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Calcium, Angiotensin Converting Enzyme Inhibitors, and Branched Chain Amino Acids Contribute to the Anti-Obesity Effects of Milk

Anna C. Herweyer
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Anna C. Herweyer entitled "Calcium, Angiotensin Converting Enzyme Inhibitors, and Branched Chain Amino Acids Contribute to the Anti-Obesity Effects of Milk." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Michael B. Zemel, Major Professor

We have read this thesis and recommend its acceptance:

Gary E. Truett, Jung Han Kim

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

__Linda Painter_____
Interim Dean of Graduate Studies

(Original signatures are on file with official student records.)

CALCIUM, ANGIOTENSIN CONVERTING ENZYME INHIBITORS AND BRANCHED CHAIN AMINO ACIDS CONTRIBUTE TO THE ANTI-OBESITY EFFECTS OF MILK

A Thesis
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Anna C. Herweyer
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ABSTRACT

Dietary calcium exerts an anti-obesity effect, and dairy exerts about twice the activity as supplemental calcium. Milk contains angiotensin converting enzyme inhibitors (ACEi) and a high concentration of branched chain amino acids (BCAA), both of which may contribute to an anti-obesity effect through effects on lipid metabolism and muscle protein synthesis, respectively. To test this hypothesis, aP2-agouti transgenic mice were maintained for 6 weeks on an obesigenic soy based diet and then randomized into an *ad libitum* control group and energy restricted (70% of *ad lib*) diets containing soy protein (0.4% Ca), non-fat dry milk (NFDM; 1.2% Ca) or Ca-depleted NFDM (0.4% Ca) for an additional 6 weeks; the soy diet was provided with or without an ACEi and BCAA supplement and provided at either 0.4 or 1.2% Ca. The NFDM group exhibited ~2-fold greater reduction in body weight and fat than the energy restricted group ($p<0.01$), and the Ca-depleted milk group exerted 60% of the effect of intact milk ($p<0.01$); this effect was replicated by the ACEi/BCAA supplemented diet, and addition of calcium to this diet resulted in further weight and fat loss. Overall, the Ca, ACEi and BCAA content of milk accounted for ~90% of its anti-obesity activity. Moreover, diets with native or supplemented BCAA attenuated muscle loss. These data indicate that Ca is responsible for ~40% of the anti-obesity effect of milk while BCAA and ACEi are responsible for much of the additional activity. The results of this study have significance for dieters wishing to achieve optimal body composition. Dairy components are seen here to help with fat loss and lean maintenance during calorie restriction, which may then further aid maintenance of weight loss.

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CHAPTER 1. INTRODUCTION

The prevalence of obesity has grown dramatically over the past three decades. In the early 1960s only 31.5% of the American population was classified as overweight, and 13.3% as obese. Now, less than 50 years later, almost 35% of the population is overweight, and another 30% are obese[1]. Overweight and obesity are highly correlated with other chronic diseases as well. Many people with a body mass index over 25 also have hypertension, hyperlipidemia, insulin resistance, and hyperglycemia[1]. People with any three of these problems are at increased risk of morbidity and mortality[2]. Many lines of research have been conducted in recent years to find mechanisms of these disorders, and therefore possible cures or lifestyle interventions that could be used to stop the downward spiral of chronic disease in the United States.

One recent line of research in the field of obesity is the role of calcium in modulating lipid metabolism. Intracellular calcium ($[Ca^{2+}]i$) stimulates *de novo* lipogenesis and inhibits lipolysis in both human adipocytes and obesity-prone transgenic mice[3-5]. Similarly, low calcium diets induce fat gain and inhibit fat loss in mice, whereas high calcium diets impede energy storage[6] Several epidemiological studies have shown a strong negative correlation between calcium intake and body fat and obesity[7-13] and milk products enhance the effect of calcium on fat metabolism[14], [15, 16].

The compounds in milk that enhance the anti-obesity effect of calcium are not yet known. However, there are two classes of bioactive compounds that show promise. The first of these compounds, angiotensin converting enzyme inhibitors (ACEi) have long been found in milk[17, 18]. ACEi stop the formation of angiotensin II which has been shown to inhibit preadipocyte differentiation[19], possibly contributing to insulin resistance, and to

stimulate hypertrophy in mature human adipocytes[20] through stimulation of fatty acid synthase (FAS) gene expression and activity, leading to increased triglyceride content of adipocytes. Thus, we have hypothesized that ACEi derived from milk could inhibit the formation of AII, thereby decreasing the availability of the substrate to increase triglyceride synthesis and storage. The second of these compounds is leucine, one of the branched chain amino acids (BCAA). Leucine has been shown to stimulate muscle protein synthesis via the mTOR and other pathways[21, 22] leading to muscle maintenance during calorie restriction[23, 24].

Consequently, the objective of this study was to determine the contributing roles of calcium, ACEi and BCAA to the anti-obesity effect of milk. Specifically, we compared differing sources of protein and differing levels of calcium, ACEi and BCAA in calorie-restricted diets in their effects on weight and fat loss, and regulation of fat metabolism and gene expression in transgenic mice.

CHAPTER 2. LITERATURE REVIEW

I. Mechanisms of adipogenesis, fatty acid synthesis and lipolysis

Key to elucidating the cause of obesity is to understand the mechanisms that lead to fat storage and release. Adipose tissue mass is determined at three levels of regulation: adipogenesis, fatty acid synthesis and lipolysis. The first phase in this cycle is that of adipogenesis- the creation and lipid-expansion of new fat cells. Preadipocyte differentiation has primarily been studied *in vitro* because the complex nature of adipose tissue makes *in vivo* studies difficult. Differentiation is stimulated by insulin and glucocorticoid[25], which is a phosphodiesterase (PDE)- inhibitor, and further stimulated by growth hormone, CCAAT/enhancer binding protein α (C/EBP-α), and peroxisome proliferator-activated receptor-γ (PPAR-γ), among other factors, reviewed by Gregoire[26]. The second part of adipose metabolism is fatty acid synthesis leading to hypertrophy of adipocytes. This process is promoted by increased levels of intracellular glucose via the action of insulin and inhibited by glucagon and a high-fat diet. It is also stimulated by glucocorticoid, and allosterically by high levels of cytosoilic citrate. Citrate is a precursor for malonyl-CoA, which when combined with acetyl-CoA begins the fatty acid synthase (FAS) cycle. The last part of lipid metabolism is lipolysis. This pathway is usually stimulated by adrenaline or noradrenaline[27]. Adenylyl cyclase forms cyclic adenosine monophosphate (AMP), which goes on to stimulate hormone sensitive lipase (HSL). HSL is the enzyme responsible for breaking a triacylglycerol into a monoacylglycerol. Cyclic AMP is inactivated by PDE, which changes the messenger into non-cyclic AMP.

II. Role of calcium in obesity

A. Agouti mice- a model for obesity

In order to study the mechanisms leading to obesity, researchers need an in-vitro model. One mouse model that displays a similar pattern of obesity as humans is the agouti yellow-mouse. The agouti gene encodes a 131-amino acid protein[28]. Normal expression of agouti protein occurs transiently in the hair follicles of infant mice, acting as a melanocortin receptor antagonist, resulting in a subapical yellow band of the otherwise black hair[29]. Initially it was found that mice over-expressing agouti protein systemically have yellow fur, become obese, and are insulin resistant[29]. These metabolic problems were explained when later it was discovered that the agouti gene product regulates calcium signaling, and that it inhibits lipolysis by means of a calcium-dependent mechanism[4, 30].

Humans express a protein that is 80% homologous to mouse agouti protein called agouti signaling protein or ASIP. This protein is expressed mainly in adipose and pancreas tissue[31, 32]. Because mouse agouti protein is known to act chiefly by antagonizing melanocortin receptors, it has been hypothesized that these proteins are also the chief effector of ASIP's actions in human adipose metabolism. Scientists created mice in the 1990s to over-express agouti specifically in the adipose tissue of yellow mice under control of the aP2 promotor[33], imitating to the normal expression of ASIP in human adipose tissue to be used as a model to explore obesity.

B. Calcium signaling in lipid metabolism

Using the agouti mouse model as well as cultured adipocytes, the role of calcium signaling in adipocyte lipid metabolism has been further elucidated. It was found that

lipolysis stimulated by either ACTH or forskolin is inhibited in both human and 3T3-L1 adipocytes while intracellular calcium ($[Ca^{2+}]i$) is elevated[4] . Later it was found that this is effected via activation of phosphodiesterase 3B and resulting reduction of cyclic AMP[5]. Correspondingly, it was found that agouti gene product also stimulates lipogenesis via fatty acid synthase stimulation[34] through increased $[Ca^{2+}]i$[35]. This led to a milestone study that combined work in human adipocytes, an *in vivo* study of weight, core temperature and lipid metabolism change in obesity-prone mice fed diets with differing amounts of calcium and dairy, and an epidemiological study of the NHANES III data set[10]. In the first part of this study, cultured human adipocytes showed sustained dose-responsive increases in $[Ca^{2+}]i$ to both 1,25-dihydroxyvitamin-D_3 [$1,25(OH)_2D$] and PTH, and a corresponding decrease in basal and stimulated lipolysis. It was concluded that the key hormones that regulate serum calcium levels also regulate adipocyte calcium channels. Both $1,25(OH)_2D$ and PTH are known to become elevated in individuals consuming low amounts of calcium; therefore, it is possible that low calcium diets induce calcitrophic hormones which then induce increases in $[Ca^{2+}]i$, resulting in lipid accumulation via stimulation of lipogenesis and inhibition of lipolysis. $1,25(OH)_2D$ has been shown to augment cortisol production via 11-β- hydroxysteroid dehydrogenase-1[36], which has in turn been implicated in adipogenesis, particularly in visceral fat depots[37]. Although this enzyme appears to be less active in the livers of obese individuals, 11-β-HSD-1 has been shown to be more active in the adipose tissue in humans[38] and fatty rats, which only become obese in the presence of glucocorticoid[39]. It has since been shown that $1,25\ (OH)_2D$ stimulates cellular Ca^{2+} influx via non-genomic actions[40, 41]. It is possible that the actions of $1,25(OH)_2D$ on cortisol production and adipogenesis are

accomplished via calcium signaling. It remained to be found how these cellular effects played out in the body, whether animal or human.

C. Animal experiments linking calcium intake to lower body weight

In multiple experiments using mice, Zemel and associates found that dietary calcium inhibits *de novo* lipogenesis, and increases lipolysis, whether the mice were being fed *ad libitum*, on calorie restricted diets, or regaining weight lost after calorie restriction[10, 42, 43]. It was also found that diets high in dairy had an even more profound effect on these measures of adipocyte metabolism than did calcium supplementation alone[10, 42, 43]. In the first of these experiments, Zemel utilized aP2-*agouti* transgenic mice, which exhibit a similar pattern of obesity-related genes as humans[10]. On a standard AIN-93G diet these mice are normal weight, but if fed a diet high in sucrose and fat (obesigenic diet), they rapidly become moderately obese[44]. These mice were fed *ad libitum* for six weeks either a obesigenic diet with low (0.4%) or high (1.2%) levels of calcium carbonate, or the same diet with 50% or 100% of the protein replaced with non-fat dry milk to provide 1.2% and 2.4% calcium, respectively. The mice with elevated calcium intake gained significantly less weight than those on the control, low calcium diet. Of these, the mice consuming the most dairy with the highest calcium intake gained the least weight. FAS gene expression and activity were significantly lower in the calcium and dairy mice than the control mice. Likewise, lipolysis was significantly higher in these mice[10]. Thus it was concluded that calcium and dairy have a profound effect on fat metabolism in both limiting *de novo* lipogenesis and by increasing lipolysis.

The second of these experiments studied of the effect of calcium and dairy on body composition and fat metabolism during energy restriction[42]. Animals were fed an

obesigenic, low-calcium diet for 6 weeks, then were divided into five diet groups, four of which were fed the same diets as those in the previous study[10], but at 70% of the kcal consumed by the fifth *ad libitum* group. Mice maintained on the high-calcium diet lost more weight and fat than those on the restricted basal diet, and those consuming the higher dairy diets lost even more weight. Adipocyte $[Ca^{2+}]i$ decreased substantially from the *ad libitum* diet with the high-calcium diets, but not at all with simple kcal restriction. FAS activity and expression decreased significantly with added calcium, dairy and kcal restriction. Furthermore, lipolysis was significantly increased by the high-calcium diet over both basal diets, and even further by both dairy diets.

The third experiment studied the effect of calcium and dairy products in weight regain[43]. Obesity was induced in transgenic mice, followed then by a calcium-supplemented cereal energy-restricted diet to produce weight loss in all the mice. This weight loss was followed by a weight-regain phase. Mice were fed *ad libitum* one of five diets including the obesigenic *ad libitum* and calcium carbonate-supplemented cereal diets of the first two phases, as well as a milk protein supplemented diet, a yogurt-based diet, and a low calcium cereal diet. The results of this study showed a significantly smaller weight regain in mice fed the high-calcium and dairy-based diets than those fed either of the control diets. It also showed that the high-calcium diet group gained significantly less fat than the *ad libitum* and cereal control groups, and that both dairy groups gained even less than the calcium group, echoing the results from the previous two studies. This study again showed a marked inhibition of FAS and an increase in lipolysis in the calcium diet and even more profound effects in the dairy-based diet. All these results occurred despite the fact that the mice were eating the same amount of

calories, indicating that calcium and other components of dairy do in fact affect energy partitioning.

Although the effects of high calcium diets on obesity prevention were firmly established in mice, it remained to be studied how calcium and dairy intake affects humans' weight and body fat distribution.

D. Clinical trials of the role of calcium in weight loss

The findings from animal studies prompted researchers to begin to study the effect of a high calcium diet in humans. In one study, 32 obese but otherwise healthy adults were randomized to one of three weight loss diets for 24 weeks[45]. The control diet provided a 500 kcal/day deficit, low calcium and dairy intake plus a placebo supplement, providing 400-500 mg/day calcium. A calcium-supplemented diet was identical to the control diet, except that the placebo was replaced with an 800 mg/day calcium supplement, providing a total of 1200-1300 mg/day calcium. The last diet group was assigned a 500 kcal/day deficit, high-dairy diet plus a placebo supplement, providing 1200-1300 mg/day calcium from a dietary rather than supplemental source. At the end of the trial all subjects lost weight, however, the weight loss in the higher calcium groups was markedly greater than that of the low calcium group. The low calcium group lost an average of 6.4% of their body weight and 8.1% of their body fat, whereas the calcium-supplemented group lost 8.6% and 11.6% and the high-dairy lost 10.9% and 14.1% of their body weight and body fat, respectively. The higher calcium groups also lost much more trunk fat as a percentage of their total fat loss. Of these two diets, however, the dairy group again showed a significantly greater fat loss than the calcium group, with

66.2% of their total fat loss coming from the trunk region as opposed to 50.1% total fat being lost from the trunk in the calcium-supplemented group.

In another study[16], 38 healthy overweight subjects were placed on one of two 500-calorie deficit diets: the first low in calcium (400-500 mg/day) and dairy and the second providing three six-ounce servings of yogurt a day (1100 mg calcium/day). After 12 weeks, all subject lost weight, however, those subjects consuming the yogurt diet lost 22% more weight, 61% more fat and 31% less lean mass than the low calcium group. The yogurt group also had a profound decrease in trunk fat (81% more than the control group), waist circumference and blood pressure, and a significant increase in lipolysis of 22.3%. A trial of dairy products on body composition and weight loss in African-Americans showed similar results[15]. On a eucaloric diet, the group consuming the low calcium (500 mg/d) low-dairy diet had no changes in body composition, but those who ate three servings of dairy each day (1200 mg Ca/d) had decreased body fat by about 2 kg, trunk fat by about 1 kg, as well as lowered insulin and blood pressure. Similarly, when these diets were modified to be calorie restricted in a second part of the clinical study, the high-dairy group lost about twice as much weight and fat as the low-dairy diet. Lean weight was maintained much better in the high-dairy group in that they lost only 13% as much as the low-dairy group. A significant drop in fasting insulin was observed with the high-dairy diet, and this group also lost over 4 kg of trunk fat compared to less than 1 kg lost by the low-dairy group.

Not all clinical trials conclude that calcium has an effect on weight loss, however. In a study by Bowen*, et al.*[46], 50 overweight people placed on either a diet high in dairy protein and calcium (2400mg/d), or a diet high in mixed protein / low calcium (500mg/d).

This study found that there was no difference between weight, fat, trunk fat or lean loss between the groups after 16 weeks of energy restriction. It is difficult to compare this study to the others discussed, however, because both of the experimental diets were high in protein (34%), which has been shown to enhance weight loss, and all groups lost about 9 kg during the 12 weeks of energy restriction, maximizing the amount of weight loss possible, leaving little room for calcium to have any apparent effect. Furthermore, the baseline calcium intake of all of these groups was over 700 mg per day, about 200mg higher than other trials[15, 16, 45] studying calcium's role in weight loss. Another study[47] compared diets with differing levels of calcium and fiber in 90 obese subjects for 48 weeks. This study also found no difference in weight and fat loss between the diet groups; however, the authors speculated that the lack of significant difference was due to the "threshold effect" which could not be observed in this trial due to lack of a low-calcium diet group. The idea that a minimum amount, or threshold, of calcium intake is required to maintain sufficiently low calcitrophic hormones and $[Ca^{2+}]i$ to keep lipid accumulation low was first suggested by Thompson *et al*. and has been supported by several studies[16, 45, 47-49]. Based on these studies, it has been hypothesized that this threshold is about 600mg of calcium per day.

E. Retrospective studies linking calcium to weight loss

Longitudinal, reanalysis of bone-density and other studies conclude that calcium and dairy have an effect on body composition. A longitudinal study of pre-school children[12] showed that those with the highest dairy, calcium, and monounsaturated fat consumption tended to have the lowest body fat as measured by dual-energy x-ray absorptiometry (DEXA). In a later study of 8 year olds, it was found that calcium intake

was again negatively associated with percentage of body fat[13]. A second study completed soon after Zemel's NHANES III analysis also found that calcium is related to body composition in young women [8]. The study was originally designed to assess the relationship between exercise and body composition, but the study design was easily adapted to assess the relationship between dietary factors and body composition. Subjects in this study were sedentary white females who were partitioned either to a control group or an exercise group for two years. Fifty-four of these women had enough dietary records for the researchers to reanalyze results to determine if a relationship existed between calcium intake and body composition as determined by DEXA. Statistical regression calculations showed that the ratio dietary calcium to energy (mg/kcal) showed significant negative correlation to body weight and fat; in other words, the more calcium a person consumes relative to calories, the less that person is likely to weigh. The investigators noted that the effect of calcium in this case was specific to dairy calcium, but it is possible that there are other components in dairy that affect body composition, or that there is a threshold level of calcium needed to have any effect, and that dietary sources of the nutrient are essential to achieving this level. Along the same lines, in 2000 the Osteoporosis Research Center reanalyzed their studies from the past 12 years and found that among 780 women of differing ages, there were significant negative correlations between body weight and calcium intake, and that for young women, calcium intake below the median equated to an odds ratio of 2.25 for being overweight[9].

The exercise intervention study [8] also found that total calcium but not energy intake predicts changes in fat mass in those women with intakes lower than the median amount of calories, but that energy and not total calcium predicts changes in fat mass in

women with higher caloric intake. In assessing the difference between the effect of calcium on body composition between the higher and lower calorie intake groups, Lin *et al.* hypothesize that higher energy intakes could overwhelm the effect of calcium. This is significant when considering the role of calcium in weight loss diets.

A recent study on calcium supplementation and weight change after 10 years[50] found that in men, calcium from the diet, supplements or both sources has little effect on weight change between the ages of 45 and 57 years, but that in women of the same age current and past supplement use are predictors for weight change. Women who took the most calcium gained the lease amount of weight. Interestingly, dietary calcium did not have a significant effect on weight gain, but dietary plus supplementary calcium did show a significant trend of weight maintenance in the higher quartiles of intake.

Despite these findings, some researchers have found that calcium has no effect on weight loss. A study on the effect of supplemented calcium on weight and fat loss in pre- and post-menopausal women found little correlation between the mineral and change in body composition[51]. This study may differ from many of the others because the mean baseline calcium intake of the groups was higher than 600 mg/day, the suggested "threshold." Also, all the calcium was supplemented via pill form, rather than through adding dairy products to the diet. Nonetheless, it raises the question of what levels of intake and in what form calcium becomes effective in promoting healthy body composition, and in what populations calcium supplementation is most beneficial.

F. Epidemiological support for the anti-obesity effect of calcium

The findings from the first published animal study using the conceptual framework of intracellular calcium as a modulator of adipocyte lipid metabolism

prompted Zemel *et al.* to investigate epidemiological evidence for a relationship between calcium or dairy and weight. In the first of these studies, the NHANES III data set was analyzed by dividing the sample (380 women and 7114 men) into quartiles of dairy consumption[10]. Compared to the lowest quartile of dairy consumption with an odds ratio (OR) of being in the highest quartile of body fat set at 1.00, the highest quartile for dairy consumption had an OR of 0.16 for being in the highest body fat quartile. This took into account both energy intake and physical activity, indicating that for any level of either of these parameters, those people who consumed the most dairy were at a much lower risk of being in the highest body fat quartile.

As increasing numbers of studies on calcium's inverse relationship with overweight were published, the researchers conducting the CARDIA prospective study reevaluated the results from the first ten years of their study (1985-1995) for any relationship between calcium and the indicators of metabolic syndrome [7]. Of all of the foods and nutrients assessed, only dairy, fiber and protein showed any association with metabolic syndrome. Researchers found that for overweight individuals, "there was a consistent reduction in incidence for each of the four components with increasing categories of dairy intake" but that the relationship was weaker in normal-weight individuals. They also found that incidence of metabolic syndrome decreased by more than 50% in overweight individuals with the highest dairy intakes from those with the lowest dairy intakes. Little evidence for confounding by other dietary and lifestyle factors were found when they were factored into odds ratios models. When the odds ratio for having metabolic syndrome was set at 1 for overweight individuals with the lowest dairy intake, researchers found highly significant odds ratio of 0.28 for people with the

highest dairy intake in a model including demographics, lifestyle factors as well as dietary fiber and protein. Similar results were found regardless of whether other dietary factors were included in models.

Mirmiran *et al.* found that subjects in the Tehran Lipid and Glucose Study who had the 462 subjects studied, those who had the highest BMIs had the lowest consumption of dairy products, and the group with the highest dairy consumption had the highest proportion of normal weight people[52]. The HERITAGE study of the interaction between calcium intake and BMI separated sex and race for statistical analysis[53]. It found that black and white men and white women tended to have decreasing incidences of overweight and obesity as calcium consumption went up, but that black women had increasing incidence of overweight and obesity as calcium intake increased. Not only was increased calcium consumption correlated with lower body weight in three of four groups, but it also showed an inverse relationship with abdominal adiposity in black men and white women.

Another prospective study, the Quebec Family Study, followed changes in self-reported eating patterns and change in weight, body fat, skinfold thickness and waist circumference over a six year follow-up period [54]. People who ate less fat and more fruit and milk between the first and second data collection times tended to have a decrease in weight and other body fat indicators over the interim. When change in physical activity was accounted for in the researchers' statistical model, the effect of milk was no longer apparent, indicating that increased energy expenditure can overwhelm the effect of increased milk consumption, much like the findings of Lin *et al*[8].

III. Role of the renin-angiotensin system in obesity

Although the preponderance of the data indicates that dairy exerts a greater effect on body weight and body composition than can be explained by its calcium content, the dairy components responsible for this additional activity have not yet been identified. However, two components of dairy seem the most likely to account for this difference. Dairy has long been recognized to contain both angiotensin converting enzyme inhibitors (ACEi)[55] and a high concentration of the branched chain amino acids (BCAA). ACEi are primarily associated with antihypertensive action by inhibiting the major step in the renin-angiotensin system[17, 18]. It has been shown that at least two of the antihypertensive proteins found in milk are absorbed intact in adults[56], and that regular ingestion of sour milk containing these peptides results in significantly lower blood pressure[57, 58]. Studies of the DASH diet, a diet high in fruit, vegetables, and milk, have commonly attributed the hypotensive effect to increased calcium intake[59, 60]; however, it is likely that more than that one nutrient in milk is the cause of this effect. Indeed, it was found in one meta-analysis that dietary sources of calcium had a two-fold greater effect versus supplemented calcium on blood pressure reduction[61, 62].

Over the past twenty years, it has been shown that adipose tissue contains all of the components of the renin-angiotensin system[63, 64], and is therefore capable of contributing significantly to the body's pool of AII thereby increasing blood pressure. Animal models confirm the link between adiposity and hypertension. Mice lacking adipose-produced angiotensinogen (AGT) have significantly lower blood pressure than wild type mice, and are markedly smaller and less fat than wild-type mice[65, 66]. Angiotensin type 2 (AT_2) receptor null mice are also significantly less fat with smaller

adipocytes than wild type mice, and have lower blood pressure even while maintained on a high-fat diet[67]. Likewise, Zucker fatty rats display increased levels of adipocyte-produced AGT after weaning compared to lean rats, which show no change in production of AGT after weaning[68] concurrent with the largest growth in adipose tissue, indicating that the hormone is linked to adipose tissue hypertrophy.

It has also been shown that preadipocytes respond to angiotensin II (AII) by differentiating into mature fat cells[69, 70], creating a feedback loop resulting in adipocyte differentiation, AGT/AII production, preadipocyte stimulation, adipocyte differentiation. Likewise, AII has been shown to increase lipogenesis in human adipocytes and 3T3-L1 adipocytes[20]. Some researchers[71] have attributed the link between AGT and adiposity to AGT's final product, AII, based on *in vitro* studies linking AII to preadipocyte differentiation. However, Janke *et al*. found that locally produced AII has an anti-differentiation effect[19]. One study using human adipocytes found no correlation between production of AGT and cell size, obesity phenotype or blood pressure; however, people with a specific single nucleotide polymorphism tended to have higher blood pressure, and exhibited significantly smaller adipocytes, which tended to produce more AGT[72]. It is difficult to draw the line between species differences, and differences in AII receptors when describing the role of the RAS in adipose tissue enlargement as mice, rats and human adipocytes have all shown different responses via different receptors to the hormone.

AII has also been shown to increase FAS gene transcription[20] and activity[73] in adipose cell lines, but to have no effect on lipolysis in human subjects[74]. ANG is converted into AII via angiotensin converting enzyme. If the effect of ANG on adipose

hypertrophy is indeed brought about by its conversion to AII, and this hormone's subsequent effects on adipocyte growth, differentiation and metabolism, then the inhibition of this enzyme would be beneficial in controlling adiposity as well as blood pressure.

IV. Branced-chain amino acids in protein synthesis and weight loss

A. Branced-chain amino acids in protein synthesis

Not only does milk contain bioavailable ACEi, it also contains a large amount of the branched-chain amino acids, valine, leucine and isoleucine. These amino acids are found in high proportion in the whey component of milk[75]. Whey comprises 20% of cow milk and 80% of human milk. The most noteworthy BCAA is leucine. Leucine stimulates protein synthesis via the mammalian target of rapamycin (mTOR) pathway by various initiation factors including eukaryotic initiation factor (eIF) 4F, a translation initiator, and via unknown mechanisms through eIF4G via its association to eIF4E (reviewed by Kimball and Jefferson[22]). Leucine also inhibits the action of eIF4E binding protein 1, which is a main inhibitor of translation initiation (reviewed in [76]). Insulin, which is released in response to elevated plasma glucose, is another major player in protein synthesis, also through the mTOR pathway [77]. The combined effect of insulin and leucine on the mTOR and other pathways makes dairy, which contains leucine and sugars, a likely player in muscle protein synthesis.

B. Leucine in energy restriction

Diets high in protein and the BCAA leucine have been shown to aid in building and repairing muscle, and to favor fat loss concurrent with muscle sparing during energy restriction. Rats maintained on a food restricted diet supplemented with leucine lost the

same amount of weight as their control group on a calorie restricted AIN-93M diet, but lost significantly more fat mass while maintaining lean mass[78]. A study of wrestlers on hypocaloric diets found that those subjects consuming a BCAA diet lost significantly more weight and fat than those consuming a control, high protein, low protein, and high carbohydrate diets, and they lost significantly more visceral fat tissue than the other diet groups[24]. Similarly, a study of high-altitude trekkers found that those consuming a high BCAA diet lost weight as the placebo group did, but they tended to gain muscle mass, whereas the placebo group lost lean mass[23]. The effects of leucine on protein synthesis could also account for greater fat and/or weight loss and greater maintenance of lean mass seen in multiple studies of high-protein vs. high-carbohydrate weight loss diets[21, 79, 80]. Diets with a lower carbohydrate to protein ratio also result in lowered serum lipids and insulin[80-83], indicating a role for BCAAs, in contributing to gluconeogenesis, stabilizing insulin, and protein synthesis, thereby increasing energy demands from lipids. These muscle-sparing effects are only seen when protein, especially leucine, is supplied in excess of what is needed for protein turnover, and can be used in stimulating protein synthesis, and gluconeogenesis.

CHAPTER 3. CALCIUM, ANGIOTENSIN CONVERTING ENZYME INHIBITORS AND BRANCHED CHAIN AMINO ACIDS CONTRIBUTE TO THE ANTI-OBESITY EFFECTS OF MILK

I. Abstract

Recent prospective and epidemiological studies as well as reports from our laboratory have shown that dietary calcium inhibits weight and fat gain, and promotes weight loss in mice and humans. Furthermore, dairy sources have been shown to exhibit a nearly two-fold greater anti-obesity effect than calcium supplementation alone. Dairy is known to contain other bioactive components including angiotensin converting enzyme inhibitors (ACEi) and branched chain amino acids (BCAA). Adipose tissue has been shown to contain all the elements of the renin-angiotensin system, and to respond to angiotensin II by promoting adipocyte differentiation. One of the BCAA, leucine, has been shown repeatedly to promote muscle protein synthesis in individuals fed eucaloric or hypocaloric diets. **Methods**: To test the hypothesis that ACEi and BCAA contribute to milk's anti-obesity effects, we used aP2-agouti transgenic mice, which simulate human obesity. All mice were maintained for six weeks on an obesigenic soy based diet, then divided into seven groups including one *ad libitum* control group and the remaining six groups on 70% calorie restriction. The restricted diets were as follows: basal diet, non-fat dried milk (NFDM)-based, depleted calcium NFDM-based, calcium replete NFDM-based, low-calcium soy-based with ACEi and BCAA added, and high-calcium soy-based with ACEi and BCAA added. At the end of the experiment body weight, fat and muscle weight, adipocyte intracellular calcium, lipolysis, as well as fatty acid synthase (FAS) and 11-β- hydroxysteroid dehydrogenase-1 (11-β-HSD) expression were measured.

Results: All mice on calorie restriction lost weight and fat, with the greatest change noted in the NFDM and calcium-replete milk-based groups. Soy-based diets with ACEi and BCAA supplemented lost more weight and fat than the basal soy diet. Mice eating milk basal diets either gained or maintained muscle mass, while those eating soy based diets with added ACEi and BCAA gained gastrocnemius mass, and lost less soleus mass than the restricted basal group. Intracellular calcium was elevated in all the low calcium diet groups while lipolysis was increased in all of the milk-based groups, with the greatest increase in the high-calcium milk groups. FAS expression was decreased significantly in all groups with ACEi and BCAA, particularly in the high-calcium groups. 11-β-HSD expression was also decreased in the milk groups and high-calcium groups. **Conclusion**: Milk, with and without calcium causes a marked anti-obesity effect when consumed by aP2-agouti transgenic mice. Furthermore, ACEi and BCAA contribute to the alterations in lipid metabolism in this mouse model.

II. Introduction

Previous research has shown the importance of calcium signaling in modulating cellular lipid metabolism. Intracellular calcium ($[Ca^{2+}]i$) stimulates *de novo* lipogenesis and inhibits lipolysis in both human adipocytes and obesity prone transgenic mice[3-5]. Similarly, low calcium diets induce fat gain and inhibit fat loss in mice, whereas high calcium diets impede energy storage[6]. Acute calcium intake by normal weight adults has been associated with increased fat oxidation[84]. Several epidemiological studies have shown a strong negative correlation between calcium intake and body fat and obesity[7-13]. It has also been suggested that calcium forms soaps with fat in the gut, thereby decreasing fat absorption and storage[85]. Furthermore, it has been shown by our laboratory that a

high calcium diet fed to transgenic mice suppresses Ca^{2+} signaling thereby affecting lipid storage via increased lipolysis and decreased lipogenesis and stimulation of adipocyte apoptosis[86].

Milk products enhance the effect of calcium on fat metabolism. *Ad libitum* feeding of aP2-agouti transgenic mice with a high calcium soy-based diet was associated with lesser weight gain than a low calcium diet, but mice fed a milk based diet gained even less weight than was explained by the calcium content alone. Similarly, clinical trials have shown consistent evidence that hypocaloric diets providing 1000 mg or more of calcium from milk have a greater anti-obesity effect than either supplementing a the diet with calcium carbonate[14], or providing less than 500 mg of calcium [15, 16].

The compounds in milk that enhance the anti-obesity effect of calcium are not yet known. However, there are two classes of bioactive compounds that show promise. Milk has long been known to contain angiotensin converting enzyme inhibitors (ACEi)[17, 18]. These proline-rich proteins are best known for their ability to lower blood pressure by inhibiting the conversion of angiotensin I into the potent vasoconstrictor angiotensin II (AII), and by inhibiting the breakdown of the vasodilator bradykinin. A study from our laboratory testing the anti-hypertensive effect of milk found weight-loss to be an unexplained side effect of the high dairy diet[10]. Since the publication of that study, it has been shown that adipocytes and preadipocytes contain all the elements of the renin-angiotensin system, and thus have the ability to contribute to the body's pool of AII[63]. Massiera *et al*. showed that mice producing no angiotensinogen in their adipose tissue had significantly lower blood pressure and fat weight than wild type mice, and mice overproducing angiotensinogen had significantly higher blood pressure and fat weight

than wild type mice[66]. It has also been shown that AII stimulates adipocytes to produce prostacyclin which then induces preadipocyte differentiation[69]. It is possible that ACEi found in milk could impede adipose tissue enlargement by interfering with adipose tissue hyperplasia. It has been shown that low doses of AII stimulate fatty acid synthase (FAS) gene expression and activity leading to increased triglyceride content of adipocytes[20]. Thus, we hypothesized that ACEi derived from milk could stop the formation of AII, thereby decreasing the availability of the substrate to increase triglyceride synthesis and storage.

The whey fraction of milk is also known to contain a high proportion of branched chain amino acids. Of these, leucine stimulates protein synthesis through the insulin-dependent mammalian target of rapamycin (mTOR) pathway, and through yet unknown insulin-independent pathways, most likely mediated by the phosphorylation of eukaryotic initiation factor 4G[22]. Leucine also acts as the primary nitrogen donor in the conversion of pyruvate to alanine, the key process in glucose recycling[87]. Glucose recycling is essential in gluconeogenesis used in glycemic control. When weight loss occurs, it is usually a loss of both fat and lean body mass. As lean mass is lost, resting metabolic rate decreases. Stimulation of protein synthesis through the mTOR pathway by leucine/BCAA administration is posited to attenuate lean body loss, and therefore help maintain metabolism and enhance fat loss. A further benefit of a leucine rich diet is better glycemic control contributing to satiety.

Consequently, the objective of this study was to determine the contributing roles of calcium, ACEi and BCAA to the anti-obesity effect of milk. Specifically, we compared differing sources of protein and differing levels of calcium, ACEi and BCAA

in calorie-restricted diets in their effects on weight and fat loss, and regulation of fat metabolism and gene expression in aP2-agouti transgenic mice.

III. Materials and methods

A. Mice and diets

This experiment utilized aP2-agouti transgenic mice, as used in previous experiments in our laboratory[6, 88]. These mice exhibit a pattern of agouti and leptin expression similar to that of humans[33]. They also are similar to humans in that they are of normal weight when fed an optimal diet, but gain weight and fat on an obesigenic diet. These characteristics make aP2- agouti transgenic mice an excellent model for studying diet-induced obesity.

1. Phase I

Seventy-eight male mice between the ages of 5 and 9 weeks were used for this experiment. In the first phase, mice were maintained on a modified AIN93G low calcium (0.4%) soy-protein diet with sucrose as the sole carbohydrate and lard supplementing the diet to provide 25% kcal from fat, for six weeks (Table 1).

2. Phase II

At the end of this period the mice were randomized to seven diet groups, such that the mean age and mean weight of each group were not statistically different. One group of mice (n=9) was maintained on the basal diet fed *ad libitum*. The remaining groups of mice were fed energy-restricted (70% kcal *ad lib*) diets as follows: 1) basal diet

Table 1: Basal Diet Composition

	Protein (Soy)	Carbohydrate	Fat	Fiber	Calcium	Leucine	Isoleucine	Valine
Grams %	14.4	64.8	11	5	0.4	1.14	0.69	0.71
Kcal %	14	62	24					

(n=9); 2) basal diet modified by replacing all soy protein with non-fat dry milk (NFDM) and native calcium level maintained at 1.2% (n=10)*;* 3) a Ca-depleted NFDM protein diet, with calcium reduced to a level of 0.4% (n=10)*;* 4) milk-protein diet as above, with calcium ($CaCO_3$) added back to the Ca-depleted NFDM to bring Ca to 1.2% (n=10); 5) low calcium basal diet supplemented with branched chain amino-acids (BCAA) and ACE inhibitors from whey (TruCal, Glambia, Wisconsin) equivalent to that in the NFDM diets (n=10); 6) high calcium basal diet with BCAA and ACE inhibitors added as above, and calcium supplemented to 1.2% using $CaCO_3$ (n=10) (Table 2). Food intake and spillage were measured daily for the second six-week phase of this experiment; body weight was measured weekly.

At the end of the second six-week phase, all the mice were sacrificed by exsanguination via cardiac puncture while under pentobarbital/phenytoin anesthesia. Epididymal, retroperitoneal, perirenal and subscapular fat depots were collected, immediately weighed, and frozen or used in immediate analysis as described below. Real time RT-PCR was used to analyze FAS and 11β- hydroxysteroid dehydrogenase (11-β-HSD) expression in adipose tissues. All animal care and sacrifices were carried out in accordance with the University of Tennessee Institutional Animal Care and Use Committee.

Table 2: Phase II Mouse Diets

	Ad Libitum	*Restricted Diets*					
	Basal	Basal	NFDM	Ca Depleted	Ca Replete	Low Ca, ACEi/BCAA	High Ca, ACEi/BCAA
Protein Source	Soy	Soy	Milk	Milk	Milk	Soy	Soy
Calcium (%)	0.4	0.4	1.2	0.4	1.2	0.4	1.2
Leucine (%)	1.14	1.14	1.33	1.33	1.33	1.33	1.33
Isoleucine (%)	0.69	0.69	0.82	0.82	0.82	0.84	0.84
Valine (%)	0.71	0.71	0.91	0.91	0.91	0.92	0.92

B. Lipolysis

Immediately after being excised, perirenal fat was incubated for two hours at 37º C. Both basal and stimulated glycerol release was measured using a one-step fluorometric method, as previously described[89]. The final data were normalized to DNA, as above.

C. Calcium imaging

Adipocyte intracellular calcium ($[Ca^{+2}]i$) measurement was performed as previously described[6, 90]. Briefly, retroperitoneal adipose tissue was first washed several times with Hank's Balanced Salt Solution (HBSS), minced into small pieces, and digested with 0.8 mg/ml type I collagenase in a shaking water bath at 37 ^{o}C for 30 min. Adipocytes were then filtered through sterile 500-μm nylon mesh and cultured in Dulbecco's Modified Eagle's Medium (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. Prior to $[Ca^{2+}]i$ measurement, adipocytes were pre-incubated 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 μmol/L) in the same buffer in the dark for 1 h at 37 ^{o} C. Adipocytes were rinsed with HBSS three times to remove extracellular dye and then post-incubated 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 Corporation, Ashland, MA). The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu 4915 CCD camera (300w Xenon, Intracellular Imaging Inc, Cincinnati, OH). Fluorescent images were captured alternatively at excitation wavelength of 340 nm and 380 nm with an emission wavelength of 520 nm. $[Ca^{2+}]i$ was calculated by using a ratio equation as described previously[6, 90].

D. Total RNA extraction

RNA was extracted from retroperitoneal fat using the Ambion ToTALLY RNA kit (Ambion, Inc., Austin TX) according to the manufacturer's instructions. RNA was resuspended in nuclease free water before quantification using the ND-1000 Spectrophotometer (NanoDrop Technologies Inc., DE), and frozen at –80ºC in aliquots of 10 ng/μl for use in real time RT-PCR.

E. Polymerase chain reaction

Gene expression for FAS and 11-β-HSD, normalized to their corresponding 18S expression was measured by real time reverse transcriptase- polymerase chain reaction (RT-PCR) using the 7300 Real Time PCR system with TaqMan universal PCR Master

Mix, no AmpErase UNG (2X), and reverse transcriptase and RNAse inhibitor from the TaqMan 1000 Reaction Core Reagent Kit (all from Applied Biosystems, Foster City, CA), according to manufacturers instructions.

Standard curves were made for each gene of interest by pooling 1µl of each sample (concentration 10 ng/µl), then using ten-fold dilutions for each progressive standard point ranging from 100 ng to 0.5 pg total RNA. RT-PCR reaction mixture contained TaqMan universal PCR Master Mix, no AmpErase UNG (2X), murine leukemia virus reverse transcriptase (50 U/µl), RNAse inhibitor, and 20X Assays-on-Demand Gene Expression Assay Mix for FAS, 11-β-HSD and 18S (all from Applied Biosystems, Foster City, CA). Polymerase chain reaction was performed according to instructions of the 7300 Real Time PCR system (Applied Biosystems) using 50 to 100ng total RNA per sample plated in Thermo-Fast 96Detection plates (ABgene, Epsom, Surrey, UK). Samples and standards were run in triplicate for best replication and standard error.

F. Statistical analysis

Data were evaluated for statistical significance using ANOVA, and significantly different group means were then further analyzed using the least significant difference (LSD) test. Variables that did not follow the normal distribution were transformed according to accepted statistical practices before ANOVA. Analyses were performed with SPSS (SPSS Inc, Chicago, Ill).

IV. Results

A. Weight Change

All the mice on restricted diets lost weight. The groups fed high calcium milk based diets lost the most weight (~14 g, $p<0.01$) with no significant difference between them (Fig. 1). The mice eating low calcium diets containing either native or supplemented ACEi and BCAA lost 1.66 to 3.03 g less weight than their respective high calcium counterparts. Mice eating the high calcium soy-based diet supplemented with ACEi and BCAA lost significantly less weight than mice on either the regular or the calcium replete milk diets, but more than those fed the restricted basal diet.

B. Fat Pad Mass

All of the milk fed groups lost significantly more fat than the restricted basal group (Fig. 2). Of note, the calcium depleted milk diet group lost ~34% ($p<0.001$) more fat than the restricted basal group. Addition of ACEi/BCAA to soy diets also resulted in significant weight loss compared to the restricted basal group; this effect was further enhanced by calcium.

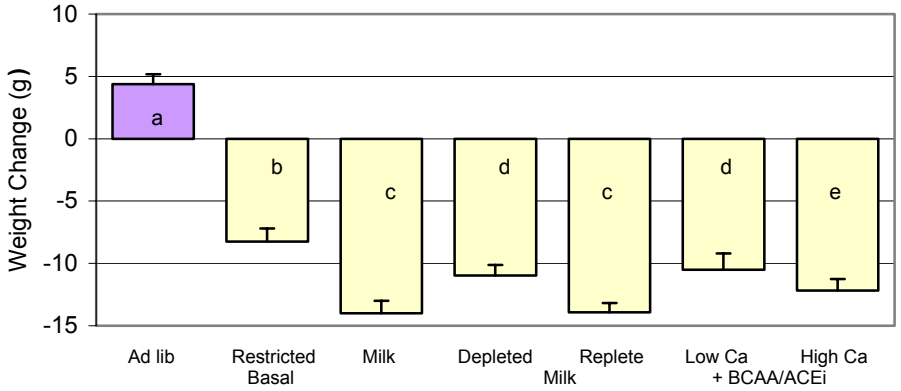

Figure 1: Effects of Calcium, ACEi and BCAA on weight change. Non-matching superscripts denote significant different, $p<0.01$.

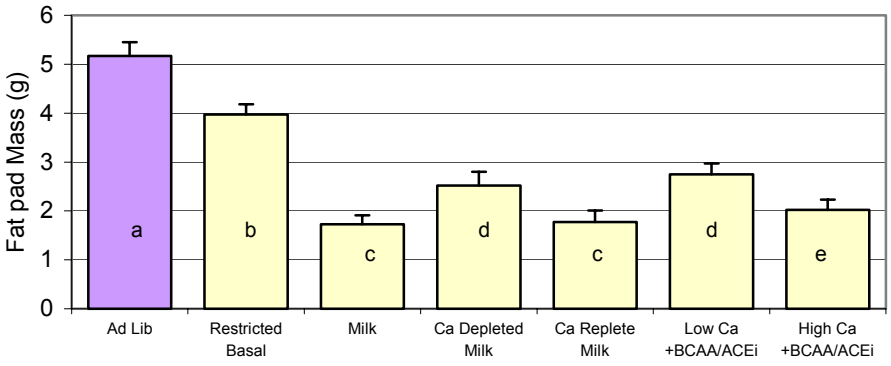

Figure 2: Effects of Calcium, ACEi and BCAA on fat pad mass. Non-matching letters denote signifant difference, $p<0.001$.

C. Gastrocnemius Mass

Mice fed *ad libitum* had an average gastrocnemius weight of 0.08 g (Fig. 3). Mice fed the basal restricted diet had significantly lower gastrocnemius weight of 0.072 g ($p<0.01$). Mice consuming diets high in either native or supplemented BCAA had significantly higher muscle weights than those eating food low in BCAA.

D. Soleus Mass

Mice fed a restricted basal diet lost a significant amount of soleus mass compared to *ad lib* fed mice ($p<0.05$) (Fig. 4). Groups that were fed soy diets supplemented with ACEi/BCAA also lost muscle mass, but to a lesser extent than the low BCAA diet. Mice fed milk-based diets maintained or gained soleus muscle mass during energy restriction.

E. Adipocyte intracellular calcium

All mice fed low calcium diets had significantly higher $[Ca^{2+}]i$ than mice fed high calcium diets ($p<0.01$) (Fig. 5). Mice on all restricted diets had significantly lower $[Ca^{2+}]i$ than mice fed ad libitum ($p<0.01$).

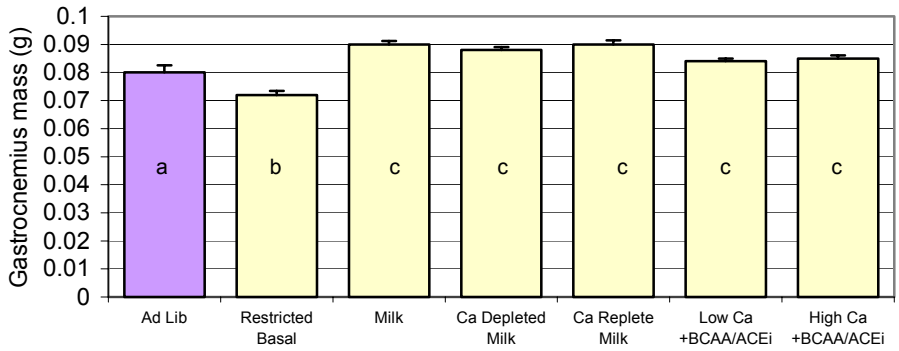

Figure 3: Effects of Calcium, ACEi and BCAA on gastrocnemius mass. Non-matching superscripts denote significant different, $p<0.01$.

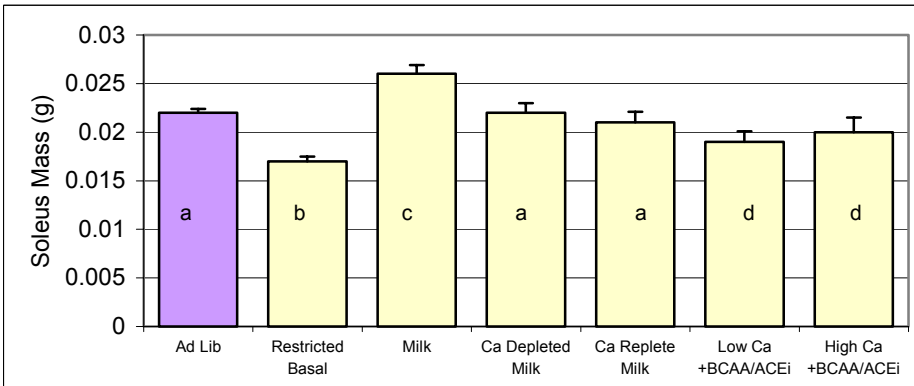

Figure 4: Effects of Calcium, ACEi and BCAA on soleus mass. Non-matching superscripts denote significant different, $p<0.05$.

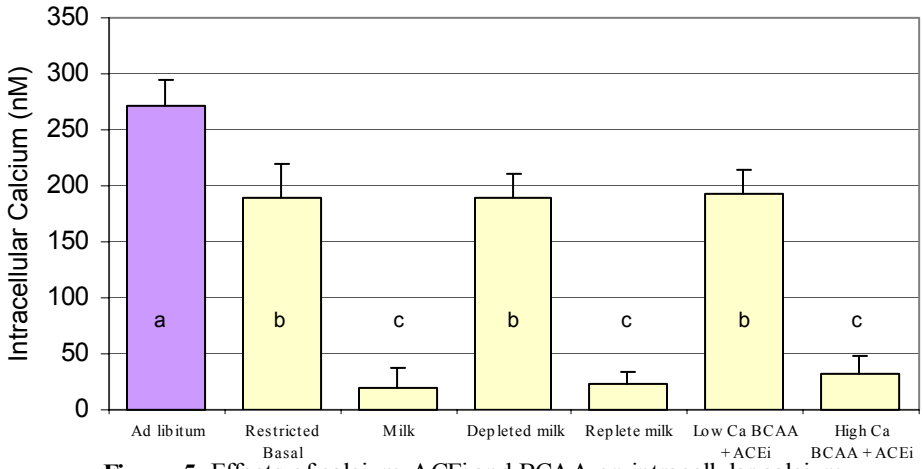

Figure 5: Effects of calcium, ACEi and BCAA on intracellular calcium. Non-matching superscripts denote significant difference $p<0.01$.

F. Lipolysis

Glycerol release increased with all of the restricted diets (Fig. 6). Milk-based diets caused the greatest increase in lipolysis over the restricted basal diet, with higher calcium content augmenting this increase ($p<0.05$). Addition of ACEi/BCAA did not increase lipolysis in soy fed groups.

G. FAS gene expression

FAS expression of the restricted basal group was not significantly different from that of the ad libitum group ($p<0.05$) (Fig. 7). The groups with low calcium showed about a 25% decrease in FAS expression from the restricted basal group, with no significant difference between these two groups. FAS expression in the high calcium milk groups dropped a further 60% compared to the depleted milk group, totaling a drop in expression of ~75% compared to the restricted basal group. There was no significant difference in FAS expression between the low and high calcium +BCAA/ACEi groups, although there was a trend ($p<0.10$).

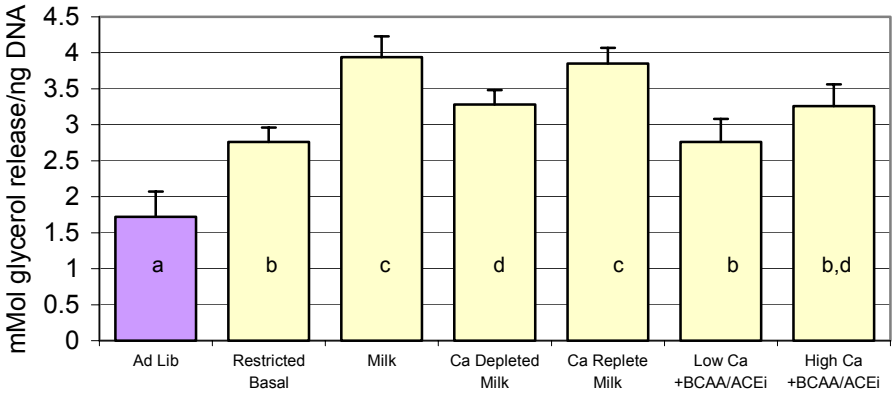

Figure 6: Effects of Calcium, ACEi and BCAA on lipolysis. Non-matching superscripts denote significant different, $p<0.05$.

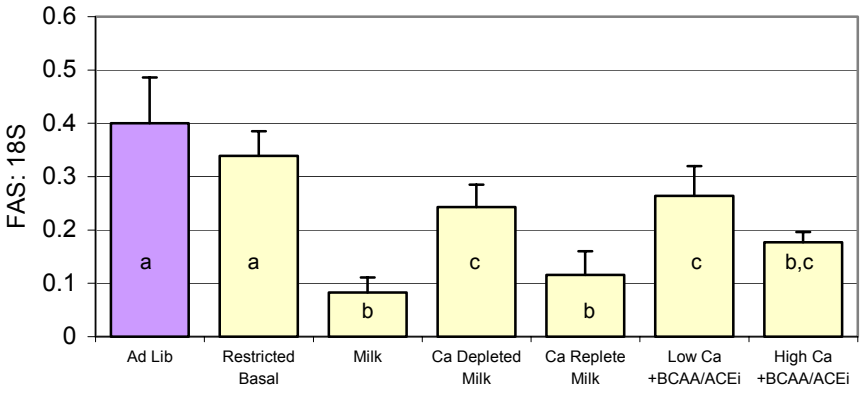

Figure 7: Effects of Calcium, ACEi and BCAA on fatty acid synthase expression. Non-matching superscripts denote significant different, $p<0.05$.

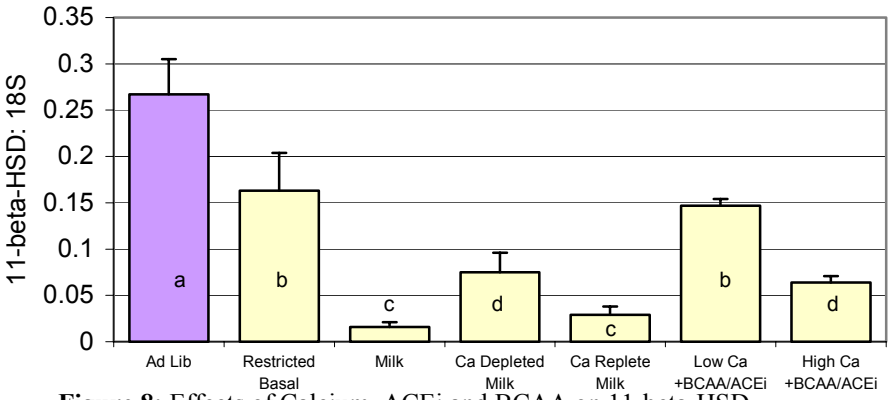

Figure 8: Effects of Calcium, ACEi and BCAA on 11-beta-HSD expression. Non-matching superscripts denote significant different, $p<0.02$.

H. 11-β-HSD gene expression

All energy restricted groups experienced a drop in 11-β-HSD expression compared to the *ad lib* fed group ($p<0.02$) (Fig. 8). The calcium depleted diet group showed a significant decrease in 11-β-HSD expression compared to the restricted basal group; this effect was enhanced by 60-80% in high calcium dairy groups. These animals had about a 90% decrease in 11-β-HSD expression compared to the restricted basal group. Addition of ACEi/BCAA to soy diets did not change 11-β-HSD expression; however, addition of calcium to this diet did have a significant effect ($p<0.02$).

V. Discussion

This study was designed to assess the roles of calcium, ACEi and BCAA in body composition and adipose tissue metabolism. The results demonstrate that milk's anti-obesity effect is largely due to calcium, ACE inhibitors and branched chain amino acids. Milk, both intact and calcium replete, caused the largest weight loss in mice, and this effect was replicated to a lesser extent by the soy diet with added calcium and ACEi/BCAA (Fig. 1). The observed changes in body weight were due largely to

corresponding changes in fat mass (Fig. 2). This experiment is novel in its use of calcium-depleted milk. The effects of dietary calcium are clearly seen in the comparison of the depleted vs. replete milk diet groups and of the soy diets/ACEi/BCAA diets with and without added calcium. When comparing the restricted diet groups it is apparent that calcium is responsible for ~35% of milk's effect on fat loss, the ACEi/BCAA combination is accountable for ~54%, leaving the remaining 10% of milk's effect unaccounted for.

Consistent with our previous data[10, 43], $[Ca^{2+}]i$ was decreased in all high-calcium diet groups, indicating that the source of calcium provided in this experiment had no effect on $[Ca^{2+}]i$. $[Ca^{2+}]i$ is increased in response to high levels of circulating calcitriol[10], and promotes FAS expression and activity, as well as inhibiting lipolysis. This study corresponds with previous data in that groups consuming the most dietary calcium have the lowest $[Ca^{2+}]i$[42, 43], the highest levels of lipolysis[10, 16, 43], and the lowest FAS expression[10, 43]. Milk also produces a non-calcium related weight reduction which appears to be due in large part to ACEi and BCAA. This effect is clearly unrelated to calcium-signaling, as it is apparent despite low levels of $[Ca^{2+}]i$.

Although about 50% of milk's anti-obesity effect is due in part to both ACEi and BCAA (Fig. 2), data from the present study cannot distinguish which plays a greater role. However, we previously found that the dairy ACE inhibitor given alone was only responsible for 10-15% of the effect of milk[91], suggesting that the large effects of the ACEi/BCAA combination in the present study are mostly attributable to the supplemental BCAA. Data from this study indicate that neither BCAA nor ACE inhibition exerts any effect on 11-β-HSD expression[92]. However, since AII has been shown to stimulate

cortisol release[36], ACE inhibition may still exert an effect on central adiposity. AII also affects adipocytes in their differentiation[69] and lipid accumulation[20] phases. Although AII has classically been considered an important regulator of blood pressure and fluid and electrolyte balance[93, 94], its link to differentiation and lipid accumulation is well established[64, 95]. AII works via AT_2 receptor to induce prostacyclin production by adipocytes[69]. This then induces preadipocytes to differentiate. AII also stimulates cortisol release via the AT_1 receptor[36], which also induces adipocyte differentiation[25]. In addition, FAS gene expression is up-regulated by AII resulting in increased activity of the FAS enzyme and lipid accumulation[20]. ACE inhibition, therefore, decreases the amount of preadipocyte differentiation and fatty acid synthesis and storage.

Although the primary effect of the BCAAs would appear to be maintenance of muscle mass, BCAA supplementation exerted a substantial effect on adiposity. Although the mechanism of this effect is not yet clear, protein synthesis is an energetically expensive process, requiring 2 ATPs and 2 GTPs for each peptide bond formed. It is likely that BCAAs play a role in decreased FAS expression by using the body's pool of energy producing substrates rather than allowing them to enter the fatty acid synthesis pathway. Accordingly, we propose that the effects of BCAA on adiposity may be due to the energy demand resulting from increased protein synthesis.

The BCAA appear to have contributed to muscle maintenance during calorie restriction. The fact that mice consuming all three of the milk-based diets gained gastrocnemius muscle mass during calorie restriction was unexpected. However, it is notable that the mice on all restricted diets appeared to be highly active compared to those on the *ad libitum* diet. Activity was not measured, *per se*, but those caring for the

animals reported a noticeable difference in the mice's waking, sleeping, and moving habits. It has been shown repeatedly that exercise causes temporary muscle protein catabolism, but when adequate protein and energy are supplied, anabolism returns, promoting muscle gain[96-98]. Leucine contributes significantly to muscle anabolism via the mTOR pathway leading to protein synthesis[76, 99, 100]. Likewise, leucine also has a muscle sparing effect during energy restriction[21, 80, 101, 102]. During weight loss studies comparing the effects of differing amounts of dietary protein on body composition, it was found that high protein diets promoted fat and weight loss, and attenuated lean tissue loss[21, 80].

Changes in soleus weights showed a different pattern than gastrocnemius. While the depleted and replete milk diet groups neither lost nor gained soleus mass, the intact milk group gained soleus mass compared to the *ad libitum*- fed group, and the soy groups with added dairy components had soleus weights that fell between the *ad libitum* and the restricted basal group muscle weights. The difference between the results for the different muscles may have to do with the nature of the activity and the muscles being exercised. Mice on restricted diets tended to bounce on their hind limbs, exercising their gastrocnemius but not their soleus muscles. The soleus muscle made of slow-twitch fibers, and is used primarily in posture rather than movement, therefore exerting less of a demand on body leucine supply than its fast-twitch counterpart, gastrocnemius. Because the soleus muscle was not being exercised, it did not experience the same gain in size as the gastrocnemius, and may therefore be viewed as a better indicator of the BCAA's influence on non-exercised muscle. Alternately, the gastrocnemius muscle is glycolytic, using large amounts of glucose during exercise, requiring the body to maintain blood

glucose via gluconeogenesis. Usually during exercise and starvation this process is supplied by breaking down lean tissue; however, a diet high in the BCAA, which are used preferentially in gluconeogenesis, provides the body with alternative substrates for maintaining glucose. Recent studies have shown that voluntary endurance exercise concurrent with diets supplemented with amino acids favors increase in both muscle types[103]. The question remains as to why the milk diet group gained soleus muscle weight while the other diet groups either maintained or lost muscle weight, even though consuming comparable quantities of BCAA. There may be a synergistic relationship between the leucine in milk and the unidentified active dairy component

In conclusion, we have shown that calcium, ACEi and BCAA all contribute to the anti-obesity effect of milk by decreasing weight and fat mass through increased lipolysis, and increasing or maintaining muscle mass. We have also demonstrated that FAS and 11-β-HSD expression are decreased by a calcium- dependent mechanism, but also by the ACEi/BCAA mixture and unidentified components in milk. Additional studies will be needed to separate the effects of ACEi from BCAA, and to determine the identities of the unknown compounds responsible for altering fat metabolism.

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