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I am submitting herewith a dissertation written by Xiaocun Sun entitled “Role of Mitochondrial Uncoupling Protein 2 (UCP2) in Modulation of Adiposity”. I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

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Role of Mitochondrial Uncoupling Protein 2 (UCP2) in Modulation of Adiposity

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Xiaocun Sun
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To my family and friends who have motivated and supported me throughout the completion of this dissertation.

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Abstract

Obesity is a disorder of energy balance in which energy intake exceeds energy expenditure. Methods to control obesity through limiting energy intake have had limited success, and it is widely recognized that energy expenditure must also be increased if long-term weight loss is to be achieved. Uncoupling proteins (UCPs) are a family of integral membrane proteins of mitochondrial inner membrane, where they uncouple the process of mitochondrial respiration from oxidative phosphorylation, diminishing the resulting production of ATP and decreasing the metabolic efficiency of the organism. Thus, UCPs provide new molecular targets for increasing energy expenditure. Unlike the other UCP family members, UCP2 is ubiquitously expressed, with the highest level in white adipose tissue. Stimulation of mitochondrial uncoupling in adipocytes *in vitro* demonstrates a direct inhibitory effect on lipogenesis and suppression on lipolysis via a calcium dependent mechanism, indicating a potential role of UCP2 in regulation of adiposity.

Previous studies demonstrate that intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) plays a key role in metabolic disorders associated with obesity. $[\text{Ca}^{2+}]_i$ can clearly be modulated by the calcitrophic hormone $1\alpha, 25\text{-dihydroxyvitamin D}_3$ ($1\alpha, 25\text{-(OH)}_2\text{-D}_3$), which appears to have both genomic and non-genomic action in adipocytes. $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ modulates adipocyte Ca^{2+} signaling directly, resulting in an increased lipogenesis and decreased lipolysis. In addition, $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ plays a genomic role in regulating adipocyte

UCP2 expression levels, indicating that the regulation of UCP2 and the resulting increased core temperature may contribute to increased rates of energy dissipation. Accordingly, the suppression of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ by increasing dietary calcium attenuates adiposity by decreasing triglyceride accumulation in the adipocytes: increasing dietary calcium results in a net reduction in fat mass in the absence of caloric restriction, a marked augmentation of body weight and fat loss during energy restriction, and an inhibition of weight and fat regain after food restriction in mice.

Although these anti-obesity effects of dietary calcium are due, in part, to enhanced metabolic rate and thermogenic processes, it is also possible that a loss of adipocytes would result in a deficit cells for lipid esterification as the body recovers. Physiological doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibit apoptosis in differentiated 3T3-L1 adipocytes, and the suppression of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ *in vivo* by increasing dietary calcium stimulates adipocyte apoptosis in refeeding following energy restriction in aP2 transgenic mice, indicating that the stimulation of adipocyte apoptosis contributes to adiposity reduction after high calcium diet administration. UCP2 plays a direct role in modulating adipocyte apoptosis by inducing mitochondrial potential collapse and inhibiting ATP production. Overexpression of UCP2 in adipocyte stimulates apoptosis while inhibition of mitochondrial uncoupling either by chemical inhibitor GDP or by siRNA duplexes suppresses adipocyte apoptosis. Accordingly, suppression of UCP2 by physiologically low doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$, which can be induced by low dietary calcium, decreases apoptosis. Although $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ causes dose-dependent stimulation on $[\text{Ca}^{2+}]_i$ levels, low doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ decrease mitochondrial calcium accumulation while a high

dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ induces markedly greater increase in $[\text{Ca}^{2+}]_i$ and stimulates calcium storage in mitochondria. Bay K 8644, which is a Ca^{2+} ionophore and can mimic the stimulatory effect of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on $[\text{Ca}^{2+}]_i$ without exerting effects on UCP2, causes a dose-dependent increases in apoptosis and mitochondrial calcium accumulation, indicating that stimulation of pharmaceutical high dose $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on apoptosis is a calcium-dependent effect.

In summary, present data support the concept that dietary calcium exerts anti-obesity effects in aP2 transgenic mice under conditions of varying nutrient status. In addition, this study extended our observation that dietary calcium not only regulates adipocyte size by decreasing lipid accumulation, but also modulates adipocyte number by stimulating apoptotic death. These anti-obesity effects of dietary calcium are attributable to the up-regulation of UCP2, which stimulates energy expenditure, fat utilization and adipocyte apoptosis in white adipose tissue. Accordingly, these data indicate an important role of increasing dietary calcium in prevention and management of obesity.

Table of Contents

Part One: Literature Review.....	1
1.1 Introduction.....	2
1.2 Obesity: General Aspects.....	5
1.2.1 Prevalence of Obesity.....	5
1.2.2 Pathology of Obesity.....	7
1.2.2.1 Genetics of Obesity.....	7
1.2.2.2 Endocrine Disorders of Obesity.....	8
a. Cortisol.....	9
b. Insulin	10
c. Growth Hormone.....	12
1.2.3 Dietary Management of Obesity.....	13
1.2.3.1 Dietary Fat.....	14
1.2.3.2 Dietary Carbohydrate.....	16
1.2.3.3 Dietary Protein.....	19
1.2.3.4 Dietary Calories.....	21
1.3 Role of Intracellular Calcium in Obesity.....	21
1.3.1 Mouse Agouti and Obesity.....	21
1.3.2 Intracellular Calcium and Obesity Related Metabolic Disorder	26
1.3.3 Dietary Calcium and Obesity.....	31
1.3.3.1 Dietary Calcium Intake and Obesity.....	31
1.3.3.2 Mechanism of Dietary Calcium Intake in Regulation of Obesity.....	32
a. Dietary Calcium, $1, 25(\text{OH})_2\text{D}_3$ and Intracellular Calcium	32
b. Intracellular Calcium and Adipocyte Lipid Metabolism.....	34

c.1 α , 25(OH) $_2$ D $_3$ and Adipocyte Energy Homeostasis.....	35
1.4 Role of UCPs in Regulation of Obesity.....	38
1.4.1 Thermogenesis and Obesity.....	38
1.4.2 UCP Family.....	39
1.4.3 UCPs and Obesity.....	43
1.4.3.1 UCPs and Thermogenesis	43
1.4.3.2 UCPs and Fatty Acid Metabolism	46
1.4.3.3 Role of UCPs in Regulation of Insulin Secretion.....	49
1.4.3.4 Role of UCPs in Regulation of Intracellular Calcium.....	50
1.4.3.5 Role of UCPs in Regulation of Reactive Oxygen Species.....	52
1.5 Regulation of Aipocyte Cellularity.....	54
1.5.1. Regulation of Adipocyte Size.....	54
1.5.1.1. Lipogenesis.....	54
1.5.1.2. Lipolysis.....	57
1.5.2. Regulation of Adipocyte Number.....	60
1.5.2.1. Adipogenesis.....	60
1.5.2.2. Adipocyte Depletion.....	66
Literature Cited	70

Part Two: Interactive Effects of Calcium-Fortified Breakfast Cereal and of Milk on Adiposity and Weight Loss in a Transgenic Mouse Model of Obesity.....	140
2.1 Abstract.....	141
2.2 Introduction.....	142
2.3 Materials and Methods.....	144

2.3.1 Animals and Diets.....	144
2.3.2 Core Temperature.....	146
2.3.3 Adipocyte Intracellular Ca ²⁺ ([Ca ²⁺] _i) Measurement.....	147
2.3.4 Fatty Acid Synthase (FAS) Activity Assay.....	148
2.3.5 Lipolysis Assay.....	148
2.3.6 Northern Blot Analysis.....	148
2.3.7 Statistical Analysis.....	149
2.4 Results.....	149
2.5 Discussion.....	159
Literature Cited	164

Part Three: Calcium and Dairy Inhibit of Weight and Fat Regain during *Ad Libitum* Feeding Following Energy Restriction in Ap2-Agouti Transgenic Mice.....170

3.1 Abstract.....	171
3.2 Introduction.....	172
3.3 Materials and Methods.....	174
3.3.1 Animals and Diets.....	174
3.3.2 Core Temperature.....	176
3.3.3 Adipocyte [Ca ²⁺] _i Measurement.....	177
3.3.4 Fatty Acid Synthase (FAS) Activity Assay.....	178
3.3.5 Lipolysis Assay.....	178
3.3.6 Total RNA Extraction.	178
3.3.7 Quantitative Real-time PCR.....	179
3.3.8 Carnitine Palmitoyltransferase (CPT) Assay.....	180
3.3.9 Statistical Analysis	181
3.4 Results.....	181

3.5 Discussion.....	191
Literature Cited	201
Part Four: Effects of Mitochondrial Uncoupling on Adipocyte Intracellular Ca ²⁺ and Lipid Metabolism.....	208
4.1 Abstract.....	209
4.2 Introduction.....	210
4.3 Materials and Methods.....	211
4.3.1 Culture and Differentiation of 3T3-L1 Adipocytes.....	211
4.3.2 Fatty Acid Synthase (FAS) Activity Assay.....	211
4.3.3 Lipolysis Assay.....	212
4.3.4 [Ca ²⁺] _i Measurement.....	212
4.3.5 Total RNA Extraction	213
4.3.6 Quantitative Real-time PCR.....	214
4.3.7 Statistical Analysis.....	214
4.4 Results.....	215
4.5 Discussion.....	224
Literature Cited	229
Part Five: Role of Uncoupling Protein 2 (UCP2) Expression and 1 α , 25 Dihydroxyvitamin D ₃ (1 α , 25-(OH) ₂ -D ₃) in Modulating Adipocyte Apoptosis.....	235
5.1 Abstract.....	236
5.2 Introduction.....	237

5.3 Materials and Methods.....	239
5.3.1 Culture and Differentiation of 3T3-L1 Adipocytes.....	239
5.3.2 UCP2 Transfection.....	240
5.3.3 Determination of Mitochondrial Membrane Potential.....	240
5.3.4 ATP Measurement.....	241
5.3.5 Measurement of Cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) and Mitochondrial Calcium ($[\text{Ca}^{2+}]_m$).....	241
5.3.6 Total RNA Extraction.....	243
5.3.7 Quantitative Real-time PCR.....	243
5.3.8 Animals and Diets.....	244
5.3.9 Statistical Analysis.....	246
5.4 Results.....	246
5.5 Discussion.....	259
Literature Cited	266

Part Six: Mechanisms of Dual Effects of 1α , 25-Dihydroxyvitamin D_3 on Adipocyte Apoptosis.....273

6.1 Abstract.....	274
6.2 Introduction.....	275
6.3 Materials and Methods.....	276
6.3.1 Culture and Differentiation of 3T3-L1 Adipocytes.....	276
6.3.2 UCP2 Transfection.....	276
6.3.3 siRNA Preparation and Transfections.	277
6.3.4 Western Blot Analysis.....	278
6.3.5 Determination of Mitochondrial Membrane Potential.....	278
6.3.6 Measurement of Cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) and Mitochondrial Calcium ($[\text{Ca}^{2+}]_m$).....	279
6.3.7 Total RNA Extraction.	280

6.3.8 Quantitative Real-time PCR.....	280
6.3.9 Statistical Analysis.....	281
6.4 Results.....	281
6.5 Discussion.....	291
Literature Cited.....	297
 Part Seven: Summary and Conclusion.....	 301
 Vita.....	 303

List of Table

Table	Page
Table 3-1 Effect of dietary calcium and dairy refeeding on serum insulin, leptin and glucose concentrations of aP2-agouti transgenic mice.	194

List of Figures

Figures		Pages
Figure 2-1	Effects of high calcium diets on weight gain in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	150
Figure 2-2	Effects of high calcium diets on fat pad weight (sum of four fat fads) in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	151
Figure 2-3	Effects of high calcium diets on adipocyte $[Ca^{2+}]_i$ in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	152

Figure 2-4	High calcium diets accelerate body weight loss in energy restricted aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	154
Figure 2-5	Effects of high calcium diets on fat pad mass (sum of four fat pads) in energy restricted aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	155
Figure 2-6	Effects of high calcium diets on fatty acid synthase activity in energy restricted aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	156
Figure 2-7	Effects of high calcium diets on fatty acid synthase mRNA in energy restricted aP2-agouti transgenic mice. Data are expressed as fatty acid synthase (FAS): actin ratio. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	157

Figure 2-8	Effects of high calcium diets on basal lipolysis in energy restricted aP2-agouti transgenic mice. Glycerol release was used to measure lipolysis, as described in Material and Methods. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	158
Figure 2-9	Effects of high calcium diets on core temperature in energy restricted aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	160
Figure 3-1	Effect of dietary calcium and dairy on weight and fat mass regain in aP2 transgenic mice. (A) body weight and (B) fat pads weight. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	182
Figure 3-2	Effect of dietary calcium and dairy refeeding on lipid metabolism in aP2 transgenic mice. (A) adipose basal lipolysis (B) FAS expression. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	184

Figure 3-3	Effect of dietary calcium and dairy refeeding on adipocyte intracellular calcium in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p<0.05$.	185
Figure 3-4	Effect of dietary calcium and dairy refeeding on liver CPT activity in aP2 transgenic mice. Data are expressed as mean $\pm$ SE (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p<0.05$.	187
Figure 3-5	Effect of dietary calcium and dairy refeeding on adipose UCP2 expression in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p<0.05$.	188
Figure 3-6	Effect of dietary calcium and dairy refeeding on UCP3 expression in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p<0.05$.	189
Figure 3-7	Effect of dietary calcium and dairy refeeding on core temperature in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p<0.05$.	190

Figure 3-8	Effect of dietary calcium and dairy refeeding on PPARα expression in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of p<0.05.	192
Figure 3-9	Effect of dietary calcium and dairy refeeding on PPARγ expression in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of p<0.05.	193
Figure 4-1	Effect of DNP on intracellular calcium in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with or without DNP (25 μ M, 50 μ M), DNP plus KCl (40mM) or DNP plus lanthanum (1mM). Data are expressed as mean $\pm$ SE (n=12). Difference letters above the bars indicate a significant difference at level of p<0.05.	216
Figure 4-2	Effect of FCCP on intracellular calcium in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with or without FCCP (1 μ M, 5 μ M), FCCP plus KCl (40mM) or FCCP plus lanthanum (1mM). Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of p<0.05.	217

Figure 4-3 Effect of DNP on lipolysis in 3T3-L1 adipocytes.

Differentiated 3T3-L1 adipocytes were treated with or without DNP (25 μ M), DNP plus insulin (30nM), DNP plus isoproterenol (50nM) or DNP plus lanthanum (1mM). Lipolysis was determined as glycerol release by enzymatic fluorescence method, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

218

Figure 4-4 Effect of FCCP on lipolysis in 3T3-L1 adipocytes.

Differentiated 3T3-L1 adipocytes were treated with or without FCCP (1 μ M, 5 μ M), FCCP plus insulin (30nM), FCCP plus isoproterenol (50nM) or FCCP plus lanthanum (1mM). Lipolysis was determined as glycerol release by enzymatic fluorescence method, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

219

Figure 4-5 Effect of DNP on fatty acid synthase (FAS) activity in 3T3-L1 adipocytes.

Differentiated 3T3-L1 adipocytes were treated with or without DNP (25 μ M), Insulin (30nM), Lanthanum (1mM), DNP plus insulin or DNP plus lanthanum. FAS activity was measured as the oxidative rate of NADPH, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

220

- Figure 4-6 Effect of FCCP on fatty acid synthase (FAS) activity in 3T3-L1 adipocytes.** Differentiated 3T3-L1 adipocytes were treated with or without FCCP (1 μ M, 5 μ M), Insulin (30nM), Lanthanum (1mM), FCCP plus insulin or FCCP plus lanthanum. FAS activity was measured as the oxidative rate of NADPH, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of $p<0.05$. 221
- Figure 4-7 Effect of DNP on fatty acid synthase (FAS) expression in 3T3-L1 adipocytes.** Differentiated 3T3-L1 adipocytes were treated with or without DNP (25 μ M), Insulin (30nM), Lanthanum (1mM), DNP plus insulin or DNP plus lanthanum. FAS mRNA was measured by real time PCR, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Difference letters above the bars indicate a significant difference at level of $p<0.05$. 222
- Figure 4-8 Effect of DNP on fatty acid synthase (FAS) activity in 3T3-L1 adipocytes.** Differentiated 3T3-L1 adipocytes were treated with or without DNP (25 μ M), ATP (10nM), Lanthanum (1mM), DNP plus ATP, DNP plus lanthanum or Lanthanum plus ATP. FAS activity was measured as the oxidative rate of NADPH, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Difference letters above the bars indicate a significant difference at level of $p<0.05$. 223

- Figure 4-9** **A proposed scheme for the integration of the regulation of lipid metabolism by mitochondrial uncoupling.** Mitochondrial uncoupling increases $[Ca^{2+}]_i$ by activating membrane calcium channels and thereby inhibits lipolysis via a Ca^{2+} -dependent pathway. Mitochondrial uncoupling has dual effects on lipogenesis: it inhibits FAS activity via a Ca^{2+} -independent mechanism and stimulates of FAS expression via a Ca^{2+} -dependent metabolism. 228
- Figure 5-1** **Caspase-1 expression in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes.** Primary-cultured adipocytes were treated with or without $1\alpha, 25-(OH)_2-D_3$ (0.1nM, 1nM, 5Nm, 10nM, and 100nM) and DNP (25 μ M and 50 μ M) for 24 h for mRNA levels. Caspase-1 mRNA levels were measured by real-time PCR and these measurements were conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$. 247
- Figure 5-2** **Caspase-3 expression in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes.** Primary-cultured adipocytes were treated with or without $1\alpha, 25-(OH)_2-D_3$ (0.1nM, 1nM, 5Nm, 10nM, and 100nM) and DNP (25 μ M and 50 μ M) for 24 h for mRNA levels. Caspase-1 mRNA levels were measured by real-time PCR and these measurements were conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$. 248

- Figure 5-3** **Bcl-2/Bax expression ratio in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes.** Primary-cultured adipocytes were treated with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (0.1nM, 1nM, 10nM, and 100nM) and DNP (25 μ M and 50 μ M) for 24 h for mRNA levels. Caspase-1 mRNA levels were measured by real-time PCR and these measurements were conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p<0.05$. 250
- Figure 5-4** **Effect of dietary calcium on caspase-1 expression white adipose tissue of aP2-agouti transgenic mice.** Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Difference letters above the bars indicate a significant difference at level of $p<0.05$. 251
- Figure 5-5** **Effect of dietary calcium on caspase-3 expression white adipose tissue of aP2-agouti transgenic mice.** Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Difference letters above the bars indicate a significant difference at level of $p<0.05$. 252
- Figure 5-6** **Effect of dietary calcium on Bcl-2/Bax expression in white adipose tissue of aP2-agouti transgenic mice.** Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Difference letters above the bars indicate a significant difference at level of $p<0.05$. 253

- Figure 5-7 Mitochondrial potential in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes.** Primary-cultured adipocytes were treated with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (0.1nM, 1nM, 5nM, 10nM, and 100nM) for 4h before assay. Mitochondrial potential were measured using fluorospectrometry as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p<0.05$. 255
- Figure 5-8 ATP production in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes.** Primary-cultured adipocytes were treated with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (1nM, 5nM, 10nM, and 100nM) for 4h before assay. ATP levels were measured using a microplate luminometer as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p<0.05$. 256
- Figure 5-9 Intracellular calcium levels in 3T3-L1 and UCP2 transfected 3T3 L1 adipocytes.** Primary-cultured adipocytes were treated with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (1nM, 5nM, 10nM, and 100nM) instantly before assay. Intercellular calcium levels were measured using a dual wavelength fluorescence microscope as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p<0.05$. 257

- Figure 5-10 Mitochondrial calcium levels in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes.** Primary-cultured adipocytes were treated with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (1nM, 5nM, 10nM, and 100nM) instantly before assay. Mitochondrial calcium levels were measured using a dual wavelength fluorescence microscope as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). 258
- Figure 5-11 Schematic illustration of effects and mechanisms of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on adipocyte apoptosis.** Physiological low doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ restore mitochondrial potential and ATP production by suppressing UCP2, thereby inhibiting apoptosis. By contrast, high dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ induces mitochondrial calcium overload and stimulates apoptosis. 264
- Figure 6-1 Western blot with anti-mouse UCP2 antibody of cell lysates harvested 48hrs after siRNA transfection for evaluation of UCP2 protein reduction (upper panel).** The internal control actin is shown in the low panel. Blot shown is representative of three similar experiments. 282
- Figure 6-2 Mitochondrial potential in 3T3-L1 and siRNA transfected 3T3-L1 adipocytes (UCP2 ko).** Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$. 283
- Figure 6-3 Caspase-3 expression in 3T3-L1 and siRNA transfected 3T3-L1 adipocytes (UCP2 ko).** Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$. 285

Figure 6-4	Effect of mitochondrial uncoupling inhibitor GDP on mitochondrial potential in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	286
Figure 6-5	Effect of mitochondrial uncoupling inhibitor GDP on caspase-3 expression in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	287
Figure 6-6	Effect of calcium channel ionophore Bay K 8644 on intracellular calcium levels in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	288
Figure 6-7	Effect of mitochondrial uncoupling inhibitor GDP and calcium channel ionophore Bay K 8644 on mitochondrial potential in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	289
Figure 6-8	Effect of calcium channel ionophore Bay K 8644 on caspase-3 expression in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	290
Figure 6-9	Effect of mitochondrial uncoupling inhibitor GDP and calcium channel ionophore Bay K 8644 on caspase-3 expression in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.	292

Figure 6-10 **Effect of calcium channel ionophore Bay K 8644 on**
mitochondrial calcium in 3T3-L1 adipocytes. Data are 293
expressed as mean $\pm$ SE (n =6).

Part One

Literature Review

1.1 Introduction

The worldwide prevalence of obesity is increasing at an alarming rate, with major adverse consequences for human health (1-2). Obesity results from a chronic imbalance between energy intake and expenditure (3-4). This imbalance can result from either overeating or a constitutional decrease in one or several of the components of energy expenditure, including resting metabolic rate, diet-induced thermogenesis, or exercise-related thermogenesis. Methods to control obesity through limiting energy intake have had modest success (5). In addition, metabolic rate is decreased with extreme calorie restriction. This decrease persists after the dieting period has ended, often leading to rapid weight regain (6). It is widely recognized that energy expenditure must be increased if long-term weight loss is to be achieved.

Uncoupling proteins (UCPs) are a family of integral membrane proteins of mitochondrial inner membrane, where they uncouple the process of mitochondrial respiration from oxidative phosphorylation, diminishing the resulting production of ATP and decreasing the metabolic efficiency of the organism (7-8). Thus, UCPs provide new molecular targets for increasing energy expenditure. It is widely acknowledged that the brown adipose tissue (BAT) mitochondrial uncoupling protein (UCP1) plays a pivotal role in adaptive thermogenic responses (9). Two homologues of UCP1 (UCP2 and UCP3) have recently been identified and population-based genetic studies have linked them with basal metabolic rate, while *in vitro* studies report that both have proton transport activity and may thus be involved in regulation of energy homeostasis and hence obesity (10). The function of UCPs may not be restricted to thermoregulation and there is now

substantial data linking UCP expression levels to fatty acid metabolism and fatty acid flux into various tissues (11). One hypothesis indicates that UCP2 and UCP3 may function as a free fatty acid transporter and thereby increase fatty acid utilization. Unlike the other family members, UCP2 is ubiquitously expressed, with the highest level in white adipose tissue. Lipid metabolism of adipose tissue is affected by the energy status of fat cells (12). Previous experiments indicated the dependence of both lipogenesis and lipolysis on ATP levels in adipocytes (13). Thus, UCP-induced respiratory uncoupling in adipocytes that results in stimulation of energy dissipation and depression of ATP synthesis may contribute to the control of lipid metabolism. This hypothesis, however, requires further investigation.

Previous studies of the agouti gene demonstrate a key role for intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in metabolic disorders associated with obesity by regulating adipocyte lipid metabolism and triglyceride storage(14). Increased $[\text{Ca}^{2+}]_i$ results in stimulation of lipogenic gene expression and lipogenesis and suppression of lipolysis, resulting in adipocyte lipid filling and increased adiposity. $[\text{Ca}^{2+}]_i$ can clearly be modulated by the calcitrophic hormone $1\alpha, 25\text{-dihydroxyvitamin D}_3$ ($1\alpha, 25\text{-(OH)}_2\text{-D}_3$), which appears to have both genomic and non-genomic action in adipocytes. $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ modulates adipocyte Ca^{2+} signaling via a non-genomic action, resulting in increased lipogenesis and decreased lipolysis (15-17). In addition, $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ plays a genomic role in regulating adipocyte UCP2 expression levels, indicating that the regulation of UCP2 and the resulting increased core temperature may contribute to increased rates of energy dissipation (18). Accordingly, we recently demonstrated that the increased $1\alpha, 25\text{-(OH)}_2\text{-$

D₃ produced in response to low-calcium diets stimulates adipocyte Ca²⁺ influx and, consequently, promotes adiposity. In contrast, the suppression of 1 α , 25-(OH)₂-D₃ by increasing dietary calcium attenuates adiposity by decreasing triglyceride accumulation in the adipocytes, resulting in a net reduction in fat mass in the absence of caloric restriction, a marked augmentation of body weight and fat loss during energy restriction (15-17). However, a key problem in the management of obesity is the maintenance of reduced body weight and fat content following successful weight loss. How an individual responds to changes of energy and nutrient status from fasting to refeeding may have important long-term consequences with respect to the retention of body fat. Thus, the effects of dietary calcium on prevention of weight regain needs to be explored.

Although the anti-obesity effects of dietary calcium are due, in part, to an enhanced metabolic rate and thermogenic processes, it is also possible that a loss of adipocytes would result in a deficit cells for lipid esterification as the body recovers from energy restriction. Clinically, the duration of maintaining at a lower body weight after dietary treatment of obese human seemed to be inversely proportional to the estimated number of adipocytes in adipose tissue (19-20). Thus, there seems to be some association with the severity of the obesity and with the cellular characteristics of adipose tissue. Recent data indicate that [Ca²⁺]_i may play a key role in modulating sensitivity to the apoptotic stress (21). Several of the functional groups of molecules involved in apoptosis, including caspases and the Bcl-2 family, are [Ca²⁺]_i responsive (22-23). Given that [Ca²⁺]_i can be modulated by 1 α , 25-(OH)₂-D₃, the role of 1 α , 25-(OH)₂-D₃ in apoptosis should be explored. In addition, UCP2 has been demonstrated to function as

mitochondrial uncoupler of oxidative phosphorylation and thus reduce efficiency of ATP synthesis. Consequently, it is reasonable to propose that UCP2 may stimulate apoptosis in adipocytes. We have previously demonstrated that $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibits UCP2 expression in human adipocyte via a genomic mechanism (18), further implying that $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ may play a role in adipocyte apoptosis. Although high doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ have been reported to induce apoptosis in various tumor cells, the regulation of apoptosis by physiological doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ remains unclear.

Accordingly, present studies expanded our previous observations by broadly exploring the effect of dietary calcium on lipid metabolism and energy partitioning under different nutritional status. I also investigated the direct effect and mechanisms of mitochondrial uncoupling on lipogenesis and lipolysis as well as the role of $[\text{Ca}^{2+}]_i$ in this process. Finally, the effect and mechanism of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ and UCP2 on adipocyte apoptosis were investigated in both *in vivo* and *in vitro* environments.

1.2 Obesity: General Aspects

1.2.1 Prevalence of Obesity

The prevalence of obesity in the United States has grown substantially over the past decade. Currently, approximately 20% of males and 25% females are considered obese based on a body mass index (BMI) of 30 or more. (24-28). Three percent of U.S. adults have a BMI of 40 or more, equivalent to approximately 100 lbs overweight (24-26). In 2000, approximately 44 million adults in the U.S. were overweight or obese, reflecting

an increase of 74 percent since 1991 (27). Overweight and obesity are significant and growing problems in children as well. Over the past decade or so, the proportion of children and adolescents who are overweight or obese in has increased significantly. Results from the 1999-2000 National Health and Nutrition Examination Survey (NHANES) (28), using measured heights and weights, indicate that an estimated 15 percent of children and adolescents ages 6-19 years are overweight, representing a 40 percent increase from the overweight estimates of 11 percent obtained from NHANES III (1988-94) (29-30).

Overweight and obese individuals (BMI of 25 and above) are at increased risk for physical ailments such as type II diabetes mellitus, insulin resistance/glucose intolerance, heart disease and stroke, hypertension, cancer, obstructive sleep apnea, osteoarthritis, and psychological disorders (31-32). Using relative hazards associated with elevated BMI in six United States studies (Alameda County Health Study, Framingham Heart Study, Tecumseh Community Health Study, American Cancer Society Cancer protection Study 1, and Nurses Health Study,) the national distribution of adult BMI, and the estimates of population size and total deaths from the same period, Mokdad *et al* calculated that the annual number of deaths attributable to overweight in 2000 was over 490,000 (33). Although obesity was rarely listed as the cause of death, it is now more frequently noted as the associated comorbidity.

Overweight and obesity and their associated health problems have substantial economic consequences for the U.S. health care system (34). The increasing prevalence of overweight and obesity is associated with both direct and indirect costs. Direct health

care costs refer to preventive, diagnostic, and treatment services related to overweight and obesity (for example, physician visits and hospital and nursing home care). Indirect costs refer to the value of wages lost by people unable to work because of illness or disability, as well as the value of future earnings lost by premature death (35). In 1995, the total (direct and indirect) costs attributable to obesity amounted to an estimated \$99 billion (36-37). In 2000, the total cost of obesity was estimated to be \$117 billion (\$61 billion direct and \$56 billion indirect) (36-37). Most of the cost associated with obesity is due to type 2 diabetes, coronary heart disease, and hypertension (36-37).

1.2.2 Pathology of Obesity

1.2.2.1 Genetics of Obesity

Obesity is determined by genetic and environmental factors acting through the physiological mediators of energy intake and energy expenditure. It is likely that genes involved in weight gain increase the susceptibility of an individual to the development of obesity when exposed to environmental conditions that favor a positive energy balance (38).

Adoption, twin and family studies have shown that human obesity has a genetic component (39-41). Body weight, however, is the archetypal polygenic trait, a quantitative phenotype that usually fails to display a Mendelian pattern of inheritance because it is influenced by many different loci, and this complex etiology makes the search for obesity genes especially challenging (42). Until recently, the analysis of candidate genes (known genes identified a priori on the basis of their effects in animal

models or suspected physiological involvement in a particular disorder) was the primary strategy used in the search for potential obesity genes (43-44). The ninth annual human obesity gene map identifies more than 70 specific gene variants thought to cause a person to become obese (45), including leptin gene and its receptor, pro-opiomelanocortine (POMC), prohormone convertase 1 (PC1), mutations in the melanocortin receptor 4, and more recently, *lpin1*. Genome scanning is an alternative but complex approach by conducting linkage analysis using a series of anonymous polymorphisms over the entire genome (46). This approach offers the potential of identifying genes previously unsuspected of having an influence on the phenotype of interest. To date, the results of two genome scans for obesity-related phenotypes have been reported, one in Mexican Americans (47), and the other in Indians (48), but no obvious candidate genes have been mapped to either chromosomal region. Results from reported genome-wide linkage studies suggest that serum leptin levels are significant linked to chromosome 2p21 (49-51) and this region of human chromosome 2 includes the POMC gene (52), in which loss-of-function mutations have been demonstrated as rare Mendelian causes of obesity in humans. Other than the locus on 2p there are no other loci which have been highlighted in more than one of the genome wide scans reported to date.

1.2.2.2 Endocrine Disorders of Obesity

Obesity is associated with multiple alterations in the endocrine system, including abnormal circulating blood hormone concentrations, which can be due to changes in the

pattern of their secretion and/or metabolism, altered hormone transport and/or action at the level of target tissue. The following discussion focuses on cortisol, insulin and growth hormone.

a. Cortisol

Patients with Cushing's syndrome display a number of clinical features that resemble those seen in patients with the metabolic syndrome (syndrome X) (53), including abdominal obesity, insulin resistance, impaired glucose homeostasis, hypertension, and lipid abnormalities. These similarities led to the hypothesis that a dysregulation of the hypothalamic-pituitary adrenal (HPA) axis could potentially be a cause for abdominal obesity and its different metabolic consequences. Adipose tissue is involved in the metabolism of cortisol. The enzyme 11 beta-hydroxysteroid dehydrogenase-1 (11β -HSD-1), which converts cortisone to cortisol, is expressed in adipose tissue (54). It appears that in obesity more cortisol is derived from cortisone due to the increased activity of this enzyme, which could simply be due to increased fat mass (54). Urine studies in obesity also show an increase in the ratio of tetrahydrocortisol to tetrahydrocortisone indicating a relative increase in the pathways leading to cortisol formation (through the action of 11β -HSD-1) (55). Although the serum concentration of cortisol and the 24 hour urine free cortisol excretion are essentially normal or slightly elevated in obesity,, it is possible that there is an increase in the local production of cortisol in the fat tissue and this in turn could lead to increased local action of cortisol with the subsequent metabolic consequences known to occur in obesity. The cortisol response to a variety of stimuli such as corticotropin releasing hormone (CRH), or meal

ingestion is increased in obesity (56). An increase in cortisol response to ACTH has also been seen in obese men and obese premenopausal women, but not in obese postmenopausal women (57), indicating a possible role for estrogens in that response. A decrease in the mineralocorticoid receptor response to circulating corticosteroids was suggested by Jessop *et al* as an explanation for the decreased HPA axis to glucocorticoid feedback in obesity (58).

b. Insulin

Obesity is strongly associated with insulin resistance and is a risk factor for type 2 diabetes (59). Visceral abdominal fat is inversely correlated with insulin sensitivity, and visceral hepatic glucose production and reduced peripheral glucose uptake are commonly seen in abdominal obesity (60-61). Insulin levels are elevated in obese individuals and a high correlation exists between insulin secretion and BMI (62). The hyperinsulinemia seen in obese subjects is mainly due to increased insulin secretion. In some patients, decreased insulin clearance can also contribute to the elevated insulin levels. When impaired glucose tolerance develops, the insulin secretory dynamics are altered with a progressive decrease in the ability of the pancreas to maintain normal glucose homeostasis, initially in response to glucose load, and ultimately in the basal state.

The exact mechanism(s) by which obesity contributes to the development of insulin resistance is the subject of extensive research. In human insulin resistance itself and hyperinsulinemia can precede the development of obesity (63). Substances originating in the adipose tissue and capable of exerting distant or local actions may

explain the link between obesity and insulin resistance. Free fatty acids (FFA) are released in excess by abdominal fat (64-66). Increased concentrations of FFA cause insulin resistance and decreased glucose uptake by impairing insulin mediated glucose transport. Impairment at the level of the insulin receptor and insulin receptor substrate-1 (IRS-1) are likely reasons for the impaired insulin mediated glucose uptake (67). Tumor necrosis factor-alpha (TNF- α) is another potential link between obesity and insulin resistance (68-69). TNF- α has been shown to alter basal and glucose stimulated insulin secretion and to produce insulin resistance in isolated cell lines (70-71). Similar conclusions can be drawn in regard to leptin. Leptin *in vitro* is capable of inhibiting insulin release but its role in the pathogenesis of insulin resistance in humans has not been confirmed (72). Resistin, a peptide isolated from the mouse, is produced by the adipose tissue and its expression is decreased by peroxisome proliferator-activated receptor gamma (PPAR γ) agonists (73-74). Serum levels of resistin are elevated in obese mice and immunoneutralization of this peptide improves insulin sensitivity in the mouse. mRNA levels of resistin in freshly isolated human adipocytes are very low but the role of resistin in human obesity and insulin resistance is unclear at this point. Ghrelin, a potent growth hormone secretagogue, is produced mainly by the stomach. Administration of ghrelin results in increased growth hormone (GH) release, hyperglycemia, and decrease insulin concentrations (75). Tschöp *et al*, found a negative correlation of circulating ghrelin levels with insulin, leptin levels, and percent body fat (76). Thus, Ghrelin is yet another peptide which may turn out to have a role in the pathogenesis of insulin resistance accompanying obesity.

c. Growth Hormone

Obesity is typically accompanied by a decrease in GH levels and increase in growth hormone binding protein (GHBP) levels (77-78). An inverse relation exists between GH levels and percent fat mass. Another major determinant of GH level is age; GH levels fall with increasing age and recombinant human growth hormone, rhGH, has been studied as a supplement to counteract the effects of aging on body composition (79). The reduction in GH levels in obesity is believed to result from decreased pituitary release of the hormone. In animal models of genetic obesity there is a decrease in the number of somatotroph cells in the pituitary gland. GH responses to a variety of stimuli, including GHRH, arginine, and GHRPs (growth hormone releasing peptides) have been shown to be reduced in obesity (80). Similarly it has been found that GHBP levels are increased in obesity, suggesting a negative feedback mechanism on GH secretion. Other possible mechanisms included in the altered GH response in obesity are FFA and leptin, both of which are increased in obesity (81-82). Lee *et al*, showed that reduction in free fatty acids concentrations in obese subjects through use of Acipimox leads to increased GH response to GH-releasing hormone. In animals, leptin has an inhibitory role on GH secretion from the pituitary gland through its effects on GHRH and neuropeptide Y (NPY) at the hypothalamus level.

Most of GH promoting effects are mediated by insulin- like growth factor-1 (IGF-1). GH also has effects independent of IGF-1. Serum IGF-1 concentration represents the most accurate reflection of growth hormone biologic activity (83). In spite of the reduced GH levels seen in obesity, IGF-1 levels do not appear to be significantly different

between obese and non obese subjects (84). Individuals with visceral obesity, even in the absence of total body obesity, may, on the other hand, have lower IGF-1 levels than comparable individuals with no abdominal obesity. The serum concentration of unbound or free IGF-1 is increased in obese subjects (85). Similarly it has been found that GHBP levels are increased in obesity. This suggests that lower levels of GH are accompanied by increased peripheral sensitivity to GH accounting for the normal IGF-1 levels. Insulin could result in increased peripheral sensitivity to GH, reduced IGFBP-1 levels and increased (to within normal range) IGF-1 in spite of decreased GH secretion by the somatotroph (86). High free IGF-1 levels in this case exert a negative feedback mechanism on GH secretion. This hypothesis supports a peripheral rather than a central mechanism by which GH release is decreased in obesity.

1.2.3 Dietary Management of Obesity

Obesity results from the accumulation of excessive calories as body fat. Accordingly, the goal for dietary management of obesity is to reduce calorie intake from diet. In addition, once the obese individual has lost body fat, this healthier weight must be maintained. Thus the energy deficit phase must be followed by a weight maintenance phase. Paradoxically, the slimmer individual's energy needs are lower to stay in energy balance than they were prior to weight reduction (87-89). The primary reason for this is that some loss of lean as well as fat tissue inevitably occurs on reducing diets. Thus metabolically active tissue is reduced and resting metabolism is decreased. Also, it takes less effort to move the lighter body, and the energy cost of physical activity is thus reduced. The implications are that a slight decrease in energy intake from prior levels and

an increase in energy output will be necessary during weight maintenance. Thus there is a need for continued attention to these factors on the part of the physician and patient. All too often the weight maintenance phase is neglected or ignored, and weight is regained over the long term. Although the macronutrient composition of the diet does not appear to play a major role in overall weight loss, there is much interest in the macronutrient composition of reducing diets, as discussed in the next section (90).

1.2.3.1 Dietary Fat

Balanced deficit weight reduction diets that are moderate to low in fat (20-30% calories) are so called because maintain a reasonable balance between macronutrients similar to that recommended in the Food Group Pyramid and the Dietary Guidelines for Americans (91). The introduction of low-fat diets (<30% fat calories) for the prevention and treatment of obesity was based on the link established between dietary fat and body fatness. Observational and mechanistic studies show that because fat possesses a lower satiating power than carbohydrate and protein, a diet rich in fat can increase energy intake (92-93). The propensity to gain weight is enhanced in susceptible persons, particularly sedentary people who have a genetic predisposition to obesity. On the other hand, empirical evidence from dietary intervention trial shows that increase in carbohydrate: fat ratio achieved by low fat intervention is effective in producing spontaneous weight loss (94). Meta-analyses of weight change occurring on *ad libitum* low-fat diets in intervention trials consistently demonstrate a highly significant weight loss of in normal-weight and overweight subjects (95). Very low fat diets (<10% fat calories) such as the Pritikin diet (96) and the Ornish diet (97), are advocated not only for

weight reduction but also for improving cardiovascular risk profiles. The Ornish diet, which is very low in fat (13% of calories) and saturated fat, very high in carbohydrate (81% of calories) and very high in fiber (38 gm) with a program that includes nonsmoking, exercise and behavior modification was shown to reduce some cardiovascular risk factors in a limited long term study. However, whether the percentage of dietary fat in diet plays an important role in the rising prevalence of overweight and its treatment has been repeatedly debated in recent years. Willett has argued that there is no evidence that energy density has an important role on long-term weight control and thus that the importance of fat-restriction in dietary treatment is unproven (98).

Even on reducing diets, needs for small amounts (e.g. 3-6 gm) of essential fatty acid (linoleic or arachidonic acid) are present, and some fat is also necessary as a carrier for the fat-soluble vitamins (99). Therefore the diet should not be devoid of fat. However, because fat is calorically dense, it is usually decreased to increase bulk and reduce energy.

High fat reducing diets (55-65%) are usually also low or very low in carbohydrate (<200 gm carbohydrate per day) (100). There is some evidence that free-living overweight people who self-select high fat low carbohydrate diets which they eat *ad libitum* consume fewer calories and lose weight. Also overweight people who consume such diets under experimental conditions lose weight. When high fat low carbohydrate reducing diets are fed they also tend to cause ketosis. They may also result in decreased blood lipids, decreased blood glucose and insulin and decreased blood pressure, but only if weight is lost. Over the short term (a few days or a week) high fat, low carbohydrate,

ketogenic diets cause a greater loss of body water than body fat, but water balance is restored when carbohydrate levels increase or when the diet ends (101).

1.2.3.2. Dietary Carbohydrate

Carbohydrate needs are at least 50 gm per day. At least 100 gm carbohydrate, and preferably 55% or more of total energy intake should be provided for diets that are over 800 kcal per day (91). On reducing diets under experimental conditions with equicaloric reducing diets, both diets very high in sugars and diets very high in complex carbohydrates as starch have similar effects in bringing about weight loss (102). However, from the practical standpoint, since many products that are high in sugar are also high in fat and calories, sugars are usually limited on reducing diets.

Low-carbohydrate diets (<100mg carbohydrate) may be accompanied by greater weight loss than other diets and this concept is supported by recent evidence. A recent comparison of a low carbohydrate diet with a low fat diet in patients with severe obesity found that patients on the low carbohydrate diet lost more weight than patients on a calorie-restricted, fat-restricted diet, and had greater improvement in insulin sensitivity and triglyceride levels (103). A second study also found a similarly greater weight loss using a low carbohydrate diet without adverse effects on lipids, glucose, or insulin (104). Both diets are associated with reduced caloric consumption. Furthermore, Foster *et al* compared a low-carbohydrate, high-protein, high-fat diet with a low-calorie, high-carbohydrate, low-fat (conventional) diet in a recent clinical trial and they demonstrated that the low-carbohydrate diet produced a greater weight loss than did the conventional

diet for the first six months (105). In addition, the low-carbohydrate diet was associated with a greater improvement in some risk factors for coronary heart disease. However, these studies were conducted in a relatively short period of time and longer and larger studies are required to determine the long-term safety and efficiency of low carbohydrate diets.

Notably, the effect of low carbohydrate diets in promoting weight loss may be partially attributable to the loss of water. Glycogen is hydrated with 2 to 3 grams of water per gram of glycogen, while fat stores contain only 0.5 grams of water per gram of fat. Thus, high fat, low-carbohydrate diets deplete muscle and liver glycogen stores rapidly and facilitate rapid weight (water) loss (101). Also, some low carbohydrate diets are ketogenic and may lead to excessive protein breakdown to maintain blood glucose levels unless protein intakes are increased. When the body must rely on degradation of protein's carbon skeletons to preserve blood glucose levels (via gluconeogenesis), the catabolism of the protein is accompanied by loss of water. For every gram of protein (or glycogen) that is broken down, 3 grams of water are released, causing rapid weight loss but also a state of relative dehydration. This rapid weight loss may contribute to the popularity of very low carbohydrate diets. Furthermore, initial weight loss on low carbohydrate diets may be enhanced by an exaggerated diuresis related to decreased postprandial serum insulin levels. Insulin promotes water retention through its antinatriuretic effects. Some behavioral impact also contributes to the effect of weight loss. When individuals commit themselves to a weight-loss program, they change usual patterns of eating and commit themselves to paying attention to what they eat and avoiding unplanned eating episodes,

and thereby decrease energy intake. Furthermore, although such diets produce rapid weight loss due to polyuria in the short run, refeeding carbohydrates causes water retention and weight gain (101). The effect low carbohydrate diets on blood cholesterol remains controversial. A study conducted by LaRosa and colleagues examined the effect of lipid changes after following the Atkins low-carbohydrate, high-fat diet for four weeks (106). Despite a weight loss, total cholesterol insignificantly increased. Significant changes in LDL cholesterol triglycerides, free fatty acids and uric acid were seen. However, some evidence exhibited an opposite effect (107-108), with low carbohydrate diets decreasing blood cholesterol.

The glycemic index (GI) is a dietary concept, originally developed for the therapy of diabetes, which has recently become popular in weight management. The GI is a property that describes the blood glucose response resulting from consumption of a defined amount of carbohydrate (usually 50 grams) from a given food, relative to the same amount of carbohydrate from a control food (usually white bread) (109). The basic premise is that more moderate blood glucose and metabolic responses will sustain satiety and energy balance to a greater extent than would larger metabolic shifts over the course of the day (110). Physiological studies demonstrate that consumption of high GI meals induce a sequence of hormonal changes that limit availability of metabolic fuels in the postprandial period and cause overeating. Short-term feeding studies consistently show less satiety or greater voluntary energy intake after consumption of high compared to low GI meals (111). However, it is still unclear if the glycemic index offers sustained

advantages to patients in planning menus and in learning to control food intake compared to other weight reduction methods.

1.2.3.3. Dietary Protein

The Recommended Dietary Allowance for protein is 0.8 gm/kg/day, but most Americans eat approximately 15% of their total caloric intake from protein, or about 1.2 gm/kg per day (91). Protein requirements do not decline and may actually rise on hypocaloric diets, and especially on low carbohydrate diets, when protein needs sharply increase above the levels needed for energy balance. When energy intakes are insufficient, some amino acids are used to maintain blood glucose levels and others must be used for energy, so overall protein requirements increase (112). High protein reducing diets are those that provide more than 1.6 gm/kg/ideal body weight per day. Self-prescribed high protein reducing diets varying in their composition from about 28-65% of energy, providing 71-162 gm of protein per day, are currently popular as a strategy for losing weight. They are usually quite low in their carbohydrate content (113). High protein diets have been demonstrated to induce successful weight loss and other beneficial effects (114-115).

There is emerging evidence that additional metabolic roles for some amino acids require plasma intracellular levels above minimum needs for protein synthesis. Branched chain amino acids (leucine, isoleucine, and valine), or BCAA's, are a group of essential amino acids that play important roles in protein synthesis and energy production. In humans, about 15-25% of total protein intake is BCAA's, and dairy products are

particularly high in them (116). BCAA's make up 35-40% of the essential amino acids in body protein and 14% of the total amino acids in skeletal muscle (117). One of the most important BCAA's is leucine and estimates of dietary requirements for leucine range from 1-12 g daily (118). Leucine stimulates skeletal muscle protein synthesis through multiple independent mechanisms, including increasing insulin secretion as well as stimulating mammalian target of rapamycin (mTOR) pathways (119-120). However, the potential to participate in these additional metabolic processes of leucine is limited by its availability and the first priority being provided to new protein synthesis (121). Accordingly, only diets that provide BCAAs in general, and leucine specifically, at levels that exceed requirements for protein synthesis can fully support the intracellular leucine concentrations required to support additional signaling pathways (122-124). Consequently, the abundance of leucine in diet is of particular interest because it plays a distinct role in protein metabolism and plays a pivotal role in the translation initiation of protein synthesis.

However, whether a high protein diet is a safe approach for obesity management is questionable. Many of these diets advocate very high intakes of protein from meat and other foods that are also often high in saturated fat, cholesterol and sodium while they are low in dietary fiber, potassium, calcium, and magnesium, increasing cardiovascular risk (125). The purine content of meat, poultry, seafood, egg, seeds and nuts is high and increases uric acid levels and risk of gout in susceptible persons. The effect of dietary protein on calcium absorption remains controversial. Some studies demonstrate that high protein load may increase urinary calcium loss if it is not buffered (126-127) while

clinical trial conducted by Heaney suggests that dietary protein exerts no effect on calcium absorption (128).

1.2.3.4 Dietary Calories

Low calorie diets of 1000-1200 calories for women and 1200-1600 calories per day for men are currently recommended by the National Institutes of Health for weight loss in most individuals (129). The rationale is that on such regimens a deficit of approximately 500-1000 Calories per day will be created, which should result in slow, progressive weight loss of 1-2 pounds per week. The dietary composition is similar in macronutrients to that of the National Heart, Lung and Blood Institute's (NHLBI) Step 1 diet to decrease risks of high blood cholesterol and blood pressure. The National Institute of Health's Obesity Initiative recently sponsored an evidence-based review of low calorie diets. It found that on the average diets such as these reduced body weight by an average of 8% over 3-12 months of treatment, and that the losses were accompanied by decreases in abdominal fat (129).

1.3 Role of Intracellular Calcium in Obesity

1.3.1 Mouse Agouti and Obesity

Agouti originally referred to the wild-type pigmentation pattern of mouse hair, with a predominately black hair shaft with a subapical yellow segment (130). The mouse agouti gene, mapped to chromosome 2, encodes a 131 amino acid paracrine molecule that

is secreted from cells of the hair follicle (131-132). Agouti protein functions as a competitive antagonist of α -melanocyte-stimulating-hormone (α -MSH) binding to the melanocortin-1 receptor (MC-1R), causing melanocytes to switch from the synthesis of black pigment (eumelanin) to yellow pigment (phaeomelanin), which in turn results in the characteristic pigmentation pattern of wild-type mice (133). However, in the absence of α -MSH, agouti also affects basal melanogenesis of melanoma cells in culture, suggesting that agouti may have an additional physiological function (134). The human homolog of the agouti gene is 85% identical to the mouse gene and encodes a protein of 132 amino acids with a consensus signal peptide (135). The major difference between mouse and human agouti is the expression pattern. Whereas mouse agouti is only transiently expressed in the hair follicle during neonatal development, human agouti is expressed in diverse tissues; primarily adipose tissue followed by the testis, heart, liver, kidney, ovary, and foreskin.

Dominant mutations in the mouse agouti gene confer a pleiotropic syndrome characterized by obesity, mild hyperphagia, decreased thermogenesis, hyperinsulinemia, peripheral insulin resistance, impaired glucose tolerance, hyperglycemia, increased susceptibility to cancer and yellow hair (136). Molecular analysis of six dominant agouti alleles (A^y , A^{sy} , A^{vy} , A^{iapy} , A^{hvy} and A^{iy}) demonstrated that mutations in promoter regions result in ubiquitous expression of normal agouti protein (137-140). Expressing the wild-type agouti cDNA under the control of a ubiquitous promoter in mice resulted in the dominant obesity syndrome, indicating ectopic overexpression of the wild-type agouti gene is the cause of the obesity syndrome.

The actual mechanism whereby the ubiquitous expression of agouti causes yellow obese syndrome is not clear yet and both peripheral and central mechanism have been proposed.

Ectopic expression of agouti may antagonize signal transduction cascades that are mediated by melanocortin receptors present in tissues other than the skin (141). Agouti modulation of pigmentation is mediated by MC-1R (142), which is primarily expressed in melanocytes, leading to the hypothesis that melanocortin receptor antagonism may be responsible for the metabolic effects of agouti in yellow obese syndrome. Four other melanocortin receptors have been cloned: MC-2R is expressed in the adrenal cortex and adipose tissue (143); MC-3R is expressed in the hypothalamus and limbic systems in the brain (143), and in the placenta and gut; MC-4R is expressed throughout the brain (144), and MC-5R is ubiquitously expressed (145). To test the hypothesis that agouti protein may antagonize these other melanocortin receptors, the receptors were expressed in human embryonic kidney cells and the ability of recombinant agouti protein to antagonize alpha-melanocyte stimulating hormone (alpha-MSH) signaling was determined by assaying for adenylyl cyclase activity (133). These experiments demonstrated that the agouti protein is a high affinity antagonist of MC-1R, MC-2R and MC-4R, but not of MC-3R or MC-5R. However, only disruption of MC-4R results in an obesity syndrome that is markedly similar to that seen in mice with dominant agouti locus mutations, suggesting that chronic antagonism of MC-4R by the agouti protein may be a central mechanism of the agouti-induced obesity syndrome (146).

Recent studies demonstrate that melanocortin/MC-4R signaling play a key role in the regulation of food intake and energy homostasis (147). MC-4R is abundantly

expressed in hypothalamic paraventricular nucleus (PVN), dorsomedial nucleus and ventromedial nucleus, which are major hypothalamic regions involved in regulating feed behavior, body weight and energy homeostasis (148). The importance of the central melanocortin system in the regulation of energy balance is highlighted by studies in transgenic animals and humans with defects in this system. Mice with a targeted disruption of MC-4R exhibit an obesity syndrome that is remarkably similar to that seen in mice with dominant agouti mutations, suggesting that chronic antagonism of MC-4R by the agouti protein may be a central mechanism of the agouti-induced obesity syndrome (146). In addition, agonist of MC-4R was shown to inhibit feeding in *A^y/a* mice, whereas a melanocortin antagonist stimulated feeding (149). Furthermore, diet-induced obesity in mice causes a down regulation of MC-4R while food restriction up-regulates its expression (150). In humans, the obesity phenotype, including BMI, fat mass and body fat content was highly associated with restriction fragment polymorphisms of MC-4R in the Quebec family study (151). In addition, mutations in MC-4R gene have been identified in severely obese human subjects (152). These recent experiments strongly support a role for neural melanocortin receptor(s) in regulating nutrient intake and energy balance;

Although agouti is not normally expressed in the central nervous system, the melanocortin system may modulate energy homeostasis through the actions of two other endogenous antagonist agouti related protein (AGRP) and α -MSH, POMC cleavage product (153). POMC is expressed at only two locations in the brain: the arcuate nucleus of the hypothalamus (ARC) and the nucleus of the tractus solitarius (NTS) of the brainstem. AGRP has been identified primarily in the arcuate nucleus of the

hypothalamus of the rat and primate with neuronal projections to the paraventricular hypothalamic nucleus (154). AGRP exhibits high degree of homology to agouti, with the highest degree of identity in the cysteine-rich carboxyl terminus (155). The AGRP protein was demonstrated pharmacologically to competitively antagonize the MC-3R and MC-4R brain melanocortin receptors, and when ectopically expressed or Central Nervous System administration, resulted in an obese phenotype (156-157). AGRP is co-localized in NPY containing neurons, and when administered centrally to NPY-/- knockout mice, results in increased food intake (158). α -MSH is the endogenous ligand for MC-4R. Previous studies demonstrate that MC-4R agonists or antagonists suppressed or stimulated food intake, respectively. It has been shown that the blockade of MC-4R due to the ectopic expression of agouti protein or the overexpression of AGRP induces hyperphagia and obesity. The MC-4R and POMC knockout mice also express an obese phenotype (159). Thus, the importance of the melanocortin system in the regulation of feeding behavior and body weight is generally accepted.

Agouti also appears to exert significant peripheral effects by targeting ion channels, thereby causing an increase in intracellular free calcium ($[Ca^{2+}]_i$) in multiple peripheral tissues, particularly in adipose tissue and pancreatic β cells (160-161).

Agouti protein contains a cysteine-rich carboxyl terminus, with the number and spacing of cysteine residues are extremely similar to those of toxins from snails (ω -conotoxins) and spiders (plectoxins) that are known to function by targeting ion channels (162-163). This cysteine-rich terminus appears to have functional activity equivalent to the full-length agouti. Analysis of the three-dimensional structure formed by the C-terminus of the agouti protein also showed a high similarity to those toxins (164).

Furthermore, recombinant agouti protein stimulates a significant increase in $[Ca^{2+}]_i$ influx in skeletal muscle myocytes and adipocytes and this effect was not mediated by antagonizing MC-R, indicating a direct targeting of Ca^{2+} channel (160).

1.3.2 Intracellular Calcium and Obesity Related Metabolic Disorder

Agouti protein appears to stimulate lipogenesis in adipocytes (165). The mRNA levels of fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD), two key enzymes in *de novo* fatty acid synthesis and desaturation respectively, were dramatically increased in obese (A^{vy}) mice relative to lean (a/a) controls. Treatment of adipocytes with recombinant agouti protein *in vitro* increased FAS and SCD expression and activity. Strong correlation between the degree of agouti expression and both $[Ca^{2+}]_i$ levels and body weight have been demonstrated in mice, indicating that agouti may modulate adiposity via a $[Ca^{2+}]_i$ dependent mechanism (166). Humans also have an agouti gene, and the mouse and human agouti proteins are 87% identical over the cysteine rich C-terminus, suggesting that agouti may be functionally similar in both species. Unlike the mouse, however, the human agouti gene is normally expressed primarily in adipose tissue. The presence of human agouti in adipose tissue is intriguing in light of the fact that recombinant murine agouti protein not only causes an increase in $[Ca^{2+}]_i$ in cultured adipocytes, but also increases fatty acid synthase expression and activity, and stimulates the accumulation of triglycerides in a $[Ca^{2+}]_i$ -dependent manner. Claycombe *et al.* reported that FAS promoter contains an agouti response element that also responds to KCl, a membrane depolarization agent (167). Furthermore, calcium channel inhibition results in significant decreases in adipose tissue mass and adipocyte lipogenesis in obese

transgenic mice that express the agouti gene in a ubiquitous manner (168). Moreover, the human adipocyte agouti is up-regulated during differentiation (169). In addition, agouti expression is highly correlated with FAS expression and activity in human adipose tissue which stimulates Ca^{2+} influx (167). These results indicate that the agouti protein may regulate fatty acid metabolism by acting directly on adipose tissue.

Agouti protein also plays a role in regulation of lipolysis via a Ca^{2+} dependent mechanism (170-171). Recombinant agouti protein inhibits both basal and agonist stimulated lipolysis in human adipocytes. Increasing Ca^{2+} influx through either voltage- or receptor-operated Ca^{2+} channels also inhibits lipolysis and this effect is blocked by Ca^{2+} channel antagonists, indicating that this anti-lipolytic effect is mediated by calcium signaling. The mechanism underlying the anti-lipolytic effect of Ca^{2+} has recently been demonstrated to be mediated by increased activation of phosphodiesterase 3B, resulting in reduced cAMP levels and consequently, inhibition of hormone sensitive lipase activity. However, considerable species variability exists in the lipolytic response to melanocortins (172). Recent studies suggested that agouti may also inhibit lipolysis by antagonizing ACTH. ACTH and α -MSH are potent lipolytic hormones (173). The adipocyte MC-2R exhibits properties similar to the adrenocortical ACTH receptor and couples to activation of adenylyl cyclase (174-175). Both ACTH and α -MSH bind to mouse adipocytes, but only ACTH elevates cAMP and stimulates lipolysis. Therefore, agouti antagonism of ACTH to adipocytes may lead to an inhibition of lipolysis and/or stimulation of lipogenesis.

Insulin resistance is of major pathogenic importance in several common human disorders, including type 2 diabetes and obesity. The human homologue of agouti is

expressed in both adipose tissue and pancreas, and may therefore exert important peripheral effects. Administration of AGRP caused significant increases in plasma insulin concentrations and fat pad mass, and transgenic mice expressing agouti exhibited hyperinsulinemia and hyperglycemia (176), indicating an interaction between insulin and agouti-induced obesity syndrome. Since agouti stimulates Ca^{2+} in several cell types and Ca^{2+} is the proximal signal for insulin release, it is possible that agouti may exert effect on pancreatic β -cells to modulate Ca^{2+} signaling and stimulates Ca^{2+} release. Indeed, agouti is expressed in pancreas and potently stimulates insulin release via a Ca^{2+} dependent mechanism (177).

One of the potential factors that may account for or contribute to insulin resistance is a sustained high level of $[\text{Ca}^{2+}]_i$ in the insulin target cells. Draznin *et al* demonstrated an optimal range of $[\text{Ca}^{2+}]_i$ for the stimulatory effect of insulin on glucose transport in isolated rat adipocytes, with the levels beyond this range causing marked decrease in insulin sensitivity (178). Similarly, elevations of $[\text{Ca}^{2+}]_i$ induced by incubation of adipocytes with depolarizing concentrations of K^+ or parathyroid hormone resulted in diminished cellular responsiveness to insulin (179). High levels of the steady state $[\text{Ca}^{2+}]_i$ have been observed in adipocytes of patients with obesity, type 2 diabetes, skeletal muscles of agouti rats, and hepatocytes of diabetic rats (180), and an inverse correlation between the whole body insulin sensitivity and the $[\text{Ca}^{2+}]_i$ of peripheral blood cells has been observed in human studies (181). It has been postulated that high $[\text{Ca}^{2+}]_i$ in the insulin target cells may diminish cellular responsiveness to insulin and thus contribute to the development of insulin resistance (182). The mechanism whereby the high levels of $[\text{Ca}^{2+}]_i$ interfere with insulin action on glucose metabolism remains largely unknown.

Insulin-receptor binding is not affected, and receptor tyrosine kinase activity is only minimally affected. Recruitment of glucose transporter, glucose transporter (GLUT) 4, is also not influenced. Begum *et al* have demonstrated in isolated rat adipocytes that an elevation in $[Ca^{2+}]_i$ induces inhibition of phosphoserine phosphatase activity through an activation of inhibitor 1 (183). Accordingly, sustained elevations in $[Ca^{2+}]_i$ may result in insulin resistance by preventing dephosphorylation of insulin-sensitive target protein(s) within the cell. Reduction in insulin-induced dephosphorylation of glycogen synthase and GLUT4 supports this possibility (184-185).

Alemzadeh *et al.* have reported that diazoxide, a drug that activates the β cell K_{ATP} channel and subsequently reduces $[Ca^{2+}]_i$ and inhibits insulin release, exerts an antiobesity effect in obese Zucker rats and hyperinsulinemic obese adults (186-187). Sulfonylureas such as glibenclamide are insulin secretagogues widely used to stimulate insulin secretion in the treatment of non-insulin-dependent diabetes mellitus. Sulfonylurea interact with specific, high affinity receptors (SUR) on the pancreatic beta-cell to close ATP-sensitive K^+ channels, depolarize the cell, activate Ca^{2+} influx through voltage-dependent Ca^{2+} channels, and trigger insulin secretion (188). SUR agonists also been demonstrated to exert direct effects on adipocytes. Draznin *et al.* reported that glibenclamide increased $[Ca^{2+}]_i$ in isolated rat adipocytes by promoting Ca^{2+} influx through voltage-dependent Ca^{2+} channels, while this effect was blocked by a Ca^{2+} channels antagonist (182). Moreover, glibenclamide has been reported to potentate peripheral insulin effects in isolated adipocytes (189). Consistent with this, human adipocytes express SUR and exhibit a glibenclamide dose-responsive increase in $[Ca^{2+}]_i$ (190). Moreover, glibenclamide exerts lipogenic and antilipolytic effects in human

adipocytes, whereas diazoxide completely blunts each of these effects. These data demonstrate that SUR in adipocyte regulates $[Ca^{2+}]_i$ and thereby exerts coordinated control over lipogenesis and lipolysis (190).

Agouti may also participate in the regulation of adipogenesis. Mynatt *et al* demonstrated substantial increases in PPAR- γ and signal transducer and activators of transcription (STATs) expression in the fat pads of the aP2-agouti mice and in cultured adipocytes that have been exposed to recombinant agouti protein (191-192). The major transcription factors involved in adipocyte gene regulation include PPAR γ , proteins belonging to the CCAAT/enhancer-binding protein (CEBP) family, and adipocyte determination and differentiation dependent factor 1, also known as sterol regulatory element binding protein (193). Recent studies have also suggested that the STAT family of transcription factors may also be important in fat cells (194). A STAT family member shows a distinct pattern of activation by cytokines and, upon nuclear translocation, can regulate the transcription of particular genes in cell- or tissue-specific manners. In fat cells, the expression of STAT1, STAT5A, and STAT5B is highly induced during differentiation and correlates with lipid accumulation. Agouti-induced regulation of STAT1, STAT3, and PPAR- γ expression results in the regulation of various genes associated with the adipocyte phenotype. $[Ca^{2+}]_i$ also plays a regulatory role in adipogenesis. $[Ca^{2+}]_i$ appears to promote and accelerate preadipocyte differentiation program(s), thereby inducing the adipocyte phenotype in the late stage of differentiation (195-196). To achieve this, increasing $[Ca^{2+}]_i$ causes a marked increase in the expression of PPAR- γ , and subsequently accelerates adipocyte differentiation by directly acting upon and eliciting late differentiation gene expression, such as aP2, steroyl-CoA

desaturase (SCD-1), phosphoenolpyruvate carboxykinase (PEPCK), and FAS. On the other hand, in late differentiation, preadipocytes become more committed to terminal differentiation after the expression of several critical differentiation transcriptional factors, such as CCAAT/enhancer binding protein beta and delta (C/EBP- β and C/EBP- δ), PPAR γ , and sterol regulatory element binding protein-1/adipocyte determination and differentiation factor-1 (SREBP-1/ADD-1). $[Ca^{2+}]_i$ may synergize with these transcriptional factors to promote the differentiation program by stimulating late differentiation gene expression.

1.3.3 Dietary Calcium and Obesity

1.3.3.1 Dietary Calcium Intake and Obesity

Human studies show an inverse association between calcium intake and body weight, body fat and the risk of becoming obese (197). The US Department of Agriculture's Nationwide Food Consumption Survey from 1987 to 1988 showed that the average dietary calcium intake in the United States was far below the suggested optimal calcium intake, and that persons with the lowest calcium intakes tended to have the highest body weight (198-199). When stratified by ethnic group, the non-Hispanic black population, which has one of the highest prevalence of obesity in the United States, was also found to have a lower mean daily calcium intake than either the Hispanic white population or the non-Hispanic white population. Using data from the first National Health and Nutrition Examination Survey (NHANES I), McCarron found a statistically significant inverse association between calcium intake and body weight (199). More

recently, Zemel *et al* demonstrated a strong inverse association between the relative risk of obesity and calcium intake for participants of NHANES III (200). The relative risk of high body adiposity was found to be greatest in those with the lowest calcium intake and was progressively lower as calcium intake increased. Animal models have provided mechanistic insight as to how low calcium intakes could influence body fat stores. Studies in the 1980s in spontaneous hypertensive rats found a lower net weight gain in the rats fed a high-calcium diet than in the rats fed a low-calcium diet, respectively (201). Shortly afterward, it was observed that diets high in both dietary calcium and sodium induced favorable changes in the total body fat content of spontaneous hypertensive rats and Wistar-Kyoto rats (202). More recently, we studied transgenic mice that overexpression agouti gene, specifically in adipocytes under the control of the aP2 promoter. We examined the effects of various calcium intakes on weight gain and acceleration of weight loss over 6 wk. Weight gain and fat-pad mass on obesity-promoting diets were significantly reduced by high calcium diets, and high calcium also promoted weight loss during energy restriction (200). These data suggest that, an increase in dietary calcium decreases body weight gain during *ad libitum* and accelerates weight loss during energy restriction.

1.3.3.2 Mechanism of Dietary Calcium Intake in Regulation of Obesity

a. Dietary Calcium, 1, 25-(OH)₂-D₃ and Intracellular Calcium

Dietary calcium supplementation has been reported to decrease the $[Ca^{2+}]_i$ concentration in various cell types in rodents and human (203-204). Calcium supplements

significantly increased the cell Ca-ATPase activity and decreased intracellular Na^+ concentration (205-206). The decrease in Na^+ results in an increased driving force for Na^+ - Ca^{2+} and Na^+ - H^+ exchange mechanisms, which have a central role in controlling the $[\text{Ca}^{2+}]_i$ level, and consequently reduce reduced free cytosolic Ca^{2+} concentration(207).

Dietary calcium reduces parathyroid secretion of parathyroid hormone (PTH) and renal production of 1, 25-dihydroxyvitamin D_3 (1, 25-(OH) $_2$ - D_3). Petrov *et al* demonstrate that oral calcium supplementation in man causes a reduction in plasma concentration of PTH and 1, 25-(OH) $_2$ - D_3 (208). According to Basile *et al*, increasing dietary calcium intake increases urinary calcium excretion and reduces plasma 1, 25-(OH) $_2$ - D_3 , but total and ionized serum Ca^{2+} and plasma 25-(OH)- D_3 are unchanged (209). 1, 25-(OH) $_2$ - D_3 also increases radio-labeled calcium uptake in various cell types. Accordingly, the decrease in $[\text{Ca}^{2+}]_i$ observed after dietary calcium supplement could be attributable to a reduced cellular influx of Ca^{2+} , resulting from suppression of circulating levels of 1, 25-(OH) $_2$ - D_3 and/or PTH. Indeed, 1, 25-(OH) $_2$ - D_3 and PTH have both been shown to stimulate a significant and sustained increase in $[\text{Ca}^{2+}]_i$ concentrations in primary cultures of adipocytes (210-211). Data from our laboratory recently demonstrated that 1α , 25-(OH) $_2$ - D_3 increased $[\text{Ca}^{2+}]_i$ whereas the specific membrane vitamin D receptor antagonist 1β -hydroxyvitamin D_3 blocked vitamin D-stimulated increases in $[\text{Ca}^{2+}]_i$ (212), indicating that a non-genomic actions of 1α , 25-(OH) $_2$ - D_3 via a putative membrane vitamin D receptor plays a significant role in 1, 25-(OH) $_2$ - D_3 -induced increases in $[\text{Ca}^{2+}]_i$.

b. Intracellular Calcium and Adipocyte Lipid Metabolism

High-calcium diets attenuate adipocyte lipid accretion and weight gain during overconsumption of an energy-dense diet and increase lipolysis and preserve thermogenesis during caloric restriction, thereby markedly accelerating weight loss (15-16). Previous studies of the agouti gene demonstrate a key role for intracellular Ca^{2+} in regulating adipocyte lipid metabolism and TG storage (213). The role of the increased $[\text{Ca}^{2+}]_i$ in stimulation of lipogenesis has been explored using fatty acid synthase, a key enzyme of *de novo* lipogenesis (166-167). An agouti/ Ca^{2+} response sequence has been mapped to the -435 to -415 region of the FAS promoter and this sequence is upstream of insulin response element. This location consistent with the observed additive effects of agouti and insulin on FAS gene transcription (166, 214). Increasing $[\text{Ca}^{2+}]_i$ in adipocytes via either receptor- or voltage-mediated Ca^{2+} channel activation stimulated FAS gene and expression and consequently resulted in stimulation of FAS activity. In contrast, $[\text{Ca}^{2+}]_i$ influx inhibition by a Ca^{2+} channel antagonist suppressed lipogenesis, indicating that $[\text{Ca}^{2+}]_i$ stimulates lipogenesis directly. In addition to activating lipogenesis, recent data also indicate that increasing $[\text{Ca}^{2+}]_i$ also contributes to increased triglyceride stores by inhibiting lipolysis (170) as reviewed in the previous section.

Animal studies using aP2 transgenic mice confirmed these observations *in vivo*. We recently demonstrated that the increased calcitriol produced in response to low-calcium diets stimulates adipocyte Ca^{2+} influx and, consequently, promotes adiposity by both inhibiting lipolysis and stimulating lipogenesis. Accordingly, suppressing calcitriol levels by increasing dietary calcium is an attractive target for obesity interventions (15-

16). In support of this concept, transgenic mice expressing the agouti gene specifically in adipocytes (a human-like 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 (15). Further, low-calcium diets impede body fat loss whereas high-calcium diets markedly accelerate fat loss in transgenic mice subjected to caloric restriction (16). Dairy sources of calcium exert markedly greater effects in attenuating weight and fat gain and accelerating fat loss. This augmented effect of dairy products is likely due to additional bioactive compounds in dairy that act synergistically with calcium to attenuate adiposity (215). These concepts are confirmed by both epidemiological and clinical data (216-217), which demonstrate that increasing dietary calcium results in significant reductions in adipose tissue mass in obese humans in the absence of caloric restriction and markedly accelerates the weight and body fat loss secondary to caloric restriction, whereas dairy products exert significantly greater effects.

c. 1α , 25-(OH) $_2$ -D $_3$ and Adipocyte Energy Homeostasis

1α , 25-(OH) $_2$ -D $_3$ appears to play an important role in modulating adipocyte lipid metabolism and energy homeostasis via both genomic and non-genomic actions. 1α , 25-(OH) $_2$ -D $_3$ generates rapid, non-genomic signal transduction, including modulation of calcium channels, via a putative membrane vitamin D receptor (mVDR) in adipocytes and thereby participates in the regulation of lipid metabolism (17). Thus suppression of 1α , 25-(OH) $_2$ -D $_3$ with high dietary calcium induces reduction in body fat (15-16).

Although this anti-obesity effect of dietary calcium appears to be due, in part, to effects on lipolysis and lipogenesis, it is also possible that a loss of adipocytes would result in a cellular deficit lipid esterification as the body recovers from energy restriction. Thus, a reduced number of available adipocytes may not be able to store excess energy coming from rebound in food intake. Moreover, generating new cells would require extra energy that would contribute to a further metabolic enhancement. Recent data also indicate that $[Ca^{2+}]_i$ may play a key role in modulating cell's sensitivity to the apoptotic stress (21). Several of the functional groups of molecules involved in apoptosis are $[Ca^{2+}]_i$ responsive. $[Ca^{2+}]_i$ has been demonstrated to modulate the role of proteases, including caspase-3, caspase-9 and Ca^{2+} -dependent endonucleases (22-23). Scorrano *et al.* also showed a key interaction occurs between Bcl-2 family protein and calcium signaling in the execution of apoptosis (218). Their data demonstrated a critical role of BAX and BAK in maintaining of homeostatic concentration of $[Ca^{2+}]_i$ in endoplasmic endo-reticulum (ER) and mitochondria, which control the apoptotic fate of cells responding to $[Ca^{2+}]_i$ dependent stimuli. Numerous studies also showed that thapsigargin, which can induce sustained $[Ca^{2+}]_i$ elevation by inhibiting sacro-endoplasmic reticulum Ca^{2+} -ATPase (SERCA), triggers apoptosis in various cell types (219-221). These data indicate a key role of $[Ca^{2+}]_i$ in the apoptosis process. Given that $[Ca^{2+}]_i$ can be modulated by $1\alpha, 25-(OH)_2-D_3$, the role of $1\alpha, 25-(OH)_2-D_3$ in apoptosis should be explored. Although high dose of $1\alpha, 25-(OH)_2-D_3$ has been reported to induce apoptosis in various tumor cells (222-225), the regulation of apoptosis by physiological doses of $1\alpha, 25-(OH)_2-D_3$ remains unclear.

$1\alpha, 25\text{-(OH)}_2\text{-D}_3$ also generates genomic actions via a nuclear receptor in a manner similar to the other members of steroid hormone superfamily. Recent study further demonstrated that $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ exerts an inhibitory effect on basal, isoproterenol, and fatty acid-stimulated UCP2 expression via a genomic action (226). This inhibition is mediated by nVDR. The mVDR agonist and antagonist fail to exert their actions to either mimic or prevent the $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibition of UCP2 expression, whereas nVDR knockout by antisense oligonucleotide prevented the inhibitory effect of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on adipocyte UCP2 expression. UCP2 is ubiquitously expressed, with the highest level in white adipose tissue, and has been shown to stimulate mitochondria proton leak and therefore exhibit a potential role in thermogenesis, energy metabolism, and obesity (12). In addition, considerable progress has been made in elucidating a pivotal role of mitochondria playing in the coordination, initiation, and execution of apoptotic cell death (227). Three general mechanisms have been proposed, including 1) disruption of oxidative phosphorylation ATP production; 2) regulation of the apoptotic proteases; and 3) alteration of cellular reduction-oxidation potential. Because UCP2, which is highly expressed in white adipose tissue, has been demonstrated to function as mitochondrial uncoupler of oxidative phosphorylation, it is reasonable to propose that UCP2 may stimulate apoptosis in adipocytes. Accordingly, the obesity-promoting effect of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ may also attributable to its suppression of UCP2 expression. However, the effect and mechanism of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ and UCP2 in regulation of adipocytes apoptosis is not yet well understood.

1.4 Role of UCPs in Regulation of Obesity

1.4.1 Thermogenesis and Obesity

Obesity results from energy intake exceeding energy expenditure over time (228). The biochemical mechanisms responsible for the regulation of energy expenditure and the efficiency of energy utilization are poorly understood. Possible ways to increase energy expenditure include increasing physical activity and energy dissipation as heat by futile metabolic cycles. Thermogenesis in mammals, comprising obligatory thermogenesis and regulatory thermogenesis, is a spontaneous mechanism allowing body temperature of most mammals to be maintained at around 37°C in cold environments (229-230). Obligatory thermogenesis corresponds to resting energy expenditure and minimal biological work essential for life in resting conditions, in absence of food intake, and at thermoneutrality. Regulatory thermogenesis, also called adaptive thermogenesis, corresponds to extra heat induced by exposure to the cold or food intake. Regulatory thermogenesis can occur in muscle in the form of shivering thermogenesis, and in brown adipose tissue and some other tissues too as non-shivering thermogenesis, such as postprandial excess heat production. Rothwell *et al* postulated decades ago that non-shivering thermogenesis could be utilized by the organism not only to protect against cold stress, but also to maintain energy homostasis in response to caloric stress (231). It followed that obesity could rise from abnormalities in this mechanism of regulation and that pharmacological stimulation of non-shivering thermogenesis could be an effective mechanism for reducing obesity (228). This hypothesis is very attractive because 1) the production of heat is fundamental to the concepts of energy balance and the regulation of

body weight; 2) non-shivering thermogenesis is a physiological system dedicated to heat production; and 3) potential to develop agonists that are specific for non-shivering thermogenesis exist.

The endocrine factors regulating basal thermogenesis are not well known, although that it has been shown that thyroid hormones play a major role in modulating resting energy expenditure (232). Such evidence comes from analysis of patients exhibiting hypo- or hyperthyroid activity, and is confirmed by experimental work on animals (233-234). The sympathetic nervous system and noradrenaline are the major regulators of adaptive and cold induced thermogenesis (235-236). Recent data point out the thermogenic effect of leptin, acting via the brain and activation of sympathetic fibers (237).

1.4.2 UCP Family

At the cellular level, thermogenesis results from loss of energy during a number of biochemical processes coupling endergonic and exergonic reactions, and linked in particular to ATP hydrolysis and ion transport (232). Uncoupling of mitochondrial respiration from ADP phosphorylation is a highly thermogenic mechanism existing in brown adipose tissue (BAT) (238). Unlike white adipose tissue, BAT is characterized by rich vascularization, which is responsible for its color, a network of sympathetic fibers around every cell and numerous mitochondria with dense cristae (239). In response to acute cold exposure, the sympathetic nerve-endings of BAT release noradrenaline (NA). This NA binds to membrane β -adrenergic receptors, stimulating lipolysis and the release of FFA. FFAs released *in situ* are used as substrates for the respiratory chain and they

apparently also activate thermogenesis in BAT (240). The brown adipocytes are also rich in the UCP1, which uncouples respiration from phosphorylation and leads to the dissipation of energy as heat. Although in large mammals BAT is present in neonates and hardly detectable in adults, the presence of large amounts of UCP has been shown in perirenal adipose tissue of adult human in certain conditions. During the past 10y, the interest of several pharmaceutical groups has focused on β_3 -adrenoceptor agonists, which mimic the action of noradrenaline and could be useful in stimulating UCP1 and increasing energy expenditure in obese patients (241). Unfortunately, these drugs often proved to have undesirable side-effects on the cardiovascular system and other tissues associated with agonist activity at β_1 and β_2 - adrenoceptors, or have a poor bioactivity (242). In addition to catecholamines, several hormones activate UCP1 gene transcription, including thyroid hormones, retinoids and ligands of PPARs (243-245).

It was known for a long time that only 70% of the energy released from respiratory substrate oxidation was recovered as ATP and that respiring mitochondria release heat (246). Measurement of proton leaks in liver and muscle mitochondria favored a role for proton leak in the less than perfect coupling of O_2 consumption to ATP synthesis (247). Rolfe and Brown quantified various processes composing mammalian resting metabolic rate (RMR). For the sum metabolism of various tissues, ~10% was estimated as being attributable to non-mitochondrial oxygen consumption and ~20% to oxygen consumption for maintaining the mitochondrial membrane potential against the leak of protons (246). The existence of proton leak suggested that proteins leaked to UCP1 might be involved (248). Despite a compelling rationale in support of a role for UCP1 in the regulation of body weight (249-251), this idea has been viewed as

questionable, because brown adipocytes are thought to be rare in adults human. Instead, white adipose tissue (WAT) and skeletal muscle are the two major peripheral tissues that regulate energy homostasis and thermogenesis in adult mammals (252-253). However, newly identified uncoupling protein homologs (UCP2, UCP3, UCP4, UCP5, avUCP, HmUCP) have been found in various animal tissues (254-259). In particular, in both rodents and human, UCP2 is expressed in a variety of tissues such as WAT, BAT, skeletal muscle, heart, placenta, brain, stomach, kidney, lung and liver. UCP2 is 60% homologous to UCP1. In contrast to the ubiquitous expression of UCP2, UCP3 is dominantly expressed in skeletal muscles of human and rodents. The amino acid sequence of human UCP3 is 57% identical to human UCP1 and 73% identical to human UCP2. This high level of homology with UCP1 suggested a possible uncoupling activity of UCP2 and UCP3. Several reports provide both direct and indirect evidence of a role of UCP2 and UCP3 in the *in vivo* regulation of energy production and expenditure (260-262). Such a role has been elucidated *in vitro*, where they both mediate mitochondrial proton leak in an identical fashion, and their overexpression leads to reduction of mitochondrial membrane potential (263-264).

Genetic strategies have also clarified the functional role of UCP2 and UCP3 in modulation of energy metabolism. Markers encompassing the UCP2/3 locus have revealed significant linkage to resting energy expenditure in a French-Canadian cohort and in Pima Indians (265-266). A polymorphism in UCP2 exon 8 has demonstrated an association with body mass index (BMI) and leptin in specific populations (267-268). Numerous sequence variants have also identified in UCP3. Particular attention has

focused on a splice site mutation in UCP3 intron 6 detected in African-American subjects, which results in reduced fatty acid oxidation (269).

So far two mechanisms have been proposed for the mitochondrial uncoupling (270). The fatty acid protonophore model of UCP-mediated H^+ flux was first introduced by Skulachev and Garlid (271-272). In this mechanism, UCP doesn't conduct protons but catalyzes flip-flop of the anionic head group of fatty acid from the matrix leaflet to the outer leaflet of the inner membrane. Transport of the anion is driven by the high, inside-negative membrane potential ($\Delta\psi$). After the carboxyl head group has crossed the membrane, it picks up a proton, and the protonated fatty acid spontaneously and rapidly flip-flops back to the matrix side, where deprotonation completes the cycle. The net result of the cycle is delivery of protons with charge to the matrix. Thus, fatty acids behave as cycling protonophores by virtue of the fact that UCP permits the anionic charge to move across the inner membrane. The other model, however, assumes that UCP transports proton directly (273). Winkler and Klingenberg demonstrate that fatty acids are buffering cofactors that operate in conjunction with resident H^+ -conducting amino acids. Mutation of two histidine residues in UCP1 causes loss of H^+ transport. This model is supported by the evidence that alkylsulfonates are competitive inhibitors of fatty acid induced proton transport via UCP. In both models, fatty acids appear to be obligatory for UCP activity.

1.4.3 UCPs and Obesity

1.4.3.1 UCPs and Thermogenesis

Thermogenesis appears to be a major function of UCP1, the uncoupling protein homolog that is restricted to BAT (251). In rodents, neonatal, or hibernating mammals, which have large amount of BAT associated UCP1 activity, energy substrate are used to generate heat, limiting ATP production. Thus transgenic mice with brown fat ablation have decreased metabolic rate and hypothermic and these mice are obese (274-275). In addition, the elevated expression of both UCP2 and UCP3 in ground squirrels during hibernation has been cited to advocate a role for these UCPs in mammalian nonshivering thermoregulation (276).

Recent studies demonstrate that the synthesis of UCP2 and UCP3 is positively controlled by hormonal regulators, including thyroid hormone and leptin (277-278). These studies reinforce the putative role for UCP2 and UCP3 in energy expenditure and are in agreement with the genetic linkage of the UCP2 and UCP3 locus to resting metabolic rate in rodents and human (279). Early studies demonstrated T3 increases the transcription of UCP1 by acting at thyroid hormone response elements (TREs) in the enhancer located 2.3kb upstream of the promoter (280). However, in hyperthyroid animals, BAT becomes inactive, with reduced UCP1 expression, suggesting that T3 induced thermogenesis in other tissues reduces the need for BAT thermogenesis (281). Since UCP1 shares high sequence similarities with UCP2 and UCP3, it is reasonable to propose that UCP2 and UCP3 have related function. Administration of T3 to rodents leads to increases in the expressions of UCP2 and UCP3 in heart and skeletal muscle

(282-283). In association with the alteration in the thyroid status, increases occur in UCP3 mRNA expression in skeletal muscle and in mitochondrial uncoupling activity. The latter result has been confirmed *in vivo* by a non-invasive method (nuclear magnetic resonance spectroscopy) in both rats and humans (284-285). In hypothyroid rats given a single injection of T3, a strict correlation in terms of time course has been shown among the induced increase in UCP3 protein levels in gastrocnemius muscle, the decrease in mitochondrial respiratory efficiency and the increase in the RMR of the whole animal (286). In that study, the maximal increase in mitochondrial UCP3 density was reached at 65 h after the T3 injection, and the increase in RMR showed the same time course. At the same time point, mitochondria isolated from gastrocnemius muscle showed a significantly higher non-phosphorylating respiration rate, whereas the membrane potential showed a decrease, clearly indicating the occurrence of uncoupling. In contrast, chronic hypothyroidism decreases the UCP3 expression in skeletal muscle from rat. These data provide *in vivo* evidence that UCP3 has the potential to act as a molecular determinant in the influence of T3 over RMR. All these data support that T3-promoted increase in thermogenesis via mitochondrial uncoupling in skeletal muscle.

In contrast to these observations, it has been reported that UCP3 null mice given a 4 day course of T3 (at 100 µg/100 g body weight/day) show the same increase in RMR as wild-type controls, a finding that does not seem to support the involvement of UCP3 in the T3-induced increase in metabolic rate (287). Among the possible reasons for the discrepancy are the very high doses of T3 used in UCP3-knockout studies (which could have overstimulated other thermogenetic mechanisms) and the possible conversion of T3 to other thermogenic iodothyronines, such as 3,5-diiodothyronine.

Leptin is believed to play a role in regulation of UCPs as well. Exogenous leptin, administered to rodents, results in reduced adipose mass, which is only in part explained on the basis of decreased food intake, implying an effect of the peptide to increase energy expenditure (288). Indeed, leptin has been demonstrated to increase core body temperature, stimulate sympathetic nerve activity, and increase norepinephrine turnover in BAT (289). Scarpace *et al* demonstrated that peripheral administration of leptin induced both oxygen consumption and UCP1 expression (290). Chronic injection of leptin in C57BL/6J was shown to induce UCP1 and UCP2 expression in brown and white adipose tissue and lead to depletion of lipid from these fat depots (291). Using an adenovirus expression vector to introduce leptin, Zhou *et al* observed similar effects in rats (292). Furthermore, leptin prevents the decrease in UCP1 and UCP2 expression caused by fasting and stimulates energy utilization in ob/ob mice by inducing UCP activity (293). Consistent with these observations, lactation is a natural model of chronic negative energy balance, and lactating rats have decreased adaptive thermogenesis, a significant reduction in UCP1 and UCP3 protein levels as well as low circulating leptin levels (294). Low circulating leptin levels during lactation could be the cause of the decrease in UCP1 and UCP3 expression. Indeed, leptin replacement during mid-lactation significantly increased UCP1 and UCP3 mRNA expression. One major difference between fasted and lactating rats is that circulating FFA levels are elevated during fasting but reduced during lactation due to the increased FFA uptake by mammary tissue. This difference may be a reason for the divergent effects of fasting and lactation on UCP3 expression in skeletal muscle despite the low serum leptin levels in these conditions. The remarkable increase in UCP3 expression during fasting agrees with previous reports

(295). Skeletal muscle is the primary tissue responsible for the clearance of dietary glucose and lipids from circulation. During fasting, when the overall strategy is to conserve energy and to spare glucose for use by the brain, the fuel requirement of skeletal muscle is diminished, and its predominant fuel is shifted toward lipids. UCP3 is likely to be specifically involved in regulating the use of lipids as fuel substrates in skeletal muscle. This hypothesis is supported by previous evidence showing an up-regulation of UCP3 in skeletal muscle under conditions where the preference for fuel substrate is shifted toward lipids, such as in fasting and high fat feeding (295). Furthermore, mice lacking UCP3 showed a tendency toward impairment in the starvation-induced shift in fatty acid metabolism (296).

1.4.3.2 UCPs and Fatty Acid Metabolism

The function of UCPs may not be restricted to thermoregulation and there is now a number of data linking UCP expression levels to fatty acid metabolism and fatty acid flux into various tissues. A high fat diet increased UCP2 gene expression in adipose tissue of mice (297). Starvation, which increases the level of circulation free fatty acids, markedly increases the level UCP2 expression in both white adipose tissue and skeletal muscle (298). In humans, the expression level of UCP2 is up-regulated during fasting in skeletal muscle and white adipose tissue of lean and obese subjects and shows a positive correlation with the percentage of body fat (299-300). Evidence of uncoupling, lower mitochondrial potential, and increased mitochondrial volume has recently been observed in rat pancreatic islets cultured for 1-2 days with palmitate (301). Similarly, long-term exposure to free fatty acids increases UCP2 expression in hepatocytes (302). These data

strongly support a regulation of UCP2 expression by fatty acids and also indicate that UCP2 activity could be related to fatty acid oxidation. The regulatory effect of fatty acids on UCP transcription may be attributable to PPARs, which can be activated upon binding fatty acid ligands. A PPAR response element (PPRE) has been identified in the UCP promoter region (303-304). In rats or lean and db/db mice, *in vivo* PPAR γ activation can induce expression of UCP-1, -2, and -3 in BAT (305), and chronic-intense PPAR γ activation may cause BAT to assume white adipose tissue-like phenotype with increased UCP-2 levels (305). PPAR α activation in mice is sufficient to induce liver UCP-2 expression (306). Since PPARs are also involved in the regulation of adipocyte differentiation and lipid oxidation, it is possible that PPARs are the key regulators that coordinate the activation of lipid metabolism and thermogenetic activity of adipocytes. Furthermore, administration of pyretic compounds such as lipopolysaccharides to mice induced significant increases in UCP2 expression in liver, indicating a potential role of UCP2 in infection and inflammation, both which are also highly thermogenetic processes (307).

However, several studies also show that UCP2 expression is up-regulated in obese animals and humans. A higher level of UCP2 expression has been found in skeletal muscle of obese versus lean individuals and UCP2 expression is positively correlated with percentage of body fat (308). In ob/ob and db/db mice, which are well characterized model of obesity associated with increased body weight and decreased energy expenditure, up-regulation of UCP2 expression has been observed (309-310), suggesting that enhanced UCP2 expression in obesity could be a compensatory response. In fact, UCP2 appears to function as a free fatty acid transporter and thereby increases fatty acid

utilization (311), as previously discussed. Indeed, impairment in the oxidation but not in the uptake of free fatty acids by muscle has been found in obesity. An increased content of UCP2, on the other hand, could function as a compensatory mechanism that could favor the outward translocation of fatty acids. The export of the fatty acid anion thus permits continued rapid fatty acid oxidation in the face of an oversupply. A similar hypothesis has been proposed for that UCP3, suggesting that it may function as a fatty acid anion exporter in muscle and brown adipose tissue when fatty acids are the predominant fuel substrates (312).

Early studies indicate that lipogenesis is dependent on mitochondrial ATP production. Therefore, mitochondrial uncoupling in adipocytes may have an inhibitory effect on lipogenesis by stimulating energy dissipation and depressing ATP production. Rognstad and Katz *et al* investigated the effect of chemical mitochondrial uncoupler, 2, 4-dinitrophenol, on glucose metabolism in epididymal fat from rat (313). Addition of 2, 4-dinitrophenol resulted in depressed synthesis of fatty acids and increased lactate, indicating that inhibition of fatty acid synthesis probably resulted from limited availability of intramitochondrial ATP for the carboxylation of pyruvate. In addition, ATP/ADP ratio has been demonstrated to affect pyruvate carboxylase activity (312). In agreement with this hypothesis, overexpression of UCP2 in adipocyte and induction of UCP1 and UCP3 in adipose tissue by pharmacological treatments results in reduction of lipid accumulation (314). Expression of UCP1 from the adipose specific promoter in aP2-UCP1 transgenic mice attenuates both genetic and diet-induced obesity (315).

Lipolysis also depends on the energy status of adipocytes (316-317). Cold temperature and high-calorie diets stimulate the sympathetic nervous system, leading to

the release of noradrenaline. The noradrenaline bound to adipocyte β_3 -adrenaline receptors stimulates hormone sensitive lipase via phosphorylation by protein kinase A. The receptor is not desensitized by noradrenaline, and thus delivers a sustained signal via Gs-protein to stimulate adenylate cyclase (AC). The AC synthesizes cAMP (cyclic AMP) and activates protein kinase (318). Protein kinase A also phosphorylates CREB (cAMP responsive element-binding protein), the trans-element for transcription of the messenger RNA for UCP1, UCP2 and UCP3 (319). A decrease of intracellular ATP, elicited in white adipocytes in vitro by uncoupling inhibitors of the mitochondrial respiratory chain, counteracted the stimulation of lipolysis by catecholamines. Incubation of isolated adipocytes with lipolytic hormones resulted in an up to a decrease of their intracellular ATP level and inhibition of lipolysis itself (320). More recently, ATP was shown to be required for translocation of hormone sensitive lipase from cytoplasm to the surface of lipid storage droplets and for phosphorylation of HSL and other proteins that are involved in lipolysis in adipocytes (321-323).

1.4.3.3 Role of UCPs in Regulation of Insulin Secretion

ATP levels are also essential for insulin signaling (324-325). The signal is altered at the level of the interaction of insulin with its receptor, as well as downstream from receptor. Like other cells, pancreatic β cells metabolism of glucose yields ATP through oxidative phosphorylation, and the generation of ATP is tightly coupled to the metabolism of glucose. In pancreatic β cells, an increase in ATP/ADP ratio associated with increased glucose metabolism promotes insulin release. The proposed mechanism involves closure of the K_{ATP} channel in response to the increased ratio causing membrane

dephosphorylation, Ca^{2+} influx and insulin secretion (326). Defects in oxidative phosphorylation would be expected to impair insulin release. Thus, UCP2 may be a negative regulator of insulin secretion, in view of its high expression in this cell type and its potential uncoupling action, as suggested by the decrease in glucose stimulated insulin secretion that follows its overexpression in isolated rat pancreatic islets (327). Consistent with this, mice lacking UCP2 had significantly lower glucose levels and higher insulin secretion rates under basal conditions and intravenous glucose administration (328). In contrast, in ob/ob mice, a model of type II diabetes, hyperinsulinemia, hyperglycemia, exhibits a significant up-regulation of UCP2 (325). When mice lacking UCP2 were crossed with ob/ob mice, the resulting reduction of UCP2 expression in the ob/ob animals was associated with a marked improvement in glucose tolerance (329). These results suggest that the level of UCP2 gene expression in the pancreatic beta cells is an important determinant of the sensitivity of insulin secretion to changes in glucose.

1.4.3.4 Role of UCPs in Regulation of Intracellular Calcium

$[\text{Ca}^{2+}]_i$ is a ubiquitous intracellular messenger involved in many cellular processes (330-331). To generate such complex Ca^{2+} signals, cells rely on the rapid release of the Ca^{2+} from storage as well as on the controlled Ca^{2+} influx from the extracellular compartment. Ca^{2+} concentration is maintained at extremely low levels by expelling Ca^{2+} ions to the exterior and by compartmentalization of Ca^{2+} stores. Ca^{2+} accumulation and protection of cells against Ca^{2+} overload has been considered a role of mitochondria. When exposed to an elevated local cytosolic Ca^{2+} , mitochondria will take up Ca^{2+} through an electrogenic uniporter, and this action is driven by the mitochondrial

membrane potential generated by oxidative phosphorylation. This process causes a depolarization of mitochondrial potential, providing a mechanism that serves to couple energy supply and demand to cell function. For instance, increased work by the myocyte is inextricably linked to an increase in $[Ca^{2+}]_i$, which in turn signals an increased ATP requirement to the mitochondria (332).

Since mitochondria play a key role in regulation of $[Ca^{2+}]_i$ homeostasis, alteration of mitochondrial function may influence $[Ca^{2+}]_i$ level. Pharmacological agents have been useful in identifying mitochondrial contributions to depolarization-evoked $[Ca^{2+}]_i$ responses in intact cells. Proton ionophores, such as DNP, depolarize the inner mitochondrial membrane and reduce the electrochemical driving force for calcium uptake, suppressing mitochondrial calcium accumulation (333). In isolated cardiac myocytes, metabolic inhibition, such as ischaemia, has generally been reported to induce an initial increase in cytosolic Ca^{2+} concentration, which appears to be associated with the depletion of intracellular ATP (334). Artificially suppression of ATP production using DNP induced a biphasic increases in $[Ca^{2+}]_i$, with the initial increase resulting from calcium release from mitochondria after depolarization and the secondary increase representing calcium release from SR. Although mitochondria will take up an imposed calcium load, when the load is intense and prolonged, it may precipitate opening of a large conductance pathway through the mitochondrial inner membrane known as the permeability transition pore (PTP). This may be a catastrophic event for the cell, leading to leakage of mitochondrial contents into cytosol, and possibly culminating in cell death, either through mitochondrial uncoupling and ATP depletion or by release of apoptosis-inducing factors (335).

$[Ca^{2+}]_i$ appears to regulate oxidative phosphorylation and various reactions of oxidative phosphorylation are possible targets for calcium activation. Some intra-mitochondrial Krebs cycle enzymes such as pyruvate dehydrogenase and isocitrate dehydrogenase are calcium-sensitive (336). Activation of these enzymes results in a substantial stimulation of the substrate oxidation subsystem. In addition, calcium may directly stimulate the respiratory chain and activation of calcium influx also result activation of ATP synthase and adenine nucleotide translocase. (337-338). Previous studies suggest that the increase in the cycling of calcium across the mitochondrial membrane will result in an increase in proton leak (339). However, this effect appears not to be mediated by direct up-regulation of UCP2 expression since increase in $[Ca^{2+}]_i$ has no effect on UCP2 expression.

1.4.3.5 Role of UCPs in Regulation of Reactive Oxygen Species

Reactive oxygen species (ROS) have been implicated in the pathogenesis of a number of clinical conditions, including atherosclerosis and chronic complications of diabetes mellitus (340-341). Recent data also indicate that manipulation of mitochondrial UCPs may be of interest for controlling the level of ROS (342). Mitochondrial production of ROS is modulated by mitochondrial potential, which is itself controlled by the level of coupling of respiration to ADP phosphorylation. BPQ (benzo[a]pyrene-1,6-quinone), which is a mitochondrial redox cycling agent that increases mitochondrial ROS production, up-regulates UCP-2 mRNAs in primary cultures of normal rat hepatocytes. Moreover, mild uncoupling of oxidative phosphorylation decreases mitochondrial ROS production. Duval *et al* demonstrate that murine endothelial cells (CRL 2181) pretreated

by antisense oligonucleotides directed against UCP-2 mRNA exhibited a significant and specific increase in membrane potential and intracellular ROS level compared with control scrambled or anti-UCP-1 and -UCP-3 antisense oligonucleotides (343). In agreement with this, UCP-ablated mice show more resistance to infection by *Toxoplasma gondii*, an intracellular parasite that may infect the brains of normal animal and cause death (344). The tentative conclusion of this study is that the macrophages in the absent of UCP2 have a greater capacity to produce ROS. There is also evidence of a role for UCP1 and UCP3 in the protection of tissues against oxidative damage by decreasing in the mitochondrial production of ROS (345). The superoxides themselves have been demonstrated to produce proton leak in mitochondria (346). Since changes in oxidative metabolism in cells and tissues provoke marked changes in free radical synthesis, the UCPs could represent important mechanism of control and limitation of these ions which have deleterious effects on cellular machinery.

TNF α may play a key role in regulation of ROS production. Tumor necrosis factor α (TNF α) inhibits mitochondrial electron transport chain activity, increasing the interaction of electrons with O₂ to generate superoxide anion and other mitochondrial ROS (347). In contrast, uncoupling proteins are known to increase mitochondrial electron transport chain activity, limiting electron interactions with O₂ and reducing O₂ formation during mitochondrial respiration (348). Accordingly, increases in UCP-2 might help mitochondria in cells to compensate for the TNF-related inhibition of electron transport chain activity. Indeed, in obese mice, rats, and humans, increased serum TNF α has been demonstrated to correlate with adiposity UCP-2 mRNA levels increase in cardiac muscle, fat and liver (337-338). In addition, normal rats treated with lipopolysaccharide, a potent

inducer of $\text{TNF}\alpha$, exhibited UCP2 expression increases and this effect is inhibited by neutralizing anti- $\text{TNF}\alpha$ antisera (346). However, this adaptive mechanism may come at a risk. By enhancing proton conductance across the inner mitochondrial membrane, UCP-2 partially depolarizes this membrane and decreases the efficiency of ATP synthesis. Inefficient mitochondrial ATP synthesis could cause energy failure and cell death if ATP requirements increase abruptly (349). Thus, increased UCP-2 activity could transform a survival advantage into a cause of cell death.

1.5 Regulation of Adipocyte Cellularity

1.5.1. Regulation of Adipocyte Size

1.5.1.1. Lipogenesis

The plasticity of adipose tissue mass is a function of both adipocyte size and number (350). An increase in adipose tissue mass can occur by hypertrophic growth. This increase in size occurs primarily by lipid accumulation within the cell. Lipogenesis is very responsive to the changes in diet (351). A diet rich in carbohydrates stimulates lipogenesis in both liver and adipose tissue, leading to elevated postprandial plasma triglyceride levels (352). Fasting reduces lipogenesis in adipose tissue, which, combined with an increased rate of lipolysis, leads to net loss of triglycerides from fat cells. High fat diets, in contrast, decreases lipogenesis by suppressing lipogenic gene expression in liver and adipose tissue, including that of FAS and SCD (353). It has been proposed that

the dietary nutrient may be translated into changes in hormonal secretion and altered expression levels of lipogenic genes (354).

Insulin is probably the most important hormonal factor influencing lipogenesis. By increasing the uptake of glucose in the adipose cell via recruitment of glucose transporters to the plasma membrane, as well as activating lipogenic and glycolytic enzymes via covalent modification, insulin potently stimulates lipogenesis (354). Insulin also has long-term effects on the expression of lipogenic genes, probably via the transcription factor sterol regulatory element binding protein-1 (SREBP-1) (355-357). In addition, insulin causes SREBP-1 to induce the expression and activity of glucokinase, thereby increasing the concentration of a glucose metabolite that supposedly mediates the effects of glucose on lipogenic gene expression (358). Another endocrine/autocrine factor connected with triglyceride synthesis is acylation stimulating protein (ASP) which is produced by adipose tissue and appears to act via an autocrine loop (359). Numerous *in vitro* studies have shown that ASP stimulates triglyceride accumulation in adipose cells (360-362). This is achieved by an increase in triglyceride synthesis, as well as by a simultaneous decrease in adipose tissue lipolysis. Leptin may also be involved in lipogenesis. There is a growing consensus that leptin limits fat storage not only by inhibiting food intake, but also by affecting specific metabolic pathways in adipose and other tissues (363).

Evidence that has been gathered over the past few years indicates that the effects of various nutrients and hormones on the expression of lipogenic genes are mediated by the SREBPs (364). Studies in transgenic mice that overexpress SREBP-2 in the liver suggested that SREBP-2 stimulates the expression of genes involved in cholesterol

metabolism, such as the LDL receptor, farnesyl pyrophosphate synthase and HMG-CoA reductase genes (365). Interestingly, mice that overexpress SREBP-1a or SREBP-1c in liver exhibit a dramatic build-up of hepatic triglycerides and elevated expression levels of lipogenic genes. This led to the conclusion that SREBP-1 activates genes connected with lipogenesis in liver. SREBP-1 probably has a somewhat different role in adipose tissue. In white adipose tissue of these mice, expression of genes implicated in cholesterol metabolism were markedly elevated, whereas the expression of genes implicated in fatty acid and triglyceride synthesis remained unchanged (366).

In vitro studies have clearly established the importance of the upstream stimulatory factors (USFs) in the regulation of the fatty acid synthase promoter by insulin (367). USFs are ubiquitous bHLH-leucine zipper transcription factors that are able to interact as homo- and/or heterodimers with E boxes of CANNTG sequence (368). Such an E box is present in the promoter of FAS (367). Mutations that weaken binding of USF1 and USF2 to this E box abolish the insulin-dependent activation of the FAS promoter (369). Recent studies with mice lacking USF1 and/or USF2 have provided compelling evidence that USF1 and USF2 are involved in mediating the stimulatory effect of insulin/glucose on fatty acid synthase expression (370). Finally, glucose may regulate expression of lipogenic genes via a carbohydrate response transcription factor (ChoRF), which has yet to be cloned. Specific response elements that bind this transcription factor have been identified in the promoter of target genes, such as pyruvate kinase (371).

An important transcription factor in adipose tissue is the nuclear hormone receptor PPAR γ (372). Despite its name, this protein is not endogenously activated by

peroxisome proliferators but by fatty acids and their eicosanoid derivatives, as well as by drugs of the thiazolidinedione class. PPAR γ is a part of the adipocyte differentiation program. To date, only a limited number of genes are known to be regulated by PPAR γ in adipose tissue. These encode the adipocyte fatty acid binding protein, lipoprotein lipase, fatty acid transport protein (FATP), acyl-CoA synthetase, phospho-enol pyruvate carboxykinase and the fasting-induced adipose factor FIAF/PPAR γ angiopoietin related gene (PGAR) (373). Based on the identities of these genes, coupled with the observation that PPAR γ expression is stimulated by insulin and by SREBP-1 (374-375), one would expect PPAR γ to have not only an adipogenic effect, but also a lipogenic effect. This is supported by clinical data, showing that patients taking synthetic PPAR γ activators frequently gain weight (376). Furthermore, heterozygous PPAR γ mutant mice exhibit smaller fat stores on a high fat diet (377).

Recent studies demonstrate that increased lipogenesis combined with reduced oxidation is associated with lipotoxicity (378). When lipids accumulate in nonadipose tissues during overnutrition, fatty acids enter deleterious pathways such as ceramide production, which, through increased nitric oxide formation, causes apoptosis of lipid-laden cells, such as pancreatic β -cells and cardiomyocytes (389-382). Lipotoxicity of pancreatic β -cells, myocardium, and skeletal muscle leads to type 2 diabetes, cardiomyopathy, and insulin resistance respectively (381).

1.5.1.2. Lipolysis

Lipolysis is the chemical decomposition and release of fat from adipose tissue (382). This process predominates over lipogenesis when additional energy is required.

The triglycerides within the adipocyte are acted upon by a multi-enzyme complex called hormone sensitive lipase (HSL), which hydrolyzes the triglyceride into free fatty acids and glycerol (383). These lipases act consecutively on triglycerides, diglycerides, and monoglycerides. Once triglycerides are hydrolyzed to fatty acids and glycerol, fatty acids enter the common free fatty acid pool where they may be re-esterified, undergo beta-oxidation, or be released into the circulation as substrates for skeletal muscle, cardiac muscle, and liver. HSL is expressed in both WAT and BAT, although more is known about its function in WAT.

In WAT, hydrolysis of triacylglycerols occurs through three consecutive reactions and is catalyzed by two enzymes: HSL catalyses the hydrolysis of triacylglycerols (TGs) and diacylglycerols (DGs) and the participation of monoglyceride lipase is required to hydrolyse monoglycerides (384). Although HSL is generally considered as the rate-limiting enzyme in lipolysis, this concept has recently been challenged. Disruption of the HSL gene has shown that the overall rate of lipolysis was unaffected, suggesting that there is another lipase with considerable activity towards TGs in WAT (385). However, HSL appears to be rate-limiting for DGs, as loss of HSL in mice results in accumulation of diglycerides (386). Paradoxically, in HSL-knockout mice, obesity is not a consequence. In fact, it was seen that WAT mass decreases in most parts of the body (387). Both *de novo* free fatty acid (FFA) synthesis and triacylglycerol synthesis were reduced in HSL-knockout mice (388). In a separate experiment, HSL overexpression in mice resulted in a decreased rate of lipolysis (389). Clearly, no simple relationship exists between HSL and the WAT mass and the involvement of other molecules is essential to explain this part of WAT metabolism (390). HSL is also the intracellular lipase in brown adipose tissue (391).

Fatty acids mobilized from BAT through the action of HSL are used in non-shivering thermogenesis. It has been interesting to note that HSL has been shown to retain high enzyme activity at low temperatures, which is probably a critical property in hibernating animals.

HSL activity is regulated by lipolytic (catecholamines, ACTH) and antilipolytic (insulin, adenosine) hormones (392). Catecholamines (such as adrenaline and noradrenaline) and ACTH (adrenocorticotrophic hormone), otherwise known as corticotrophin, active lipolysis whereas insulin exerts an anti-lipolytic effect. Adrenaline, synthesized in the adrenal gland, binds to β -adrenergic receptors. This binding induces conformational changes in the septa-spanning receptor, resulting in activation of adenylate cyclase, which in turn, the hydrolysis of ATP to form cAMP. Cyclic AMP has various targets, one of which is protein kinase A (PKA). The two main targets for PKA-mediated phosphorylation in the adipocyte are HSL and perilipins, which result in a large increase in lipolysis. Perilipins (predominant isoform in adipocytes is perilipin A) coat the surface of lipids, thereby interfering with the access of HSL to the triglyceride substrate. PKA-mediated perilipin phosphorylation removes the perilipin from the lipid surface. Hormone-sensitive lipase, which is also phosphorylated by PKA, can then access the TG and hydrolyse it to glycerol and FFAs. It was once thought that Ser 563 is the regulatory site (phosphorylation site) (393), although further study found that mutation of Ser 563 didn't abolish PKA-induced of HSL (394). Other serines (565, 659 and 660) have since been considered to be possible activation sites. To prevent the accumulation of FFAs having a negative feedback inhibitory effect on HSL, compounds such as adipocyte lipid binding protein (ALBP) is associated with HSL as part of a

“lipolytic complex” (395). ALBP sequesters FFAs and thereby increases the hydrolytic activity of HSL.

Insulin, the most important inhibitor of catecholamine-induced lipolysis, induces phosphorylation and activation of phosphodiesterase type 3B (PDE 3B), leading to a decrease in the cAMP levels and therefore a decrease in the PKA activity (396-397). The signaling pathway leading to PDE 3B activation involves the insulin receptor, insulin receptor substrates, phosphatidylinositol-3 kinase and probably protein kinase B as well. Specific inhibition of PDE 3B blocks the antilipolytic effect of insulin, indicating that cAMP degradation is the main mechanism by which insulin antagonizes catecholamine-induced lipolysis. The phosphorylation of PDE 3B is associated with the phosphorylation of Ser-302 in rat adipocyte, catalyzed by an insulin-stimulated protein serine kinase (PDE3IK) (398). A molecule called lipotransin has been proposed to be involved in docking HSL to the lipid droplet surface and to play a role in the arrest of lipolysis by insulin (399).

1.5.2. Regulation of Adipocyte Number

1.5.2.1. Adipogenesis

In mammals, WAT formation begins before birth (401). The chronology of WAT appearance, however, is strictly dependent on the species as well as the adipose depot. White adipose tissue expansion takes place rapidly after birth as a result of increased fat cell size as well as an increase in fat cell number (402). Even at the adult stage, the potential to generate new fat cells persists (403). It has been demonstrated that fat cell number can increase when rats are fed a high-carbohydrate or high-fat diet (404). An

increase in fat cell number is also observed in severe human obesity (405). Moreover, early differentiation markers of adipocyte differentiation can be detected even in adipose tissue derived from very old mice (406). Fat cell precursors isolated from adult WAT of various species, including humans, can be differentiated *in vitro* into mature adipocytes (407-408). Several studies on multipotent clonal cell lines have suggested that the adipocyte lineage derives from an embryonic stem cell precursor with the capacity to differentiate into the mesodermal cell types of adipocytes (409-410). That potential to acquire new fat cells from fat cell precursors throughout the life span is now undisputed.

In vitro systems have been extensively used to study adipocyte differentiation, leading to a dissection of the molecular and cellular events taking place during the transition from undifferentiated fibroblast-like preadipocytes into mature round fat cells (411). The committed preadipocyte maintains the capacity for growth but has to withdraw from the cell cycle before conversion to adipose. In preadipose cell lines as well as in primary preadipocytes, growth arrest but not cell confluence or cell-cell contact appears to be required for adipocyte differentiation. After growth arrest at confluence, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through subsequent differentiation steps. Studies on preadipose cell lines have shown that growth-arrested cells undergo at least one round of DNA replication and cell doubling. This has been proposed to lead to the clonal amplification of committed cells. Growth arrest and clonal expansion accompanied by complex changes in the pattern of gene expression (401).

Expression of lipoprotein lipase (LPL) mRNA has often been cited as an early sign of adipocyte differentiation (412). Lipoprotein lipase is secreted by mature

adipocytes and plays a central role in controlling lipid accumulation (413). At least two families of transcription factors, C/EBP and PPAR γ , are induced early during adipocyte differentiation (415). PPAR γ is largely adipocyte specific and is expressed at low but detectable levels in preadipocytes (373). Its expression rapidly increases after hormonal induction of differentiation. The subsequent decrease of C/EBP- β and C/EBP- δ in early to mid stages of differentiation is concomitant with the induction of C/EBP- α mRNA (416). This increase in C/EBP- α expression occurs slightly before the expression of adipocyte-specific genes. Another transcription factor induced very early during adipocyte differentiation is SREBP-1c/ ADD1 (adipocyte determination and differentiation factor 1), a bHLH-leucine zipper protein that is involved in cholesterol metabolism and may also participate in adipocyte gene expression (417). During the terminal phase of differentiation, adipocytes in culture markedly increase *de novo* lipogenesis and acquire sensitivity to insulin (418). The activity, protein, and mRNA levels for enzymes involved in triacylglycerol metabolism including ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, SCD1, glycerol-3-phosphate acyltransferase, glycerol-3-phosphate dehydrogenase, FAS, and glyceraldehyde-3-phosphate dehydrogenase increase (407). Glucose transporters, insulin receptor number, and insulin sensitivity increase (407). During adipocyte differentiation, there is a loss of β 1-adrenergic receptors and an increase in the β 2- and the β 3-subtypes, resulting in an increase in total adrenergic receptor number. In addition to increases in mRNAs for proteins directly related to lipid metabolism, adipocytes also synthesize other adipose tissue-specific products. These include aP2, an adipocyte-specific fatty acid binding protein (419); FAT/CD36, a putative fatty acid transporter (420); and perilipin, a lipid

droplet-associated protein (421). In addition, adipocytes produce a number of secreted products, including monobutyrin, an angiogenic agent; adipsin, a homolog of the serine protease complement factor D; Acrp30/AdipoQ; PAI-1; angiotensinogen II; and leptin (422-423).

In mammals, adipose tissue is a multi-depot organ made of two tissue types, the white and brown adipose tissues, which collaborate in partitioning the energy contained in lipids between thermogenesis and the other metabolic functions (424). Recent data have stressed the plasticity of the adipose organ in adult animals (349). Indeed, under peculiar conditions fully differentiated, white adipocytes can transdifferentiate into brown adipocytes, and vice versa (425-426). The ability of the adipose organ to interconvert its main cytotypes in order to meet changing metabolic needs may be pertinent to the physiopathology of obesity and related to therapeutic strategies.

The adipose tissue expansion that occurs during the development of obesity is initially characterized by fat cell hypertrophy (427-428). However, adipocytes do not have an unlimited capacity for expansion, and increases in fat cell number do occur. The development of hyperplastic adipose tissue noted in both genetic and diet-induced obesity is associated with the most severe forms of obesity and has the poorest prognosis for treatment (428-429). Understanding the mechanisms that regulate the growth of adipose tissue may enhance efforts to develop successful prevention and treatment strategies to limit accumulation of excess body fat. Chumlea *et al* estimated adipocyte number and adipocyte size in nonobese adults 20 to 50 yr of age (430). Women had greater percentage body fat and larger adipocytes than men in every age group except the oldest (45-50 yr). Adipocyte number, total body fat, and percentage body fat are each positively

correlated with age in both sexes. Adipocyte size is not correlated with age but is positively correlated with total and percent body fat in men and women irrespective of age. These cross-sectional data suggest that adipocyte number, rather than being stable during adulthood, increases with age and is associated with corresponding increases in total and percentage body fat. Noppa et al. demonstrated a significant relation between body build in childhood and prevalence and degree of obesity in adulthood in women (431). In addition, there was a significant relationship between the degree of obesity and total fat cell number when age of onset was kept constant, but not between age of onset and total fat cell number when degree of obesity was kept constant indicating that adipocyte number is attributable to obesity. Animal studies also confirmed that adipocyte hyperplasia is associated with obesity. Obst *et al.* assessed fat depot cellularity and cell number and lipid per cell for three fat depots in overfed dietary obesity resistant S 5B/Pl (S) and susceptible Osborne-Mendel (OM) rats (432). They found that both the size and the number of adipocytes was associated with overfeeding-induced obesity. However, other studies have the opposite observation. One study demonstrated that, when put obese woman underwent controlled 1000 kcal diets for 6-month, weight gaining patients showed a significantly higher mean fat cell number than while weight losing patients had a higher mean adipose cell weight than the weight gaining and weight stable patients (433).

The processes involved in adipocyte hypertrophy are relatively well understood, but much less is known about adipocyte hyperplasia. The mature adipocyte has little capacity for cell division and the hyperplastic capacity of adipose tissue resides in a population of fibroblast-like adipocyte precursor cells (434). The origin of these cells and

the processes involved in their commitment to the adipocyte lineage is not known. Growth factors, in particular the bone morphogenetic proteins (BMP), are likely to be involved in regulating commitment to the adipocyte lineage. *In vitro* studies have shown that once committed to the adipocyte lineage, the proliferation and differentiation of adipocyte precursor cells is regulated by a number of different growth factors (435-436). A number of these growth factors are expressed in adipocyte precursor cells *in vitro* and may have an autocrine-paracrine role (437). Others, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are more likely to have an endocrine role (438). The precise role that each growth factor plays in regulating adipocyte development *in vivo* is poorly understood. It is likely that adipocyte hyperplasia occurs in a localized fashion; adipocytes are not randomly distributed in mammals but are organized in identifiable depots. In addition, adipose tissue growth appears to occur within these depots through local recruitment of pluripotent precursors rather than through generation of new loci.

The hypothesis that a critical fat cell size precedes adipocyte hyperplasia is not a new one. Increases in mean fat cell size have been noted in mice and several strains of rats fed high-fat diets. Studies of adipose tissue cellularity in the genetically obese Zucker rat also indicated that hypertrophy develops before hyperplasia (428,439). Based on these observations, Faust proposed that attainment of a critical fat cell size initiated the events that lead to proliferation of adipocytes (440). A more recent study conducted by Brenda *et al* demonstrated that proliferative activity of conditioned medium obtained from all depots in obese Zucker rats was positively correlated to subsequent changes in fat cell number, indicating that enlarged adipocytes secrete growth factors that induce

preadipocyte proliferation (441). Considine *et al* report that mature adipocytes from both lean and obese subjects release a factor that stimulates the growth of preadipocyte-enriched and dedifferentiated adipocyte-enriched cell cultures (442). They demonstrate that mature adipocytes from obese patients stimulate the growth of preadipocyte-enriched cultures to a greater extent than those from lean individuals. There is now a preponderance of evidence that adipose tissue is a source of growth factors such as IGF-I, IGF binding proteins (443), TNF α (444), angiotensin II (445), and macrophage colony-stimulating factor (MCSF) (446) that are capable of stimulating proliferation. The relative importance of these autocrine/paracrine factors in the normal control of preadipocyte proliferation is unknown. In addition, the proliferative response of preadipocytes to the paracrine milieu is undoubtedly modulated by neural inputs to fat tissue and/or serum factors (447). Together, these multiple regulatory controls orchestrate overall and region-specific adipose tissue cellularity responses associated with the development of hyperplastic obesity.

1.5.2.2. Adipocyte Depletion

Large increases in fat stores involve an increase in adipocyte number via the replication and differentiation of preadipocytes, with the resultant cell gain widely regarded as irreversible. A reduction in fat tissue mass generally involves the loss of stored lipids by lipolytic processes (448). However, there is now growing evidence that decreases in adipose tissue mass in humans may also result from a loss of fat cells through programmed cell death, especially in condition when large amounts of fat are lost (449). In cultured adipocytes, apoptosis may be induced by serum deprivation or

exposure to TNF- α and HIV protease inhibitors (450-453). Studies in rodents demonstrate that induced weight and fat loss such as starvation, dietary conjugated linoleic acid (CLA), injection of neuropeptide Y-Y5 receptor antisense oligo or intracerebroventricular administration of leptin results in apoptosis of fat cells (454-459). Prins *et al* reported that human adipocytes undergo apoptosis following growth factor deprivation or mild heat injury *in vitro*, thus suggesting a cellular mechanism by which normal adipocyte loss could occur *in vivo* (460). Fat cell apoptosis was demonstrated in patients with tumor cachexia (461) and in HIV-patients during treatment with protease inhibitors (462). Patients with acquired forms of lipodystrophy (Lawrence Syndrome, Barraquer-Simons Syndrome) show an immunologically mediated loss of fat cells probably by apoptosis (463).

Up to now, general mechanisms for apoptosis induction in human adipocytes have not been intensively investigated. Little is known about either the physiologic regulators of cell death or the apoptotic sensitivity of preadipocytes versus adipocytes. Using the well-established immortalized murine 3T3-L1 cell line model of adipogenesis, Papineau *et al* reported that as adipogenesis ensues, these cells acquire resistance to apoptosis induced by growth factor deprivation (464). Expression of 2 cell survival genes, neuronal apoptosis inhibitor protein (NAIP) and Bcl-2, increased, consistent with the differentiation-dependent effect on survival (465). Others have confirmed the apoptotic sensitivity of 3T3-L1 preadipocytes (466-468). Although 3T3-L1 preadipocytes are a valuable experimental model, they do have distinctive attributes compared with human preadipocytes in primary culture, beyond the obvious species difference. 3T3-L1 preadipocytes are aneuploid, as well as embryonal in origin, and such features could

influence cell survival pathways. Death receptors (CD95, TRAIL-R1 and R2, and TNFR1) are expressed in human fat cells and that apoptosis can be induced by specific ligands (469). In addition, inhibition of the auto-/ paracrine actions of IGF-1 dramatically sensitizes human adipocytes for death-ligand induced apoptosis. Qian et al demonstrated that injection of leptin could dramatically up-regulate the expression of PPAR γ but decrease TNF α in young rat (470). Lower TNF α levels were probably a secondary effect of reduced fat mass and increased PPAR γ maybe attributable to leptin induced apoptosis, as PPAR γ is associated with apoptosis in cancer cells and in white adipose tissue from rodents treated with thiazolidinediones (471-472). Since PPAR γ is a powerful promoter of preadipocyte differentiation, the minor change of the WAT weight may be the combined results of increased adipocyte recruitment and adipocyte apoptosis. Supplementing mature adipocytes in culture with leptin induced expression of angiopoietin-2(Ang-2), which antagonizes angiogenesis, and caused apoptosis of adipose tissue endothelial cells (473). The apoptotic process also is modulated by energy states. Mitochondrial dysfunction causes ATP depletion, ion dysregulation, mitochondrial and cellular swelling, activation of degradative enzymes, plasma membrane failure, and cell death (474). Augmentation of cellular ATP by manipulation of the glucose content in the cultures led to an increase in survival from death signaling. *In vitro* experiments indicated the dependence of both lipogenesis and lipolysis on ATP levels in adipocytes (475). Thus, respiratory uncoupling in adipocytes that results in stimulation of energy dissipation and depression of ATP synthesis may contribute to the control of lipid metabolism, adiposity, and insulin sensitivity. This notion is supported by the observation that the expression of UCPs in adipocytes as well as some protonophoric anion transporters reduce adiposity

(476-477). Consistent with this, recent studies demonstrated a negative correlation between expression of UCPs in adipocytes and accumulation of white fat (478). These data suggested that energy status may also play a role in regulation of adiposity via apoptosis.

Based on the preceding literature review, the research described in this dissertation was designed to a) determine the role of dietary calcium and dairy on adipocyte metabolism and energy modulation during energy restriction and energy repletion; b) determine the role of mitochondrial uncoupling on adipocyte lipid metabolism; c) determine the role and mechanism of dietary calcium and 1, 25 (OH)₂-D₃ in modulating adipocyte deletion via apoptosis.

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Part Two

Interactive Effects of Calcium-Fortified Breakfast Cereal and of Milk on Adiposity and Weight Loss in a Transgenic Mouse Model of Obesity

2.1 Abstract

We previously demonstrated a regulatory role for intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in adipocyte metabolism. Recently, we reported that increasing dietary calcium, by suppressing calcitriol-mediated increases in $[\text{Ca}^{2+}]_i$ attenuates adipocyte lipid accretion and weight gain during over-consumption and accelerates fat loss during energy restriction. Notably, dairy sources of calcium exert consistently greater effects. The present study was conducted to extend these observations by determining the efficacy of a calcium-fortified cereal, alone or with a small amount of milk, in attenuation of weight and fat gain in the *aP2-agouti* transgenic mouse. Male mice placed on a basal low calcium (0.4%)/high fat (25 energy %)/high sucrose diet for six weeks exhibited ~2-fold increases in $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ ($p < 0.001$) and 25- 30% decreases in weight gain ($p < 0.03$) and total fat pad mass compared to the basal diet ($p < 0.001$), while food consumption was unaffected. Interestingly, addition of sufficient non-fat 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 substantially greater reductions in adipocyte $[\text{Ca}^{2+}]_i$ (63%, $p < 0.001$), weight gain (45%, $p < 0.002$) and fat pad mass (39%, $p < 0.0001$). In a follow-up study, both the calcium-fortified cereal and cereal plus milk diets significantly accelerated weight and fat loss secondary to a fixed level of energy

restriction, with the cereal plus milk diet exerting markedly greater effects. Thus, a calcium-fortified breakfast cereal is effective in reducing adiposity in this model of obesity, while addition of a small amount of milk significantly amplifies this effect.

2.2 Introduction

A substantial body of evidence has emerged over the last three years in support of an “anti-obesity” effect of dietary calcium and calcium-rich foods (1-3). Increasing dietary calcium in the absence of caloric restriction appears to result in a re-partitioning of dietary energy from adipose tissue to lean body mass, resulting in a net reduction in fat mass in both mice and humans (1-2), while increasing dietary calcium intake during energy restriction results in a marked augmentation of body weight and fat loss in both mice and humans (1). These observations are supported by epidemiological observations from NHANES III (2), the Quebec Family Study (4) and the CARDIA study (5). Data from NHANES III demonstrate an 84% reduction in the probability of being in the highest quartile of body fat in those in the highest quartile versus those in the lowest quartile of calcium and dairy intake (2). Similarly, data from the CARDIA study demonstrate a significant inverse relationship between dairy consumption and each of the components of the Insulin Resistance Syndrome (IRS), including obesity, during a 10-year follow-up of young adults who were overweight (body mass index ≥ 25 kg/m²) at baseline, with the odds of developing IRS 72% lower among overweight individuals in the highest (≥ 35 times per week) versus the lowest (< 10 times per week) category of

dairy consumption; the cumulative incidence of obesity was reduced from 64.8% in the lowest dairy consuming group to 45.1% in the highest dairy consuming group ($p < 0.001$)(5).

Interestingly, the source of dietary calcium has a substantial impact on the magnitude of calcium's effect on adiposity, with dairy sources of calcium producing 50-70% greater effects on fat loss during energy restriction in both mice and humans. Consistent with this observation, analysis of NHANES III demonstrates a significantly stronger inverse relationship between dietary calcium and adiposity when the number of dairy servings is included in addition to the level of calcium consumption (2). Moreover, the inverse relationship between dietary calcium and IRS in the CARDIA study was explained solely by dairy intake, whereas the inverse association between dairy consumption and IRS was not altered by adjustment for calcium, indicating an effect of dairy consumption independent of calcium intake (5).

A compelling mechanism for the anti-obesity effect of dietary calcium was provided by studies of the mechanism of action of the *agouti* gene in regulating murine and human adipocyte metabolism (6-11). These studies demonstrated a key role for intracellular Ca^{2+} in the regulation of adipocyte metabolism, resulting in modulation of adipocyte triglyceride stores (7-10). Since intracellular Ca^{2+} can clearly be regulated by calcitrophic hormones, including $1,25\text{-(OH)}_2\text{-D}_3$ and parathyroid hormone, suppression of these hormones by increasing dietary calcium may facilitate re-partitioning of dietary energy from adipose tissue to lean body mass and thermogenesis. In support of this concept, we have demonstrated that human adipocytes possess membrane (non-genomic)

vitamin D receptors which transduce a rapid intracellular Ca^{2+} response to $1,25\text{-(OH)}_2\text{-D}_3$ (12); consequently, $1,25\text{-(OH)}_2\text{-D}_3$ treatment of human adipocytes results in coordinated activation of fatty acid synthase expression and activity and suppression of lipolysis, leading to an expansion of adipocyte lipid storage (2). Moreover, $1,25\text{-(OH)}_2\text{-D}_3$ acts via the adipocyte nuclear vitamin D receptor to inhibit the expression of uncoupling protein 2 (UCP2) (13), while suppression of $1,25\text{-(OH)}_2\text{-D}_3$ levels by feeding high calcium diets to mice results in increased adipose tissue UCP2 expression and increased thermogenesis (14), suggesting that high calcium diets may also affect energy partitioning by suppressing $1,25\text{-(OH)}_2\text{-D}_3$ -mediated inhibition of adipocyte UCP2 expression.

Although the foregoing mechanism provides a plausible explanation for an anti-obesity effect of dietary calcium, there is as of yet no compelling mechanism to explain the differential effects between dairy and non-dairy sources of calcium. Accordingly, the present study was conducted to further explore the interaction between dairy and non-dairy sources of calcium in modulating adiposity in a previous established transgenic mouse model of diet-induced obesity.

2.3 Materials and Methods

2.3.1 Animals and Diets

We utilized transgenic mice which express *agouti* specifically in adipose tissue under the control of the aP2 promoter (15), similar to the human pattern of agouti signaling protein (ASIP) expression. These mice are not obese when fed standard rodent

chow, but develop mild to moderate obesity when fed high sucrose and/or high fat diets (16).

Phase I

40 Six-week-old male aP2-agouti transgenic mice from our colony were randomized to four groups, as follows. The basal group were placed on a modified AIN 93-G diet with suboptimal calcium (0.4%), sucrose as the sole carbohydrate source, and fat increased to 25% of energy with lard; a Ca-fortified cereal diet group, using both sucrose and fortified cereal as carbohydrate sources, increased dietary calcium three-fold to 1.2%, with macronutrient adjustments to ensure identical carbohydrate, protein and fat levels with the basal diet; a Ca-fortified cereal plus non-fat milk diet group, in which 25% of the protein was replaced by skim fat dry milk and dietary calcium was increased to 1.3%; a cereal control group utilized the same amount of a non-calcium fortified cereal as used in the Ca-fortified cereal group with macronutrients adjusted. Food intake and spillage were measured daily while body weight, core temperature and blood glucose were measured weekly. At the conclusion of the 6 wk feeding period, animals were killed by exsanguination under pentobarbitol (65mg/kg body weight) anesthesia, and blood was collected via cardiac puncture for glucose and insulin measurements. Fat pads (epididymal, perirenal, abdominal, and subscapular) and liver were dissected immediately, weighed, frozen in liquid nitrogen and stored at -80°C for later analysis. $[Ca^{2+}]_i$ was measured immediately in abdominal adipocytes obtained as described below. Fatty acid synthase activity and mRNA levels were measured in abdominal fat, and lipolysis was measured in freshly obtained perirenal fat as described below.

Phase II

This study was divided into two 6-week stages. In the first 6-week stage, 50 Six-week-old aP2-agouti transgenic mice were placed on the basal diet described above to induce obesity. At the end of this stage, five representative mice were killed for measurement of fat pad mass, adipocyte $[Ca^{2+}]_i$, fatty acid synthase activity and mRNA levels, and lipolysis in abdominal fat. The remaining 45 diet-induced obese transgenic mice were assigned to five groups as follows. One group was continued *ad lib* on the same low-calcium (0.4%) basal diet with no modification, while the other four groups received the four diets used in phase I (basal, Ca-fortified cereal, Ca-fortified cereal plus milk, cereal control) at 70% of the energy level of the *ad lib* four groups using a pair-feeding protocol. Diets were administered daily, and body weight, core temperature and blood glucose was monitored every 5 days. At the end of second stage, all mice were killed by exsanguinations under pentobarbital anesthesia (65mg/kg body weight). Blood obtained via cardiac puncture was used for insulin and glucose measurements. Fat pads were collected for intracellular Ca^{2+} , fatty acid synthase activity and mRNA levels, lipolysis as indicated for phase I.

2.3.2 Core Temperature

Core temperature was used as an indirect metabolic index to determine the effect of dietary calcium on thermogenesis. Temperature was measured via a thermocouple (Columbus Instruments, Columbus, Ohio) weekly. The probe was inserted a constant distance (1.8 cm) into the rectum of each mouse. After stabilization (10 s), the

temperature was recorded every 5 s for 30 s. All core temperature measurements were performed between 8:00 and 9:00 A.M.

2.3.3 Adipocyte Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) Measurement

Adipose tissue was first washed several times with Hank's Balanced Salt Solution (HBBS), minced into small pieces, and digested with 0.8 mg/ml type I collagenase in a shaking water bath at 37° 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. $[\text{Ca}^{2+}]_i$ in isolated mouse adipocytes was measured by using a fura-2 dual wavelength fluorescence imaging system. Prior to $[\text{Ca}^{2+}]_i$ 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 $\mu\text{mol/L}$) in the same buffer in dark for 1 h at 37° 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, Mass.). The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu 4915 CCD camera. Fluorescent images were captured

alternatively at excitation wavelength of 340 and 380 nM with an emission wavelength of 520 nM. $[Ca^{2+}]_i$ was calculated by using a ratio equation.

2.3.4 Fatty Acid Synthase (FAS) Activity Assay

FAS activity was determined spectrophotometrically in crude cytosolic extracts of mouse adipose tissue. Mouse abdominal fat pads were homogenized in 250 mmol/L sucrose solution containing 1 mmol/L ethylenediamine-tetraacetic acid (EDTA), 1 mmol/L dithiothreitol (DTT), and 100 μ mol/L phenylmethylsulfonyl fluoride (PMSF) (pH 7.4). The homogenate was centrifuged at 18,500 X g for 1 h, and the supernatant was used for measuring oxidation rate of NADPH. The final FAS activity was normalized to DNA, which was measured by CyQUANT cell proliferation assay kit according to manufacturer's instruction (Parkard instrument Company, Inc., Downers Grove, IL).

2.3.5 Lipolysis Assay

Perirenal adipose tissue was immediately dissected and incubated for 4h. Glycerol released into the culture medium was used to measure lipolysis, as described previously (17). Glycerol was measured using a one-step enzymatic fluorimetric method (18). The final lipolysis was normalized by DNA, as described above.

2.3.6 Northern Blot Analysis

Total RNA from mouse abdominal adipose tissue was extracted by using $CsCl_2$ density centrifugation, run in 1% agarose gel, and transferred to nylon membrane (New England Nuclear, Boston, Mass.). The membrane was hybridized with FAS and β -actin

(as a loading control) cDNA probes that were radiolabeled by using a random primer method. Unbound probe was removed by rinsing the membrane with 2X SSC / 0.1% SDS for 30 min at room temperature and 0.1X SSC / 0.1% SDS for 45 min at 55 °C. Finally, the membrane was exposed to X-ray film (New England Nuclear) at -80 °C. Autoradiographs were quantitated densitometrically, and all blots were stripped and reprobed with β -actin as a loading control.

2.3.7 Statistical Analysis

All data are presented expressed as mean $\pm$ SE. Data were evaluated for statistical significance by one- way analysis of variance (ANOVA), and significantly different group means were then separated by the least significant difference (LSD) test by using SPSS (SPSS Inc, Chicago, IL).

2.4 Results

We first investigated the effect of high calcium diet on attenuation of weight gain. There were no significant differences in food intake among the *ad libitum*-fed groups. Nonetheless, weight gain was reduced by 20% and 37% in the animals fed the Ca-fortified cereal and fortified cereal plus milk diets, respectively, when compared to those fed the basal low calcium diet ($P < 0.015$) (**Figure 2-1**). Consistent with this, fat pad mass was reduced by 24% and 41% by the calcium-fortified cereal and cereal plus milk diets, respectively ($P < 0.01$) (**Figure 2-2**). **Figure 2-3** demonstrates that the high calcium diet groups exhibited significant decreases in adipocyte $[Ca^{2+}]_i$, with decreases of 44% and

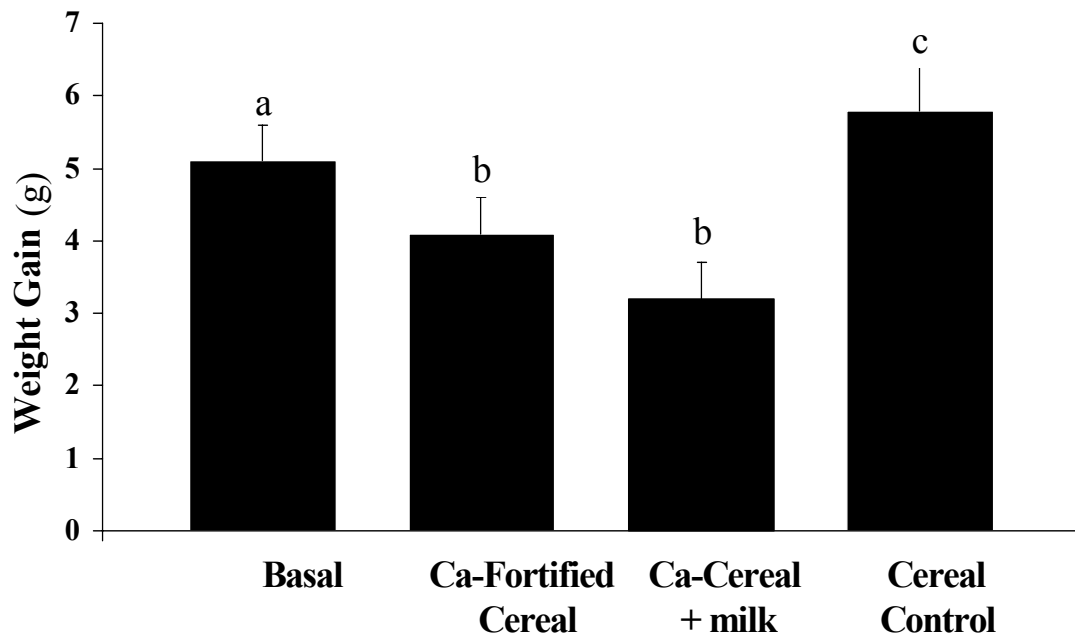


Figure 2-1. Effects of high calcium diets on weight gain in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

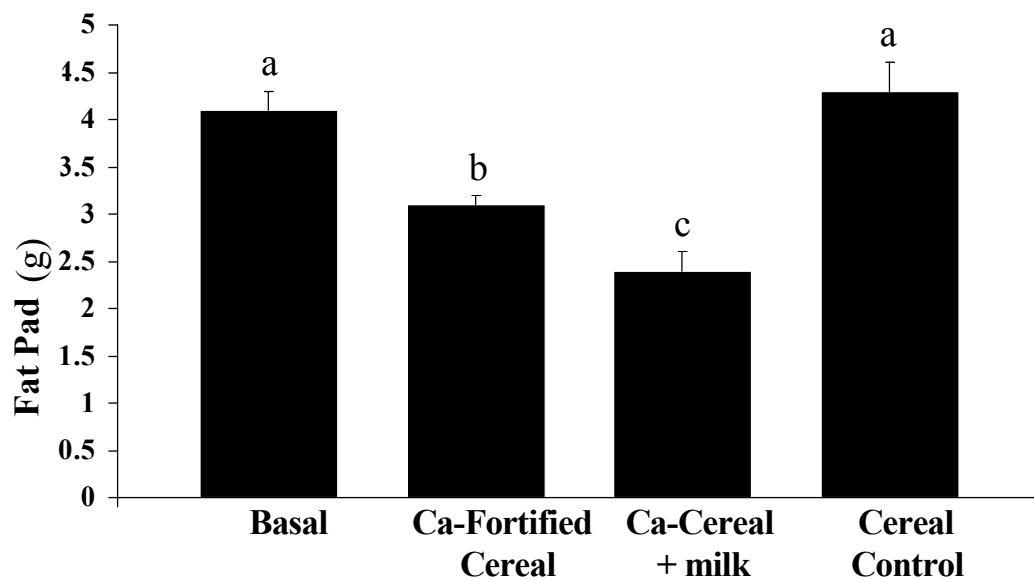


Figure 2-2. Effects of high calcium diets on fat pad weight (sum of four fat fads) in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

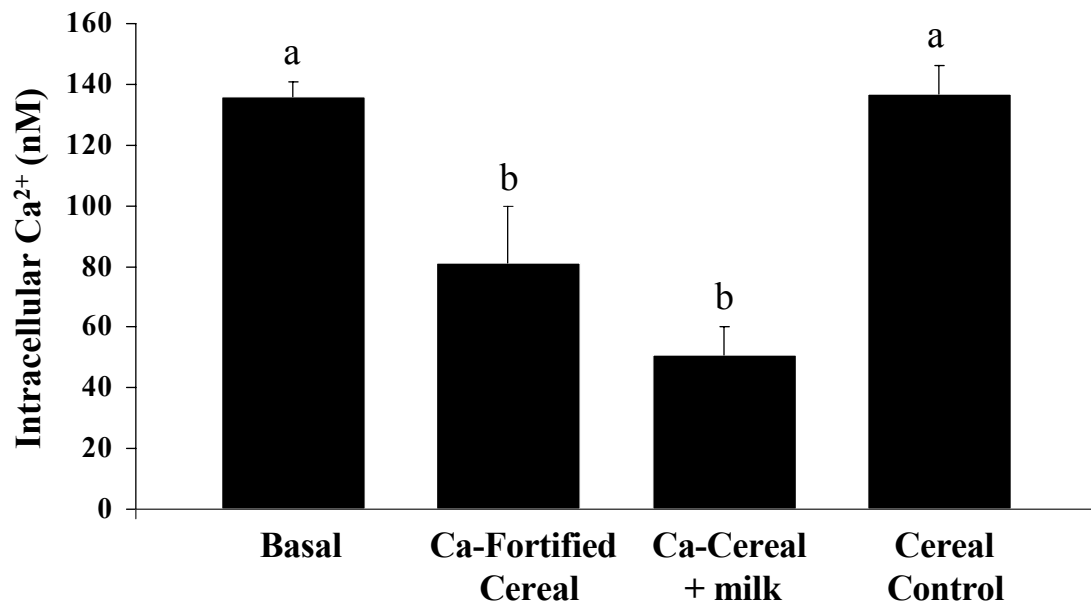


Figure 2-3. Effects of high calcium diets on adipocyte $[Ca^{2+}]_i$ in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

63% induced by the calcium-fortified cereal and cereal plus milk diets, respectively ($p<0.001$).

To study the effect of the high calcium diets on acceleration of weight loss, aP2-agouti mice were first placed on the *ad lib* basal low calcium / high fat / high sucrose diet to induce mild to moderate obesity and then randomized to the same dietary treatments used in Phase I, with energy restricted to 70% of an additional group maintained on the basal diet *ad libitum* using a pair-feeding protocol. Energy restriction on the low calcium diet resulted in a 6% body weight loss. However, greater weight reductions of 14% and 19% were observed in the Ca-fortified cereal group and fortified cereal plus milk groups, respectively ($p<0.05$) (**Figure 2-4**). A similar pattern was noted for fat loss during energy restriction, with a 21% decrease in fat pad mass on the low calcium diet, versus a 43% decrease on the Ca fortified cereal diet and a 64% decrease on the fortified cereal plus milk diet ($p<0.01$) (**Figure 2-5**).

Figure 2-6 shows that Ca-fortified cereal diet and fortified cereal plus non-fat milk diet caused 28% and 47% decreases, respectively, in FAS activity ($p<0.05$ vs. basal energy-restricted group). Similarly, the two high calcium diets caused corresponding decreases in adipocyte FAS mRNA, with 67% and 84% decreases on the Ca-fortified cereal diet and fortified cereal plus non-fat milk diet, respectively (**Figure 2-7**). Increasing dietary calcium on the energy-restricted diets caused a corresponding increase in lipolysis. The basal energy-restricted diet increased basal lipolysis by only 5% (NS) while the Ca-fortified cereal and fortified cereal plus non-fat dry milk diet stimulated lipolysis by 34% and 57%, respectively ($p<0.05$) (**Figure 2-8**).

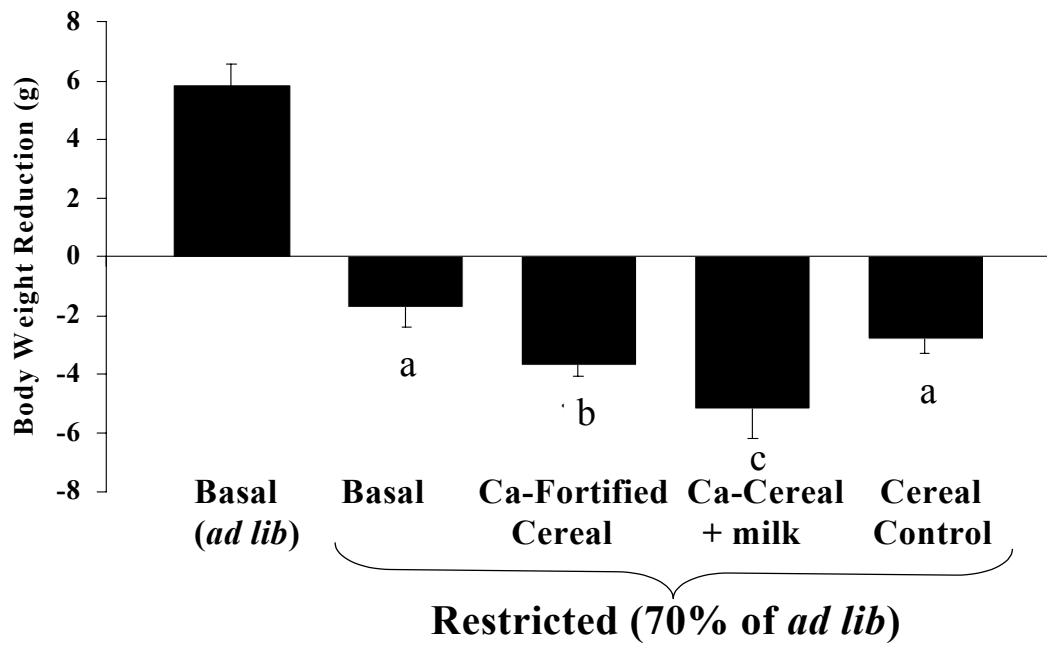


Figure 2-4. High calcium diets accelerate body weight loss in energy restricted aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

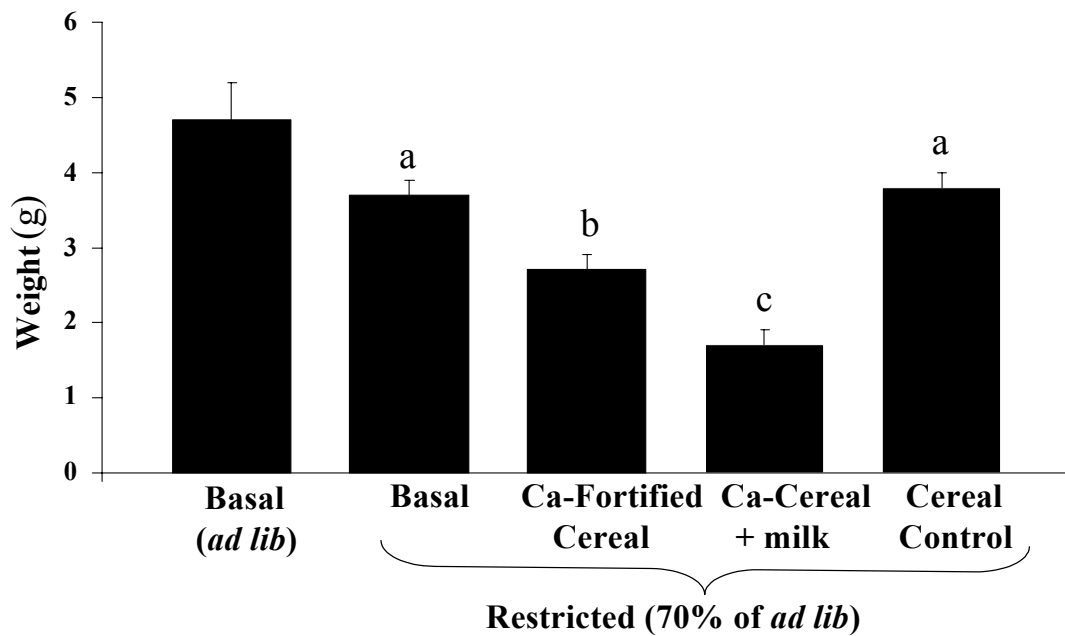


Figure 2-5. Effects of high calcium diets on fat pad mass (sum of four fat fads) in energy restricted aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

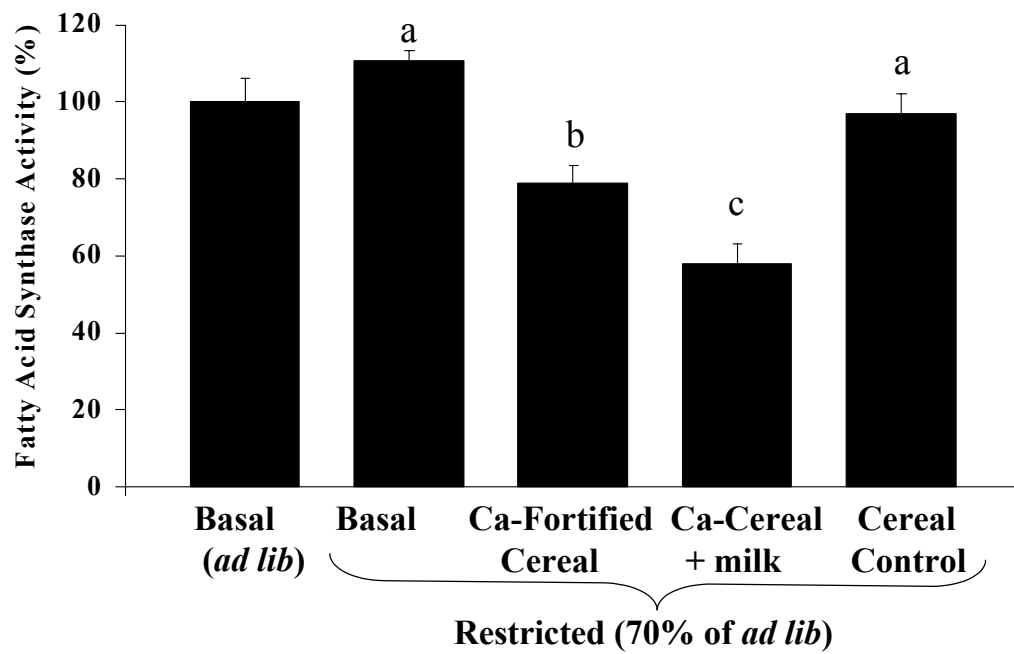


Figure 2-6. Effects of high calcium diets on fatty acid synthase activity in energy restricted aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

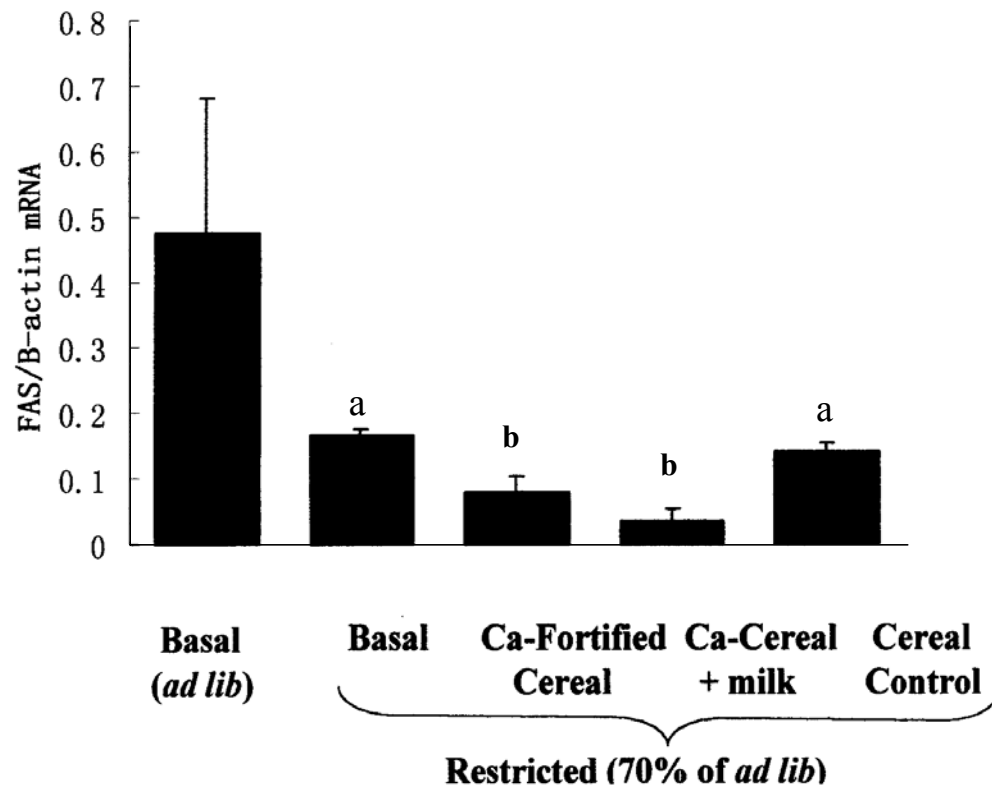


Figure 2-7. Effects of high calcium diets on fatty acid synthase mRNA in energy restricted aP2-agouti transgenic mice. Data are expressed as fatty acid synthase (FAS): actin ratio. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

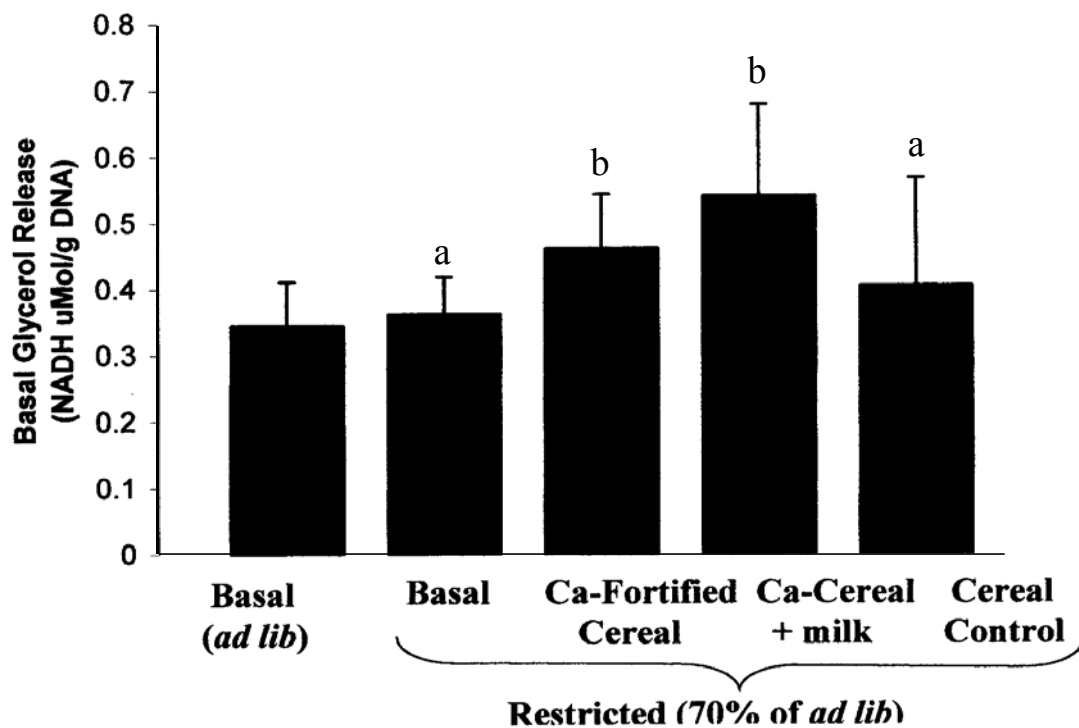


Figure 2-8. Effects of high calcium diets on basal lipolysis in energy restricted aP2-agouti transgenic mice. Glycerol release was used to measure lipolysis, as described in Material and Methods. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n = 8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

This apparent shift in the efficiency of energy utilization was reflected in the effects of the dietary treatments on core temperature. As expected, energy restriction significantly reduced core temperature (**Figure 2-9**). However, the Ca-fortified cereal diet and fortified cereal plus milk diets caused significant increases in core temperature compared to the animals fed the basal low calcium diet during energy restriction (**Figure 2-9**).

2.5 Discussion

Consistent with our previous reports, administration of basal low calcium diet caused a significant increase in body weight, fat pad mass and basal adipocyte $[Ca^{2+}]_i$ in aP2-agouti transgenic mice. In contrast, high-calcium diets caused a significant reduction in body weight, fat-pad mass, and basal adipocyte $[Ca^{2+}]_i$ in energy-restricted aP2 mice. Further, administration of high-calcium diets suppressed lipogenesis and stimulated lipolysis and thermogenesis in energy-restricted aP2 mice. Notably, inclusion of non-fat dry milk in the diet markedly amplified the effect.

We have previously shown that *agouti* stimulates Ca^{2+} influx (7, 19) and promotes energy storage in human adipocytes by coordinately stimulating the expression and activity of fatty acid synthase, a key enzyme in *de novo* lipogenesis and inhibiting lipolysis in a Ca^{2+} -dependent fashion (7, 8, 20). Thus, adipocyte $[Ca^{2+}]_i$ modulates energy storage, suggesting that adipocyte $[Ca^{2+}]_i$ is a logical target for modulation of adiposity.

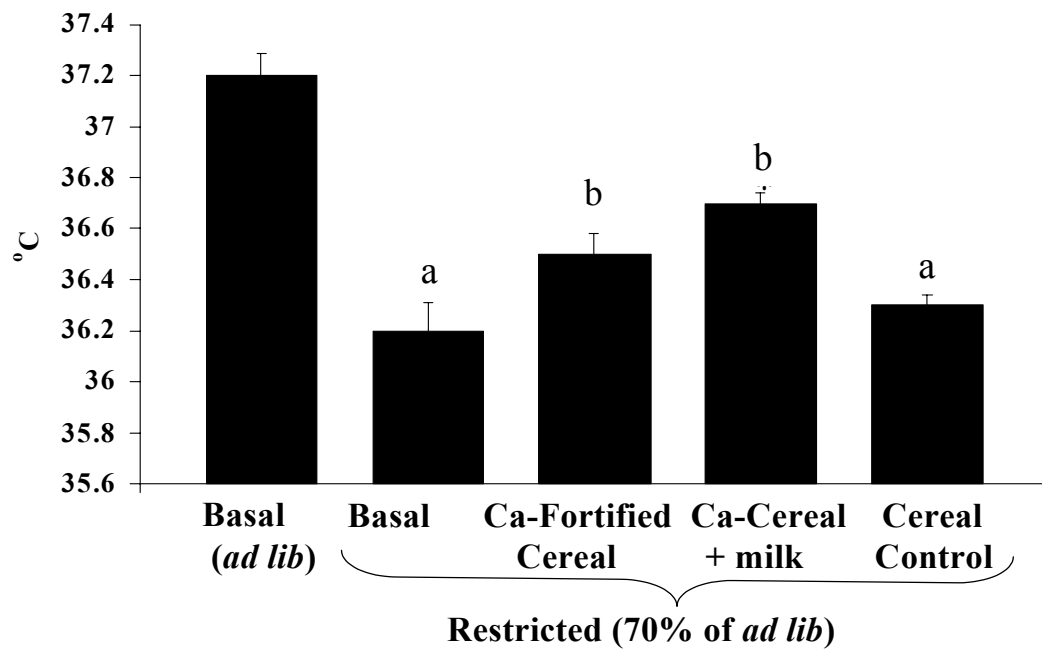


Figure 2-9. Effects of high calcium diets on core temperature in energy restricted aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

We have recently demonstrated that 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂-D₃) exerts a rapid action on human adipocytes to cause a sustained increase in [Ca²⁺]_i and a corresponding marked inhibition of lipolysis and stimulation of fatty acid synthase expression as well as activity (1, 2, 12). Further, increased circulating level of 1, 25-(OH)₂-D₃ was found in obese humans(21-25). In present study, we sought to decrease [Ca²⁺]_i by utilizing an increase in dietary calcium to suppress 1, 25-(OH)₂-D₃ level. This approach has previously been demonstrated to suppress 1, 25-(OH)₂-D₃ levels in multiple studies (26-28). Accordingly, we proposed that dietary calcium suppression of 1, 25-(OH)₂-D₃ would reduce adipocyte [Ca²⁺]_i and thereby inhibit triglyceride accumulation. Increasing dietary calcium resulted in increased adipocyte lipolysis and suppression of lipogenesis, similar to our previous observations (1-3). Moreover, these data extend our previous observations by demonstrating that dietary calcium not only attenuates diet-induced obesity but also accelerates weight loss and fat mass reduction secondary to caloric restriction in established obesity.

It is noteworthy that dairy and elemental calcium exerted qualitatively comparable effects; however, quantitatively, the effects were significantly different. Calcium in the form of dairy exerted a markedly greater effect on lipid metabolism and corresponding body-weight/adipose tissue mass reduction compared with a like quantity of elemental calcium. The effects of the high calcium cereal were similar to those described previously for calcium carbonate, producing significant attenuation of diet-induced obesity and accelerating body weight and fat loss during caloric restriction (1). However, the increase in dietary calcium from 1.2% to 1.3% by adding small amount of non-fat dry milk

doubled the rate of fat loss. Similar results were reported from utilization of yogurt as a calcium source to increase dietary calcium from 0.4% to 1.2% (29).

Thus, it is clear that dairy contains additional factors which augment the anti-obesity effect of calcium. This is consistent with the recent report from the CARDIA study, in which the inverse relationship between dairy consumption and each of the components of the insulin resistance syndrome, including obesity, was explained solely by dairy intake, and was not altered by adjustment for calcium, indicating an effect of dairy consumption independent of calcium intake (5).

Although the anti-obesity effect of dairy is clearly attributable to other factor(s) in addition to calcium, the identity of these factors is not yet clear. Although conjugated linoleic acid (CLA) has been demonstrated to attenuate adiposity (30-33), these effects are primarily attributable to the trans-10, cis-12 isomer of CLA, while the predominant isomer present in dairy is cis-9, trans-11 (30). Moreover, since non-fat dry milk was the source of dairy used in present study, as well as in our previous studies (1,2), no appreciable CLA could have been present in this fat-free product.

Alternatively, it is possible that one or more of the compounds in whey may contribute to the observed anti-obesity effect. Indeed, whey is a rich source of bioactive compounds (34) which may act independently or synergistically with calcium to attenuate lipogenesis, accelerate lipolysis and /or affect nutrient partitioning between adipose tissue and skeletal muscle. Notably, milk proteins have been reported to contain significant angiotensin converting enzyme (ACE) activity (35, 36). Furthermore, recent data demonstrated that adipocytes have an autocrine/paracrine rennin-angiotensin system and that adipocyte lipogenesis is regulated partially by angiotensin II (37). Indeed,

inhibition of the rennin-angiotensin system mildly attenuates obesity in rodents, and limited clinical observations support this concept in hypertensive patients treated with ACE inhibitors (37, 38).

In summary, high-calcium diets exert potent effects in enhancing reduction of body weight and fat pad mass in both *ad lib* fed and energy-restricted aP2-*agouti* transgenic mice. High-calcium diets suppressed adipocyte $[Ca^{2+}]_i$, stimulated lipolysis, inhibited lipogenesis and caused a corresponding increase in core temperature. Consequently, dietary calcium facilitates reduction of fat tissue mass and body weight by modulating energy partitioning. Moreover, dairy products contain other bioactive components which markedly amplify this effect, although the identity of this component(s) is not yet clear.

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Part Three

Calcium and Dairy Inhibit of Weight and Fat Regain during *Ad Libitum* Feeding Following Energy Restriction in Ap2-Agouti Transgenic Mice

3.1 Abstract

We have previously demonstrated that dietary calcium suppression of calcitriol reduces adipocyte Ca^{2+} , suppresses lipogenesis and increases lipid utilization during energy restriction. Notably, dairy calcium sources exert markedly greater effects. To determine the effects of dietary calcium and dairy on energy partitioning during subsequent re-feeding, we induced obesity in aP2-agouti transgenic mice with a high fat/high sucrose diet, then restricted energy intake on a high calcium (1.3%) diet for 6-weeks to induce fat loss, and then provided *ad libitum* access to the low calcium (0.4%) diet or to high (1.3%) calcium diets which utilized either calcium-fortified foods or dairy (milk or yogurt) for 6-weeks. Re-feeding the low calcium diet caused re-gain of all weight and fat, while all high calcium diets reduced weight gain by 53% ($p<0.01$). All high calcium diets stimulated adipose tissue UCP2 and skeletal muscle UCP3 expression and increased core temperature, but only the dairy diets elicited a marked (>10 -fold, $p<0.001$) increase in skeletal muscle PPAR α expression. The high calcium diet produced a 50% increase in lipolysis, a 63% decrease in fatty acid synthase (FAS) and prevented 55% of the fat regain ($p<0.001$), while the high dairy diets stimulated lipolysis by 271%, decreased FAS by 81% and prevented 85% of total fat regain ($p<0.01$). Thus, high Ca diets elicit a shift in energy partitioning and reduction of weight gain during re-feeding, with dairy Ca sources exerting markedly greater effects.

3.2 Introduction

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

Previous data from this laboratory demonstrate that adipocyte intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) plays a key role in disorders associated with obesity (5-9). Our understanding of the effects of $[\text{Ca}^{2+}]_i$ derives from studies of the mechanism of action *agouti*, an obesity gene expressed in rodent and human adipocytes (5,9). We have demonstrated that recombinant *agouti* protein stimulates the expression and activity of fatty acid synthase (FAS)(10-11), and inhibits lipolysis in both human and murine adipocytes (12-13). We have also demonstrated that *agouti* is expressed in human pancreas, and stimulates both Ca^{2+} -signaling and insulin release in human pancreatic islets, thereby contributing to hyperinsulinemia (14-15). This coordinated regulation of lipid metabolism by *agouti* appears to be responsible for the accumulation of triglyceride in adipose tissue. Further, these effects of *agouti* can be mimicked by stimulation of Ca^{2+} influx with KCl and blocked by $[\text{Ca}^{2+}]_i$ channel antagonists (16-17), indicating that strategies directed at reducing adipocyte intracellular $[\text{Ca}^{2+}]_i$ would also be expected to reduce triglyceride

accumulation in adipose tissue. Recent data from our laboratory demonstrates that calcitrophic hormones, such as 1, 25-(OH)₂-D₃, cause a significant, sustained increase in [Ca²⁺]_i in primary cultured human adipocytes and a corresponding inhibition of lipolysis (18). Consistent with this, we also reported that suppression of 1, 25-(OH)₂-D₃ by feeding high calcium diets inhibits [Ca²⁺]_i influx and subsequently attenuates diet-induced adiposity by simultaneously stimulating lipolysis and inhibiting lipogenesis (19-20), suggesting that dietary calcium may not only attenuate the diet-induced development of adiposity but also promote weight loss in established obesity. We also demonstrated that dairy sources of calcium exert substantially greater anti-obesity effects than calcium carbonate (19-20). Compared with calcium carbonate, using dairy as a calcium source induces a significantly greater attenuation of diet-induced obesity and acceleration of weight and fat loss during energy restriction in both mice and humans (9, 19-21). Thus dairy appears to contain additional bioactive compounds which provide an attenuation of adiposity beyond that food with calcium (5).

We have now used an experimental model to further investigate the effect of [Ca²⁺]_i in regulating lipid metabolism in aP2 transgenic mice under conditions of varying nutrient status: *ad libitum*, energy restriction and refeeding. The purpose of this study was to investigate the effect of dietary calcium content and sources in regulating lipid metabolism and energy partitioning in response to energy repletion following deprivation.

3.3 Materials and Methods

3.3.1 Animals and Diets

This study was divided into three phases. The first phase was designed to induce weight gain and fat accretion, the second phase to induce weight and fat loss, and the third phase to determine the role of calcium and dairy in attenuating weight and fat gain during re-feeding. We utilized aP2-*agouti* transgenic mice as a model of diet-induced obesity, as previously described (19, 20). These mice are useful models for diet-induced obesity in a genetically susceptible human population in that they are not obese on a standard AIN-93G diet, but develop mild to moderate obesity when fed high sucrose and/or high fat diets (19-20). Indeed, it is applicable that some individuals have manifested a much greater increase of weight fat than others, strongly suggesting the possibility that there is a subgroup that is genetically susceptible to obesity and a different subgroup that is relatively resistant.

Phase I

Six-week old aP2 transgenic mice were studied in a six-week obesity induction period on the basal low calcium/high sucrose/high fat diet, as follows.

60 aP2-*agouti* transgenic mice from our colony were placed at 6 weeks of age on a modified AIN 93 G diet with suboptimal calcium (calcium carbonate, 0.4%), sucrose as the sole carbohydrate source and providing 64% of energy, fat increased to 25% of energy with lard and protein(casein purified high nitrogen) provided 11% of energy.

Animals were studied for six weeks, during which food intake and spillage were measured daily and body weight, fasting blood glucose, food consumption and core temperature were assessed weekly. At the end of phase I, five representative animals were sacrificed to collect blood, fat pads, liver and muscle.

Phase II

At the end of the phase one, the 55 animals remaining from phase I were placed on a high calcium diet with energy intake limited to 70% of that found during *ad libitum* consumption. The high calcium diet used in this phase consisted of the basal diet with the addition of sufficient calcium-fortified cereal to bring the calcium content of the diet to 1.3%, with the macronutrients adjustments as described previously (19, 20). Animals were then studied for six weeks, during which food intake and spillage were measured daily and body weight, fasting blood glucose, food consumption and core temperature were assessed weekly. At the end of phase II, five animals were sacrificed as following to collect blood, fat pads, liver and muscle.

Phase III

At the end of the phase II, the 50 animals remaining from phase II were further randomized into five *ad libitum*-fed groups, as follows: **1) basal:** modified AIN 93 G diet with suboptimal calcium (0.4%), as described in phase I, **2) high-Ca cereal:** the high-Ca- (1.3%) cereal diet described in phase II; **3) high-Ca-cereal plus dry milk:** a high calcium cereal plus non fat milk diet. 1.2% calcium was derived from calcium fortified cereal and an additional 0.1% calcium content was derived from non-fat dry milk, with

20% protein was replaced by non-fat dry milk **4) yogurt:** a high calcium (1.3 %) diet in which sufficient spary-dried non-fat yogurt was added to the basal diet to increase calcium to 1.3%, with 100% protein was replaced by non-fat dry yogurt **5) cereal control:** the basal diet with a calcium-free control cereal (identical to the calcium-fortified cereal used in diet 3, but without the calcium fortification) with a final calcium concentration of 0.4% and macronutrients adjusted to match the other diets. 10 animals /group were studied for six weeks, during which food intake and spillage were measured daily and body weight, fasting blood glucose, food consumption and core temperature were assessed weekly. At the conclusion of the study, all mice were sacrificed under isoflurane anaesthesia and blood collected via cardiac puncture, and fat pads, soleus and gastrocnemius muscles were immediately excised, weighed and used for further study, as described below.

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

3.3.2 Core Temperature

Core temperature was used as an indirect metabolic index to determine the effect of the diets on thermogenesis. Temperature was measured via a thermocouple (Columbus Instruments, Columbus, Ohio) weekly. The probe was inserted a constant distance (1.8 cm) into the rectum of each mouse. After stabilization (10s), the temperature was recorded every 5 s for 30 s. All core temperature measurements were performed between 8:00 and 9:00 A.M.

3.3.3 Adipocyte $[Ca^{2+}]_i$ Measurement

Adipose tissue was first washed several times with Hank's Balanced Salt Solution (HBBS), minced into small pieces, and digested with 0.8 mg/ml type I collagenase in a shaking water bath at 37° C for 30 min. Adipocytes were then filtered through sterile 500- μ m nylon mesh and cultured in 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 μ mol/L) in the same buffer in dark for 1 h at 37° 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, Mass.). The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu 4915 CCD camera. Fluorescent images were captured alternatively at excitation wavelength of 340 and 380 nm with an emission wavelength of 520 nm. $[Ca^{2+}]_i$ was calculated by using a ratio equation.

3.3.4 Fatty Acid Synthase (FAS) Activity Assay

FAS activity was determined spectrophotometrically in crude cytosolic extracts of mouse adipose tissue. Mouse abdominal fat pads were homogenized in 250 mmol/L sucrose solution containing 1 mmol/L ethylenediamine-tetraacetic acid (EDTA), 1 mmol/L dithiothreitol (DTT), and 100 μ mol/L phenylmethylsulfonyl fluoride (PMSF) (pH 7.4). The homogenate was centrifuged at 18,500 X g for 1 h, and the supernatant was used for measuring oxidation rate of NADPH. The final FAS activity was normalized to DNA, which was measured by CyQUANT cell proliferation assay kit according to manufacturer's instruction (Parkard instrument Company, Inc., Downers Grove, IL).

3.3.5 Lipolysis Assay

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

3.3.6 Total RNA Extraction

Total cellular RNA isolation kit (Ambion Inc., Austin, TX) was used to extract total RNA from mouse abdominal adipose tissue and soleus muscle according to manufacturer's instruction.

3.3.7 Quantitative Real-time PCR

Mouse abdominal adipose tissue FAS, PPAR gamma, UCP2 mRNA, and mouse soleus muscle UCP3 and PPAR alpha mRNA were quantitatively measured using a Smart Cycler Real Time PCR System (Cepheid, Sunnyvale, CA) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg, NJ). The primers and probes for mouse genes are as follows. FAS: forward primer: 5'-CCCAGAGGCTTGTGCTGACT-3', reverse primer: 5'-CGAATGTGCTTGGCTTGGT-3', probe: 5'-CCGATCTGGAATCCGCACCGG-3'; PPAR alpha: forward primer: 5'-CGATACTGTCCTCCTTGATGA-3', reverse primer: 5'-CGCGTGTGATAAAGCCATTG-3', probe: 5'-ACGCGATCAGCATCCCGTCTTTG-3'; PPAR gamma forward primer: 5'-GCCTATGAGCACTTCACAAGAAATT-3', reverse primer: 5'-TGCGAGTGGTCTTCCATCAC-3', probe: 5'-TCTGCCCCACCAACTTCGGAATCAG-3'; UCP2 forward primer: 5'-GCGTTCTGGGTACCATCCTAAC-3', reverse primer: 5'-GCGACCAGCCCATTGTAGA-3', probe: 5'-GCCTATGAGCACTTCACAAGAAATT-3'; UCP3 forward primer: 5'-CCTCTACGACTCTGTCAAGCAGTTCTAC-3', reverse primer: 5'-CAGCCTGCCAGAATCCTGAT-3', probe: 5'-CGCTGGAGTGGTCCGCTCCCT-3'.

Pooled mouse abdominal adipose tissue total RNA was serial-diluted in the range of 1.5625-25 ng and used to establish a standard curve of desired gene; total RNAs for unknown samples were also diluted in this range. Reactions of quantitative RT-PCR for standards and unknown samples were also performed according to the instructions of Smart Cycler System (Cepheid, Sunnyvale, CA) and TaqMan Real

Time PCR Core Kit (Applied Biosystems, Branchburg, NJ). The mRNA quantitation for each sample was further normalized using the corresponding 18s or cyclophilin quantitation, with 18S forward primer: 5'-AGTCCCTGCCCTTTGTACACA-3', reverse primer: 5'-GATCCGAGGGCCTCACTAAAC-3', probe: 5'-CGCCCGTCGCTACTACCGATTGG-3'; Cyclophilin forward primer: 5'-GGTGGAGAGCACCAAGACAGA-3', reverse primer: 5'-GCCGGAGTCGACAATGATG-3', probe: 5'-AGCCGGGACAAGCCACTGAAGGAT-3'.

3.3.8 Carnitine Palmitoyltransferase (CPT) Assay

Liver samples were first homogenized in 10 volumes (wt/vol) of 0.25M sucrose/0.2mM EDTA (PH 7.5) in a glass tissue grinder to isolate hepatocyte mitochondria. The tissue homogenates were then centrifuged for 12minutes at 750g to collect the supernatant, which was further centrifuged for 12 min at 6,700g. These centrifugations were repeated twice and the pellet was then suspended in 4 ml of mitochondrial suspension buffer (70 mM sucrose, 220mM mannitol, 2mM HEPES buffer, 1mM EDTA, PH 7.4). CPT activity was determined by measuring the initial rates of total CoASH formation by the DTNB reaction from palmitoyl CoA by mitochondria individually with l (-)-carnitine and d (+)-carnitine using a spectrophotometry method. Final data were normalized to cytosolic protein, which was assayed by the method of Bradford.

3.3.9 Statistical Analysis

All data were evaluated for normality of distribution and equality of variance prior to statistical analysis. Variables with skewed distribution were ln-transformed. Outcomes are shown as means and SD and were evaluated for statistical significance by one-way analysis of variance (ANOVA), and significantly different group means were then separated by the least significant difference (LSD) test by using SPSS (SPSS Inc, Chicago, IL.).

3.4 Results

Body weight, weight regain and fat pad weight data are summarized in figure 1. Although we did not observe significant difference in food consumption among the five group of mice during the refeeding following energy restriction (average daily food intake per mice during *ad libitum* refeeding: basal group: 3.5 ± 0.3 g/day, high-Ca cereal group: 3.7 ± 0.3 g/day, high-Ca cereal plus dry milk: 3.8 ± 0.3 g/day, yogurt group: 3.9 ± 0.4 g/day and cereal control group: 3.7 ± 0.3 g/day, $p > 0.05$), there was a striking effect of diet on weight gain ($p < 0.05$) (**Figure 3-1 A**) and fat pad weight (**Figure 3-1 B**) ($p < 0.04$). Mice re-fed the low calcium diets exhibited weight gain and an increase in fat pad mass. In contrast, mice on the high calcium diets exhibited approximately 50% of this regain, with increases of body weight and fat pad mass. Further, the dairy-based high calcium diets were more effective in preventing fat gain, as the high dairy diets prevented 85% of the fat gain while the high calcium diet prevented 55% of the fat gain.

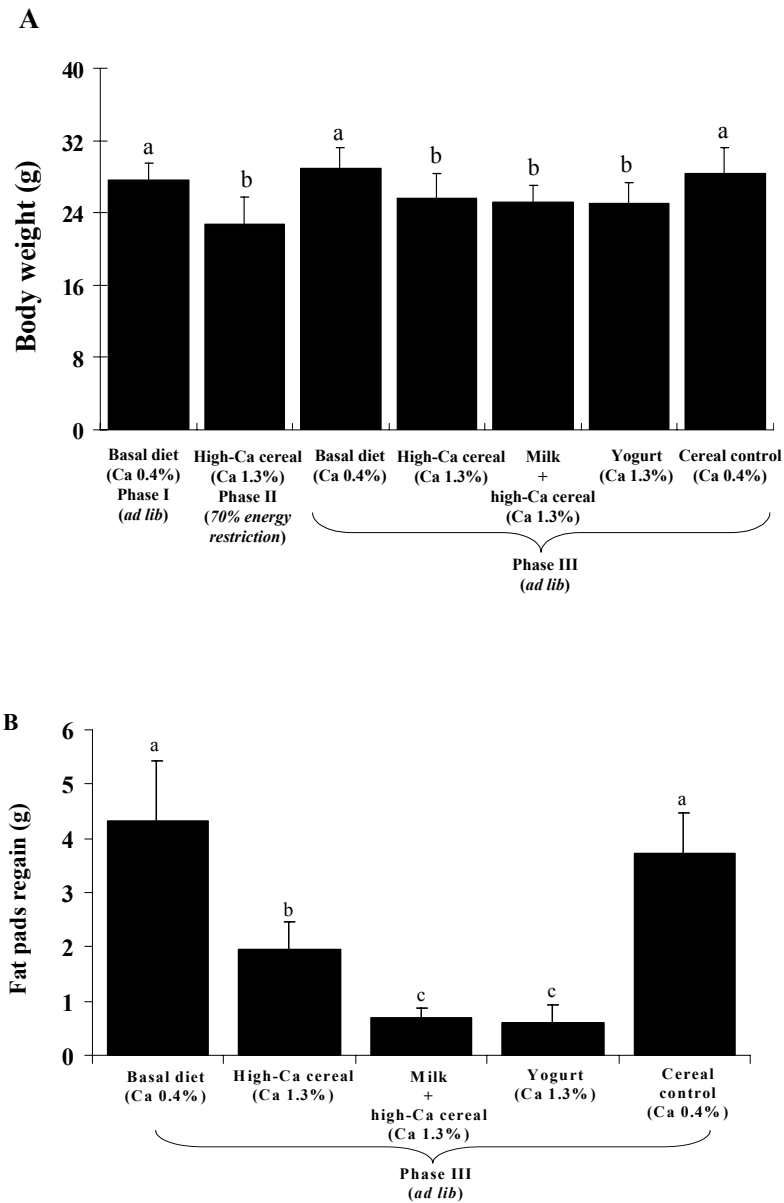


Figure 3-1. Effect of dietary calcium and dairy on weight and fat mass regain in aP2 transgenic mice. (A) body weight and (B) fat pads weight. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

Figure 3-2 shows adipose tissue lipolysis, FAS expression and FAS activity. We found a slight decrease (20%, $p<0.05$) in lipolysis in mice on 70% restricted high calcium cereal diet compared to the high sucrose, high fat, low calcium diet fed *ad libitum* (**Figure 3-2 A**). Upon refeeding after energy restriction, the mice on the high calcium diets exhibited a significantly higher level of lipolysis than mice on the low calcium diets ($p<0.05$), and the dairy-based high calcium diets exhibited a significantly greater effect than the non-dairy source ($p<0.001$). Adipose tissue FAS activity and expression responded to dietary manipulations in an inverse fashion to lipolysis responses. **Figure 3-2 B** show that energy restriction with high-Ca fortified cereal diet decreased FAS expression and activity by 99% and 91% respectively (FAS activity data not shown). Upon refeeding, the low calcium diets restored FAS activity and expression back to the basal level (cereal control diet) or higher (low calcium basal diet, $p<0.03$). In contrast, the groups on high calcium diets exhibited marked lower level of adipose FAS activity and expression. Furthermore, the mice re-fed the dairy-based high calcium diets exhibited a significantly lower level of FAS expression than those fed the non-dairy-based high calcium diet ($p<0.001$).

Energy restriction in aP2 mice for 6 wks using high calcium diet resulted in a 79% decrease in adipocyte $[Ca^{2+}]_i$ ($380 \pm 43\text{nM}$ vs. $79 \pm 10\text{nM}$). Upon refeeding, the low calcium diets induced a 4 to 5-fold ($p<0.001$) increase in adipocyte $[Ca^{2+}]_i$, (**Figure 3-3**), while there was no significant increase on the high calcium diets, suggesting that diet-induced dysregulation of adipocyte $[Ca^{2+}]_i$ is associated with diet-induced adiposity in aP2-*agouti* transgenic mice.

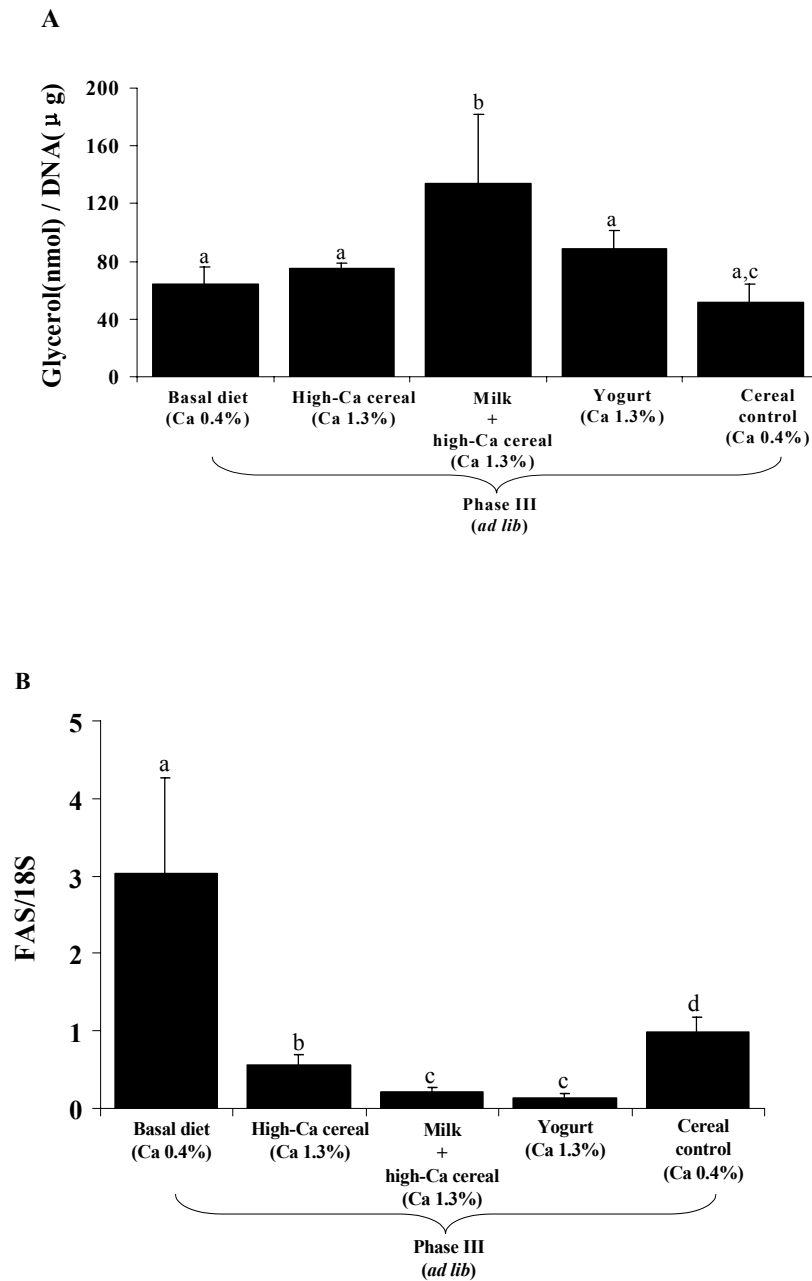


Figure 3-2. Effect of dietary calcium and dairy refeeding on lipid metabolism in aP2 transgenic mice. (A) adipose basal lipolysis, (B) FAS expression. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

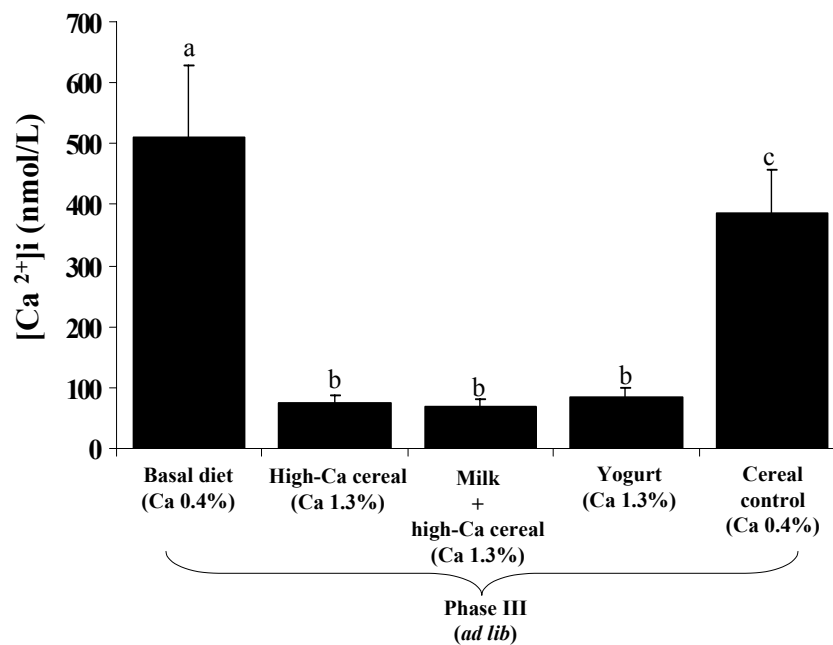


Figure 3-3. Effect of dietary calcium and dairy refeeding on adipocyte intracellular calcium in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

To determine if shifts in liver fat oxidation potentially contributed to changes in adiposity, we also examined liver CPT activity (**Figure 3-4**). Upon refeeding, treatment of aP2-transgenic mice with the low calcium diets resulted in a slight increase in liver CPT activity by 44% and 56% while the high calcium diets increase CPT activity by 150%. However, there was no significant effect of the source of calcium on liver CPT activity.

The shift in energy metabolism in refeeding was also confirmed further by dietary-induced increase in UCP2 expression in adipose tissue (**Figure 3-5**) and UCP3 (**Figure 3-6**) expression in skeletal muscle. Upon refeeding, the high calcium diets induced 8 to 19-fold increases in UCP2 expression in white adipose tissue and 30 to 34-fold increases in UCP3 expression in skeletal muscle, compared with the low calcium diets ($p < 0.001$). Furthermore, the dairy-based high calcium diets exerted greater effects on adipose tissue UCP2 expression than the non-dairy based high calcium diet, although a comparable difference between calcium sources was not found for skeletal muscle UCP3 expression.

We also found dietary-calcium induced increases in core temperature, with increases on refeeding the high-calcium, high-calcium plus milk and yogurt respectively (**Figure 3-7**). This increase, coupled with the lack of difference in food intake in refeeding, suggests a shift in efficiency of energy metabolism from energy storage to thermogenesis.

This shift in energy metabolism was also evident in studies of the peroxisome proliferator-activated receptor alpha (PPAR α), a transcriptional factor which regulates

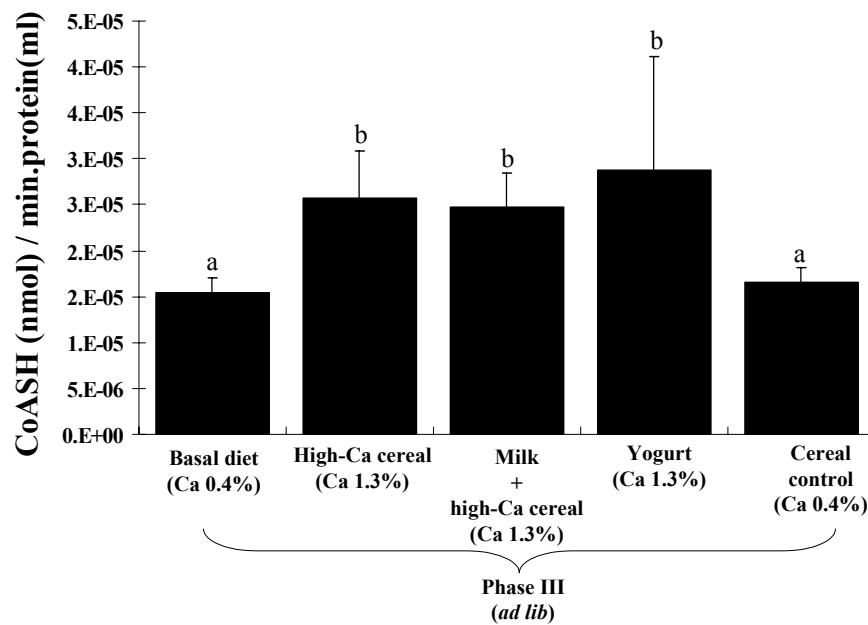


Figure 3-4. Effect of dietary calcium and dairy refeeding on liver CPT activity in aP2 transgenic mice. Data are expressed as mean $\pm$ SE (n=5 for phase I and phase II, n=8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

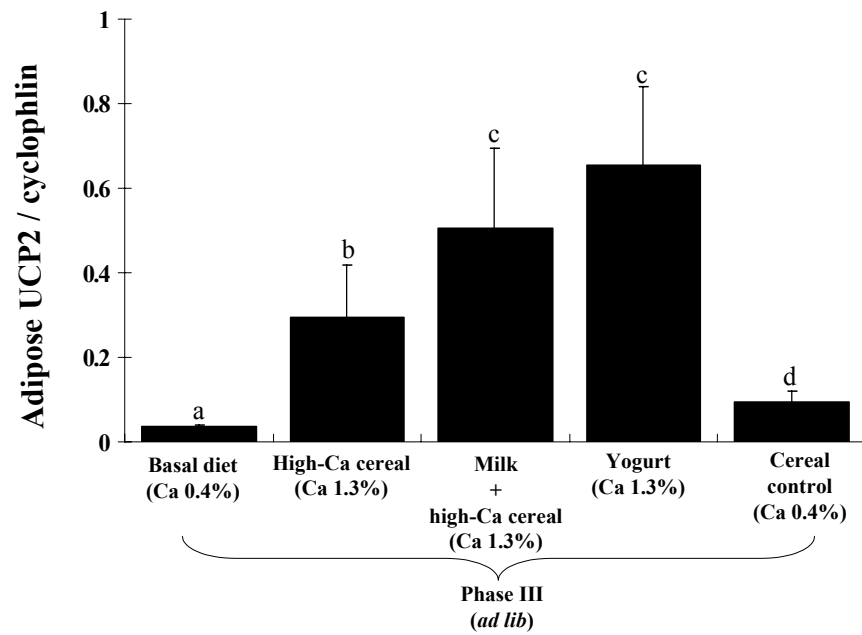


Figure 3-5. Effect of dietary calcium and dairy refeeding on adipose UCP2 expression in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

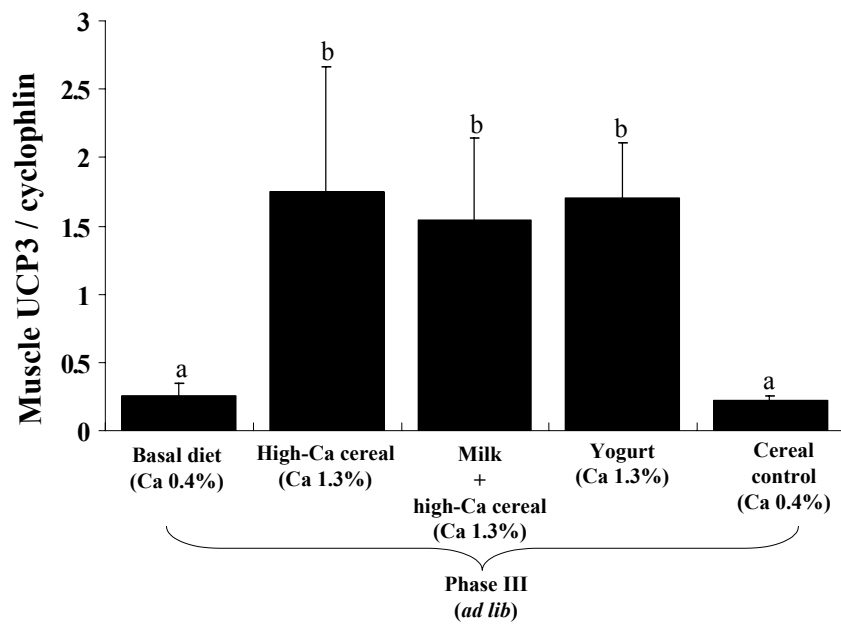


Figure 3-6. Effect of dietary calcium and dairy refeeding on UCP3 expression in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

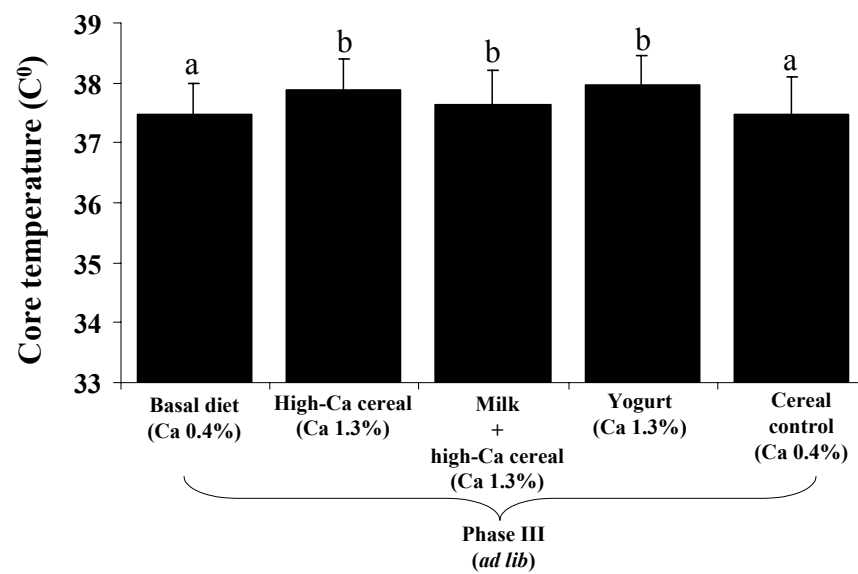


Figure 3-7. Effect of dietary calcium and dairy refeeding on core temperature in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

cellular lipid catabolism Refeeding with the dairy-based high calcium diets resulted in a marked (> 10 -fold) increase in skeletal muscle PPAR α compared to the low calcium diets (**Figure 3-8**, $p<0.03$), while the non-dairy calcium-enriched diet exerted no effect on PPAR α expression. **Figure 3-9** indicates that mice refed high calcium diets exhibited significant lower level of PPAR γ expression, suggesting dietary calcium may also inhibit fat mass regain by suppressing adipogenesis.

Serial measurements of plasma glucose and insulin demonstrate a diabetogenic effect of the basal high fat, high sucrose and low calcium diet, with an increase in fasting glucose from 121 ± 23 to 169 ± 16 mg/dl and a corresponding degree of compensatory hyperinsulinemia and hyperleptinemia. On refeeding after fasting, hyperglycemia, hyperleptinemia and hyperinsulinemia were observed in mice on low calcium diet but not in mice on high calcium diets (**Table 3-1**).

3.5 Discussion

It is well accepted that energy balance are core factor involved in the obesity epidemic, with increases in energy intake contribute to a net positive energy balance and progressive weight gain. However, it is also well known that obesity is a complex genetic trait, with multiple genes interacting to confer relative resistance or susceptibility to positive energy balance. Similarly, specific micro- or macronutrients, dietary patterns, or both may modulate the same metabolic pathways affected by these genetic factors and thereby alter nutrient and energy partitioning. This concept is supported a potential body

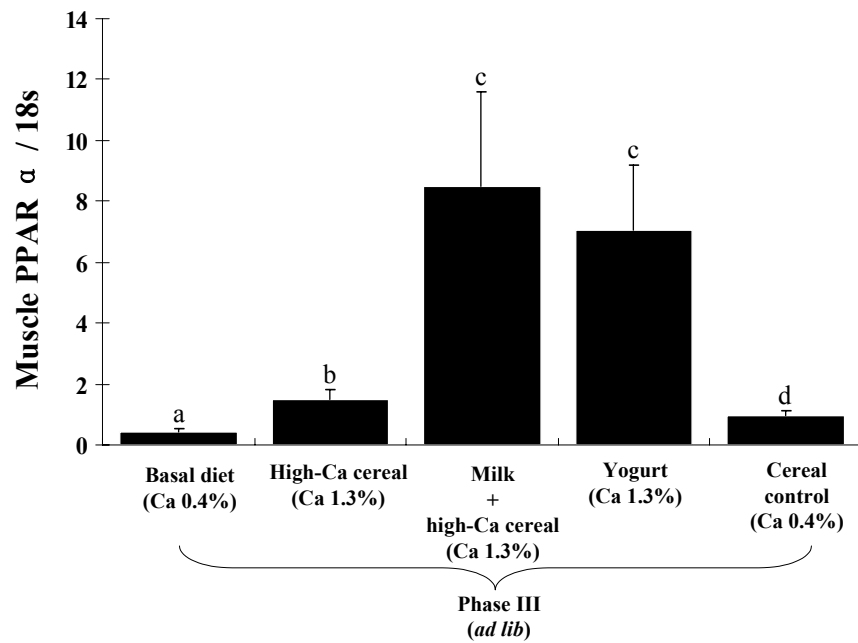


Figure 3-8. Effect of dietary calcium and dairy refeeding on PPAR α expression in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

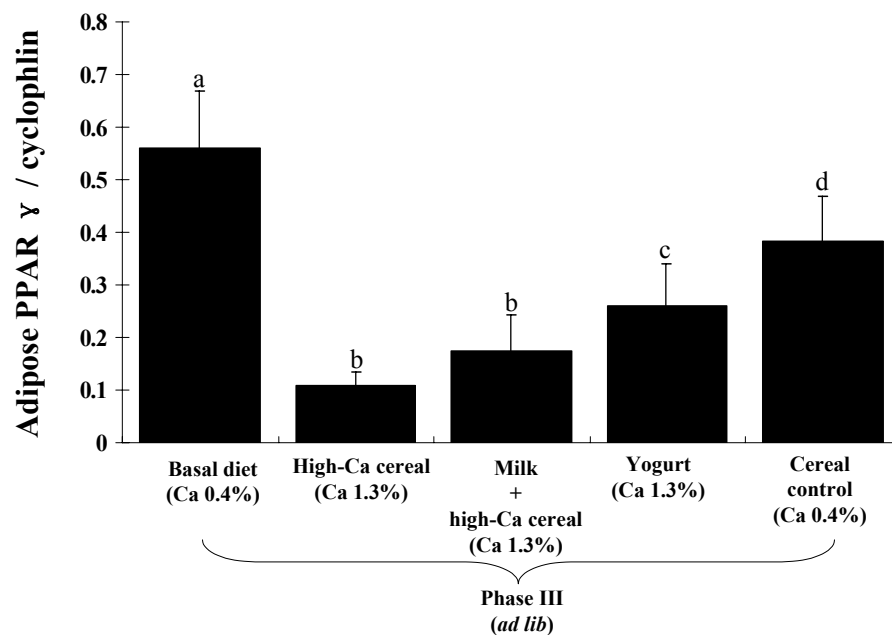


Figure 3-9. Effect of dietary calcium and dairy refeeding on PPAR γ expression in aP2 transgenic mice. Data are expressed as mean $\pm$ SEM (n=5 for phase I and phase II, n =8 for phase III). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

Table 3-1. Effect of dietary calcium and dairy refeeding on serum insulin, leptin and glucose concentrations of aP2-agouti transgenic mice.

Groups	Insulin (ng/ml)	Leptin (ng/ml)	Glucose (mg/dl)	I:G ratio($\times 10^{-6}$)
I-Basal	9.12 $\pm$ 3.21	13.15 $\pm$ 4.18	169.42 $\pm$ 16.98	0.07 $\pm$ 0.19
II-Calcium Cereal	1.20 $\pm$ 0.46 ^{ab}	4.38 $\pm$ 3.39 ^{ab}	127.93 $\pm$ 18.61 ^{ab}	0.01 $\pm$ 0.02 ^{ab}
III-Basal	12.12 $\pm$ 7.43	17.12 $\pm$ 6.13	145.30 $\pm$ 19.32 ^b	0.12 $\pm$ 0.38
III-Calcium Cereal	3.28 $\pm$ 0.20 ^{ab}	8.77 $\pm$ 0.86 ^{ab}	131.00 $\pm$ 15.07 ^b	0.03 $\pm$ 0.01 ^{ab}
III-Clacium Cereal+Milk	2.86 $\pm$ 0.25 ^{ab}	6.33 $\pm$ 1.13 ^{ab}	112.9 $\pm$ 10.59 ^{ab}	0.03 $\pm$ 0.02 ^{ab}
III-Yogurt	2.20 $\pm$ 0.34 ^{ab}	6.40 $\pm$ 2.21 ^{ab}	129.8 $\pm$ 17.12 ^{ab}	0.02 $\pm$ 0.02 ^{ab}
III-Cereal Control	4.46 $\pm$ 0.84 ^{ab}	14.07 $\pm$ 4.46	151.4 $\pm$ 20.10 ^b	0.04 $\pm$ 0.04 ^{ab}

^a p<0.05 vs. III-Basal *ad lib*.

^b p<0.05 vs. I-Basal *ad lib*

* Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =10).

Statistical significance is as indicated in each panel.

of evidence emerged in recent years which indicated that dietary calcium and dairy products may play a role in modulation of adiposity independent of energy restriction, with high dietary calcium and addition of dairy products attenuate weight and fat gain without energy restriction and stimulates weight and fat loss during energy restriction. However, the effect of dietary calcium and dairy products on prevention of fat and weight regain is not yet clear.

The present study demonstrates that dietary calcium exerts marked effects in the regulation of weight and fat regain in aP2-agouti transgenic mice, as the high calcium diets inhibited weight and fat regain while the low calcium diets promoted fat and weight regain. Notably, dairy source of calcium appears to exert greater effects than elemental calcium. Although there were no significant diet related difference in energy intake during *ad libitum* refeeding following energy-restriction, the mice refed the low calcium diets showed a rapid, pronounced regain of weight and fat which exceeded that lost during energy restriction. In contrast, the mice refed the high calcium diets regained only 50% of the weight lost during energy restriction period, suggesting that dietary calcium inhibits weight and fat regain by decreasing efficiency of energy utilization during rebound food ingestion.

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

25-(OH)₂-D₃ has been previously shown to stimulate [Ca²⁺]_i in multiple cell types, including vascular smooth muscle cells, pancreatic β cells and adipocytes (15, 17, 18, 20). We demonstrated that 1,25-(OH)₂-D₃ also acts on human adipocytes to cause a rapid sustained increase in [Ca²⁺]_i and a coordinated activation of FAS and inhibition of lipolysis (5, 18, 20). Increased 1,25-(OH)₂-D₃ has also been observed in obese humans, further suggesting that 1,25-(OH)₂-D₃ may exert effects in human energy metabolism(20). Consistent with this concept, we recently demonstrated that dietary calcium not only attenuates diet-induced obesity but also accelerates weight loss and fat mass reduction secondary to caloric restriction in established obesity in both mice (19) and human (9). In present study, antagonism of [Ca²⁺]_i was again achieved through a dietary intervention by increasing dietary calcium. We proposed that suppression of 1, 25-(OH)₂-D₃ dietary calcium would reduce adipocyte [Ca²⁺]_i and thereby regulates lipid metabolism in response to different energy status. Indeed, the present study extended our previous observations by demonstrating that dietary calcium not only attenuates diet-induced obesity and accelerates weight loss and fat mass reduction in energy restriction in established obesity, but also inhibit weight and fat mass regain during refeeding secondary to energy restriction.

Notably, increasing dietary calcium not only modulates energy petitioning by inhibiting lipogenesis and stimulating lipolysis, but also stimulates UCP2 and UCP3 expression and causes an increase in core temperature, an indirect metabolic index associated with thermogenesis during refeeding after energy restriction. This may further contribute to the observed attenuation of weight and fat pad regain during refeeding. This concept is supported by our previous observation that 1, 25-(OH)₂-D₃ inhibits UCP2

expression in human adipocytes, indicating that suppression of $1, 25\text{-(OH)}_2\text{-D}_3$ by increasing dietary calcium may stimulate UCP2 induced thermogenesis and fat oxidation and thereby result in decreased energy efficiency.

The function of UCPs is not restricted to thermoregulation, as a growing body of evidence links UCP expression to fatty acid metabolism and fatty acid flux in various tissues (23-24). Previous studies have demonstrated that free fatty acids increase UCP2 and UCP3 expression in adipocytes, pancreatic beta cells and myocytes (25-28). These data strongly support a regulation of UCP2 and UCP3 expression by fatty acids and also indicate that UCPs activity could be related to fatty acid oxidation. In fact, UCP2 and UCP3 may function as free fatty acid transporter and thereby increases fatty acid utilization (29-30).

The regulation of UCP transcription is attributable a group of ligand-regulated-transcription factors such as peroxisome-proliferation-activated factors, which can be activated upon binding fatty acid ligand (31). Since PPARs are also involved in the regulation of adipocyte differentiation and lipid oxidation (31), it is possible that PPARs are the key regulators which coordinate the activation of lipid metabolism and oxidation in adipocytes. In fact, a PPAR response element (PPRE) has been identified in the UCP promoter region (32-33), and PPAR α activation in mice is sufficient to induce liver UCP-2 expression (34). Consistent with this, our data showed that high calcium diets induced increases in UCP2 and UCP3 expression were accompanied by marked increases in PPAR α expression during refeeding, indicating that high dietary calcium may stimulates UCPs via a PPAR α -dependent mechanism. Consistent with the effect of dietary calcium on stimulating thermogenesis and energy partitioning via up-regulation of UCPs, we also

observed high calcium diets caused a significant increase in hepatic activity of CPT during refeeding, suggesting that dietary calcium may stimulate a diversion of hepatic fatty acid metabolism towards oxidation pathway during *ad libitum* refeeding. These data suggest that dietary calcium reduces lipid accumulation via energy repartitioning and dissipation, resulting in less regain of fat during *ad libitum* refeeding.

We also found that refeeding high calcium diets caused a significant decrease in PPAR γ expression in white adipose tissue. Unlike PPAR α , which is highly expressed in liver and skeletal muscle and modulates fatty acid oxidation, PPAR γ is mainly restricted to white and brown adipose tissue and regulates adipogenesis (35). Accordingly, these present data suggest the possibility that dietary calcium may also inhibit adiposity by decreasing adipogenesis during food re-administration.

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

Additional factors in dairy responsible for this effect besides calcium have been recently discussed. Several investigators have reported that conjugated linoleic acid (CLA), a component in dairy product and meat is an effective regulator of body fat accumulation and retention (36). However, the dairy diet utilized in this study was from non-fat dry milk and fat-free yogurt, which does not contain CLA, indicating that additional effect cannot be attributed to CLA. Other components of dairy which may contribute to these anti-obesity effects include branch chain amino acids and small bioactive peptides. Our preliminary data in mice isolated most of the additional bioactivity of dairy products to the whey fraction (unpublished data), which containing high proportion of branch chain amino acids (BCAAs, leucine, isoleucine, and valine). BCAAs, especially leucine, play a specific metabolic role in regulation of energy metabolism and muscle protein synthesis (37-38). In skeletal muscle, leucine stimulates protein synthesis and inhibits protein catabolism through multiple independent mechanisms (39-40). Leucine stimulates protein synthesis through the mammalian target of rapamycin (mTOR) pathways, 70-kDa ribosomal protein S6 kinase activity, and enhances eIF4E-binding protein phosphorylation and the association of eukaryotic initiation factor eIF4E with eIF4G (41-42). Accordingly, dairy products provide sufficient BCAAs to be able to maintain the high concentration of intracellular leucine required by these signaling pathways. Milk proteins have also been reported to contain significant angiotensin converting enzyme (ACE) activity (43-44). Furthermore, recent data demonstrate that adipocytes have an autocrine/paracrine rennin-angiotensin system and that adipocyte lipogenesis is regulated partially by angiotensin II (45). Moreover, inhibition of the rein-angiotensin system mildly attenuates obesity in rodents and clinical

observations in hypertensive patients treated with ACE inhibitors support this concept (21). In addition, our recent data demonstrate that dairy-derived ACE inhibitor augments the anti-obesity effects of calcium in aP2 transgenic mice.

In summary, high-calcium diets exert potent effects in weight maintenance during refeeding following energy-restriction in aP2-agouti transgenic mice. High-calcium diets suppressed adipocyte $[Ca^{2+}]_i$, stimulated lipolysis, inhibited lipogenesis and a corresponding increase in core temperature. Consequently, dietary calcium facilitates reduction of fat tissue mass and body weight in refeeding by modulating energy metabolism, which serves to reduce energy storage and increase beta-oxidation. This concept that calcium modulation of adiposity is consistent with the observations obtained epidemiologically in the NHANES III data set , Quebec Family Study; CARDIA and a recent clinical trial (20, 46, 47, 9). Moreover, dairy source of calcium exerts greater effects o preventing regain compared to supplementary calcium carbonate.

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Part Four

Effects of Mitochondrial Uncoupling on Adipocyte Intracellular Ca^{2+} and Lipid Metabolism

This part is a slightly revised version of a paper by the same name published in the *Journal of Nutritional Biochemistry* in 2003 by Xiaocun Sun, Michael B. Zemel:

Xiaocun Sun, Michael B. Zemel. Effect of mitochondrial uncoupling on adipocyte intracellular Ca^{2+} and lipid metabolism, *Journal of Nutritional Biochemistry* 14 (2003) 219-226.

4.1 Abstract

Previous data from this laboratory demonstrate that increased intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) coordinately regulates human and murine adipocyte lipid metabolism by stimulating lipogenesis and inhibiting lipolysis. However, recent data demonstrate metabolic uncoupling increase $[\text{Ca}^{2+}]_i$ but inhibit lipogenesis by suppressing fatty acid synthase (FAS) activity. Accordingly, we have evaluated the interaction between mitochondrial uncoupling, adipocyte $[\text{Ca}^{2+}]_i$, and adipocyte lipid metabolism. Pretreatment of 3T3-L1 cells with mitochondrial uncouplers (DNP or FCCP) amplified the $[\text{Ca}^{2+}]_i$ response to depolarization with KCl by 2-4 fold ($p < 0.001$), while this increase was prevented by $[\text{Ca}^{2+}]_i$ channel antagonism with lanthanum. Mitochondrial uncouplers caused rapid (within 4hr) dose-dependent inhibition of FAS activity ($p < 0.001$), while lanthanum caused a further additive inhibition. The suppression on FAS activity induced by uncoupler DNP was reversed by addition of ATP. Mitochondrial uncouplers increased FAS expression significantly while $[\text{Ca}^{2+}]_i$ antagonism with lanthanum decreased FAS expression ($P < 0.001$). In contrast, mitochondrial uncouplers independently inhibited basal and isoproterenol-stimulated lipolysis (20-40%, $p < 0.001$), while this inhibition was

fully reversed by lanthanum. Thus, mitochondrial uncoupling exerted short-term regulatory effects on adipocyte $[Ca^{2+}]_i$ and lipogenic and lipolytic systems, serving to suppress lipolysis via a Ca^{2+} -dependent mechanism and FAS activity via a Ca^{2+} -independent mechanism.

4.2 Introduction

The discovery of novel homologues of the brown adipose tissue mitochondrial uncoupling protein (UCP), such as UCP2, has provoked intensive study of these mitochondrial proteins and the role that they may play in energy metabolism (1). Since lipogenesis is dependent upon mitochondrial ATP production (2), mitochondrial uncoupling in adipocytes may contribute to control of lipid metabolism.

Previous data from our laboratory demonstrates that intracellular calcium ($[Ca^{2+}]_i$) plays a key role in the regulation of adipocyte energy metabolism (3). Increased $[Ca^{2+}]_i$ stimulates the expression and activity of fatty acid synthase (FAS), a key enzyme in de novo lipogenesis, and inhibits basal and agonist-stimulated lipolysis in both human and murine adipocytes(4-5). However, little is known of the mitochondrial regulation of $[Ca^{2+}]_i$ and lipid metabolism in adipose tissue. Accordingly, in the present study, the role of mitochondrial uncoupling in regulating lipolysis and lipogenesis as well as intracellular calcium have been examined in differentiated 3T3-L1 adipocytes. We used two different mitochondrial uncouplers to induce uncoupling effect on adipocytes mitochondria (6-7). We report that mitochondrial uncoupling increases $[Ca^{2+}]_i$ and down-regulates lipolysis via a calcium-dependent mechanism. In contrast, the effect of

mitochondrial uncoupling on fatty acid synthase is more complex, with up-regulation of FAS expression via a calcium-dependent mechanism, and suppression of FAS activity via a Ca^{2+} -independent mechanism.

4.3 Materials and Methods

4.3.1 Culture and Differentiation of 3T3-L1 Adipocytes

3T3-L1 preadipocytes were incubated at a density of 8000 cells/cm² (10 cm² dish) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics at 37°C in 5% CO₂ in air. Confluent preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM-F10 (1:1, vol/vol) medium supplemented with 1% FBS, 1 μM dexamethasone, IBMX (0.5 mM) and antibiotics (1% BSA). Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium. Cultures were refed every 2–3 days. Cell viability was measured via trypan blue exclusion.

4.3.2 Fatty Acid Synthase (FAS) Activity Assay

Adipocytes were incubated for 4 h in the presence of 0-50 μM 2,4-dinitrophenol (DNP) or 0-5 μM carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma, St Louis, MO) with or without insulin (30nM) and with or without lanthanum(1mM) (Sigma, St Louis, MO) . Subsequently, the cells were returned to serum free medium (containing 1% BSA) for 48 hours. Fatty acid synthase activity was measured in cytosolic

extracts by measuring the oxidation rate of NADPH, as described previously (8-10). Adipocytes were then homogenized in 250 mmol/L sucrose solution containing 1 mmol/L ethylenediamine-tetraacetic acid (EDTA), 1mmol/L dithiothreitol (DTT), and 100 μ mol/L phenylmethylsulfonyl fluoride (PMSF) (pH 7.4). The homogenates were centrifuged at 18,500 X g for 1h and then incubated in phosphate buffer and acetyl-CoA, with or without ATP (10nM) (Sigma, St Louis, MO), for 15 minutes, and the supernatant was used for measuring oxidation of NADPH right after adding malonyl-CoA. The final FAS activity was normalized to nucleic acids, which was measured by CyQUANT cell proliferation assay kit (Parkard instrument Company, Inc., Downers Grove, IL) according to manufacturer's instruction.

4.3.3 Lipolysis Assay

Adipocytes were incubated for 2 h in the presence of 0-50 μ M DNP or 0-5 μ M FCCP with or without insulin (30nM) and with or without lanthanum (1mM), and with or without isoproterenol (50nM). Glycerol released into the culture medium was determined as an indicator for lipolysis, using a one-step enzymatic fluorometric method (11). Glycerol release data was normalized for cellular nucleic acids as described above.

4.3.4 $[Ca^{2+}]_i$ Measurement

$[Ca^{2+}]_i$ in 3T3-L1 adipocytes was measured using a fura-2 dual-wavelength fluorescence imaging system. Preadipocytes were plated in 35-mm dishes (P35G-0-14-C, MatTek). Prior to $[Ca^{2+}]_i$ measurement, cells were preincubated with or without different concentration 0-50 μ M DNP or 0-5 μ M FCCP with or with or without lanthanum(1mM)

for one hour. Cells were then put in serum-free medium overnight and rinsed with HEPES balanced salt solution (HBSS) containing the following components (in mM): 138 NaCl, 1.8 CaCl₂, 0.8 MgSO₄, 0.9 NaH₂PO₄, 4 NaHCO₃, 5 glucose, 6 glutamine, 20 HEPES, and 1% bovine serum albumin. Cells were loaded with fura-2 acetoxymethyl ester (fura-2 AM) (10 μM) in the same buffer for 2 h at 37°C in a dark incubator with 5% CO₂. To remove extracellular dye, cells were rinsed with HBSS three times and then postincubated at room temperature for an additional 1 h for complete hydrolysis of cytoplasmic fura-2 AM. The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu model 4915 charge-coupled device (CCD) camera. Fluorescent images were captured alternatively at excitation wavelength of 340 and 380 nm with an emission wavelength of 520 nm. After establishment of a stable baseline, the response to 40mM KCl was determined. [Ca²⁺]_i was calculated using a ratio equation as described previously(12). Each analysis evaluated responses of 5 representative whole cells. Images were analyzed with InCyt Im2 version 4.62 imaging software (Intracellular Imaging, Cincinnati, OH). Images were calibrated using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR) to create a calibration curve in solution, and cellular calibration was accomplished using digitonin (25 μM) and pH 8.7 Tris-EGTA (100 mM) to measure maximal and minimal [Ca²⁺]_i levels respectively.

4.3.5 Total RNA Extraction

Total cellular RNA isolation kit (Ambion Inc., Austin, TX) was used to extract total RNA from 3T3-L1 cells according to manufacturer's instruction.

4.3.6 Quantitative Real-time PCR

3T3-L1 adipocyte FAS mRNA was quantitatively measured using a Smart Cycler Real Time PCR System (Cepheid, Sunnyvale, CA) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg, NJ). The primers for mouse FAS are as follows. Forward primer: 5'-CCCAGAGGCTTGTGCTGACT-3' reverse primer: 5'-CGAATGTGCTTGGCTTGGT-3'. Pooled 3T3-L1 adipocyte total RNA was serial-diluted in the range of 1.5625-25 ng and used to establish a FAS standard curve; total RNAs for unknown samples were also diluted in this range. Reactions of quantitative RT-PCR for standards and unknown samples were also performed according to the instructions of Smart Cycler System (Cepheid, Sunnyvale, CA) and TaqMan Real Time PCR Core Kit (Applied Biosystems, Branchburg, NJ). The FAS mRNA quantitation for each sample was further normalized using the corresponding 18s quantitation, with a forward primer: 5'-AGTCCCTGCCCTTTGTACACA-3' and a reverse primer: 5'-GATCCGAGGGCCTCACTAAAC-3'.

4.3.7 Statistical Analysis

All data are expressed as mean $\pm$ SE. Data were evaluated for statistical significance by one-way analysis of variance (ANOVA), and significantly different group means were then be separated by the least significant difference test by using SPSS (SPSS Inc, Chicago, IL).

4.4 Results

Both mitochondrial uncouplers increased KCl-stimulated $[Ca^{2+}]_i$ ($p<0.001$). Antagonism of calcium channels with the non-specific inhibitor lanthanum decreased both basal and stimulated $[Ca^{2+}]_i$ significantly ($p<0.001$) and reversed the stimulation of $[Ca^{2+}]_i$ induced by mitochondrial uncouplers (**Figures 4-1 and 4-2**).

Mitochondrial uncoupling with DNP or FCCP significantly inhibited lipolysis (**Figures 4-3 and 4-4**). This effect was attenuated by insulin while lanthanum completely reversed the suppression by mitochondrial uncouplers or/and insulin. These results indicate that metabolic uncouplers suppress lipolysis via a calcium dependent mechanism. In short-term (4 hours) treatment with DNP, FAS activity was decreased significantly by mitochondrial uncoupling ($p<0.001$) (**Figure 4-5**). However, lanthanum was unable to reverse this short-term suppression, indicating that this short-term suppression of FAS activity by DNP was calcium-independent. Similar results were observed with a long-term (48hours) treatment with both mitochondrial uncouplers, with lanthanum causing a further suppression (**Figures 4-5 and 4-6**). However, both mitochondrial uncouplers significantly increased FAS expression and this effect was reversed by lanthanum (**Figure 4-7**). Thus, metabolic uncoupling has a dual effect on FAS: to decrease activity via a Ca^{2+} -independent mechanism and to increase FAS expression via a Ca^{2+} -dependent mechanism. The addition of ATP reversed the DNP-induced suppression of FAS activity, indicating that this effect is secondary to ATP depletion (**Figure 4-8**).

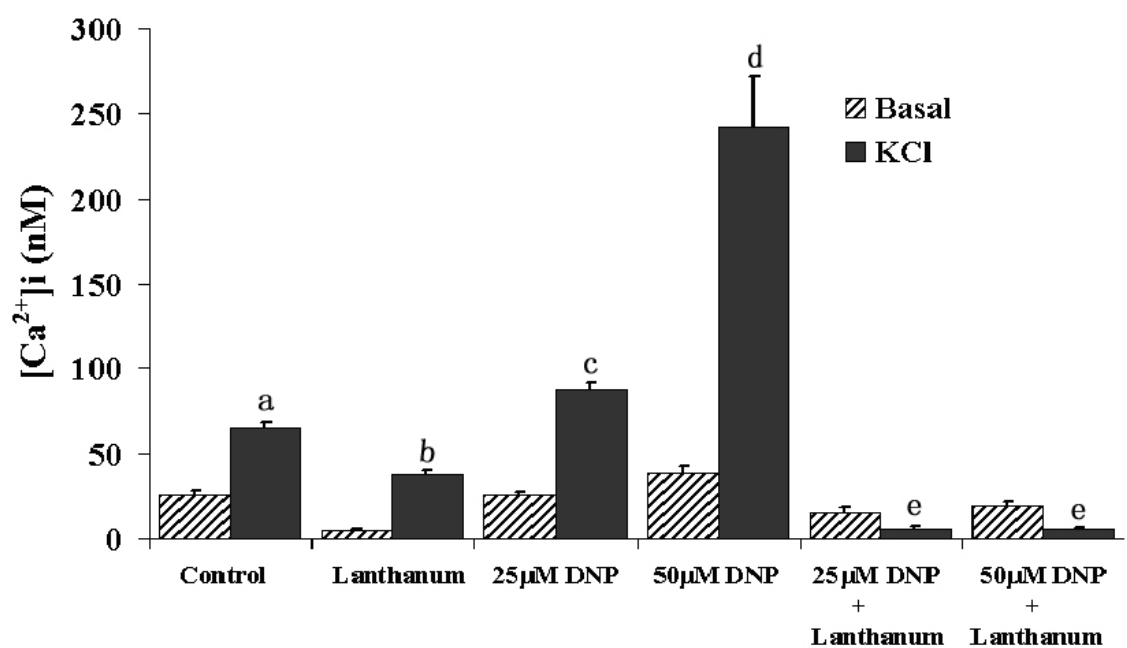


Figure 4-1. Effect of DNP on intracellular calcium in 3T3-L1 adipocytes.

Differentiated 3T3-L1 adipocytes were treated with or without DNP (25µM, 50µM), DNP plus KCl (40mM) or DNP plus lanthanum(1mM). Data are expressed as mean ± SE (n=12). Difference letters above the bars indicate a significant difference at level of p<0.05.

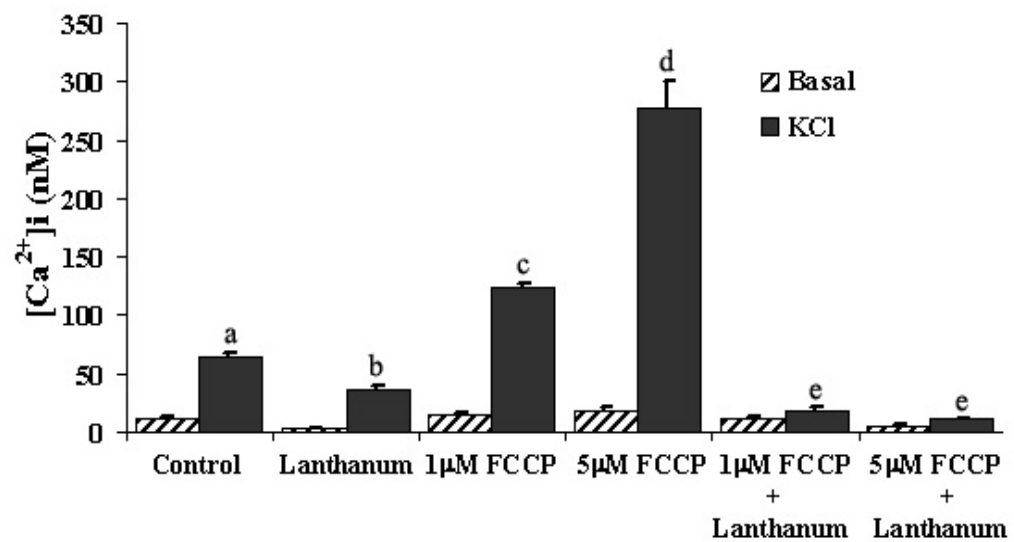


Figure 4-2. Effect of FCCP on intracellular calcium in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with or without FCCP (1µM, 5µM), FCCP plus KCl (40mM) or FCCP plus lanthanum (1mM). Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

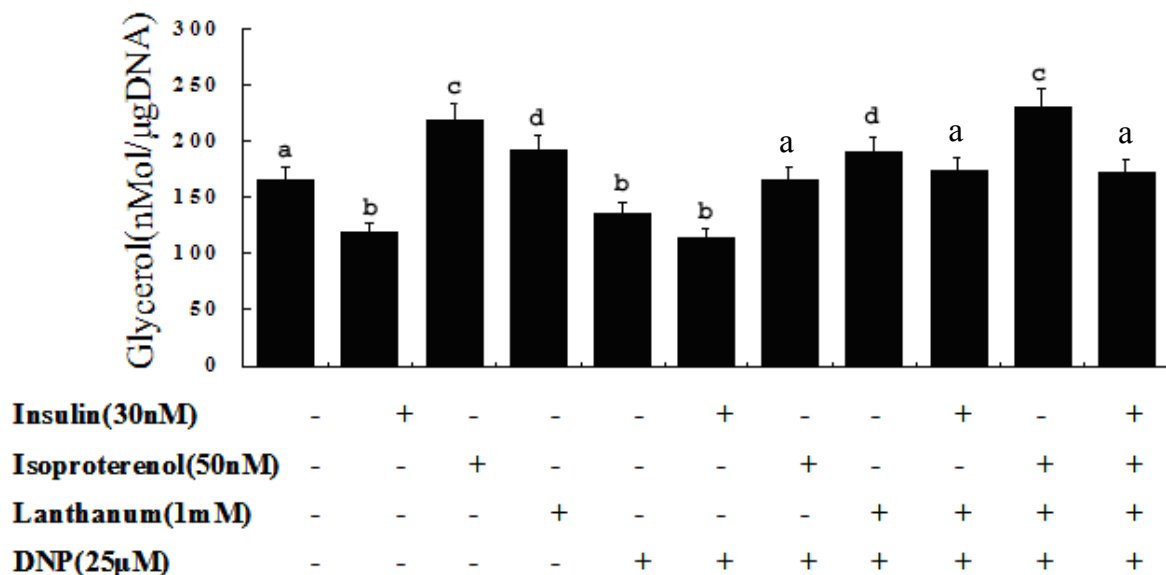


Figure 4-3. Effect of DNP on lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with or without DNP (25μM), DNP plus insulin (30nM), DNP plus isoproterenol (50nM) or DNP plus lanthanum (1mM). Lipolysis was determined as glycerol release by enzymatic fluorescence method, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

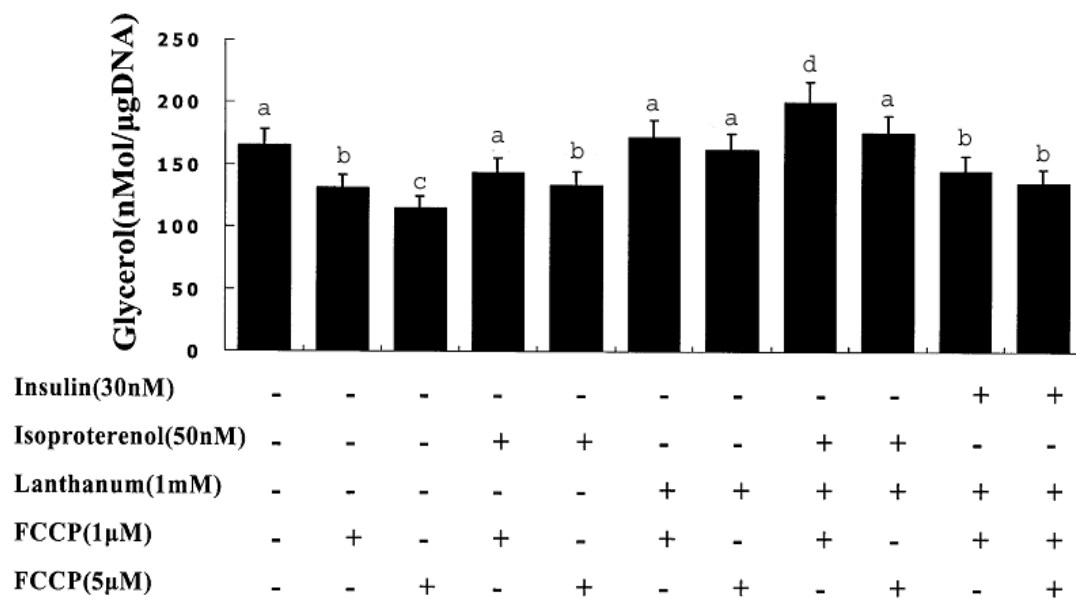


Figure 4-4. Effect of FCCP on lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with or without FCCP (1μM, 5μM), FCCP plus insulin (30nM), FCCP plus isoproterenol (50nM) or FCCP plus lanthanum (1mM). Lipolysis was determined as glycerol release by enzymatic fluorescence method, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

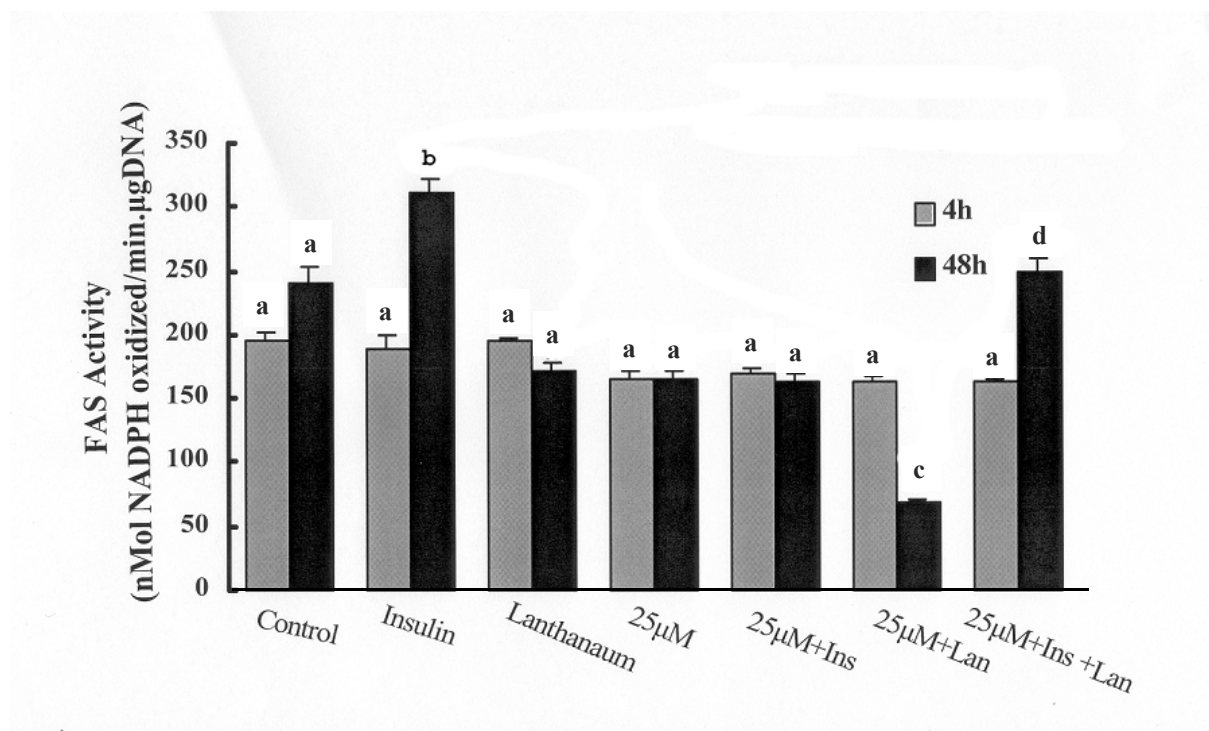


Figure 4-5. Effect of DNP on fatty acid synthase (FAS) activity in 3T3-L1 adipocytes.

Differentiated 3T3-L1 adipocytes were treated with or without DNP (25µM), Insulin (30nM), Lanthanum(1mM), DNP plus insulin or DNP plus lanthanum. FAS activity was measured as the oxidative rate of NADPH, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of $p<0.05$.

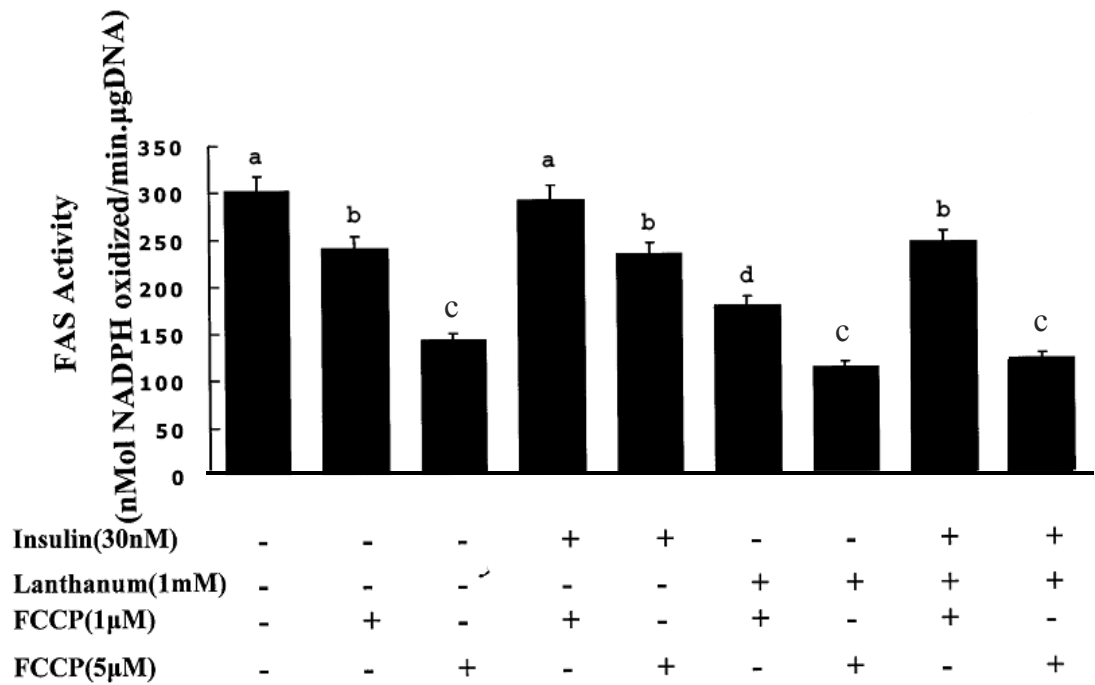


Figure 4-6. Effect of FCCP on fatty acid synthase (FAS) activity in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with or without FCCP (1µM, 5µM), Insulin (30nM), Lanthanum (1mM), FCCP plus insulin or FCCP plus lanthanum. FAS activity was measured as the oxidative rate of NADPH, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

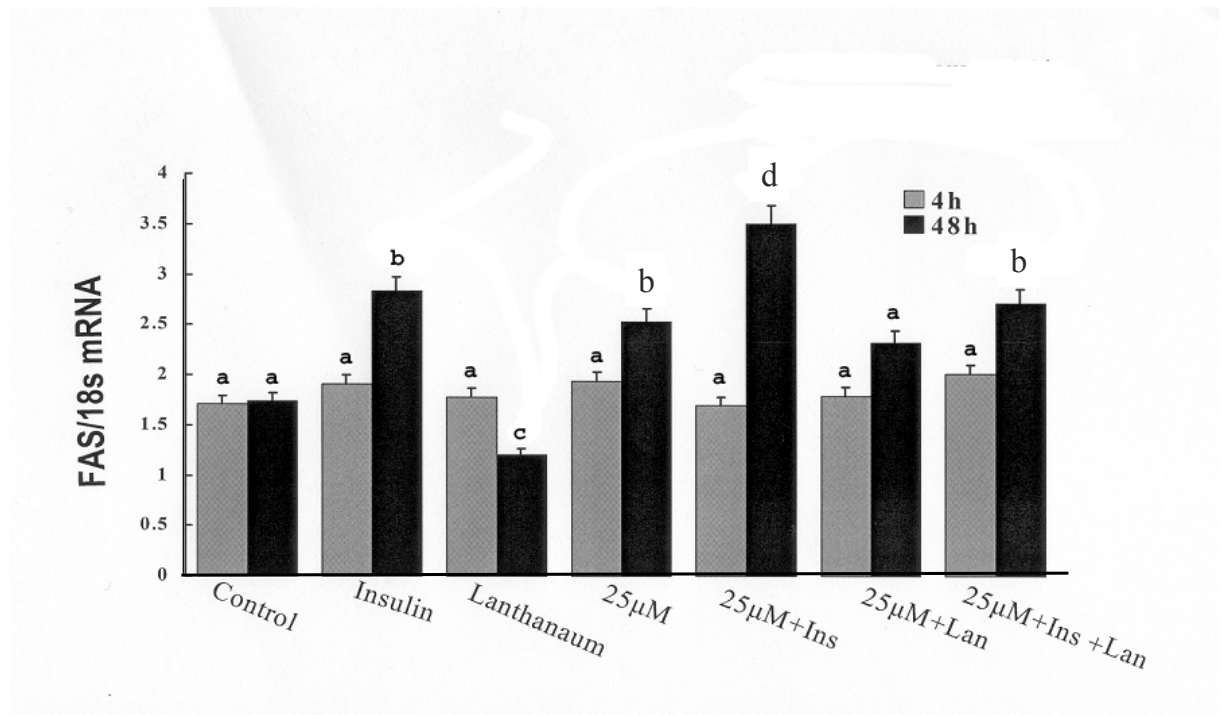


Figure 4-7. Effect of DNP on fatty acid synthase (FAS) expression in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with or without DNP (25μM), Insulin (30nM), Lanthanum (1mM), DNP plus insulin or DNP plus lanthanum. FAS mRNA was measured by real time PCR, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

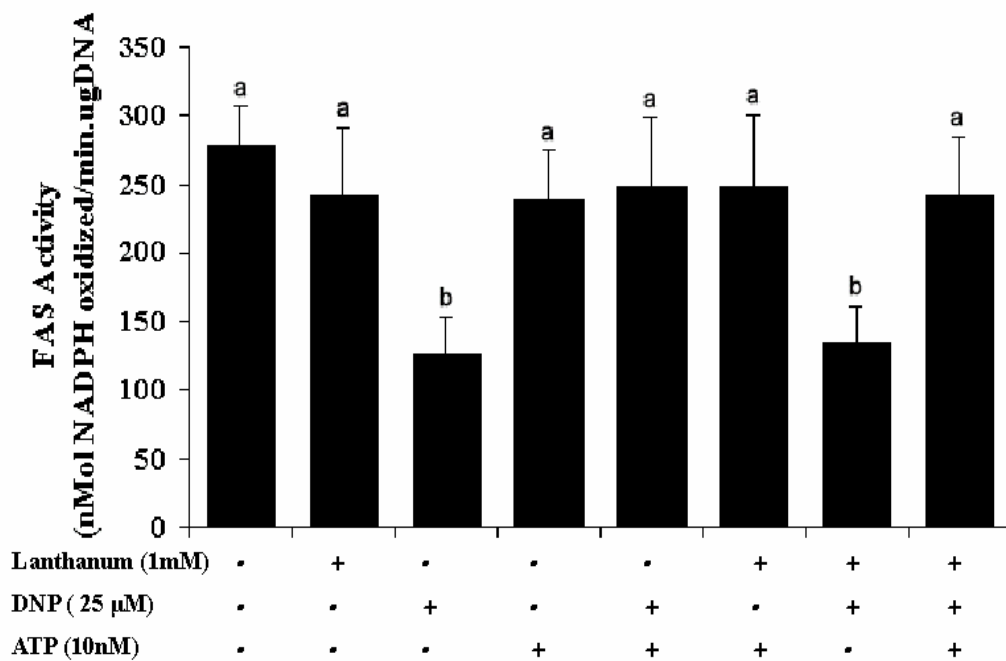


Figure 4-8. Effect of DNP on fatty acid synthase (FAS) activity in 3T3-L1 adipocytes.

Differentiated 3T3-L1 adipocytes were treated with or without DNP (25μM), ATP (10nM), Lanthanum(1mM), DNP plus ATP, DNP plus lanthanum or Lanthanum plus ATP. FAS activity was measured as the oxidative rate of NADPH, which was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

4.5 Discussion

Several studies have reported that mitochondrial uncoupling regulates lipid metabolism both in vivo and in vitro (13-15). However, the mechanism mediating this effect remains unclear. Our previous data suggested intracellular calcium is an important regulator of both lipogenesis and lipolysis. Accordingly, We investigated whether there is an effect of mitochondrial uncoupling on intracellular calcium level and whether $[Ca^{2+}]_i$ is the mediator for the regulation of lipid metabolism by mitochondrial uncoupling.

The role of the mitochondria in regulation of $[Ca^{2+}]_i$ homeostasis has been widely investigated. Several observations suggest that $[Ca^{2+}]_i$ is regulated by mitochondria in an energy-linked process (16-18). In addition, experimental evidence indicates a positive association between mitochondrial calcium and mitochondrial ATP production (19-22). Our results suggest that mitochondrial uncoupling contributes to the control of membrane calcium channel activity in differentiated 3T3-L1 adipocytes. We report that mitochondrial uncouplers dose-responsively increase $[Ca^{2+}]_i$ in adipocytes. This regulation of $[Ca^{2+}]_i$ level by mitochondrial uncouplers in adipocytes appears to involve membrane calcium channels, as this effect can be completely blocked by non-specific calcium channel antagonist (lanthanum). Although Makowska *et al.* previously reported that mitochondrial uncoupler depressed the rate of calcium influx in vitro (23), this inhibitory effect of uncoupler on intracellular calcium was observed on store-operated calcium channel rather than voltage-gated calcium channel (VGCC). However, an important relevant role of VGCCs is to serve as highly regulated mechanisms to

deliver calcium ions into specific intracellular locales for a variety of calcium-dependent processes (24). Thus, the observation from present work demonstrated that mitochondrial uncoupler regulates VGCC by increasing the calcium influx. Additionally, Lynch *et al* have shown that calcium was released from mitochondrial as a result of DNP dissipation of the H^+ gradient (25). Consequently, we reasoned that intracellular calcium may be a trigger in the effect of mitochondrial uncoupling on lipid metabolism.

Previous studies have demonstrated that lipolysis depends on the energy status of adipocytes (26-27). A decrease in intracellular ATP elicited in white adipocytes *in vitro* by inhibitors of the mitochondrial respiratory chain reversed the stimulation of lipolysis by catecholamines (28). More recently, ATP was shown to be required for translocation of HSL from cytoplasm to the surface of lipid storage droplets and for phosphorylation of HSL and other proteins that are involved in lipolysis in adipocytes (29). Incubation of isolated adipocytes with lipolytic hormones resulted in up to 50% decrease of their intracellular ATP level and in inhibition of lipolysis itself (29). In addition, The intracellular ATP is also essential for insulin signaling (30-31), which is altered at the level of the interaction of insulin with its receptor (32). In present study, we also showed that mitochondrial uncoupling inhibits lipolysis and this effect is via a calcium dependent mechanism. Previous data from our laboratory demonstrate that increasing $[Ca^{2+}]_i$ inhibits adipocyte lipolysis. This effect is primarily mediated by activation of adipocyte phosphodiesterase 3B and a reduction of cAMP levels, leading to a decrease in hormone sensitive lipase phosphorylation and, consequently, an inhibition of lipolysis (32). In the present study, increased $[Ca^{2+}]_i$ and decreased lipolysis were observed concomitantly with two individual mitochondrial uncouplers, while inhibition of Ca^{2+} influx prevented

the suppression of lipolysis. Therefore, $[Ca^{2+}]_i$ appears to mediate the effect of mitochondrial uncouplers on lipolysis. In addition, the mitochondrial uncoupling results in reduced adipocytes lipolysis in response to isoproterenol or insulin, suggesting a reduction in adipocytes cAMP levels.

We also found that mitochondrial uncoupling has dual effects on a key lipogenic system, fatty acid synthase. The suppression of FAS activity induced by short-term treatment with mitochondrial uncouplers indicates a non-genomic effect of mitochondrial uncoupling on FAS. This effect is a calcium-independent since lanthanum is unable to reverse the suppression. The stimulation of FAS expression was induced by long-term treatment with mitochondrial uncouplers and this stimulation of FAS expression can be fully reversed by lanthanum, indicating that a genomic effect of mitochondrial uncoupling on FAS expression is $[Ca^{2+}]_i$ -dependent, consistent with our previous reports of $[Ca^{2+}]_i$ regulation of FAS expression(8-9). To our surprise, FAS activity was suppressed by long-term treatment with mitochondrial uncouplers and addition of lanthanum induced a further suppression on FAS activity, which suggests that there is both a calcium dependent and a non-genomic, Ca^{2+} -independent effect similar to that observed in the short-term treatment. This non-genomic effect of suppression on FAS activity by mitochondrial uncoupling may result from the shortage of the substrates (e.g. NADPH and ATP) secondary to reduced mitochondrial energy with mitochondrial uncouplers (33). For each molecule of acetate incorporated into long-chain FA, two molecules of NADPH are necessary in the reaction catalyzed by FA synthase. Moreover, mitochondrial production of ATP is required for synthesis of oxalacetate by carboxylation

of pyruvate and subsequent synthesis of citrate in mitochondria and for FA synthesis and esterification in cytoplasm (34-35). The ATP/ADP ratio has been demonstrated to affect pyruvate carboxylase activity directly (35-36). Although the *in vitro* assay system used in this study provides acetyl-CoA, malonyl -CoA and NADPH, it does not provide exogenous ATP. Therefore, reduced availability of intramitochondrial ATP and the a reduced ATP: ADP ratio secondary to uncoupling may be responsible for the observed reduction in activity (37-38). To further test this hypothesis, we found that addition of ATP reversed the uncoupler-induced suppression on FAS activity, indicating that inhibition of FA synthesis by uncouplers probably resulted from limited availability of intramitochondrial ATP.

In conclusion, mitochondrial uncoupling participates in the regulation in lipid metabolism and $[Ca^{2+}]_i$. **Figure 4-9** depicts a proposed scheme for the integration of this regulation, as follows. Mitochondrial uncoupling increases $[Ca^{2+}]_i$ by activating membrane calcium channels and thereby inhibits lipolysis via a Ca^{2+} -dependent pathway. Mitochondrial uncoupling has dual effects on lipogenesis: it inhibits FAS activity via a Ca^{2+} -independent mechanism and stimulates of FAS expression via a Ca^{2+} -dependent metabolism.

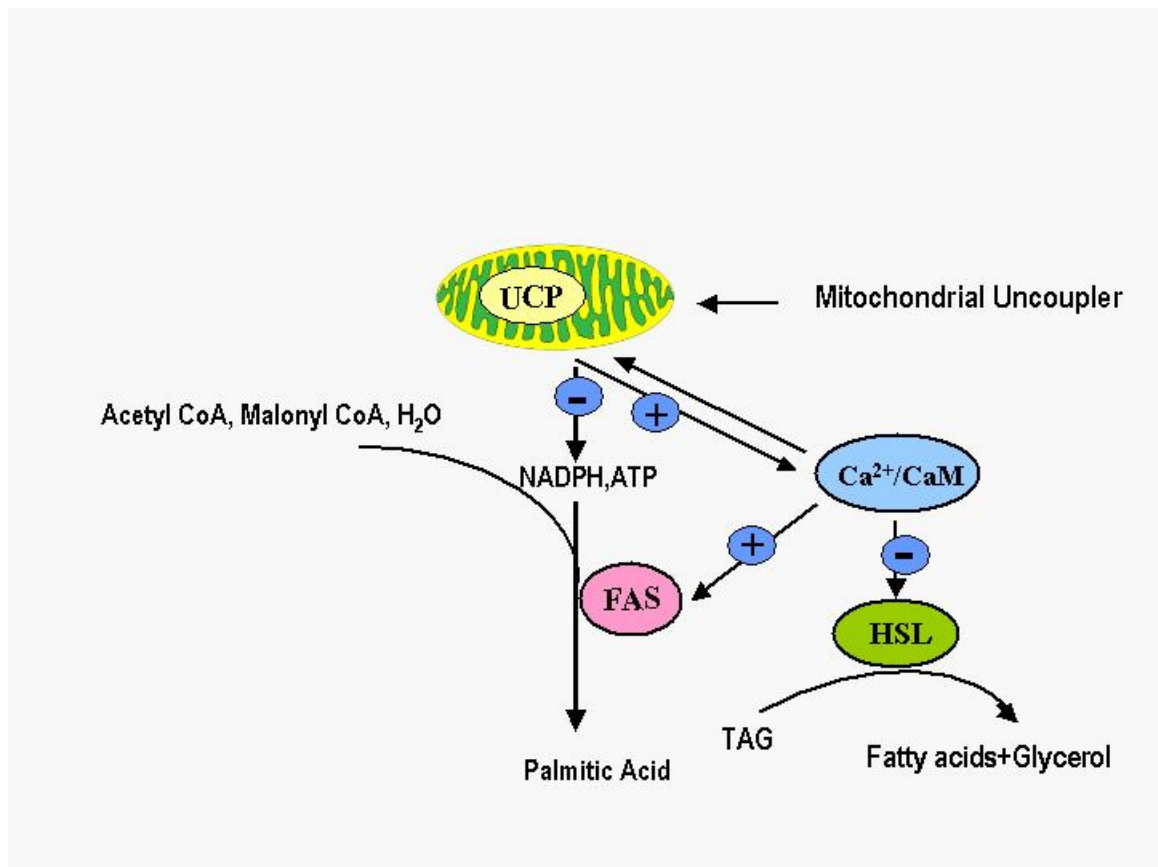


Figure 4-9. A proposed scheme for the integration of the regulation of lipid metabolism by mitochondrial uncoupling. Mitochondrial uncoupling increases $[Ca^{2+}]_i$ by activating membrane calcium channels and thereby inhibits lipolysis via a Ca^{2+} -dependent pathway. Mitochondrial uncoupling has dual effects on lipogenesis: it inhibits FAS activity via a Ca^{2+} -independent mechanism and stimulates of FAS expression via a Ca^{2+} -dependent metabolism.

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Part Five

Role of Uncoupling Protein 2 (UCP2) Expression and 1 α , 25 Dihydroxyvitamin D₃ (1 α , 25-(OH)₂-D₃) in Modulating Adipocyte Apoptosis

This part is a slightly revised version of a paper by the same name published in the FASEB Journal in 2004 by Xiaocun Sun, Michael B. Zemel:

Xiaocun Sun, Michael B. Zemel. Role of uncoupling protein 2 (UCP2) expression and $1\alpha, 25$ -dihydroxyvitamin D_3 in modulating adipocyte apoptosis, FASEB Journal (2004) [Epublication ahead of print]

5.1 Abstract

We previously found that $1\alpha, 25$ -(OH) $_2$ - D_3 modulates adipocyte lipid metabolism via a Ca^{2+} -dependent mechanism and inhibits adipocyte UCP2 expression, indicating that the anti-obesity effects of dietary calcium are mediated by suppression of $1\alpha, 25$ -(OH) $_2$ - D_3 levels. However, because UCP2 reduces mitochondrial potential, we have evaluated the roles of UCP2, mitochondrial uncoupling and $1\alpha, 25$ -(OH) $_2$ - D_3 in adipocyte apoptosis. Overexpressing UCP2 in 3T3-L1 cells induced marked reductions in mitochondrial potential ($\Delta\psi$) and ATP production ($p < 0.01$), increases in the expression of caspases ($p < 0.05$) and a decrease in Bcl-2/Bax expression ratio ($p < 0.01$). Physiological doses of $1\alpha, 25$ -(OH) $_2$ - D_3 (0.1-10 nM) restored mitochondrial $\Delta\psi$ in LI-UCP2 cells and protected against UCP2 overexpression-induced apoptosis ($p < 0.01$), while a high dose (100 nM) stimulated apoptosis in 3T3-L1 and LI-UCP2 cells ($p < 0.05$). $1\alpha, 25$ -(OH) $_2$ - D_3 stimulated cytosolic Ca^{2+} dose-dependently in both 3T3-L1 and LI-UCP2 cells. However, physiological doses suppressed mitochondrial Ca^{2+} levels by ~50% while the high-dose increased mitochondrial Ca^{2+} by 25% ($p < 0.05$); this explains stimulation of apoptosis by

the high dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$. Using high calcium diets to suppress $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ stimulated adipose tissue apoptosis in aP2 transgenic mice ($p<0.01$), suggesting that increasing dietary calcium stimulates adipose apoptosis and thereby further contributes to an anti-obesity effect of dietary calcium.

5.2 Introduction

Previous data from this laboratory demonstrate a key role for intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in the regulation of adipocyte metabolism and lipid storage(1-3). Because $[\text{Ca}^{2+}]_i$ can clearly be modulated by calcitrophic hormones, including $1\alpha, 25\text{-dihydroxyvitamin D}_3$ ($1\alpha, 25\text{-(OH)}_2\text{-D}_3$), these data provide a theoretical framework to explain an anti-obesity effect of high calcium diet (4). Indeed, increasing dietary calcium in the absence of caloric restriction appears to result in a re-partitioning of dietary energy from adipose tissue to lean body mass, resulting in a net reduction in fat mass in both mice and humans, while increasing dietary calcium intake during energy restriction results in a marked augmentation of body weight and fat loss in both mice and humans(4-6).

We have also found that high calcium diets attenuate the decreases in core temperature which otherwise occur with energy restriction in mice(4), and that dietary calcium up-regulates uncoupling protein 2(UCP2) expression in white adipose tissue(7). We recently extended this concept by demonstrating that $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ directly suppresses UCP2 expression in isolated human adipocytes (8), indicating that the up-

regulation of UCP2 induced by dietary calcium may result from the loss of inhibition of UCP2 expression by $1\alpha, 25\text{-(OH)}_2\text{-D}_3$.

Recent data also indicate that $[\text{Ca}^{2+}]_i$ may play a key role in modulating sensitivity to the apoptotic stress(9-12). Several of the functional groups of molecules involved in apoptosis, including caspases and the Bcl-2 family, are $[\text{Ca}^{2+}]_i$ responsive(15-18). $[\text{Ca}^{2+}]_i$ has been demonstrated to modulate the role of proteases, including caspase-3, caspase-9 and Ca^{2+} -dependent endonucleases(9,19-22). Scorrano *et al.* also showed a key interaction occurs between Bcl-2 family protein and calcium signaling in the execution of apoptosis (23). Their data demonstrate a critical role of BAX and BAK in maintaining of homeostatic concentration of $[\text{Ca}^{2+}]_i$ in endoplasmic endo-reticulum(ER) and mitochondria, which control the apoptotic fate of cells responding to $[\text{Ca}^{2+}]_i$ dependent stimuli. Several studies have also shown that thapsigargin, which can induce sustained $[\text{Ca}^{2+}]_i$ elevation by inhibiting sacro-endoplasmic reticulum Ca^{2+} -ATPase(SERCA), triggers apoptosis in various cell types(24-26). These data indicate a key role of $[\text{Ca}^{2+}]_i$ in the apoptosis process. Given that $[\text{Ca}^{2+}]_i$ can be modulated by $1\alpha, 25\text{-(OH)}_2\text{-D}_3$, the role of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ in apoptosis should be explored. Although high dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ has been reported to induce apoptosis in various tumor cells (27-30), the regulation of apoptosis by physiological doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ remains unclear.

Considerable progress has been made in elucidating a pivotal role of mitochondria playing in the coordination, initiation, and execution of apoptotic cell death (31-33). Three general mechanisms have been proposed, including 1) disruption of oxidative

phosphorylation ATP production; 2) regulation of the apoptotic proteases; and 3) alteration of cellular reduction-oxidation potential. Because UCP2, which is highly expressed in white adipose tissue, has been demonstrated to function as mitochondrial uncoupler of oxidative phosphorylation thus reduce efficiency of ATP synthesis, it is reasonable to propose that UCP2 may stimulate apoptosis in adipocytes. Accordingly, in the present study, the effect and mechanisms of mitochondrial uncoupling and $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on apoptosis in adipocytes were examined. We report here that 1) mitochondrial uncoupling induces apoptosis in differentiated 3T3-L1 cells; 2) low-doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibit apoptosis in differentiated 3T3-L1 cells in a dose-dependent manner while high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ stimulates apoptosis; and 3) high calcium diets, which suppress $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ levels *in vivo*, stimulate adipose tissue apoptosis in aP2 transgenic mice.

5.3 Materials and Methods

5.3.1 Culture and Differentiation of 3T3-L1 Adipocytes

3T3-L1 preadipocytes were incubated at a density of 8000 cells/cm² (10 cm² dish) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics (adipocyte medium) at 37°C in 5% CO₂ in air. Confluent preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM-F10 (1:1, vol/vol) medium supplemented with 1% FBS, 1μM dexamethasone, IBMX (0.5 mM) and antibiotics (1% Penicillin-Streptomycin). Preadipocytes were maintained in this

differentiation medium for 3 days and subsequently cultured in adipocyte medium. Cultures were re-fed every 2–3 days to allow 90% cells to reach fully differentiation before conducting chemical treatment. Chemicals were freshly diluted in adipocyte medium before treatment. Cells were washed with fresh adipocyte medium, re-fed with medium containing the different treatments, and incubated at 37°C in 5% CO₂ in air before analysis. Cell viability was measured via trypan blue exclusion.

5.3.2 UCP2 Transfection

UCP2 full-length cDNAs was amplified by RT-PCR using mRNAs isolated from mouse white adipose tissues. The PCR primers for this amplification are shown as follows: UCP2 forward, 5'-GCTAGCATGGTTGGTTTCAAG-3', reverse, 5'-GCTAGCTCAGAAAGGTGAATC-3'. The PCR products were then subcloned into pcDNA4/His expression vectors. The linearized constructs were transfected into 3T3-L1 preadipocytes using lipofectamine plus standard protocol (Invitrogen, Carlsbad, CA). After 48 hrs of transfection, cells were split and cultured in selective medium containing 400 ug/ml zeocin for the selection of resistant colonies. Cells were fed with selective medium every 3 days until resistant colonies could be identified. These resistant foci were picked, expanded, tested for expression, and frozen for future experiments.

5.3.3 Determination of Mitochondrial Membrane Potential

Adipocytes were incubated with adipocyte medium for 4h with or without 1 α , 25-(OH)₂-D₃. Mitochondrial membrane potential was analyzed fluorometrically with a lipophilic cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol

carbocyanine iodide) using a mitochondrial potential detection kit (Biocarta, San Diego, CA). Mitochondrial potential was determined as the ratio of red fluorescence (excitation 550 nm, emission 600 nm) and green fluorescence (excitation 485 nm, emission 535 nm) using a fluorescence microplate reader.

5.3.4 ATP Measurement

Adipocytes were incubated with adipocyte medium for 4h with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$. ATP levels were measured using ENLITEN® Total ATP Rapid Biocontamination Detection Kit (Promega, Madison, MI) according to the manufacturer's instruction. Cellular ATP was extracted as described previously (34), and was using a microplate luminometer (Labsystem, Helsinki, Finland). The integration time of the luminometer was set at 1s with normal gain.

5.3.5 Measurement of Cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) and Mitochondrial Calcium ($[\text{Ca}^{2+}]_m$)

$[\text{Ca}^{2+}]_c$ in adipocytes was measured using a fura-2 dual-wavelength fluorescence imaging system. Cells were plated in 35-mm dishes (P35G-0-14-C, MatTek). Prior to $[\text{Ca}^{2+}]_c$ measurement, cells were put in serum-free medium overnight and rinsed with HEPES balanced salt solution (HBSS) containing the following components (in mM): 138 NaCl, 1.8 CaCl_2 , 0.8 MgSO_4 , 0.9 NaH_2PO_4 , 4 NaHCO_3 , 5 glucose, 6 glutamine, 20 HEPES, and 1% bovine serum albumin. Cells were loaded with fura-2 acetoxymethyl ester (fura-2 AM) (10 μM) in the same buffer for 2 h at 37°C in a dark incubator with 5% CO_2 . To remove extracellular dye, cells were rinsed with HBSS three times and then post-incubated at room temperature for an additional 1h for complete hydrolysis of

cytoplasmic fura-2 AM. The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu model 4915 charge-coupled device (CCD) camera. Fluorescent images were captured alternatively at excitation wavelengths of 340 and 380 nm with an emission wavelength of 520 nm. After establishment of a stable baseline, the responses to $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ was determined. $[\text{Ca}^{2+}]_c$ was calculated using a ratio equation as described previously. Each analysis evaluated responses of 5 representative whole cells. Images were analyzed with InCyt Im2 version 4.62 imaging software (Intracellular Imaging, Cincinnati, OH). Images were calibrated using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR) to create a calibration curve in solution, and cellular calibration was accomplished using digitonin (25 μM) and pH 8.7 Tris-EGTA (100 mM) to measure maximal and minimal $[\text{Ca}^{2+}]_c$ levels respectively.

Rhod-2/AM was used for qualitative measurement of $[\text{Ca}^{2+}]_m$ (35). Cells were incubated with the Ca^{2+} sensitive fluorescent indicator rhod-2 AM (1-1.5 μM), for at least 1 h at 37°C in 5% CO_2 in humidified air. Since rhod-2 AM consists of a cationic rhodamine molecule, it accumulates preferentially inside the mitochondria due to their negative membrane potential. After loading, the cells were kept in rhod-2 AM-free standard solution for at least 1 h to allow conversion of the dye to its Ca^{2+} -sensitive, free acid form. Images of $[\text{Ca}^{2+}]_m$ were acquired at the wavelength of 552nm excitation and 590nm emission using the intracellular imaging system described above. Since rhod-2 is not a ratiometric dye, its fluorescence intensity was not calibrated to obtain absolute values of $[\text{Ca}^{2+}]_m$. Instead, only relative values were recorded as fluorescence signal (F) relative to the control value (F0). After baseline calcium concentration was measured, the

1, 25(OH)₂ D₃ was immediately applied to the cells and the calcium concentration was measured again.

5.3.6 Total RNA Extraction

A total cellular RNA isolation kit (Ambion, Austin, TX) was used to extract total RNA from 3T3-L1 cells according to manufacturer's instruction.

5.3.7 Quantitative Real-time PCR

Adipocyte caspase-1, caspase-3, Bcl-2 and Bax mRNA were quantitatively measured using a Smart Cycler Real Time PCR System (Cepheid, Sunnyvale, CA) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg, NJ). The primers and probe sets were ordered from Applied Biosystems TaqMan® Assays-on-Demand™ Gene Expression primers and probe set collection according to manufacture's instruction. Pooled 3T3-L1 adipocyte total RNA was serial-diluted in the range of 1.5625-25 ng and used to establish a standard curve; total RNAs for unknown samples were also diluted in this range. Reactions of quantitative RT-PCR for standards and unknown samples were also performed according to the instructions of Smart Cycler System (Cepheid, Sunnyvale, CA) and TaqMan Real Time PCR Core Kit (Applied Biosystems, Branchburg, NJ). The mRNA quantitation for each sample was further normalized using the corresponding 18s quantitation, with a forward primer: 5'-AGTCCCTGCCCTTTGTACACA-3' , and a reverse primer: 5'-GATCCGAGGGCCTCACTAAAC-3'.

5.3.8 Animals and Diets

This study was divided into three phases. The first phase was designed to induce weight gain and fat accretion, the second phase studied acceleration of weight and fat loss on the obese animals induced by phase one, and the third phase determined attenuation of regaining back of weight and fat. The study was conducted in the aP2 transgenic mice used in the studies described previously. These mice exhibit a normal pattern of leptin gene expression and activity as well as a pattern of agouti gene expression similar to that found in humans. Therefore, these mice are useful models for diet-induced obesity in a genetically susceptible human population in that they are not obese on a standard AIN-93G diet, but develop mild to moderate obesity when fed high sucrose and/or high fat diets (4).

Phase I

Six-week old aP2 transgenic mice were studied in a six-week obesity induction period on a basal low Calcium/high sucrose/high fat diet.

60 aP2 transgenic mice from our colony were placed at 6 weeks of age on a modified AIN 93 G diet with suboptimal calcium (0.4%), sucrose as the sole carbohydrate source, and fat increased to 25% of energy with lard. Animals were studied for six weeks, during which food intake and spillage were measured daily, and body weight and food consumption were assessed weekly. At the end of phase I, five representative animals were sacrificed as following to collect fat pads.

Phase II

At the end of the phase one, the 55 animals remaining from phase I were put into high calcium diet but the energy intake was limited to 70% of that found in the *ad libitum* fed during phase I. The high calcium diet used in this phase was made of the basal diet with the addition of sufficient calcium to bring the calcium content of the diet to 1.3%. Animals were maintained on this diet for six weeks, during which food intake and spillage were measured daily and body weight as well as food consumption were assessed weekly. At the end of phase II, five animals were sacrificed as following to collect fat pads.

Phase III

At the end of the phase II, the 50 animals remaining from phase II were further randomized into five groups, as following: 1) Basal-refed group were put on high sucrose/high fat/low calcium basal diet *ad lib* 2) High-Ca cereal-refed group were put on high-Ca-(1.3%)cereal diet *ad lib*. 3) High-Ca-cereal plus dry milk group were put on high calcium cereal plus non fat milk diet *ad lib*, in which 1.2% calcium content comes from fortified cereal and 0.1% calcium content comes from non fat milk; 4)Yogurt refed group were put on high calcium (1.3 %)-yogurt diet *ad lib*. 5) Cereal control refed group were put on low calcium (0.4%)-cereal diet *ad lib*. 10 animals /group were studied for six weeks, during which food intake and spillage were measured daily and body weight and food consumption were assessed weekly. At the conclusion of the study, all mice were

sacrificed under isoflurane anaesthesia and fat pads were immediately excised, weighed and snap-frozen in liquid nitrogen for subsequential assessment of gene expression.

The animal portion of this study was approved from an ethical standpoint by the University of Tennessee, Institutional Animal Care and Use Committee.

5.3.9 Statistical Analysis

All data are expressed as mean $\pm$ SE. Data were evaluated for statistical significance by one-way analysis of variance (ANOVA), and significantly different group means were then separated by the least significant difference test by using SPSS (SPSS Inc, Chicago, IL.).

5.4 Results

All apoptosis signaling pathways converge on a common pathway of cell destruction that is activated by a family of cysteine proteases (caspases) that cleave protein at aspartate residues. Accordingly, we first studied the effect of 1α , $25\text{-(OH)}_2\text{-D}_3$ and DNP on caspase-1 and caspase-3 expression in differentiated 3T3-L1(control) and UCP2 transfected 3T3-L1 cells. **Figures 5-1 and 5-2** demonstrate that physiological low-doses of 1α , $25\text{-(OH)}_2\text{-D}_3$ (0.1nM, 1nM, 5nM and 10nM) inhibited Caspase-1 and caspase-3 expression respectively ($p<0.05$), indicating reduced apoptosis in both control and UCP2 transfected 3T3-L1 cells. In contrast, a high-dose of 1α , $25\text{-(OH)}_2\text{-D}_3$ (100nM) stimulated both caspase-1 and caspase-3 expression in both control and UCP2 transfected 3T3 L1 cells ($p<0.01$), indicating a pro-apoptotic effect. Although 1α , $25\text{-(OH)}_2\text{-D}_3$

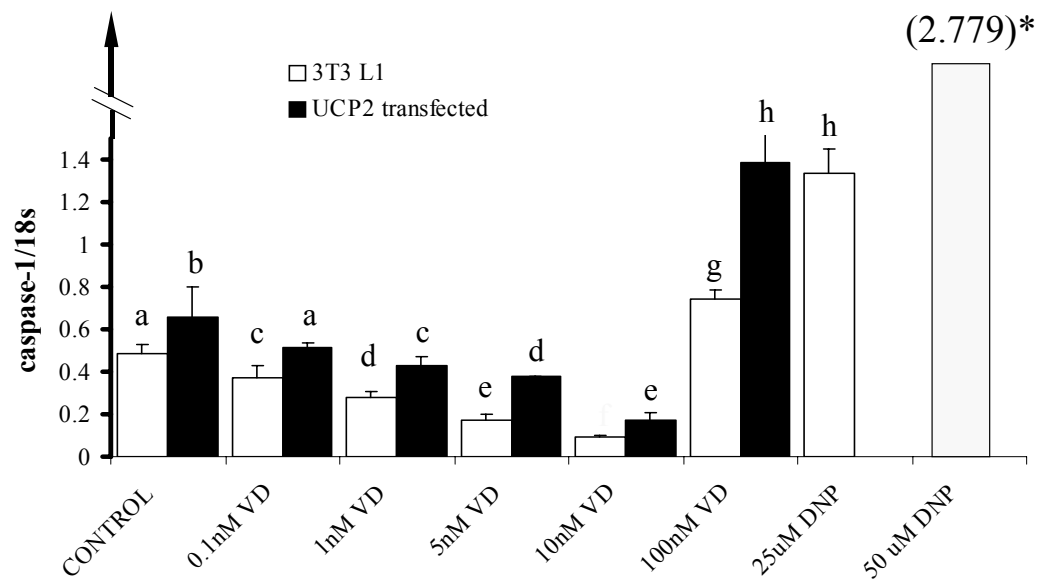


Figure 5-1. Caspase-1 expression in 3T3 L1 and UCP2 transfected 3T3-L1 adipocytes. Primary-cultured adipocytes were treated with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (0.1nM, 1nM, 5nM, 10nM, and 100nM) and DNP (25 μ M and 50 μ M) for 24 h for mRNA levels. Caspase-1 mRNA levels were measured by real-time PCR and these measurements were conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

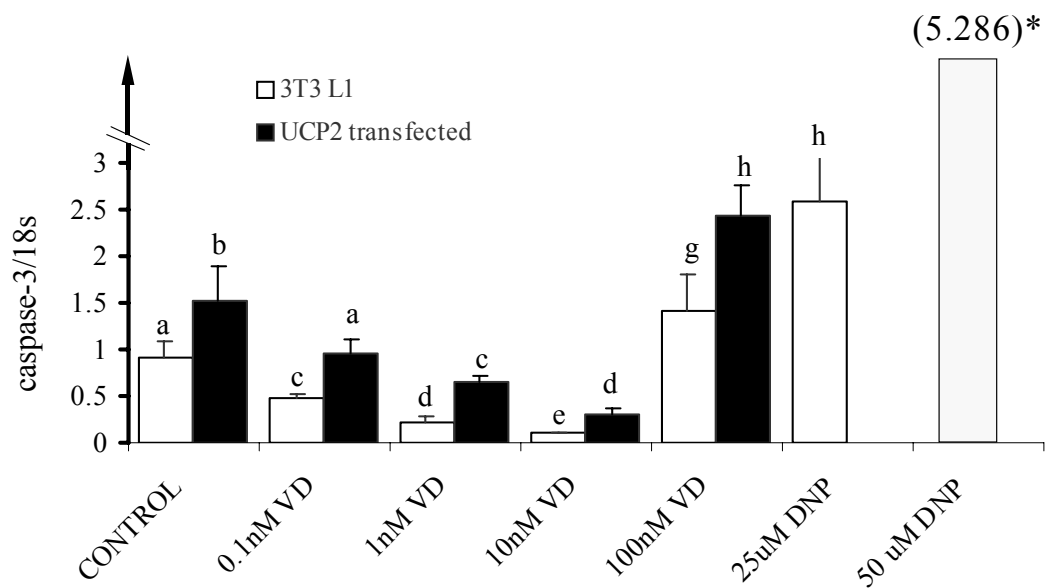


Figure 5-2. Caspase-3 expression in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes. Primary-cultured adipocytes were treated with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (0.1nM, 1nM, 5nM, 10nM, and 100nM) and DNP (25 μ M and 50 μ M) for 24 h for mRNA levels. Caspase-3 mRNA levels were measured by real-time PCR and these measurements were conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

exerted similar effects on caspases-1 and 3 expression in 3T3-L1 and UCP2 transfected 3T3-L1 cells, the basal expression levels of these two caspases were significantly higher in the DNP treated and UCP2 transfected cells in compared to control cells($p<0.05$), suggesting that mitochondrial uncoupling stimulates apoptosis in cultured 3T3-L1 cells.

Both Bcl-2 and Bax are apoptotic proteins belonging to Bcl-2 family. Due to their completely different roles in apoptotic signaling pathway, the ratio of protective Bcl-2 to apoptotic Bax is widely used to determine the susceptibility to apoptosis by regulating mitochondrial function following an apoptotic stimuli. **Figure 5-3** shows the effect of $1\alpha, 25-(\text{OH})_2\text{-D}_3$ on Bcl-2/ Bax expression ratio. We found that the lower levels of $1\alpha, 25-(\text{OH})_2\text{-D}_3$ induced a dose-dependent increase in Bcl-2/Bax ratio($p<0.01$), indicating a protective role of lower level of $1\alpha, 25-(\text{OH})_2\text{-D}_3$ against apoptosis. In contrast, the high dose of $1\alpha, 25-(\text{OH})_2\text{-D}_3$ resulted in a significant decrease in the Bcl-2/Bax ratio in control and UCP2 transfected 3T3-L1 cells($p<0.01$). In addition, the DNP treated and UCP2 transfected cells showed significantly lower ratio of Bcl-2/Bax than non-transfected 3T3-L1 cells ($p<0.02$), indicating mitochondrial uncoupling induced a decrease in protection from apoptotic death.

Since physiological low-dose of $1\alpha, 25-(\text{OH})_2\text{-D}_3$ appears to inhibit adipocyte apoptosis, suppression of $1\alpha, 25-(\text{OH})_2\text{-D}_3$ with high calcium diet may stimulate apoptosis. Accordingly, we compared caspase-1 and caspase-3 expression as well as Bcl-2/Bax expression ratio in aP2 transgenic mice fed diets with different calcium content after energy restriction. As shown in the **Figure 5-4, 5-5 and 5-6**, we found significant higher increases in caspase-1 and caspase-3 expression ($p<0.001$), and decreases in Bcl-2/Bax ratio in mice fed high calcium diets(Ca 1.3%) than mice fed low calcium diets(Ca

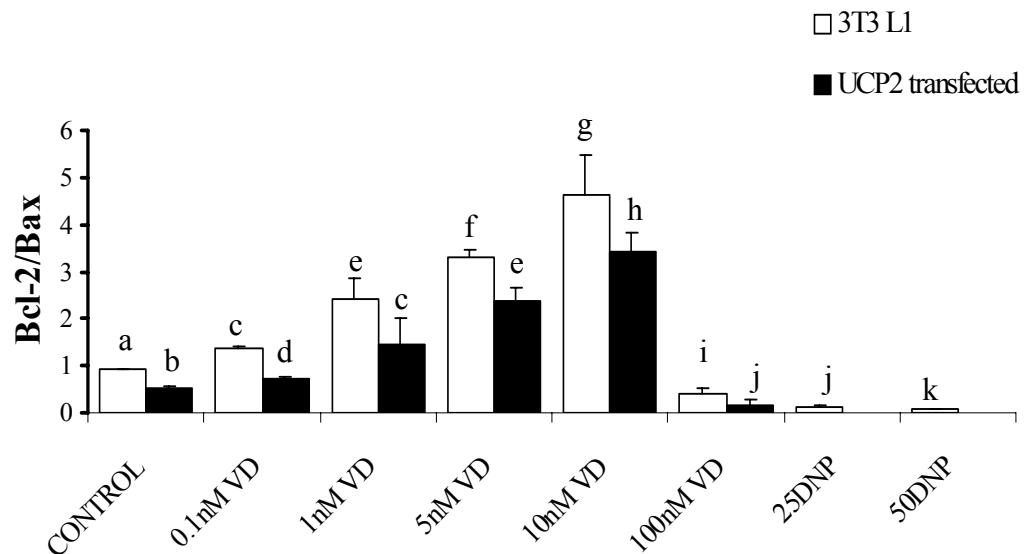


Figure 5-3. Bcl-2/Bax expression ratio in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes. Primary-cultured adipocytes were treated with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (0.1nM, 1nM, 10nM, and 100nM) and DNP (25 μ M and 50 μ M) for 24 h for mRNA levels. Caspase-1 mRNA levels were measured by real-time PCR and these measurements were conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

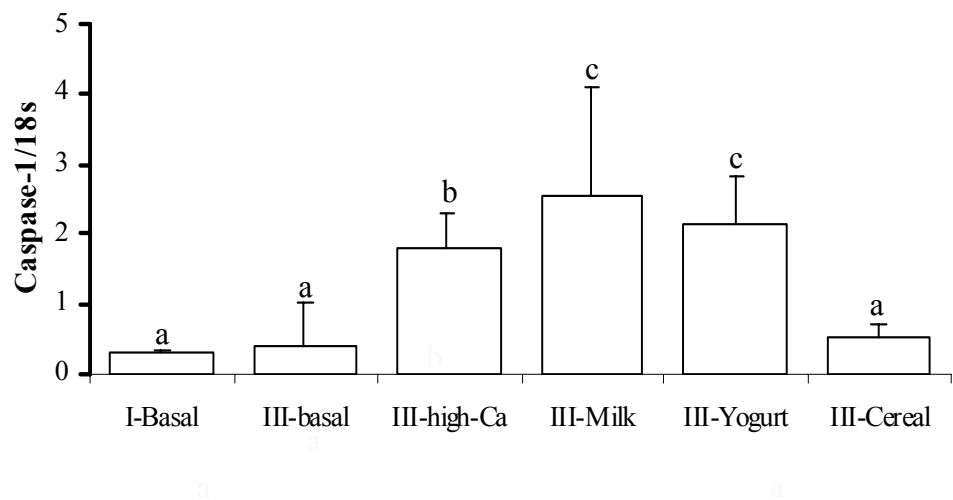


Figure 5-4. Effect of dietary calcium on caspase-1 expression white adipose tissue of aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

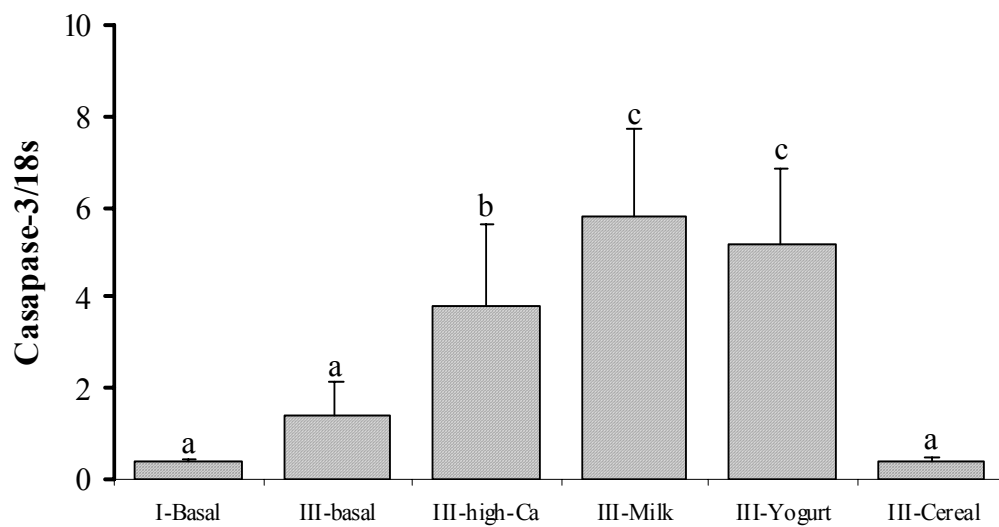


Figure 5-5. Effect of dietary calcium on caspase-3 expression white adipose tissue of aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

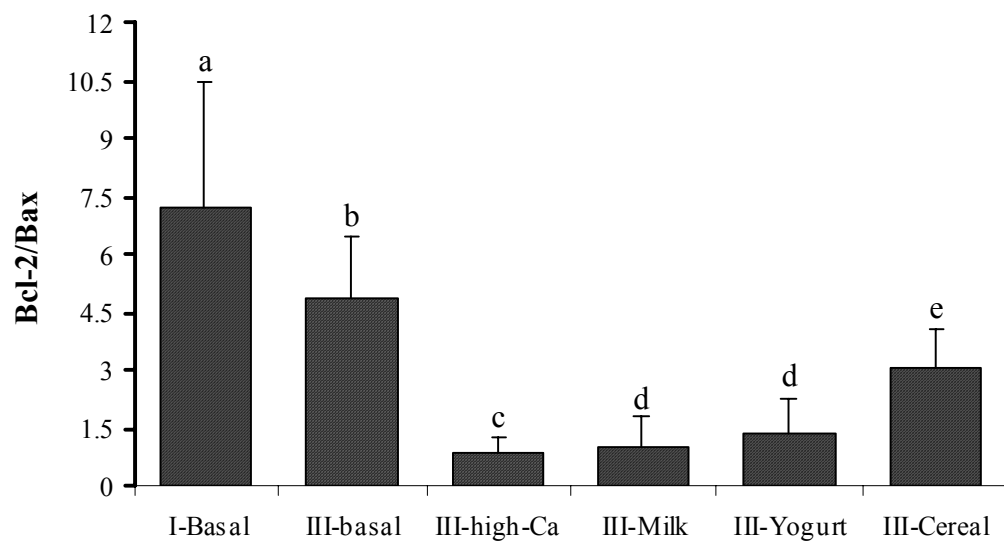


Figure 5-6. Effect of dietary calcium on Bcl-2/Bax expression in white adipose tissue of aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =8). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

0.4%) ($p < 0.01$). These data suggest that suppression $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ physiologically *in vivo* may stimulate adipocytes apoptosis.

Figure 5-7 shows the effect of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on mitochondrial potential in 3T3 L1 and UCP2 stably transfected 3T3 L1 cells. We found that lower-doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ significantly increased mitochondrial potential in all three groups of cells whereas the high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ caused mitochondrial potential collapse. Consistent with these data, ATP production was markedly increased in response to low-dose $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ treatment ($p < 0.01$)(**Figure 5-8**), while the high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibited ATP production ($p < 0.05$). These effects are consistent with our previous observation that low-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibited UCP2 expression(6). Thus, by inhibiting UCP2 expression, the low-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ restored mitochondrial potential and increased ATP production.

$1\alpha, 25\text{-(OH)}_2\text{-D}_3$ also stimulated Ca^{2+} influx in both control and UCP2 transfected cells. **Figure 5-9** demonstrates a dose-dependent increase in cytosolic calcium levels in response to $1\alpha, 25\text{-(OH)}_2\text{-D}_3$. Notably, the high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ induced a 10-fold higher increase in calcium influx than the lower doses.

Figure 5-10 shows the effect of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on $[\text{Ca}^{2+}]_m$ levels in control and UCP2 transfected cells. Low-doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ decreased mitochondrial calcium in both type of cells while the high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ significantly stimulated $[\text{Ca}^{2+}]_m$. Since mitochondrial calcium overload might trigger apoptosis by inducing mitochondrial collapse and cytochrome c release, the pro-apoptotic effect of the high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ is most likely a result of the stimulation of $[\text{Ca}^{2+}]_m$. In contrast,

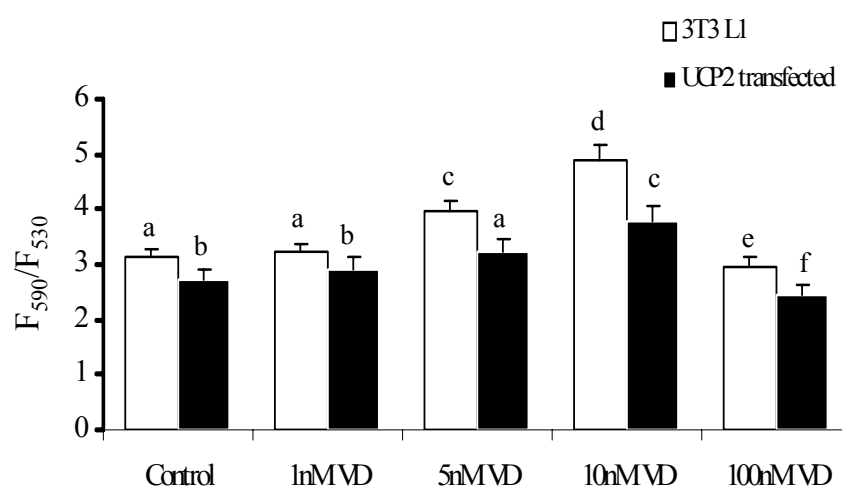


Figure 5-7. Mitochondrial potential in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes. Primary-cultured adipocytes were treated with or without $1\alpha, 25-(\text{OH})_2\text{-D}_3$ (0.1nM, 1nM, 5nM, 10nM, and 100nM) for 4h before assay. Mitochondrial potential were measured using fluorospectrometry as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

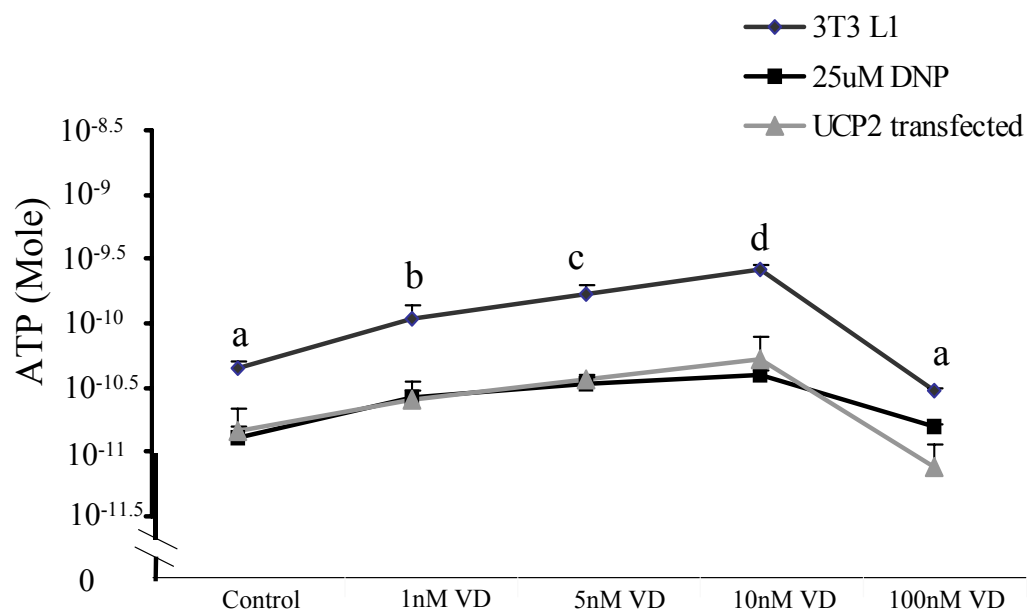


Figure 5-8. ATP production in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes. Primary-cultured adipocytes were treated with or without $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (1nM, 5nM, 10nM, and 100nM) for 4h before assay. ATP levels were measured using a microplate luminometer as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

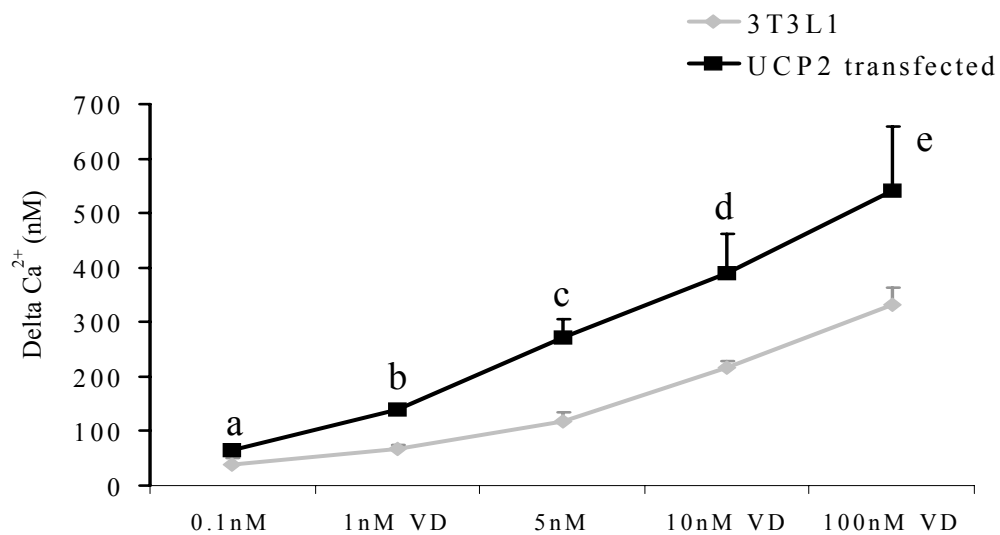


Figure 5-9. Intracellular calcium levels in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes. Primary-cultured adipocytes were treated with or without $1\alpha, 25-(OH)_2-D_3$ (1 nM, 5 nM, 10 nM, and 100 nM) instantly before assay. Intracellular calcium levels were measured using a dual wavelength fluorescence microscope as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

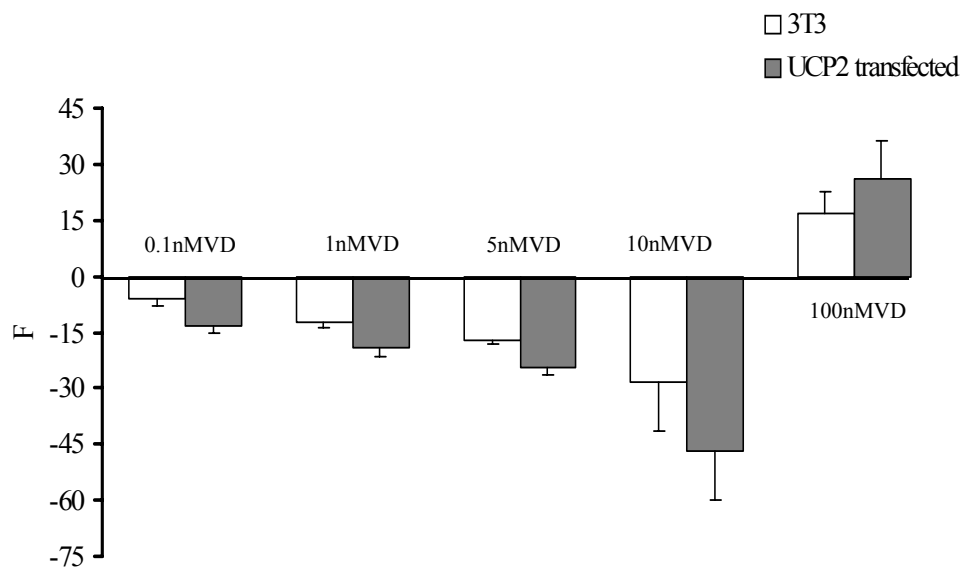


Figure 5-10. Mitochondrial calcium levels in 3T3-L1 and UCP2 transfected 3T3-L1 adipocytes. Primary-cultured adipocytes were treated with or without $1\alpha, 25-(\text{OH})_2\text{-D}_3$ (1nM, 5nM, 10nM, and 100nM) instantly before assay. Mitochondrial calcium levels were measured using a dual wavelength fluorescence microscope as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n=6).

the decreases in $[Ca^{2+}]_m$ observed in response treatment lower doses of $1\alpha, 25-(OH)_2-D_3$ indicate that, in addition to the inhibitory effect on UCP2, reducing the mitochondrial calcium load may contribute to the anti-apoptotic effect of low doses of $1\alpha, 25-(OH)_2-D_3$.

5.5 Discussion

Obesity is characterized by an increase in both adipocyte size and number. Adipose tissue is thus linked to the dynamic role played by adipocytes. However, the concept of adipocyte deletion by apoptosis as a significant contributor to adipose tissue loss during weight reduction is a relatively recent one. Qian et al showed that rat adipocytes underwent apoptosis following brain administration of leptin and thereby demonstrated that leptin induced weight loss was a result not only of an decrease in food intake and increase in energy expenditure, but also of adipocyte depletion (36). Niesler et al. found a depot specific susceptibility to apoptosis in human preadipocytes. Omental preadipocytes were more susceptible to apoptotic stimuli than were subcutaneous preadipocytes, indicating a possible mechanism for regulation of adipocyte tissue distribution (37). Several studies also demonstrated that conjugated linoleic acid (CLA) reduces body fat content in part by inducing adipocyte apoptosis, and that some factors, including PPARs (38), UCPs (39), leptin (39) and TNF- α (40), are involved in CLA modestly induced adipocyte apoptosis. However, data on the mechanisms and regulation of adipocyte apoptosis are still limited.

Previous data from our laboratory have demonstrated that $1\alpha, 25-(OH)_2-D_3$ appears to modulates adipocyte lipid and energy metabolism via both genomic and non-

genomic mechanisms(3,4,7,8). We have reported that $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ plays a direct role in the modulation adipocyte Ca^{2+} signaling(8), resulting in an increased lipogenesis and decreased lipolysis(1,5,8). In addition, $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ also plays a role in regulating human adipocyte UCP2 mRNA and protein levels, indicating that the suppression of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ and the resulting up-regulation of UCP2 may contribute to increased rates of lipid oxidation(7). Accordingly, the suppression of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ by increasing dietary calcium attenuates adipocyte triglyceride accumulation and caused a net reduction in fat mass in both mice and humans in the absence of caloric restriction (4-5), a marked augmentation of body weight and fat loss during energy restriction in both mice and humans(4-5), and a reduction in the rate of weight and fat regain after food restriction in mice(4, 41). Although these anti-obesity effects of dietary calcium are appear to be due, in part, to effects on lipolysis and lipogenesis(1,4), it is also possible that a loss of adipocytes would results in a cellular deficit lipid esterification as the body recovers from energy restriction. Thus, a reduced number of available adipocytes may not be able to store excess energy coming from rebound in food intake. Moreover, generating new cells would require extra energy that would contribute to a further metabolic enhancement. Accordingly, present study demonstrates that lower (physiological) doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibit apoptosis in differentiated 3T3-L1 adipocytes, and the suppression of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ *in vivo* by increasing dietary calcium stimulates adipocyte apoptosis during refeeding following energy restriction in aP2 transgenic mice, suggesting that the stimulation of adipocyte apoptosis contributes to reduced adipose tissue mass after administration of high calcium diets.

Key feature of apoptosis involves the proteolytic caspases (42) as well as apoptotic proteins Bcl-2 and Bax (43). Activation of Bax induces apoptosis by disturbing mitochondrial electron transport chain, counters the death repressor activity of Bcl-2, and promotes the release of cytochrome c into the cytoplasm (44-45). This, in turn, activates caspases that initiate execution of apoptotic death. Bcl-2, which is localized to mitochondria, inhibits Bax induced apoptosis, and consequently, the Bcl-2/Bax ratio can be used to determine the susceptibility to apoptosis. In the present study, we found that lower doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (0.1nm, 1nm, 5nm and 10nm) dose-dependently inhibit caspase-1 and caspase-3 gene expression, and increase the Bcl-2/Bax expression ratio in control and UCP2 transfected 3T3-L1 adipocytes, indicating an inhibition of apoptosis. We also found that UCP2 transfected 3T3-L1 adipocytes have higher basal level of caspase-1 and caspase-3 expression but a lower Bcl-2/Bax ratio than non-transfected 3T3-L1 cells. These results are consistent with our observation that UCP2 stimulates apoptosis by inducing mitochondrial potential collapse and inhibiting ATP production. We previously demonstrated that $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibits UCP2 expression in human adipocytes (7). Consistent with our this observation, we have found $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ functionally inhibits UCP2 action by increasing mitochondrial potential and ATP production in both non-transfected and UCP2 transfected 3T3-L1 cells. Thus, the suppression of apoptosis induced by $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ is, in part, mediated by inhibiting UCP2 expression and activity. Accordingly, we proposed that the suppression of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ secondary to consumption of high calcium diets may stimulate adipocytes apoptosis *in vivo*. We evaluated this concept in aP2 transgenic mice undergoing re-feeding following energy restriction, and found a significant increases in white adipose

tissue apoptosis in mice re-fed high calcium diets(1.3%) compared to low calcium diets (0.4% Ca). These results further confirm that suppression of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ stimulates apoptosis in white adipose tissue and suggest that this effect also contribute to the anti-obesity effect of dietary calcium.

In contrast, very high doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$, which are three to four fold higher than physiological levels, exert the opposite effect. We found that a high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (100nM) actually stimulated caspase-1 and caspase-3 expression and inhibited Bcl-2/Bax ratio, a complete reversal of the effect of lower doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on apoptosis. Although lower doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ caused moderate increases in cytosolic $[\text{Ca}^{2+}]$ levels in adipocytes, the high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ induced an extreme elevation, and excessive intracellular calcium has been reported to be a pro-apoptotic factor(45-46). Ca^{2+} is a ubiquitous intracellular messenger involved in many cellular processes. To generate such complex Ca^{2+} signals, cells rely on the rapid release of the Ca^{2+} storage (such as ER/SR and mitochondria) as well as on the controlled Ca^{2+} influx from the extracellular medium upon stimuli, and strive to maintain the Ca^{2+} concentration at extremely low levels by expelling Ca^{2+} ions to the exterior and by compartmentalization of Ca^{2+} intracellular stores under physiological conditions of rest. Mitochondria take up Ca^{2+} through the uniporter on the inner membrane at the expense of $\Delta\psi$ (48). Mitochondria are often located close to the ER, and therefore exposed to the Ca^{2+} release by the Inositol-1,4,5-trisphosphate receptor(IP3R) and ryanodine receptor (RyR). The high Ca^{2+} levels achieved at these contact sites favors Ca^{2+} uptake into mitochondria. Because of their tight coupling to ER Ca^{2+} store, mitochondria are highly susceptible to abnormalities in Ca^{2+} signaling (49). Recent evidence suggests that the

amount of Ca^{2+} going through mitochondria is crucial in triggering a Ca^{2+} -dependent apoptosis response, probably by the opening of a sensitized state of permeability transition pore (PTP) (50). Scorrano *et al* reported that mouse embryonic fibroblasts deficient in Bax were protected from apoptosis because of reducing resting concentration of calcium in ER and decreased uptake of Ca^{2+} by mitochondria from the ER(23). Expression of SERCA, on the other hand, corrected $[\text{Ca}^{2+}]_{\text{er}}$ and $[\text{Ca}^{2+}]_{\text{m}}$, thereby restoring apoptotic death (51). In the present study, we also monitored the mitochondrial Ca^{2+} concentration and found that low-doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ decreased mitochondrial Ca^{2+} levels in a dose-dependent manner while high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ markedly stimulates the elevation of $[\text{Ca}^{2+}]_{\text{m}}$, indicating that the increase $[\text{Ca}^{2+}]_{\text{m}}$ is associated with the induction of apoptosis by high-dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$. We have previously reported that mitochondrial uncoupling stimulated $[\text{Ca}^{2+}]_{\text{c}}$ level, which may in turn induce an increase in $[\text{Ca}^{2+}]_{\text{m}}$ to maintain cytoplasmic calcium homeostasis. Low-doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ induce dose-dependent depletion $[\text{Ca}^{2+}]_{\text{m}}$ by inhibiting UCP2, thereby protecting adipocytes from apoptotic death. High-doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$, on the other hand, increases $[\text{Ca}^{2+}]_{\text{c}}$ to extreme level, which may increase both $[\text{Ca}^{2+}]_{\text{m}}$ and $[\text{Ca}^{2+}]_{\text{er}}$. Since ER can open their Ca^{2+} release channel in response to elevations in $[\text{Ca}^{2+}]_{\text{c}}$ and contribute to Ca^{2+} induced Ca^{2+} release (CICR), this may further increases $[\text{Ca}^{2+}]_{\text{m}}$ and calcium overload in mitochondria in turn triggers apoptosis.

In summary, the present study demonstrates dual effects of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on apoptotic death. These are summarized in **Figure 5-11** as follows: by inhibiting UCP2 and decreasing mitochondrial calcium, lower (physiological) doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$

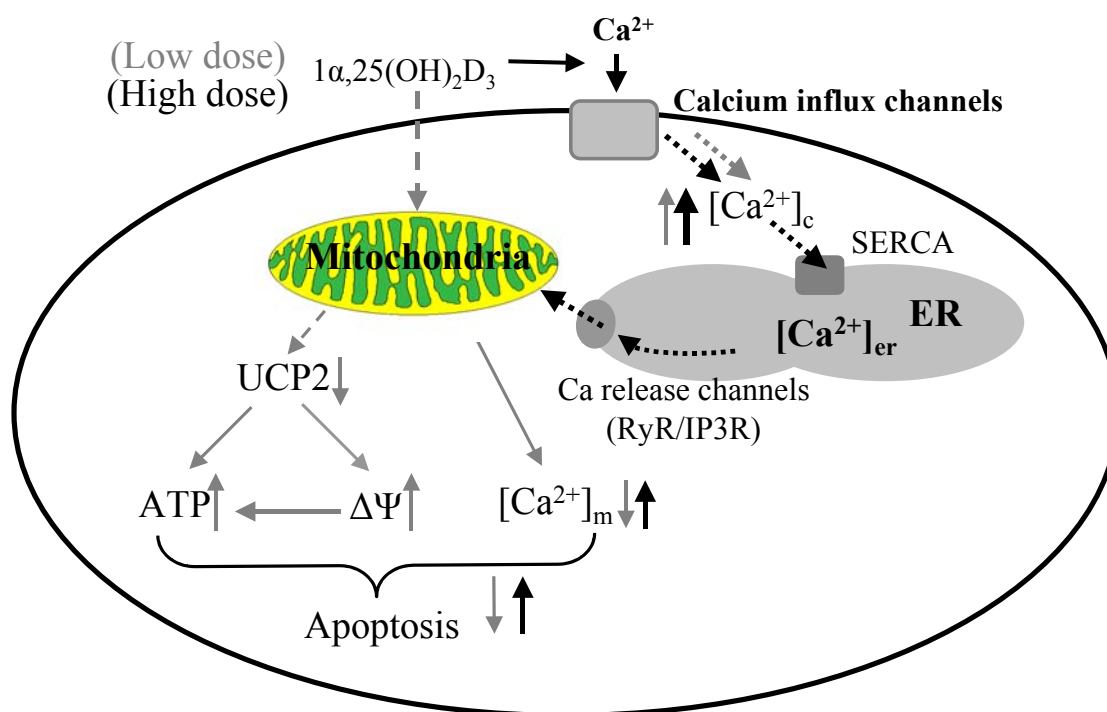


Figure 5-11. Schematic illustration of effects and mechanisms of $1\alpha, 25\text{-(OH)}_2\text{D}_3$ on adipocyte apoptosis. Physiological low doses of $1\alpha, 25\text{-(OH)}_2\text{D}_3$ restore mitochondrial potential and ATP production by suppressing UCP2, thereby inhibiting apoptosis. By contrast, high dose of $1\alpha, 25\text{-(OH)}_2\text{D}_3$ induces mitochondrial calcium overload and stimulates apoptosis.

inhibit apoptosis in adipocytes, while suppression of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ using high calcium diets stimulates adipose apoptosis in mice. In contrast, a high dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ stimulates mitochondrial calcium overload and apoptosis in adipocytes. These results indicate that dietary calcium not only regulates adipocytes size by decreasing lipid accumulation, but also modulates adipocyte number by stimulating apoptotic death. Thus, these results provide a new mechanism in support of the “anti-obesity” of dietary calcium.

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Part Six

Mechanisms of Dual Effects of 1α , 25-Dihydroxyvitamin D₃ on Adipocyte Apoptosis

6.1 Abstract

We have previously shown that $1\alpha, 25$ -Dihydroxyvitamin D_3 ($1\alpha, 25$ -(OH) $_2$ - D_3) has dual effects on apoptotic death in adipocytes: physiological doses of $1\alpha, 25$ -(OH) $_2$ - D_3 inhibit adipocyte apoptotic death while suppression of $1\alpha, 25$ -(OH) $_2$ - D_3 using high calcium diets stimulates adipose apoptosis in mice; In contrast, pharmacological doses of $1\alpha, 25$ -(OH) $_2$ - D_3 stimulate apoptosis. We have also demonstrated that $1\alpha, 25$ -(OH) $_2$ - D_3 inhibits expression of mitochondrial uncoupling protein 2 (UCP2), which plays a key role in regulation of mitochondrial potential and apoptosis. We therefore hypothesized that low doses of $1\alpha, 25$ -(OH) $_2$ - D_3 inhibit adipocyte apoptotic death by inhibiting UCP2 expression while high doses stimulate adipocyte apoptotic death via a calcium dependent mechanism. In the current study, Ca^{2+} ionophore Bay K 8644(BK8644) induced 3-4 fold of increases in $[Ca^{2+}]_i$ (within the response range to physiological low doses of $1\alpha, 25$ -(OH) $_2$ - D_3) and dose-dependently stimulated caspase-3 expression by 94%-260% ($p<0.01$). In contrast, GDP, a potent inhibitor of mitochondrial uncoupling, decreased $[Ca^{2+}]_i$, and increased mitochondrial potential, and suppressed caspase-3 expression in a dose dependent manner(47%-80%, $p<0.05$). To address the independent effect of mitochondrial uncoupling, pretreatment of 3T3-L1 cells with BK 8644 prevented GDP-induced decreases in $[Ca^{2+}]_i$ but preserved the effect on GDP stimulated mitochondrial potential. GDP suppressed caspase-3 expression by 45% ($p<0.05$) in BK8644 pretreated cells. Transfection of dsRNA specific for UCP2 mRNA into 3T3-L1 cells suppressed UCP2 expression by 70%, and caused a 52% increase in mitochondrial potential but a 58% decrease in caspase-3 expression ($p<0.05$), indicating a direct role of mitochondrial

uncoupling in adipocyte apoptosis. These data further demonstrate that physiological doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibit apoptosis and this effect is attributable to the inhibitory effect on mitochondrial uncoupling. In contrast, high doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ stimulate apoptosis via a calcium-dependent mechanism.

6.2 Introduction

A substantial body of recent evidence demonstrate that dietary calcium regulates adiposity (1-5). High dietary calcium, by suppressing $1\alpha, 25\text{-(OH)}_2\text{-D}_3$, decreases intracellular calcium and thereby inhibits lipogenesis and stimulates lipolysis (4-8), while low dietary calcium intakes increase $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ and thereby causes an increase in adiposity (9). Although the anti-obesity effect of dietary calcium appears to be primarily attributable to the regulatory effect of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on intracellular calcium on adipocyte lipid accumulation, regulation on adipocyte apoptosis may also involved.

We have recently shown that $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ has dual effects on adipocyte apoptosis, with low (within the physiological range) doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$, which can be induced by low calcium diets in vivo, inhibiting apoptosis and pharmacologically high doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ stimulating apoptosis(10). The mechanism of these dual effects on adipocyte apoptosis induced by $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ is yet to be determined. However, we have previously demonstrated that $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ inhibits mitochondrial uncoupling protein 2 (UCP2) expression in cultured human adipocyte via a calcium independent mechanism(11). Because UCP2, which is highly expressed in white adipose tissue, has been founded to function as mitochondrial uncoupler of oxidative

phosphorylation and thus reduce efficiency of ATP synthesis (12), it is reasonable to propose that UCP2 may stimulate adipocyte apoptosis. Accordingly, the inhibition of UCP2 by low doses of 1α , 25-(OH) $_2$ -D $_3$ may suppress apoptosis. This observation, coupled with the observation that intracellular calcium plays a key role in apoptosis (13), led us to test the hypothesis that low dose of 1α , 25-(OH) $_2$ -D $_3$ inhibits apoptosis by suppressing UCP2 expression while high dose of 1α , 25-(OH) $_2$ -D $_3$ stimulates apoptosis via a calcium dependent pathway.

6.3 Materials and Methods

6.3.1 Culture and Differentiation of 3T3-L1 Adipocytes

3T3-L1 preadipocytes were incubated at a density of 8000 cells/cm ^{2} (10 cm ^{2} dish) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics at 37°C in 5% CO $_2$. Confluent preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM-F10 (1:1, vol/vol) medium supplemented with 1% FBS, 1 μ M dexamethasone, IBMX (0.5 mM) and antibiotics (1% penicillin/streptomycin). Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium. Cultures were refed every 2–3 days. Cell viability was measured via trypan blue exclusion.

6.3.2 UCP2 Transfection

UCP2 full-length cDNAs were amplified by RT-PCR using mRNAs isolated from mouse brown and white adipose tissues, respectively. The PCR primers for this

amplification are shown as follows: UCP2 forward, 5'-GCTAGCATGGTTGGTTTCAAG-3', reverse, 5'-GCTAGCTCAGAAAGGTGAATC-3'. The PCR products were then subcloned into pcDNA4/His expression vectors. The linearized constructs were transfected into 3T3-L1 preadipocytes using lipofectamine plus standard protocol (Invitrogen, Carlsbad, CA). After 48 hrs of transfection, cells were split and cultured in selective medium containing 400 ug/ml zeocin for the selection of resistant colonies. Cells were fed with selective medium every 3 days until resistant colonies were identified. These resistant foci were picked, expanded, tested for expression, and frozen for future experiments.

6.3.3 siRNA Preparation and Transfections

Duplex siRNA corresponding to UCP2 gene was designed and chemically synthesized by Invitrogen. The following gene-specific sequences were used successfully: Si-UCP2 sense 5'-GCCUCUACGACUCUGUCAA-3' and antisense 5'-UUGACAGAGUCGAGGC-3'. Transfection of siRNA was performed with OligofectamineTM reagent (Invitrogen Life Technologies, CA) in 6-well plates. Briefly, Oligofectamine diluted in DMEM was applied to the duplex siRNA mixture, and the formulation was continued for 20min to allow the Oligofectamine-oligonucleotide complex to form. Per well, 800µl of plating medium and 10ul of 20µM duplex siRNA duplex siRNA formulated with 4µl of Oligofectamine were applied in a final volume of 1ml. Cells were then incubated at 37°C in a CO₂ incubator for 4hrs. 500 ul of DMEM medium containing 3x serum was added to transfection mixture after transfection, and cells were incubated for 48 h~72h.

6.6.4 Western Blot Analysis

Adipocytes were harvested and sonicated in a homogenization buffer containing 50 mM Tris-HCl (PH 7.4), 250mM sucrose, 1mM EDTA, 1mM dithiothreitol, 1% (v/v) Triton X, 10% protease cocktail (Sigma). Cell homogenates were incubated on ice for 1h to solublize all proteins, and the insoluble protein was removed by centrifugation at 12,500g at 4⁰C for 15 min. Homogenate intrafratant protein from equal numbers of cells (determined via protein quantitation using Bradford method as described previously) was boiled in Laemmli sample buffer and subject to 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins on the gels were transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The transferred membranes with proteins were blocked, washed, incubated with 1:1000 dilution of anti-UCP2 polyclonal antibody or 1:500 dilution of anti-actin polyclonal antibody (Santa Cruz Biotechnology, CA) followed by peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA). Visualization was detected with chemiluminescence reagent, using the Western Blotting Luminal Reagent(Santa Cruz Biotechnology, CA).

6.3.5 Determination of Mitochondrial Membrane Potential

Mitochondrial membrane potential were analyzed spectrophotometrically with a lipophilic cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide) using a mitochondrial potential detection kit (Biocarta, San Diego, CA).

6.3.6 Measurement of Cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) and Mitochondrial Calcium ($[\text{Ca}^{2+}]_m$)

$[\text{Ca}^{2+}]_c$ in adipocytes was measured using a fura-2 dual-wavelength fluorescence imaging system. Cells were plated in 35-mm dishes (P35G-0-14-C, MatTek). Prior to $[\text{Ca}^{2+}]_c$ measurement, cells were put in serum-free medium overnight and rinsed with HEPES balanced salt solution (HBSS) containing the following components (in mM): 138 NaCl, 1.8 CaCl_2 , 0.8 MgSO_4 , 0.9 NaH_2PO_4 , 4 NaHCO_3 , 5 glucose, 6 glutamine, 20 HEPES, and 1% bovine serum albumin. Cells were loaded with fura-2 acetoxymethyl ester (fura-2 AM) (10 μM) in the same buffer for 2 h at 37°C in a dark incubator with 5% CO_2 . To remove extracellular dye, cells were rinsed with HBSS three times and then post-incubated at room temperature for an additional 1h for complete hydrolysis of cytoplasmic fura-2 AM. The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu model 4915 charge-coupled device (CCD) camera. Fluorescent images were captured alternatively at excitation wavelengths of 340 and 380 nm with an emission wavelength of 520 nm. After establishment of a stable baseline, the responses to $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ was determined. $[\text{Ca}^{2+}]_c$ was calculated using a ratio equation as described previously. Each analysis evaluated responses of 5 representative whole cells. Images were analyzed with InCyt Im2 version 4.62 imaging software (Intracellular Imaging, Cincinnati, OH). Images were calibrated using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR) to create a calibration curve in solution, and cellular calibration was accomplished using digitonin (25 μM) and pH 8.7 Tris-EGTA (100 mM) to measure maximal and minimal $[\text{Ca}^{2+}]_c$ levels respectively.

Rhod-2/AM was used for qualitative measurement of $[Ca^{2+}]_m$ (14). Cells were incubated with the Ca^{2+} sensitive fluorescent indicator rhod-2 AM (1-1.5 μ M), for at least 1 h at 37°C in 5% CO_2 in humidified air. Since rhod-2 AM consists of a cationic rhodamine molecule, it accumulates preferentially inside the mitochondria due to their negative membrane potential. After loading, the cells were kept in rhod-2 AM-free standard solution for at least 1 h to allow conversion of the dye to its Ca^{2+} -sensitive, free acid form. Images of $[Ca^{2+}]_m$ were acquired at the wavelength of 552nm excitation and 590nm emission using the intracellular imaging system described above. Since rhod-2 is not a ratiometric dye, its fluorescence intensity was not calibrated to obtain absolute values of $[Ca^{2+}]_m$. Instead, only relative values were recorded as fluorescence signal (F) relative to the control value (F_0).

6.3.7 Total RNA Extraction

A total cellular RNA isolation kit (Ambion, Austin, TX) was used to extract total RNA from 3T3-L1 cells according to manufacturer's instruction.

6.3.8 Quantitative Real-time PCR

Adipocyte caspase-3 was quantitatively measured using a Smart Cycler Real Time PCR System (Cepheid, Sunnyvale, CA) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg, NJ). The primers and probe sets were designed and synthesised by Applied Biosystems TaqMan® Assays-on-Demand™ Gene Expression primes and probe set collection according to manufacture's instruction. Pooled 3T3-L1 adipocyte total RNA was serial-diluted in the range of 1.5625-25 ng and used to establish a standard curve; total RNAs for unknown samples were also diluted in this range.

Reactions of quantitative RT-PCR for standards and unknown samples were also performed according to the instructions of Smart Cycler System (Cepheid, Sunnyvale, CA) and TaqMan Real Time PCR Core Kit (Applied Biosystems, Branchburg, NJ). The mRNA quantitation for each sample was further normalized using the corresponding 18s quantitation, with a forward primer: 5'-AGTCCCTGCCCTTTGTACACA-3' and a reverse primer: 5'-GATCCGAGGGCCTCACTAAAC-3'.

6.3.9 Statistical analysis

All data are expressed as mean $\pm$ SE. Data were evaluated for statistical significance by one-way analysis of variance (ANOVA), and significantly different group means were then separated by the least significant difference test by using SPSS (SPSS Inc, Chicago, IL.).

6.4 Results

We have previously shown that the increase of mitochondrial uncoupling by either chemical uncouplers or UCP2 overexpression in 3T3-L1 cells stimulates apoptosis, with significant increases in caspase-1 and caspase-3 expression as well as decrease in Bcl-2/Bax expression ratio (10). Consistent with this observation, we found in this study that suppression of UCP2 by siRNA transfection in 3T3-L1 cells caused a 70% decrease in UCP2 expression and a 52% increase in mitochondrial potential ($p < 0.05$) (**Figures 6-1 and 6-2**), indicating that suppression of UCP2 increases adipocyte mitochondrial potential. Inhibition of UCP2 expression in transfected cells also induced a 58% decrease

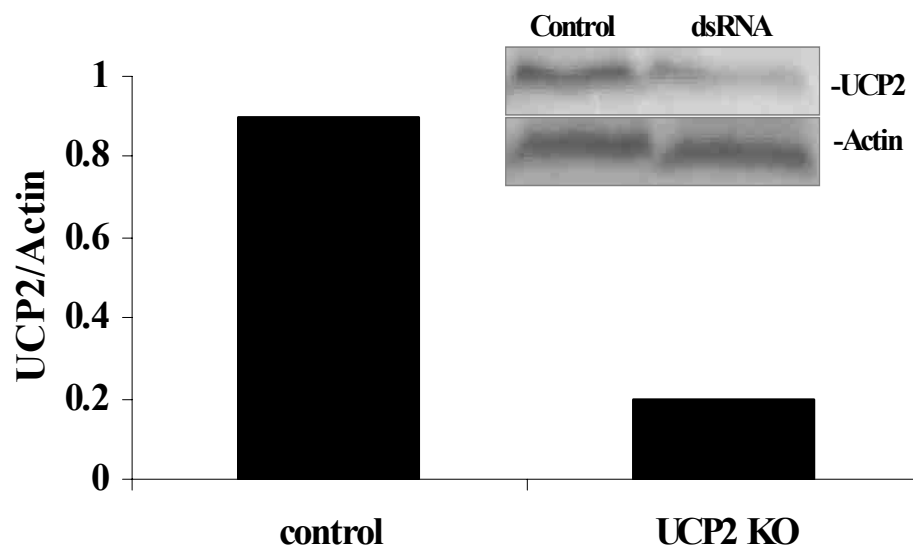


Figure 6-1. Western blot with anti-mouse UCP2 antibody of cell lysates harvested 48hrs after siRNA transfection for evaluation of UCP2 protein reduction (upper panel). The internal control actin is shown in the low panel. Blot shown is representative of three similar experiments.

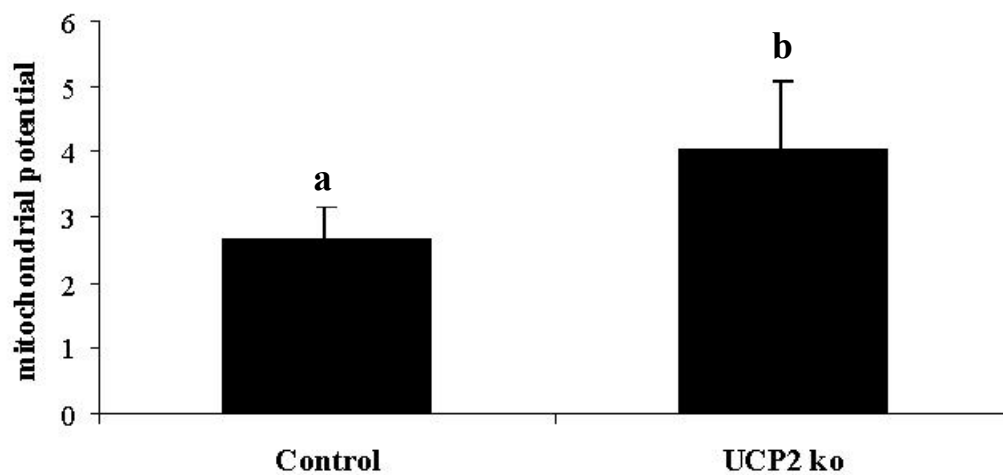


Figure 6-2. Mitochondrial potential in 3T3-L1 and siRNA transfected 3T3-L1 adipocytes (UCP2 ko). Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

in caspase 3 expression ($p<0.01$) (**Figure 6-3**). These data suggest that UCP2 plays a direct role in stimulation of adipocyte apoptosis. Accordingly, suppression of UCP2 expression decreases apoptotic stress.

Although we demonstrated that overexpression of UCP2 favors adipocyte apoptosis while suppression of UCP2 decreases apoptotic stress (10), it is not clear whether other UCP isoforms may also be regulated correspondingly during UCP2 manipulation and thereby affect apoptosis. Accordingly, we further used GDP as a general mitochondrial uncoupling inhibitor to investigate the effect of mitochondrial uncoupling inhibition on apoptosis. GDP at 100 μ M and 500 μ M increased mitochondrial potential by 27% and 48% ($p<0.05$) respectively (**Figure 6-4**), confirming an inhibitory effect of GDP on mitochondrial uncoupling. **Figure 6-5** shows that GDP treatments inhibited apoptosis in 3T3-L1 cells, with 47%-81% decreases in caspase-3 expression ($p<0.01$), indicating that general inhibition of mitochondrial uncoupling suppresses adipocyte apoptosis.

Bay K 8644 (BK 8644) is a Ca^{2+} ionophore which stimulated adipocyte Ca^{2+} influx in a dose-dependent manner (**Figure 6-6**). This stimulation of BK8644 (1nM-100nM) on $[\text{Ca}^{2+}]_i$ is of comparable magnitude to the effect of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$. However, BK8644 exerted no effect on mitochondrial potential or UCP2 expression (**Figure 6-7**). This result is consistent with our previous observation that $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ regulates UCP2 expression via a calcium-independent mechanism(10). Notably, BK 8644 stimulated caspase-3 expression dose-dependently (**Figure 6-8**), indicating that increases in intracellular calcium influx independently stimulate adipocyte apoptosis. Consistent

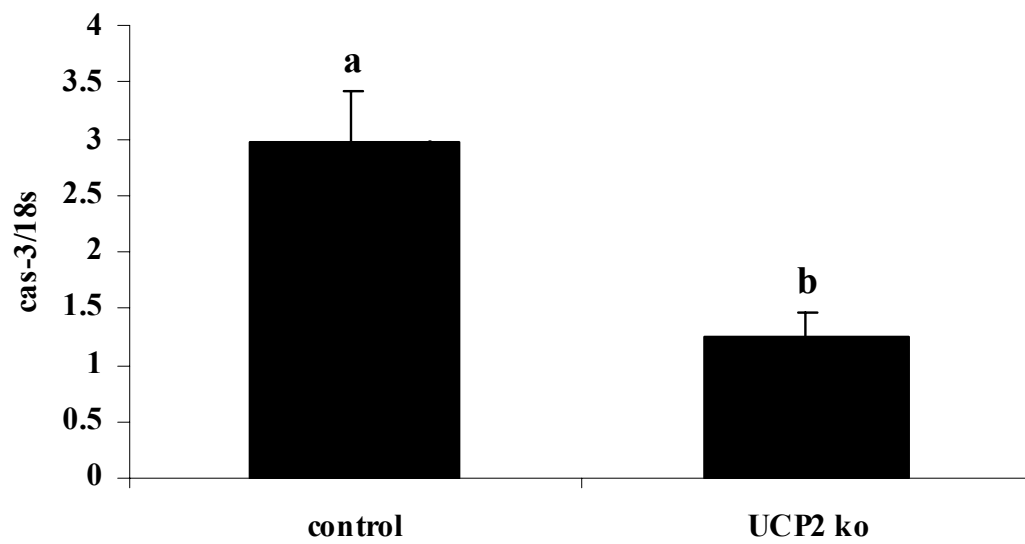


Figure 6-3. Caspase-3 expression in 3T3-L1 and siRNA transfected 3T3-L1 adipocytes (UCP2 ko). Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

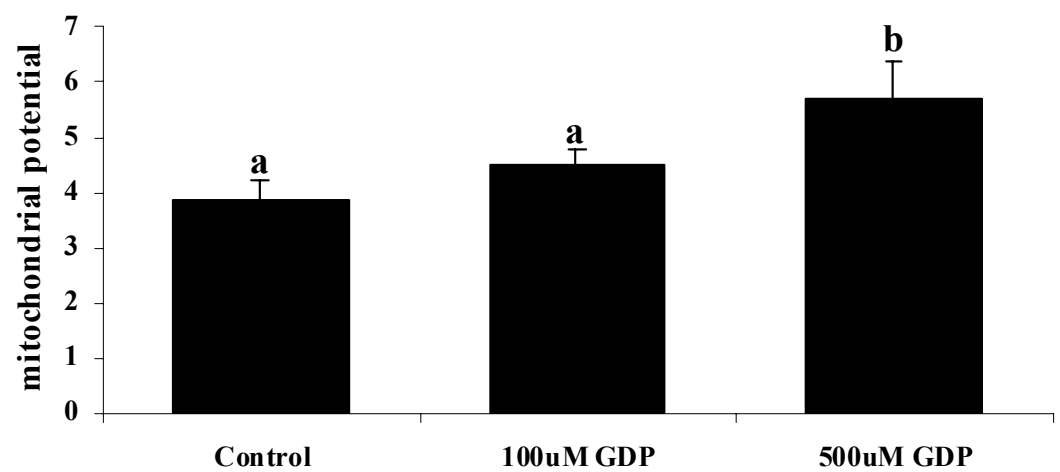


Figure 6-4. Effect of mitochondrial uncoupling inhibitor GDP on mitochondrial potential in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

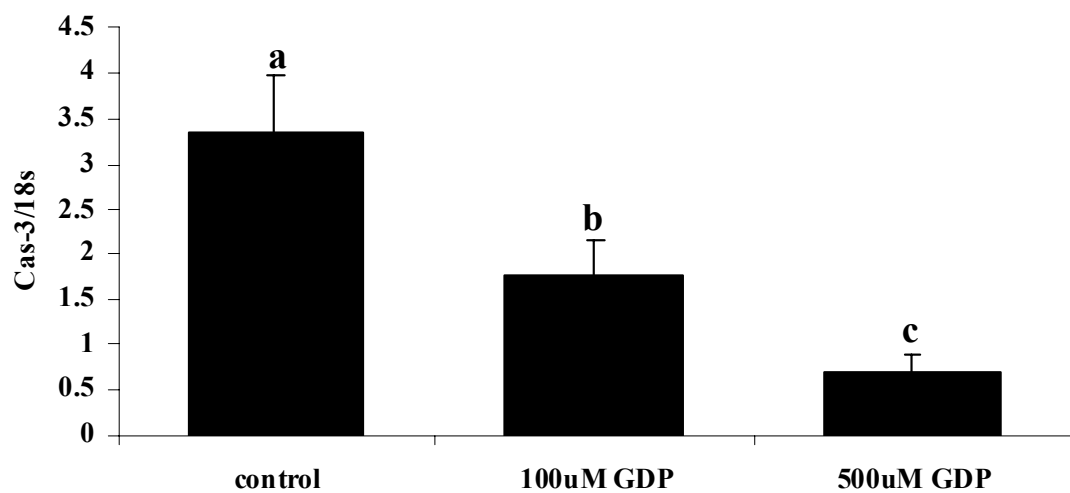


Figure 6-5. Effect of mitochondrial uncoupling inhibitor GDP on caspase-3 expression in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

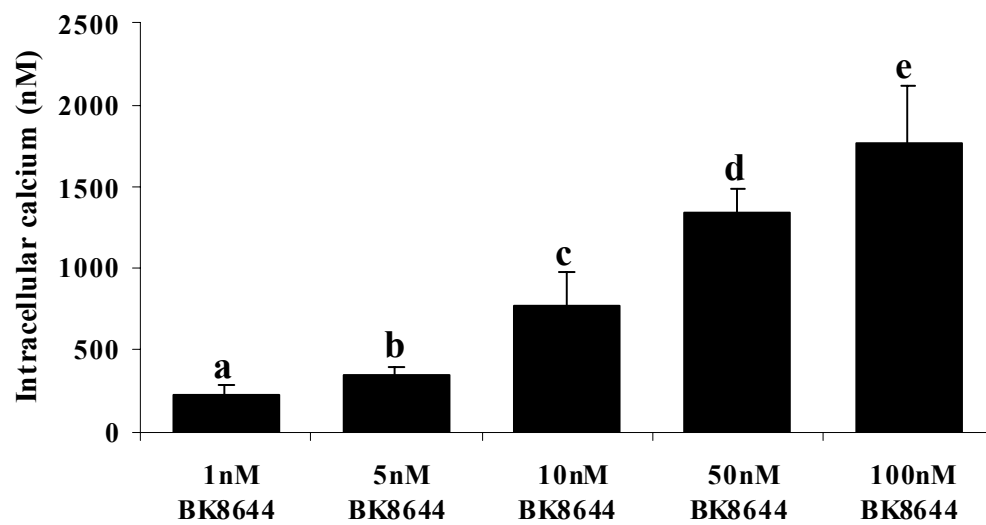


Figure 6-6. Effect of calcium channel ionophore Bay K 8644 on intracellular calcium levels in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

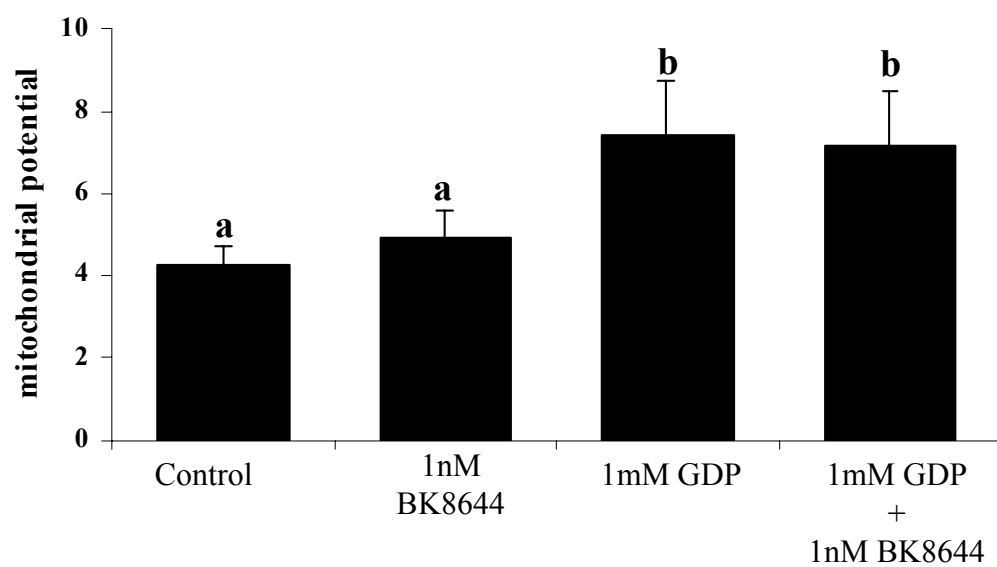


Figure 6-7. Effect of mitochondrial uncoupling inhibitor GDP and calcium channel ionophore Bay K 8644 on mitochondrial potential in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

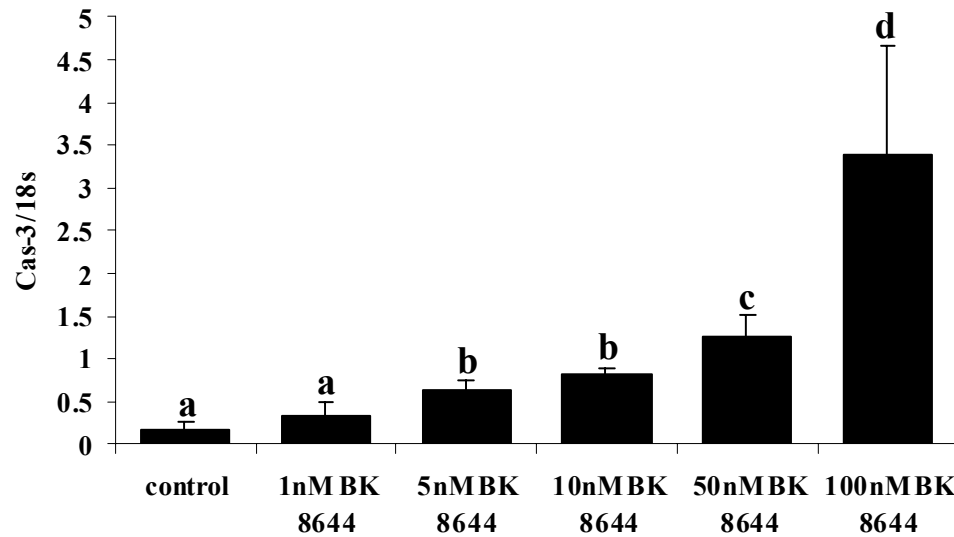


Figure 6-8. Effect of calcium channel ionophore Bay K 8644 on caspase-3 expression in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

with our previous finding that mitochondrial uncoupling increases cytosolic calcium level (15), inhibition of mitochondrial uncoupling with GDP decreased $[Ca^{2+}]_i$ level significantly (data not shown). Combining 1mM GDP with 1nM BK 8644 exerted no effect on $[Ca^{2+}]_i$ but induced similar magnitude of increases in mitochondrial potential as observed in GDP alone (**Figure 6-7**), indicating that the regulation of mitochondrial potential is calcium independent. However, addition of Bay K 8644 caused a significant increase in caspase-3 expression (**Figure 6-9**), suggesting that $[Ca^{2+}]_i$ stimulates apoptosis independent of mitochondrial potential.

Stimulation of calcium influx by BK 8644 increased mitochondrial calcium (**Figure 6-10**) ($p < 0.05$). Although $1\alpha, 25-(OH)_2-D_3$ induced similar effects on $[Ca^{2+}]_i$ increase as BK8644, the opposite effect on mitochondrial calcium was observed with low dose of $1\alpha, 25-(OH)_2-D_3$ (0.1nM to 10nM) pretreated cells, with significant decreases in mitochondrial calcium. The high dose of $1\alpha, 25-(OH)_2-D_3$, however, caused similar increases in mitochondrial calcium as observed with BK 8644.

6.5 Discussion

We investigated the effects of the calcium channel ionophore BK 8644, a mitochondrial uncoupling inhibitor (GDP) and UCP2 silencing using siRNA transfection on apoptosis. BK 8644, which exerted no effect on mitochondrial uncoupling, caused a dose dependent increase in intracellular calcium as well as mitochondrial calcium storage, and stimulated caspase-3 expression, indicating that increases in intracellular calcium influx, along with mitochondrial calcium load, stimulate apoptosis. Inhibition of

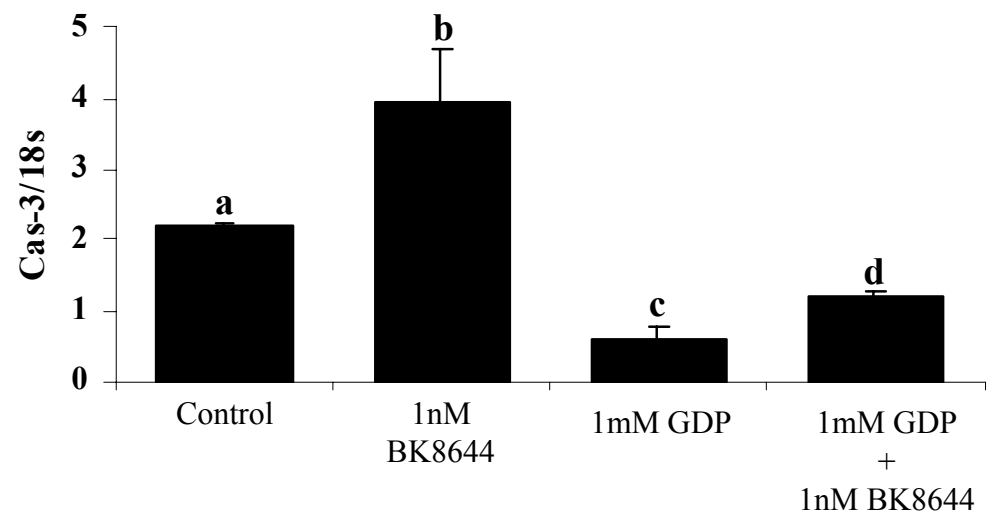


Figure 6-9. Effect of mitochondrial uncoupling inhibitor GDP and calcium channel ionophore Bay K 8644 on caspase-3 expression in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6). Different letters above the bars indicate a significant difference at level of $p < 0.05$.

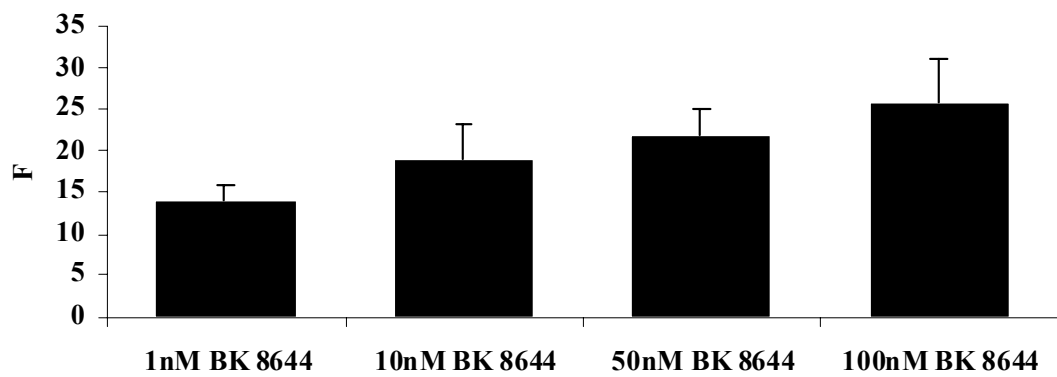


Figure 6-10. Effect of calcium channel ionophore Bay K 8644 on mitochondrial calcium in 3T3-L1 adipocytes. Data are expressed as mean $\pm$ SE (n =6).

mitochondrial uncoupling with GDP decreased intracellular calcium and mitochondrial potential and thereby inhibited apoptosis. Consistent with our previous data that the mitochondrial uncoupler 2, 4-dinitrophenol (DNP) stimulates apoptosis (10), these results indicate a direct role of mitochondrial uncoupling in stimulating of apoptosis. Specific suppression of UCP2 also decreased apoptosis while UCP2 overexpression caused the opposite effect. Accordingly, the inhibitory effect of low dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ on apoptosis may be mediated by its suppression of UCP2 expression (11). Although $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ increases $[\text{Ca}^{2+}]_i$, and such increases are often associated with apoptosis, only the very high dose ($>100\text{nM}$) of $1, 25\text{(OH)}_2\text{D}_3$ stimulated apoptosis. This result indicates that the pro-apoptotic effect of intracellular calcium with the treatment of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ may be overwhelmed by its anti-apoptotic effect induced by suppression of UCP2.

Early studies by Kaiser and Edelman demonstrated that glucocorticoid-stimulated apoptosis is associated with enhanced $[\text{Ca}^{2+}]_i$ influx (16), providing the first evidence that increased $[\text{Ca}^{2+}]_i$ might be involved in triggering apoptosis. This hypothesis was further supported by the observation that increased level of inositol 1,4,5-triphosphate(InsP3) receptor in lymphocytes stimulates apoptosis while IP3R-deficient cells are resistant to T-cell receptor (TCR)-induced apoptosis(17-18). Calcium was also linked to the release of arachidonic acid from cells triggered to undergo apoptosis by various chemotherapeutic agents (19), suggesting that calcium is important to many cPLA2-dependent apoptotic responses. Consistent with this, the present study confirms that increases in intracellular calcium influx stimulate apoptosis by increasing caspase-3 expression. This result may explain the pro-apoptotic effect of high dose $1\alpha, 25\text{-(OH)}_2\text{-D}_3$. In addition, calcium increases precede the cytolysis of the targets of cytotoxic T cells, indicating rapid,

sustained intracellular calcium increase is required for the apoptotic process (20). Although $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ induced dose-dependent increases in $[\text{Ca}^{2+}]_i$, our data indicate that the magnitude of this increases in $[\text{Ca}^{2+}]_i$ may determine whether apoptosis results. Previous studies demonstrate that intracellular calcium storage sites also appear to be affected, as the cytosolic calcium undergo changes in response to apoptotic stimuli (21-22). During apoptotic stress, the mitochondrial calcium pool may also be affected, since mitochondrial potential, which plays a key role in maintaining mitochondrial calcium homeostasis, drops very early during apoptotic death (23). Decreased mitochondrial potential reduces ATP production and causes cytochrome c leakage and mitochondrial swelling (24). Accordingly, mitochondrial uncoupling might stimulate apoptosis by decreasing mitochondrial potential. This hypothesis is further supported by our observations that the chemical uncoupler DNP decreases adipocyte mitochondrial potential and stimulates apoptosis (10) while in present study, the mitochondrial uncoupling inhibitor GDP causes the opposite effects. Consistent with this, overexpression of UCP2 stimulated apoptotic proteases while suppression of UCP2 decreased expression of these genes, indicating that UCP2 plays a positive role in regulation of apoptosis. $1\alpha, 25\text{-(OH)}_2\text{-D}_3$, which has been found to inhibit UCP2 expression via a calcium-independent pathway (11), suppressed apoptosis at physiological low doses from 0.1nM to 10nM, suggesting that the anti-apoptotic effect of low doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ might be mediated by suppression of UCP2 expression.

In the present study, $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ induced similar effect on $[\text{Ca}^{2+}]_i$ increase as BK 8644 but the two compounds exerted different effects on mitochondrial calcium storage, as low doses $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (0.1nM to 10nM) decreased mitochondrial calcium

while BK 8644 increased mitochondrial calcium. However, the high dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ (100nM) caused similar increases in mitochondrial calcium as observed in BK 8644. Since mitochondrial calcium overload might trigger apoptosis by inducing mitochondrial potential collapse and cytochrome c release, the pro-apoptotic effect of the high dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ is most likely a result of the stimulation of mitochondrial calcium. In contrast, the decreases in mitochondrial calcium observed in response to treatment with lower dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ indicates that reduction of mitochondrial calcium load, along with the inhibitory effect on UCP2 contribute to the anti-apoptotic effect of low dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$.

Although low doses of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ may also induce apoptotic stress via a calcium dependent mechanism, this signal is counter-balanced by the suppression of UCP2, which may increase the capability to maintain intracellular homeostasis by regulating mitochondrial potential and ATP production. Indeed, many calcium channel and pumps located either on plasma membrane or intracellular organelles are ATP dependent. High dose of $1\alpha, 25\text{-(OH)}_2\text{-D}_3$, however, induced much greater increases in intracellular calcium, resulting in more rapid and stronger apoptotic signals, which could not be offset by suppression of UCP2.

In summary, physiological low dose of $1, 25\text{-(OH)}_2\text{-D}_3$ inhibits apoptosis by suppression of UCP2 expression. Since the low doses of $1, 25\text{-(OH)}_2\text{-D}_3$ used in this study are within the range of physiological levels which response to dietary calcium, the anti-obesity effect of high dietary calcium may, in addition to the previously cited regulation of lipid metabolism(1-7), may also result from suppression of apoptosis by $1\alpha, 25\text{-(OH)}_2\text{-D}_3$.

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Part Seven

Summary and Conclusion

Dietary calcium stimulates UCP2 expression in white adipose tissue and core temperature in aP2 transgenic mice during ad lib feeding, energy restriction and refeeding following energy restriction, resulting in an attenuation of fat accumulation, an acceleration of fat loss and an inhibition of weight and fat regain. In addition, UCP2 appear to regulate lipid metabolism directly and mitochondrial uncoupling induced by chemical uncouplers modulates both lipogenesis and lipolysis. Uncoupling protein 2 also stimulates adipocyte apoptosis by decreasing mitochondrial potential and ATP production, and disturbing cytosolic and mitochondrial calcium homeostasis. In addition, inhibition of UCP2 expression with physiological low doses of $1\alpha, 25(\text{OH})_2\text{D}_3$ inhibits adipocyte apoptosis. Accordingly, suppression of $1\alpha, 25(\text{OH})_2\text{D}_3$ by increasing dietary calcium stimulates UCP2 expression and thus favors adipocyte apoptotic death.

In conclusion, adipocyte UCP2 plays an important role in modulation of adiposity through regulating mitochondrial potential gradient, energy metabolism and cellular calcium homeostasis, thus results in modulation of both adipocyte lipid metabolism and adipocyte numbers, thereby regulating total adiposity. Thus, UCP2 appear to be a potential molecular target for obesity management.

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