X., Yan, Y. S. & Wang, S. R. (2003) Expression of uncoupling protein-2 mRNA in diet-induced obesity-resistant rats. *Zhonghua Yufang Yixue Zazhi* 37: 446-449.
- Cadenas, S., Buckingham, J. A., Samec, S., Seydoux, J., Din, N., Dulloo, A. G. & Brand, M. D. (1999) UCP2 and UCP3 rise in starved rat skeletal muscle but mitochondrial proton conductance is unchanged. *FEBS Lett.* 462: 257-260.
- Boss, O., Hagen, T. & Lowell, B. B. (2000) Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. *Diabetes* 49: 143-156.
- Schrauwen, P., Hoeks, J., Schaart, G., Kornips, E., Binas, B., Vusse, G. J., Van Bilsen, M., Luiken, J. J., Coort, S. L., Glatz, J. F., Saris, W. H. & Hesselink, M. K. (2003) Uncoupling protein 3 as a mitochondrial fatty acid anion exporter. *FASEB J.* 17: 2272-2274.
- Ferre, P. (2004) The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes* 53: S43-S50.
- Acin, A., Rodriguez, M., Rique, H., Canet, E., Boutin, J. A. & Galizzi, J. P. (1999) Cloning and characterization of the 5' flanking region of the human uncoupling protein 3 (UCP3) gene. *Biochem. Biophys. Res. Commun.* 258: 278-283.
- Kelly, L. J., Vicario, P. P., Thompson, G. M., Candelore, M. R., Doebber, T. W., Ventre, J., Wu, M. S., Meurer, R., Forrest, M. J., Conner, M. W., Cascieri, M. A. & Moller, D. E. (1998) Peroxisome proliferator-activated receptors gamma and alpha mediate in vivo regulation of uncoupling protein (UCP-1, UCP-2, UCP-3) gene expression. *Endocrinology* 139: 4920-4927.
- Grav, H. J., Tronstad, K. J., Gudbrandsen, O. A., Berge, K., Fladmark, K. E., Martinsen, T. C., Waldum, H., Wergedahl, H. & Bergem, R. K. (2003) Changed energy state and increased mitochondrial beta-oxidation rate in liver of rats associated with lowered proton electrochemical potential and stimulated uncoupling protein 2 (UCP-2) expression: evidence for peroxisome proliferator-activated receptor-alpha independent induction of UCP-2 expression. *J. Biol. Chem.* 278: 30525-30533.
- Tontonoz, P., Hu, E. & Spiegelman, B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* 79: 1147-1156.
- Pariza, M. W., Park, Y. & Cook, M. E. (2001) The biologically active isomers of conjugated linoleic acid. *Prog. Lipid Res.* 40: 283-298.
- Layman, D. K. (2003) The role of leucine in weight loss diets and glucose homeostasis. *J. Nutr.* 133: 261S-267S.
- Kimbball, S. R. & Jefferson, L. S. (2002) Control of protein synthesis by amino acid availability. *Curr. Opin. Clin. Nutr. Metab. Care* 2002 5: 63-67.
- Rieu, I., Sornet, C., Bayle, G., Prugnaud, J., Pouyet, C., Balage, M., Papet, I., Grizard, J. & Dardevet, D. (2001) Leucine-supplemented meal feeding for ten days beneficially affects postprandial muscle protein synthesis in old rats. *J. Nutr.* 133: 1198-1205.
- MacLean, D. A., Graham, T. E. & Saltin, B. (1994) Branched-chain amino acids augment ammonia metabolism while attenuating protein breakdown during exercise. *Am. J. Physiol.* 267: E1010-E1022.
- Lynch, C. J., Halle, B., Fujii, H., Vary, T. C., Wallin, R., Damuni, Z. & Hutson, S. M. (2003) Potential role of leucine metabolism in the leucine-signaling pathway involving mTOR. *Am. J. Physiol.* 285: E854-E863.
- Liu, Z., Jahn, L. A., Long, W., Fryburg, D. A., Wei, L. & Barrett, E. J. (2001) Branched chain amino acids activate messenger ribonucleic acid translation regulatory proteins in human skeletal muscle, and glucocorticoids blunt this action. *J. Clin. Endocrinol. Metab.* 86: 2136-2143.
- FitzGerald, R. J., Murray, B. A. & Walsh, D. J. (2004) Hypotensive peptides from milk proteins. *J. Nutr.* 134: 980S-988S.
- Fuglsang, A., Nilsson, D. & Nyborg, N. C. (2003) Characterization of new milk-derived inhibitors of angiotensin converting enzyme in vitro and in vivo. *J. Enzyme Inhib. Med. Chem.* 18: 407-412.
- Toblli, J. E., Cao, G., DeRosa, G., Di Gennaro, F. & Forcada, P. (2004) Angiotensin-converting enzyme inhibition and angiogenesis in myocardium of obese Zucker rats. *Am. J. Hypertens.* 17: 172-180.
- Chagnon, Y. C., Chen, W. J., Perusse, L., Chagnon, M., Nadeau, A., Wilkison, W. O. & Bouchard, C. (1997) Linkage and association studies between the melanocortin receptors 4 and 5 genes and obesity-related phenotypes in the Quebec Family Study. *Mol. Med.* 3: 663-673.
- Pereira, M. A., Jacobs, D. R., Jr., Van Horn, L., Slattery, M. L., Kartashov, A. I. & Ludwig, D. S. (2002) Dairy consumption, obesity, and the insulin resistance syndrome in young adults: the CARDIA Study. *J. Am. Med. Assoc.* 287: 2081-2089.