Symposium: Dairy Product Components and Weight Regulation

Mechanisms of Dairy Modulation of Adiposity[1]

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ABSTRACT Dietary calcium plays a pivotal role in the regulation of energy metabolism, in that we have found high calcium diets to attenuate adipocyte lipid accretion and weight gain during periods of overconsumption of an energy-dense diet and to increase lipolysis and preserve thermogenesis during caloric restriction, thereby markedly accelerating weight loss. Our studies of the *agouti* gene in obesity and insulin resistance demonstrate a key role for intracellular Ca^{2+} in regulating adipocyte lipid metabolism and triglyceride storage, with increased intracellular Ca^{2+}, resulting in stimulation of lipogenic gene expression and lipogenesis, and suppression of lipolysis, resulting in adipocyte lipid filling and increased adiposity. Moreover, we have recently demonstrated that the increased calcitriol produced in response to low calcium diets stimulates Ca^{2+} influx in human adipocytes and thereby promotes adiposity. Accordingly, suppressing calcitriol levels by increasing dietary calcium is an attractive target for the prevention and management of obesity. In support of this concept, transgenic mice expressing the *agouti* gene specifically in adipocytes (a humanlike pattern) respond to low calcium diets with accelerated weight gain and fat accretion, whereas high calcium diets markedly inhibit lipogenesis, accelerate lipolysis, increase thermogenesis and suppress fat accretion and weight gain in animals maintained at identical caloric intakes. Further, low calcium diets impede body fat loss, whereas high calcium diets markedly accelerate fat loss in transgenic mice subjected to caloric restriction. Notably, dairy sources of calcium exert markedly greater effects in attenuating weight and fat gain and accelerating fat loss. This augmented effect of dairy vs. supplemental calcium is likely attributable to additional bioactive compounds in dairy that act synergistically with calcium to attenuate adiposity; among these are angiotensin converting enzyme inhibitory peptides, which limit angiotensin II production and thereby limit angiotensin II stimulation of adipocyte lipogenesis. These concepts are confirmed by both epidemiological and clinical data, which similarly demonstrate that dairy products exert a substantially greater effect on both fat loss and fat distribution compared to an equivalent amount of supplemental calcium. J. Nutr. 133: 252S–256S, 2003.

KEY WORDS: • *calcium* • *obesity* • *dairy* • *adipocyte* • *vitamin D*

A substantial body of evidence has emerged over the last 3 y to support what appears to be a very unlikely concept: that dietary calcium may play a role in the regulation of energy metabolism and in modulating obesity risk. We first observed an "antiobesity" effect of dietary calcium accidentally, during the course of a study investigating the antihypertensive effect of dairy products in African-Americans. We noted that adding calcium-rich dairy foods (yogurt) to the daily diet resulted in significant reductions in body fat and circulating insulin (1) in addition to a sustained reduction in intracellular Ca and an antihypertensive effect (2,3). This observation has now been supported by a number of epidemiological and clinical studies reviewed by Teegarden (4) and Heaney (5) in this issue of the journal, as well as by recently published observations from the CARDIA study demonstrating an inverse relationship between dairy consumption and all components of the insulin resistance syndrome, including body mass index (BMI) (6).

Although the initial observation of dairy modulation of adiposity was inexplicable to us at the time it was first made (~1990), our recent studies of the mechanism of action of the *agouti* gene in obesity and insulin resistance have provided a compelling mechanism that has now been confirmed in a series of studies described in this review. These data demonstrate a key role for intracellular Ca^{2+} in the regulation of both murine and human adipocyte metabolism, resulting in modulation of adipocyte triglyceride stores, as described below. Because intracellular Ca^{2+} can clearly be modulated by calcitrophic hormones, including 1,25-$(OH)_2$-D and parathyroid hormone, these data provide a theoretical framework to explain an "antiobesity" effect of dairy products. The next portion of this review is devoted to a review of these findings.

[1] Presented as part of the symposium "Dairy Product Components and Weight Regulation" given at the 2002 Experimental Biology meeting on April 21, 2002, New Orleans, LA. The symposium was sponsored in part by Dairy Management Inc. and General Mills, Inc. The proceedings are published as a supplement to *The Journal of Nutrition.* Guest editors for the symposium were Dorothy Teegarden, Department of Foods and Nutrition, Purdue University, West Lafayette, IN, and Michael B. Zemel, Departments of Nutrition and Medicine, The University of Tennessee, Knoxville, TN.

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Agouti, intracellular calcium and obesity

Most of the data describing the role of intracellular Ca^{2+} in human adipocyte metabolism derive from our studies of the mechanism of action of *agouti*, the first of the obesity genes to be cloned (7). The C-terminal region of agouti protein, which retains full functional activity relative to the intact protein in an in vitro assay system (8), exhibits a striking spatial homology in both number and spacing of cysteine residues to spider and snail venoms (ω-conotoxins, plectoxins), which target Ca^{2+} channels (9). Accordingly, the C-terminus may form a three-dimensional structure that is functionally similar to these venoms and may thereby have as a primary function modulation of Ca^{2+} transport. Indeed, we have reported that obese *agouti* mutant mice (viable yellow, A^{vy}) mice exhibit increases in both steady-state intracellular Ca^{2+} and Ca^{2+} influx in several tissues (10,11). This increase in intracellular Ca^{2+} was closely correlated with both the degree of ectopic agouti expression and body weight (8), suggesting the possibility of a causal mechanism between intracellular Ca^{2+} and obesity in these animals. Because A^{vy} mice exhibit elevated rates of adipocyte lipogenesis and increased adipocyte size relative to lean controls (12,13), we have explored the links between agouti, intracellular Ca^{2+} and regulatory enzymes in lipid metabolism.

Intracellular calcium regulates adipocyte lipid metabolism

Recombinant agouti protein directly increased Ca^{2+} influx and steady-state intracellular Ca^{2+} in a variety of cell types, including both murine and human adipocytes (10,11). This regulation occurs in response to physiologically meaningful concentrations of agouti (EC_{50} of 18–62 nM, depending on cell type) and, although studies in HEK-293 cells demonstrate the dependence of this effect on the presence of intact melanocortin receptors, it is not dependent on melanocortin receptor antagonism (10). The role of these increases in Ca^{2+} in lipogenesis has been explored using fatty acid synthase (FAS), given that this multifunctional enzyme is highly regulated by nutrients and hormones and is a key enzyme in de novo lipogenesis. FAS expression and activity are markedly increased in A^{vy} relative to control mice (14), and nanomolar concentrations of agouti protein stimulate nearly twofold increases in FAS gene expression and activity and triglyceride accumulation in 3T3-L1 adipocytes as well as in human adipocytes, similar to the maximal increases stimulated by insulin (13). These increases are mediated by a distinct agouti/Ca^{2+} response sequence in the FAS promoter (15). This sequence maps to the −435 to −415 region of the FAS promoter and is upstream of the insulin response element, which maps to −67 to −52, consistent with the observed additive effects of agouti and insulin on FAS gene transcription (15). Further, we recently reported that agouti exerts a regulatory effect on human FAS expression in vivo, and that there is a strong correlation between agouti (the human homolog, *agouti signaling protein, ASIP*) expression and FAS expression in adipose tissue obtained from normal volunteers (16). Moreover, a significant positive correlation was recently reported between adipose tissue *ASIP* mRNA and BMI in women (17), although an as of yet inexplicable sexual dimorphism exists such that an inverse relationship exists between *ASIP* and BMI in men (17). This agouti modulation of FAS transcription appears to be mediated via intracellular Ca^{2+}, given that it can be inhibited by Ca^{2+} antagonism (14,15,18) and can be mimicked in the absence of agouti by either receptor- or voltage-mediated Ca^{2+} channel activation (19).

In addition to activating lipogenesis, recent data also indicate that increasing intracellular Ca^{2+} may also contribute to increased triglyceride stores by inhibiting lipolysis. Increasing Ca^{2+} influx with either arginine vasopressin or epidermal growth factor was reported to inhibit lipolysis in rat adipocytes in a Ca^{2+} dose-responsive fashion (20). Further, we have shown that the agouti gene product similarly inhibits lipolysis in human adipocytes via a Ca^{2+}-dependent mechanism (21). This inhibition can also be mimicked in the absence of agouti by either receptor- or voltage-mediated Ca^{2+} channel activation (21). The antilipolytic effect of intracellular Ca^{2+} is attributed to a direct activation of phosphodiesterase 3B, resulting in a decrease in cAMP (adenosine 3′,5′-cyclic monophosphate) and, consequently, reduced ability of agonists to stimulate phosphorylation and activation of hormone-sensitive lipase (22). Thus, agouti regulation of adipocyte intracellular Ca^{2+} appears to promote triglyceride storage in human adipocytes by exerting a coordinated control of lipogenesis and lipolysis, serving to simultaneously stimulate the former and inhibit the latter.

However, it is important to note that agouti interaction with insulin is required for the full expression of agouti-induced obesity. Agouti and insulin exert independent, additive effects on FAS transcription and lipogenesis (15). Because increased intracellular Ca^{2+} is the proximate signal for insulin release, and agouti regulates Ca^{2+} in several cell types (10), it is reasonable to speculate that agouti may stimulate insulin release as well. Indeed, we recently found that agouti is expressed in human pancreas and stimulates Ca^{2+} signaling in rat, hamster and human pancreatic β-cells (23). Further, hyperplasia of β-cells precedes the development of obesity in agouti mutant mice, suggesting that hyperinsulinemia may be a direct effect of agouti acting on the pancreas and that the combination of this hyperinsulinemia and agouti-stimulated adipocyte Ca^{2+} influx may lead to obesity. In support of this concept, transgenic mice expressing agouti at high levels in adipose tissue under the control of the aP2 promoter become obese if they were also hyperinsulinemic as a result of either exogenous insulin or a high sucrose diet, whereas hyperinsulinemia was without effect in nontransgenic littermate controls (24–26). Given that humans exhibit a similar pattern of adipocyte agouti expression (27), similar agouti/insulin/Ca^{2+} interactions may result in excessive adipocyte triglyceride storage.

Taken together, these data indicate that regulation of adipocyte and pancreatic intracellular Ca^{2+} may be an important target for the development of therapeutic strategies for the prevention and treatment of obesity (18). This concept is summarized in **Figure 1**.

To further evaluate this hypothesis, agouti-expressing transgenic mice were treated with high doses of a Ca^{2+} channel antagonist, nifedipine. This treatment resulted in an 18% reduction in fat pad mass and completely normalized the agouti-induced hyperinsulinemia over a 4-wk treatment period in the transgenic mice, but was without effect in the nontransgenic littermate controls (28). Thus, adipocyte and/or pancreatic β-cell Ca^{2+} appear to be reasonable therapeutic targets for the treatment and/or prevention of obesity.

We recently extended this concept by demonstrating that human adipocytes express a sulfonylurea receptor (SUR) that exerts a regulatory effect on the Ca^{2+} channel and, consequently, modulates adipocyte lipid accumulation (19,29). Compounds acting on the pancreatic SUR to increase (e.g., glinbeclamide) or decrease (e.g., diazoxide) intracellular Ca^{2+} (indirectly, via a K^+-ATP channel) cause corresponding increases and decreases in weight gain, although these effects

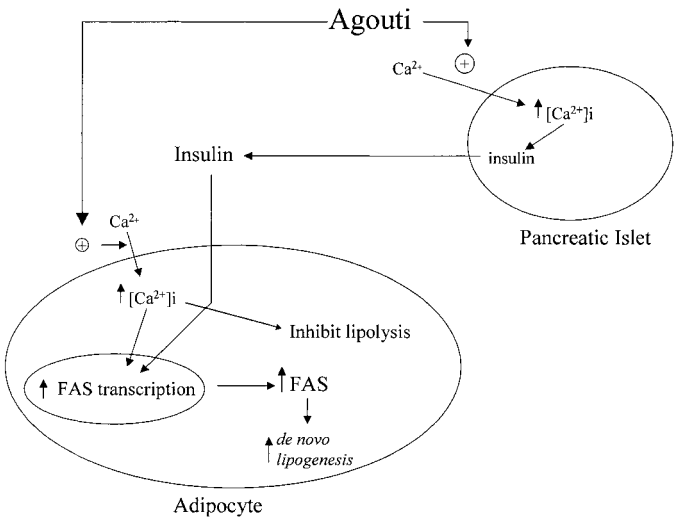

FIGURE 1 Ca^{2+}-mediated mechanisms of agouti regulation of adiposity.

have previously been attributed to the effects of these compounds on circulating insulin. However, the identification of SUR expression in human adipocytes (19) suggests that it may modulate adipocyte Ca^{2+} flux and thereby regulate lipid metabolism. Indeed, the SUR agonist glinbeclamide increases human adipocyte intracellular Ca^{2+} and thereby causes marked increases in lipogenic enzyme activity and inhibition of lipolysis. Moreover, inhibition of the adipocyte SUR–regulated Ca^{2+} channel with diazoxide completely prevented each of these effects. Accordingly, the adipocyte SUR may represent a new target for the development of pharmacological interventions in obesity (19). In support of this concept, diazoxide has been demonstrated to exert significant antiobesity effects in both obese Zucker rats and hyperinsulinemic obese adults (30–32). Although this effect was attributed to actions on pancreatic β-cell insulin release, we subsequently found diazoxide treatment to significantly suppress adipose tissue fatty acid synthase and lipoprotein lipase in obese Zucker rats (29).

Role of 1,25-dihydroxyvitamin D in regulating adipocyte Ca^{2+} and lipid metabolism

On the basis of these findings, and on our earlier observations of dietary calcium-induced reductions in adiposity, we proposed that the elevations in 1,25-dihydroxyvitamin D [1,25-(OH_2)-D], which occur in response to low calcium diets, may stimulate adipocyte Ca^{2+} influx and thereby increase adiposity. Indeed, we recently reported that 1,25-(OH_2)-D stimulates significant Ca^{2+} influx and sustained dose-responsive increases in steady-state intracellular Ca^{2+} in primary cultures of human adipocytes (1). Moreover, 1,25-(OH_2)-D treatment of human adipocytes resulted in a coordinated activation of FAS and inhibition of lipolysis, similar to the action of agouti on these cells (1,33). Consequently, suppression of 1,25-(OH_2)-D with high calcium diets would be anticipated to reduce adipocyte intracellular Ca^{2+}, inhibit FAS and activate lipolysis, thereby exerting an antiobesity effect. This concept is summarized in **Figure 2**.

Dietary calcium modulation of adiposity

This concept was confirmed in transgenic mice expressing agouti in adipose tissue under the control of the aP2 promoter. Mice placed on low calcium (0.4%)/high fat/high sucrose diets for 6 wk exhibited marked increases in adipocyte lipogenesis, inhibited lipolysis and accelerated increases in body weight and adipose tissue mass. However, high calcium (1.2%) diets reduced lipogenesis by 51% and stimulated lipolysis three- to fivefold, resulting in 26–39% reductions in body weight and adipose tissue mass (1). The magnitude of these effects depended on the source of dietary calcium, with dairy sources of calcium exerting significantly greater effects than that of calcium carbonate.

These data have significant implications for the prevention or attenuation of diet-induced obesity but do not directly address the issue of whether high calcium diets will exert any effect on established obesity. Accordingly, we next conducted a study to extend these findings by determining whether dietary calcium and calcium-rich dairy products reduce metabolic efficiency and accelerate fat loss secondary to caloric restriction in the same mouse model after dietary induction of obesity (34). Mice (aP2-agouti transgenic) similar to those used in the studies described above were used. They were fed the same basal low (0.4%) Ca/high fat/high sucrose diet for 6 wk used in the preceding study.

Administration of the low calcium (0.4%)/high-fat/high sucrose diet to aP2-a mice for 6 wk resulted in a nearly twofold increase in adipocyte $[Ca^{2+}]i$, with a corresponding body weight gain of 29% ($P < 0.001$) and a twofold increase in total fat pad mass ($P < 0.001$), demonstrating that diet-induced dysregulation of adipocyte $[Ca^{2+}]_i$ is associated with increased adiposity in aP2-a mice.

All three calcium diets, including high calcium diet (1.2% Ca derived from $CaCO_3$), medium dairy diet (1.2% Ca derived from nonfat dry milk replacing 25% of protein) and high dairy diet (2.4% Ca derived from nonfat dry milk replacing 50% of protein), caused a 50% decrease in adipocyte $[Ca^{2+}]_i$ ($P < 0.001$), whereas $[Ca^{2+}]_i$ in adipocytes from mice maintained on the energy-restricted basal low calcium diet remained at the same elevated level as that of ad libitum animals.

Although energy restriction resulted in a body weight loss of 11%, markedly greater weight reductions of 19, 25 and 29% were observed in the high calcium, medium and high dairy groups, respectively ($P < 0.01$ vs. basal energy-restricted group). Consistent with this, energy restriction caused only 8% lower fat pad mass (not significant), compared to the basal

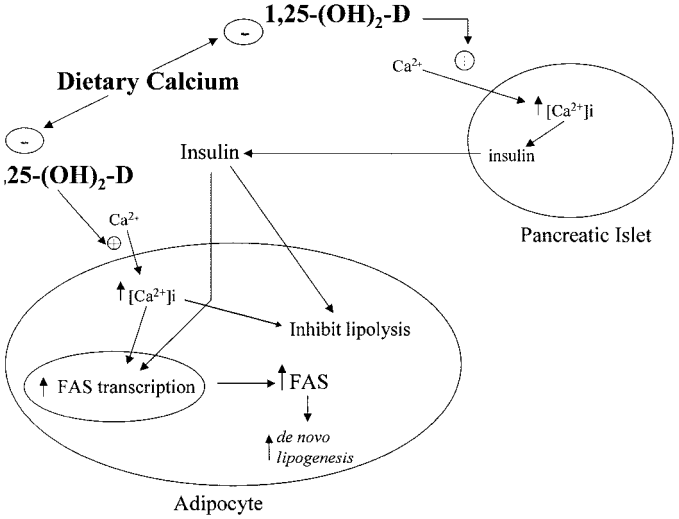

FIGURE 2 Dietary calcium modulation of adiposity is mediated via 1,25-(OH_2)-D regulation of Ca^{2+} flux.

diet ad libitum group, whereas the high calcium diet caused a 42% decrease ($P < 0.001$), which was further reduced by 60 and 69% by the medium and high dairy diets ($P < 0.001$ vs. basal energy-restricted group), respectively.

The high calcium diet caused a 35% decrease in FAS activity ($P < 0.05$ vs. basal energy-restricted group), which was further reduced by 63 and 62% by the medium and high dairy diets ($P < 0.05$), respectively; FAS mRNA followed a similar trend. Increasing dietary calcium caused a corresponding increase in lipolysis. Although the basal energy-restricted diet did not affect adipocyte lipolysis, the high calcium diet caused 77% stimulation in lipolysis ($P < 0.05$), which was further increased in the medium and high dairy diet groups ($P < 0.05$ vs. basal energy-restricted group). Increased lipolysis, coupled with decreased lipogenesis, may represent a metabolic state in which the efficiency of energy metabolism is shifted from energy storage to energy expenditure.

This shift in energy metabolism was further confirmed by a dietary calcium–induced increase in core temperature. All three high calcium diets exerted significant stimulatory effects on core temperature, whereas the basal energy-restricted diet did not affect core temperature. A possibly physiological basis underlying the increased core temperature is that the expression of uncoupling protein 2 (UCP2), was upregulated in white adipose tissue, with an 80% increase in all three high calcium diets ($P < 0.05$). However, the role of UCP2 in thermogenesis is not clear, and further study is required to address the precise mechanism whereby dietary calcium regulates UCP2 expression. Although this upregulation of UCP2 expression may result directly from inhibition of $[Ca^{2+}]_i$, it is also possible that it is merely a result of increased substrate (fatty acid) flux secondary to increased lipolysis. However, we recently found 1,25-$(OH)_2$-D to directly suppress UCP2 expression in isolated human adipocytes, and that this effect is independent of fatty acid flux (35).

Collectively, these data demonstrate that high Ca diets suppress adipocyte $[Ca^{2+}]_i$ and thereby reduce energy storage and increase thermogenesis during energy restriction, with greater effects exerted by dairy calcium than by elemental calcium.

We recently studied the efficacy of a calcium-fortified breakfast cereal, alone or with a small amount of milk, in attenuation of weight and fat gain in the aP2-*agouti* transgenic mouse (36). Male mice placed on a basal low calcium (0.4%)/high fat (25 energy %)/high sucrose diet for 6 wk exhibited nearly twofold increases in $[Ca^{2+}]_i$ and both visceral and subcutaneous fat mass. However, addition of a calcium-fortified breakfast cereal sufficient to increase dietary calcium to 1.2% with macronutrient adjustments to ensure identical carbohydrate, protein and fat levels with the basal diet resulted in a 41% decrease in adipocyte $[Ca^{2+}]_i$ ($P < 0.001$) and 25–30% decreases in weight gain ($P < 0.03$) and total fat pad mass compared to that of the basal diet ($P < 0.001$), whereas food consumption was unaffected. Comparable decreases were found in both subcutaneous and visceral fat compartments. A second control group, which received the basal diet supplemented with the same amount cereal without calcium fortification (with macronutrient adjustment), was not significantly different from the basal control group.

We also found the calcium-fortified cereal to have similar effects in markedly accelerating weight and fat loss secondary to caloric restriction in these mice. Interestingly, addition of sufficient nonfat dried milk to bring the calcium content of the Ca-fortified cereal diet to from 1.2 to 1.3% (with macronutrient adjustment) resulted in substantial amplification of these effects. Thus, a calcium-fortified breakfast cereal is effective in reducing adiposity and accelerating fat loss during caloric restriction in this model of obesity, whereas the use of milk with the cereal significantly amplifies this effect.

We recently confirmed the utility of calcium-rich diets in accelerating fat loss during a 6-mo clinical trial in obese patients (37). Thirty-two obese adults were maintained for 24 wk on balanced deficit diets (500 kcal/d deficit) and randomized to control (0–1 serving/d and 400–500 mg Ca/d supplemented with placebo), high calcium (control diet supplemented with 800 mg Ca/d) or high dairy (3–4 servings of low fat dairy products/d, total Ca intake of 1200–1300 mg/d). Control patients lost 6.4 ± 2.5% of their body weight, which was increased by 26% on the high calcium diet and 70% (to 10.9 ± 1.6%) on the high dairy diet ($P < 0.01$). Fat loss (via DEXA) followed a similar trend, with the high calcium and high dairy diets augmenting the fat loss found on the low calcium diet by 38 and 64%, respectively ($P < 0.01$).

An unexpected finding was a marked change in the distribution of body fat loss (35). Patients on the low calcium diet lost 5.3 ± 2.3% of their trunk (abdominal region) fat on the low calcium diet. This was increased to 12.9 ± 2.2% on the high calcium diet and 14.0 ± 2.3% on the high dairy diet ($P < 0.025$ vs. low calcium and high calcium diets). Consequently, fat loss from the abdominal region represented 19.0 ± 7.9% of the total fat lost on the low calcium diet, and this was increased to 50.1 ± 6.4% of the fat lost on the high calcium diet ($P < 0.001$) and 66.2 ± 3.0% on the high dairy diet ($P < 0.001$). Thus, increasing dietary calcium not only accelerates weight and fat loss secondary to caloric restriction, but also shifts the distribution of fat loss to a more favorable pattern, with more fat lost from the abdominal region on the high calcium diet. Moreover, dairy products exert a substantially greater effect on both fat loss and fat distribution compared to an equivalent amount of supplemental calcium.

Role of additional dairy-derived bioactive compounds

The foregoing experimental animal and human study data clearly support a beneficial role for dietary calcium in weight management, but markedly greater effects are evident from dairy vs. nondairy sources of calcium. Although the additional components of dairy responsible for the differential effects between calcium and dairy products are not yet known, current work is under way to determine their identity. At present, preliminary data suggest that this additional activity resides in the whey fraction of milk. Whey is recognized as a rich source of bioactive compounds (38), which may act independently or synergistically with the calcium to attenuate lipogenesis, accelerate lipolysis and/or affect nutrient partitioning between adipose tissue and skeletal muscle. Notably, whey proteins were recently reported to contain significant angiotensin converting enzyme (ACE) activity (39,40). Although ACE inhibitory activity may appear to be more relevant to an antihypertensive effect than to an antiobesity effect of dairy, recent data demonstrate that adipocytes have an autocrine/paracrine renin-angiotensin system, and that adipocyte lipogenesis is regulated in part by angiotensin II [reviewed in Morris et al. (41)]. Indeed, inhibition of the renin-angiotensin system mildly attenuates obesity in mice, and limited clinical observations support this concept in hypertensive patients treated with ACE inhibitors (41). Thus, it is possible that whey-derived ACE-inhibitory activity may contribute to the antiobesity effect of dairy. However, it is also possible that other whey bioactive compounds may contribute or, alternatively, that a synergistic effect of multiple factors, along with the aforementioned effects of the calcium, are responsible.

In conclusion, a growing body of evidence now clearly demonstrates a beneficial role for dietary calcium in the partitioning of dietary energy, resulting in reductions in body fat and an acceleration of weight and fat loss during energy restriction. Interestingly, dairy sources of calcium exert substantially greater effects than supplemental or fortified sources of calcium. There is a strong theoretical framework in place to explain the "antiobesity" effects of dietary calcium; however, the mechanism of dairy augmentation of this antiobesity effect is not yet clear, although it may be mediated by whey peptides. These data have important implications for the prevention of both pediatric and adult obesity, especially in light of the marginal calcium intakes exhibited by the majority of the population and the population-based data indicating protection from obesity and the insulin resistance syndrome in populations consuming greater amounts of dairy products (6,42).

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