To the graduate council:

I am submitting herewith a dissertation written by Hang Shi entitled "Role of intracellular Ca^{2+} in modulation of adiposity". I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy, with a major in Human Ecology.

Michael B. Zemel, Ph.D., Major Professor

We have read this dissertation
and recommend its acceptance:

Naïma Moustaïd-Moussa, Ph.D.

Jay Whelan, Ph.D.

John Koontz, Ph.D.

Accepted for the council:

Vice Provost and
Dean of Graduate Studies

ROLE OF INTRACELLULAR Ca^{2+} IN MODULATION OF ADIPOSITY

A Dissertation
Presented for the
Doctor of Philosophy
Degree
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Hang Shi
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DEDICATION

This dissertation is dedicated to my wife Bingzhong Xue for her deepest love, and for her spiritual and intellectual support, and to my parents, Guangrong Shi and Yulan Gu, for all their sacrifices in bringing me up, and for their moral support and uncompromising principles that guide my life.

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ABSTRACT

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays a key role in metabolic disorders associated with obesity and insulin resistance. Previous data from this laboratory demonstrated that increasing $[Ca^{2+}]_i$ via stimulation of either receptor or voltage-mediated calcium channels 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. Therefore, increasing $[Ca^{2+}]_i$ appears to promote adipocyte triglyceride accumulation by exerting a coordinated control over stimulation of lipogenesis and inhibition of lipolysis. These data suggest that adipocyte $[Ca^{2+}]_i$ is a logical target for pharmacological/nutritional regulation of adiposity. However, the role of $[Ca^{2+}]_i$ in human adipocyte differentiation, an important contributor to adiposity, is unknown. Moreover, specific adipocyte $[Ca^{2+}]_i$ targets need to be further explored. Accordingly, our studies were designed to (1) determine the role of $[Ca^{2+}]_i$ in human adipocyte differentiation, and (2) identify adipocyte targets which regulate $[Ca^{2+}]_i$ and determine strategies for modulating these targets. We demonstrated that increasing preadipocyte $[Ca^{2+}]_i$ in early stages of differentiation suppresses human adipocyte differentiation, while increasing $[Ca^{2+}]_i$ late promotes human adipocyte differentiation. To antagonize adipocyte Ca^{2+} influx by blocking Ca^{2+} channels, we identified a potential receptor target, the adipocyte sulfonylurea receptor (SUR), which mediates adipocyte $[Ca^{2+}]_i$ signaling and corresponding increased lipogenesis and decreased lipolysis. Antagonism of this receptor reverses all these actions. We further extended this approach to dietary regulation of adipocyte $[Ca^{2+}]_i$. We demonstrated that $1\alpha,25\text{-}(OH)_2\text{-}D_3$, which is regulated by dietary calcium, also stimulates adipocyte $[Ca^{2+}]_i$, promotes lipogenesis, and inhibits

lipolysis, thereby resulting in triglyceride accumulation, via a rapid non-genomic action mediated by a putative membrane vitamin D receptor (mVDR). These actions can be mimicked by 1α,25-$(OH)_2$-lumisterol$_3$, a specific agonist for mVDR, and completely prevented by 1β,25-$(OH)_2$-D_3, a specific antagonist for the mVDR. This led to a hypothesis that dietary calcium suppression of 1α,25-$(OH)_2$-D_3 would reduce adipocyte $[Ca^{2+}]_i$ and thereby inhibit triglyceride accumulation by coordinated control over lipogenesis and lipolysis. We further confirmed this hypothesis in transgenic mice over-expressing the *agouti* gene specifically in adipose tissue, similar to the pattern of human expression. Our data demonstrated that suppression of 1α,25-$(OH)_2$-D_3 by increasing dietary calcium decreases adipocyte $[Ca^{2+}]_i$, stimulates lipolysis, inhibits lipogenesis in aP2-agouti transgenic mice and that dietary calcium not only attenuated diet-induced obesity but also accelerated weight loss and fat mass reduction secondary to caloric restriction. Notably, high calcium diets also increased white adipose tissue uncoupling protein 2 (UCP2) expression and core temperature. To determine whether this up-regulation of UCP2 is a direct effect of inhibition of 1α,25-$(OH)_2$-D_3, we evaluated the role of 1α,25-$(OH)_2$-D_3 in regulating human adipocyte UCP2 expression. We demonstrated that 1α,25-$(OH)_2$-D_3 exerts an inhibitory effect on white adipocyte UCP2 expression and that this effect is mediated via a genomic action (nuclear vitamin D receptor; nVDR). Accordingly, these data demonstrate that SUR-mediated Ca^{2+} signaling and 1α,25-$(OH)_2$-D_3-mediated genomic and non-genomic pathways play important roles in regulation of adipocyte lipid and energy metabolism, thereby contributing to modulation of adiposity, and that antagonism of adipocyte $[Ca^{2+}]_i$ by blocking these

pathways may represent an effective approach for development of therapeutic intervention in obesity.

TABLE OF CONTENTS

PART 1. INTRODUCTION..... 1
Introduction.....2
Literature Cited.....7

PART 2. LITERATURE REVIEW.....12

I. Obesity: An Epidemic Disease.....13
A. Prevalence of Obesity.....13
B. Obesity as A Medical Problem.....13
a. Effects of Obesity on Mortality.....13
b. Effects of Obesity on Morbidity.....14
1. Obesity and Insulin Resistance/Type II Diabetes.....14
2. Obesity and Other Metabolic Disorders.....18

II. Genetics of Obesity.....20
A. Overview.....20
a. Genetics of Obesity in Humans.....20
b. Genetics of Obesity in Animal Models.....23
1. Single Genes.....23
1.1 Obese (ob/ob) Mouse, Diabetic (db/db) Mouse, and Fatty (fa/fa)Rat.....24
1.2 Fat (fat/fat) Mouse.....25
1.3 Tubby (tub/tub) Mouse.....26
2. Polygenic Obesity in Animal Models.....27
B. The Mouse Agouti Gene and Its Role in Obesity.....28
a. Agouti Gene and Its Mutation.....28
b. Mechanisms of Agouti Signaling in Obesity.....29
1. Central Effects of Agouti in Obesity: Role of Melanocortin Receptor.....29
2. Peripheral Actions of Agouti in Obesity: Role of Intracellular Ca^{2+}.....33

III. Role of Intracellular Ca^{2+} in Obesity.....37
A. Syndrome X: Role of Intracellular Ca^{2+}.....37
a. Intracellular Ca^{2+} and Insulin Resistance/Hyperinsulinemia.....37
b. Intracellular Ca^{2+} and Hypertension.....40
c. Intracellular Ca^{2+} and Coronary Heart Disease.....43
B. Role of Intracellular Ca^{2+} in Obesity.....45
a. Calcium Homeostasis and Obesity.....45
b. Intracellular Ca^{2+} and Adipocyte Lipid Metabolism.....46
c. Intracellular Ca^{2+} Mobilizing Agents in Obesity.....48
1. Sulfonylurea and Sulfonylurea Receptor.....49

2. 1,25-Dihyroxyvitamin D_3 and Dietary Calcium.....52

IV. Role of Adipose Tissue in Obesity.....59
A. Adipocyte Hyperplasia.....60
a. Adipocyte Origin.....61
b. Program of Adipocyte Differentiation.....64
c. Transcriptional Control of Adipocyte Differentiation.....66
1. PPARγ.....66
2. C/EBP Family.....69
3. SREBP1/ADD1.....72
4. Transcriptional Cascade in Adipogenesis.....73
d. Hormonal and Cellular Regulation of Adipocyte Differentiation.....76
1. Insulin, IGF-1 and Other Growth Factors.....76
2. Tumor Necrosis Factor-α (TNF-α) and Other Cytokines.....78
3. Nuclear Hormone Superfamily.....79
4. Prostaglandins.....80
5. Intracellular Secondary Messengers.....81
B. Adipocyte Hypertrophy.....84
a. Regulation of De Novo Lipogenesis in Adipocytes.....84
1. Fatty Acid Synthase (FAS).....86
2. Nutritional and Hormonal Regulation of FAS.....87
2.1 Nutritional and Hormonal Regulation of FAS In Vivo.....88
2.2 Molecular Mechanism Mediating FAS Gene Regulation.....90
b. Regulation of Lipolysis in Adipocytes.....94
1. Hormone Sensitive Lipase (HSL) in Adipocyte Lipolysis.....95
2. Nutritional and Hormonal Regulation of HSL.....100
2.1 Nutritional Regulation of HSL.....100
2.2 Hormonal Regulation of HSL.....100
2.3 Lipolysis in Obesity and Type II Diabetes.....103

V. Role of Thermogenesis in Obesity.....105
A. Adaptive Thermogenesis and Respiratory Uncoupling.....105
a. Adaptive Thermogenesis.....105
b. Respiratory Uncoupling.....106
B. Uncoupling Protein (UCP) Family.....108
a. Role of UCP1 in Thermogenesis and Energy Expenditure.....108
1. UCP1-Mediated Proton Transport.....108
2. Regulation of UCP1 Activity and Expression.....110

2.1 Physiological Regulation of UCP1 In Vivo.....110
2.2 Transcriptional Control of UCP1 Expression.....112
3. Role of UCP1 in Energy Expenditure and Obesity.....115
b. UCP2 and UCP3: Potential Regulators of Energy Metabolism.....118
1. Cloning of UCP1 Homologues with Sequence Similarity.....118
2. Proton Transport Activity of UCP2 and UCP3.....120
3. Regulation of UCP2 and UCP3 Expression.....121
3.1 Physiological Regulation of UCP2 and UCP3....121
3.2 Transcriptional Control of UCP2 and UCP3 Expression.....122
4. Physiological Functions of UCP2 and UCP3.....123
4.1 Regulators of Energy Expenditure and Adiposity.....124
4.2 Regulators of Insulin Secretion.....125
4.3 Regulators of Fatty Acid Utilization.....126
4.4 Regulators of Reactive Oxygen Species.....127

Literature Cited.....129

PART 3. ROLE OF INTRACELLULAR CALCIUM IN HUMAN ADIPOCYTE DIFFERENTIATION.....225
I. Abstract.....226
II. Introduction.....227
III. Materials and Methods.....229
A. Culture and Differentiation of Human Preadipocytes.....229
B. Intracellular Ca^{2+} Measurement.....229
C. Triglyceride Content Assay.....230
D. Glycerol-3-Phosphate Dehydrogenase Activity Assay.....231
E. Protein Assay.....231
F. Northern Blot Analysis.....231
G. Statistical Analysis.....232
IV. Results.....233
V. Discussion.....247

Literature Cited.....252

PART 4. ROLE OF THE SULFONYLUREA RECEPTOR IN REGULATING HUMAN ADIPOCYTE METABOLISM.....257
I. Abstract.....258
II. Introduction.....259
III. Materials and Methods.....261
A. Isolation and Culture of Human Adipocytes.....261

B. Reverse Transcription-Polymerase Chain Reaction.....261
C. Northern Blot Analysis.....262
D. Intracellular Ca^{2+} Measurement.....262
E. Fatty Acid Synthase and Glycerol-3-Phosphate-Dehydrogenase Activity Assay.....263
F. Lipolysis Assay.....263
G. Statistical Analysis.....264
IV. Results.....265
V. Discussion.....274

Literature Cited.....278

PART 5. DIETARY CALCIUM ATTENUATES DIET-INDUCED OBESITY IN aP2-AGOUTI TRANSGENIC MICE.....282
I. Abstract.....283
II. Introduction.....284
III. Materials and Methods.....286
A. Isolation and Culture of Human Adipocytes.....286
B. Animals and Diets.....286
C. Core Temperature.....287
D. Intracellular Ca^{2+} Measurement.....288
E. Lipolysis.....288
F. Fatty Acid Synthase Activity and mRNA Levels.....288
G. Statistical Analysis.....289
IV. Results.....290
V. Discussion.....302

Literature Cited.....305

PART 6. DIETARY CALCIUM ACCELERATES BODY WEIGHT LOSS IN CALORIC RESTRICTED aP2-AGOUTI TRANSGENIC MICE.....309
I. Abstract.....310
II. Introduction.....312
III. Materials and Methods.....314
A. Animals and Diets.....314
B. Core Temperature.....315
C. Mouse Adipocyte Intracellular Ca^{2+} Measurement.....316
D. Fatty Acid Synthase Activity Assay.....317
E. Lipolysis Assay.....317
F. Northern Blot Analysis.....317
G. Statistical Analysis.....318
IV. Results.....319
V. Discussion.....331

Literature Cited.....337

PART 7. 1α,25-DIHYDROXYVITAMIN D_3 MODULATES HUMAN ADIPOCYTE METABOLISM VIA NON-GENOMIC ACTION.....343
I. Abstract.....344
II. Introduction.....346
III. Materials and Methods.....348
A. Culture of Human Adipocytes.....348
B. Human adipocyte Intracellular Ca^{2+} Measurement.....349
C. Fatty Acid Synthase and Glycerol-3-Phosphate-Dehydrogenase Activity Assay.....350
D. Lipolysis Assay.....350
E. Northern Blot Analysis.....351
F. Statistical Analysis.....351
IV. Results.....352
V. Discussion.....361

Literature Cited.....366

PART 8. 1α,25-DIHYDROXYVITAMIN D_3 INHIBITS UNCOUPLING PROTEIN 2 EXPRESSION IN HUMAN ADIPOCYTES.....373
I. Abstract.....374
II. Introduction.....376
III. Materials and Methods.....378
A. Culture of Human Adipocytes.....378
B. Northern Blot Analysis.....379
C. Western Blot Analysis.....379
D. Reverse Transcription-Polymerase Chain Reaction.....380
E. Antisense Oligodeocynucleotide (ODN) Transfection.....381
F. Statistical Analysis.....381
IV. Results.....382
V. Discussion.....395

Literature Cited.....401

PART 9. SUMMARY AND CONCLUSIONS.....413
Summary and Conclusions.....414

Vita.....416

LIST OF TABLES

1. Effects of calcium and dairy products on fat pad mass in transgenic mice expressing agouti in adipose tissue under the control of the aP2 promoter.....299

2. Effects of 6-week administration of high calcium diets on core temperature in energy-restricted aP2-agouti transgenic mice.....327

LIST OF FIGURES

1. The effects of thapsigargin, A23187, and KCl on human preadipocyte intracellular calcium ($[Ca^{2+}]_i$).....234

2. The effects of the thapsigargin and A23187 treatment at 23-24 h of differentiation on human adipocyte triglyceride accumulation.....236

3. The effects of the thapsigargin and A23187 teatemtn at 23-24 of differentiation on human adipocyte glycerol-3-phosphate dehydrogenase (GPDH) activity.....237

4. The effects of the thapsigargin and A23187 treatment at 47-48 h of differentiation on human adipocyte triglyceride accumulation.....239

5. The effects of KCl and agouti protein treatment during 0-48 h of differentiation on triglyceride accumulation.....240

6. The effects of KCl and agouti protein treatment during 0-48 h of differentiation on GPDH activity.....241

7. The effects of KCl and agouti protein treatment during 0-48 h of differentiation on human preadipocyte PPARγ expression.....242

8. The effects of the thapsigargin and A23187 treatment at 71-72 h of differentiation on triglyceride accumulation.....243

9. The effects of KCl and agouti protein treatment during 48-72 h of differentiation on triglyceride accumulation.....244

10. The effects of KCl and agouti protein treatment during 48-72 h of differentiation on human preadipocyte PPARγ expression.....246

11. Detection of the expression of SUR gene in human adipocyte by RT-PCR.....266

12. Detection of the expression of SUR mRNA in human adipocyte by Northern Blot Analysis.....267

13. Effects of glibenclamide on stimulation of $[Ca^{2+}]_i$ in human adipocytes.....268

14. The dose-responsive effect of glibenclamide on $[Ca^{2+}]_i$ in human adipocytes.....269

15. The effect of glibenclamide on fatty acid synthase (FAS) activity in human adipocytes.....270

16. The effect of glibenclamide in GPDH activity in human adipocytes.....272

17. The effect of glibenclamide on lipolysis in human adipocytes.....273

18. Effects of 1,25-$(OH)_2$-D_3 and parathyroid hormone on $[Ca^{2+}]_i$ in human adipocytes.....291

19. Effects of 1,25-$(OH)_2$-D_3 on forskolin-stimulated lipolysis in human adipocytes.....293

20. Effects of calcium and dairy products on 6 week weight gain in transgenic mice expressing agouti adipose tissue under the control of the aP2 promoter.....294

21. Effects of calcium and dairy products on core temperature in transgenic mice expressing agouti in adipose tissue under the control of the aP2 promoter.....295

22. Effects of calcium and dairy products in adipocyte FAS activity, FAS mRNA, and lipolysis in transgenic mice expressing agouti in adipose tissue under the control of the aP2 promoter.....296

23. Effects of calcium and dairy products on fasting plasma glucose and insulin levels in transgenic mice expressing agouti in adipose tissue under the control of the aP2 promoter.....300

24. Effects of 6-week administration of basal low-calcium (0.4)/high-fat/high-sucrose diet on body weight, total fat pad mass, and basal adipocyte $[Ca^{2+}]_i$ in aP2-agouti transgenic mice.....320

25. Effects of 6-week administration of high-calcium diets on weight reduction, fat pad mass, and basal adipocyte $[Ca^{2+}]_i$ in energy-restricted aP2 agouti transgenic mice.....322

26. Effects of 6-week administration of high-calcium diets on FAS activity, FAS mRNA, and lipolysis in energy-restricted aP2-agouti transgenic mice.....325

27. Effects of 6-week administration of high-calcium diets on abdominal adipose tissue uncoupling protein 2 expression in energy-restricted aP2-agouti transgenic mice.....328

28. Effects of dietary calcium on plasma insulin levels.....330

29. The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on stimulation of human adipocyte $[Ca^{2+}]_i$.....353

30. The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on FAS activity and expression in human adipocytes.....355

31. The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on GPDH activity in human adipocytes.....358

32. The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on human adipocyte lipolysis.....359

33. The effect of 1α,25-$(OH)_2$-D_3 on basal UCP2 mRNA and protein level in human adipocytes.....383

34. The effect of 1α,25-$(OH)_2$-D_3 on isoproterenol-stimulated UCP2 mRNA and protein level in human adipocytes.....385

35. The effect of 1α,25-$(OH)_2$-D_3 on fatty acid-stimulated UCP2 mRNA in human adipocyte.....387

36. The effects of 1α,25-$(OH)_2$-D_3 and its membrane vitamin D receptor (mVDR) agonist and antagonist on basal UCP2 mRNA in human adipocytes.....389

37. The effects of 1α,25-$(OH)_2$-D_3 and its mVDR agonist and antagonist on isoproterenol-stimulated UCP2 mRNA in human adipocytes.....390

38. The effects of 1α,25-$(OH)_2$-D_3 and its mVDR agonist and antagonist on fatty acid-stimulated UCP2 mRNA in human adipocytes.....391

39. Detection of the expression of nuclear vitamin D receptor (nVDR) in human adipocytes by RT-PCR and western blot.....392

40. Nuclear vitamin D receptor (nVDR) knockout by antisense oligodeoxynucleotide (ODN) prevented the inhibitory effect of 1α,25-$(OH)_2$-D_3 on adipocyte UCP2 expression.....393

VOLUME

1

PART 1

INTRODUCTION

INTRODUCTION

Obesity is an increasingly prevalent, costly and serious medical problem in most of the world. The third National Health and Nutrition Examination Survey (1988-1994) shows that more than half of American adults are overweight and nearly one-fourth are clinically obese, with 97 million overweight or obese adults in the U.S (1). Obesity results from an interaction between genetics and environment (such as diet and physical activity) (1). Unfortunately, strategies to modify the environment factors have met with little success. Two-thirds of patients who lose weight initially regain weight in one year, and almost all do so in five years (2). Therefore, modulation of body weight is apparently resisted by biological mechanisms.

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays a key role in metabolic disorders associated with obesity and insulin resistance (3-5). Recent data from this laboratory demonstrate a pivotal role for $[Ca^{2+}]_i$ in the regulation of adipocyte lipid metabolism (6, 7). The studies of the mechanism of action of the *agouti* gene in obesity have demonstrated that recombinant *agouti* protein acts on a novel response element to stimulate the expression and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis, and inhibit basal and agonist stimulated lipolysis in a Ca^{2+}-dependent manner (8-11). These effects of *agouti* in promoting lipogenesis and suppressing lipolysis can be mimicked by Ca^{2+} channel activation and reversed by Ca^{2+} channel antagonism (9-11). Therefore, increasing adipocyte $[Ca^{2+}]_i$ appears to promote triglyceride accumulation by exerting a coordinated control over stimulation of lipogenesis and inhibition of lipolysis, resulting in an obese

phenotype. These data suggest that adipocyte $[Ca^{2+}]_i$ is a logic target for pharmacological/nutritional regulation of adiposity.

Although the role of $[Ca^{2+}]_i$ in adipocyte lipid metabolism has been investigated, several issues still need to be further addressed. $[Ca^{2+}]_i$ has also been implicated in regulation of murine adipocyte differentiation (12). However, the role of $[Ca^{2+}]_i$ in human adipocyte differentiation, an important contributor to human adiposity in vivo (13-16), is not known. Moreover, exploiting adipocyte $[Ca^{2+}]_i$ as a potential target for the development of intervention in obesity needs to be explored.

Antagonism of $[Ca^{2+}]_i$ by either blocking Ca^{2+} channels or inhibiting Ca^{2+} agonists is a logical approach for development of therapeutic intervention in obesity. Indeed, calcium channel blockade has been proven to reduce body weight and fat pad mass effectively in several animal studies (17-19). Previous data from this laboratory demonstrated that treating transgenic mice over-expressing agouti with a Ca^{2+} channel antagonist (nifendipine) resulted in significant decreases in adipocyte lipogenesis and corresponding adipose tissue mass (17). Similarly, Ca^{2+} channel antagonists, such as nifendipine, verapamil and felodipine, caused decreases in body weight and fat pad mass in obese SHHF/Mcc-fa^{cp} rats (18, 19).

Sulfonylureas, such as glibenclamide, are insulin secretagogues widely used to stimulate insulin secretion in the treatment of type II diabetes mellitus. These drugs bind to sulfonylurea receptor (SUR) of pancreatic β cells and then block the conductance of K_{ATP}

channels, thereby resulting in depolarization and secondary Ca^{2+} influx via L-type Ca^{2+} channels, which in turn triggers insulin release (20). The β cell receptor for sulfonylureas, sulfonylurea receptor1, has been cloned (20).

Patients using sulfonylureas for type II diabetes develop obesity, while diazoxide, a drug that activates the β cell K_{ATP} channel and inhibits sulfonylureas, exerts an anti-obesity effect in hyperinsulinemic obese human (21) and Zucker rats (22). However, these effects were previously attributed to direct actions of diazoxide on β cells, rather than direct effects of diazoxide on adipocyte metabolism. Nonetheless, these SUR agonists and antagonist may also exert direct effect on adipocyte $[Ca^{2+}]_i$ and lipid metabolism, resulting in body weight regulation.

On the other hand, identifying and characterizing hormones that modulate $[Ca^{2+}]_i$ is a logical approach for elucidating novel mechanism underlying the modulation of adipocyte. 1α,25-dihyroxyvitamin D_3 (1α,25-$(OH)_2$-D_3) was originally believed to solely function via a nuclear vitamin D receptor (nVDR) to generate genomic action (23). However, this concept was challenged by recent studies revealing that it also generates rapid, non-genomic signal transductions including stimulation of $[Ca^{2+}]_i$ via a putative membrane vitamin D receptor (mVDR) in a wide variety of cells (24-28). Although the role of 1α,25-$(OH)_2$-D_3 in modulating adipocyte Ca^{2+} signaling and lipid metabolism has not been explored, several lines of evidence demonstrate that circulating 1α,25-$(OH)_2$-D_3 levels are elevated in obese humans (29-32).

During the course of a previous clinical trial investigating the anti-hypertensive effect of calcium in obese African-Americans, it was noted that increasing daily calcium intake from 400-1000 mg/day for 1 year resulted in a 4.9 Kg reduction in body fat (33). This led to a hypothesis that increasing 1α,25-$(OH)_2$-D_3 secondary to low calcium diets stimulates adipocyte Ca^{2+} influx and thereby increase lipid storage. If this is correct, then increasing dietary calcium should be able to suppress 1α,25-$(OH)_2$-D_3 and thereby inhibit adipocyte $[Ca^{2+}]_i$ and lipid storage.

Accordingly, our studies were designed to:

I. Determine the role of $[Ca^{2+}]_i$ in human adipocyte differentiation.

II. Determine the physiological regulation of adipocyte Ca^{2+} influx and its impact on adipocyte metabolism, as follows:

(A) Sulfonylurea receptor (SUR): This study was designed to determine direct role of the human adipocyte SUR in regulating adipocyte Ca^{2+} signaling and metabolism, and develop approaches for the selective modulation of this adipocyte Ca^{2+}channel.

(B) 1α,25-$(OH)_2$-D_3: This study was designed to (a) determine the role of 1α,25-$(OH)_2$-D_3 in modulation of adipocyte Ca^{2+} signaling and lipid metabolism and whether this modulation is via novel rapid non-genomic action mediated by the putative mVDR and (b) determine the role of dietary calcium suppression of 1α,25-$(OH)_2$-D_3 in attenuating diet-induced obesity and accelerating weight loss secondary to caloric restriction.

III. Determine the role of 1α,25-$(OH)_2$-D_3 in regulating human adipocyte uncoupling protein 2 (UCP2) expression and whether this regulation is via the classic genomic action mediated by the nVDR.

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PART 2

LITERATURE REVIEW

I. OBESITY: AN EPIDEMIC DISEASE

A. PREVALENCE OF OBESITY

Obesity, defined as body mass index (BMI) greater than 30 kg/m^2, is an increasingly prevalent, costly and serious medical problem in the developed countries (1). National surveys in the United States have shown a striking population-wide increase in prevalence of overweight (BMI $\geq$25.0) and obesity (BMI $\geq$30.0) over the short period of time between 1980 and 1994 (1). The third National Health and Nutrition Examination Survey (1988-1994) shows that more than half of American adults are overweight and nearly one-fourth are clinically obese, with 97 million overweight or obese adults in U.S. (1). This survey also demonstrates that the number of overweight children and adolescents in the United States has markedly increased, and that 11% of children and adolescents are overweight (2). Moreover, the prevalence in developing countries is also increasing at an alarming rate (3).

B. OBESITY AS A MEDICAL PROBLEM

Obesity is closely associated with the development of other serious chronic diseases, including diabetes mellitus, hypertension, dyslipidemia, coronary heart disease and several forms of cancer. Obesity-related diseases cause more than 300,000 deaths annually in the United States alone (1).

a. Effect of Obesity on Mortality

Life insurance data first demonstrated the relationship of increased body weight and central fat to high death rate (4). Subsequent epidemiological studies confirmed that

increasing degrees of overweight and obesity are important predictors of decreased longevity (5). The overall relationship between mortality and BMI adjusted for age shows an acceleration of the mortality risk above a BMI of 30 kg/m^2 (6). This is further illustrated by the work of Manson et al. who demonstrated the association between BMI and both overall mortality and mortality from specific diseases in a cohort study, showing that the risk of death was 1.3 in those with a BMI 25-26.9, 1.6 in those with a BMI 27-28.9 and doubled for those with a BMI of 29.0-31.9 (7). Moreover, recent evidence indicates that a weight loss of more than 9 kg is associated with a 25% reduction in all causes of mortality (8).

Regional fat distribution has an important additional role in excess death (9). The relationship of body fat distribution to overall mortality was demonstrated by the Gothenburg study showing that mortality was increased with a high ratio of waist circumference to hip circumference as the index of central fatness (10).

b. Obesity-Associated Morbidity

Obesity and overweight are associated with increased risk of insulin resistance/type II diabetes, coronary heart disease (CHD), dyslipidemia, hypertension, respiratory disease, gall bladder disease and several types of cancer (1).

1. Obesity and Insulin Resistance/Type II Diabetes

The association of obesity with type II diabetes has been recognized for decades. More than 80% of type II diabetics are obese (1). Both prospective and retrospective studies

have demonstrated that the risk of type II diabetes increases as BMI increases in both genders and in populations at high risk for both conditions (11, 12, 13). Moreover, body fat distribution exerts a marked influence on the risk of type II diabetes, as abdominal visceral obesity has a strong association with type II diabetes (14, 15).

Type II diabetes is preceded by insulin resistance, a defect in the response of muscle, adipose tissue and liver to insulin-mediated glucose transport, metabolism, and storage. Insulin resistance in obesity is manifested by decreased insulin-stimulated glucose transport and metabolism in insulin-sensitive tissues (16). These functional defects in obesity may result from impaired insulin signaling, including insulin binding to its receptor, receptor phosphorylation and kinase activity, phosphorylation of insulin receptor substrate (IRS), and activation of PI-3 kinase, and subsequent down-regulation of GLUT4, the major insulin-responsive glucose transporter (16). Indeed, the expression of various insulin signaling molecules is reduced in skeletal muscle in morbid obesity (17). Moreover, adipocytes from obese humans exhibit reduced insulin receptor substrate-1 (IRS-1) expression, resulting in decreased PI3-kinase activity (18). In addition, decreased expression of GLUT4 or impaired GLUT4 translocation, docking, and vesicle fusion is evident in skeletal muscle and adipocytes from obese subjects (19).

The mechanism whereby increased energy storage in adipocyte promotes insulin resistance in the other tissue such as skeletal muscle and liver is not clear. However, adipose tissue functions as an endocrine organ that expresses and secretes several molecules that exert multiple endocrine effects. Several of these, including free fatty

acids (FFAs) (20), tumor necrosis factor alpha (TNF-α) (21), resistin (22, 23), and adiponectin (24-26), may contribute to insulin resistance in obesity, as follows.

- Free Fatty Acids

Elevated FFAs impair insulin-stimulated glucose transport by inhibiting GLUT4 expression (27). FFAs also inhibit glucose oxidation (28, 29) and glycogen synthase activity (30) in skeletal muscle, thereby reducing glucose utilization. Moreover, FFAs stimulate hepatic gluconeugenesis and thereby increase glucose output (31). Intra-abdominal adipocytes are more lipolytically active due to their abundant adrenergic receptors mediating vigorous response to noradrenaline. This would contribute to an exaggerated release of FFAs from abdominal adipocytes into portal system (32, 33), thereby promoting insulin resistance. This is thought to explain why abdominal fat depots are much more strongly linked to insulin resistance than are peripheral (gluteal/subcutaneous) fat depots.

- TNF-α

TNF-α is over-expressed in obese humans and animals (34). This cytokine stimulates lipolysis and the resulting increases in FFAs may thereby contribute to insulin resistance (35). Moreover, TNF-α has been shown to impair insulin signaling through phosphorylation of IRS-1 at serine residues (36). In addition, TNF-α is able to down-regulate GLUT4 expression, thereby decreasing insulin-stimulated glucose transport (37, 38). This is supported by the studies demonstrating that TNF-α knockout mice exhibit improved insulin sensitivity (39).

- Resistin

Recently, Steppan et al. identified an adipocyte-derived hormone named resistin which exerts an effect on insulin resistance and potentially links obesity to diabetes (22). Resistin gene expression is induced during adipocyte differentiation, and the resistin protein is specifically expressed and secreted by adipocytes (22). Circulating resistin level is markedly increased in both genetic and diet-induced obesity mice (22). Moreover, administration of resistin impairs glucose tolerance and insulin action in normal mice (22). By contrast, immunoneutralizaton of resistin improves blood glucose and insulin action in the animal models of type II diabetes (22). In addition, thiazolidinediones (TZDs), a class of anti-diabetic drugs, have been demonstrated to inhibit resistin expression and thereby exert their anti-diabetic effects (22).

- Adiponectin

Another adipocyte-derived hormone adiponectin, also known as Acrp30, plays an important role in insulin resistance (24-26). Decreased expression and circulating levels of adiponectin correlate with insulin resistance in diabetic animal models (24). Conversely, caloric restriction increases the levels of this protein (25). In contrast to resistin, TZDs increase adiponectin expression in adipocytes (24), indicating that this protein may also mediate the anti-diabetic effects of TZDs. Adiponectin decreases insulin resistance by decreasing triglyceride content in muscle and liver in obese mice (24). This effect results from increased expression of molecules involved in both fatty acid combustion and energy dissipation in muscle (24). Moreover, insulin resistance in

lipoatrophic mice was completely reversed by the combination of physiological dose of adiponectin and leptin (24).

2. Obesity and Other Metabolic Disorders

In addition to type II diabetes, obesity is also strongly associated with other metabolic disorders, including coronary heart disease (CHD), hypertension, and dyslipidemia, which are closely related and interact with each other (1).

Data from the Nurse Health Trial demonstrate that risk for CHD is increased 3.3-fold in women with a BMI greater than 29 kg/m^2, compared with the risk for women with a BMI less than 21 kg/m^2 (40). Similar relationship between increased BMI and CHD risk has been observed in men (41). A weight gain of 5-8 Kg may be translated to an increased CHD risk of 25% (41). Physiologically, the increased fat mass could increase circulating blood volume which in turn contributes to an increase in the left ventricular preload and a subsequent increase in cardiac output (42). The volume expansion and increased cardiac output may further lead to an increase in wall stress and structural changes of the heart such as an enlarged left ventricular cavity dimension and myocardial hypertrophy (42).

It is well known that dyslipidemia plays an important role in the development of CHD. Dyslipidemia is manifested by high total cholesterol, high triglycerides, low high-density lipoprotein cholesterol (HDL), and elevated low-density lipoprotein cholesterol (LDL) (1). A positive correlation between BMI and high total cholesterol, high triglycerides and high LDL has been demonstrated repeatedly (1). Moreover, an inverse relationship

between BMI and HDL has also been well documented (1). A meta-analysis shows that 1 Kg weight loss corresponds to a 1% reduction of total cholesterol and LDL, a 1% increase of HDL, and a 3% reduction of triglyerides (43). It is noteworthy that obesity-related insulin resistance contributes to the pathological development of dyslipidemia, which may further cause CHD (44).

Obesity and hypertension are significant risk factors for the development of cardiovascular disease. A large number of population-based studies have documented a strong association between obesity and hypertension in both sexes, in all age groups, and for virtually every geographic and ethnic group (45). Data from NHANES III show that the age-adjusted prevalence of high blood pressure increases progressively with higher levels of BMI in both men and women. The large international study of salt (INTERSALT) carried out in more than 10,000 people reported that a 10 Kg higher body weight is associated with 3.00 mm Hg higher systolic and 2.3 mm Hg higher diastolic blood pressure (46). The pathophysiology underlying the development of hypertension associated with obesity includes associated increases in vascular resistance, blood volume, cardiac output, and sodium retention. These cardiovascular abnormalities associated obesity are believed to be related to a combination of obesity-related insulin resistance/hyperinsulinemia, increased sympathetic nervous system activity, and alterations of the rennin-angiotensin system (1).

II. GENETICS OF OBESITY

A. OVERVIEW

Obesity is a multifactorial disease with a potent genetic component. Several mutations in rodent obesity genes that segregate as mendelian traits have been cloned; subsequently, corresponding homologous mutations have been identified as rare causes of human obesity (47). Phenotypes shared between the human and animals with homologous obesity mutations exhibit conserved physiological pathways. Cloning and characterization of these obesity genes and gene products reveal novel molecular and biochemical mechanisms underlying the obesity development and thereby provides potential targets for therapeutic interventions in obesity. On the other hand, quantitative genetic approaches for human population-based genetic studies and mouse-inbred strains have led to the genetic maps for obesity in humans and mice. This assists in not only identifying those genetic loci that fail to display a mendelian inheritance pattern and function in a gene-gene or gene-environment interaction manner to contribute to a polygenic trait, but also in identifying the human population with a genetic susceptibility to obesity.

a. Genetics of Obesity in Humans

Human obesity is a polygenic trait, a quantitative phenotype that fails to display mendelian inheritance pattern because it is controlled by many different loci (47). Whether or not these genetic propensities are expressed may depend on appropriate environmental variables. It is believed that human obesity results from interaction between genes and environment. Several lines of evidence support a model in which

susceptibility to obesity is determined largely by genetic factors, but the environment determines phenotype expression (47).

Genetic factors account for a substantial portion of variation in human obesity. This is supported by numerous studies of genetic epidemiology of human obesity (48-52). The most convincing evidence for the genetic transmission of human obesity comes from twin studies. If the heritability quotient value 0.0 indicates no inheritance and 1.0 represents complete inheritance for twin study data, the heritability quotients from twin studies of human obesity range from 0.4 to as high as 0.98 (48-50). Family studies and adoption studies have also consistently shown that 40-70% of the variation in obesity-related phenotypes, such as BMI, skinfold thickness and fat mass, is heritable (51). Moreover, many segregation analyses, which evaluate the evidence and pattern of transmission for a major gene based on observed patterns of phenotypic inheritance, demonstrate that major genes that influence the obesity-related phenotypes account for as much as 40% of the variation in BMI and fat mass (51, 52).

Emphasis on human genetics of obesity has shifted from the question of whether human obesity has genetic components to which specific genes are responsible for specific obesity phenotypes. The search for human obesity genes is a challenging task. Two strategies, the candidate gene approach and the genome scan approach, are now used to identify human obesity gene (47, 51). The candidate gene approach is used to screen obese human patients for mutations in candidate genes selected on the basis of the mouse genetic studies. Using this strategy, two children with defects in leptin have been

identified; these patients are homozygous for loss of function mutations and exhibit phenotypes of morbid obesity and hyperphasia (53, 54). Similarly, a leptin receptor mutation has also been reported (55). Moreover, the importance of the melanocortin pathway in the control of energy homeostasis in human has been demonstrated by identification of obese patients with mutations in pro-opiomelanocortin (POMC) and melanocortin-4 receptor (MC4R). Two children with loss of function mutations in POMC gene exhibit impaired melanocortin signaling and display obesity and altered pigmentation phenotypes (56). In addition, several groups have reported MC4R mutations, including base deletion-induced frameshift and base substitution missense mutations, as well as haploinsufficiency mutations (57-61). The metabolic phenotypes caused by MC4R mutations in human are similar to those caused by impaired melanocortin signaling in mice. Other mutations of candidate genes identified in human obesity include PPARγ (62) and prohormone convertase 1 (PC1) (63).

Single gene mutations as causes of obesity in humans is so rare that identification of these mutations is unable to address genetic causes in population as a whole. Therefore, genetic linkage and association analyses of candidate genes with obesity are more effective in this regard. The statistical support for linkage has been presented in the form of a LOD score (logarithm of the likelihood ratio for linkage) (47, 51). This has yielded intriguing insights into the genetics of human obesity. Numerous DNA polymorphisms at or near candidate genes have been identified to be associated with the quantitative trait of BMI or other measures of body fatness (51).

Genetic determinants of variation in human obesity and related phenotypes are likely to be multiple and interacting, with most single variants generating a moderate effect on obesity (51). Therefore, the genome scan approach is an effective approach to identify these individual genetic loci contributing to obesity. In a whole genome scan, linkage analysis is conducted using a series of DNA markers or polymorphisms, spaced at relatively constant intervals over the whole genome, to identify quantitative trait loci (QTL) associated with the obesity phenotypes. This approach offers the potentials to identify previously unknown genes affecting the phenotypes of interest. Results from four reported genome-wide linkage studies that examined obesity and related phenotypic traits in Mexican-Americans, Pima-Indians and African-Americans have identified several loci that show positive evidence for linkage (47, 64-66). For instance, significant linkage of serum leptin levels and fat mass to short arm (p) of chromosome 2 at band 21 (2p21) was demonstrated in Mexican-Americans (64, 65). However, no obvious candidate genes have been so far mapped to any chromosome QTL regions.

b. Genetics of Obesity in Animal Models

1. Single Genes

The genes responsible for mutations which produce well characterized obesity in rodent models have been cloned and characterized. These genetic obese animal models include yellow (A^y/a) obese mouse, obese (*ob/ob*) mouse, diabetic (*db/db*) mouse, Zucker fatty (*fa/fa*) rat, Tubby (*tub/tub*), Fat (*fat/fat* or cpe^{fat}/cpe^{fat}) mouse.

1.1 Obese (*ob/ob*) Mouse, Diabetic (*db/db*) Mouse, and Fatty (*fa/fa*) Rat

The phenotypic expression of the obese syndrome is similar across three mutations that cause obesity in the ob/ob mouse, db/db mouse, and the fa/fa rat. These animals exhibit hyperphasia, morbid early-onset obesity, decreased energy expenditure, insulin resistance/hyperinsulinemia (67). Coleman conducted an elegant parabiosis experiment with *ob/ob* and *db/db* mice, suggesting that *ob/ob* mouse lacks a weight-regulating factor and *db/db* mouse lacks a molecule responding to this factor (68). Parabiosis studies suggested that the *fa/fa* rat was similar to the *db/db* mouse in its failure to respond to a circulating factor.

Using positional cloning strategy, the mouse *ob* gene was cloned (69) and shown to encode a 4.5 kilobase mRNA transcript with a highly conserved 167 amino acid. Analysis of this gene product revealed characteristics consistent with a secretory peptide, which is also classified as a cytokine-like protein without homology with other known cytokines. The mutation was identified as a base substitution that introduces a stop codon, resulting in a premature-truncated protein. Leptin is primarily expressed in white adipose tissue, but also in placenta and gastric epithelium at lower levels. Leptin circulates as a 16-kDa protein and plasma leptin levels are highly correlated with adipose tissue mass. Administration of leptin via either intraperitoneal or intracerebroventricular injection results in a significant correction in obese and diabetic syndrome in *ob/ob* but not *db/db* mice (70). Accordingly, leptin appears to function as a body weight modulator.

The complete non-response of *db* mouse to leptin and identical phenotype of *ob* and *db* mouse suggest that *db* locus encodes the leptin receptor (ob-R). The ob-R was first isolated from mouse choroid plexus cDNA library by expression cloning strategy (71). This receptor belongs to a single transmemberane receptor of class I cytokine family and binds leptin with nanomolar affinity. Alternative splicing results in at least 5 different isoforms of ob-R (ob-Ra, ob-Rb, ob-Rc, ob-Rd, ob-Re) (72-74). All these isoforms share identical extracellular ligand binding domain, but only ob-Rb encodes all intracellular motifs capable of activating JAK (janus kinase)-STAT (signal transducers and activators of transcription) signal transduction (72-74). A base substitution mutation of db gene in db/db mouse introduces a stop codon that prevents translation of the long intracellular domain of the receptor (72-74). A similar mutation is present in the *fa/fa* rat (73, 74). Thus, these studies confirm the original prediction from parabiosis studies that there was a circulating factor to which the *db/db* and *fa/fa* rat could not respond.

The leptin receptor is expressed at high levels in hypothalamic neurons (72). Therefore, the hypothalamus is the primary target tissue which leptin act upon to exert its energy-regulatory effects (73, 74). Leptin responsive neurons in hypothalamus express neuropeptides that are modulated by leptin and interact with each other in controlling feeding behavior and energy balance.

1.2 Fat (fat/fat or cpe^{fat}/cpe^{fat}) Mouse

The homozygous fat mouse with recessive fat gene mutation, unlike the *ob/ob* mouse, the *db/db* mouse, and the *fa/fa* rat, exhibits late-onset obesity. These mice develop marked

hyperinsulinemia prior to the development of obesity (75, 76). Therefore, the fat mouse is an interesting animal model for studying the insulin resistance/hyperinsulinemia and obesity interrelationship (75, 76). The fat gene, located on mouse chromosome 8, has been shown to encode carboxypeptidase E (77). A single base mutation that results in a serine to proline substitution at position 202 severely reduces the activity of carboxypeptidase E in both pancreas and pituitary (77). This enzyme is required for cleavage of two arginine residues from the B chain of proinsulin during pro-enzyme processing. The impairment in processing of proinsulin causes hyperproinsulinemia in the fat mouse. In addition, a number of prohormones and proneuropeptides, including POMC, require carboxypeptidase E for cleavage of paired dibasic residues from carboxy terminus to yield the biologically active form of peptides. Accordingly, the obesity phenotype of fat mouse is likely to result from a complex pattern of alterations in activity or secretion of neuropeptides, such as POMC, in hypothalamus (75-78).

1.3 Tubby (tub/tub) Mouse

Homozygous mice with a mutation at the tub locus develop late-onset obesity and insulin resistance (75-78). Using positional cloning, tub gene has been cloned and mapped to mouse chromosome 7 (79). Tubby mice have a naturally occurring slicing site mutation at the junction of the 3' coding exon. A recent study show that tubby proteins localize to the plasma membrane by binding to phosphatidylinositol 4,5-bisphosphate (PIP2) and function as transcriptional factors that translocate to the nucleus in response to receptor-mediated activation of G protein α_q ($G\alpha_q$) (80).

2. Polygenic Obesity in Animal Models

Unlike the single-gene mutations in the aforementioned animal models, human obesity is polygenic, likely to have significant genetic heterogeneity, and clearly involves environmental factors (50). Thus, animal models of polygenic obesity may be used to investigate the genetic mechanisms underlying the development of obesity involved in gene-gene interaction and gene-environment interaction.

Using polymorphic genetic markers covering the whole mouse genome, quantitative trait loci (QTL) mapping is performed to identify the genetic loci associated with specific traits involved in obesity or obesity-related phenotype in inbred mouse strains (47, 51). These inbred strains of mice are useful, as different strains encompass a wide range of adiposity. Thus, these strains can be intercrossed to generate an F1 generation, and the segregation of the trait in F1 backcross or in F1 intercross can be followed and linked to the segregation of polymorphic genetic markers (47, 81, 82). QTL mapping can be used to target the specific obesity phenotypes caused by gene-gene interaction or gene-environment interaction. For instance, West et al. used two strains of mice (AKR/J x SWR/J) that differ in their sensitivity to diet-induced obesity (83, 84), while Warden et al. used BSB model (C57BL/6J x *Mus spretus* strain) whose body fat composition varies from 1% to 50% (85, 86). Both studies have mapped QTLs on chromosomes corresponding to specific phenotypes. So far, over 70 loci for polygenic obesity have been identified from more than 15 different crosses (47).

B. THE MOUSE AGOUTI GENE AND ITS ROLE IN OBESITY

a. Agouti Gene and Its Mutation

Agouti gene was the first obesity gene cloned (87). This gene, mapped to mouse chromosome 2, encodes a 131-amino acid protein with a consensus signal peptide (87-90). The agouti gene is normally involved in coat color regulation in mice, with transient expression in neonates, resulting in a pigmentation pattern classically called agouti (88-90). Agouti is transiently expressed in neonatal skin, secreted by follicular cells, and exerts a paracrine action on melanocytes. This results in a competitive antagonism of α-melanocyte-stimulating hormone (α-MSH) binding to the melanocortin-1 receptor (MC1-R), which in turn causes a switch from the synthesis of α-MSH-stimulated black pigment (eumelanin) to that of default yellow pigment (phaeomelanin) (91, 92). This produces the characteristic pigmentation pattern of wild-type mice, a predominantly black hair shaft with a subapical yellow segment.

A number of dominant mutations at agouti locus results in ectopic expression in all tissues throughout the life of the mouse (89-91). These mutations result in not only an altered pigmentation pattern, but also a pleiotropic syndrome characterized by adult-onset obesity, hyperphagia, hyperinsulinemia, insulin resistance, impaired glucose tolerance, and decreased thermogenesis (89-91). Molecular analysis of dominant agouti alleles, such as lethal yellow (A^y) and viable yellow (A^{vy}), demonstrates that mutations in promoter regions result in ubiquitous expression of normal agouti protein (88, 89). This was confirmed by overexpressing agouti cDNA under control of a ubiquitous promoter in

transgenenic mice (93). These animals recapitulated the phenotypes of dominant mutants (93).

b. Agouti Signaling

Although the genetic defect in the agouti yellow mouse syndrome clearly involves ectopic expression of the agouti gene, the actual mechanism whereby the overexpressed agouti causes the yellow obese syndrome is not clear. Both central and peripheral effects of agouti on obesity syndrome have been extensively explored. Several lines of evidence suggest that both central effects of agouti on energy intake, mediated by melanocortin signaling pathway, and peripheral effects of agouti on adipocyte lipid metabolism and pancreatic β cell insulin/amylin secretion, mediated by intracellular Ca^{2+}, may contribute to agouti yellow mouse syndrome.

1. Central Actions of Agouti: Role of Melanocortin Receptor

The initial observation that agouti modulation of pigmentation is mediated by antagonism of MC-1R (87, 92) has led to the hypothesis that melanocortin receptor antagonism may be responsible for the metabolic effects of agouti in yellow obese syndrome.

MC1-R belongs to melanocortin receptor family of 7-transmanbrane G protein coupled receptors, which includes 5 members (94, 95). MC-1R, the α-MSH receptor, is primarily expressed in melanocytes and regulates pigmentation. MC2-R, the adrenocorticotropin hormone (ACTH) receptor, is expressed in adrenal cortex and in adipose tissue (96). MC3-R is expressed in hypothalamus and limbic system, as well as in placenta and gut

(97). MC4-R is expressed widely in the brain, with a high expression in region of hypothalamus involved in regulation of energy intake and body weight (98). MC5-R is expressed ubiquitously and appears to be involved in thermoregulation and immunomodulation (99-101). Lu et al investigated the potential of agouti to antagonize the binding of α-MSH to each of these melanocortin receptors. They demonstrated that agouti protein potently antagonizes MC1-R, MC2-R and MC4-R, but exerts little or no effect on MC3-R and MC5-R (92). Of these receptors antagonized by agouti, both loss and gain of function mutations targeting MC-1R affect coat color without producing significant metabolic effects (94), whereas disruption of MC2-R results in familial glucocorticoid deficiency (102). Therefore, only MC4-R remains as a likely target to mediate agouti-induced obesity syndrome.

The role of melanocortin/MC4-R signaling pathway in regulation of food intake and energy homeostasis had not been explored until the cloning and characterization of the agouti gene. MC4-R is abundantly expressed in the hypothalamic paraventricular nucleus, dorsomedial nucleus and ventromedial mucleus, major hypothalamus regions involved in regulating feeding behavior, body weight and energy homeostasis (103-106). Huszar et al (107) reported that disruption of MC4-R in mice resulted in an adult-onset obesity syndrome associated with hyperphagia, hyperinsulinemia/insulin resistance, which recapitulated several characteristic features of dominant agouti mutation. Moreover, Fan et al (108) demonstrated that intracerebroventricular administration of MTII, a potent MC4-R agonist, caused a dose-dependent inhibition of food intake in four murine models of obesity and hyperphagia. This inhibition was reversed by

coadministration of SHU9119, a potent melanocortin antagonist. The effect of MC4-R on feeding was confirmed by the study demonstrating that MC4-R knockout mice did not respond to the anorectic actions of MTII (109). Further evidence from Harrold et al (110) showed that food restriction caused a selective up-regulation of MC4-R density in rat hypothalamus, whereas diet-induce obesity resulted in a down-regulation of MC4-R, suggesting that MC4-R plays an important role in the control of feeding behavior.

Several studies demonstrate that MC4-R may also play a role in human obesity. Recent data from Chagnon et al (57) demonstrated a significant association between restriction fragment polymorphisms of MC4-R and obesity phenotype, including body mass index (BMI), fat mass, and percent body fat, in the Quebec family study. Moreover, Yeo et al (58) and Vaisse et al (59) described a severely obese child and adult, respectively, with a base deletion or insertion induced frameshift mutation in MC4-R gene. Both patients are heterozygous. In addition, three allelic variants at MC4-R locus have been identified in obese human by polymorphism analysis (60). One variant from extremely obese person was cloned and found to be severely impaired in ligand binding and signaling (60), raising the possibility that this variant of MC4-R gene may contribute to development of obesity. Hinney et al (61) screened the coding region of MC4-R gene in both extremely obese and normal-weight people. Several mutations, including base deletion induced frameshift, base substitution (both mutations yield truncated MC4-R), and missense mutation, have been identified in extremely obese subjects with BMIs above 99^{th} percentile. Further study from the same group (111) detected a haploinsufficiency mutation in MC4-R gene, which was believed to be responsible for the extreme obesity.

Although agouti is not normally expressed in the central nervous system, agouti related protein (AGRP) (also known as agouti related transcript, ART), a homolog of agouti, was identified as an endogenous antagonist for MC4-R (112). AGRP is abundantly expressed in arcuate neurons and its expression is markedly elevated in obese mouse model (112). Ubiquitous expression of AGRP in transgenic mice recapitulates the phenotypes of obesity syndrome found in MC4-R knockout mice (113, 114). AGRP exhibits 25% identity to agouti, with the highest degree of identity in the cysteine-rich carboxyl terminus (112). As carboxyl terminus of agouti was believed to act as functional domain for antagonizing MC4-R (115, 116), a similarity between carboxyl terminal regions of the two proteins suggests similar functions for this domain in interacting with MC4-R. This was verified by demonstrations that carboxyl terminus of AGRP was critical for its antagonism of α-MSH at MC4-R (117, 118). Consistent this, intracerebroventricular administration of a carboxyl-terminal fragments of AGRP increased food intake in rats (119). This effect was mimicked by the MC4-R antagonist SHU9119.

The endogenous ligand for MC4-R is α-MSH, a peptide derived from proopiomelanocortin (POMC) (sequential cleavage of this protein also generates melanocortin peptides ACTH, β and γ MSH as well as opioid-receptor ligand β endorphin) (103). α-MSH appears to bind to MC4-R with a high affinity and act as a strong agonist in regulation of food intake and body weight. POMC gene is expressed in arcuate nucleus and then projects α-MSH to MC4-R-expressing cells, such as the paraventricular nucleus, resulting in a decreased food intake (104). Yaswen et al (120) recently generated a POMC-knockout mouse that developed severe obesity, similar to

that of the MC4-R knockout mouse. 2-week treatment of these POMC-null mice with α-MSH agonist caused more than 40% of weight loss. Consistent with this, a patient with severe early-onset obesity caused by POMC gene mutation was recently reported (56). The phenotype of this patient is similar to that found in POMC-null mice.

2. Peripheral Actions of Agouti: Role of Intracellular Ca^{2+} ($[Ca^{2+}]_i$)

In addition to the central effects of agouti on food intake and body weight regulation, available evidence also demonstrate that agouti protein also exerts potent pleiotropic effects in peripheral tissue, particularly in adipocytes and pancreatic β cells (121, 125, 126, 131) . Although chronic antagonism of central MC4-R may be responsible for the hyperphasia in yellow agouti mutants, the efficiency of energy utilization, such as preferential energy partitioning into adipose tissue, is also markedly increased in these mice (121). For example, adipose tissue expression of fatty acid synthase (FAS), the key enzyme in *de novo* lipogenesis, is significantly elevated in yellow obese mice (121). Moreover, MC4-R is expressed in several peripheral tissues, including adipose tissue (57). It is therefore possible that agouti-induced obesity syndrome may also involve its action at peripheral melanocortin receptors. In addition, transgenic mice specifically overexpressing agouti in adipose tissue under the control of aP2 promoter become obese in response to a concomitant hyperinsulinemia induced by either exogenous insulin administration (122) or a high sucrose diet (123), while their non-transgenic littermates do not. Recent data indicate that these peripheral actions of agouti in preferential energy partitioning in adipose tissue are mediated by modulation of $[Ca^{2+}]_i$.

Agouti protein contains a cysteine-rich carboxyl terminus, which bears functional activity equivalent to the full-length agouti in melanocortin receptor antagonism (124). All 10 cysteines at this domain are involved in forming disulfide bonds (124). The spacing and pattern of disulfide bonds is highly homologous to that of spider and snail neurotoxin venoms, which primarily target Ca^{2+} channels. Therefore, the structural similarity of C-terminus between agouti and these venoms suggests a similar function of Ca^{2+} channel modulation for the agouti C-terminal domain. Indeed, A^{vy} mice exhibit increased steady-state $[Ca^{2+}]_i$ and Ca^{2+} influx in adipose tissue and skeletal muscle (125, 126). There is a strong correlation between $[Ca^{2+}]_i$ levels and the degree of agouti expression and body weight (126) in these mice. Since adipocyte lipogenesis is markedly elevated in A^{vy} mice, it is tempted to speculate that agouti may modulate adipocyte lipid metabolism via modulating $[Ca^{2+}]$i.

Recombinant agouti protein causes a dose-responsive increase in $[Ca^{2+}]$i in a variety of cells, including both human and murine adipocytes (125, 126). Although the agouti stimulation of $[Ca^{2+}]_i$ requires a functional melanocortin receptors, which are expressed in both human adipocytes and 3T3-L1 (57), this action is not mediated by MCR antagonism (125). The physiological consequence of this elevation in $[Ca^{2+}]_i$ was addressed by the studies of adipocyte lipogenesis. Agouti stimulates the expression and activity of fatty acid synthase (FAS) via a Ca^{2+}-dependent manner, and this action can be mimicked by Ca^{2+} channel activation and reversed by Ca^{2+} channel antagonism (121). These actions are mediated by an agouti/Ca^{2+} response element on FAS promoter region (127). Moreover, recent evidence shows that agouti expression is highly correlated with

in vivo FAS expression and activity, suggesting that agouti protein, which is normally expressed in human adipose tissue, may play a role in adipocyte metabolism (128).

Agouti protein inhibits basal and agonist-stimulated lipolysis in human adipocytes via a Ca^{2+}-dependent mechanism (129). The mechanism underlying the anti-lipolytic effect of $[Ca^{2+}]_i$ was recently demonstrated to be increased $[Ca^{2+}]_i$ activation of phosphodiesterase 3B, resulting in reduced cAMP levels and, consequently, inhibition of hormone sensitive lipase activity (130). Accordingly, agouti regulation of adipocyte $[Ca^{2+}]_i$ appears to promote triglyceride accumulation in adipocyte by exerting a coordinated control over stimulation of lipogenesis and inhibition of lipolysis, serving to simultaneously stimulate the former and inhibit the latter.

It is noteworthy that hyperinsulinemia is necessary for complete expression of the obese phenotype in transgenic mice expressing agouti in adipose tissue under control of the aP2 promoter, indicating an interaction between insulin and agouti in agouti-induced obesity syndrome. Consistent with this, there is an additive effect of agouti and insulin on FAS transcription and lipogenesis (127). Since increased $[Ca^{2+}]_i$ is the proximate signal for insulin release and agouti stimulates $[Ca^{2+}]_i$ in several cell types, it is possible that agouti may exert a direct effect on pancreatic β cells to modulate Ca^{2+} signaling and stimulate insulin release. Indeed, agouti is expressed in human pancreas and potently stimulates and insulin release in RIN-5F, HIT-T15 and human pancreatic β cells via Ca^{2+}-dependent mechanism (131). Therefore, agouti stimulation of insulin release may contribute to the hyperinsulinemia/insulin resistance commonly present in yellow obese mice, and interact

with agouti to promote obesity phenotype. Moreover, agouti also stimulates amylin secretion from pancreatic β cells (132) via Ca^{2+}-dependent mechanism. This may contribute to the development of amyloidogenesis, leading to impaired β cell function and diabetes.

Therefore, agouti modulation of adipocyte lipid metabolism via $[Ca^{2+}]_i$, coupled with pancreatic insulin release via $[Ca^{2+}]_i$, may contribute to the peripheral actions of agouti in promoting obesity syndrome in yellow obese mouse. This was confirmed by demonstrating that treatment of agouti-overexpressing transgenic mice with nifedipine, a Ca^{2+} channel antagonist, markedly reduced the fat pad mass and corrected the agouti-induced hyperinsulinemia (133). Accordingly, antagonism of adipocyte and pancreatic β-cell $[Ca^{2+}]_i$ may represent a target for the development of therapeutic interventions in obesity.

III. ROLE OF INTRACELLULAR Ca^{2+} IN OBESITY

A. SYNDROME X: ROLE OF INTRACELLULAR Ca^{2+}

Obesity is closely related to other metabolic disorders, including insulin resistance/hyperinsulinemia, hypertension and cardiac hypertrophy. These diseases are integrated into a metabolic syndrome referred to as "Syndrome X" (134). To explain these tightly interacting abnormalities, Resnick et al (135) proposed a unifying "ionic hypothesis", in which a common cell lesion underlying these disorders in different tissues was characterized, in part, by elevations in steady-state intracellular $[Ca^{2+}]_i$ levels.

a. Intracellular Ca^{2+} and Insulin Resistance/Hyperinsulinemia

$[Ca^{2+}]_i$ plays an important role in insulin resistance. Dysregulation of $[Ca^{2+}]_i$ in multiple tissues, including adipose tissue, skeletal muscle and pancreas, may cause insulin resistance. Sustained increase in $[Ca^{2+}]_i$ have been observed in adipocytes, skeletal muscle cells, erythrocytes, and platelets in patients with insulin resistance (136-140). Draznin et al. demonstrated an optimal range of $[Ca^{2+}]_i$ for maximizing insulin-stimulated glucose transport in isolated adipocytes, with increases beyond this range causing marked decreases in insulin sensitivity (136). Consistent with this, adipocytes isolated from NIDDM patients exhibit high levels of $[Ca^{2+}]_i$ associated with a decreased insulin-stimulated glucose uptake (136-138), while pretreatment of these adipocytes with the Ca^{2+} channel antagonist verapmil prevented this $[Ca^{2+}]_i$-mediated inhibition of insulin-stimulated glucose transport (136). A^{vy} mice with marked insulin resistance/hyperinsulinemia exhibit elevated $[Ca^{2+}]_i$ in skeletal muscle, and the degree of elevation is closely correlated with the degree to which the mutant traits are expressed in

individual animals (126). Since agouti protein is able to stimulate $[Ca^{2+}]_i$ in cultured and freshly isolated skeletal myocytes, it is reasonable to speculate that agouti promotes insulin resistance in A^{vy} mice through its ability to increase $[Ca^{2+}]_i$ (126).

Two calcitrophic hormone parathyroid hormone (PTH) and 1,25-$(OH)_2$-D_3, which respond to suboptimal calcium intake, are also involved in insulin resistance. PTH is elevated in insulin resistant patients and stimulates Ca^{2+} influx in adipocytes (141), suggesting a role for this hormone in insulin signaling in adipocytes. 1,25-$(OH)_2$-D_3 has been shown to stimulate Ca^{2+} (142) and insulin release (143, 144) in pancreatic β-cells, suggesting that 1,25-$(OH)_2$-D_3 may play a role in the development of hyperinsulinemia. This increased $[Ca^{2+}]_i$ induced hyperinsulinemia may further aggravate insulin resistance.

Since increasing $[Ca^{2+}]$i appears to mediate the development of insulin resistance, antagonism of $[Ca^{2+}]_i$ in the insulin sensitive tissue would be predicted to improve insulin sensitivity. Indeed, $[Ca^{2+}]$i antagonism in obese elderly patients markedly increased insulin sensitivity of adipocytes isolated from abdominal biopsies (145). Similarly, antagonism of $[Ca^{2+}]_i$ with the L-type calcium channel blocker nirendipine significantly improved insulin sensitivity and glucose tolerance in spontaneously hypertensive rats (146). Moreover, antagonism of $[Ca^{2+}]_i$ with Ca^{2+} channel blockers (nitrendipine, diltiazem, or ampodipine) improved glucose tolerance and reduced fasting and glucose-stimulated circulating insulin levels in patients with insulin resistance, obesity and hypertension (147-149).

The mechanisms whereby dysregulation of $[Ca^{2+}]_i$ causes insulin resistance are not well defined. There is an association between decreased GLUT4 and increased $[Ca^{2+}]_i$ in skeletal muscle in type II diabetic patients (150). GLUT4 activity can be modulated by phosphorylation at serine residue. Phosphorylation of GLUT4 results in inactivation of GLUT4, while dephosphorylation of GLUT4 leads to activation of this protein. Therefore, increased $[Ca^{2+}]_i$ may contribute to insulin resistance by inhibiting the dephosphorylation of GLUT4 and thereby decreasing its activity (151). This was confirmed by demonstration that increasing Ca^{2+} influx by either membrane depolarization or parathyroid hormone treatment increased phosphorylation of GLUT4, whereas antagonism of Ca^{2+} influx with L-type calcium channel blocker nitrendipine maintained active and dephosphorylated state of GLUT4 (151). These effects of increased $[Ca^{2+}]_i$ on hyperphosphorylation of GLUT4 appeared to be mediated by $[Ca^{2+}]_i$-induced phosphorylation and activation of inhibitor 1, which in turn inhibited phosphoserine phosphorylase 1(PP1) (152). This inactivation of PP1 by increased $[Ca^{2+}]_i$ therefore caused hyperphosphorylation of GLUT4 and inhibited its activity.

In addition to the effects of $[Ca^{2+}]_i$ regulation of GLUT4 on insulin resistance, Ca^{2+}-regulated calmodulin has been shown to interact with IRS-1 and thereby modulate insulin signaling (153). Alternatively, it is also possible that increased $[Ca^{2+}]_i$ may result in insulin resistance via activation of protein kinase C (PKC). The activation of PKC is accomplished by the translocation of PKC from the cytosol to the plasma membrane for association with diacylglycerol (DAG). $[Ca^{2+}]_i$ plays a key role in this process. The

activated PKC may phosphorylate and inactivate the insulin receptor (IR) β-subunits, resulting in an inhibition of insulin signaling (141, 154).

b. Intracellular Ca^{2+} and Hypertension

Sustained elevations of $[Ca^{2+}]_i$ have been observed in vascular smooth muscle cells, platelets, erythrocytes, lymphocytes, aorta, and renal cells in either patients with hypertension or hypertensive rodents (140, 155-161). Increased $[Ca^{2+}]_i$ in platelets has been demonstrated to augment platelet aggregation in response to epinephrine (162). Most importantly, the role of $[Ca^{2+}]_i$ in regulating vascular tone and blood pressure has been well established (135, 163, 164). Depolarization of vascular smooth muscle cell membrane results in Ca^{2+} influx via voltage-dependent L or T type of Ca^{2+} channels. This, in turn, results in cytosolic Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum (SR) via activating ryanodine-sensitive Ca^{2+} channels on the SR membrane, thereby magnifying and spreading the initial, localized $[Ca^{2+}]_i$ throughout the cytosol. Subsequently, elevated Ca^{2+} triggers a cascade of molecular reaction of calmodulin and myosin light-chain kinase, ultimately leading to myofilament shortening and vasoconstriction (135, 163, 164). It is noteworthy that increased $[Ca^{2+}]_i$ induced by the other cellular events, such as hormonal receptor signaling-mediated generation of inositol triphosphate (IP3), is also able to cause the similar vasoconstriction (135). Antagonism of $[Ca^{2+}]_i$ in vascular smooth muscle cells causes vasodilation and reduces blood pressure. L-type Ca^{2+} antagonists, such as nitrendipine and nifendipine, are widely used to treat hypertension clinically. Moreover, thiazolidinediones, a group of insulin sensitizing

agents, have been demonstrated to block calcium channel in vascular smooth muscle and reduce blood pressure (165-167).

Factors that regulate $[Ca^{2+}]_i$ in vascular smooth muscle cells may modulate vasoconstriction and blood pressure. Insulin regulation of vascular smooth $[Ca^{2+}]_i$ is responsible for insulin regulation of vascular tone (168). Insulin attenuates both voltage and receptor-mediated Ca^{2+} influx in vascular smooth muscle cells (168). Inhibition of Ca^{2+} influx by insulin attenuates vasoconstriction in artery cells (169). Further, physiological concentrations of insulin inhibit either arginine vasopressin (AVP), angiotensin II (AII), or norepinephrine induced elevation in $[Ca^{2+}]_i$ (170). The insulin inhibition of sarcoplasmic Ca^{2+} release caused by either AVP, AII or norepinephrine is mediated by insulin stimulation of Ca^{2+}-ATPase activity in smooth muscle cells (171). Reduced Ca^{2+}-ATPase activity has been observed in type II diabetic patients (172). Insulin stimulates Ca^{2+}-ATPase-mediated Ca^{2+} efflux and causes vascular relaxation (173), and vascular smooth muscle cell Ca^{2+} efflux is impaired in both insulin resistant rats and streptozotocin-treated rats (156, 174, 175). This insulin stimulation of Ca^{2+}-ATPase is exerted via the up-regulation of Ca^{2+}-ATPase expression, as reduced Ca^{2+}-ATPase expression has been observed in vascular smooth muscle from insulin resistant rats (176) and insulin directly stimulates vascular smooth muscle Ca^{2+}-ATPase expression (177). These data suggest that impaired insulin action, due to a defect in insulin signaling caused by insulin resistance, may result in decreased vascular smooth muscle Ca^{2+}-ATPase expression and activity (177). This in turn results in decreased vascular smooth muscle Ca^{2+} efflux and increased vasoconstriction. Thus, the role of

insulin in regulation of vascular $[Ca^{2+}]_i$ and vascular tone provides the mechanism whereby insulin resistance and hypertension are closely associated disorders.

PTH and 1,25-$(OH)_2$-D_3 may also play important roles in regulation of vascular tone and hypertension. 1,25-$(OH)_2$-D_3 stimulates Ca^{2+} influx in a variety of cells (178, 179), including vascular smooth muscle cells (180), via a rapid non-genomic action. This action is believed to be mediated via a putative membrane vitamin D receptor (mVDR) rather than the classic nuclear vitamin D receptor (nVDR) (178, 179). Therefore, 1,25-$(OH)_2$-D_3 exerts a vasoconstrictive effect, serving to increase peripheral vascular resistance (135, 181, 182). However, the role of PTH in regulation of vascular tone is more complicated. There are two mechanisms for the action of PTH at cellular level. In addition to stimulation of Ca^{2+} influx, PTH also stimulates synthesis of cAMP by activating adenylyl cyclase (141). Therefore, although PTH does stimulate Ca^{2+} influx in vascular smooth muscle, it also exhibits vasodilatory and hypotensive effects (183, 184). Resnick (185) proposed that PTH may exert two independent effects on regulation of vascular tone: an acute vasodilatory effect due to the burst of cAMP generation and a chronic vasoconstrictory effect due to a persistent increase in $[Ca^{2+}]_i$. Moreover, parathyroid hypertensive factor (PHF), a circulating hypertensive factor of parathyroid origin, stimulates vascular smooth muscle Ca^{2+} influx, potentiates vasoconstrictive responses to other pressor factors and increases blood pressure (186, 187). The hyperparathyroidism associated with over-production of these calcitrophic hormones exerts physically detrimental impacts on vascular smooth muscle cells, due to their capability of stimulation of Ca^{2+} influx in these cells. This is manifested with the

deposition of calcium in the wall of arteries from patients with primary or secondary hyperparathyroidism (188).

Accordingly, low calcium diets, which stimulate the over-production of calcitrophic hormones, would be expected to stimulate vasoconstriction and increase blood pressure, while high calcium diets, which suppress the calcitrophic hormone levels, would be expected to maintain vasodilation and reduce blood pressure. This concept has been consistently confirmed by numerous epidemiological studies and clinical trials. McCarron et al. reported that low calcium diet is associated with hypertension and that dietary calcium intake is inversely correlated with the risk of being hypertensive in American adults (189, 190). This suggests that dietary calcium intervention is a practical approach in prevention or treatment of hypertension. Indeed, the DASH (Dietary Approaches to Stop Hypertension) trial demonstrates that increasing dietary calcium with low-fat dairy, combined with increased fruit and vegetable consumption, exerted profound effects on reduction of blood pressure. The effects of this calcium-rich combination diet in hypertensive group are comparable to those observed in pharmacological trials in mild hypertension (182, 191). These data indicate that dietary calcium exerts a potent effect on prevention or treatment of hypertension in the context of specific dietary patterns.

c. Intracellular Ca^{2+} and Coronary Heart Disease (CHD)

Dysregulation of $[Ca^{2+}]_i$ contributes to the development of insulin resistance and hypertension, both of which are co-morbidities and high risk factors of CHD. In addition,

$[Ca^{2+}]_i$ *per se* plays an important role in the pathogenesis of atherosclerosis (141, 193, 194). Sustained elevations of $[Ca^{2+}]_i$ have been observed in vascular smooth muscle cells, platelets, erythrocytes, lymphocytes, aorta, cardiomyocytes, and renal cells in patients with CHD (135, 155-161), while the elevation in calcitrophic hormones secondary to suboptimal calcium intake stimulate increases in $[Ca^{2+}]_i$ in these cells (141). As a consequence, increased $[Ca^{2+}]_i$ in these cells promotes smooth muscle cell and macrophage migration into the intima, facilitates platelet aggregation, enhances endothelial cell proliferation, stimulates macrophage to become foam cells (141). Atherosclerosis is initialized by the influx of Ca^{2+} into atherogensis-involved cells and accelerated by massive filtration of Ca^{2+} in vascular smooth muscle cells and completed by final Ca^{2+} deposition and calcification in vascular wall (141, 193). This was confirmed by clinical evidence that Ca^{2+} channel blockers are able to prevent arterial calcium overlaod and atherosclerosis (194). Moreover, formation of coronary plaques induced in vitamin D-intoxicated rats is highly sensitive to Ca^{2+} channel antagonists (141, 194). The pathogenesis of coronary plaques in this animal model resembles that in humans, suggesting an important role of $[Ca^{2+}]_i$ in the development of human atherosclerosis. Both cholesterol and LDL stimulate Ca^{2+} influx via L-type Ca^{2+} channels in vascular smooth muscle cells (141, 195, 196), while oxidized LDL, a primary atherogenic factor, causes a sustained increase of $[Ca^{2+}]_i$ in cultured aortic endothelial cells (197). Free radicals, anther group of atherogenic factors, have also been shown to modulate Ca^{2+} channels (198), while increased $[Ca^{2+}]_i$ in turn stimulates formation of free radicals such as lipid peroxides (199). Formation of free radicals during ischemia reperfusion, hyperoxia, and phagocytosis is associated with increased $[Ca^{2+}]_i$ (200). Further, the

detrimental effects of free radicals on cell functions are, at least in part, mediated by their modulation of $[Ca^{2+}]_i$ (201). Myocardial reperfusion injury is markedly attenuated by Ca^{2+} channel antagonists (202).

B. ROLE OF INTRACELLULAR Ca^{2+} IN OBESITY

a. Calcium Homeostasis and Obesity

Obesity is associated with alterations in calcium homeostasis. In a general profile of plasma calcium composition, 50% of total plasma calcium is ionized, 35% is albumin-bound, and the rest is complexed with anions. Serum calcium concentration is typically 2.25-2.5 mM, with 1.25 mM ionized Ca^{2+}. Only the ionized calcium is biologically active and can be regulated by calcium-homeostatic hormones, including PTH, 1,25-$(OH)_2$-D_3, and calcitonin. Decreased serum ionized Ca^{2+}, increased urinary calcium excretion, and increased calcitrophic hormone PTH and 1,25-$(OH)_2$-D_3 have been frequently observed in obese humans (203-206). Serum calcium levels are relatively stable. However, serum albumin, phosphate, and bicarbonate levels are decreased in obesity, while citrate, lactate, fatty acids and urate levels are increased (204). This causes the concentration of less readily resorbable anion in the renal ultrafilturate, resulting in increased urinary calcium excretion and thereby decreased serum ionized Ca^{2+} (207, 208). This decreased ionized Ca^{2+} subsequently stimulates calcitrophic hormone synthesis and release (204). An inverse correlation has been found between serum ionized Ca^{2+} levels and BMI/percent of body weight (204, 209). By contrast, weight reduction results in normalization of serum phosphate, bicarbonate, lactate, and fatty acid levels, leading to increases in serum ionized Ca^{2+} and corresponding decreases in calcitrophic hormones (210).

b. Intracellular Ca^{2+} and Adipocyte Lipid Metabolism

$[Ca^{2+}]_i$ plays an important role in the regulation of adipocyte lipid metabolism. Draznin et al (136-138) first reported that obese patients exhibited an elevation in basal adipocyte $[Ca^{2+}]_i$. The physiological consequence of this elevation in $[Ca^{2+}]_i$ was addressed by the following studies involved in adipocyte lipogenesis and lipolysis. Avasthy et al (211) demonstrated that treatment of rat adipocytes with A23187, a calcium ionophore, caused a marked increase in both basal and insulin-stimulated lipogenesis, while this action was completely prevented by the calcium channel antagonist verapamil and by the calmodulin inhibitor calmidazolium (211).

In addition to lipogenesis, several lines of evidence demonstrated an inhibitory effect of $[Ca^{2+}]_i$ on adipocyte lipolysis (212-216). Modulation of $[Ca^{2+}]_i$ plays a role in mediating both lipolytic and anti-lipolytic actions of hormones in adipocytes (213-216). The anti-lipolytic effect of epidermal growth factor (EGF) is mediated by an increase in $[Ca^{2+}]_i$. The EGF-stimulated $[Ca^{2+}]_i$ increases interaction between Gi and adenylyl cyclase, resulting in a reduction in cAMP and thereby an inhibition in lipolysis (215, 216). Alternatively, thyroid hormone modulates adipocyte lipolysis by increasing $[Ca^{2+}]_i$, which involves alterations in phosphodisesterase activity (213). In addition, increasing $[Ca^{2+}]_i$ by the calcium ionophore A23187 in hamster adipocytes stimulates phosphodisesterase and decreases cAMP levels, resulting in an inhibition of lipolysis (214).

$[Ca^{2+}]_i$ mediated increase in lipogenesis and inhibition in lipolysis were further explored in the studies of agouti. A^{vy} mice exhibit increases in both steady-state $[Ca^{2+}]_i$ and Ca^{2+} influx in several tissues (125, 126). The increase in $[Ca^{2+}]_i$ is correlated with the degree of ectopic agouti expression and body weight (125). Moreover, A^{vy} mice exhibit elevated adipocyte fatty acid synthase expression, suggesting that adipocyte *de novo* lipogenesis is increased in these animals (121). In addition, *in vitro* studies show that recombinant agouti protein causes a dose-responsive increase in $[Ca^{2+}]_i$ in a variety of cells, including both human and murine adipocytes (125, 126). The physiological consequence of this elevation in $[Ca^{2+}]_i$ was addressed by the studies of adipocyte lipogenic genes and activity. Agouti stimulates the expression and activity of fatty acid synthase (FAS) in a Ca^{2+}-dependent manner, and this action can be mimicked by Ca^{2+} channel activation and reversed by Ca^{2+} channel antagonism (121). These actions are mediated by an agouti/Ca^{2+} response element on FAS promoter region (127). Moreover, recent evidence shows that agouti expression is highly correlated with *in vivo* FAS expression and activity, suggesting that agouti protein, which is normally expressed in human adipose tissue, may play a role in human obesity (128).

In addition to the effect of agouti on stimulation of lipogenesis, agouti protein inhibits basal and agonist-stimulated lipolysis in human adipocytes via a Ca^{2+}-dependent mechanism (129). The mechanism underlying the anti-lipolytic effect of $[Ca^{2+}]$i was recently demonstrated to be increased $[Ca^{2+}]_i$ activation of phosphodiesterase 3B, resulting in reduced cAMP levels and, consequently, inhibition of hormone sensitive lipase activity (130). Accordingly, agouti regulation of adipocyte $[Ca^{2+}]_i$ appears to

promote triglyceride accumulation in adipocyte by exerting a coordinated control over stimulation of lipogenesis and inhibition of lipolysis, serving to simultaneously stimulate the former and inhibit the latter.

Accordingly, antagonism of $[Ca^{2+}]_i$ by either blocking Ca^{2+} channels or inhibiting Ca^{2+} agonists is a logical approach for development of therapeutic intervention in obesity. Indeed, calcium channel blockade has been proven to reduce body weight and fat pad mass effectively in several animal models. Kim et al. previously reported that treating transgenic mice overexpressing agouti with a Ca^{2+} channel antagonist (nifedipine) resulted in significant decreases in adipocyte lipogenesis and reduction in adipose tissue mass (133). Similarly, Ca^{2+} channel antagonists, such as nifendipine, verapamil, felodipine, and benidipine, caused decreases in body weight and fat pad mass in obese SHHF/Mcc-fa^{cp} rats and obese mice (217, 218).

c. Intracellular Ca^{2+} Mobilizing Agents in Obesity

There are many nutritional, hormonal, and pharmacological factors that modulate adipocyte $[Ca^{2+}]_i$ and may, therefore, modulate adipocyte lipid metabolism. In addition to agouti, these include sulfonylureas (219), calcitrophic hormone PTH (220, 221) and 1,25-$(OH)_2$-D_3 (220, 222), arginine vasopressin, agiotensin II, and glutamate, etc. Therefore, identifying and characterizing the factors that modulate adipocyte $[Ca^{2+}]_i$ is a logical approach for elucidating novel mechanism involved in modulating adiposity and identifying targets for the development of therapeutic interventions in obesity.

1. Sulfonylurea and Sulfonylurea Receptor

We recently demonstrated that human adipocytes express a sulfonylurea receptor (SUR), which mediates adipocyte $[Ca^{2+}]_i$ signaling and, consequently, modulates adipocyte lipid metabolism (219).

Sulfonylureas, such as glibenclamide, are insulin secretagogues widely used to stimulate insulin secretion in the treatment of non-insulin-dependent diabetes mellitus. These insulin secretagogues bind to the sulfonylurea receptor (SUR) of pancreatic β cells and then block the conductance of an ATP- dependent potassium channel (K_{ATP} channel) (223-225). The attenuation of potassium current by blocking this channel depolarizes the β cells and thereby induces Ca^{2+} entry via L-type calcium channels (226), leading to increased insulin secretion (227). The SUR is a member of the ATP- binding cassette proteins, with multiple membrane-spanning domains and two nucleotide- binding folds (225). The SUR itself does not form the ion-conducting part of the K_{ATP} channel. However, SUR combines and interacts with an inward-rectifier K^+ channel (Kir6.2) to generate K_{ATP} channel (228). Notably, endosulfine, an endogenous ligand of SUR, has also been cloned and is able to mimic the effects of sulfonylureas and thereby exhibit physiological functions, including inhibiting K_{ATP} channels currents and stimulating insulin release in β cell (229).

Patients treated with glibenclamide frequently experience weight gain as a side effect. Conversely, diazoxide, which inhibits SUR by activating K_{ATP} channels, exerts an antiobesity effect in obese Zucker rats and hyperinsulinemic obese humans (230-232).

These effects of glibenclamide and diazoxide on body weight have been attributed to their effect on circulating insulin rather than to any direct effect on adipocytes (230-232). However, the identification of SUR expression in human adipocytes suggests that it may modulate adipocyte $[Ca^{2+}]_i$ and corresponding lipid metabolism (219). Indeed, Adipocytes exhibited corresponding glibenclamide dose-responsive increase in $[Ca^{2+}]_i$. Moreover, glibenclamide exerted lipogenic and antilipolytic effects in human adipocytes, with a marked increase in fatty acid synthase and glycerol-3-phosphate dehydrogenase activities and a remarkable decrease in glycerol release, while diazoxide significantly blunted each of these effects (219). These data demonstrate that SUR in human adipocyte regulates $[Ca^{2+}]_i$ and thereby exerts coordinate control over lipogenesis and lipolysis. Accordingly, the human adipocyte SUR appears to represent an important target for further development of therapeutic intervention in obesity.

Consistent with our findings, SUR agonists have previously been demonstrated to exert direct effects on adipocytes. Draznin et al (136, 233) reported that glibenclamide increased $[Ca^{2+}]_i$ in isolated rat adipocytes in a dose-dependent manner by stimulating Ca^{2+} influx through voltage-dependent Ca^{2+} channels, while this effect was blocked by nitrendipine. Moreover, glibenclamide has been reported to potentiate peripheral insulin effect in isolated adipocytes (234,235). By contrast, Rajan et al (236) were unable to identify high affinity SUR in either isolated rat adipocytes or 3T3-L1 adipocytes. Moreover, they were unable to inhibit $^{86}Rb^{+}$ efflux (a surrogate for K_{ATP} channel activity) or increase $[Ca^{2+}]_i$ with glibenclamide. The reason for this discrepancy is not clear. However, several other investigators have reported both specific binding of sulfonylurea

and specific post-receptor effects in murine and rat adipocytes. For example, rat adipocytes exhibit specific, saturable glibenclamide binding (K_D of 1-3 μM), which is displaced by other sulfonylureas, and sulfonylurea treatment of isolated rat adipocytes potentiates insulin receptor of glucose transport (234,235,237). Further, Muller et al. (238,239) reported that glimepiride exhibits specific binding to 3T3-L1 and rat adipocytes, resulting in an insulin-mediated stimulation of glucose transport and non-oxidative glucose disposal. These effects were attributed to sulfonylurea-induced inhibition of cAMP level and protein kinase A activity. Thus, rodent adipocytes appear to exhibit SUR binding and functional response to this binding.

Interestingly, the human chromosome region encoding both the SUR and the associated inward rectifying K^+ channel, 11p15.1 (240) also contains the human homolog of Tubby, a locus responsible for severe obesity in mice (241). Moreover, a significant association was recently reported between an exon 22 allelic variant of the SUR gene and obesity in French Caucasians (242). In addition, linkage between the SUR region and a subgroup of morbidly obese families was also noted (242). Thus, the SUR locus may contribute to genetic susceptibility to obese (242). Whether this contribution is based on alteration in insulin secretion or an extrapancreatic role of the SUR is unknown. However, our data suggest a potential role for the human adipocyte SUR in modulating energy storage and thereby potentially contribute to obesity.

It is noteworthy that both electrophysiological and pharmacological evidence demonstrates that K_{ATP} channels present in different tissues, such as pancreas, muscle and brain, belong to different subtypes and show different binding affinity to sulfonylureas, thereby resulting in different efficacy (243). Accordingly, it is possible that SURs expressed in pancreatic β cells and adipocytes are also different subtypes and have different sensitivities to sulfonylureas. If so, it may be possible to exploit these differences to make adipocyte specific SUR agonists and antagonists.

2. 1,25-$(OH)_2$-D_3 and Dietary Calcium

It is now well recognized that 1α,25-$(OH)_2$-D_3, the biologically active form of the vitamin D, generates biological responses via both genomic and nongenomic pathways. 1α,25-$(OH)_2$-D_3 is a highly conformationally flexible molecule and is able to rotate around its 6, 7 carbon-carbon bond of the B ring. This rotation allows generation of a continuum of potential ligand shapes ranging from the steroid like 6-s-cis conformation to the extended 6-s-trans conformation (179, 244). Thus, this conformational flexibility of 1α,25-$(OH)_2$-D_3 in a structure and function context underlies the mechanism whereby 1α,25-$(OH)_2$-D_3 generates both genomic and non-genomic cellular responses.

1α,25-$(OH)_2$-D_3 was originally believed to solely function via a nuclear receptor in a manner similar to the other members of steroid hormone superfamily, resulting in a biological response (245). Briefly, 1α,25-$(OH)_2$-D_3 binds to and activates a specific nuclear hormone receptor, nVDR. The activated nVDR then interacts with another

nuclear receptor RXR and forms a heterodimer complex. This complex functions as a transcriptional factor to act on the direct repeat response element named DR-3 in the promoter region of target genes, thereby stimulating or suppressing transcription of those genes encoding for proteins that carry out a variety of functions (245). Indeed, recent studies of nVDR knockout mouse showed that mice lacking nVDR exhibit a vitamin D deficient phenotype, including impaired bone formation and growth retardation (246, 247), demonstrating that nVDR mediated genomic action exhibits an important biological function. However, a high calcium/phosphorus/lactose diet fed at 16 days of age in nVDR knockout mice allows normal mineral ion homeostasis and thereby rescues the vitamin D deficient phenotype (248), suggesting that an alternative pathway may compensate to preserve intestinal calcium absorption and bone development and modeling in the absence of a functional nVDR. In fact, several lines of evidence demonstrated that 1α,25-$(OH)_2$-D_3 stimulates intestinal transcaltachia (249-253), the rapid stimulation of Ca^{2+} transport in intestinal epithelium, which may contribute to the intestinal calcium absorption in these nVDR ablated mice, although this may not be sufficient to prevent the phenotype of vitamin D deficiency in these animals fed with normal calcium diets. Moreover, several studies demonstrated that 1α,25-$(OH)_2$-D_3 is able to induce rapid non-genomic effects in nVDR-null cells or cells from nVDR knockout mice (254, 255), further indicating that the genomic model of 1α,25-$(OH)_2$-D_3 action is not complete.

1α,25-$(OH)_2$-D_3 also generates rapid, nongenomic signal transduction via a putative membrane vitamin D receptor (mVDR) in a wide variety of cells (178), including

modulation of calcium channels (142, 180, 249-253, 256-258), production of diacyl glycerol and inositol triphosphate induced by activation of phospholipase (259), activation of protein kinase C (260), and stimulation of MAP kinase (261-263). The non-genomic action of 1α,25-$(OH)_2$-D_3 has been confirmed by studies of analogs of 1α,25-$(OH)_2$-D_3. These analogs have been extensively examined for their ability to generate genomic or non-genomic responses (179, 264). For instance, 1α,25-dihyroxylumisterol$_3$, a 6-s-cis-locked analog of 1α,25-$(OH)_2$-D_3, preferentially exerts a non-genomic action, including stimulation of Ca^{2+} influx, via the putative mVDR (265, 266). Another A-ring diasteromer analog, 1β,25-dihyroxyvitamin D_3, has been demonstrated to antagonize this action but has no effects on the nVDR (267, 268). Most importantly, recent pursuit of physical existence of the putative mVDR provides the further evidence to strengthen these findings. Nemere et al (253) first demonstrated a specific binding protein for 1α,25-$(OH)_2$-D_3 in the membrane of chick intestinal epithelium, which mediates the stimulation of calcium transport. Moreover, Nemere et al (260) subsequently identified a membrane associated protein with a high binding affinity for 1α,25-$(OH)_2$-D_3, which mediated the rapid non-genomic regulation of PKC. Further, Baran et al (269) recently found another plasma membrane-bound protein with a specific and saturable binding for 1α,25-$(OH)_2$-D_3, which was identified as annexin II by sequence analysis and Western Blot. They further demonstrated that annexin II mediated the rapid effects of 1α,25-$(OH)_2$-D_3 on $[Ca^{2+}]_i$. However, it is not known whether these identified binding proteins for 1α,25-$(OH)_2$-D_3 are the same protein or different receptors mediating similar non-genomic functions.

In addition to the direct stimulation of Ca^{2+} influx, 1α,25-$(OH)_2$-D_3 also increases the expression of the CaT1 (270), a recently cloned Ca^{2+} calcium channel involved in capacitative calcium entry (CCE) activated by intracellular Ca^{2+} release (271, 272).

We further extend these observations by demonstrating that 1α,25-$(OH)_2$-D_3 elicits both genomic and non-genomic action in adipocytes, resulting in modulation of lipid and energy metabolism (220, 222, 273). We recently demonstrated that 1α,25-$(OH)_2$-D_3 stimulates adipocyte $[Ca^{2+}]_i$, promotes lipogenesis and inhibits lipolysis via a rapid non-genomic action (220, 222). These actions can be mimicked by 1α,25-dihyroxylumisterol$_3$, a specific mVDR agonist, and can be prevented by 1β,25-dihyroxyvitamin D_3, a specific mVDR antagonist. We further demonstrated that 1α,25-$(OH)_2$-D_3 exerts an inhibitory effect on basal, isoproterenol and fatty acid stimulated adipocyte UCP2 expression via a genomic action (273). mVDR agonist and antagonist fail to exert their actions to either mimic or prevent 1α,25-$(OH)_2$-D_3' inhibitory effect on adipocyte UCP2 expression, while nVDR knockout by antisense oligodeoxynucleotide (ODN) prevents the inhibitory effect of 1α,25-$(OH)_2$-D_3 on adipocyte UCP2 expression (unpublished data).

Regardless of genomic or non-genomic action, 1α,25-$(OH)_2$-D_3 exhibits an important role in modulation of obesity. Evidence shows that 1α,25-$(OH)_2$-D_3 is involved in preadipocytes differentiation. 1α,25-$(OH)_2$-D_3 up-regulates nVDR expression in 3T3-L1 preadipocytes (274) and exerts an inhibitory effect on 3T3-L1 preadipocyte differentiation (275-278). This inhibition is mediated via either competition of nVDR

with other differentiation transcriptional factors with structural similarity such as RXR and PPARγ on DNA response elements (278) or mVDR-mediated Ca^{2+} influx. However, unlike the differentiation of 3T3-L1 preadipocyte which is an embryo-origin cell line, the differentiation of OB17 preadipocyte, an adult-origin cell line, is stimulated by 1α,25-$(OH)_2$-D_3 (279, 280). In addition to differentiation, 1α,25-$(OH)_2$-D_3 also promotes the expression and secretion of lipoprotein lipase in cultured adipocytes (281), indicating that 1α,25-$(OH)_2$-D_3 may enhance adipocyte lipid accumulation by increasing fatty acid uptake.

The role of 1α,25-$(OH)_2$-D_3 in obesity has been documented in human population-based studies. nVDR gene polymorphism is associated with the susceptibility to obesity in humans with early-onset type II diabetes (282). Moreover, Several lines of evidence demonstrate that circulating 1α,25-$(OH)_2$-D_3 level is elevated in obese humans (203-206, 283-285). Since increased circulating 1α,25-$(OH)_2$-D_3 exerts a negative feedback on the hepatic synthesis of 25-(OH)-D_3, a negative association between BMI and circulating 25-(OH)-D_3, as expected, is observed in humans (286, 287). It is noteworthy that 1α,25-$(OH)_2$-D_3 induced hyperinsulinemia may be also involved in the development of obesity in humans. 1,25-$(OH)_2$-D_3 has been shown to stimulate Ca^{2+} (142) and insulin release (143, 144) in pancreatic β-cells, indicating that 1,25-$(OH)_2$-D_3, elevated with the disturbance of calcium homeostasis commonly seen in human obesity, may contribute to development of hyperinsulinemia via a non-genomic action. Moreover, genetic

epidemiological studies show that nVDR gene polymorphism influences insulin secretion and susceptibility to IDDM in Indian Asians (288) and Bangladeshi Asians (289).

Accordingly, antagonism of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ and its genomic and/or non-genomic pathways may represent a promising approaches for development of interventions in obesity. Since increasing dietary calcium has been demonstrated to suppress $1\alpha,25\text{-}(OH)_2\text{-}D_3$ levels in several studies (135, 141, 290), we test the hypothesis that dietary calcium suppression of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ would reduce adipocyte $[Ca^{2+}]_i$ and thereby inhibit triglyceride accumulation by coordinated control over lipogenesis and lipolysis. Our data demonstrated that suppression of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ by increasing dietary calcium decreased adipocyte intracellular Ca^{2+}, stimulated lipolysis, inhibited lipogenesis, increased adipocyte uncoupling protein 2 (UCP2) expression and core temperature in aP2- agouti transgenic mice and that dietary calcium not only attenuated diet-induced obesity but also accelerated weight loss and fat mass reduction secondary to caloric restriction (220, 291). Consistent with our findings, Metz et al (292) and Bursey et al (293) previously reported that increased dietary calcium reduced body weight and body fat composition in spontaneously hypertensive rats, Wistar-Kyoto rats and lean and obese Zucker rats.

Recent data suggest that this approach may be relevant to humans. Population-based observation in NHANES III demonstrated a profound reduction in the odds of being in the highest quartile of adiposity associated with increases in calcium intake (220). This is

supported by earlier clinical trial demonstrated that increasing daily calcium intake from 400-1000 mg/day for one year resulted in a 4.9 Kg reduction in body fat in obese African-Americans (220). Consistent with this, a randomized clinical trial demonstrated a greater weight loss in patients maintained on a milk-based diet with high calcium for 16 weeks, compared with those on conventional hypocaloric diet at same energy intake (294). Recently, these findings were further confirmed and extended by Davis et al. (295). They reported that a significant negative association between calcium intake and body weight was observed in two cross-sectional and two longitudinal studies, and that the odds ratio of being overweight was 2.25 for women in lower half of calcium intakes (295). Further, they found a significant weight loss in subjects maintained on a calcium supplement in a double-blinded, placebo-controlled, randomized clinical trial (295).

IV. ROLE OF ADIPOSE TISSUE IN OBESITY

Adipose tissue is the body's largest energy storage organ where energy is stored as triglyceride (TAG). When total energy intake exceeds the energy expenditure, excessive energy is deposited in adipose tissue in the form as TAG. The adipocyte TAG is either derived from chyromicroms (CM) and very-low-density lipoproteins (VLDL) or synthesized via de novo lipogenesis from carbohydrate precursor such as glucose. TAG from circulating CM and VLDL is hydrolyzed by lipoprotein lipase (LPL) located on the capillary walls of adipose tissue to generate free fatty acids (FFAs) and monoacylglycerol. FFAs are then transported into adipocytes by specific fatty acid transport proteins and re-esterified to TAG. On the other hand, excess glucose initiates the *de novo* lipogenesis by generating acetyl CoA. Acetyl CoA is used as the substrates to synthesize the long-chain fatty acids, which are further esterfied to TAG. During nutritional deprivation, adipocytes undergo lipolysis to release energy. Intracellular TAG is hydrolyzed to FFAs and glycerol by hormone sensitive lipase. These products leave adipocytes and are transported via the circulation to energy-consuming tissues (296).

Adipose tissue has long been considered as a passive energy-storage depot as described above. However, data emerging over the past decade have established a new role for adipose tissue as a functional endocrine/paracrine/autocrine organ. Adipocytes express and secrete numerous peptide hormones and cytokines, as follows. Leptin plays a key role in regulating body weight and energy balance. Tumor necrosis factor alpha (TNF-α) (21), resistin (22, 23), and adiponectin (24-26), contribute to insulin resistance/NIDDM.

Angiotensin II regulates vascular tone, adipocyte lipogenesis and differentiation (297). Plasminogen-activator inhibitor-1 is involved in vascular functions and cardiovascular heart disease (16). Adipose tissue also secretes active steroid hormones, such as estrogen and cortisol (298, 299), the immune system-related protein adipsin (300), and prostaglandins (297). These bioactive molecules link adipocyte biology to the systematic functions of diverse organ through the body, indicating that local changes of adipocyte functions or mass may dynamically influence systematic metabolism.

A. ADIPOCYTE HYPERPLASIA

Adipocyte differentiation occurs in both prenatal and postnatal states. It was originally believed that individuals are born with a fixed complement of adipocytes that developed prenatally and persisted throughout life without gaining new cells. However, it is now recognized that adipogenesis occurs throughout the lifespan. Adipogenesis may result from either normal cell turnover or the requirement for more fat mass upon excessive energy storage (301, 302). Indeed, although adipocyte size is variable corresponding to the changes of amount of stored lipid, the physical space for a single adipocyte to store lipid is ultimately limited. On the other hand, animals always continue to store fat as long as energy intake exceeds nutritional requirement, suggesting a requirement for de novo differentiation of adipocytes to accommodate this excessive energy (301).

Increased fat mass can arise from an increase in adipocyte size, number, or both. It was originally believed that only early-onset obesity was associated with adipocyte hyperplasia, while maturity-onset obesity was believed to result solely from adipocyte

hypertrophy (303, 304). This concept was challenged by later studies demonstrating that the potential to acquire new adipocytes continues through adulthood (305-309). Direct evidence from studies of rats fed with a high caloric diet demonstrates that ^{3}H-thymidine incorporation into new adipocytes occurs throughout life (305). Moreover, expression of specific early differentiation genes has been reported to express in adipose tissue from very old mice (306). In addition, fat cell precursors, such as stromal-vascular cells, isolated from adult human adipose tissue can be fully differentiated into mature adipocytes in vitro (307, 308). Further, adipocyte development has been shown to depend on fat precursor cell recruitment *in vivo* (307). Human population-based study shows that an increase in fat cell number appears to be well correlated with severity of human obesity in adult life (309). Although the relative contribution of adipocyte hypertrophy versus hyperplasia to human adiposity is unknown, the capacity to generate new adipocytes from cells persists throughout life span and clearly plays a role in regulating fat tissue mass.

a. Adipocyte Origin

The development of adipocyte origin *in vivo* is not well defined. However, studies on pluripotent stem cell lines in vitro demonstrate that the adipocyte lineage derives from the embryonic stem cell precursors, which possess the potentials to differentiate into mesodermal cell types, including adipocytes, chondrocytes, osteoblasts, and myocytes (310). This was confirmed by demonstration that demethylating agents induce murine embryonic stem cells to differentiate into adipocytes, myocytes, and chondrocytes simultaneously (310, 311). Moreover, there is evidence that the same marrow-derived

stromal cells give rise to both bone and fat cells (312, 313). This conversion of stromal cells into specific cell lineages depends on different culture conditions that could selectively promote the differentiation of one cell type over another. For instance, TGF-β superfamily proteins act as potent osteogenic agonist to stimulate bone cell formation, while these proteins inhibit adipocyte differentiation (310, 313, 314). By contrast, PPARγ agonists function as potent adipogenic factors to stimulate adipocyte differentiation, while they inhibit bone cell formation (310, 313, 314). Although the transcriptional pathways in the late stages of adipocyte differentiation have been well defined, the early molecular events that promote the determination of primitive mesenchymal precursor cells to the adipocyte lineage cells remain unknown. There are no clear molecular markers identified so far to indicate the determination of the transition from primitive mesenchymal cells to committed adipocyte lineage cells. However, a recent study shows that GATA-deficient embryonic stem cells exhibit enhanced capacity to differentiate into adipocyte (315), indicating that down-regulation of GATA transcriptional factors sets the stage for the transition of embryonic stem cells to adipocytes. Since the initial steps of adipogenesis from primitive mesenchymal cells are not clear, pre-determined clonal cell lines, such as preadipocyte cell lines, and primary-cultured stromal vascular precursor cells isolated from adipose tissue are used to study adipogenesis. These preadipocyte cell lines and primary-cultured preadipocytes are already committed to adipocyte lineage, although they are morphologically fibroblasts and represent different stages of adipocyte development (310). The most frequently used cell models are 3T3-L1 and 3T3-F442A, which are clonally derived from 3T3-Swiss cells isolated from disaggregated 17 to 19 day mouse embryo (310, 316-318), and Ob17 cells, which are adipocyte precursors

isolated from epididymal fat pads of adult ob/ob mice (319). When these cells are cultured in appropriate conditions containing fetal bovine serum (FBS), methylisobutylxanthine (MIX), dexamethasone (not required for 3T3-F442A differentiation), insulin, and T_3 (required for Ob17 cell differentiation), they are able to acquire morphological and biochemical characteristics of adipocytes and become fully differentiated.

Morphological methods such as microscopic investigation have been successfully utilized to study the early development of adipose tissue *in vivo*. At early developmental stages of human fetus, cells with the potential to develop into adipocytes are morphologically indistinguishable from those with the potentials to develop into other cell types (320). The human fat organ initially appears between 14^{th} and 16^{th} week of gestation (320, 321). The first indication of adipogenesis is evident as an aggregation of mesenchymal cells associated with the formation of blood vessels. The first primitive organ structures where fat cells aggregate are known as fat lobules (320, 322). Although the number of fat lobules remains constant after 23^{th} week of gestation, the size of these lobules grows during the following several weeks of gestation (320, 322). During stages of the early fat development, adipose tissue predominantly exists as the multilobular forms (320, 322). Interestingly, the morphological development in vitro resembles that in vivo. Preadipocyte differentiation from either preadipocyte cell lines or primary-cultured preadipocytes tends to form adipocyte clusters (320). This not only occurs in adipocyte differentiation stages but also persists in mature adipocyte culture, such as Matrigel-cultured mature adipocytes with a three-dimensional culture model mimicking in vivo

adipocyte-growing condition (323, 324). At the beginning of the third trimester, adipocytes exist in the major fat depots but remain rather small. At birth, body fat accounts for 16% of body weight (320, 322).

b. Program of Adipocyte Differentiation

The first stage in the adipocyte differentiation is cell growth arrest, a required event for further differentiation process (301, 310). At this point, preadipocytes arrest at the G_0/G_1 cell cycle boundary (325, 326). Although cell-cell contact or cell confluence does cause preadipocyte growth arrest, cell contact inhibition *per se* is not a prerequisite for growth arrest to occur. Preadipocytes plated in at low density or cultured in methylcellulose-stabilized suspension are still able to differentiate into mature adipocytes (310, 327). Following cell growth arrest, preadipocytes must receive mitogenic or adipogenic signals to proceed further proliferation and adipogenic conversion, which can be initiated by treating confluent cells with an appropriate combination of hormone agents such as methylisobutylxanthine (MIX) and dexamethasone. Following exposure to these agents, preadipocytes undergo at least one round of cell division, so called mitotic expansion. DNA replication during this mitosis presumably alters the accessibility of gene promoter/regulatory elements to transcriptional factors that activate or suppress early regulatory genes responsible for initiating differentiation (325, 326). These mitotic events are required for cells to enter a unique growth-arrested phase of cell cycle, referred to as G_D (328-330), which is permissive for subsequent differentiation events. Once cells enter

the G_D phase, cells enter a final period of growth arrest and differentiation becomes more committed and irreversible.

Growth arrest and clonal expansion are accompanied by complex changes in the pattern of gene expression. Lipoprotein lipase (LPL) is the one of the early genes expressed during differentiation process. LPL expression appears as early as in the confluent stage of growth arrest, although biological role of its early expression is not known (325, 326, 331). Two key families of transcriptional factors, CCAAT/enhancer binding protein (C/EBP) and proliferators-activated receptor-γ (PPARγ), are expressed in the early stages of differentiation (301, 310), and play critical roles in terminal differentiation, as discussed in the following section (Transcriptional Control of Adipocyte Differentiation). PPARγ expression increases immediately following the hormonal induction of differentiation and represents an early marker of adipocyte differentiation. Its expression can be detectable on the second day of differentiation and remains high in mature adipocytes (332). Transient induction of C/EBPβ and C/EBPδ precedes that of PPARγ (333-335). The subsequent decreases of C/EBPβ and C/EBPδ are concomitant with the induction of C/EBPα expression, which appears slightly before the induction of adipocyte-specific genes (333-335). Another important transcriptional factor induced in the early stage of differentiation is sterol response element binding protein-1/adipocyte determination and differentiation factor-1 (SREBP1/ADD1), which is also involved in the induction of adipocyte-specific gene expression (336, 337). Following the expression of these early transcriptional factors, preadipocytes enter the terminal phase of differentiation. There are numerous adipocyte-functional genes which characterize the

adipocyte phenotype induced during this stage. These adipocyte functional genes include those responsible for lipid metabolism, such as fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), ATP citate lyase, malic enzyme, and glycerol-3-phosphate-dehydrogenase (GPDH) for de novo lipogenesis (310, 338-340) and hormone sensitive lipase for lipolysis, aP2 for fatty acid transport (339, 341), stearoyl-CoA desaturase-1 (SCD1) for fatty acid desaturation (340), those genes involved in glucose utilization, such as glucose transport 4 for glucose transport (GLUT4) (342), and phosphoenolpyruvate carboxykinase for gluconeugenesis (343), and those genes responsible for adipocyte signaling, such as the insulin receptor and β_2- and β_3- adrenergic receptors (310, 344, 345), and those gene encoding adipocyte secretory hormones/cytokines, such as leptin, TNFα, resistin, CD36, and Acrp30 (22-25, 310, 346), etc. By contrast, certain preadipocyte specific genes, such as preadipocyte factor-1 (pref-1) which is involved in maintaining preadipocyte phenotype, are dramatically decreased in the differentiation process (347).

c. Transcriptional Control of Adipocyte Differentiation

1. Proxisome Proliferator-Activated Receptor Gamma (PPARγ)

Cloning of PPARγ revealed it to be a member of PPAR subfamily of nuclear hormone receptor (348-350). PPAR family includes three related but quite distinct proteins, PPARγ, PPARα and PPARδ, each of which is encoded by a separate gene and shows a distinct tissue distribution (351). PPARγ is expressed in an adipose-selective fashion in both rodents and humans, with 10-30 fold higher levels in fat than in most other tissues

(350). PPARγ exists as two isoforms, PPARγ1 and PPARγ2 which differ in the N-terminus by 30 amino acids, produced by alternative promoter usage (352, 353). PPARγ1 is expressed at low levels in many tissues, but abundantly in fat, while PPARγ2 is abundantly and selectively expressed in fat (352-354).

The binding of ligands to PPARγ remarkably enhances its transcriptional activity. Two classes of PPARγ ligands so far have been identified. One is a family of synthetic compounds, thiazolidinedions (TZDs), which are used to increase insulin sensitivity clinically (355). These compounds bind to PPARγ with high affinity and potently increase its transcriptional activity (355). Searches for the natural ligands for PPARγ have revealed that fatty acids and eicosanoid derivatives, such as linoleic acid, linolenic acid, prostaglandin J2, D2 and other metabolites, are able to bind and activate this receptor (356-358). The studies of Gal4-reporter gene activation demonstrated that the most potent natural ligand is 15-deoxy $\Delta^{12,14}$ PG J2 (356). Although all of these molecules are considered as potential ligands in vivo, several ligand-receptor binding assays show that their binding affinities are low, in the range of 2-50 μM. Indeed, this is inconsistent with the higher affinities that most nuclear receptors possess for their endogenous ligands (Kd at low nM level) (348). Recent study of X-ray crystal structure revealed a large binding pocket in PPARγ, which may provide easier access for ligands and explain the diversity of ligands for PPARγ (359).

PPARγ plays a key role in transcriptional control of adipocyte-specific gene expression, which include aP2, PEPCK, LPL, GLUT4, and leptin, etc. Ligand-activated PPARγ heterodimerizes with RXR and this heterodimerization complex acts on a conserved DNA element DR-1 (direct repeat of the hormone response element separated by one base) like sequence in these adipocyte-specific genes (352). PPARγ-dependent adipogenic transcription was first characterized on the transactivation of adipocyte-specific enhancer of aP2 gene (350, 352). PPARγ also transactivates PEPCK gene expression during preadipocyte cell line differentiation (343). This PPARγ induced PEPCK expression has recently been extended in vivo, where activation of PEPCK promoter in adipose tissue depends on PPARγ binding site, whereas expression of this gene in other tissues does not (360).

It is now well recognized that PPARγ per se plays a central role in transcriptional control of adipogenesis. PPARγ was expressed at low but detectable levels in preadipocytes and its expression was markedly increased early in adipocyte differentiation (350, 352). Retroviral expression of PPARγ in fibroblasts caused an adipocyte phenotype in these fibroblasts, including morphological changes, lipid accumulation, and adipocyte-specific gene expression. However, a point mutation in the zinc finger of PPARγ required for DNA binding ablated its capability of differentiation induction (361). Moreover, treatment with selective high-affinity PPARγ agonists such as thiazolidinediones (TZDs) further increased the potency of PPARγ on differentiation (362, 363). Conversely, synthetic antagonists for PPARγ inhibit adipocyte differentiation (364). Consistent with

this, a dominant negative mutant of PPARγ inhibited PPARγ agonist-induced adipogenesis (365)

The role of PPARγ in adipogenesis has also been demonstrated in PPARγ knockout mice. The homozygous null mutation is lethal in gestation, resulting from defective placenta development (366, 367). Barak et al. created chimeric embryos using a combination of wild-type tetraploid cells which contribute to the development of extraembryonic tissues (366). One PPARγ-null homozygous mutant developed to term, but succumbed to a variety of metabolic disorders by post-natal day 5. This animal lacked adipose tissue, indicating that PPARγ is required for adipose tissue development in vivo (366). Moreover, Rosen (368) et al created chimeric mice derived from both wild-type ES cells and PPARγ$^{-/-}$ ES cells. In these animals, PPARγ null ES cells do not contribute to the formation of fat tissue, but participate in the development of the other tissues. In addition, PPARγ is also required for the in vitro differentiation of adipocytes from ES cells (368).

2. CCAAT Enhancer Binging Protein (C/EBP) Family

C/EBP proteins were first transcriptional factors demonstrated to play an important role in adipocyte differentiation. These transcriptional factors contain a basic transactivation domain and a leucine zipper motif, which are responsible for homo- or heterodimerization with other transcriptional factor (301, 310, 333). C/EBPs are expressed not only in adipose tissue, but also in other tissue, such as liver, which actively metabolize lipid (333).

The expression of C/EBPs during adipocyte differentiation exhibits a distinct pattern. C/EBPβ and C/EBPδ are transiently expressed early in response to hormonal stimulation, whereas C/EBPα is expressed just preceding the induction of most adipocyte specific gene (333-335), suggesting that this protein may be responsible for the expression of these adipocyte marker genes (330). Several adipocyte-specific genes, such as stearoyl CoA desaturase-1 (SCD-1), GLUT4, aP2, PEPCK, leptin, and insulin receptor, contain CEBPα binding site in their promoter regions (310, 333, 369-373).

Ectopic expression of C/EBPβ is sufficient to induce the differentiation of 3T3-L1 cells in the absence of hormone stimulation, while differentiation hormonal agents are required to induce differentiation in the C/EBPδ-overexpressed 3T3-L1 cells, but adipogenesis in these cells is accelerated (374). Similar result is observed in the NIH-3T3 cells overexpressing C/EBPβ. C/EBPβ is also able to determine primitive mesenchymal cells to the adipocyte lineage and promote adipogenic in these cells (375). By contrast, either C/EBPβ- or C/EBPδ-deficient embryonic fibroblasts exhibited slightly reduced adipogenic potential, while cells lacking both C/EBPδ and C/EBPβ were severely inhibited from differentiating into adipocytes (376). C/EBPα is also critical to the adipogenic program. Constitutive expression of C/EBPα is sufficient to induce differentiation of 3T3-L1 cells without additional hormonal agents, while expression of antisense C/EBPα mRNA in 3T3-L1 preadipocytes prevents differentiation (377, 378). Moreover, C/EBPα has been shown to promote adipogenesis in a variety of mouse fibroblasts which have little or no spontaneous capacity to differentiate into adipocytes (379).

Mouse models lacking C/EBPs illustrate the importance of this family of transcriptional factors in adipogenesis. Mice lacking either C/EBPβ or C/EBPδ usually survive post-natally without significant developmental or physiological defects. These animals develop normal white adipose tissue, although their brown fat exhibits slight reduction in lipid accumulation and UCP1 expression (376). However, the double-knockout mice (C/EBP$\delta^{-/-}$ and C/EBP$\beta^{-/-}$) have a low survival rate (15%), but the double knockout survivors exhibit reduced brown and white fat. Interestingly, the reduction in brown fat results from impaired lipid accumulation, while the reduction in white fat involves decreased cell number (376). Generation of C/EBPα-deficient mice confirms the physiological role of C/EBPα in adipose tissue differentiation. A high percentage of these knockout mice die from hypoglycemia as a result of a failure to activate hepatic gluconeugenesis postnatally (373, 380, 381). This is caused by the C/EBPα deficiency in the liver, where it regulates genes involved in energy metabolism including glucose. However, phenotype rescue by injection of glucose is able to increase survival rate. These C/EBPα-null mice do not have subcutaneous inguinal white fat. Although the interscapular brown fat is still present, lipid accumulation is dramatically reduced in this tissue (373, 380, 381). Moreover, the role of C/EBP family in adipogenesis is further demonstrated by functional knockout of all members of C/EBP in white adipose tissue. Transgenic mice expressing a dominant-negative basic leucine zipper protein, termed B-ZIP protein, under the control of aP2 promoter exhibit lipidystrophy (382). The dominant negative mutant B-ZIP proteins overexpressed in adipose tissue dimerize with all member proteins of C/EBP family and abolish their DNA-binging abilities. These mice

are unable to develop white fat, demonstrating critical roles of C/EBP family proteins in adipose tissue development.

3. Sterol Response Element Binding Protein-1/Adipocyte Determination and Differentiation Factor-1 (SREBP1/ADD1)

SREBP1/ADD1 is a group of basic helix-loop-helix (bHLH) leucine zipper transcriptional factors (310). SREBP1/ADD1 displays a dual DNA binding specificity, binding to both an E-box motif and non-E-box (SRE-1) motif element. The SREBP family consists of three members, SREBP-1a, 1c, and 2, which are encoded by 2 independent genes. In human and mouse, SREBP-1a and 1c are generated from the same gene by the usage of alternative transcription start sites (383). ADD1, cloned from a rat cDNA library, is a homologue of human SREBP-1c (384). SREBP1/ADD1 has been shown to regulate expression of numerous genes encoding proteins involved in both cholesterol and fatty acid metabolism (310, 385-388)). SREBP1/ADD1 is an inactive molecule bound to the membrane of the endoplasmic reticulum. Once cells sense a cholesterol depletion, SREBP1/ADD1 undergoes proteolytic cleavage and translocates to the nucleus to regulate expression of target genes (389). Although all three SREBPs are capable of modulating similar gene expression, regulation of fatty acid biosynthesis is primarily mediated by SREBP-1a and SREBP-1c, which is the predominant form in adipose tissue in vivo (385).

The expression of SREBP1 is detectable on the second day of 3T3L-1 cell differentiation (336). Ectopic expression of a constitutive active form of SREBP-1c increases expression

of adipocyte marker genes and enhance the differentiation in the presence of PPARγ agonists in NIH-3T3 cells (336). Overexpression of SREBP-1c in preadipocytes increases the transcriptional activity of PPARγ on aP2 promoter by increasing the endogenous ligands (390). Moreover, the ectopic expression of a dominant-negative form of this transcriptional factor that contains a point mutation in the DNA-binding domain suppresses the expression of adipocyte marker genes and inhibits differentiation (336).

4. Transcriptional Cascade in Adipogenesis

Addition of specific hormones initiates a cascade of transcriptional events that account for the expression of adipocyte-functional genes. Transient expression of C/EBPδ and C/EBPβ precedes that of PPARγ (375). These C/EBPs have been demonstrated to mediate the expression of PPARγ through C/EBP binding sites in the PPARγ promoter region (353, 391). Moreover, C/EBPδ and C/EBPβ also induce C/EBPα expression via a consensus sequence in its promoter (392). Once induced, C/EBPα and PPARγ appear to cross-regulate each other to maintain their gene expression levels, although the C/EBPδ and C/EBPβ levels decrease after early transient expression (393).

SREBP1/ADD1 is also induced early during differentiation. This transcriptional factor appears to up-regulate PPARγ expression (391). Moreover, ectopic expression of SREBP-1c in preadipocytes increases the transcriptional activity of PPARγ by producing the endogenous ligands (390). Therefore, SREBP1/ADD1 plays an important role in transcriptional events mediating adipogenesis.

During the late stages of terminal differentiation, PPARγ and C/EBPα play the major role in regulating the gene expression of proteins responsible for the development of functional mature adipocytes. C/EBPδ and C/EBPβ are able to directly regulate adipocyte-functional gene expression, as there are a number of these genes that bear active consensus sequence for either C/EBPα or PPARγ. It is also possible that C/EBPδ and C/EBPβ indirectly regulate expression of other adipocyte-functional genes via the activation of intermediary transcriptional factors that act on those genes (385).

It is noteworthy that preadipocytes express a number of inhibitory proteins that may function to maintain the preadipocyte phenotype. Therefore, these inhibitory factors must be down-regulated or inactivated to allow differentiation process to proceed. Pref-1 is an inhibitor of adipocyte differentiation (347). It is synthesized as a plasma membrane protein containing 6 EGF repeats. Pref-1 is abundantly expressed in preadipocytes, but is not detectable in mature adipocytes (347). Down-regulation of pref-1 is required for adipogenic conversion, while constitutive expression of pref-1 inhibits differentiation. That was confirmed by demonstrating that application of antisense pref-1 to preadipocytes markedly increases adipogenesis (347). However, pref-1 expression is not detectable in stromal-vascular fraction in mouse adipose tissue in vivo (393), suggesting a different expression pattern of this gene in vivo. Moreover, recent studies demonstrate novel signaling pathways involved in the inhibition of adipogenesis. Wnt signaling has been demonstrated to be a molecular switch that controls adipogenesis (394). Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of PPARγ and C/EBPα expression (394). Conversely, disruption of Wnt signaling increases

cell adipogenesis. In addition, family of GATA transcriptional factors also plays a role in modulation of adipogenesis (315). Down-regulation of these transcriptional factors sets the stage for terminal differentiation. By contrast, constitutive expression of GATA suppresses adipocyte differentiation and traps cells at the preadipocyte stages. This inhibitory effect on adipogenesis is mediated by direct inhibition of PPARγ (315). Further, several lines of evidence shows that suppression of AP-2a and sp1 expression during differentiation is required for PPARγ and C/EBPα expression (395, 396).

Adipogenic transcriptional factors may also modulate adipogenesis by interacting with cell cycle proteins to affect preadipocyte cell cycles. Activation of PPARγ causes cell cycle withdraw by inhibiting the DNA-binding and transcriptional activity of the E2F-DP complex (397). This alteration in E2F/DP DNA binding is secondary to the decreased expression of protein phosphorylase 2A, which results in increased phosphorylation of DP-1, thereby preventing E2F DNA binding (397). Moreover, since retinoblastoma (pRB) is able to bind and sequester E2F, increased pRB expression or activity is expected to inhibit E2F DNA binding and transcriptional activity. Indeed, knockout of either pRB activity or expression inhibits adipogenesis (398), whereas either activation or overexpression of two members of pRb protein, p107 and p130, promotes adipogenesis (399). In addition to sequestering E2F, pRB is also able to directly interact with adipogenic transcriptional factors, such as C/EBPs and MyoD, to modulate adipocyte genes involved in terminal differentiation (400). pRB is inactivated via phosphorylation exerted by a dimmer complex comprising a regulatory subunit cyclin and a catalytic subunit cyclin-dependent kinase (cdk). This complex is further modulated by cyclin-

dependent kinase inhibitors; phosphorylation of cyclin/cdk complex by cdk inhibitor inactivates its activity. Recent studies demonstrate that cdk inhibitors, including p18 (INK4c), p21 (Cip1), and p27 (Kip1), are up-regulated during adipocyte differentiation (401), suggesting that cdk inhibitors may play a role in cell cycle withdraw and maintaining the irreversible growth arrest which defines the adipocyte terminal differentiation. This up-regulation of cdk inhibitor is mediated by PPARγ. Either ligand activation or ectopic expression of PPARγ increases the expression of these cdk inhibitor proteins or even increases the stability of these proteins (401). Further, the p38 MAP kinase pathway, activated by stress signal for cell growth arrest, is also involved in adipogenesis. Treatment of preadipocytes with p38 MAP kinase inhibitor during the early differentiation stages blocks adipogenesis (402). Moreover, ectopic expression of a dominant-negative p38 inhibits adipogenesis. This action is mediated by inhibition of C/EBPβ, which contains a consensus site for p38 phosphorylation and serves as a substrate for p38 MAP kinase (402).

d. Hormonal and Cellular Regulation of Adipogenesis

1. Insulin, IGF-1 and Other Growth Factors

Addition of insulin to differentiation media can promote adipogenesis and also increase lipid accumulation in adipocytes (403). Although preadipocytes do express few insulin receptors (404), insulin may potentiate adipocyte differentiation via binding to the IGF-1 receptor. This may explain why the pharmacological doses of insulin are required to achieve the stimulatory effects on adipogenesis.

IGF-1 is a potent inducer of adipogenesis. It is a critical component of fetal bovine serum for adipocyte differentiation, as supplementation of this factor enables differentiation to proceed in serum-free medium (405). Moreover, IGF-1 has been shown to stimulate 3T3-L1 preadipocyte differentiation in a dose-dependent manner (406). IGF-1 secreted from Ob17 cells could act in an autocrine/paracrine fashion to induce differentiation (407).

Moreover, IGF-1 mediates the adipogenic effect of growth hormone, which is required for preadipocyte cell line differentiation (310). Growth hormone stimulates expression and secretion of IGF-1 in preadipocytes, which in turn act on adipogenesis. Growth hormone also sensitizes cells to the mitogenic effects of IGF-1 for clonal expansion and thereby promotes differentiation (310).

IGF-1 and insulin may stimulate adipogenesis by activating downstream signal transduction. Activation of ras by insulin or IGF-1 has been demonstrated to mediate the stimulatory effects on adipogenesis. This was confirmed by demonstration that ectopic expression of ras promotes adipogenesis in the absence of hormonal stimulation (408), whereas inactivation of ras inhibits adipogenesis (409). However, the timing of ras activation during differentiation is crucial in determining whether it exerts a stimulatory or inhibitory effect on differentiation, as this small G protein is a potent mediator of MAP kinase pathway, whose activation leads to inhibition of differentiation. Moreover, PKB/Akt may also function as a down-stream signal for insulin/IGF-1 stimulated adipogenesis. Ectopic expression of PKB in 3T3-L1 preadipocytes induces spontaneous differentiation (410).

Unlike insulin/IGF-1, other growth factors are generally believed to be inhibitors of adipogenesis, due to their mitogenic effects on cell growth. Epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and TGF-β have been consistently shown to inhibit adipogenesis in a variety of preadipocyte cell lines and primary-cultured preadipocytes in different species (411, 412). Transgenic mice overexpressing TGF-α exhibit reduced body fat by 50% (413), while TGF-β has been shown to inhibit adipogenesis in vivo (414). However, basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) exhibit either no effects, inhibitory effects, or slightly stimulatory effects on adipogenesis, depending on the cell origin, developmental stage, and culture condition (see review 310). This inhibitory effect on adipogenesis is likely to be mediated by growth factor activation of MAP kinase pathways, such as erk 1 and 2. These kinases are able to phosphorylate PPARγ at N-terminal serine residues and thereby inhibit its transcriptional activity (415-417). Moreover, MAP kinases also phosphorylate RXR, the heterodimerization partner of PPARγ, and inhibit its dimerization with PPARγ (418).

2. Tumor Necrosis Factor-α (TNF-α) and Other Cytokines

A number of cytokines, including TNF-α and IL-1, have been shown to inhibit adipogenesis (301). TNF-α is a potent inhibitor of adipocyte differentiation. Moreover, it also suppresses the expression of some adipocyte-specific gene expression in newly differentiated adipocytes, serving to dedifferentiate these cells (419-421). This inhibitory effect on adipogenesis is mediated by down-regulation of C/EBPα and PPARγ (421, 422). This may explain the parallel down-regulation of those adipocyte-specific genes,

such as aP2 and GLUT4, which contain binding sites for PPARγ and C/EBPα in their promoters.

3. Nuclear Hormone Superfamily

The members of nuclear hormone superfamily that exert effects on adipocyte differentiation include glucocorticord, 3,3',5-triiodothyronine (T_3), retinoic acid (RA), and 1,25-dihroxyvitamine D_3. It is believed that these hormones act on differentiation via activating nuclear hormone receptors, belonging to a large nuclear receptor superfamily including PPARγ. Dexamethasone (Dex), a synthetic glucocorticoid, has long been used to induce differentiation in preadipocyte cell lines and primary cultured preadipocytes in different species (310). Addition of Dex to the differentiation cocktail is either required for differentiation or at least accelerates the differentiation process, depending on the origin of the cells (310). Dex has been shown to induce C/EBPs expression, while cells overexpressing PPARγ or C/EBPs still require the addition of Dex to be fully differentiated (301, 310). Recently, Sul et al. demonstrated that Dex promotes adipogenesis via inhibiting pref-1 expression, while constitutive expression of pref-1 blocks the adipogenic effect of Dex (347).

RA influences adipocyte differentiation in a biphasic manner. Administration of pharmacologic doses of RA inhibits adipogenesis, while treatment with physiological doses of RA increases adipogenesis. The inhibitory effect of RA on adipogenesis is mediated by inhibition of C/EBPβ, C/EBPα and PPARγ expression (310, 423, 424). In contrast to the inhibitory effect of RA used in pharmacological doses, administration of

RA in physiological concentration exerts a stimulatory effect on adipogenesis in OB17 preadipocytes (425, 426). Moreover, RA exhibits a critical role in ES cell-derived adipogenesis. Application of RA to ES cells markedly promotes adipogenic transition from ES cells to adipocytes (427).

T_3 is only required for Ob17 preadipocyte differentiation (428). There is no requirement of this hormone in the other differentiation system. 1α,25-$(OH)_2$-D_3 either promotes or inhibits adipogenesis, depending on cell models. 1α,25-$(OH)_2$-D_3 up-regulates nVDR expression in 3T3-L1 preadipocytes (274) and exerts an inhibitory effect on 3T3-L1 preadipocyte differentiation (275-278). This inhibition is mediated via either competition of nVDR with other differentiation transcriptional factors with structural similarity such as RXR and PPARγ on DNA response elements (278) or mVDR-mediated Ca^{2+} influx. However, unlike the differentiation of 3T3-L1 preadipocyte which is an embryo-origin cell line, the differentiation of OB17 preadipocyte, an adult-origin cell line, is stimulated by 1α,25-$(OH)_2$-D_3 (279, 280).

4. Prostaglandins

Both mature adipocytes and preadipocytes secrete large amounts of prostaglandins (PG). Several major PGs that exert effects on adipogenesis and lipid metabolism include $PGF_{2\alpha}$, PGI_2, PGD_2, and PGE_2 (297, 310, 348, 430-434). PGE_2 is a potent anti-lipolytic compound, suggesting that it may contribute to lipid accumulation in terminal adipogenesis. PGD_2 and its derivative PGJ_2 are endogenous ligands for PPARγ and therefore promote adipogenesis via a PPARγ-dependent mechanism (348). PGI_2

stimulates Ob1771 cell differentiation in an autocrine/paracrine manner (412). This action may be mediated by stimulation of cAMP (429). Moreover, PGI_2 is also an agonist for PPARα, PPARδ, and PPARγ (297), suggesting that PGI_2 may exert its adipogenic effect by activation of PPARs. In addition, angiotensin II stimulates PGI_2 secretion from differentiated Ob1771 cells by binding to AT_2 receptor. The secreted PGI_2 in turn acts on preadipocytes to promote differentiation in a paracrine manner (430), indicating that PGI_2 may play a physiological role in adipogenesis *in vivo*. By contrast, $PGF_{2\alpha}$ exerts inhibitory effect on adipogenesis in various preadipocyte cell lines and primary preadipocytes (431, 432). This was confirmed by demonstration that FP receptor ($PGF_{2\alpha}$ receptor) agonists potently inhibit 3T3-L1 preadipocyte differentiation (433, 434).

5. Intracellular Secondary Messengers

The role of cAMP in adipogenesis has been well demonstrated in the application of methylisobutylxanthine (MIX) in adipocyte differentiation. MIX is a component required in the differentiation medium for most types of preadipocyte differentiation. MIX causes intracellular cAMP accumulation by simultaneously inhibiting phosphodiesterase and stimulating adenylyl cyclase via blocking protein G_i (310). MIX has been shown to induce adipogenesis by increasing C/EBPβ. Several studies have demonstrated that MIX induction of adipogenesis is mediated via increasing cAMP. Synthetic cAMP analogs can replace MIX for adipocyte differentiation (435) and increasing cAMP through activation of adenylyl cyclase by forskolin can induce differentiation in the absence of MIX (436). Recently, the mechanism of cAMP stimulation of adipogenesis was addressed by demonstration that activated CREB (cAMP response element binding protein) is required

and sufficient to induce adipogenesis, indicating that CREB, the cAMP-activated transcriptional factor, mediates the cAMP-stimulated adipocyte differentiation (435).

The G protein family members, including $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$, are involved in regulation of adipogenesis. Either suppression of $G_{\alpha s}$ by $G_{\alpha s}$ antisense knockout or ectopic expression of constitutively active $G_{\alpha i}$ dramatically accelerated adipogenesis. These effects were exerted at ambient or elevated cAMP levels, suggesting that the effects of G protein on adipogenesis are independent of G modulation of adenylyl cyclase (437, 438). This was confirmed by further evidence demonstrating that the specific domains of $G_{\alpha s}$ and $G_{\alpha i}$ responsible for adipogenesis are distinct from those interacting with adenylyl cyclase (438). Activation of $G_{\alpha q}$ appears to down-regulate adipocyte differentiation. $G_{\alpha q}$ activates phospholipase Cβ, resulting in production of diacylglycerol and further activation of PKC, a negatively regulatory signal in adipogenesis (310, 440).

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is also an important cellular signaling mediating adipogenesis. Ntambi et al. have reported that increasing $[Ca^{2+}]i$, by either inhibiting Ca^{2+}-ATPase or stimulating Ca^{2+} influx, inhibits the early stages of murine adipocyte differentiation (441). $[Ca^{2+}]_i$ exerted this inhibitory effect by blocking the postconfluent mitotic phase and mediating sustained levels of *c-myc* expression (441). This sustained *c-myc* expression precludes cell entry to the G_D stage necessary for subsequent differentiation. Consistent with this, stimulation of FP receptor by $PGF_{2\alpha}$, an inhibitory prostaglandin in adipogenesis, causes a transient increase in $[Ca^{2+}]_i$ and in turn activates calcium/calmodulin-dependent kinase (CaM kinase), resulting in an inhibition of

adipocyte differentiation (431). This anti-adipogenic effect of $PGF_{2\alpha}$ can be reversed by KN-62, a CaM kinase inhibitor (431). However, other studies demonstrate that activation of CaM kinase stimulates adipogenesis, while inhibition of CaM Kinase with KN-62 or KN-93 prevents the adipogenic conversion. A possible explanation to this discrepancy is that the effect of $[Ca^{2+}]_i$ on adipogenesis depends on the timing of $[Ca^{2+}]_i$ stimulation during differentiation process. Our recent data confirm that increasing $[Ca^{2+}]_i$ exerts a biphasic regulatory role in human adipocyte differentiation, serving to inhibit the early stages of differentiation, while promote the late stages of differentiation and lipid filling (441). The mechanism whereby $[Ca^{2+}]_i$ undergoes the role transition during human adipocyte differentiation is unknown. However, cAMP also plays a discordant role in adipocyte differentiation and mature adipocyte metabolism. Increasing cAMP promotes adipocyte differentiation, while it inhibits expression and activity of fatty acid synthase (442, 443), a key enzyme in de novo lipogenesis, and stimulates lipolysis in mature adipocytes. Moreover, there is a significant interaction between the calcium and cAMP signaling pathways. Structural and functional studies have demonstrated that adenylyl cyclases are associated with the site of Ca^{2+} entry into the cell and that Ca^{2+} entry causes a marked inhibition of type V and VI adenylyl cyclases, thereby reducing cAMP levels in several cell types (444). Alternatively, recent data from our laboratory demonstrated that increasing adipocyte $[Ca^{2+}]_i$ stimulates phosphodiesterase 3B activity, resulting in reduced cAMP levels (130). Accordingly, increasing $[Ca^{2+}]_i$ in the early stages of differentiation may suppress pre-adipocyte cAMP levels and thereby inhibit differentiation. By contrast, a $[Ca^{2+}]_i$-induced decrease in cAMP late in differentiation

up-regulates lipogenesis and down-regulates lipolysis, thereby promoting adipocyte maturation and lipid filling.

B. ADIPOCYTE HYPERTROPHY

The adipocyte TAG is either derived from circulating lipoproteins or synthesized via de novo lipogenesis from carbohydrate. *De novo* lipogenesis essentially transfers the energy from carbohydrates to long-chain fatty acids, which are saved as TAG. Meanwhile, TAG undergoes lipolysis to release fatty acids, which are used as energy. Therefore, adipocyte hypertrophy characterized as overloaded TAG primarily results from increased *de novo* lipogenesis and decreased lipolysis.

a. Regulation of *de novo* Lipogenesis in Adipocytes

There are several enzymes responsible for *de novo* lipogenesis. Glucose first undergoes glycolysis to generate pyruvate, which is catalyzed by glycolytic enzymes. Pyruvate is then converted to acetyl-CoA which is transported as citrate from mitochondria to cytosol. ATP-citrate lyase catalyzes citrate into acetyl-CoA, which is then converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). Fatty acid synthase (FAS) catalyzes the subsequent synthesis of palmitate from acetyl-CoA and malonyl-CoA using NADPH as reducing equivalent, which is derived from malate conversion catalyzed by malic enzyme or pentose shunt catalyzed by glucose-6-phosphate dehydrogenase or gluconate-6-phosphate dehydrogenase. ACC and FAS are two key enzymes in *de novo* lipogenesis. ACC catalyzes the rate-limiting step in fatty acid synthesis. Its enzymatic activity is

primarily regulated via short-term allosteric modification or covalent phosphorylation, although long-term regulation of ACC involved in protein amount does exist. FAS activity, however, is regulated at transcription level, which results in changes of FAS protein amount (445, 446).

Humans exhibit lower FAS activity in both liver and adipose tissue than that of rodents. This is attributed to the lower metabolic rate and higher fat/lower carbohydrate diets in human than in rodents (447). However, the physiological significance of *de novo* lipogenesis in human adipose tissue has been demonstrated. Indeed, human adipose tissue expresses substantial levels of FAS, which is subject to nutritional and hormonal regulation (448-450). In primary cultured human adipose tissue incubated with labeled glucose, 80% of glucose can be incorporated into lipid (451). This was confirmed by *in vivo* studies demonstrating that high carbohydrate diets stimulate adipocyte lipogenesis, resulting in lipid accumulation in human adipose tissue (452, 453). In fact, adipose tissue may account for up to 40% of whole body lipogenesis in nutritionally-depleted humans given hypercaloric carbohydrate diets and total fat synthesis in adipose tissue is at least equal to, if not greater than, that in liver (453). Moreover, adipocyte FAS expression and activity are elevated in genetic obesity (454). These data suggest that up-regulation of adipocyte *de novo* lipogenesis may contribute to obesity *in vivo*.

Recent studies demonstrated that both ACC and FAS as well as their metabolites may play novel roles in regulating energy homeostasis involved in energy expenditure and feeding behavior. Abu-Elheiga et al. (455) generated the mice lacking ACC2, a dominant

ACC isoform in skeletal and cardiac muscle. These ACC2-deficient mice exhibit reduced fat storage in liver and adipose tissue by 50%, although they are hyperphagic. This contributes to the increased fatty acid oxidation in the skeletal muscle. ACC2 deficiency decreases the levels of malonyl CoA, a potent inhibitor of carnitine palmitoyltransferase (CPT), resulting in increases in fatty acid transport into mitochondria and fatty acid oxidation. Moreover, these ACC2 knockout mice exhibit increased UCP3 expression in skeletal muscle, which may also contributes to fatty acid oxidation and thermogenesis. On the other hand, Loftus et al. reported that treatment of mice with FAS inhibitors results in inhibition of feeding and dramatic weight loss. These FAS inhibitors suppress the expression of neuropeptide Y in hypothalamus and act in a leptin-independent manner. These actions are attributed to increased malonyl CoA, which acts as a novel neuronal signal (456).

1. Fatty Acid Synthase (FAS)

The fatty acid synthase in bacterial and plants is a multienzyme complexes consisting of 7 discrete, but tightly associated and well organized monofunctional enzymes (445). By contrast, the animal FAS is a multifunctional enzyme with a single large polypeptide. From the N-terminus of FAS to its C-terminus, the amino acid sequence of this large polypeptide encodes β-ketoacyl synthase, acetyl-CoA transacylase, malonyl-CoA, transacylase, dehydratase, enoyl reductase, ketoacyl reductase, acyl carrier protein, and thioesterase activites, which are organized into discrete domain (445). The functional FAS consists of two identical 250 KDa subunits which act as a homodimer (445). The multifunctional organization of animal FAS is believed to result from gene fusion due to

selection of evolution, as individual domains responsible for specific function can be coordinately regulated in this way to improve whole functional efficiency (457). For example, this arrangement facilitates transferring of the product from one reaction to the active center of subsequent enzymatic step as a substrate. Moreover, regulating product of one single reaction can modulate all seven catalytic reactions in a concerted manner (445).

FAS is encoded by a single copy gene that generates a single transcript in mice and humans, and two transcripts produced by alternative usage of polyadenylation sites in rats and chickens (445). Using differential display strategy, the mouse FAS cDNA was cloned by differential hybridization of liver cDNA library with ^{32}P-labeled cDNA reverse-transcribed from mRNA derived from mice fasted or fasted and refed a high-carbohydrate diet (338). A single 8.2 Kb FAS mRNA was identified and exhibited wide tissue distribution, with the highest levels of expression in liver and adipose tissue. The human FAS cDNA was also cloned and FAS gene was mapped to 17q25 by fluorescent in situ hybridization (458, 459). Northern blot revealed that human FAS mRNA is 9.3 Kb in size and that its level varies among human tissues, with liver, adipose tissue, brain showing prominent expression (460).

2. Nutritional and Hormonal Regulation of Fatty Acid Synthase

Unlike ACC which is subject to allosteric modification and covalent phosphorylation, FAS, however, is primarily regulated at transcription level in response to nutritional and hormonal modulation.

2.1 Nutritional and Hormonal Regulation of FAS in vivo

Starvation followed by re-feeding a high carbohydrate/low fat diet is often used as paradigm for nutritional regulation of FAS expression (461). Fasting in rats results in a marked decline of the FAS synthesis, while re-feeding these fasted animals with a high carbohydrate/low fat diet increases FAS synthesis by 20-25 fold (462). Using the same starvation-re-feeding approach, similar changes are observed in mouse FAS expression (442). This starvation-re-feeding manipulation alters the circulating glucose level, which in turn induces the secretion of hormones responsible for de novo lipogenesis. It is well recognized that circulating insulin levels are elevated in animals challenged with a high-carbohydrate diet, while circulating glucagon levels are increased during nutritional deprivation with low circulating glucose levels (445, 463). These two hormones play major roles in regulating FAS expression in response to changes of nutritional status. Indeed, streptozotocin-diabetic mice exhibited low FAS expression in both liver and adipose tissue, while administration of insulin to these animal increased FAS mRNA by 2-fold within 1 hr and 19-fold within 6 hr (442). By contrast, administration of glucagon or dibutyryl cAMP to the re-feeding animals with previous fasting completely blocked the elevation of hepatic FAS expression caused by high carbohydrate diets (442). These data suggest that glucagon, mediated by cAMP, antagonizes insulin stimulation of FAS expression.

Dietary polyunsaturated fatty acid (PUFA) potently inhibits FAS activity and expression in both liver and adipose tissue. Supplement of 2% 18:2 (n-6) to a high carbohydrate, fat-free diet results in a marked inhibition of hepatic FAS activity and fatty acid synthesis

(464). The inhibitory effect of 18:2, n-6 PUFA on FAS expression has been extended to all PUFAs of both n-3 and n-6 family (465).

The nutritional and hormonal regulation of FAS expression described above was well demonstrated in the study of transgenic mice carrying the 201-kb 5'-flanking promoter region of FAS fused to a chloramphenicol acetyltransferase (CAT) reporter gene (443, 463). CAT activity was low in the fasted transgenic mice, while it was significantly up-regulated in liver and adipose tissue of fasted animals re-fed with a high carbohydrate diet. However, administration of dibutyryl cAMP to the re-fed transgenic animals prevented an increase in CAT activity. Streptozotocin-diabetic transgenic mice exhibited low CAT activity in both liver and adipose tissue. Moreover, dietary PUFA markedly suppressed CAT activity as well as endogenous FAS expression in liver and adipose tissue. Further, administration of glucocorticoid to transgenic mice increased CAT activity in adipose tissue and liver. This was confirmed by later studies that dexamethasone increased FAS activity and mRNA by increasing both transcription and mRNA stability (466, 467).

Other hormones involved in regulation of FAS expression include growth hormone (GH) and T_3. GH appears to antagonize the effect of insulin on FAS. Administration of GH decreased FAS mRNA levels in rats and pig (468, 469). T_3, however, stimulates FAS expression. Injection of thyroid hormone into rats increased hepatic FAS activity (470). In addition, hyperthyroid animals exhibit an elevation in both liver and adipocyte FAS mRNA, while hypothyroid animals exhibit a decrease in FAS expression (471, 472).

2.2 Molecular Mechanism Mediating FAS Gene Regulation

- Insulin Response Element and Transcriptional Factors

Insulin stimulates FAS mRNA and activity in both 3T3-L1 adipocytes and primary cultured human adipocytes (448, 450). This stimulatory effect is mediated by a cis-acting insulin response element (IRE) in FAS promoter region (463, 473, 474). Analysis of FAS 5' promoter deletion constructs fused to report genes demonstrated that IRE is located in the region spanning from -67 to -25. DNAase I footprinting analysis further revealed a nuclear extract-protected region from -71 to -50. Gel mobility shift assays with the sequence from -71 to -50 demonstrated a nuclear extract-binding complex in this region. Further mutation analysis in this region demonstrated that -68/-60 with a functional E box is required for binding of nuclear extracts (463). Three tandem repeats of the -68/-52 region linked to SV40 promoter with a luciferase report gene were sufficient to respond to insulin stimulation. These data demonstrate that the minimal region (-68/-52) that responds to insulin contains an E box (-65/-60) essential for transcriptional factor binding, suggesting that those transcriptional factors capable of binding to the E box (-65/-60) may mediate insulin stimulation of FAS expression.

Upstream stimulatory factor 1 and 2 (USF1 and 2), members of basic helix-loop-helix leucine zipper family of transcriptional factor, have been shown to bind to E box and function as heterodimer in the other gene promoters (475, 476). Therefore, the interaction between USFs and FAS promoter IRE was examined. Indeed, gel shift assay demonstrated that both USF1 and 2 are able to bind to FAS promoter IRE to form a binding complex which can be disrupted by cold DNA sequence known to bind to USFs

and can be super-shifted by anti-USF1 and anti-USF2 antibodies (477). Moreover, overexpression of USF1 and 2 with FAS promoter luciferase reporter construct increased insulin stimulation of FAS promoter, while overexpression of dominant negative USF mutants without DNA binding domains inhibited insulin stimulation of FAS promoter (463, 478). These data demonstrate that insulin stimulation of FAS expression is dependent on USFs binding to E-box (-65/-60). The role of USF in mediating insulin stimulation of FAS was further confirmed by the *in vivo* study of USF-knockout mice (479). These animals fed with a high carbohydrate diet exhibit impaired FAS induction, indicating that USFs mediate glucose/insulin stimulation of FAS *in vivo*.

Recent studies showed that SREBP1 also mediates insulin stimulation of FAS. FAS promoter contains two tandem SREBP1 binding sites which are split by the –65/-60 E-box. SREBP1 binding to these sites was first shown to mediate sterol modulation of FAS expression (480). However, Kim et al (386) demonstrated that SREBP1c also binds to this E box region to activate FAS transcription. Moreover, SREBP1 levels were reduced dramatically during fasting and elevated upon re-feeding of a high carbohydrate diet. These data suggest that SREBP1 may mediate insulin stimulation of FAS by binding to the E box (-60/-65).

The cellular signaling pathway mediating insulin regulation of FAS has been explored. Wortmannin, a PI3-kinase inhibitor, has been shown to block insulin stimulation of FAS promoter (481). Moreover, ectopic expression of constitutive active p110 subunit of PI3-kinase increased insulin stimulation of FAS promoter, while overexpression of dominant

negative p85 subunit suppressed insulin stimulation of FAS transcription (481). In addition, overexpression of PKB/Akt, a down-stream signal of PI-3 kinase, stimulated FAS transcription in the absence of insulin (481). These data demonstrate that PI3-kinase and down-stream PKB/Akt signaling pathway mediates insulin stimulation of FAS.

- Glucose Stimulation of FAS and PUFA Inhibition of FAS

The metabolic relationship between glucose and insulin obscure their effects on FAS expression. They may exert effects on FAS dependently and independently. Glucose is definitely required for insulin stimulation of FAS transcription. Both USFs (479) and SREBP1 (482), which mediate insulin stimulation on FAS, also have been shown to mediate glucose induced FAS expression by binding to E-box region. These data indicate that glucose and insulin do share certain common pathways in regulating FAS expression. However, glucose also exerts the regulatory effect on FAS expression via distinct pathways. For example, a glucose response element, which is distinct from either USF or SREBP1 binding sites of E-box region, has been identified in the first intron of FAS gene (483). Moreover, glucose response element binding protein, a distinct transcriptional factor, has been identified to bind to this response element and mediate glucose induced FAS expression (483). In addition, glucose is also able to increase hepatic FAS mRNA stability to increase FAS mRNA levels independent of transcription (484, 485).

Glucose-6-phosphate and its metabolites rather than glucose itself may be the signal that mediates glucose regulation of FAS. 3-O-methylglucose, a glucose analog that can be

transported into cells but is unable to be physphorylated, exerts no effects on FAS expression (486). On the other hand, 2-Deoxyglucose, an unhydrolizable glucose analog that can be transported into cells and phosphorylated, mimics the effect of glucose on FAS expression (487).

PUFA has been shown to inhibit hepatic FAS expression by decreasing the mature form of SREBP1 protein. Moreover, PUFA inhibition of FAS expression is also mediated by decreasing SREBP1 expression (488). This was confirmed by in vivo study demonstrating that dietary PUFA suppresses SREBP1 expression, decreases SREBP1 mature protein, and inhibits FAS expression (489). Therefore, PUFA may antagonize insulin stimulation on FAS expression by inhibiting SREBP1.

- Other Hormone Response Elements in FAS Promoter

Glucagon antagonism of insulin stimulation on FAS expression is mediated by cAMP. This has been confirmed by in vitro studies. Analysis of promoter deletion constructs identified a cAMP response element spanning from –149 to +68 in FAS promoter. This region contains FAS IRE that is required for cAMP inhibition of FAS (489, 490). Further mutation analysis identified an inverted CCAAT box in the –99/-92 region of FAS promoter, which is also required for cAMP responsiveness, as mutation of this box blocked cAMP inhibitory effects on FAS expression (489, 490).

T_3 has been shown to stimulate FAS expression independent of insulin. Thyroid hormone (T_3) activated thyroid hormone receptor (TR), a member of family of nuclear hormone

receptor, interacts with RXR to form a heterodimer complex. This complex binds to consensus thyroid hormone response element (TRE), resulting in activation of FAS gene transcription. Two TREs mediating T_3 stimulation of FAS transcription have been identified in FAS promoter (491).

AII also stimulates FAS expression and activity in both 3T3-L1 adipocytes and primary cultured human adipocytes (451). This action is mediated by activation of SREBP1c and IRE (492).

Agouti protein has been demonstrated to stimulate adipocyte FAS activity and expression in a Ca^{2+}-dependent mechanism (121). This agouti stimulation of FAS is exerted at transcription level. The stimulatory effects of agouti and insulin on FAS expression are independent and additive. Further studies demonstrated an agouti/Ca^{2+} response element mediating agouti stimulation of FAS transcription in FAS promoter (127).

b. Regulation of Lipolysis in Adipocytes

Lipolysis plays a pivotal role in lipid metabolism and overall energy homeostasis as well as other cellular signaling events. Fatty acids stored in the form of TGs are main source of energy during nutritional deprivation, during which adipose tissue can release up to150 g of fatty acids daily through adipocyte lipolysis, contributing to more than half of the daily caloric supply (493). In addition, lipolysis-derived fatty acids and their derivatives also participate in various cellular events involved in cell signaling and membrane

biosynthesis in most cells. For instance, fatty acid derivatives are considered as endogenous ligands for PPARγ, a key regulator of adipogenesis.

1. Hormone-Sensitive Lipase in Adipocyte Lipolysis

Adipocyte lipolysis, a process of mobilization of free fatty acids (FFA) from TG, occurs through three consecutive reactions which are catalyzed by two enzymes, hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL). HSL catalyzes the rate-limiting step in the hydrolysis of TG to diacylglycerol (DAG), and also the subsequent hydrolysis of DAG to monoacylglycerol. HSL preferentially acts upon the 1- and 3-ester bond of its acylglycerol substrates to generate two FFAs and one monoacylglycerol per TG (493, 494). The conversion of monoacylglycrol to FFA and glycerol, the last step in lipolysis, is carried out by MGL. HSL is under acute hormonal and nutritional control, while there is no evidence for any regulation of MGL (493, 494). Therefore, HSL is a key enzyme in lipid metabolism and energy homeostasis.

The HSL cDNA was first cloned from rat adipocytes (495). This cDNA predicts 768 amino acids, with a molecular weight of 84 kDa. Li et al. (496) then cloned the mouse HSL gene which spans approximately 10.4 kb and comprises 9 exons interrupted by 8 introns. Wang et al. (497) further demonstrated that mouse HSL gene is located on chromosome 7 and this mouse locus is associated with obesity. Holm et al. used the rat clone to map the human HSL gene to 19cen-q13.3 by southern analysis of DNA from human-rodent somatic cell hybrids. This human HSL gene mapping was refined by other two studies that localize the HSL gene to 19q13.1-19q13.2 using fluorescence in situ

hybridization (498, 499). Like mouse HSL gene, the human HSL gene also comprises 9 exons, encoding 775 amino acids which show 82% and 85% identity with that of rats and mouse (500), respectively.

Northern blot analysis of HSL in animals have demonstrated that HSL is expressed in a variety of tissues, including white and brown adipose tissue, all steroidogenic tissues, muscle, pancreas, etc (501-505). Several distinct mRNA species exhibiting a tissue-specific distribution have been observed (493). This is attributed to the tissue-specific expression of exon 1, which is either non-coding in adipose tissue or encodes an N-terminal extension of HSL in testis and pancreas (507, 508), resulting in a larger mRNA and protein species in these tissue.

HSL protein consists of two major structure domains: an N-terminal domain and a C-terminal catalytic domain (493, 494). The catalytic domain harbors a catalytic core composed of Ser-423, Asp-703, His-733 and a regulatory motif with four phosphorylation sites: Ser-563, Ser-565, Ser-659, Ser-660 (493, 494, 508, 509). N-terminal domain is proposed to bind to lipid droplets and interact with other proteins, such as fatty acid binding protein (FABP) (510).

Adipocyte lipolysis is subject to acute neuronal and hormonal control. Catecholamines are considered as major stimulators of lipolysis, whereas insulin is believed to be the most important anti-lipolytic hormone. The increased sympathetic output increases the release of catecholamines which in turn enhance the cellular cAMP by binding to and

activating β-adrenergic receptors, resulting in activation of PKA. Two main targets for PKA-mediated phosphorylation in lipolysis are HSL and perilipins, and phosphorylation of these two proteins markedly increases adipocyte lipolysis (511, 512). Insulin, however, is the most important physiological inhibitor of catecholamine-induced lipolysis. Insulin binds to its receptor and activates the downstream PI3-kinase pathway, leading to activation of PKB/Akt. Activated PKB/Akt further phosphorylates and activates phosphodiesterase 3B, which catalyzes the degradation of cAMP, resulting in a decrease in cAMP level and concomitant decrease of PKA activity, and consequently, inhibition of lipolysis (513, 514). This was confirmed by demonstration that specific inhibition of PDE3B completely blocks the anti-lipolytic effect of insulin (515).

HSL is regulated by reversible protein phosphorylation. Phosphopeptide mapping and amino acid sequencing analysis demonstrated that HSL is phosphorylated at two distinct serine residues, named regulatory and basal sites (512), respectively. Phosphorylation of the regulatory site (Ser-563 in rats and Ser-551 in human) by PKA activates HSL activity (511, 512). However, recent evidence showed that mutation of this regulatory site Ser-563 failed to prevent PKA-induced activation of HSL, raising the possibility that phosphorylation of other regulatory sites might be involved in activation of HSL as well. Indeed, recent studies have identified two novel PKA-mediated phosphorylation sites, Ser-659 and Ser-660, whose phosphorylation is responsible for activation of HSL in response to isoproterenol stimulation in primary cultured adipocytes (516). Further site-directed mutagenesis study demonstrated the critical roles for these two phosphorylation sites in regulation of HSL activity.

In addition to three regulatory sites phophorylated by PKA, HSL is also phosphorylated at basal site Ser-565 (rat), two residues away to the regulatory site Ser-563, by glycogen synthase kinase-4, Ca^{2+}/calmodulin-dependent kinase II, and AMP-activated protein kinase (AMPK) (517, 518). The basal site is usually phosphorylated in quiescent cells. Phosphorylation of the basal site Ser-565 by AMPK prevents the subsequent phosphorylation of regulatory site Ser-563 (519), indicating that phosphorylation of the basal site exerts an anti-lipolytic effect. This was confirmed by evidence that treatment with AICAR, a stimulator of AMPK, inhibited the catecholamine-stimulated lipolysis in primary cultured adipocytes (520).

Phosphorylated HSL undergoes regulation of dephosphorylation exerted by several protein phosphatases, including PP2A, PP2C, and PP1 (521, 522). PP2A and PP2C exhibit higher activity on dephosphorylation of both regulatory sites and basal site than PP1 does. PP1, however, may selectively act on the basal site (521).

Upon lipolytic stimulation, activation of HSL by PKA-mediated phosphorylation triggers the translocation of HSL from cytoplasmic compartment, where HSL resides in quiescent state, to the surface of the lipid droplets (523-525). Recent evidence demonstrated that phosphorylation of HSL is required for HSL to translocate and interact with lipid droplets, as mutation of regulatory phosphorylation sites at Ser-659 and Ser-660 blocks this translocation (493).

Ectopic expression of HSL increases lipolysis and reduces TG accumulation in adipocytes (526). However, HSL-knockout mice exhibit normal body weight, fat mass, and cold sensitivity (527). The white adipocytes remain 40% lipase activity. The only significant phenotypic abnormality is that HSL-deficient male mice are sterile. Although gonadal function is normal in these mice, the neutral cholesterol ester hydrolase (NCEH) activity is completely lost in the testis.

Perilipins, a family of proteins located on the surface of lipid droplets, preclude HSL binding to lipid droplets in resting cells (528). Perilipin is a single copy gene that produces three isoforms by alternative splicing. Perilipin A is the most abundant species in both adipocytes and steroidogenic cells, while the B and C isoforms reside primarily in adipocytes and steroidogenic cells, respectively. Perilipin A and B contain a common N-terminal but distinct C terminal region (528). Significant amounts of the perilipin proteins are found only on the TG-rich droplets in adipocytes and cholesterol ester-rich droplets in steroidogenic cells (528). It has been proposed that non-phosphorylated perilipins create a protective barrier to prevent HSL interaction with lipid droplets (493). Upon lipolytic stimulation, phosphorylation of perilipins by PKA causes redistribution of perilipins in lipid surface and removes these restraints, which allows HSL to interact with lipid, leading to initiation of TG hydrolysis (493). Perilipin-deficient mice exhibit hyperphagia, reduced fat mass and increased muscle mass (529). They also show elevated basal lipolysis that is resistant to β-adrenergic agonist stimulation. Moreover, these knockout mice are resistant to diet-induced obesity. In addition, breeding the perilipin$^{-/-}$ alleles into db/db mice reverses the obesity phenotype by increasing the metabolic rate of the mice.

Two proteins, ALBP (510, 528) and lipotransin (530), have recently been shown to interact with HSL at N-terminus. ALBP may function to facilitate rapid evacuation of lipolytic product such as free fatty acids. Lipotransin is a novel HSL-interacting protein that appears to translocate HSL to the lipid droplets (530). The interaction between the two depends on the phosphorylation of HSL. Therefore, lipotransin is an HSL-docking protein that may direct the hormonally regulated redistribution of HSL (530).

2. Nutritional and Hormonal Regulation of HSL

2.1 Nutritional Regulation of HSL

Prolonged fasting in rats increases adipocyte HSL mRNA, proteins, and activity, thereby resulting in increased basal lipolysis rate (531). Long-chain fatty acid CoA and intermediary metabolites exert inhibitory effects on HSL activity and lipolysis (532), while FABP blocks these inhibitory effects by removing these fatty acids away from HSL (510). The ketone bodies derived from fasting, vigorous exercise, and diabetes complications, potently inhibit HSL activity and lipolysis (533).

2.2 Hormonal Regulation of HSL

- Lipolytic Factors

Catecholamines are primary stimulators of adipocyte lipolysis. They exert the stimulatory effects via four adrenergic receptors: β1, β2, β3, and α2, which belong to superfamily of G-protein-coupled receptors with 7-ransmembrane domain (534). Activation of β receptors activates adenylyl cyclase mediated by $G_{\alpha s}$ protein, resulting in increases in cAMP levels and HSL activity, while activation of α2 receptors inhibits adenylyl cyclase

via $G_{\alpha i}$ protein, reducing cAMP levels and HSL activity. The physiological significance of existence of 4 adrenergic receptors in adipocytes is not clear. It has been suggested that β receptors play major roles in stimulation of lipolysis in stress, while α receptors predominate in regulation of lipolysis in resting state and provide the opportunity for dual regulation of adenylyl cyclase (535). Each β receptor subtype exhibits different expression pattern in adipocytes. Differentiation of 3T3-F442A cells up-regulates β3 expression, but not β2 and β3 expression (536). cAMP increases β3 expression, but suppresses β2 and β3 expression. Insulin and dexamethasone also exerts different effects on expression of different β receptor subtype (537). Each β receptor subtype also serves as different signaling role in adipocyte lipolysis. β1 receptors, which are more sensitive to catecholamines and desensitize rapidly, may mediate acute stimulation of low doses of catecholamine, while β3 receptor, which are activated by higher doses of catecholamines and resistant to desensitization, may deliver sustained signal of long-term catecholamine stimulation (534).

Other lipolytic hormones include growth hormone, glucagon, and glucocorticoids. Growth hormone and glucagon exert lipolytic effects by stimulating acute HSL enzymatic activity only, while glucocorticoids increase lipolysis by stimulating both HSL activity and expression (538).

TNF-α potently stimulates lipolysis by inhibiting perilipin expression. This was confirmed by demonstration that over-expression of perilipin blocks TNF-α stimulation of lipolysis (539).

- Anti-lipolytic Factors

In addition to insulin which is the most important physiological anti-lipolytic hormone, adenosine is also a potent physiological inhibitor of lipolysis. Intracellular cAMP is exported from cytosol to extracellular matrix and then converted to adenosine by phosphodiesterase and 5'-nucleotidase. Adenosine in turn acts on A1 adenosine receptor in an autocrine/paracrine manner (540). Thus, adenosine exerts the inhibitory effect on HSL through activation of A1 adenosine receptor, which inhibits adenylyl cyclase via coupling $G_{\alpha i}$, reducing cAMP levels (534). A1 adenosine receptor and coupling efficiency may influence lipolysis and obesity. The receptors are down-regulated in adipose tissue of obese humans (541). Moreover, the sensitivity of A1 receptor-Gi protein to GTP is 5- to 10-fold higher in adipocyte membrane from obese Zucker rats compared to lean animals (542).

Prostaglandins also exert effect on lipolysis. PGE_2 has been demonstrated to inhibit lipolysis in cultured rat adipose tissue. In contrast, PGI_2 has been shown to stimulate lipolysis in rat adipocytes (543).

2.3 Lipolysis in Obesity and Type II Diabetes

Adipocyte lipolysis is altered in obese humans. Although adipocyte lipolytic rate measured by FFAs and glycerol release or turnover in vitro or in vivo is increased in obese humans compared to lean subjects, this is attributed to the increased adipocyte size and total fat mass (544, 545). However, obese humans exhibit resistance to the stimulatory effect of catecholamine on lipolysis (546). This could be attributed to the decreased number of β-adrenergic receptors, increased $\alpha 2$-adrenergic receptors, changes in the functional balance between β- and $\alpha 2$-adrenergic receptors, or postreceptor alteration (493, 546, 547). Hellstrom et al (548) reported that impaired lipolytic action of catecholamines mainly results from reduced HSL activity without changes of HSL mRNA levels, suggesting an involvement of posttranscriptional modulation of HSL or postreceptor alteration. However, recent studies showed that catecholamine-stimulated lipolytic capacity in human adipocytes obtained from different individuals is well correlated with HSL mRNA, protein content, and activity (549), suggesting that both transcriptional and posttranscriptional mechanisms appear to be involved in determining the catecholamine stimulation of HSL activity. This was further demonstrated to be relevant to obesity, as HSL mRNA, protein, and activity as well as lipolytic rate were decreased in subcutaneous adipose tissue obtained from obese humans compared to lean controls (550).

Since elevated circulating FFAs play important role in insulin resistance/type II diabetes, regulation of HSL may contribute to the development of this metabolic disorder. Adipose tissue obtained from diabetic subjects exhibits resistance to lipolytic effects of

catecholamines, due to a decrease in β-adrenergic receptor or an increase in α2-adrenergic receptor numbers (551). On the other hand, resistance to anti-lipolytic effects of insulin may also occur in these patients. Upper-body obesity exhibits greater resistance to the anti-lipolytic effects of insulin and to the lipolytic effects of catecholamines compared to lower-body obesity, and the net lipolytic rate and basal FFA release in upper-body obese subjects are higher than those in lower-body obesity (552, 553). This is consistent with the observation that upper-body obesity is strongly correlated with insulin resistance/type II diabetes and other metabolic disorders (554). This insulin resistance-prone obesity can be further extended to those lipolytically active fat depots that have strong association with insulin resistance. For example, internal visceral fat depots with potent lipolytic capacity due to abundant expression of β-adrenergic receptors are strongly correlated with insulin resistance (555, 556).

The role of HSL in obesity and diabetes has also been documented in genetic studies in human population. Klannemark et al. demonstrated that a polymorphic marker in intron 7 of the human HSL gene is associated with abdominal obesity and type II diabetes (557). Moreover, a similar association was observed on another polymorphism marker of HSL gene with type II diabetes. In addition, transmission disequilibrium tests suggested linkage disequilibrium between the HSL gene marker and another allele or gene that increases susceptibility to abdominal obesity and type II diabetes (558).

V. ROLE OF THERMOGENESIS IN OBESITY

A. ADAPTIVE THERMOGENESIS AND RESPIRATORY UNCOUPLING

a. Adaptive Thermogenesis

Obesity is a disorder of energy homeostasis due to a chronic imbalance between energy intake and energy expenditure. Energy expenditure can be divided into three principle components: obligatory energy expenditure required for cells and organs to maintain normal physiological functions, energy expenditure caused by physical activity, and energy expenditure resulting from adaptive thermogenesis (559). Adaptive thermogenesis is defined as energy dissipated as a heat instead of being captured in ATP in response to environment temperature and diet, serving to protect organism from cold exposure or regulate energy balance after food intake.

Acute cold exposure dramatically increases oxygen consumption and metabolic rate through muscular shivering involving the synthesis and breakdown of ATP in animals. However, with the time extended to chronic cold exposure, shivering ceases and the continued high metabolism still occurs through non-shivering adaptive thermogenesis in brown adipose tissue in rodents (560). Although the effect is smaller, lowering temperature by 5 ^{o}C also causes a 7% increase in heat production in humans (561). Cold exposure-induced adaptive thermogenesis is mediated by the sympathetic nervous system. Administration of the β-adrenergic agonists to animals causes increases in thermogenesis comparable to exposure to cold, while treatment with the antagonists of the sympathetic nervous system makes animals sensitive to cold and unable to maintain body temperature (562). Moreover, mice lacking adrenaline and noradrenaline, the main

effectors of the sympathetic nervous system, as a result of dopamine β-hydroxylase gene knockout are cold sensitive (563). The central control of adaptive thermogenesis is also via hypothalamic-pituitary-thyroid axis. Thyroid hormones can either independently stimulate thermogenesis or interact with sympathetic nervous system to increase energy expenditure (564). On the other hand, diet also plays an important role in regulation of adaptive thermogenesis (559). Fasting and caloric restriction decrease metabolic rate and energy expenditure, while feeding exerts both acute and chronic stimulatory effects on metabolic rate and energy expenditure (565). As human overeats, a protective thermogenic adaptation, termed nonexercise activity thermogenesis (NEAT), dissipates excess energy to preserve leanness (566). Notably, NEAT also bears genetic background and failure to activate NEAT due to genetic susceptibility may result in obesity (566).

b. Respiratory Uncoupling

Adaptive thermogenesis is mediated by proton leak-induced respiratory uncoupling. In normal physiology of mitochondrial respiration, the mitochondrial oxidation of substrates results in reduced NADH and $FADH_2$, which donate their electrons to the electron transport chain in the mitochondrial inner membrane, where oxidative phosphorylation occurs. The chemiosmotic hypothesis proposes that electron transport along the electron transport chain is accompanied by pumping of protons into the mitochondrial intramembrane matrix, generating a proton electrochemical potential gradient ($\Delta\mu_H^+$) across the mitochondrial inner membrane. This force drives protons back to mitochondrial matrix via ATP synthase (F_0F_1-ATPas) in a reaction leading to synthesis of ATP from ADP (567). The oxidative phosphorylation is tightly regulated by the

availability of ADP, the by-product of ATP consumption. Once cells become inactive and reduce the ATP consumption, ADP levels correspondingly decrease. Subsequently, protons are unable to re-enter the matrix in the absence of ADP through ATP synthase. Electron transport along the respiratory chain continues to increase proton gradients, which in turn inhibits further substrate (NADH and $FADH_2$) oxidation (567). As a result, substrate oxidation is tightly coupled to energy needs by linking respiration to ADP availability.

However, even when ADP is not available, isolated mitochondrial still consume oxygen, a process called state 4 respiration (568). This suggests that protons leak across the inner membrane of mitochondria in the absence of ADP. Consequently, the coupling of oxidative phosphorylation is impaired by this proton leaking, leading to respiratory uncoupling. In fact, proton leaks account for a surprisingly high proportion of cellular metabolic rate, with 25% and 52% of the resting respiration rate of isolated rat hepatocytes and perfused rat skeletal muscle preparation (569-571). It has been estimated that overall proton leak contributes to 20% of standard metabolic rate (SMR), making it the largest single contributor to mammalian SMR; SMR constitutes 60-70% of total energy expenditure (572, 573).

A major consequence of the respiratory uncoupling is the activation of substrate oxidation and dissipation of oxidation energy as heat. This is important in mammalian energy balance and body weight regulation and represents research targets for obesity. Uncoupling of respiration and dissipation of energy as heat are commonly observed in

adaptive thermogenesis in rodents exposed to the cold, which is mediated by uncoupling protein 1 (UCP1) via catalyzing proton leaking in rodent brown adipose tissue.

B. UNCOUPLING PROTEIN FAMILY

a. Role of UCP1 in Thermogenesis and Energy Expenditure

1. UCP1-Mediated Proton Transport

Nicholls first demonstrated that mitochondria isolated from brown adipose tissue exhibited increased proton leak (574, 575). This proton leak was stimulated by fatty acid and inhibited by purine nucleoside di- and tri-phosphate. Using photolabelling with a radioactive purine nucleotide, a 32-kDa protein with high affinity purine nucleotide binding sites was identified. This protein, named UCP (changed to UCP1 later), was further purified and sequenced (576, 577). The mouse and rat UCP1 gene were also cloned and the mouse UCP1 was further mapped to chromosome 8 (578-580). Bouillaud et al. cloned human UCP1 which spans 13 kb with a 9 kb transcription region containing 6 exons and exhibits 79% and 80% identity with mouse and rat UCP1, respectively (581). Human UCP1 was mapped to 4q31 (582). UCP1 is expressed exclusively in mammalian brown adipose tissue, an important site of thermogenesis in rodents and neonatal humans.

UCP1 gene encodes a 306 amino acid protein which contains 6 transmembrane α-helices (583). Functional assays, immunodetection and purification studies have confirmed that functional UCP1 exists as a dimeric protein in the mitochondrial inner membrane (584). Over-expressing UCP1 in reconstituted liposomes, bacteria, yeasts, adipocytes, and skeletal myocytes resulted in proton leak and respiratory uncoupling, while brown

adipose tissue mitochondria from mice lacking UCP1 were unable to show proton leak (585), confirming the proton transport function of UCP1. UCP1-mediated proton transport is fully dependent on free fatty acids, which are considered as cofactors for this action. Proton transport of UCP1 has been shown to be activated by free fatty acids with 10 or more carbons and by saturated as well as unsaturated fatty acids, but not by esterified carboxy groups, such as acyl CoA (586-588). The mechanism whereby free fatty acids stimulate UCP1 proton transport is still not clear. However, two hypotheses have been proposed. The first model, termed the UCP1 channel model, proposes that fatty acids function as buffering cofactors, donating H^+ to the tanslocation channel formed by UCP1 with a pore-channel domain and a gating domain (567, 583, 589). The second model, known as fatty acid protonophore cycling (or flip-flop) model, suggests that UCP1 catalyzes the flip-flop transport of the protonated fatty acid head to the inner mitochondrial membrane, releasing the H^+ in the inner mitochondrial matrix, and then drives the transport of the negatively charged free fatty acid head back to the outside of mitochondrial membrane. This UCP1 action enables fatty acids to behave as cycling protonophores (567, 583, 590). In addition, coenzyme Q (CoQ) has been recently identified as a cofactor for the proton transport of UCP1. On addition of CoQ to reconstituted UCP1 from *E. Coli* inclusion bodies, fatty acid-dependent proton transport reached the same rate as with native UCP1 (591).

The proton transport activity of UCP1 is inhibited by purine nucleoside di- and tri-phosphate (GTP, GDP, ATP, ADP), but not by monophosphate (583, 584). UCP1 bears specific binding sites for these purine nucleotides, with the highest binding affinity to

GDP (592). Site-directed mutagenesis of arginine residues at 83, 182, 276 in transmembrane α-helices abolished the nucleotide binding and inhibition of UCP1 proton transport. Moreover, the binding of nucleotides to UCP1 is highly sensitive to pH. When the pH rises, the affinity of UCP1 for nucleotides declines. The glutamate residue at 190 has been demonstrated to be essential for the pH-sensitive nucleotide binding (583, 593). Further, mutagenesis of histidine residues in UCP1 indicated that a pair of histidine (His-145 and His-147) in the matrix loop is essential for proton transport (594, 595).

2. Regulation of UCP1 Activity and Expression

2.1 Physiological Regulation of UCP1 in vivo

Both acute and chronic cold exposure stimulates UCP1-mediated proton transport and thermogenesis. Acute cold exposure stimulates UCP1 activity in proton transport, while chronic cold exposure results in increases in UCP1 expression, mitochondrial biogenesis, and brown adipocyte hyperplasia and hypertrophy (596, 597). Sympathetic nerves are abundantly distributed in brown adipose tissue. Acute cold exposure stimulates sympathetic output and thereby increases adrenaline and noradrenaline release, resulting in activation of β-adrenergic receptor and cAMP-PKA-dependent pathway (598). The PKA-stimulated lipolysis causes an increase in the cytosolic free fatty acids, which serve to directly stimulate UCP1 proton transport and supply fuels for mitochondrial respiration. This is supported by evidence that addition of free fatty acids to brown adipocytes mimics the stimulatory effects of β-adrenergic stimulation of UCP1 proton transport (599). Moreover, norepinephrine and free fatty acids stimulation of proton leaks is absent in brown adipocytes from UCP1 knockout mice (600). These data indicate that

free fatty acids secondary to stimulated lipolysis mediate cold stress stimulation of UCP1 proton transport. On the other hand, chronic cold exposure up-regulates UCP1 transcription by increasing PPARγ coactivator-1 (PGC1) expression mediated by activation of β-adrenergic receptor and the cAMP-PKA-dependent pathway (601, 602). Cold stress activation of this pathway may also systematically stimulate mitochondrial biogenesis, and brown adipocyte hyperplasia and hypertrophy, resulting in increases in overall UCP1 protein content. Moreover, free fatty acids secondary to stimulated lipolysis serve as ligands for PPARγ to stimulate UCP1 expression. Regardless of acute or chronic clod stress, β_3-adrenergic receptor plays a key role in mediating the signaling pathways in thermogenesis. This receptor is expressed abundantly and predominantly in brown adipocytes and rodent white adipocytes. Appropriate selective agonists for the β_3-adrenergic receptor may play a role in modulating thermogenesis and energy balance (603).

Thyroid hormone T_3 also exerts the effect on cold exposure stimulation of thermogenesis. Type II iodothyronine 5' deiodinase, an enzyme which converts T_4 to T_3, is activated by cold-mediated β-adrenergic stimulation, leading to an increase in most potent thyroid hormone T_3. T_3 increases free fatty acids by stimulating lipolysis, and thereby acutely stimulates activity of UCP1 proton transport. Moreover, T_3 independently stimulate UCP1 transcription or synergize with β-adrenergenic system to increase UCP1 expression (564, 604, 605).

Nutritional status also regulates UCP1 activity and expression. This effect is at least partially mediated by leptin. Fasting reduces leptin expression, which in turn inhibits sympathetic activity and decreases UCP1 expression. Moreover, administration of leptin stimulates sympathetic outflow and increases expression (606, 607).

2.2 Transcriptional Control of UCP1 Expression

The molecular mechanisms mediating UCP1 gene transcription have been extensively explored. Studies have been conducted to address the mechanisms of specific expression of UCP1 in brown adipose tissue and identification of cis-acting elements in promoter regions and transcriptional factors involved in regulation of UCP1 transcription in response to physiological status. A 221-bp enhancer element, located 2.4 kb upstream of the mouse and rat UCP1 transcription start site, has been identified to be essential for brown fat-selective expression and full response to β-adrenergenic stimulation (via cAMP) (608, 609). This complex enhancer element also contains putative binding sites for the thyroid hormone receptor, retinoic acid receptor (RAR), AP-1, and PPARγ. The response elements for these transcriptional factors were identified to be functionally effective in regulating UCP1 transcription. Ligand-activated PPARγ, thyroid hormone receptor (TR), and RAR interact with RXR to form heterodimers which further act upon distinct response elements to stimulate UCP1 transcription (559, 605, 610-614). This underlies the molecular mechanisms by which cold stress, β-adrenergic agonists, PPARγ agonist thiazolidinediones, retinoid acids, and thyroid hormones up-regulate UCP1 expression *in vivo* and *in vitro*.

Although this 221-bp enhancer element is sufficient to induce specific expression of UCP1 in brown adipose tissue (608), the brown-fat selective transcriptional factors that drive this specific expression have not been identified. The PPARγ binding to the enhancer element is necessary for functional activation of this element and UCP1 transcription. Moreover, PPARγ is not brown-fat specific transcriptional factor and it also exerts a crucial effect on white adipocyte adipogenesis, indicating that this transcriptional factor simultaneously plays a role in energy storage in white adipocytes and energy dissipation in brown adipocytes. Therefore, other cofactor(s) could interact with PPARγ and therefore affect its physiological role in different cell types. The identification of a PPARγ coactivator (PGC-1) provides an explanation to this paradoxical role of PPARγ. PGC-1 was cloned using a yeast two-hybrid screening with PPARγ as bait in a brown adipose tissue cDNA library (601). PGC-1, a 90 kDa protein, is abundantly expressed in brown fat and skeletal muscle, two major sites of adaptive thermogenesis, but not in white adipose tissue (601). Its connection to adaptive thermogenesis was well demonstrated by its rapid and marked induction in brown fat and skeletal muscle upon cold exposure of mice (601 602). This cold induction of PGC-1 results from sympathetic nervous system outflow, as this effect can be mimicked by β-adrenergic stimulation (601, 602). PGC-1 also interacts with other nuclear hormone receptors, such as RAR and TR, which along with PPARγ exert stimulatory effects on UCP1 transcription.

The expression of PGC-1 in cell types other than brown adipocytes also initiates multiple adaptive thermogenic programs that make these cells perform thermogenic function as brown adipocytes do. Ectopic expression of PGC-1 in white adipocytes increases UCP1

expression and mitochondrial biogenesis (601), suggesting that PGC-1 may play a role in determining the phenotypic development of adipocyte lineages to become a brown or white adipocyte. When over-expressed in skeletal muscle, PGC-1 induces UCP2 expression and mitochondrial biogenesis (602).

In addition to interaction to PPARγ, PGC-1 is also involved in regulation of whole mitochondrial biogenetic system, such as nuclear respiratory factors (NRF) and mitochondrial transcription factor (mtTF). NRF are key factors that activate the gene expression of mitochondrial electron transport system, including cytochrome c and ATPase synthase (559). NRFs also promote the transcription of mtTF, which is expressed in nucleus and translocats to mitochondria and promotes mitochondrial gene expression (559). Cold stress stimulation of PGC-1 expression promotes NRF expression. Moreover, PGC-1 interacts and co-activates NRFs to stimulate mtTF expression (602). The increased expression of NRFs and mtTF may further promote the gene expression of whole mitochondrial biogenetic system and thereby stimulate adaptive thermogenesis.

Recent studies demonstrated that PGC-1 exerts effects in regulation of energy metabolism other than thermogenesis. PGC-1 also mediates hepatic gluconeogenesis by promoting transcription of gluconeugenic enzymes, such as phosphoenopyruvate carboxykinase and glucose-6-phosphatase (615, 616). This effect is mediated by cAMP-PKA stimulation of PGC-1 expression, the same signaling pathway as PGC-1 mediated thermogenesis, indicating a role of PGC-1 in cell type-selective coactivation of gene expression (616, 617).

In addition to transcriptional regulation mediated by β-adrenergic stimulation (cAMP), PGC-1 is also regulated at translational level (617). All nuclear-encoded mRNAs contain a 5' cap structure that is recognized by the eukaryotic translation initiation factor 4E (eIF4E) subunit of the eIF4E complex. The eIF4E-binding proteins (4E-BP), however, reversibly repress cap-dependent translation by binding to eIF4E, thus preventing the formation of the eIF4E complex. Recent reports demonstrated that disruption of mouse translational inhibitor 4E-BP1 gene markedly increases PGC-1 protein translation and UCP1 mRNA expression in white adipose tissue, and causes a corresponding increase in metabolic rate and a reduction in white fat mass (617). These finding demonstrate that 4E-BP1 is a novel thermogenic regulator that is involved in functional switch between white fat and brown fat. However, it is not known that 4E-BP1 exerts this effect at adipogenic level or mature adipocyte levels involved in PGC-1 translation and UCP1 expression.

3. The Role of UCP1 in Energy Expenditure and Obesity

The role of UCP1 in energy balance and obesity was first demonstrated in genetic obese mouse models, such as leptin-deficient ob/ob and leptin receptor-deficient db/db mouse, which exhibit reduced sympathetic outflow, decreased UCP1 expression and brown fat thermogenesis, and decreased energy expenditure (618, 619), while administration of leptin to ob/ob mice stimulates sympathetic activity, increases UCP1 expression and energy expenditure (606, 607, 620-621). Transgenic and knockout animal models further demonstrated the role of UCP1 in energy balance and obesity. Transgenic mice with ablation of brown fat as a result of over-expression of diphtheria toxin gene under control

of the UCP1 promoter exhibit decreased energy expenditure and the obese phenotype (622). However, UCP 1 knockout mice exhibit reduced cold sensitivity and thermogenic response to β-adrenergic stimulation, but do not develop obesity (623), suggesting either that UCP1 is not essential for the control of body weight or that its absence can be compensated by other dissipating pathways, such as compensatory up-regulation of UCP2. In contrast, over-expressing UCP 1 in white adipose tissue under control of aP2 promoter attenuated diet-induced obesity in mice (624). It is noteworthy that over-expression of UCP1 in white adipocytes not only dissipates stored energy but also affects energy partitioning, as adipocytes from these transgenic animals exhibit not only decreases in mitochondrial potential but also in lipogenesis (625). Consistent with this, over-expressing UCP1 specifically in skeletal muscle under control of myosin light chain promoter decreased skeletal muscle mitochondrial potential, increased oxygen consumption, increased energy expenditure, and prevented diet-induced obesity and insulin resistance in transgenic mice (626, 627).

Although the role of UCP1 in energy balance and obesity is quite convincing, unfortunately, the absence of readily detectable brown fat depots in adults have led to precluding this mode of energy expenditure as an applicable target to human obesity. In fact, brown fat is abundant and active in neonatal humans. It seemingly loses its importance with age, probably as a consequence of the control of ambient temperature. Nonetheless, brown adipose tissue does exist in adult humans to a variable extent (605). It has been reported that normal human white fat depots contain small islands of brown fat and the ratio of brown to white adipocytes is approximately 1/1000 (301, 628, 629).

Indeed, UCP1 mRNA is detectable in white-fat depot in adult human using sensitive RT-PCR (628, 630). Moreover, it is inducible in certain circumstances. There is a marked increase in UCP1 expression in individuals who have undergo prolonged exposure to cold stress, such as working in low temperatures and immersion in cold water (631, 632); this induced UCP1 abundance is, in some cases, as high as seen in cold-exposed rodents (631). Moreover, patients with pheochromocytoma, a tumor secreting catecholamines, develop large and active brown fat depots evidenced by morphological appearance and UCP1 expression (633). These observations support that brown adipocytes do exist in human adult fat depots and may represent a potentially important target for physiological and pharmacological intervention. The interest of pharmacological research has focused on agonists for β_3-adrenergic receptors, which are predominantly expressed in rodent brown and white fat, but at low levels in human white fat. However, these drugs often prove to have undesirable side-effects in the cardiovascular system and other tissues associated with agonist activity at β_1- and β_2- adrenergic receptors, or have a poor bioavailability (634).

Human genetic studies show that a polymorphic Bcl I site resulting from an A/G mutation has been described in the 5'-flanking region of the UCP1 gene (635, 636). This polymorphism was reported to correlate significantly with the percentage of body fat gain over 12 year (635). This is consistent with the notion that marginal increase/decrease in energy expenditure due to UCP1 would influence body weight regulation over time rather than exert rapid effects as being a determinant of body weight. Moreover, this polymorphic site is associated with higher weight gain in morbidly obese subjects, and a

lower weight loss due to caloric restriction (637, 638). In addition, an additive effect of this UCP1 polymorphism and the Trp64 to Tyr mutation of the β_3-adrenergic receptor gene in obese humans has been reported (637).

b. UCP2 and UCP3: Potential Regulators of Energy Metabolism

1. Cloning of UCP1 Homologues with Sequence Similarity

Since the specific expression of UCP1 in brown fat limits its respiratory uncoupling system as an applicable target for human obesity, researchers started to pursue the UCP1 homologues. In fact, substantial mitochondrial proton leaks have been observed in tissues devoid of UCP1. Adult humans who possess little active brown fat exhibit thermogenesis in skeletal muscle in response to catecholamine administration (639). Moreover, proton leaks account for 25-52% of the mitochondrial oxygen consumption in isolated rat hepatocytes and perfused rat skeletal muscle preparation (569-571). These observations led to the hypothesis that certain components such as UCP1 homologues in mitochondrial inner membrane catalyze the proton leaks in these tissues.

UCP2 and UCP3, two homologues of UCP1, have been cloned (640-643). UCP2 is 56% identical to UCP1 and is expressed ubiquitously throughout body, with the highest level in white adipose tissue (640, 641). UCP3 is 57 and 73% identical to UCP1 and UCP2, respectively, with its expression confined to brown fat and skeletal muscle (642, 643). The human UCP3 gene gives rise to two mRNAs, a long form called UCP3L and a short form called UCP3S, by alternative splicing at a polyadenylation site in intron 6. The

UCP3S mRNA lacks the last coding exon, presumably generating a truncated form of UCP3 protein lacking the sixth transmembrane domain (644).

The UCP2 and UCP3 genes have been mapped to mouse chromosome 7, rat chromosome 1, and human chromosome 11. In fact, these two genes are adjacent and located within 100 kb of chromosome region; the UCP2 gene is only 7 kb and 8 kb downstream of the UCP3 gene in mouse and human, respectively, suggesting a duplication event during gene evolution. The genomic organization of UCP2 and UCP3 genes is similar to that of UCP1 gene, with the coding sequence over 6 exons. However, the UCP2 and UCP3 genes differ from the UCP1 gene by the presence of two or one untranslated exons located on the 5' side of UCP2 and UCP3 gene, respectively (584, 585).

Recently, two additional homologues of uncoupling proteins, UCP4 and BMCP1/UCP5, were cloned (645, 646). The deduced amino acid sequences are less similar to UCP1, UCP2, and UCP3, and they are expressed in the brain. Moreover, two plant uncoupling proteins, termed StUCP in *Solanum tuberosum* and AtUCP in *Arabidopsis thaliana*, were also cloned (647-649).

All these uncoupling protein identified so far belong to a large family of inner mitochondrial membrane proteins which share a number of features (567). Each protein monomer contains approximately 300 amino acids which form a tripartite structure with three 100-amino acid repeats and each repeat possessing a mitochondrial carrier signature motif. Each functional mitochondrial carrier protein exists as homodimer and each

monomer consists of six transmembrane domains (567). The uncoupling proteins, however, exhibit higher sequence similarity to each other than to other members of the mitochondrial carrier family (567).

2. Proton Transport Activity of UCP2 and UCP3

The high structural and sequence similarity of UCP2 and 3 to UCP1 suggest that these two proteins may possess similar proton transport function. Detailed analysis of amino acid sequences indicates that UCP2 and UCP3 do contain a number of functional residues, such as Arg83, Arg182, Arg276 (650), Glu190 (593), and His214 (651), which are crucial for purine nucleotide regulation of UCP1 uncoupling activity, suggesting that purine nucleotide binding and PH sensitivity are preserved in UCP2 and UCP3. A histidine pair (His-145 and His-147) in the matrix loop of UCP1 is essential for its proton transport activity (594, 595). Unlike UCP1, UCP2 lacks both of them and UCP3 lacks His145 (594), suggesting that UCP2 and 3 may have no or reduced proton transport function, or that they use a distinct mechanism mediating proton transport. However, mitochondria from yeast over-expressing UCP1 that lacks this histidine pair exhibit normal function of proton transport (652), demonstrating that absence of this histidine pair in UCP2 and 3 may not rule out the possibility of being a functional uncoupling protein.

Functional analysis of UCP2 and UCP3 uncoupling activity demonstrated that these two proteins are able to decrease the mitochondrial potential in transformed yeast and transfected mouse myoblasts (640, 641, 652, 653). Expressing UCP2 in yeast causes a

50% increase in state 4 respiration (640), indicating that this protein may act on proton leak. Similarly, over-expressing UCP3 in yeast increases oxygen consumption comparable to that observed in UCP1 (652, 653). This was subsequently confirmed by demonstrating that UCP2 and UCP3 expressed in *Escherichia Coli* inclusion body and then reconstituted into liposomes are able to catalyze electrophoretic proton flux (654). Like UCP1, UCP2 and UCP3 require fatty acid to mediate this proton transport activity (654). Consistent with these findings, a recent study demonstrated that UCP2 and UCP3 reconstituted into vesicles is a highly active proton transporter (655). The UCP2 and UCP3- mediated proton transport activity requires fatty acid and coenzyme Q, an obligatory cofactor for proton transport by UCP1 (656). Moreover, this proton transport process is highly sensitive to purine nucleotides (655), although the others reported that UCP2 and UCP3 are not sensitive to purine nucleotide inhibition and exhibit much greater inhibition constant values compared to UCP1 (654). Therefore, all these proton transport properties for UCP2 and UCP3 resemble those for UCP1.

3. Regulation of UCP2 and UCP3 Expression

3.1 Physiological Regulation of UCP2 and UCP3 expression

Various physiological states and many nutritional and hormonal factors have been shown to regulate UCP2 and UCP3 expression. Cold exposure only modestly up-regulates UCP2 in brown fat (657) and UCP3 expression in brown fat and skeletal muscle (658, 659). High fat diet feeding causes increases in UCP2 and UCP3 expression (640, 659). Fasting and total caloric restriction, however, inhibit UCP3, but not UCP2 expression, in brown fat, and increase UCP2 and UCP3 expression in skeletal muscle (567, 584, 585).

Increased circulating levels of fatty acids are closely associated with up-regulation of UCP2 and UCP3 expression (660-663), suggesting that the regulation of UCP2 and 3 expression may be influenced by the fatty acid utilization. Administration of thermogenic hormones such as thyroid hormone (T_3) and leptin to rodents increases UCP2 and UCP3 expression (567, 659, 664-671). Moreover, PPARγ agonist (thiazolidinediones) and retinoic acids are potent stimulators of UCP2 and UCP3 expression (567,584, 585, 668, 672-676). In addition, free fatty acids, potential endogenous ligands for PPARγ, and β-adrenergic agonists, are also able to induce UCP2 and UCP3 expression in vitro and in vivo (567, 584, 585, 668, 677-680).

3.2 Transcriptional Control of UCP2 and UCP3 Expression

Unlike the UCP1 gene, the UCP2 gene promoter regions lack TATA and CAAT boxes (584, 585). Functional characterization of UCP2 promoter region has demonstrated several potent cis-acting regulatory elements including 2 PPARγ response elements (PPRE), 3 cAMP response elements (CREB-1), 2 putative thyroid hormone response elements (TRE), and MyoD and glucocorticoid receptor binding sites (681-683). These responsive elements could provide mechanisms of the positive regulation of UCP2 expression via PPARγ agonists, fatty acids (ligands of PPARγ), thyroid hormones, β-adrenergic agonists (isoproterenol). However, little is known regarding negative regulatory factors of UCP2 expression. We demonstrated that $1\alpha,25\text{-}(OH)_2\text{-}D_3$ exerts an inhibitory effect on UCP2 expression. The mechanism of this nVDR mediated inhibition of UCP2 is not known. However, the human UCP2 promoter region does contain several cis-acting negative regulatory elements, which strongly repress promoter activity (681),

although it is not clear whether nVDR acts on one of these silencers. Alternatively, nVDR may also compete with other positive transcriptional factors containing similar DNA binding domains on the responsive element binding or similar protein-protein interaction domain (such as PPARγ) on heterodimerization with the same transcriptional factor (RXR).

UCP3 gene contains a TATA box in the promoter region (584). Characterization of UCP3 promoter region has identified several cis-acting response elements, including the E-box, MyoD, PPARγ, RAR, and thyroid hormone receptor (TR) binding sites (584, 585). This is consistent with the up-regulation of UCP3 gene expression by PPARγ agonist TZDs, free fatty acids, T_3, and retinoid (659, 662, 663, 668, 669, 679, 684).

4. Physiological Functions of UCP2 and UCP3

Since UCP2 and UCP3 exhibit high sequence similarity and similar uncoupling activity with UCP1, it is tempting to speculate that these two homologues may play similar roles in regulating adaptive thermogenesis and energy expenditure. However, accumulating evidence indicates that the physiological role of UCP2 and UCP3 is more complicated than that of UCP1. UCP2 and UCP3 in different tissues and physiological states may exert various functions, even though they assume the same proton transport and uncoupling activity. However, the true physiological role of these two proteins is still not clear. The proposed physiological functions of UCP2 and UCP3 include control of thermogenesis and energy expenditure, regulation of insulin secretion, regulation of reactive oxygen species (ROS) and regulation of fatty acid utilization as substrates (567).

4.1 Regulators of Energy Expenditure and Adiposity

UCP2 is involved in regulating metabolic rate and adiposity in vivo. A high fat diet increased WAT UCP2 gene expression in obesity-resistant A/J strains but not in obesity-prone C57B6 mice, indicating UCP2 also plays a role in preventing obesity (685). A positive correlation between UCP2 mRNA expression in WAT and resting metabolic rate (RMR) has been reported in humans (686). In addition, UCP2 expression has also been shown to decrease by 28% in skeletal muscle of obese subjects (687). Consistent with this finding, a reduced UCP2 expression in visceral adipose tissue in morbidly obese subjects has been reported recently (688). A number of genetic linkage studies have also documented association between genetic markers and variants of UCP2 gene and human metabolic rate and/or adiposity (689, 690). A genetic study of markers in UCP2/UCP3 locus revealed a highly significant linkage with resting metabolic rate (689). Others reported that UCP2 gene polymorphisms are associated with sleeping metabolic rate in Pima Indians (690). Moreover, an association between UCP2 exon 8 variant and BMI was found in South Indians. A recent study showed that a common G/A polymorphism in the UCP2 promoter region is associated with increased white adipocyte UCP2 mRNA level and reduced adiposity (691). Moreover, this promoter polymorphism results in an increased transcription of a reporter gene in a human adipocyte cell line (691). However, two identified frequent polymorphism in human UCP2 gene, one in exon 4 with Ala55-Val mutation and the other corresponding to untranslated region of exon 8 with a 45 bp insertion/deletion, are not associated with obesity (692, 693).

Several studies have also demonstrated the physiological relevance of UCP 3 in obesity. In human study, a report showed a negative correlation between UCP 3 mRNA level and body mass index (BMI) in Pima Indians (694). Consistent with this, a positive correlation was found between sleeping metabolic rate and UCP 3 mRNA level (694). Moreover, a mutation in UCP 3 gene resulting in defective function of UCP 3 has been determined in African-Americans with severe obesity and type II diabetes (695). A polymorphism in splicing donor of exon 6 was identified only in African-American. This polymorphism results in a premature termination of the protein product, which lacks the sixth transmembrane domain, similar to UCP3S (the short form of UCP3) (696, 697). Individuals bearing this mutation exhibit reduced fat oxidation by 50%, suggesting a role for UCP3 in metabolic energy partitioning. Moreover, this mutation frequently exists in severely obese subjects (696). Recently, mice over-expressing human UCP3 gene in skeletal muscle were generated (698). These animals exhibit reduced fat mass and body weight, even though they consume more food than wild-type littermates. Transgenic mice also exhibit increased resting oxygen consumption in muscle, indicating an increase in energy expenditure. This was confirmed by observation that the muscle state 4 respiration, an index of proton leaking, is elevated (698).

4.2 Regulators of Insulin Secretion

Abundant expression of UCP2 in pancreatic-β cells coupled with its uncoupling activity raises the possibility that UCP2 may be involved in regulating insulin secretion. Indeed, over-expressing UCP2 in pancreatic β cells decreases ATP/ADP ratio and thereby reduces glucose-stimulated insulin secretion, while UCP2-deficient mice exhibit higher

ATP levels and increased glucose-stimulated insulin secretion, establishing that UCP2 negatively regulates insulin secretion (699-702). The inhibitory effect of UCP2 on insulin secretion is mediated by its proton transport activity, as over-expressing UCP2 in β cells decreases the mitochondrial potential, resulting in a decrease in ATP/ADP ratio (699-702). The role of UCP2 in insulin secretion bears physiological significance. UCP2 is markedly up-regulated in islets of *ob/ob* mice, a model of obesity-induced diabetes (700). Moreover, ob/ob mice lacking UCP2 have restored first-phase insulin secretion, increased serum insulin levels, and decreased levels of glycemia (700). These data demonstrate that UCP2 functions as a key component of β-cell glucose sensor, and a critical link between obesity, β-cell dysfunction, and type II diabetes. The role of UCP2 in modulation of β-cell insulin secretion further confirm UCP2 proton transport function and uncoupling activity, however, in different physiological scenarios.

4.3 Regulators of Fatty Acid Utilization

In contrast to the UCP1 expression pattern, skeletal muscle UCP2 and UCP3 mRNA levels are increased during food deprivation (660, 661), a situation in which whole body needs to save energy. This suggests that UCP2 and UCP3 in muscle might regulate fatty acid metabolism rather than thermogenesis. This was confirmed by fasting and re-feeding studies demonstrating that muscle UCP2 and UCP3 expressions are up-regulated during food deprivation (when this tissue thermogenesis is decreased, but its lipid utilization is increased), while down-regulated upon transition to re-feeding (a period of enhanced efficiency of body fat deposition) (660). The association of UCP2 and UCP3 expression with fat metabolism is strong. Starvation and lipid infusion, both of which raise the

circulating free fatty acid levels, increase UCP2 and UCP3 expression (660, 661, 678, 679). However, the role of UCP2 and UCP3 in lipid metabolism is not known. It is proposed that UCP2 and UCP3 may accelerate fatty acid β-oxidation by facilitating fatty acid transport.

Recently, Samec et al. reported that the down-regulation of skeletal muscle UCP3 expression during re-feeding is abolished during cold exposure (703). This may suggest that the necessity to defend body temperature by ensuring utilization of lipid as fuel overrides the necessity to spare lipid in favor of fat recovery (703). This also suggests that UCP2 and UCP3 may exert different functions to meet physiological priority according to different physiological condition.

4.4 Regulators of Reactive Oxygen Species (ROS)

ROS, such as O_2^-, H_2O_2, •OH, •NO, and •RO, are derived from complex I and complex III of mitochondrial respiratory chains. The ROS production is markedly increased as the proton electrochemical gradient increases. Therefore, ROS is positively correlated with mitochondrial potential. The proton transport activity of UCP2 and UCP3 raises the possibility that these two proteins may function to limit ROS production by reducing mitochondrial potential and thereby decreasing free radical intermediates which can donate its electron to oxygen to generate ROS (567, 584). This was confirmed by the studies of UCP2 and UCP3 knockout mice (703, 704). UCP2- and UCP3-deficient mice do not exhibit an obese phenotype, although UCP3 knockout mice do have increased mitochondrial uncoupling activity in skeletal muscle (703, 704). However, these

knockout animals exhibit increased ROS levels in macrophages and are resistant to deadly parasite infection (703, 704). These results agree with the hypothesis that UCP2 and UCP3 play important roles in modulating ROS production and preventing oxidative stress.

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ROLE OF INTRACELLULAR Ca^{2+} IN MODULATION OF ADIPOSITY

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VOLUME

2

PART 3

ROLE OF INTRACELLULAR CALCIUM IN HUMAN ADIPOCYTE DIFFERENTIATION

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I. ABSTRACT

Intracellular calcium ($[Ca^{2+}]_i$) modulates adipocyte lipid metabolism and inhibits the early stages of murine adipogenesis. Consequently, we evaluated effects of increasing $[Ca^{2+}]_i$ in early and late stages of human adipocyte differentiation. Increasing $[Ca^{2+}]_i$ with either thapsigargin or A23187 at 0-1 hr of differentiation markedly suppressed differentiation, with a 40-70% decrease in triglyceride and glycerol-3 phosphate dehydrogenase (GPDH) ($p < 0.005$). However, a 1-hr pulse of either agent at 47-48 hr only modestly inhibited differentiation. Sustained, mild stimulation of Ca^{2+} influx with either agouti protein or KCl-induced depolarization during 0-48 hr of differentiation inhibited triglyceride accumulation and GPDH activity by 20-70% ($p < 0.05$) and markedly suppressed peroxisome proliferator-activated receptor gamma (PPARγ) expression. These effects were reversed by Ca^{2+} channel antagonism. In contrast, Ca^{2+} pulses late in differentiation (71-72 h or 48-72 h) markedly increased these markers of differentiation. Thus, increasing $[Ca^{2+}]_i$ appears to exert a biphasic regulatory role in human adipocyte differentiation, inhibiting the early stages of differentiation while promoting the late stages of differentiation and lipid filling.

II. INTRODUCTION

Intracellular calcium $[Ca^{2+}]_i$ plays a key role in metabolic derangements associated with obesity (1-3). Increasing ($[Ca^{2+}]_i$) via stimulation of either receptor or voltage-mediated calcium channels has also been shown to stimulate the expression and activity of fatty acid synthase (FAS), a key enzyme in de novo lipogenesis, and inhibit basal and agonist-stimulated lipolysis in both human and murine adipocytes (4, 5). These effects can be reversed by calcium channel antagonism (4, 5). Therefore, increasing $[Ca^{2+}]_i$ appears to promote triglyceride accumulation in adipocytes by exerting a coordinated control over lipogenesis and lipolysis, serving to simultaneously stimulate the former and suppress the latter, resulting in lipid filling and adipocyte hypertrophy.

$[Ca^{2+}]_i$ has also been implicated in regulating adipogenesis, which has been thought to contribute to human and murine obesity (6). Ntambi et al. (7) have reported that increasing $[Ca^{2+}]_i$ by either inhibiting Ca^{2+}-ATPas or stimulating Ca^{2+} influx, inhibited the early stages of murine adipocyte differentiation. However, the role of $[Ca^{2+}]_i$ in human adipocyte differentiation is unknown and may not be inferred from the rodent model, as cells derived from different species and development stages may show distinct patterns of responsiveness to various differentiation-inducing agents. In addition, the time-course effects of $[Ca^{2+}]_i$ in early versus late stages of differentiation is not well defined. Moreover, further investigation may assist in elucidating the role transition of $[Ca^{2+}]_i$ from preadipocyte, where it exerts a inhibitory effect in murine adipocyte differentiation, to mature adipocytes, where it acts as a lipogenic and anti-lipolytic signaling factor in regulating adipocyte metabolism.

Accordingly, the present study was designed to determine the effect of increasing $[Ca^{2+}]_i$ in both early and late stages of human adipocyte differentiation. We report here that increasing $[Ca^{2+}]_i$ in early stages of differentiation suppressed human adipocyte differentiation, similar to previous reports in the murine adipocyte cell line (7). In contrast, increasing $[Ca^{2+}]_i$ in late stages of differentiation promoted human adipocyte differentiation. Consequently, our data suggest that increasing $[Ca^{2+}]_i$ exerts a biphasic regulatory role in human adipocyte differentiation, serving to inhibit the early stages of differentiation, while promote the late stages of differentiation and lipid filling.

III. MATERIALS AND METHODS

A. CULTURE AND DIFFERENTIATION OF HUMAN PREADIPOCYTES

Human preadipocytes used in this study were supplied by Zen-Bio Inc (Research Triangle, NC). These preadipocytes originated from normal human subcutaneous adipose tissue and were isolated using a collagenase digestion and centrifugation method as previously described (8, 9). Preadipocytes were inoculated in Dulbecco's Modified Eagle' Medium (DMEM) / Ham' F-10 medium (F-10) (1:1, v/v) containing 10% fetal bovine serum (FBS), 15mM Hepes and antibiotics at a density of 30,000 cells/cm^2. Confluent monolayers of preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM/F-10 (1:1, v/v) medium supplemented with 15 mM Hepes, 3% FAS, 33 μM biotin, 17 μM pantothenate, 100nM insulin, 0.25 μM methylisobutylxanthine (MIX), 1 μM dexamethasome, 1 μM BRL49653 and antibiotics with or without calcium agonists (thapsigargin and A23187). Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium in which BRL49653 and MIX were omitted. Cultures were refed every 2-3 days.

B. INTRACELLULAR Ca^{2+} MEASUREMENT

$[Ca^{2+}]_i$ in human preadipocytes was measured using a fura-2 dual wavelength fluorescence imaging system. Preadipocytes were plated in 35 mm dished with glass coverslips (P35G-0-14-C, MatTek Corporation). Prior to $[Ca^{2+}]_i$ measurement, cells were preincubated in serum-free medium overnight and rinsed with Hepes Balanced Salt Solution (HBSS) containing the following components (in mM): 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%. Cells were loaded with fura-2 acetoxymethyl ester (AM) (10 μM) in the same buffer for 2 hr at 37 ^{0}C in a dark incubator with 5% CO_2. To remove extracellular dye, cells were rinsed with HBSS 3 times and then postincubated at room temperature for an additional 1 hr 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 4915 CCD camera. Fluorescent images were captured alternatively at excitation wavelength of 340 and 380nM with an emission wavelength of 520 nM. After establishment of stable image baseline, the response to calcium agonists, thapsigargin and A23187, was determined. $[Ca^{2+}]_i$ was calculated using a ratio equation as described previously(10). Each analysis evaluated responses of 8-10 representative whole cells. Images were calibrated using a fura-2 calibrated using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR) to create a calibration curve in solution, and cellular cellular calibration was accomplished using digitonin (25 μM) and pH 8.7 Tris-EGTA (100 mM) to measure maximal and minimal $[Ca^{2+}]$i levels (10).

C. TRIGLYCERIDE CONTENT ASSAY

Human preadipocytes were incubated with calcium agonists as indicated during differentiation. Cellular triglyceride content was determined spectrophotometrically using a triglyceride assay kit (Sigma, St. Louis, MN). Cells were rinsed with Hank' Balanced Salt solution and scraped in 0.9% saline. Cell suspension was then homogenized with sonication and subject to measurement.

D. GLYCEROL-3-PHOSPHATE DEHYDROGENASE (GPDH) ACTIVITY ASSAY

GPDH activity was measured by a spectrophotometric method (11). Cells were scraped and sonicated in 250 mM sucrose solution containing 1 mM ethylenediamine-tetraacticacid (EDTA), 1 mM dithiothreitol (DTT), and 100 μM phenylmethylsulfonyl fluoride (PMSF) (pH 7.4). Homogenate was centrifuged at 18,500 X g for 1 hr and the infranatant was used for measuring oxidation rate of NADH.

E. PROTEIN ASSAY

Total cellular protein content for correction was measured by a modified Bradford method using coomassie blue dye (Pierce, Rockford, IL).

F. NORTHERN BLOT ANALYSIS

Northern blot analysis was conducted as previously described (12). Total RNA from human adipocytes was extracted using $CsCl_2$ density centrifugation, run in 1% agarose gel and transferred to nylon membrane (New England Nuclear, Boston, MA). The membrane was hybridized with a peroxisome proliferator-activated receptor gamma (PPARγ) cDNA probe radiolabeled 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 ^{0}C. Finally, the membrane was exposed to X-ray film (New England Nuclear, Boston, MA) at –80 ^{o}C. All membranes were stripped and reprobed with 18s as loading control.

G. STATISTICAL ANALYSIS

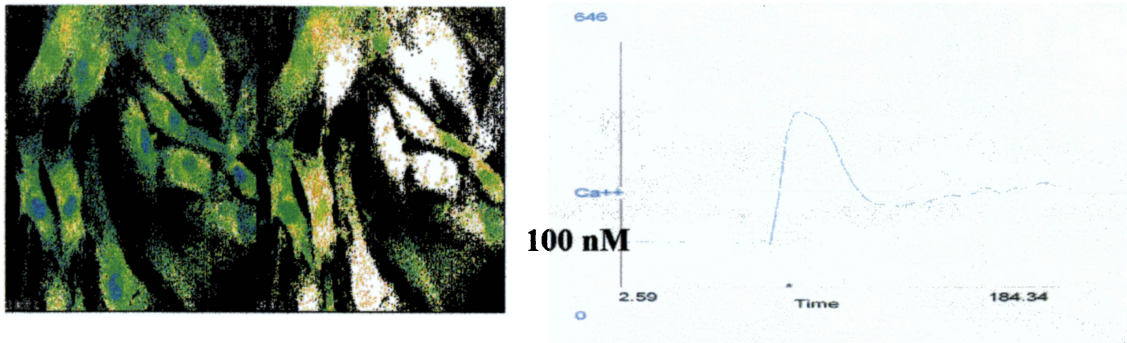
Replicates of 16 independent experiments were conducted for all triglyceride assay, 8 independent studies for GPDH activity, 10 independent experiments for the $[Ca^{2+}]_i$ measurements, and three independent experiments for the PPARγ mRNA measurements. All data are expressed as mean ± SE and evaluated for statistical significance by one way Analysis of Variance (ANOVA) using SPSS (SPSS Inc, Chicago, IL).

IV. RESULTS

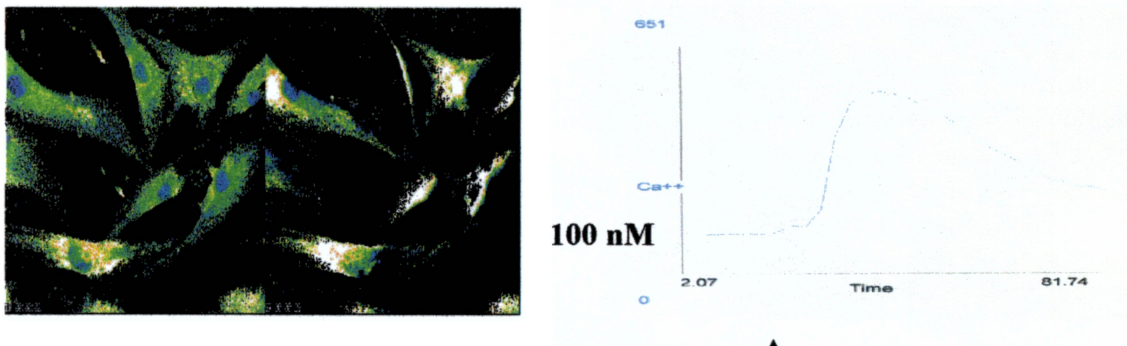
To investigate the role of $[Ca^{2+}]_i$ in human adipocyte differentiation, we first selected thapsigargin, a Ca^{2+}-ATPase inhibitor, and A23187, a calcium ionophore, as calcium agonists to stimulate $[Ca^{2+}]_i$. Both agents are widely used to mimic physiological $[Ca^{2+}]_i$ mobilization in many cell types (13-16). In this study, we demonstrate that thapsigargin and A23187 also stimulated $[Ca^{2+}]_i$ in human preadipocytes. A fura-2 dual wavelength imaging system was used to measure $[Ca^{2+}]_i$ stimulation caused by calcium agonists. **Fig. 1** (top) shows the cell fluorescence image in pseudocolor, which changed from green in baseline to yellow upon addition of 30 nM thapsigargin, indicating an increase of $[Ca^{2+}]_i$. Quantitation of this response demonstrated a 3-fold increase in $[Ca^{2+}]_i$ (baseline of 108 ± 12 nM vs. stimulated value of 456 ± 23 nM, $p<0.001$). Similar results were observed in preadipocytes treated with 2 μM A23187 (baseline of 112 ± 14 nM vs. stimulated value of 412 ± 28 nM, $p<0.001$), as shown in **Fig.1** (middle). Similarly, using KCl as a depolarizing agent caused a slow, sustained increase in $[Ca^{2+}]$i (baseline of 117 ± 15nM vs. stimulated value of 280 ± 26 nM, $p<0.001$; **Fig. 1**, bottom).

To evaluate the effect of increasing $[Ca^{2+}]_i$ in early stages of differentiation, we treated human preadipocytes with calcium agonists during the first 48 h of differentiation, using triglyceride content and GPDH activity as late differentiation markers. **Fig. 2** illustrates that a 1-h pulse with 30 nM thapsigargin or 2 μM A23187 between the 23rd and 24th hour of differentiation suppressed subsequent triglyceride accumulation by 60 and 40% ($p<0.005$), respectively. Similarly, a 1-hr treatment with 30 nM thapsigargin or 2 μM A23187 at 23-24 hr inhibited GPDH activity by 70 and 65% ($p<0.005$, **Fig. 3**),

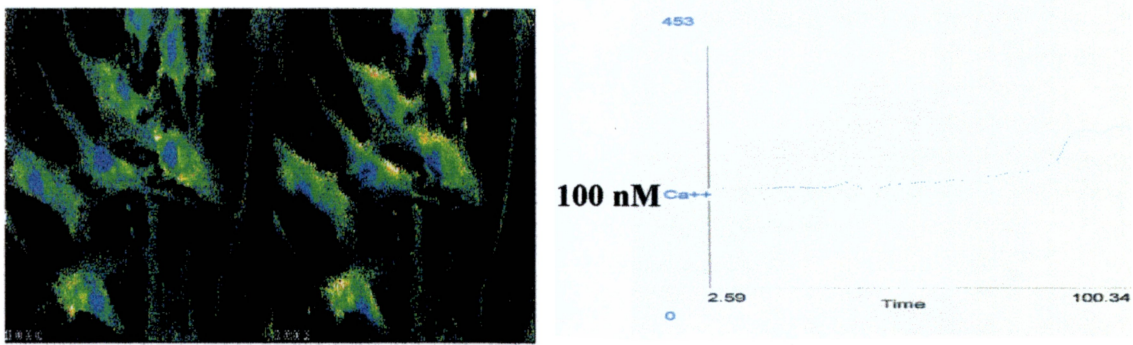
Fig 1. The effects of thapsigargin, A23187, and KCl on human preadipocyte $[Ca^{2+}]_i$. A fura-2 dual wavelength fluorescence imaging system was used to measure $[Ca^{2+}]_i$ as described in Materials and Methods. Ten independent experiments were conducted for each experiment. Left: cell fluorescence images in pseudocolor, which changes from green in baseline (right) to yellow (left) upon addition of each agonist, indicating increased $[Ca^{2+}]_i$. Quantitation of this response is shown in the graph on the right. The arrow indicates the time of addition of each agonist, as follows. Top: a 3-fold increase in $[Ca^{2+}]_i$ in response to 30 nM thapsigargin (456 ± 23 vs. 108 ± 12 nM $[Ca^{2+}]_i$, $p<0.001$). Middle: Similar results with 2 μM A23187 (412 ± 28 vs. 112 ± 14 nM $[Ca^{2+}]_i$, $p<0.001$). Bottom: addition of 20 mM KCl caused a slow, sustained increase in $[Ca^{2+}]_i$ (280 ± 26 vs. 117 ± 15 nM $[Ca^{2+}]_i$, $p<0.001$).



Thapsigargin 30 nM



A23187 2 μM



KCl 10 mM

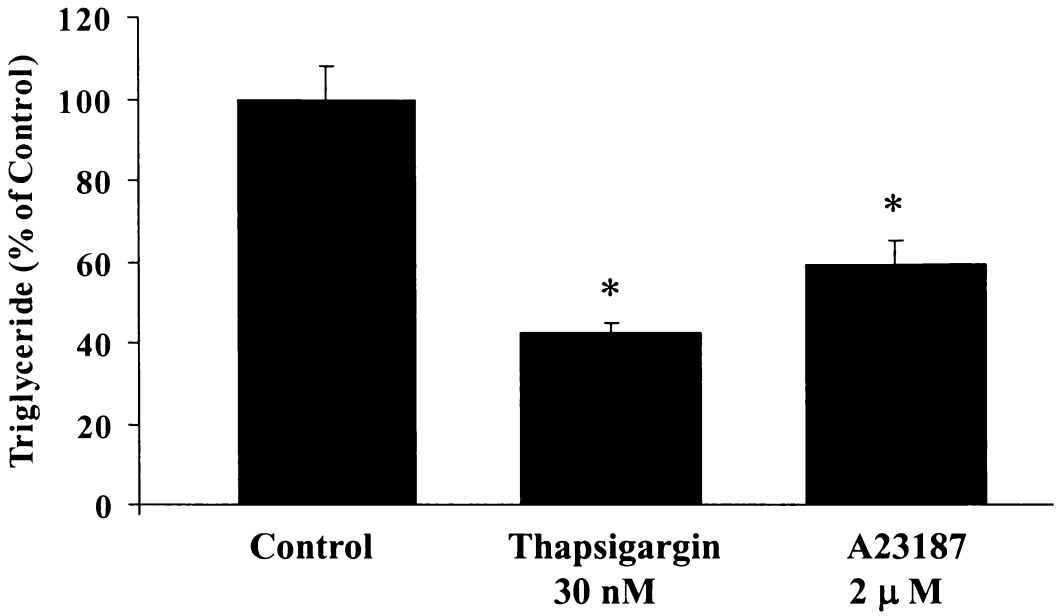


Fig. 2. The effects of the thapsigargin and A23187 treatment at 23-24 hr of differentiation on human adipocyte triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium supplemented with or without thapsigargin or A23187 as indicated. 1-hr pulse treatment of human preadipocytes with 30 nM thapsigargin or 2 μM A23187 was conducted at 23-24 hr of differentiation. Total cellular triglyceride content was measured at the end of day 8 of differentiation as described in Material and Methods. Data are normalized to percent of control (0.988 ± 0.079 $\mu g/\mu g$ protein). *$p<0.005$ vs control; n=16.

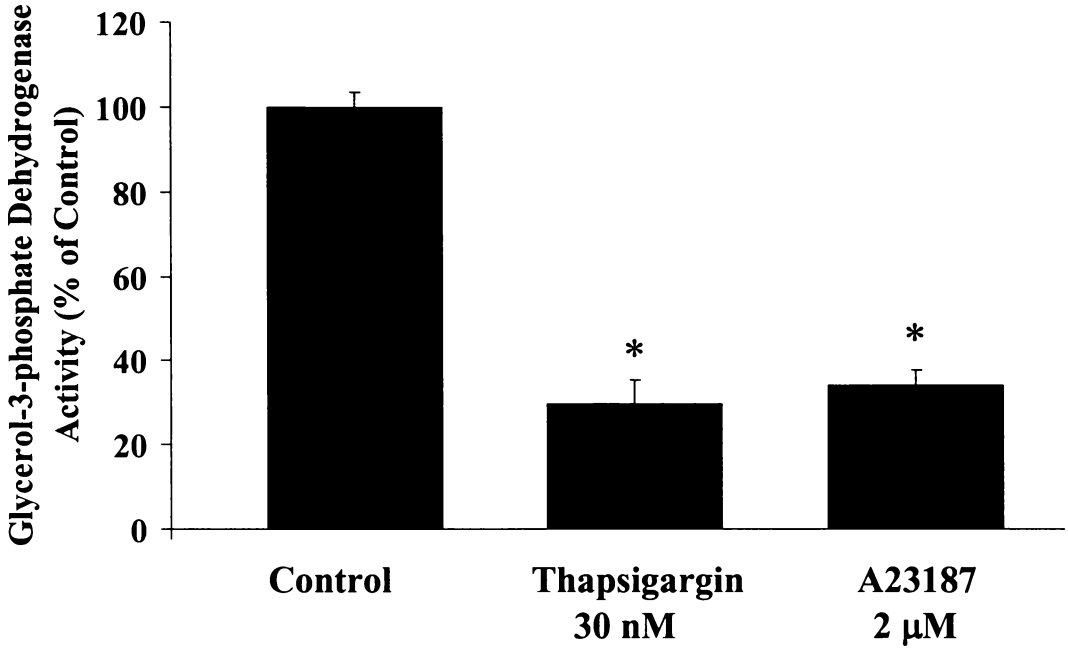


Fig. 3. The effects of the thapsigargin and A23187 treatment at 23-24 hr of differentiation on human adipocyte glycerol-3-phosphate dehydrogenase (GPDH) activity. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of thapsigargin or A23187 as indicated. A 1-hr pulse treatment of human preadipocytes with 30 nM thapsigargin or 2 μM A23187 was conducted at 23-24 hr of differentiation. GPDH activity was measured at the end of day 8 of differentiation as described in Material and Methods. Data are normalized to percent of control oxidation of NADH (23.2 ± 0.9 nmol.min-1.mg protein-1). *$p<0.005$ vs control; n=8.

respectively. However, a later 1-hr pulse of thapsigargin or A23187 at 47-48 hr of differentiation caused an inhibition in triglyceride content by only 20% ($p<0.05$, **Fig. 4**), indicating an attenuation of their inhibitory effects on differentiation.

We next utilized more physiological $[Ca^{2+}]_i$ agonists, such as KCl, a cell membrane depolarization agent, and agouti protein, which is expressed in human adipose tissue and increases $[Ca^{2+}]_i$ in several cell types (17, 18), to stimulate Ca^{2+} influx during differentiation. Agouti protein was obtained as previously described (5). **Fig. 5** shows that treatment with 10 mM KCl or 100 nM agouti protein during 0-48 hr of differentiation suppressed triglyceride content by 70 and 20% ($p<0.01$), respectively. Similarly, a long-term treatment with 10 mM KCl or 100 nM agouti during 0-48 hr of differentiation caused an inhibition in GPDH activity by 40 and 20% ($p<0.05$, **Fig. 6**), respectively. This inhibition was completely prevented by 30 nM nitrendipine, an L-type Ca^{2+} channel antagonist. Moreover, using PPARγ expression as a differentiation marker, we demonstrated that long-term treatment with 10 mM KCl or 100 nM agouti during 0-48 hr of differentiation greatly inhibited PPARγ expression, which was totally recovered by nitrendipine (**Fig. 7**).

We next assessed the effect of increasing $[Ca^{2+}]_i$ in late stages of differentiation. In contrast to early stage of treatment, a 1-hr pulse treatment with 30 nM thapsigargin or 2 μM A23187 at 71-72 hr of differentiation increased triglyceride content by 90 and 65% ($p<0.001$, **Fig. 8**), respectively. Similarly, **Fig. 9** illustrates that a sustained treatment with 10 mM KCl or 100 nM agouti during 48-72 hr of differentiation enhanced triglyceride

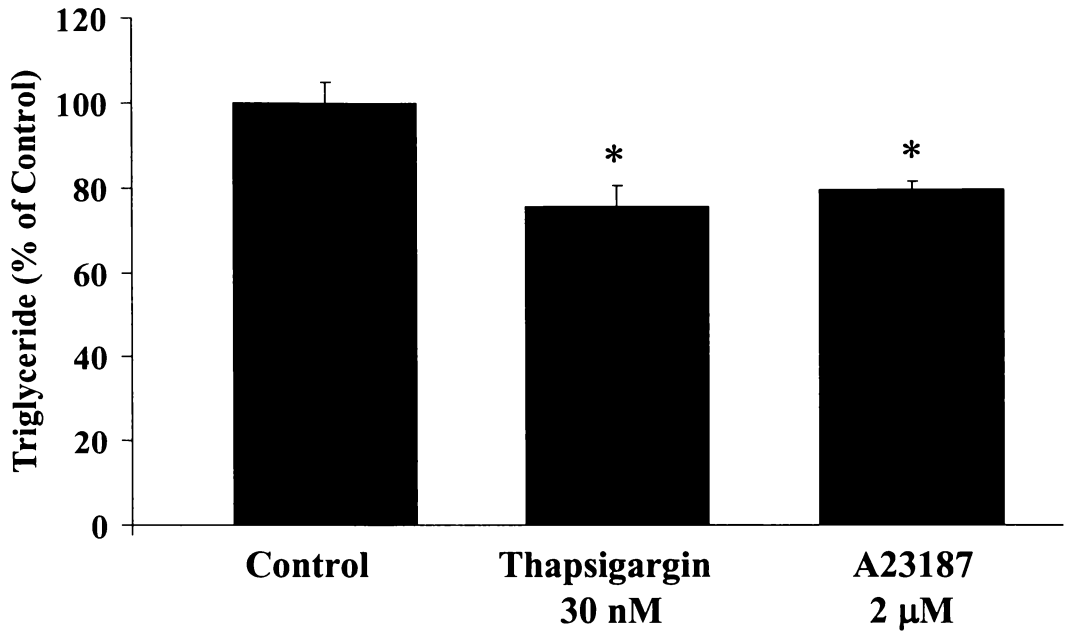


Fig 4. The effects of the thapsigargin and A23187 treatment at 47-48 hr of differentiation on human adipocyte triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium supplemented with or without thapsigargin or A23187 as indicated. A 1-hr pulse treatment of human preadipocytes with 30 nM thapsigargin or 2 μM A23187 was conducted at 47-48 hr of differentiation. Total cellular triglyceride content was measured at the end of day 8 of differentiation as described in Material and Methods. Data are normalized to percent of control (1.04 ± 0.05 μg/μg protein). *$p<0.005$ vs control; n=16.

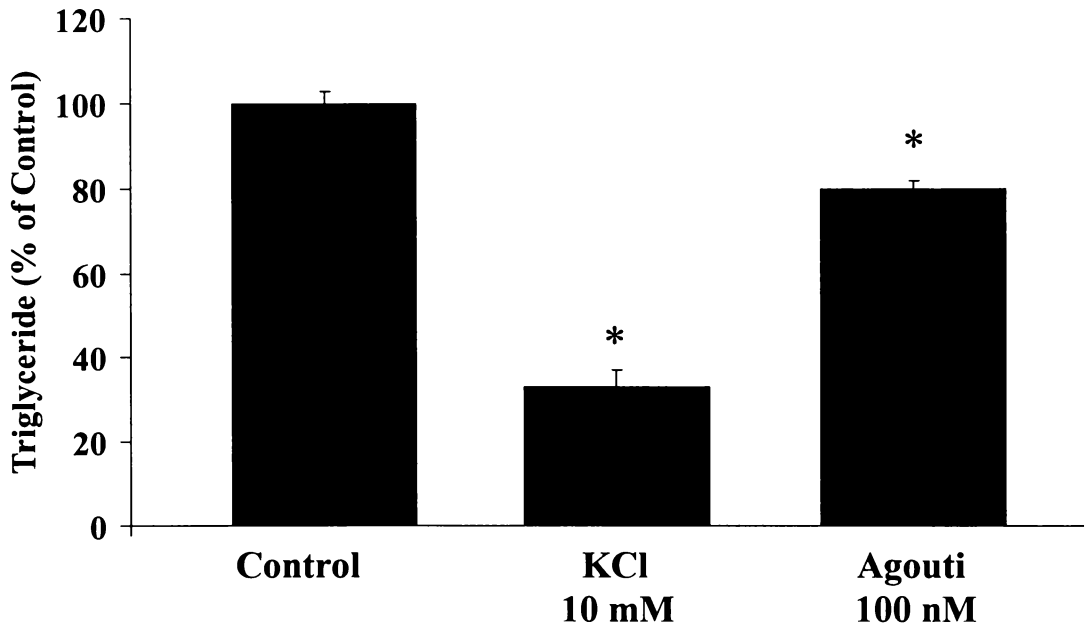


Fig. 5. The effects of KCl and agouti protein treatment during 0-48 hr of differentiation on human triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti as indicated. KCl and agouti protein were removed after 2-day treatment during 0-48 hr of differentiation. Total cellular triglyceride content was measured at the end of day 8 of differentiation as described in Material and Methods. Data are normalized to percent of control (1.46 ± 0.12 μg/ μg protein). *$p<0.01$ vs control; n=16.

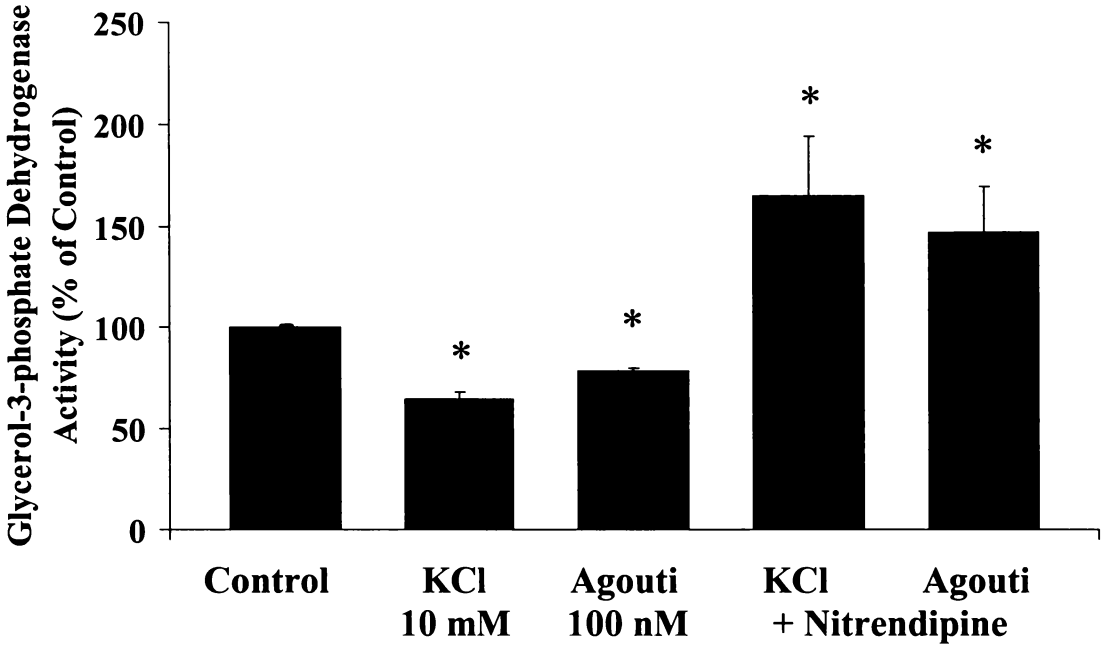


Fig. 6. The effects of KCl and agouti protein treatment during 0-48 hr of differentiation on human glycerol-3-phosphate dehydrogenase activity. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti alone, KCl or agouti plus nitrendipine as indicated. KCl and agouti protein were removed after 2-day treatment during 0-48 hr of differentiation. GPDH activity was measured at the end of day 8 of differentiation as described in Material and Methods. Data are normalized to percent of control (20.4 ± 0.2 nmol.min-1.mg protein-1). *$p<0.05$ vs. control; n=8.

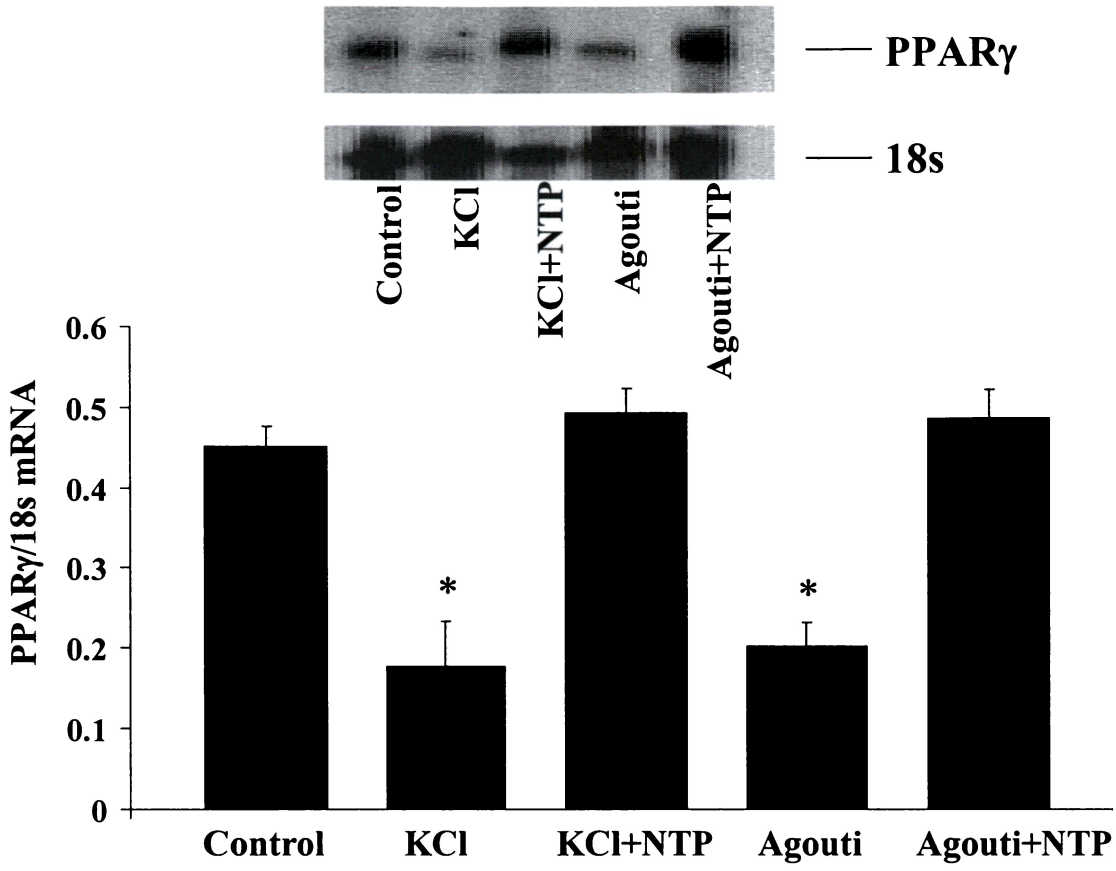


Fig. 7. The effects of KCl and agouti protein treatment during 0-48 hr of differentiation on human preadipocyte PPARγ expression. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti alone, KCl or agouti plus nitrendipine as indicated. KCl and agouti protein were removed after 2-day treatment during 0-48 hr of differentiation. Total RNA was prepared at the end of day 4. Northern blot analysis was performed as described in Materials and Methods. Blot shown is representative of 3 similar experiments. *$p<0.05$ vs. control.

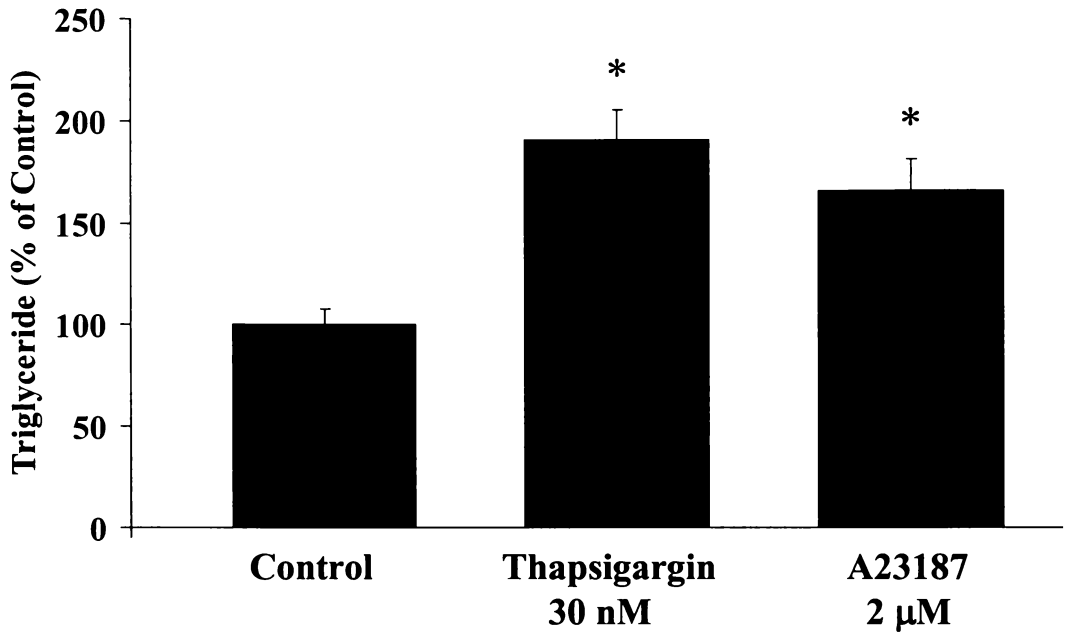


Fig. 8. The effects of the thapsigargin and A23187 treatment at 71-72 hr of differentiation on human adipocyte triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium supplemented with or without thapsigargin or A23187 as indicated. 1-hr pulse treatment of human preadipocytes with 30 nM thapsigargin or 2 μM A23187 was conducted at 71-72 hr of differentiation. Total cellular triglyceride content was measured at the end of day 8 of differentiation as described in Material and Methods. Data are normalized to percent of control (0.93 ± 0.07 μg/μg protein). *$p < 0.001$ vs control; n=16.

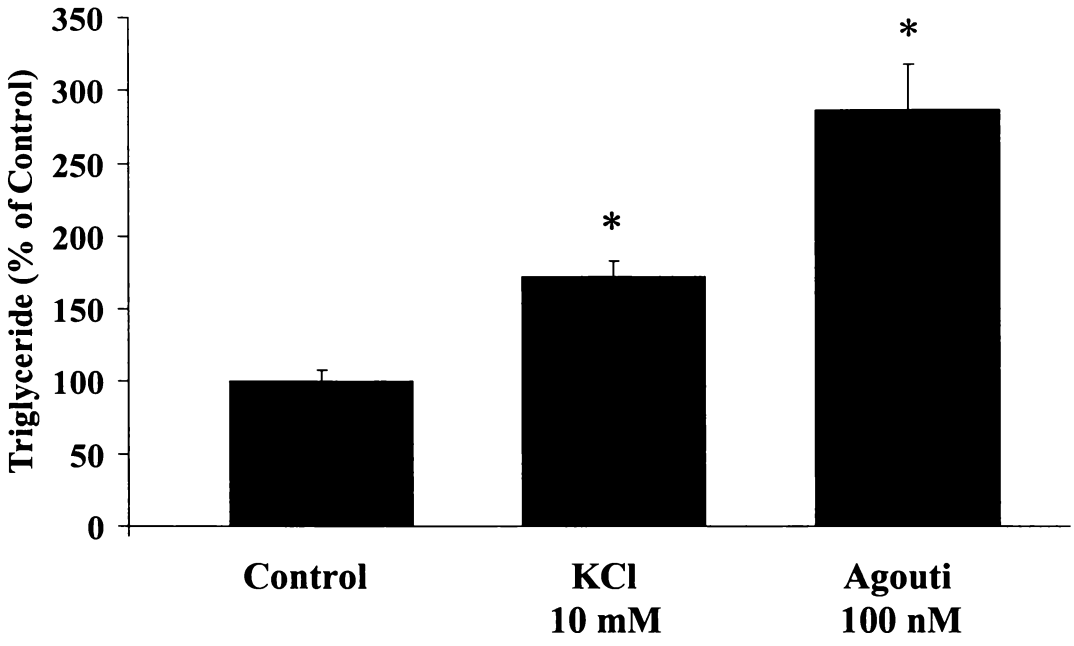


Fig. 9. The effects of KCl and agouti protein treatment during 48-72 hr of differentiation on human triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti as indicated. 1-day treatment of human preadipocytes with 10 mM KCl or 100 nM agouti was conducted during 48-72 hr of differentiation. Total cellular triglyceride content was measured at the end of day 8 of differentiation as described in Material and Methods. Data are normalized to percent of control (1.46 ± 0.12 μg/μg protein). *$p<0.01$ vs. control; n=16.

accumulation by 2 to 3 fold ($p<0.001$). Further, **Fig. 10** shows that a long-term treatment with either KCl or agouti late in differentiation caused a marked increase in PPARγ expression.

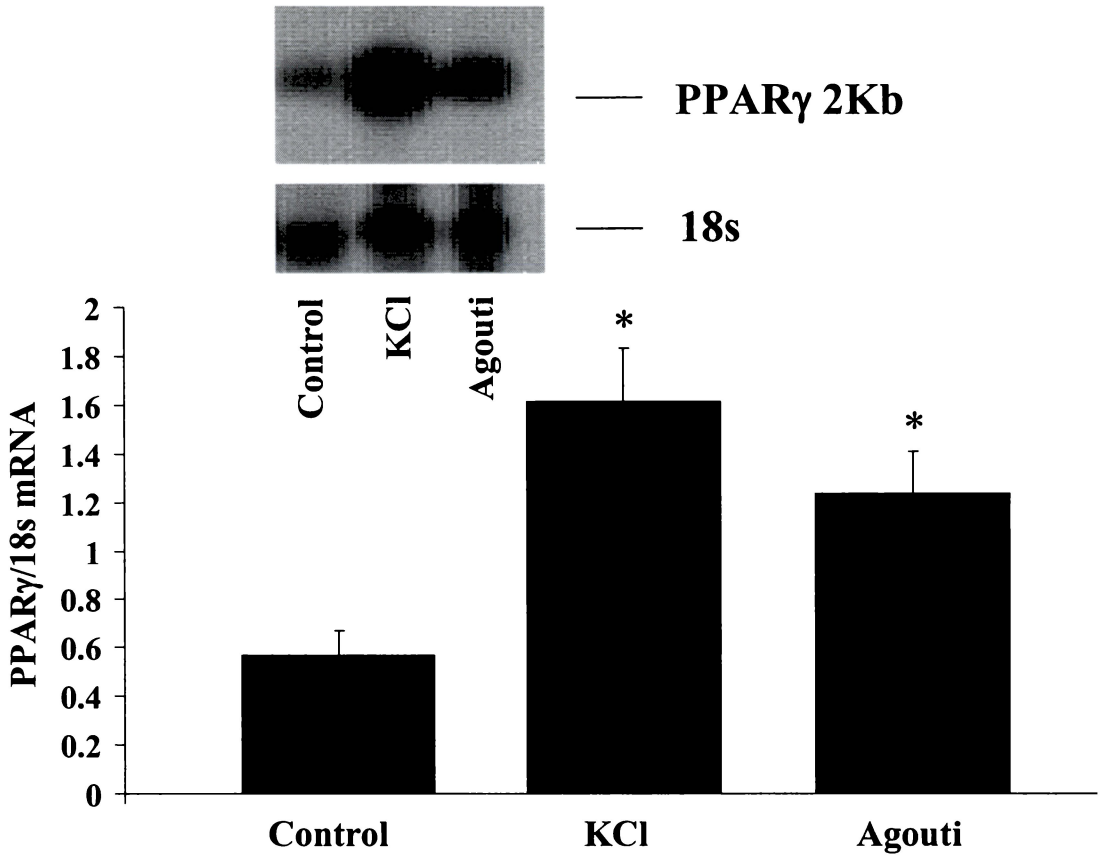


Fig. 10. The effects of KCl and agouti protein treatment during 48-72 hr of differentiation on human preadipocyte PPARγ expression. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti as indicated. 1-day treatment of human preadipocytes with 10 mM KCl or 100 nM agouti was performed during 48-72 hr of differentiation. Total RNA was prepared at the end of day 4. Northern blot analysis was conducted as described in Materials and Methods. Blot shown is representative of three similar experiments. *$p<0.05$.

V. DISCUSSION

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) appears to play a key role in metabolic disorders associated with obesity, and sustained high levels of $[Ca^{2+}]_i$ may contribute to this derangement (1-3). In this study, we demonstrate that $[Ca^{2+}]_i$ plays a regulatory role in adipogenesis, an important contributor to increased fat tissue mass. Our data suggest that increasing $[Ca^{2+}]_i$ in the early stages of differentiation inhibits human adipocyte differentiation, whereas increasing $[Ca^{2+}]_i$ in late stage promotes human adipocyte differentiation. This delineates a role transition in $[Ca^{2+}]$i, which serves to inhibit differentiation in early stages but promote differentiation in late stages.

$[Ca^{2+}]_i$ appears to promote and accelerate preadipocyte differentiation program(s), thereby inducing adipocyte phenotype in the late stage of differentiation. To achieve this, increasing $[Ca^{2+}]_i$ causes a marked increase in the expression of PPARγ (**Fig. 10**), a nuclear hormone receptor that acts as a critical transcriptional factor in adipocyte differentiation programs (19). Increased expression of PPARγ may subsequently accelerate adipocyte differentiation by directly acting upon and eliciting late differentiation gene expression, such as aP2, steroyl-CoA desaturase (SCD-1), phosphoenolpyruvate carboxykinase (PEPCK) and fatty acid synthase (FAS) (20-25). 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/EBPβ and δ), PPARγ and sterol regulatory element binding protein-1/adipocyte determination and differentiation factor-1 (SREBP-1/ADD-1) (19, 20, 26, 27). $[Ca^{2+}]_i$ may synergize with

these transcriptional factors to promote differentiation program by stimulating late differentiation gene expression. Consistent with this, $[Ca^{2+}]_i$ acts as a lipogenic and anti-lipolytic signaling factor in regulating adipocyte metabolism in both rodent and human adipocytes (4, 5). Previous reports from our laboratory demonstrated that increasing $[Ca^{2+}]_i$ stimulates the expression and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis and thereby increases triglyceride storage (4). In addition, we have also shown that increasing $[Ca^{2+}]_i$ inhibits basal and agonist-stimulated lipolysis in primary cultured human adipocytes (5). These effects can be completely blocked by calcium channel antagonism (4, 5). Therefore, increasing $[Ca^{2+}]_i$ appears to promote triglyceride accumulation in adipocytes by exerting a coordinated control over lipogenesis and lipolysis, serving to simultaneously stimulate the former and suppress the latter, thereby resulting in lipid filling and adipocyte hypertrophy. Accordingly, the lipogenic and anti-lipolytic effects of $[Ca^{2+}]_i$, coupled with increased expression of differentiation transcriptional factor such as PPARγ, may contribute to the stimulatory effect of $[Ca^{2+}]_i$ in the late stages of differentiation.

However, data from Ntambi et al (7) did not show the stimulatory effect of increasing $[Ca^{2+}]_i$ in late stages of differentiation in 3T3-L1 cells. The reason for this discrepancy is not clear. A possible explanation is that these two studies used two different cell models, 3T3-L1 derived from mouse embryo and human preadipocyte originating from human stromal-vascular cells in subcutaneous fat depot (20). Therefore, cell models derived from different species and development stage may exhibit distinct differentiation properties.

In contrast to the stimulatory effect of increasing $[Ca^{2+}]_i$ in late stages of differentiation, increasing $[Ca^{2+}]_i$ in the early stage suppresses human adipocyte differentiation. In agreement with our findings, increasing $[Ca^{2+}]_i$ has been shown to exhibit an inhibitory effect in early stages of murine adipocyte differentiation (7). $[Ca^{2+}]_i$ exerted this inhibitory effect by blocking the postconfluent mitotic phase and mediating sustained levels of c-myc expression (7). This sustained c-myc expression precludes cell entry to the G_D stage necessary for subsequent differentiation (28-30) and instead forces cells to re-enter the normal cell cycle (31). In this study, increasing $[Ca^{2+}]_i$ in early stage of differentiation caused a substantial inhibition in PPARγ expression (**Fig. 7**), which would be expected to subsequently reduce late differentiation gene expression and thereby inhibit further differentiation.

The mechanism whereby $[Ca^{2+}]_i$ undergoes this role transition during human adipocyte differentiation is unknown. However, cAMP also plays a discordant role in adipocyte differentiation and mature adipocyte metabolism. Increasing cAMP promotes adipocyte differentiation (20), while it inhibits expression and activity of fatty acid synthase (32, 33), a key enzyme in de novo lipogenesis, and stimulates lipolysis in mature adipocytes. Moreover, there is a significant interaction between the calcium and cAMP signaling pathways. Structural and functional studies have demonstrated adenylyl cyclases are associated with the site of Ca^{2+} entry into the cell and that Ca^{2+} entry causes a marked inhibition of type V and VI adenylyl cyclases, thereby reducing cAMP levels in several cell types (34). Alternatively, we have recently demonstrated that increasing human

adipocyte $[Ca^{2+}]_i$ stimulates phosphodiesterase 3B activity, resulting in reduced cAMP levels and thereby inhibit differentiation (35). Conversely, a $[Ca^{2+}]_i$-induced decrease in cAMP late in differentiation up-regulates lipogenesis and suppresses lipolysis, thereby promoting adipocyte maturation and lipid filling.

It was originally believed that only early-onset obesity was associated with adipocyte hyperplasia, while maturity-onset obesity was believed to result solely from adipocyte hypertrophy (36, 37). This concept was challenged by later studies demonstrating that the potential to acquire new adipocytes persists even at adult stage. Several lines of evidence demonstrate that an increase in fat cell number appears to be well correlated with severity of human obesity in adult life (8). Moreover, specific early differentiation genes have been reported to express in adipose tissue from very old mice (38). In addition, fat cell precursors, such as stromal-vascular cells, isolated from adult human adipose tissue can be fully differentiated into mature adipocytes in vitro (8, 39). Further, adipocyte development has been shown to depend on fat precursor cell recruitment in vivo (40). Although the relative contribution of adipocyte hypertrophy versus hyperplasia to human adiposity is unknown, the capacity to generate new adipocytes from cells persists throughout life span and clearly plays a role in regulating fat tissue mass. Our data in the present study implicate a role of $[Ca^{2+}]_i$ in modulating adipocyte hyperplasia and further affecting adipose tissue mass *in vivo*. The significance of $[Ca^{2+}]_i$ in regulating adipogenesis bears physiological basis, as there are many nutritional, hormonal and pharmaceutical factors that can modulate $[Ca^{2+}]_i$ signaling in both adipocytes and preadipocytes. In addition to the $[Ca^{2+}]_i$ mobilization agents used in this study (cell

membrane depolarization and agouti protein, an obesity gene product expressed in human adipose tissue), other endocrine and/or paracrine factors, including 1,25-dihydroxy-vitamin D, parathyroid hormone (41), angiotensin II, and arginine vasopressin (unpublished data), have also been demonstrated to mobilize adipocyte $[Ca^{2+}]_i$. Further, we recently reported that sulfonylureas, a family of insulin secretgogues widely used to stimulate insulin release in the treatment of type II diabetes, stimulates $[Ca^{2+}]_i$ and thereby modulates lipid metabolism in human adipocytes (12). Thus, $[Ca^{2+}]_i$ appears to exhibit a physiological role in regulating adipogenesis and fat tissue mass formation.

In summary, our data suggest that increasing $[Ca^{2+}]_i$ exerts a biphasic regulatory role in human adipocyte differentiation, serving to inhibit the early stages of differentiation while promoting the late stages of differentiation and lipid filling. We conclude that the role of $[Ca^{2+}]_i$ in adipocyte differentiation may not only provide insight into the mechanism of human adipogenesis and energy homeostasis, but also represent an important target for further development of therapeutic intervention in obesity.

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PART 4

ROLE OF THE SULFONYLUREA RECEPTOR (SUR) IN REGULATING HUMAN ADIPOCYTE METABOLISM

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I. ABSTRACT

A regulatory role for intracellular Ca^{2+} ($[Ca^{2+}]_i$) in adipocyte lipogenesis, lipolysis and triglyceride accumulation has been demonstrated. Compounds acting on the pancreatic sulfonylurea receptor (SUR) to increase (e.g. glibenclamide) or decrease (e.g.diazoxide) $[Ca^{2+}]_i$ cause corresponding increases and decreases in weight gain. However, these weight gain and loss effects have been attributed to insulin release rather than primary effects of these compounds on the adipocyte SUR and its associated K_{ATP} channel. Accordingly, we have now evaluated the direct role of the human adipocyte SUR in regulating adipocyte metabolism. We used RT-PCR with primers for a highly conserved region of SUR1 to demonstrate that human adipocytes express SUR1.The PCR product was confirmed by sequence analysis and used as a probe to demonstrate adipocyte SUR1 expression by northern blot analysis. Adipocytes exhibited corresponding glibenclamide dose-responsive (0-20 μM) increases in $[Ca^{2+}]_i$ ($p<0.05$). Similarly, glibenclamide (10 μM) caused a 67% increase in adipocyte fatty acid synthase (FAS) activity ($p<0.001$), a 48% increase in glycerol-3-phosphate dehydrogenase (GPDH) activity ($p<0.01$) and a 68% inhibition in lipolysis ($p<0.01$), while diazoxide (10 μM) completely prevented each of these effects. These data demonstrate that human adipocyte express a SUR which regulates $[Ca^{2+}]_i$ and, consequently, exerts coordinate control over lipogenesis and lipolysis. Accordingly, the adipocyte SUR1 may represent an important target for the development of therapeutic interventions in obesity.

II. INTRODUCTION

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays a key role in the metabolic disorders associated with obesity and insulin resistance (1-3). Recombinant agouti protein, an obesity gene product, causes a dose-responsive increase in $[Ca^{2+}]_i$ in a variety of cells (4,5), including both murine and human adipocyte. Moreover, agouti protein promotes the expression and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis, and thereby increases triglyceride storage in a Ca^{2+}- dependent manner (6). In addition, we have also shown that agouti inhibits basal and agonist-stimulated lipolysis in primary cultured human adipocytes via a Ca^{2+}- dependent mechanism (7). Therefore, increasing $[Ca^{2+}]_i$ appears to promote triglyceride accumulation in adipocytes by exerting a coordinated control over lipogenesis and lipolysis.

Sulfonylureas, such as glibenclamide, are insulin secretagogues widely used to stimulate insulin secretion in the treatment of non-insulin-dependent diabetes mellitus. Sulfonylureas depolarize pancreatic β cells by blocking K_{ATP} channels, thereby resulting in depolarization and secondary Ca^{2+} influx via L-type Ca^{2+} channels, which in turn triggers insulin release (8,9). The β cell receptor for sulfonylureas, sulfonylurea receptor1 (SUR1), has been cloned (10).

Recently, Alemzadeh et al. (11, 12) reported that diazoxide, a drug that activates the β cell K_{ATP} channel and subsequently exerts an anti-obesity effect in obese Zucker rats. Further, they recently reported that diazoxide exerted a significant anti-obesity effect in

hyperinsulinemic obese adults (13). However, this action was attributed to direct actions of diazoxide on β cells, rather than direct effects of diazoxide on adipocyte metabolism.

Accordingly, the present study was conducted to assess the direct role of the human adipocyte SUR in adipocyte metabolism. We report here that human adipocytes express SUR and exhibit corresponding glibenclamide dose-responsive increase in $[Ca^{2+}]_i$. Moreover, glibenclamide exerts lipogenic and antilipolytic effects in human adipocytes, while diazoxide completely blunts each of these effects. These data demonstrate that SUR in human adipocyte regulates $[Ca^{2+}]_i$ and thereby exerts coordinate control over lipogenesis and lipolysis.

III. MATERIALS AND METHODS

A. ISOLATION AND CULTURE OF HUMAN ADIPOCYTES

Human subcutaneous adipose tissue was obtained from patients undergoing abdominal plastic surgery with no known history of metabolic disorders. This protocol was approved by the Institutional Review Board for Human Subjects and the Committee for Research Participation of the University of Tennessee. Adipocytes were isolated as previously described (14). Briefly, adipose tissue was first washed several times with Hank's Balanced Salt Solution, minced into small pieces and digested with 0.8 mg/ml type1 collagenase in a shaking water bath at 37 ^{0}C for 30 min. Adipocytes were then filtered through a sterile 500 μm nylon mesh and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% fetal bovine serum (FBS), 100 UI/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin. Cells were cultured in suspension and maintained in a thin layer at the top of culture media, which were changed every day. Cells were maintained viable and metabolically responsive under this culture condition for 7 days.

B. REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA from human adipocytes was extracted using $CsCl_2$ density centrifugation. mRNA from human adipocyte was isolated according to manufacture's instruction (micro poly(A) pure kit, Ambion Inc, Austin, TX). RT-PCR was performed essentially as previously described (7). Briefly, 400 ng of human adipocyte mRNA was reverse-transcribed to first strand cDNA using random-hexamer and reverse transcriptase (Perkin Elmer, Norwalk, CT) and amplified by PCR (Perkin Elmer, Norwalk, CT). The PCR

conditions were as follows: initial denaturation at 94 ^{0}C for 5min, followed by 34 cycles of denaturation at 94 ^{0}C for 45s, annealing at 55 ^{0}C for 1min, and extension at 72 ^{0}C for 2 min, with a final extension step at 72 ^{0}C for 8 min with 0.5 μM 5' primer (5'-CATCATTGATGGCATTGACATCCGC) and 3' primer (5'-CTCTGGCTTATCGAACTCAAGGATGG) which correspond to nucleotide positions 4206-4230 and 4664-4689 in HSU63421 respectively. The amplified PCR products were then visualized by 1.2% agarose gel electrophoresis, purified (Geneclean kit, bio 101, Inc) and subjected to sequence analysis using ABI PRISM system (model version 2. 1. 1).

C. NORTHERN BLOT ANALYSIS

Northern blot analysis was conducted as previous described (15). Human adipocyte mRNA (4 μg/well) was run in 1% agarose gel and transferred to nylon membrane, which was hybridized with human SUR1 cDNA probes eluted from PCR products and radiolabeled using a random primer method. Unbound probe was removed by rinsing the membrane with 2X SSC for 30 min at room temperature and 0.1X SSC/ 0.1% SDS for 45 min at 60 ^{0}C.Finally, membrane was exposed to X-ray film at –80 ^{0}C.

D. INTRACELLULAR Ca^{2+} MEASUREMENT

$[Ca^{2+}]_i$ in isolated human adipocytes was determined fluorometrically as previously described (2,16). Briefly, the human adipocytes isolated as described above were incubated in DMEM medium overnight for cell recovery. Prior to $[Ca^{2+}]_i$ measurement, adipocytes were pre-incubated in serum-free medium for 2 hrs, rinsed with HBSS

solution containing the following components in mM: NaCl 138, $CaCl_2$ 1.8, $MgSO_4$ 0.8, NaH_2PO_4 0.9, $NaHCO_3$ 4, glucose 20, glutamine 6, HEPES 20 and BSA 1%. Cells were then loaded with fura-2 acetoxymethyl ester (10 μM) in the same buffer for 45 min at 37 ^{0}C in dark with continuous shaking. To remove extracellular dye, cells were rinsed with HBSS for 3 times and resuspended in this solution at a concentration of $2X10^5$ cells/ml. $[Ca^{2+}]_i$ was measured using dual excitation (340 and 380 nm) and single emission (510 nm) fluorometry. Following the establishment of stable baseline, the response to glibenclamide (10 and 20 μM) was determined. Digitonin (25 μM) and Tris/EGTA (100 mM) were used to measure maximal and minimal fluorescence to calibrate the signals, and final $[Ca^{2+}]_i$ was then calculated by the equation of Grynkiewicz et al (17).

E. FATTY ACID SYNTHASE (FAS) AND GLYCEROL-3-PHOSPHATE DEHYDROGENASE (GPDH) ACTIVITY ASSAY

Human adipocytes were incubated in 24-well plates with the treatments indicated for 48 hr. FAS and GPDH activities were determined spectrophotometrically in crude cytosolic extracts of human adipocytes by measuring the oxidation rate of NADPH or NADH respectively, as previously described (18). The protein correction was measured by a modified Bradford method using Coomassie Blue dye (Pierce, Rockford, IL).

F. LIPOLYSIS ASSAY

Human adipocytes were treated as described above, and glycerol release into the culture medium was determined as an indicator for lipolysis using a one step enzymatic fluorometric method (19). After treatment medium was obtained, $HClO_4$ was added for

deproteinization. The sample was next centrifuged to precipitate protein and the supernatant was neutralized with NaOH before glycerol assay.

G. STATISTICAL ANALYSIS

All data are expressed as mean±SE. Data were evaluated for statistical significance by analysis of variance or t-test.

IV. RESULTS

Human SUR1 is expressed in adipocytes. Using RT-PCR, we detect a 490 bp SUR1 fragment, which represents a highly conserved region in SUR gene (**Fig. 11**). Sequence analysis confirmed the identity of this PCR product, which was 100% identical to human SUR1. Interestingly, no SUR1 transcript was detectable in mRNA isolated from human preadipocytes (**Fig. 11**). The PCR product was purified and used as a probe to detect adipocyte SUR1 expression by northern blot analysis, which showed an approximately 5 Kb transcript of SUR1 mRNA in adipose tissue (**Fig. 12**).

To determine the functional significance of this adipocyte SUR, the $[Ca^{2+}]_i$ response to glibenclamide was evaluated. 5 and 10 μM glibenclamide induced sustained increases of $[Ca^{2+}]_i$ in human adipocytes in a dose-dependent manner, while pre-treatment of human adipocytes with diazoxide, the K_{ATP} channel opener, completely prevented glibenclamide-induced increases in $[Ca^{2+}]_i$ (**Fig. 13** and **14**). However, human preadipocytes did not exhibit any $[Ca^{2+}]_i$ response to glibenclamide. This is consistent with the absence of SUR1 in human preadipocytes noted above.

To study the role of adipocyte SUR1 in regulating lipogenesis, we treated human adipocyte with SUR1 agonist and antagonist using FAS and GPDH activities as lipogenic markers. 10 μM glibenclamide caused a 67% increase in adipocyte FAS activity (0.692±0.052 NADPH nM/min/mg protein vs. 1.154±0.010 NADPH nM/min/mg protein, $p<0.001$, **Fig. 15**), which was completely blocked by 10 μM diazoxide, a K_{ATP} channel activator, and partially inhibited by nitrendipine, an L-type Ca^{2+} channel antagonist.

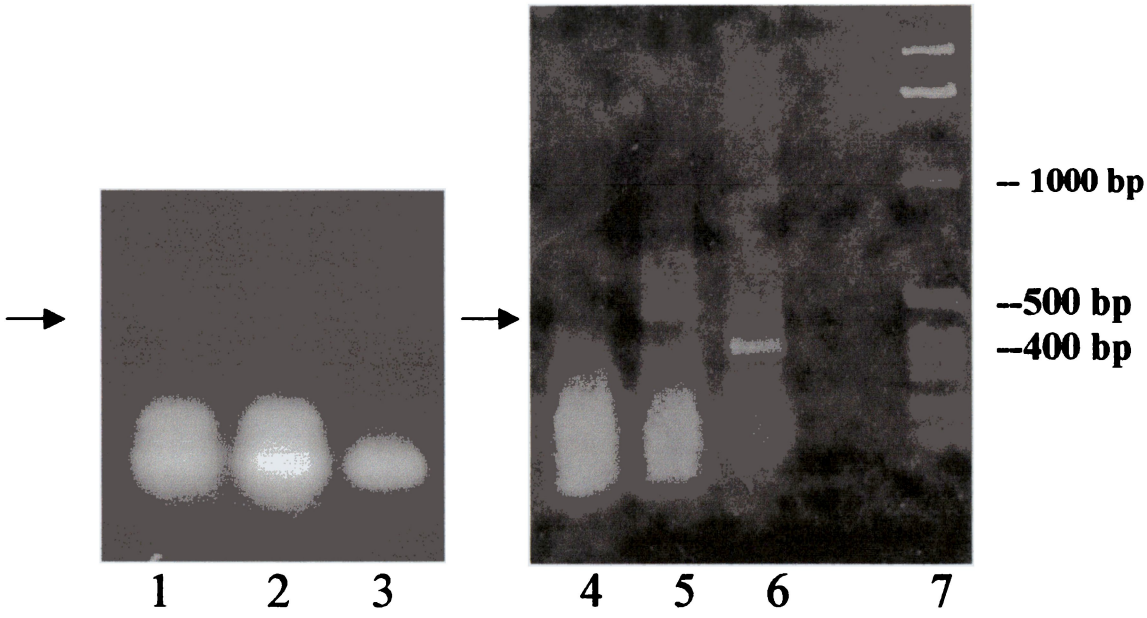


Fig 11. Detection of the expression of SUR1 gene in human adipocyte by RT-PCR. The PCR product, a 490 bp SUR1 fragment (designated by arrow), was visualized by 1.2% agarose gel electrophoresis. Lane 1: human pancreatic β cells as positive control; lane 2: human preadipocytes without reverse transcriptase; lane 3: human preadipocyte with reverse transcriptase; lane 4: human adipocytes without reverse transcriptase; lane 5: human adipocytes with reverse transcriptase; lane 6: 18s fragment amplified as RT-PCR system positive control; lane 7: DNA marker. RT-PCR was performed as described in Materials and Methods.

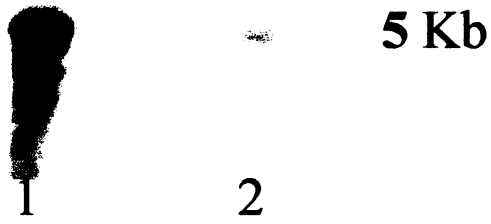


Fig 12. Detection of the expression of SUR1 mRNA in human adipocyte by northern blot analysis. A 5 Kb transcript of SUR1 mRNA was detected in human adipocyte. Lane 1: human pancreas mRNA as positive control; lane 2: human adipocyte mRNA. Northern blot analysis was conducted as described in Materials and Methods.

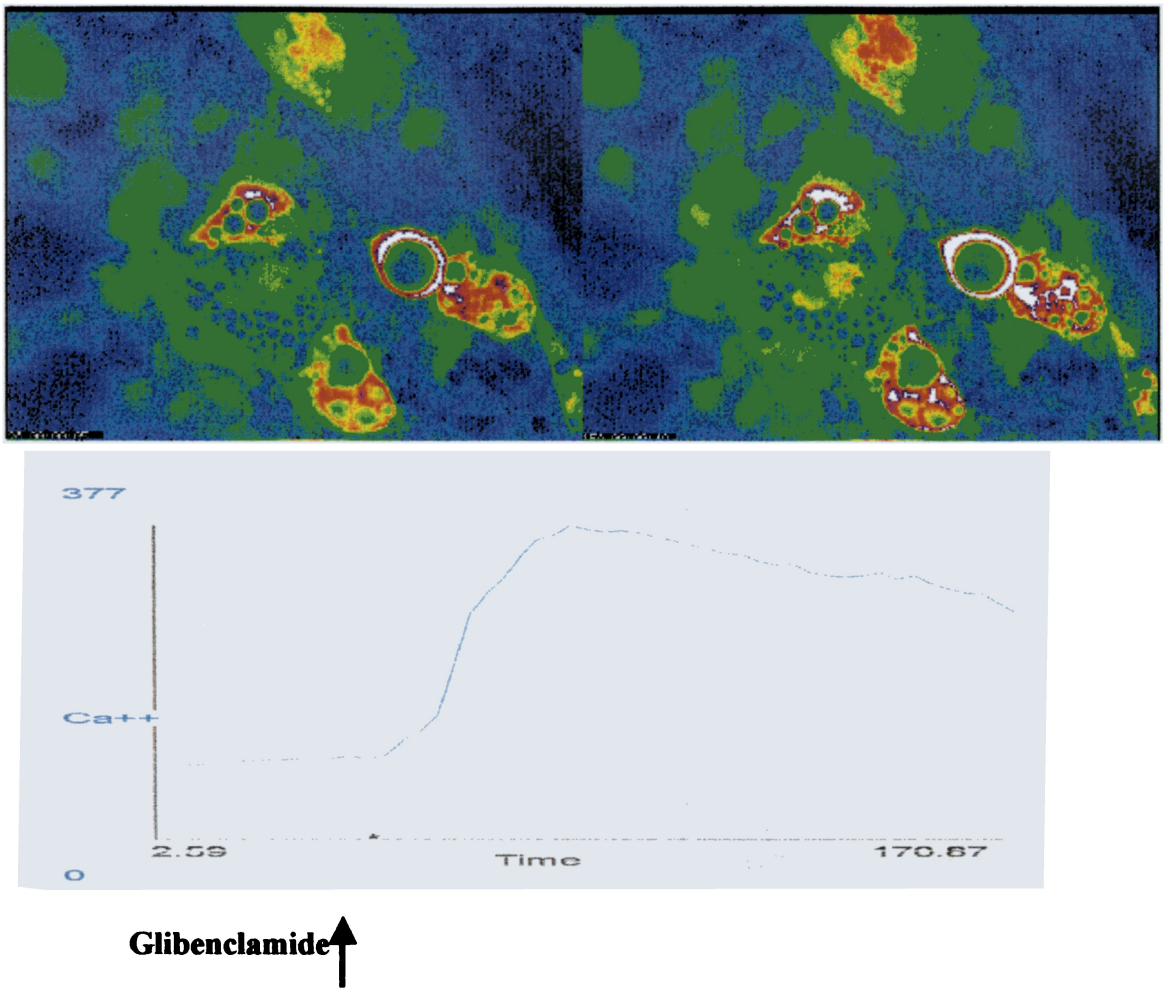


Fig 13. Effects of glibenclamide (10 μM) on stimulation of $[Ca^{2+}]_i$ in human adipocytes. $[Ca^{2+}]_i$ measurement was conducted as described in Materials and Methods. Glibenclamide was added at time designated by the arrow. The upper panel shows the cell fluorescence image in pseudocolor, which changed from green in baseline to yellow upon the addition of 10 μM glibenclamide, indicating an increase in $[Ca^{2+}]$i. Quantitation of this response by Ca^{2+} tracing demonstrated a 2.5 fold increase in $[Ca^{2+}]$i (lower panel).

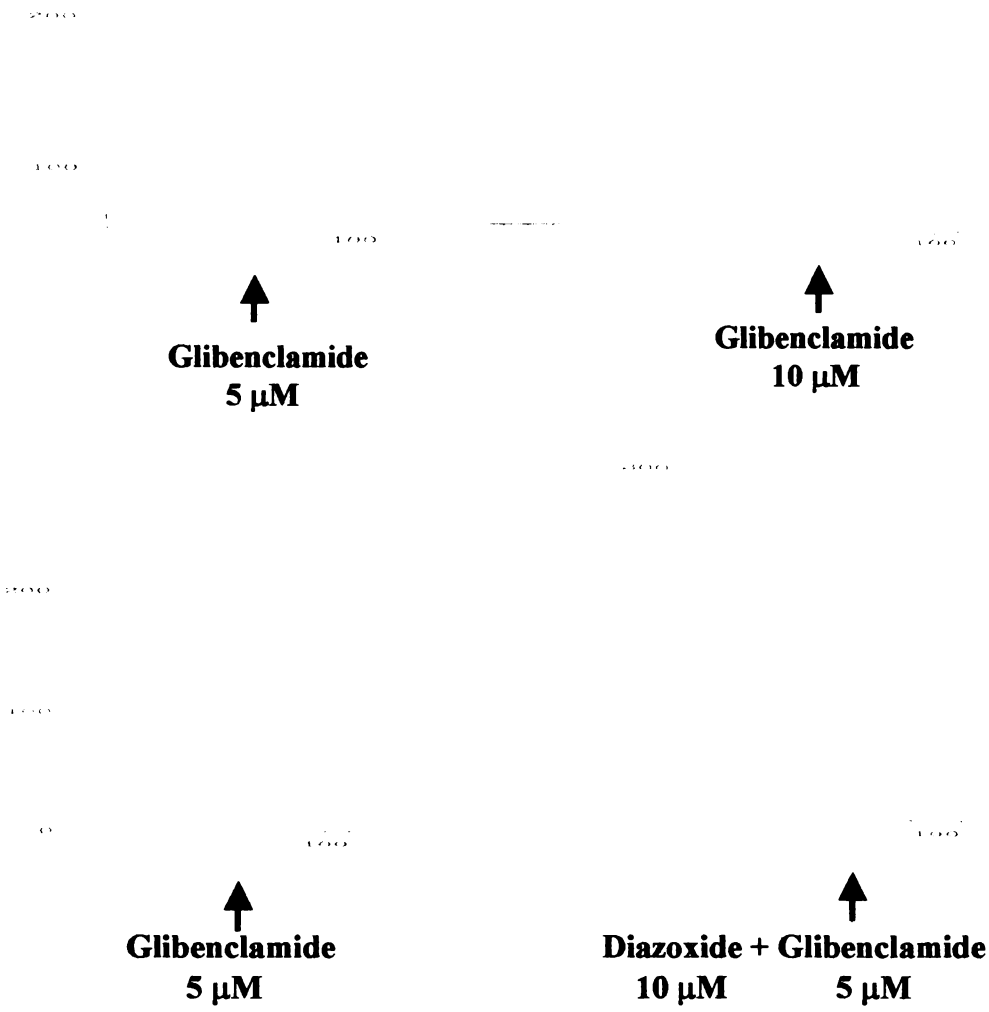


Figure 14. The dose-responsive effect of glibenclamide (5 μM and 10 μM) on $[Ca^{2+}]_i$ in human adipocytes. $[Ca^{2+}]_i$ measurement was conducted as described in Materials and Methods. Glibenclamide was added at time designated by the arrow. Upper panel: glibenclamide stimulates a dose-responsive increase in human adipocyte $[Ca^{2+}]_i$. Lower panel: pre-treatment of human adipocyte with diazoxide prevented glibenclamide-induced increases in $[Ca^{2+}]_i$.

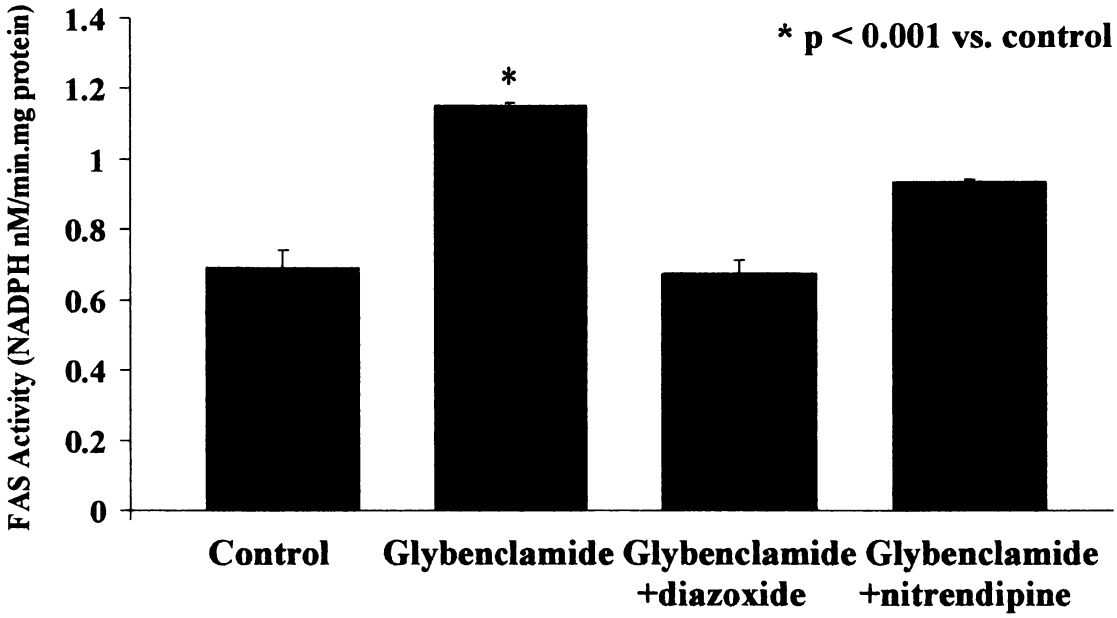


Fig 15. The effect of glibenclamide on FAS activity in human adipocytes. Human adipocytes were treated with 10 μM glibenclamide alone, 10 μM glibenclamide plus 10 μM diazoxide or 10 μM glibenclamide plus 30 nM nitrendipine for 48 hr. FAS activity was measured as described in Materials and Methods. * $p < 0.001$ vs. Control; n=8.

Similarly, 10 μM glibenclamide stimulated a 48% increase in GPDH activity (554.0±33.0 NADH nM/min/mg protein vs. 821.0±73.4 NADH nM/min/mg protein, $p<0.01$, **Fig. 16**), which was totally blocked by 10 μM diazoxide.

We next investigated the role of adipocyte SUR1 on lipolysis. 48 hr-treatment with 10 μM glibenclamide caused a 68% inhibition in lipolysis (0.193±0.050 NADH μM/mg protein vs. 0.061±0.009 NADH μM/mg protein, $p<0.05$, **Fig. 17**), which was recovered to 75% and 50% by diazoxide and nitrendipine, respectively.

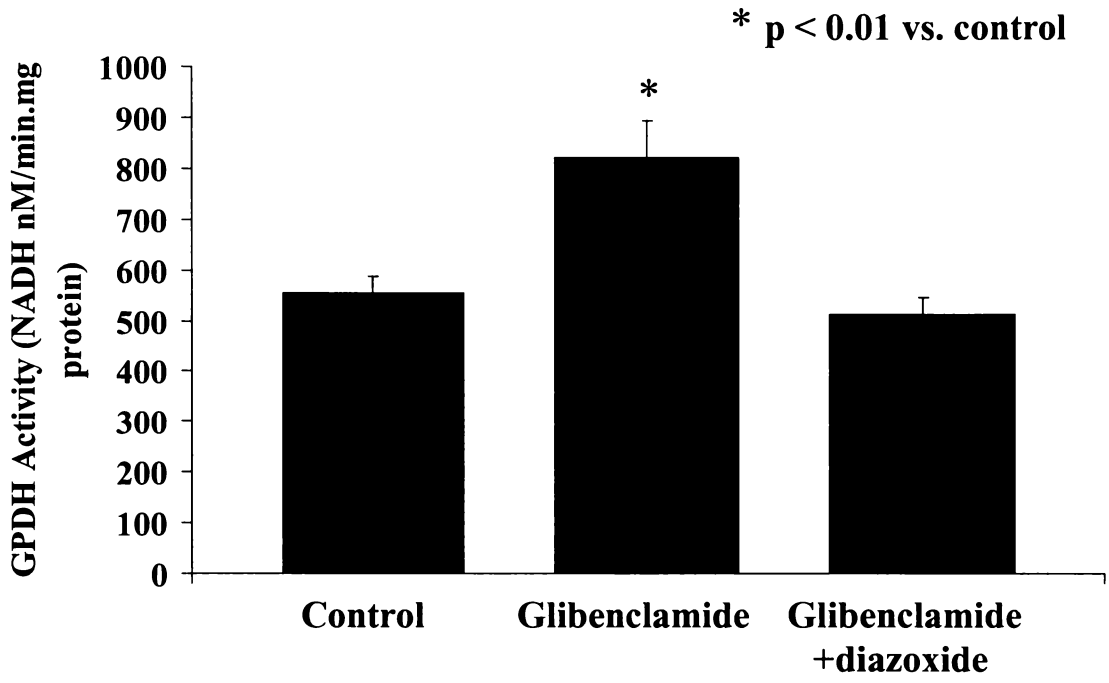


Fig 16. The effect of glibenclamide on GPDH activity in human adipocytes. Human adipocytes were treated with 10 μM glibenclamide or 10 μM glibenclamide plus 10 μM diazoxide for 48 hr. GPDH activity was measured as described in Materials and Methods. * $p < 0.01$ vs. Control; n=8.

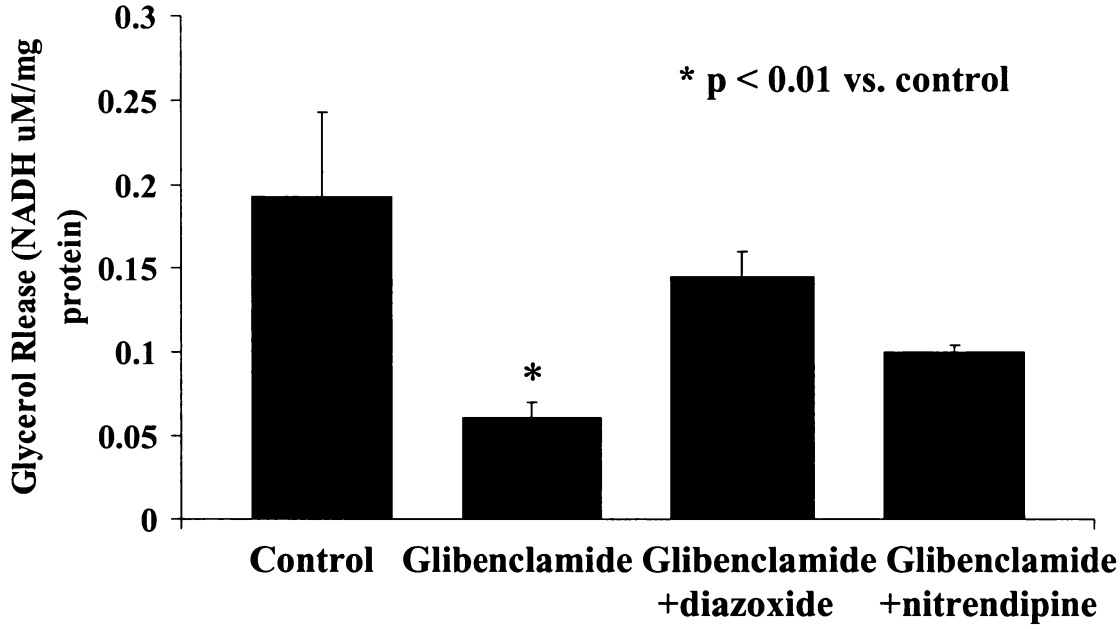


Fig 17. The effect of glibenclamide on lipolysis in human adipocytes. Human adipocytes were treated with 10 μM glibenclamide alone, 10 μM glibenclamide plus 10 μM diazoxide or 10 μM glibenclamide plus 30 μM nitrendipine for 48 hr. Glycerol release was determined as described in materials and methods. * $p < 0.01$ vs. Control; n=8.

V. DISCUSSION

We have demonstrated that the sulfonylurea receptor is expressed in human adipose tissue. We have found that this receptor responds similarly to the pancreatic form of the receptor to a typical agonist, glibenclamide. Importantly, this receptor mediates physiological response such as lipogenesis and lipolysis in the adipocytes. These results implicate the SUR in mediating energy homeostasis in an extrapancreatic tissue and suggest that the adipocyte SUR may be a suitable system for the identification of novel anti-obesity agents.

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) appears to play a key role in the metabolic disorders associated with obesity and insulin resistance and sustained high levels of $[Ca^{2+}]_i$ may contribute to these derangement (1-3). Several reports from our laboratory demonstrated that $[Ca^{2+}]_i$ modulates *de novo* lipogenesis and lipolysis in both rodent and human adipocytes (4-7). Sulfonylureas are a family of oral drugs used to promote insulin release in the treatment of type II diabetes. These insulin secretagogues bind to the sulfonylurea receptor (SUR) of pancreatic β cells and then block the conductance of an ATP-dependent potassium channel (K_{ATP} channel) (8). The attenuation of potassium current by blocking this channel depolarizes the β cells and thereby induces Ca^{2+} entry via L-type calcium channels (20), leading to increased insulin secretion (21). The SUR is a member of the ATP- binding cassette proteins, with multiple membrane-spanning domains and two nucleotide- binding folds (10). The SUR itself does not form the ion-conducting part of the K_{ATP} channel. However, SUR combines and interacts with an inward-rectifier K^+ channel (Kir6.2) to generate K_{ATP} channel (22). Thomas et al (23) reported that familial

persistent hyperinsulinemic hypoglycemia of infancy (PHHI), an autosomal recessive disorder characterized by unregulated insulin secretion, was associated with two separate SUR gene splice site mutations.

Patients treated with glibenclamide frequently experience weight gain as a side effect. Conversely, reports by Alemzadeh et al. (11-13) showed that diazoxide, which inhibits SUR by activating K_{ATP} channels, exerts an anti-obesity effect in obese Zucker rats and hyperinsulinemic obese humans. These effects of glibenclamide and diazoxide on body weight have been attributed to their effect on circulating insulin rather than to any direct effect on adipocytes (11-13). However, our data indicate that these effects may also be attributable in part to direct effects on the adipocyte SUR and K_{ATP} channel.

SUR agonists have previously been demonstrated to exert direct effects on adipocytes. Draznin et al. (2, 16) reported that glibenclamide increased $[Ca^{2+}]_i$ in isolated rat adipocytes in a dose dependent manner by promoting Ca^{2+} influx through voltage-dependent Ca^{2+} channels, while this effect was blocked by nitrendipine. Moreover, glibenclamide has been reported to potentiate peripheral insulin effect in isolated adipocytes (24, 27). These data support our observation of a direct effect of glibenclamide on adipocyte metabolism. In contrast, Rajan et al. (26) were unable to identify high affinity SUR in either isolated rat adipocytes or 3T3-L1 adipocytes. Moreover, they were unable to inhibit $^{86}Rb^{+}$ efflux (a surrogate for K_{ATP} channel activity) or increase $[Ca^{2+}]_i$ with glibenclamide. The reason for this discrepancy is not clear. However, several other investigators have reported both specific binding of sulfonylurea

and specific post-receptor effects in murine and rat adipocytes. For example, rat adipocytes exhibit specific, saturable glibenclamide binding (K_D of 1-3 μM), which is displaced by other sulfonylureas, and sulfonylurea treatment of isolated rat adipocytes potentiates insulin receptor of glucose transport (24, 25, 27). Further, Muller et al (28, 29) reported that glimepiride exhibits specific binding to 3T3-L1 and rat adipocytes, resulting in an insulin-mediated stimulation of glucose transport and non-oxidative glucose disposal. These effects were attributed to sulfonylurea-induced inhibition of cAMP level and protein kinase A activity. Thus, rodent adipocytes appear to exhibit SUR binding and functional response to this binding.

Similarly, data presented here demonstrate that SUR appears to play an important role in adipocyte metabolism. We have demonstrated that human adipocytes express SUR1 by both RT-PCR and northern blot analysis, while human preadipocytes do not. This provides a mechanism for SUR agonists and antagonists to modulate $[Ca^{2+}]_i$ levels. Indeed, glibenclamide elicited sustained increases in human adipocyte $[Ca^{2+}]_i$, while human preadipocytes were not responsive to glibenclamide. Similarly, glibenclamide (10 μM) caused a 67% increase in FAS activity, a 48% increase in GPDH activity and a 68% inhibition in lipolysis, while diazoxide (10 μM) completely prevented each of these effects. These observations are consistent with our previous reports that modulation of $[Ca^{2+}]_i$ in adipocytes may exert the coordinated regulation of lipogenesis and lipolysis (6, 7). Thus, these data suggest that previous observations of diazoxide-induced weight loss may be attributed to the effects of this compound on the adipocyte SUR rather than primarily to effects on insulin release.

Interestingly, the human chromosome region encoding both the SUR and the associated inward rectifying K^+ channel, 11p15.1 (30) also contains the human homolog of Tubby, a locus responsible for severe obesity in mice (31). Moreover, a significant association was recently reported between an exon 22 allelic variant of the SUR gene and obesity in French Caucasians (32). In addition, linkage between the SUR region and a subgroup of morbidly obese families was also noted (32). Thus, the SUR locus may contribute to genetic susceptibility to obese (32). Whether this contribution is based on alteration in insulin secretion or an extrapancreatic role of the SUR is unknown. However, our data suggest a potential role for the human adipocyte SUR in modulating energy storage and thereby potentially contribute to obesity.

We conclude that modulation of the human adipocyte SUR results in corresponding significant modulation of adipocyte energy storage. This provides a potential mechanism for previous observations of each sulfonylurea induced weight gain and diazoxide-induced weight loss. Our data suggest that diazoxide-induced antagonism of adipocyte lipogenesis and promotion of lipolysis, possibly coupled with suppression of insulin release, provide a likely mechanism for the anti-obesity effect of diazoxide. Accordingly, the human adipocyte SUR1 appears to represent an important target for further development of therapeutic intervention in obesity.

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PART 5

DIETARY CALCIUM ATTENUATES DIET-INDUCED OBESITY IN aP2-AGOUTI TRANSGENIC MICE

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I. ABSTRACT

Recent data from this laboratory demonstrate that increasing adipocyte intracellular Ca^{2+} results in a coordinated stimulation of lipogenesis and inhibition of lipolysis. We have also noted that increasing dietary calcium of obese patients for one-year resulted in a 4.9 kg loss of body fat ($p<0.01$). Accordingly, we tested the possibility that calcitrophic hormones may act on adipocytes to increase Ca^{2+} and lipid metabolism by measuring the effects of 1,25-$(OH)_2$-D in primary cultures of human adipocytes, and found significant, sustained increases in intracellular Ca^{2+} and a corresponding marked inhibition of lipolysis (EC_{50} ~50 pM; $p<0.001$), suggesting that dietary calcium could reduce adipocyte mass by suppressing 1,25-$(OH)_2$-D. To test this hypothesis, we placed transgenic mice expressing the *agouti* gene specifically in adipocytes on a low (0.4%) Ca/high fat/high sucrose diet either unsupplemented or with 25 or 50% of the protein replaced by non-fat dry milk or supplemented to 1.2% Ca with $CaCO_3$ for six weeks. Weight gain and fat pad mass were reduced by 26-39% by the three high calcium diets ($p<0.001$). The high calcium diets exerted a corresponding 51% inhibition of adipocyte fatty acid synthase expression and activity ($p<0.002$) and stimulation of lipolysis by 3.4-5.2-fold ($p<0.015$). Thus, increasing dietary calcium suppresses adipocyte intracellular Ca^{2+} and thereby modulates energy metabolism and attenuates obesity risk.

II. INTRODUCTION

Intracellular Ca^{2+} plays a key role in the metabolic disorders associated with obesity and insulin resistance (1-3). Recombinant agouti protein, an obesity gene product, stimulates Ca^{2+} influx in a variety of cells (4, 5). Agouti also stimulates the expression and activity of fatty acid synthase, a key enzyme in *de novo* lipogenesis, and inhibits basal and agonist-stimulated lipolysis in human and murine adipocytes via a Ca^{2+}-dependent mechanism (6, 7). These effects can be mimicked in the absence of agouti by either receptor or voltage-mediated Ca^{2+}channel activation and inhibited by Ca^{2+} channel antagonism (6-8), and agouti-induced obesity in transgenic mice was markedly attenuated by four-weeks of Ca^{2+} channel antagonism (9). Thus, increasing adipocyte intracellular Ca^{2+} appears to promote triglyceride storage in human adipocytes by exerting a coordinated control of lipogenesis and lipolysis, serving to simultaneously stimulate the former and inhibit the latter.

During the course of a previous, unrelated clinical trial investigating the antihypertensive effect of calcium in obese African Americans, we noted that increasing daily calcium intake from approximately 400 to 1,000 mg/day for one-year resulted in a 4.9 kg reduction in body fat. Although these data were inexplicable at the time, our recent data demonstrating regulation of adipocyte energy storage by intracellular Ca^{2+} leads to the proposal that increases in circulating calcitrophic hormones (1,25-$(OH)_2$-D and/or parathyroid hormone) secondary to low calcium diets stimulate adipocyte Ca^{2+} influx and thereby increase lipid storage. If this is correct, then increasing dietary calcium should

suppress calcitrophic hormones and thereby reduce adipocyte intracellular Ca^{2+} and lipid storage. The present study was conducted to address this concept.

III. MATERIALS AND METHODS

A. ISOLATION AND CULTURE OF HUMAN ADIPOCYTES

Human subcutaneous adipose tissue was obtained from patients with no known history of metabolic disorders undergoing abdominal plastic surgery. Adipocytes were isolated by washing, mincing, collagenase digestion and filtration as previously described (12, 17) and cultured in Dulbecco's Modified Eagle's Medium supplemented with 1% fetal bovine serum, penicillin (100 IU/mL), streptomycin (100 μg/mL) and gentamicin (50 μg/mL). Approval for this procedure was obtained from the Institutional Review Board for Human Subjects of the University of Tennessee. Cells were cultured in suspension and maintained in a thin layer at the top of the culture media, which was changed every day. Cells were studied ~72 hours following isolation, and were serum-starved prior to study.

B. ANIMALS AND DIETS

To evaluate the role of dietary calcium in regulating adiposity *in vivo*, transgenic mice expressing *agouti* specifically in adipocytes under the control of the aP2 promoter were studied. We have previously reported a characterization of these animals. Briefly, they exhibit a normal pattern of leptin expression and activity, similar to that found in humans, and exhibit a human pattern (adipocyte-specific) of *agouti* expression (10). We have found these mice to be useful models for diet-induced obesity in that they are not obese on a standard AIN-93G diet, but become obese in response to hyperinsulinemia induced by either insulin administration (10) or high sucrose diets (11). Male aP2-agouti transgenic animals from our colony were placed at six 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. They were randomized to four groups, as follows: The basal group continued this diet with no modifications; a high calcium group received the basal diet supplemented with $CaCO_3$ to increase dietary calcium three-fold, to 1.2%; a medium dairy diet, in which 25% of the protein was replaced by non-fat dry milk, and dietary calcium increased to 1.2%; and a high dairy group, in which 50% of the protein was replaced by non-fat dry milk, increasing calcium to 2.4%. Food intake and spillage was measured daily, and animals were weighed weekly. At the conclusion of the six-week feeding period, animals were euthanized by exsanguination under isofluorane anaesthesia, and blood was collected via cardiac puncture for glucose and insulin measurements. Fat pads (epididymal, perirenal, abdominal and subscapular) were dissected, immediately weighed, frozen in liquid nitrogen, and stored at –80°C. Fatty acid synthase activity and mRNA levels were measured in abdominal fat as described below.

C. CORE TEMPERATURE

Core temperature was used as an indirect metabolic index to determine if any reduction in efficiency of conversion of food energy to body weight was accompanied by increased thermogenesis (9). Temperature was measured via a thermocouple (Columbus Instruments, Columbus, Ohio). The probe was inserted a constant distance (1.8 cm) into the rectum of each animal. After stabilization (10 seconds), the temperature was recorded every 5 seconds for 30 seconds (9). All temperature measurements were made between 8:00 and 9:00 A.M.

D. INTRACELLULAR CALCIUM (HUMAN ADIPOCYTES)

Intracellular Ca^{2+} was determined flourometrically as previously described (17). Cells were washed with HEPES buffered salt solution, loaded with fura-2-acetoxymethyl ester (10 μM) for 45 minutes at 37° in the dark with continuous shaking. Cells were then rinsed three times, resuspended and intracellular Ca^{2+} measured using dual excitation (340 and 380 nm)/single emission (510 nm) fluorometry. Following the establishment of a stable baseline, the response to 1,25-$(OH)_2$-D or parathyroid hormone (10 pM - 100 nM) or their respective vehicles was determined. Digitonin (25 μM) and Tris/EGTA (100 mM, pH 8.7) were used to for calibration to calculate the final intracellular Ca^{2+}. (18).

E. LIPOLYSIS

Adipocytes were incubated for four hours in the presence or absence of forskolin (1 μM), and glycerol release into the culture medium was measured (18) to assess lipolysis. Glycerol release data was normalized for cellular protein.

F. FATTY ACID SYNTHASE ACTIVITY AND mRNA LEVELS

Immediately following sacrifice, adipose tissue was isolated and fatty acid synthase activity was measured in cytosolic extracts by measuring the oxidation rate of NADPH, as previously described (6, 9, 17). Enzyme activity was protein corrected using Coomassie Blue dye.

Total RNA was extracted by guanidine thiocyanate (GTC) lysis, cesium chloride density gradient, electrophoresed, subjected to northern blot analysis and hybridized with a radiolabelled rat cDNA probe for fatty acid synthase using standard methods (6, 12, 17). Autoradiographs were quantitated densitometrically, and all blots were stripped and re-probed with β-actin as a loading control.

G. STATISTICAL ANALYSIS

All data are expressed as mean ± SD. Data were evaluated for statistical significance by one-way analysis of variance or t-test, depending upon the number of comparisons made. All data sets with multiple comparisons were analyzed via ANOVA followed by separation of significantly different group means via test the least significant difference using SPSS-PC (v. 8.0).

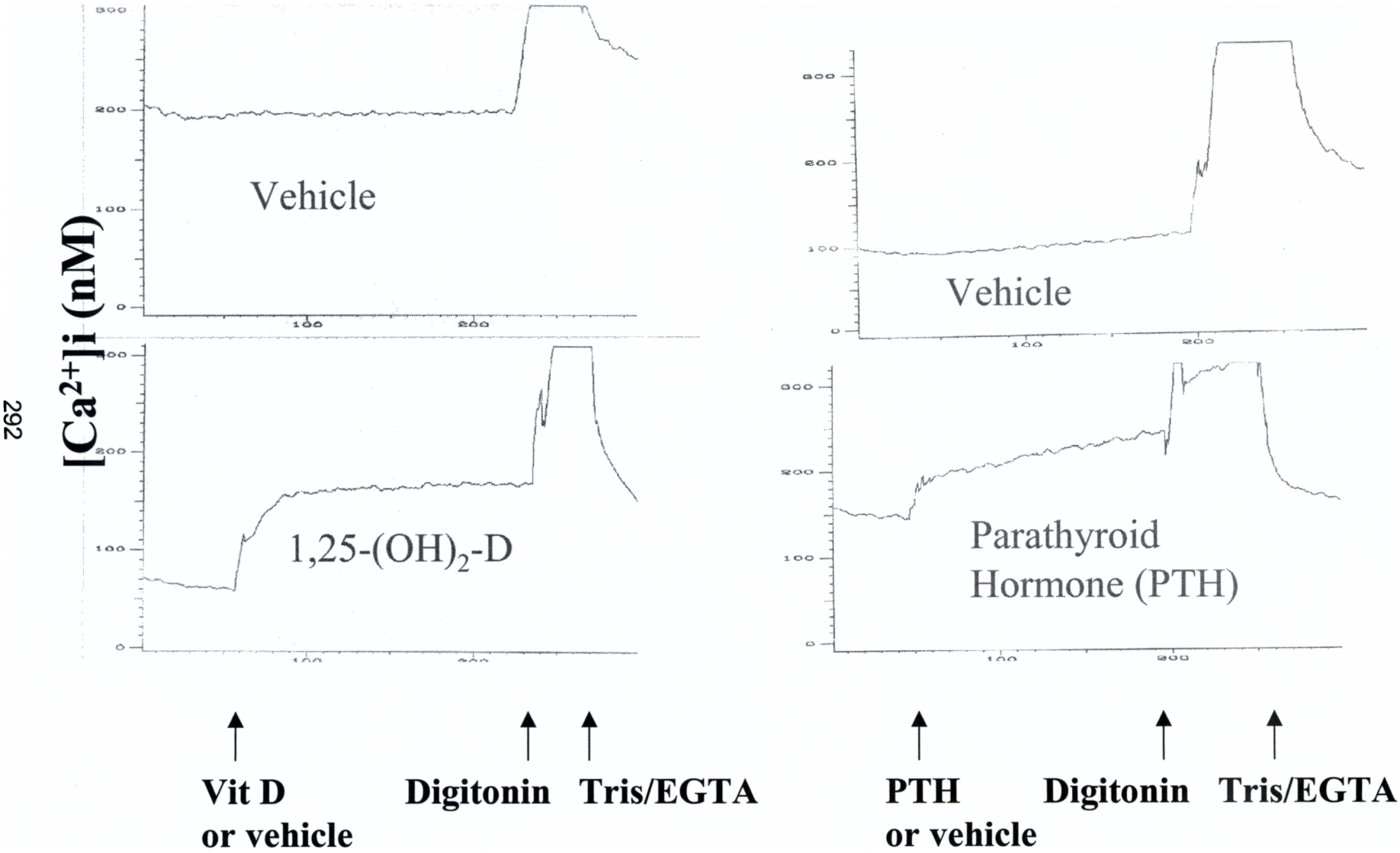
IV. RESULTS

Fig. 18 demonstrates that both 1,25-$(OH)_2$-D and parathyroid hormone (PTH) stimulate significant, sustained increases in intracellular Ca^{2+} in primary cultures of human adipocytes ($p<0.001$; $EC_{50} \sim 50$ pM for 1,25-$(OH)_2$-D and ~ 10 nM for PTH). 1,25-$(OH)_2$-D treatment also resulted in marked (83%) inhibition of forskolin-stimulated lipolysis ($p<0.001$) in human adipocytes (**Fig. 19**). PTH treatment exerted little effect on lipolysis (data not shown) despite its stimulation of an intracellular Ca^{2+} response, most likely as a result of an accompanying activation of adenylate cyclase.

Treatment of aP-2-transgenic-agouti mice with the high fat/high sucrose basal diet resulted in a weight gain of 24%, which was reduced by 26 and 29% by the high calcium and medium dairy diets, respectively ($p<0.04$) and further reduced by 39% by the high dairy diet ($p<0.04$; **Fig. 20**). These differences occurred despite the lack of any difference in food intake. Measurement of core temperature, an indirect metabolic index, reflected these observations, with ~0.5° C increases in core temperature in response to all three high calcium diets ($p<0.03$; **Fig. 21**). This increase, coupled with the lack of difference in food intake, is indicative of a shift in efficiency of energy metabolism from energy storage to thermogenesis.

This shift in energy metabolism was evident in studies of fatty acid synthase, a key enzyme in *de novo* lipogenesis which is highly sensitive to regulation by nutrients and hormones (12). The basal diet caused a 2.6-fold increase in fatty acid synthase activity, and this effect was markedly attenuated by all three high calcium diets ($p<0.002$; **Fig 22**

Fig 18. Effects of 1,25-$(OH)_2$-D (left panel) and parathyroid hormone (right panel) on intracellular calcium in human adipocytes.


[Ca2+]i (nM)
Vehicle
1,25-(OH)2-D
Vehicle
Parathyroid Hormone (PTH)
Vit D or vehicle
Digitonin
Tris/EGTA
PTH or vehicle
Digitonin
Tris/EGTA

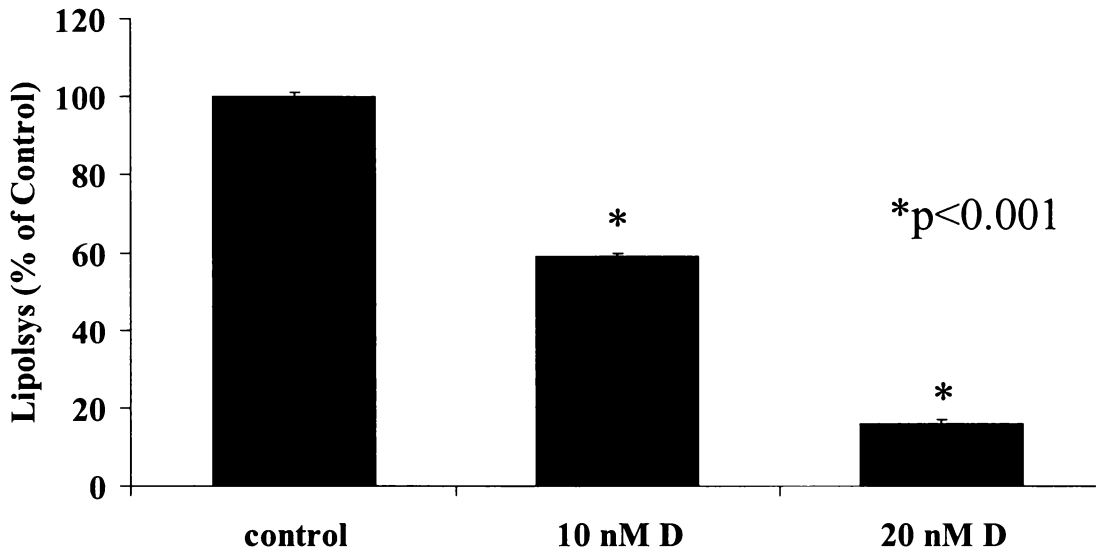


Fig 19. Effects of 1, 25-$(OH)_2$-D on forskolin-stimulated lipolysis in human adipocytes. Forskolin treatment resulted in a two fold increase in glycerol release; data shown are normalized to 100% for the forskolin-treated group. (n=6/group; $p<0.001$)

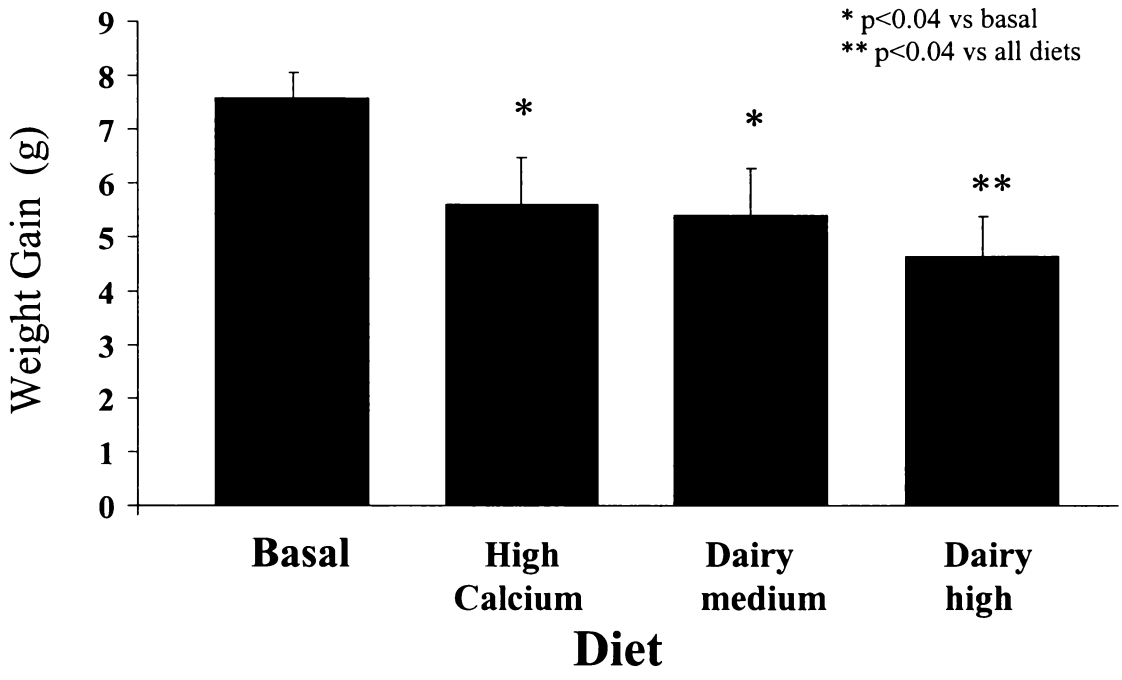


Fig 20. Effects of calcium and dairy products on 6-week weight gain in transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter.

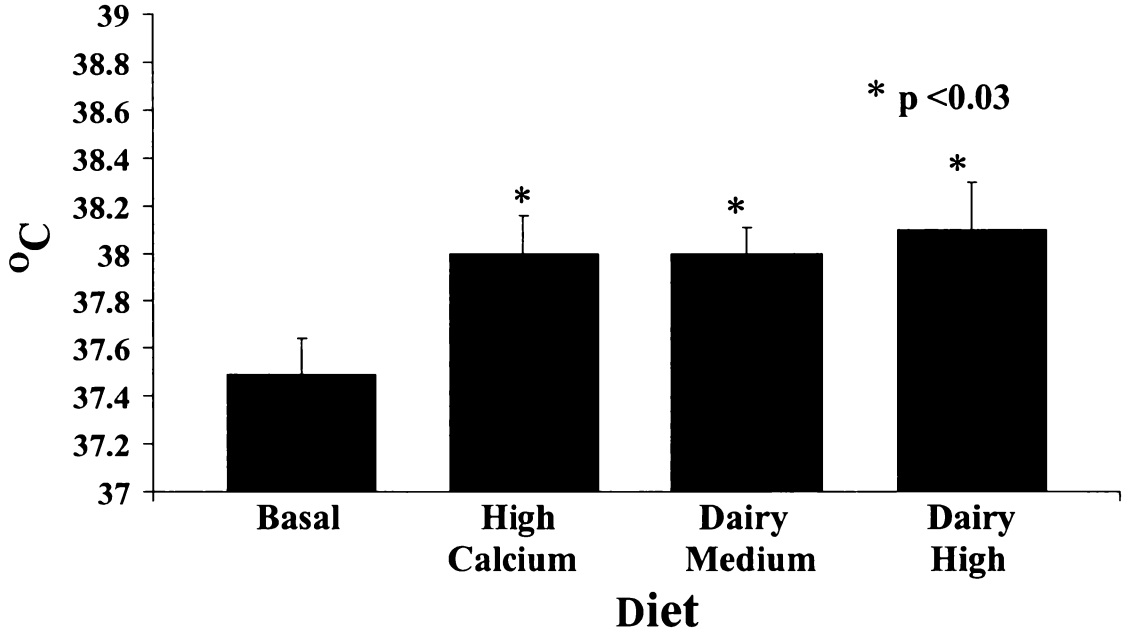
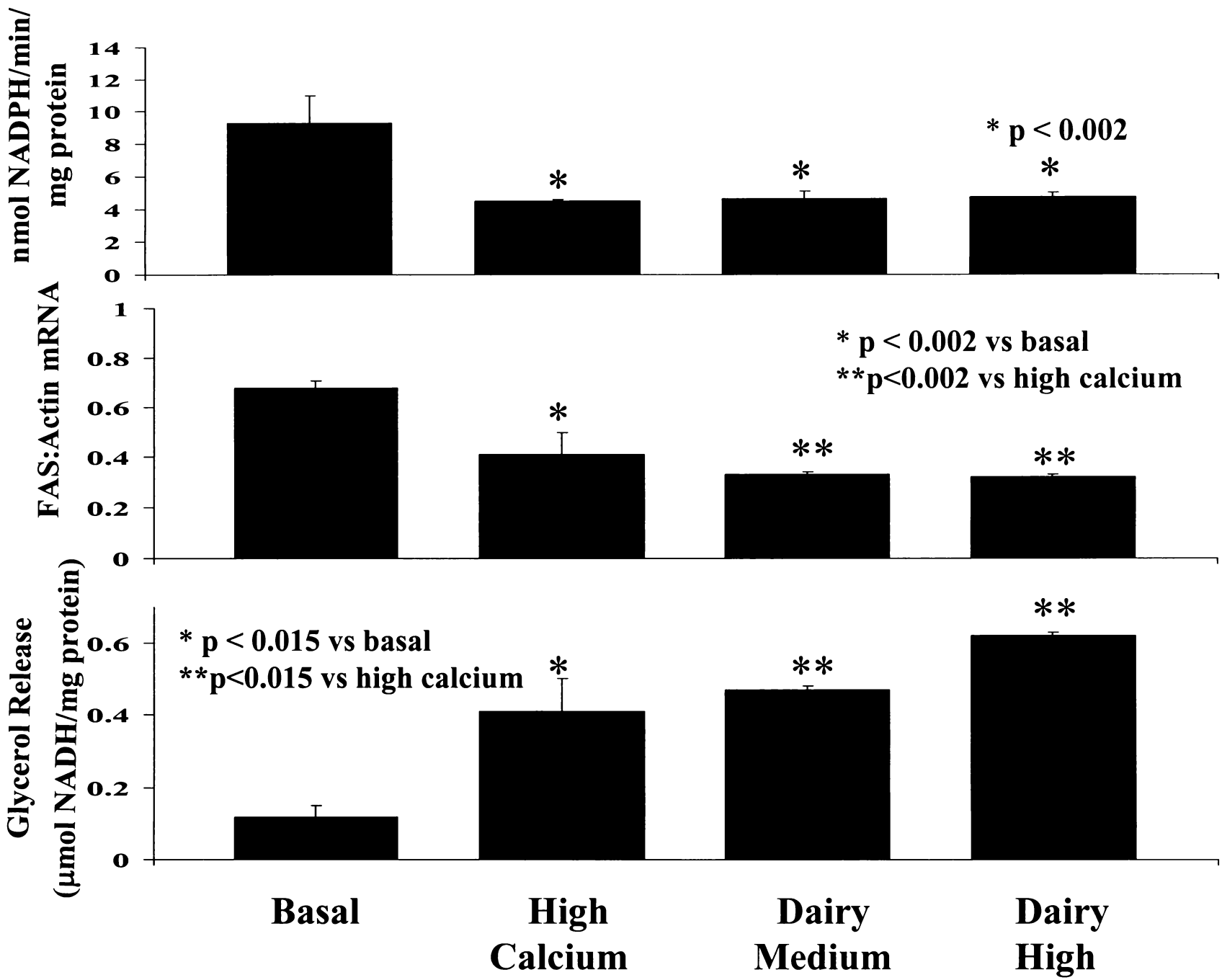


Fig 21. Effects of calcium and dairy products on core-temperature in transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter.

Fig 22. Effects of calcium and dairy products on adipocyte fatty acid synthase activity (upper panel), fatty acid synthase mRNA (middle panel; data are expressed as fatty acid synthase (FAS):actin ratio.) and lipolysis in transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter. Data are expressed as mean $\pm$ standard deviation (n = 10/group). Statistical significance is as indicated in each panel.


nmol NADPH/min/ mg protein
14
12
10
8
6
4
2
0
* p < 0.002
*
*
*
FAS:Actin mRNA
1
0.8
0.6
0.4
0.2
0
* p < 0.002 vs basal
**p<0.002 vs high calcium
*
**
**
Glycerol Release (µmol NADH/mg protein)
0.6
0.4
0.2
0
* p < 0.015 vs basal
**p<0.015 vs high calcium
*
**
**
Basal
High Calcium
Dairy Medium
Dairy High

upper). The diets caused corresponding decreases in adipocyte fatty acid synthase mRNA, with a 27% reduction on the high calcium diet and a 51% reduction on the medium and high dairy diets ($p<0.01$; **Fig. 22** middle). Adipocyte lipolysis responded to dietary manipulations in an inverse fashion to the fatty acid synthase responses. The basal diet caused a marked (67%) suppression of lipolysis ($p<0.0001$); however, lipolysis was stimulated 3.4-fold to 5.2-fold by the high calcium diets ($p<0.015$; **Fig. 22** bottom), with greater effects from the high dairy diets than from the high calcium diet. Assessment of fat pad mass following six weeks of dietary treatment provide further support for these findings. **Table 1** demonstrates that all three high calcium diets caused a 36% reduction the mass of the epididymal, abdominal, perirenal and subscapular adipose tissue compartments ($p<0.001$). Epidymal and subscapular fat pad mass was reduced by ~50% by all three diets, while the abdominal fat pads exhibited greater decreases on the medium and high dairy diets than on the high calcium diet ($p<0.001$; **Table 1**).

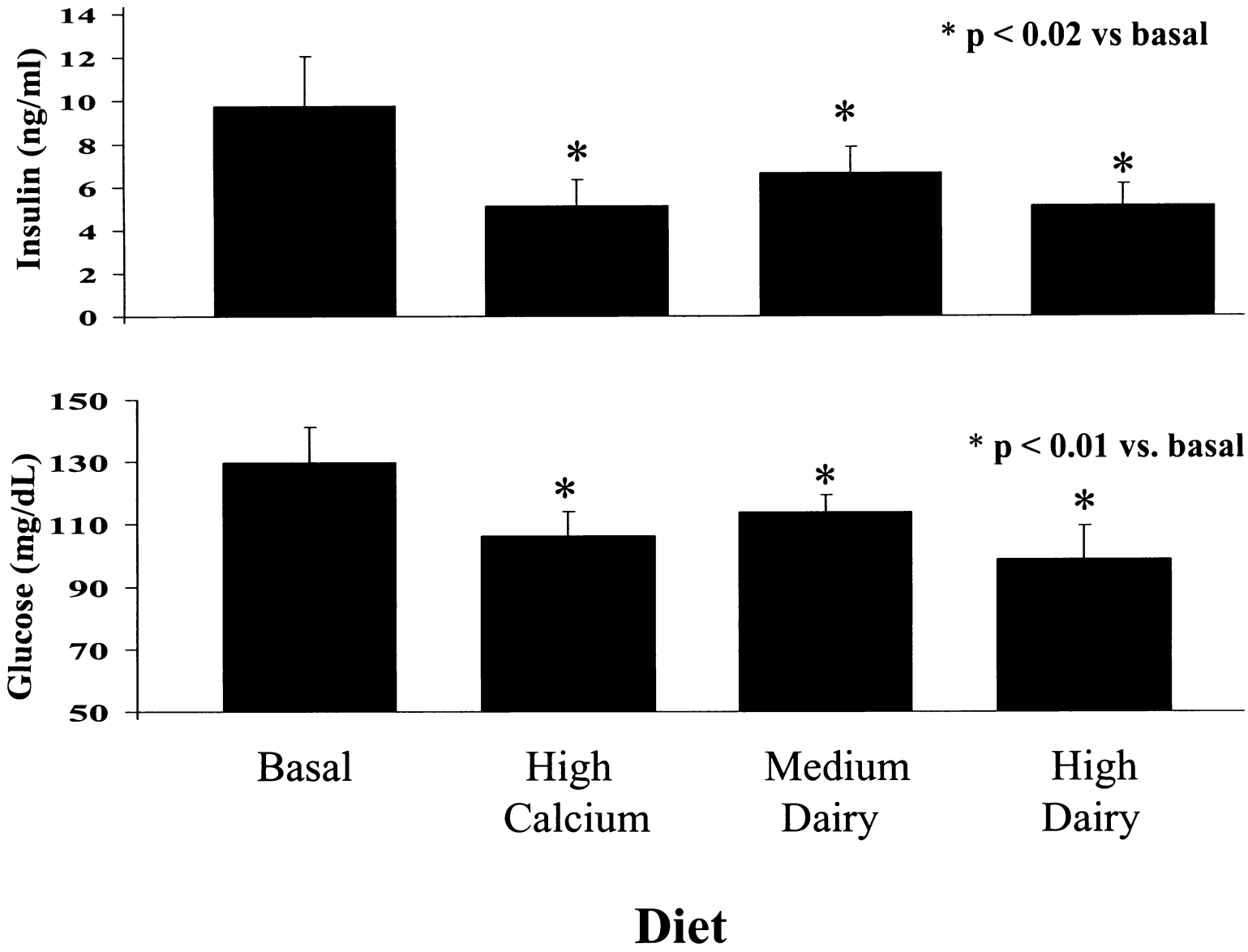
Serial measurements of plasma glucose and insulin demonstrate a diabetogenic effect of the basal high fat/high sucrose/low calcium diet, with an increase in fasting glucose from 98 ± 10 to 130 ± 11 mg/dL ($p<0.02$) and a corresponding degree of compensatory hyperinsulinemia. These increases were attenuated by the high calcium and medium dairy diets and prevented by the high dairy diet (**Fig. 23**).

Table 1. Effects of Calcium and Dairy Products on Fat Pad Mass in Transgenic Mice Expressing *Agouti* in Adipose Tissue Under Control of the aP2 Promoter

		Basal	High Calcium	Medium Dairy	High Dairy
Abdominal	(g)	2.239±0.109	1.807±0.082*	1.661±0.127**	1.680±0.113**
Perirenal	(g)	1.675±0.124	1.271±0.098*	1.172±0.123*	1.052±0.094*
Epididymal	(g)	0.198±0.036	0.110±0.017*	0.110±0.014*	0.097±0.015*
Subscapular	(g)	1.592±0.318	0.680±0.069*	0.663±0.068*	0.639±0.087*
Sum[2]	(g)	5.703±0.548	3.649±0.238*	3.705±0.276*	3.787±0.251*

[1]Data expressed as mean ± standard deviation
[2]Sum of Abdominal, perirenal, epididymal and subscapular fat pads
*$p<0.001$ vs. basal
**$p<0.001$ versus high calcium

Fig 23. Effects of calcium and dairy products on fasting plasma glucose and insulin levels in transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter. Top panel depicts insulin and the bottom panel depicts glucose.


Insulin (ng/ml)
14
12
10
8
6
4
2
0
* p < 0.02 vs basal
Glucose (mg/dL)
150
130
110
90
70
50
* p < 0.01 vs. basal
Basal
High Calcium
Medium Dairy
High Dairy
Diet

V. DISCUSSION

We have previously shown that *agouti*, an obesity gene expressed in human adipocytes, stimulates Ca^{2+} influx (4, 5) 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 (6, 7). Interestingly, the C-terminus of agouti protein, which retains functional activity in an *in vitro* assay system (20), bears a striking spatial homology in number and spacing of cysteine residues to spider and snail venoms (ω-conotoxins, plectoxins), which target Ca^{2+} channels (21). Moreover, this agouti modulation of adipocyte lipid metabolism is fully mimicked by Ca^{2+} channel agonists and inhibited by Ca^{2+} channel antagonists (6-8). In addition, treating transgenic mice overexpressing *agouti* with a Ca^{2+} channel antagonist (nifedipine) for four weeks resulted in significant decreases in adipocyte lipogenesis and an 18% reduction in adipose tissue mass (9). Thus adipocyte Ca^{2+} modulates energy storage, suggesting that adipocyte Ca^{2+} is a logical target for pharmacological and/or nutritional regulation of adiposity.

Dietary calcium modulation of intracellular calcium, mediated by suppression of calcitrophic hormones, has previously been demonstrated to attenuate the risk of hypertension, and possibly type II diabetes as well (23, 24). Intracellular calcium plays a key role in multiple related metabolic disorders, including hypertension, cardiac hypertrophy, insulin resistance and hyperinsulinemia, all of which are commonly associated with obesity. It is now well recognized that these are not merely co-morbid factors which occur secondary to obesity, but rather are part of an integrated metabolic

syndrome referred to as "Syndrome X " (25), "plurimetabolic syndrome", the "deadly quartet" (obesity, hypertriglcyeridemia, hypertension and insulin resistance/hyperinsulinemia), or "generalized cardiovascular and metabolic disease" (23,24). Regardless of terminology, a growing body of evidence suggests that these conditions are all characterized by an underlying impairment in intracellular Ca^{2+} (1-3, 23, 24, 26-28). Indeed, Resnick (23) has proposed a unifying "ionic hypothesis", in which the varying metabolic abnormalities associated with "Synrome X" represent different tissue-specific manifestations of a cellular lesion which is characterized, in part, by elevations in steady-state intracellular Ca^{2+} levels. Consistent with this concept, correcting elevations in intracellular Ca^{2+} results in clinical improvements in blood pressure, insulin resistance, platelet aggregation, and left ventricular hypertrophy (23). Our previous studies of the mechanisms of agouti-induced obesity indicate that obesity may also be, in part, a manifestation of a "lesion" in intracellular Ca^{2+} regulation (4-7, 9), and data from the present study lend further support for this hypothesis.

In order for adipocyte Ca^{2+} to serve as a logical target for nutritional regulation, human adipocytes would need to exhibit responsiveness to calcitrophic hormones. Our observation in the present study that human adipocytes respond to both parathyroid hormone and 1,25-$(OH)_2$-D with dose-responsive increases in intracellular Ca^{2+} suggests that low Ca^{2+} diets, by virtue of stimulating a calcitrophic hormone response, will increase adipocyte intracellular Ca^{2+}, while higher calcium diets will suppress this response. Accordingly, a coordinated down-regulation of lipogenesis and up-regulation of lipolysis would be predicted to result from increasing dietary calcium. Data from the

present study of transgenic mice over-expressing *agouti* in adipose tissue to mimic the human pattern of expression support this prediction, as the obesity promoting effects of the lard/sucrose-based diet were significantly attenuated on the high calcium and high dairy diets. These data are further supported by the measurements of fatty acid synthase mRNA and activity, as well as the lipolysis data. Thus, these data demonstrate that increasing dietary calcium attenuates diet-induced adiposity by modulating adipocyte intracellular Ca^{2+} and thereby coordinately regulating lipogenesis and lipolysis (6-9).

Dairy and elemental sources calcium exerted qualitatively comparable effects; however, calcium in the form of dairy exerted a greater effect on attenuating fat deposition than a comparable quantity of elemental calcium. Consistent with this, a recent randomized clinical trial demonstrated a markedly greater weight loss (7.0 vs 1.7 kg) in patients maintained on a milk-based diet for 16 weeks versus those maintained on conventional hypocaloric diet at the same level of energy intake (16). Although this difference was attributed to the novelty of the milk-based diet possibly contributing to a greater level of compliance, data presented herein suggest that this effect may also be attributable to suppression of 1,25-$(OH)_2$-D and adipocyte Ca^{2+}, with a consequent reduction in the efficiency of energy utilization. Accordingly, dietary calcium appears to modulate the efficiency of energy utilization, with low calcium diets favoring increased efficiency of energy storage and higher calcium diets reducing energy efficiency and instead favoring increased thermogenesis. This concept is further supported by our observation of reduced energy efficiency and increased core-temperature in the transgenic mice fed the higher calcium diets.

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PART 6

DIETARY CALCIUM ACCELERATES BODY WEIGHT LOSS IN ENERGY-RESTRICTED AP2-AGOUTI TRANSGENIC MICE

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I. ABSTRACT

We have previously demonstrated a regulatory role for intracellular Ca^{2+} ($[Ca^{2+}]_i$) in adipocyte lipogenesis and lipolysis, and recently reported that 1,25-$(OH)_2$-D increases adipocyte $[Ca^{2+}]_i$, causing increased lipogenesis and decreased lipolysis. We have now tested the hypothesis that suppressing 1,25-$(OH)_2$-D by increasing dietary Calcium will suppress adipocyte $[Ca^{2+}]_i$, thereby facilitating weight loss by stimulating lipolysis and inhibiting lipogenesis in calorically (Kcal)-restricted (70% of ad lib) aP2-agouti transgenic (aP2-a) mice. Mice (aP2-a) exhibiting a pattern of obesity gene expression similar to humans were fed a low (0.4%) Ca/high fat/high sucrose diet for 6-weeks, resulting in a 27% and 2 fold increase in body weight and total fat pad mass, respectively, with a 2-fold increase in adipocyte $[Ca^{2+}]_i$ ($p<0.001$). Mice were then either maintained on the same low Ca basal diet *ad lib* or Kcal-restricted (70% of *ad lib*) on this diet either unsupplemented (basal) or with 25 or 50% of the protein replaced by non-fat dry milk (medium or high) dairy or supplemented with $CaCO_3$ to 1.2% Ca for 6 weeks. Adipocyte $[Ca^{2+}]_i$ was unaffected by Kcal restriction but was markedly reduced by all three high Ca diets (290 vs. 130 nM, $p<0.001$). Body weight was reduced by 11, 19, 25, and 29% by the restricted basal, high Ca, medium dairy and high dairy diets, with corresponding decreases in fat pad mass of 8, 42, 60, and 69% vs. *ad lib* basal ($p<0.001$), respectively. The high Ca diets caused similar decreases in fatty acid synthase activity 35-63%, $p<0.001$), increases in lipolysis (2-3 fold, $p<0.001$). All three high Ca diets increased adipocyte uncoupling protein 2 expression ~2 fold ($p<0.02$), with a corresponding increase in core temperature (0.48-0.67 ^{0}C, $p<0.05$). Thus, high Ca diets suppress

adipocyte $[Ca^{2+}]_i$ and thereby reduce energy storage and increase thermogenesis during Kcal restriction.

II. INTRODUCTION

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays a key role in the metabolic disorders associated with obesity and insulin resistance (1-3). Recombinant agouti protein, an obesity gene product, causes a dose responsive increase in $[Ca^{2+}]_i$ in a variety of cells, including both human and murine adipocytes (4, 5). Agouti protein has also been shown to stimulate the expression and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis, and inhibit basal and agonist-simulated lipolysis in a Ca^{2+} dependent manner (6, 7). These effects of agouti in promoting lipogenesis and suppressing lipolysis can be mimicked by Ca^{2+} channel activation and reversed by Ca^{2+} channel antagonism (6, 7). Therefore, increasing adipocyte $[Ca^{2+}]_i$ appears to promote triglyceride accumulation in adipocytes by exerting a coordinated control over stimulation of lipogenesis and inhibition of lipolysis, thereby resulting in an obese phenotype.

Recent data from our laboratory demonstrate that 1,25-dihydroxyvitamin D (1,25-$(OH)_2$-D) causes a significant, sustained increase in $[Ca^{2+}]_i$ in primary cultured human adipocytes and a corresponding inhibition of lipolysis (8). We also reported that inhibition of 1,25-$(OH)_2$-D by increasing dietary calcium suppresses $[Ca^{2+}]_i$ and subsequently attenuates diet-induced adiposity by simultaneously stimulating lipolysis and inhibiting lipogenesis (8). Consistent with this, increasing dietary calcium from 400 to 1000 mg/day in humans for 1-year results in a 4.9 kg body fat reduction (8), suggesting that dietary calcium may not only attenuate the diet-induced development of adiposity but also promote weight loss in established obesity.

Accordingly, the present study was conducted to test the hypothesis that suppressing 1,25-$(OH)_2$-D by increasing dietary calcium inhibits adipocyte $[Ca^{2+}]_i$, and thereby accelerates weight loss secondary to caloric restriction in aP2-agouti transgenic mice exhibiting a pattern of obesity gene expression similar to humans.

III. MATERIALS AND METHODS

A. ANIMALS AND DIETS

aP2-agouti transgenic (aP2-a) mice from our breeding colony were used as an animal model in this study. This study was approved from an ethical standpoint by the University of Tennessee Institutional Animal Care and Use Committee. The characterization of these animals has been described previously (9). These transgenic mice express normal agouti protein specifically in adipose tissue under the control of the aP2 promoter (9), similar to the adipocyte-specific human pattern of agouti expression. These mice are useful animal model for the study of diet-induced obesity study, as they are not obese on a standard AIN 93-G diet, but become obese in response to hyperinsulinemia induced by either exogenous insulin administration (9) or high sucrose diet (10).

This study was divided into two 6-week stages. In the first stage, 60 6-week-old male aP2 mice were placed on a modified AIN 93-G diet with suboptimal calcium (0.4%), sucrose as the sole carbohydrate source and lard added to increase fat to 25% of energy. To evaluate this low calcium/high fat/high sucrose diet-induced obesity in these animals, we monitored the body weight every 5 days and euthanized 8 representative mice for measurement of fat pad mass and adipocyte $[Ca^{2+}]_i$ at the end of first 6 week-basal diet feeding. In the second 6-week stage, the rest of these diet-induced obese transgenic mice, weighing from 45.44 ± 0.81 g, were randomly assigned to five groups. One group was continued *ad lib* on the same low calcium (0.4%) diet with no modification, while the other four groups were maintained with energy restriction (70% of *ad lib*) as follows. The

mice in basal restriction group were placed on the basal low calcium (0.4%) diet with energy-restriction. A high calcium energy restricted group received the basal diet supplemented with calcium increased to 1.2%. Two additional groups, termed medium dairy and high dairy, were fed modified basal diet in which either 25 or 50% of protein was replaced by non-fat dry milk, with total dietary calcium increased to 1.2 or 2.4%, respectively. Diet was administrated daily and body weight was monitored every five days. At the end of second stage, all mice were euthanized with beuthanasia (concentrated pentobarbital with phenytoin) and blood was obtained via cardiac puncture for insulin and glucose measurement. Fat pads (epididymal, perirenal, abdominal and subscapular) were dissected, immediately weighed, frozen in liquid nitrogen, and stored at –80 ^{0}C. Fatty acid synthase activity and mRNA levels were measured in abdominal fat as described below.

B. CORE TEMPERATURE

Core temperature was used as an indirect metabolic index to determine if dietary calcium regulates energy metabolism associated with increased thermogenesis, an important contribution to energy expenditure. Temperature was measured via a thermocouple (Columbus Instruments, Columbus, Ohio) weekly (11). The probe was inserted a constant distantance (1.8 cm) into the rectum of each mouse. After stabilization (10 seconds), the temperature was recorded every 5 seconds for 30 seconds. All core temperature measurements were performed between 8:00 and 9:00 A.M.

C. MOUSE ADIPOCYTE INTRACELLULAR Ca^{2+} MEASUREMENT

Isolated mouse adipocytes were prepared from mouse abdominal fat depots as previously described (12) with slight modification. Briefly, adipose tissue was first washed several times with Hank's Balanced Salt Solution, minced into small pieces and digested with 0.8 mg/ml type I collagenase in a shaking water bath at 37 ^{0}C for 30 min. Adipocytes were then filtered through a 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 two hours for cell recovery.

$[Ca^{2+}]_i$ in isolated mouse adipocytes was measured using a fura-2 dual wavelength fluorescence imaging system (13). Prior to $[Ca^{2+}]$i measurement, adipocytes were pre-incubated in serum-free medium for 2 hrs and rinsed with Hepes Balanced Salt Solution (HBSS) containing the following components (in mmol/L): NaCl 138, $CaCl_2$ 1.8, $MgSO_4$ 0.8, NaH_2PO_4 0.9, $NaHCO_3$ 4, glucose 5, glutamine 6, Hepes 20, and bovine serum albumin 1%. Adipocytes were loaded with fura-2 acetoxymethyl ester (AM) (10 μmol/L) in the same buffer for 1 hr at 37 ^{0}C in a dark incubator with 5% CO_2. To remove extracellular dye, adipocytes were rinsed with HBSS 3 times and then post-incubated at room temperature for an additional 30 min for complete hydrolysis of cytoplasmic fura-2 AM. A thin layer of adipocytes was plated in 35 mm dished with glass coverslips (P35G-0-14-C, MatTek Corporation). 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

380nM with an emission wavelength of 520 nM. $[Ca^{2+}]_i$ was calculated using a ratio equation as described previously(14).

D. FATTY ACID SYNTHASE ACTIVITY ASSAY

FAS activity was determined spectrophotometrically in crude cytosolic extracts of mouse adipose tissue as previously described (15). Mouse abdominal fat pads were homogenized in 250 mmol/L sucrose solution containing 1 mmol/L ethylenediamine-tetraacticacid (EDTA), 1 mmol/L dithiothreitol (DTT), and 100 μmol/L phenylmethylsulfonyl fluoride (PMSF) (pH 7.4). Homogenate was centrifuged at 18,500 X g for 1 hr and the infranatant was used for measuring oxidation rate of NADPH.

E. LIPOLYSIS ASSAY

Following the sacrifice, mouse perirenal adipose tissue was immediately dissected and incubated for four hours. Glycerol released into the culture medium was determined as an indicator of lipolysis (7), using a one-step enzymatic fluorometric method as previously described (16).

F. NORTHERN BLOT ANALYSIS

Northern blot analysis was conducted as described (13). Total RNA from mouse abdominal adipose tissue was extracted using $CsCl_2$ density centrifugation, run in 1% agarose gel and transferred to nylon membrane (New England Nuclear, Boston, MA). The membrane was hybridized with uncoupling protein 2 (UCP2) or FAS cDNA probes that were radiolabeled 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 ^{0}C. Finally, the membrane was exposed to X-ray film (New England Nuclear, Boston, MA) at –80 ^{0}C. All membranes were stripped and reprobed with β-actin as loading control.

G. STATISTICAL ANALYSIS

All data are expressed as mean ± SE. Data are 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 using SPSS (SPSS Inc, Chicago, IL). A p value < 0.05 is considered significant.

IV. RESULTS

Administration of the low calcium (0.4%), high-fat, high sucrose diet to aP2-a mice for 6 weeks resulted in a ~100% increase in adipocyte $[Ca^{2+}]_i$ (128 ± 18 vs. 267 ± 15 nM, $p < 0.001$, lower panel, **Fig 24**), with a corresponding body weight gain of 29% ($p < 0.001$, upper panel, **Fig 24**) and two fold increase in total fat pad mass ($p < 0.001$, middle panel, **Fig 24**), demonstrating that diet-induced dysregulation of adipocyte $[Ca^{2+}]_i$ is associated with increased adiposity in aP2-a mice.

All three calcium diets, including high calcium diet (1.2% Ca derived from $CaCO_3$), medium dairy diet (1.2% Ca derived from non-fat dry milk replacing 25% of protein) and high dairy diet (2.4% Ca derived from non-fat dry milk replacing 50% of protein), caused a 50% decrease in adipocyte $[Ca^{2+}]_i$ ($p < 0.001$, **Fig 25**, lower panel), while $[Ca^{2+}]_i$ in adipocytes from mice maintained on the Kcal-restricted basal low calcium diet remained at the same elevated level as that of *ad lib* animals

Energy restriction resulted in a body weight loss by 11% ($p < 0.001$, **Fig 25**, upper panel), compared to *ad lib* group. However, markedly greater weight reductions of 19, 25, 29% were observed in the high calcium, medium and high dairy groups, respectively ($p < 0.01$ vs. basal energy-restricted group, **Fig 25**). Consistent with this, energy restriction caused an only 8% decrease in fat pad mass (not significant), compared to basal diet *ad lib* group, while the high calcium diet caused a 42% decrease ($p < 0.001$, **Fig 25**, middle panel), which was further reduced by 60 and 69% by the medium and high dairy diets ($p < 0.001$ vs. basal energy-restricted group), respectively.

Fig 24. Effects of 6-week administration of basal low Calcium (0.4%)/high fat/ high sucrose diet on body weight (top panel), total fat pad mass (middle panel) and basal adipocyte $[Ca^{2+}]_i$ (lower panel) in aP2-agouti transgenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 8/group). * $p < 0.001$ vs. before administration.

* p < 0.001 vs. Before

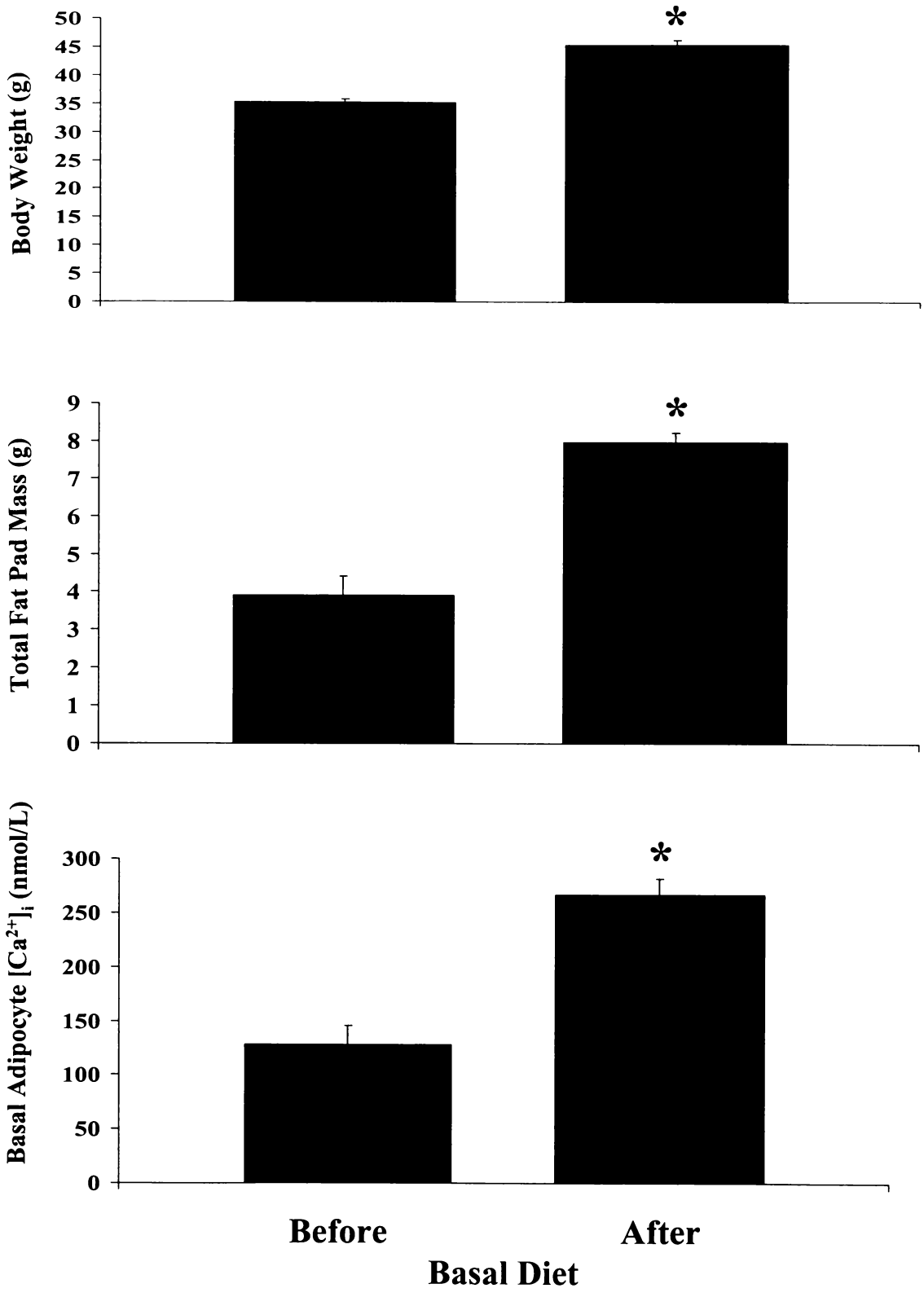


Fig 25. Effects of 6-week administration of high calcium diets on weight reduction (upper panel), fat pad mass (middle panel) and basal adipocyte $[Ca^{2+}]_i$ (lower panel) in energy-restricted (70% of *ad lib*) aP2-agouti trangenic mice. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 8).

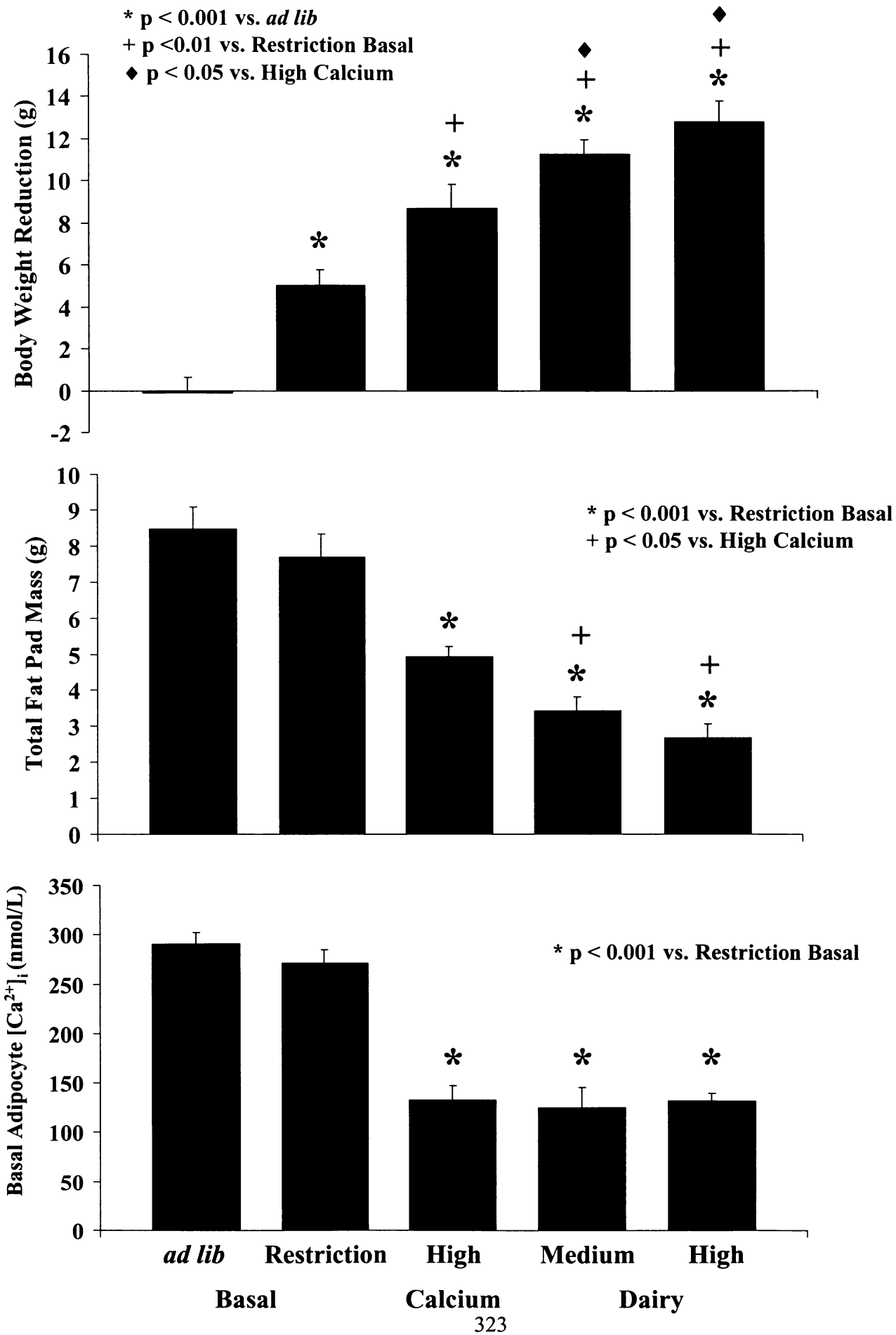
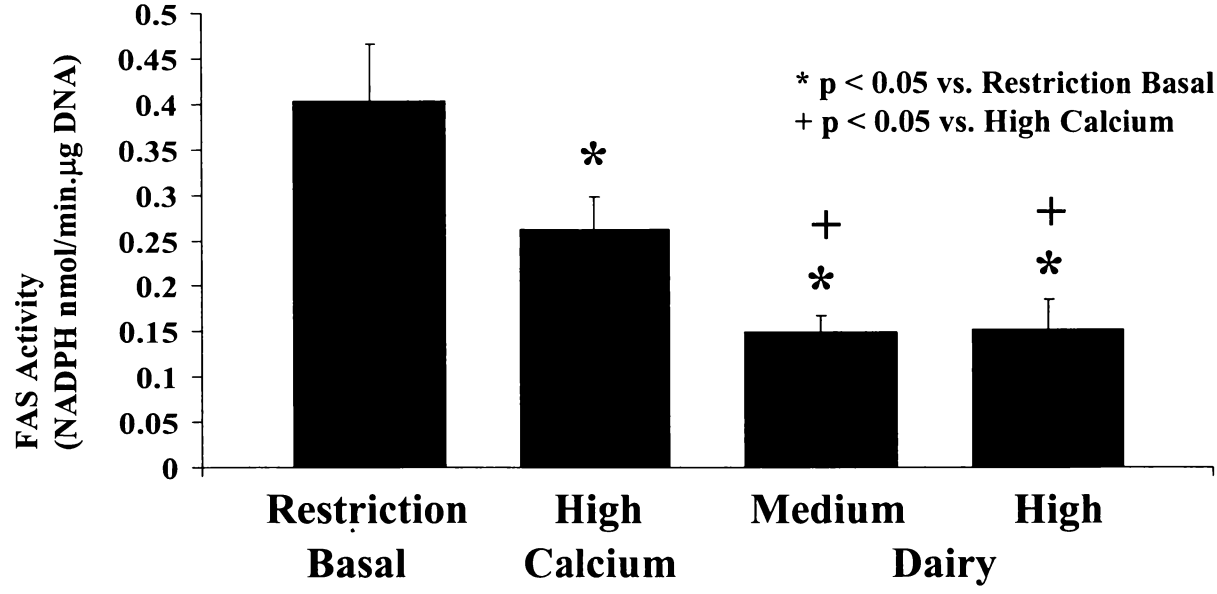

* p < 0.001 vs. ad lib
+ p <0.01 vs. Restriction Basal
♦ p < 0.05 vs. High Calcium
Body Weight Reduction (g)
16
14
12
10
8
6
4
2
0
-2
* p < 0.001 vs. Restriction Basal
+ p < 0.05 vs. High Calcium
Total Fat Pad Mass (g)
10
9
8
7
6
5
4
3
2
1
0
* p < 0.001 vs. Restriction Basal
Basal Adipocyte [Ca2+]i (nmol/L)
350
300
250
200
150
100
50
0
ad lib
Restriction
High
Medium
High
Basal
Calcium
Dairy

Fig 26 (upper panel) demonstrates that the high calcium diet caused a 35% decrease in fatty acid synthase (FAS) activity ($p < 0.05$ vs. basal energy-restricted group), which was further reduced by 63 and 62% by the medium and high dairy diets ($p < 0.05$), respectively. Similarly, the three high calcium diets caused corresponding decreases in adipocyte FAS mRNA, with 61%, 72% and 81% reductions on high calcium, medium dairy and high dairy diets, respectively ($p < 0.05$ vs. basal energy-restricted group, **Fig 26**, middle panel). Increasing dietary calcium caused a corresponding increase in lipolysis. Although the basal energy restricted diet did not affect adipocyte lipolysis, the high calcium diet caused 77% stimulation in lipolysis ($p < 0.05$. **Fig 26**, lower panel), which was further increased in the medium and high dairy diet groups ($p < 0.05$ vs. basal energy-restricted group). Increased lipolysis, coupled with decreased lipogenesis, may represent a metabolic state in which the efficiency of energy metabolism is shifted from energy storage to energy expenditure.

This shift in energy metabolism was further confirmed by dietary calcium-induced increase in core temperature. All three high calcium diets exerted stimulatory effects on core temperature, with 0.48 ^{0}C, 0.57 ^{0}C and 0.67 ^{0}C increases on the high calcium, medium dairy and high dairy diets, respectively ($p < 0.05$, **table 2**), while the basal energy restricted diet did not affect core temperature. A possibly physiological basis underlying the increased core temperature is that the expression of uncoupling protein 2 (UCP2), which has been implicated in thermogenesis (17, 18), was up-regulated in white adipose tissue, with 80% increase on all three high calcium diets ($p < 0.05$, **Fig 27**).

Fig 26. Effects of 6-week administration of high calcium diets on fatty acid synthase activity (upper panel), fatty acid synthase mRNA (middle panel) and lipolysis (lower panel) in energy-restricted (70% of *ad lib*) aP2-agouti trangenic mice. Diet and animal administration was conducted as described in Materials and Methods. Glycerol release is used to measure lipolysis, as described in Materials and Methods. Data are expressed as mean ± SE (n = 8).


FAS Activity
(NADPH nmol/min.µg DNA)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
* p < 0.05 vs. Restriction Basal
+ p < 0.05 vs. High Calcium
Restriction Basal
High Calcium
Medium Dairy
High Dairy

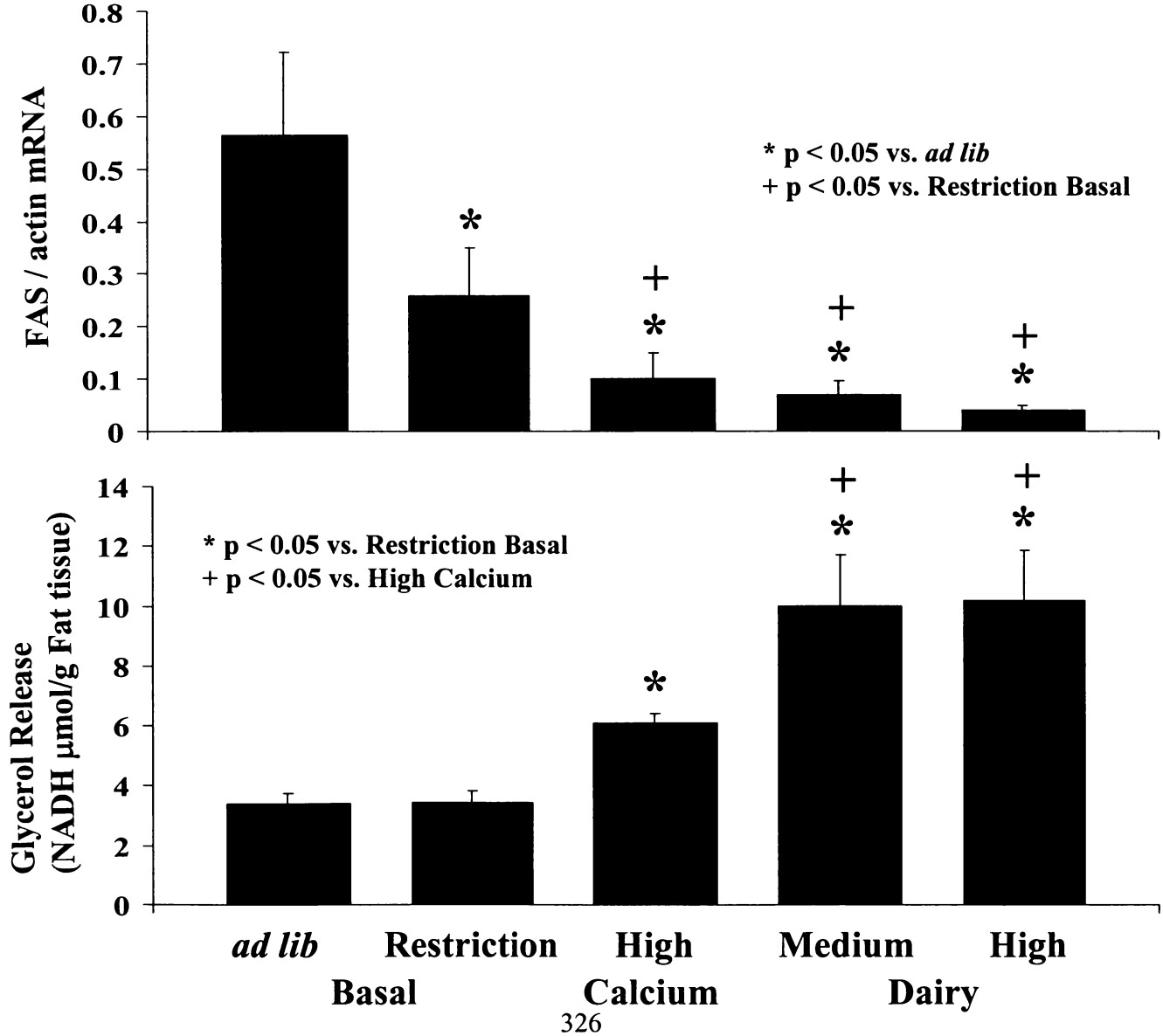

FAS / actin mRNA
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
* p < 0.05 vs. ad lib
+ p < 0.05 vs. Restriction Basal
Glycerol Release
(NADH µmol/g Fat tissue)
14
12
10
8
6
4
2
0
* p < 0.05 vs. Restriction Basal
+ p < 0.05 vs. High Calcium
ad lib
Restriction Basal
High Calcium
Medium Dairy
High Dairy

Table 2. Effects of 6-week administration of high calcium diets on core temperature in energy-restricted (70% of *ad lib*) aP2-agouti transgenic mice.

Groups (n=8)	Core temperature (°C) Before	After
Control (*ad lib*)	37.26 ± 0.24	36.56 ± 0.24
Control (restriction)	37.43 ± 0.19	37.30 ± 0.12
High Ca	37.11 ±0.24	37.59 ± 0.13*
Medium dairy	37.28 ± 0.14	37.85 ± 0.11* +
High dairy	37.27 ± 0.14	37.94 ± 0.15* +

Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n=8/group). * $p<0.05$ vs. before treatment; + $p<0.05$ vs. basal diet.

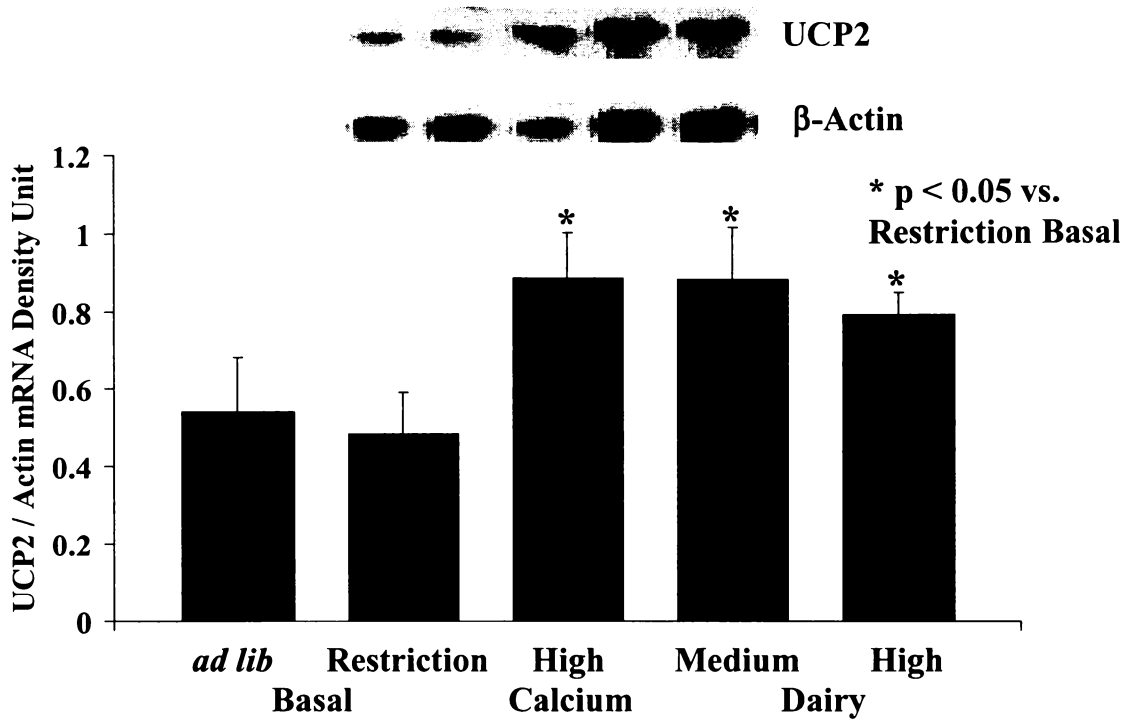


Fig 27. Effects of 6-week administration of high calcium diets on abdominal adipose tissue uncoupling protein 2 (UCP2) expression in energy-restricted (70% of *ad lib*) aP2-agouti trangenic mice. Diet and animal administration was conducted as described in Materials and Methods. Upper panel: a representative northern blot from 8 replicates; lower panel: quantitative analysis of UCP2/actin mRNA density units. Data are expressed as mean ± SE (n = 8/group). * $p < 0.05$ vs. basal diet.

The basal low calcium diet exerted a hyperinsulinemic effect, with a doubling of plasma insulin levels ($p < 0.001$, Figure 5). The mice maintained on this basal diet *ad lib* exhibited sustained levels of hyperinsulinemia, while energy restriction *per se* reduced plasma insulin by approximately 50%. The high calcium diets did not further significantly affect insulin levels, although there was a non-significant trend towards further reductions ($p=0.1$, **Fig 28**).

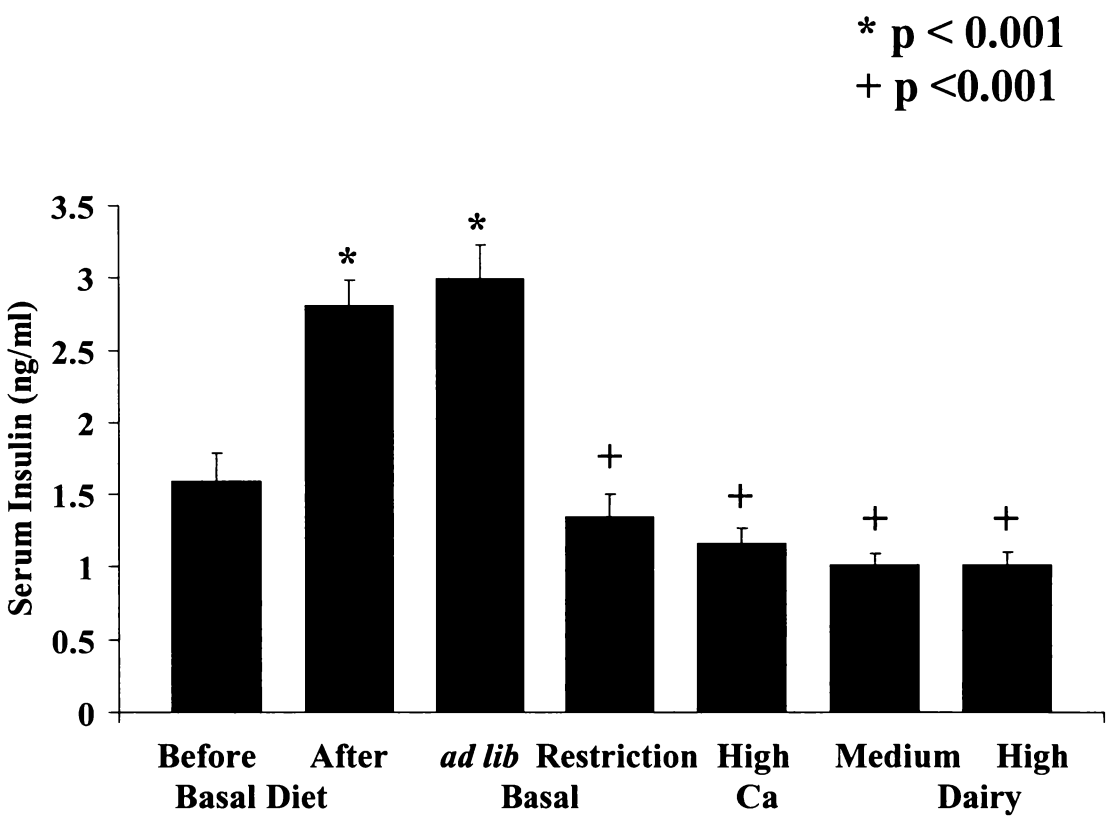


Fig 28. Effect of dietary calcium on plasma insulin levels. Diet and animal administration was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 8/group). * $p < 0.001$ vs. before basal diet treatment; + $p < 0.001$ vs. *ad lib*.

V. DISCUSSION

Obesity is closely related to other metabolic disorders, including insulin resistance/hyperinsulinemia, hypertension and cardiac hypertrophy. These diseases are integrated into a metabolic syndrome referred to as "Syndrome X" (19). To explain these tightly interacting abnormalities, Resnick (20) proposed a unifying "ionic hypothesis", in which a common cell lesion underlying these disorders in different tissues was characterized, in part, by elevations in steady-state $[Ca^{2+}]_i$ levels. Consistent with this, sustained elevations of $[Ca^{2+}]_i$ have been observed in vascular smooth muscle cells, platelets and erythrocytes in patients with hypertension (20-24), skeletal muscle cells and adipocytes in patients with insulin resistance (1, 2), and cardiomyocytes in patients with left ventricular hypertrophy (20). Moreover, antagonism of Ca^{2+} influx results in improvements in blood pressure, platelet aggregation, insulin resistance, and left ventricular hypertrophy (20).

Draznin et al (2) first reported that obese patients exhibited a elevation in basal adipocyte $[Ca^{2+}]_i$. The physiological consequence of this elevation in adipocyte $[Ca^{2+}]_i$ was addressed by our later studies demonstrating that increasing $[Ca^{2+}]_i$ via activation of either receptor or voltage-mediated calcium channels stimulates 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 (6, 7). These effects can be reversed by calcium channel antagonism (6, 7). In addition, modulation of $[Ca^{2+}]_i$ play a role in mediating both lipolytic and antilypolytic actions of hormones in adipocytes (25-28). Therefore, increasing $[Ca^{2+}]_i$ appears to promote triglyceride storage

in adipocytes by exerting coordinated control over lipogenesis and lipolysis, serving to simultaneously stimulate the former and suppress the latter, and thereby cause lipid filling.

Other endocrine and/or paracrine hormones that modulate human adipocyte $[Ca^{2+}]_i$ also modulate adipocyte lipid metabolism. Agouti protein, an obesity gene product that is expressed in human adipose tissue and stimulates Ca^{2+} influx (4, 5), simultaneously stimulates the expression and activity of fatty acid synthase (FAS) and inhibits basal and agonist-stimulated lipolysis via a Ca^{2+} dependent mechanism (6, 7). Moreover, we recently demonstrated that agouti expression is highly correlated with in vivo FAS expression and activity in human adipose tissue (29), suggesting that agouti protein, a physiological Ca^{2+} agonist, may play a role in human obesity. In addition, we have recently reported that the calcitrophic hormone 1,25-$(OH)_2$-D, which has been previously shown to stimulate $[Ca^{2+}]_i$ in both vascular smooth muscle cells and pancreatic β cells and play a role in development of hypertension and hyperinsulinemia, respectively (30, 31), also acts on human adipocytes to cause a sustained increase in $[Ca^{2+}]_i$ and a corresponding marked inhibition of lipolysis (8). Further, several lines of evidence demonstrated that circulating 1,25-$(OH)_2$-D level is increased in obese humans (32-35), further suggesting that increased 1,25-$(OH)_2$-D may exert effects in human obesity in vivo.

Accordingly, antagonism of $[Ca^{2+}]_i$ by either blocking Ca^{2+} channels or inhibiting Ca^{2+} agonists is an attractive and logical approach for development of therapeutic intervention

in obesity. Indeed, calcium channel blockade has been proven to reduce body weight and fat pad mass effectively in several animal models. We previously reported that treating transgenic mice overexpressing agouti with a Ca^{2+} channel antagonist (nifedipine) resulted in significant decreases in adipocyte lipogenesis and reduction in adipose tissue mass (11). Similarly, Ca^{2+} channel antagonists, such as nifendipine, verapamil and felodipine, caused decreases in body weight and fat pad mass in obese SHHF/Mcc-fa^{cp} rats (36, 37).

In present study, antagonism of $[Ca^{2+}]_i$ was achieved through a dietary intervention, increasing dietary calcium. This approach has previously been demonstrated to suppress 1,25-$(OH)_2$-D levels in multiple studies (20, 38). Accordingly, we proposed that dietary calcium suppression of 1,25-$(OH)_2$-D would reduce adipocyte $[Ca^{2+}]_i$ and thereby inhibit triglyceride accumulation.

Although Metz et al. (39) and Bursey et al. (40) previously reported that increased dietary calcium reduced body weight and body fat composition in spontaneously hypertensive rats, Wistar-Kyoto rats and lean and obese Zucker rats, they were unable to demonstrate the mechanism involved in this adiposity modulation. Although some investigators have attributed the effect of dietary calcium on lipid metabolism to potential inhibition of dietary fatty acid absorption, significant feed caloric losses do not result from moderate increases in dietary calcium. Indeed, data presented in this study demonstrate that increasing dietary calcium inhibits adipocyte $[Ca^{2+}]_i$, thereby facilitating weight loss by simultaneously stimulating lipolysis and inhibiting lipogenesis in energy restricted aP2-a

transgenic mice. Consistent with this, we previously found that increasing dietary attenuated the development of diet-induced obesity in aP2-agouti transgenic mice (8). Increasing dietary calcium resulted in increased adipocyte lipolysis and suppression of lipogenesis (8), similar to our observations in the present study. Accordingly, 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 to a comparable quantity of elemental calcium. The reason for this difference is not yet apparent, although it is consistent with our previous observations (8).

Interestingly, dietary calcium not only modulates lipid metabolism involved in lipogenesis and lipolysis, but also causes an increased expression of white adipose tissue uncoupling protein 2 (UCP2), which has been implicated in thermogenesis (17, 18), and a corresponding higher core temperature, an indirect metabolic index associated with thermogenesis. The contribution of thermogenesis to anti-obesity action of Ca^{2+} channel blockade has been addressed in previous studies (41, 42). However, this mechanism remains controversial in that some investigators have attributed the thermogenic effect of Ca^{2+} channel blockade to the increased function of brown adipose (41, 42). In fact, we have also previously observed an increased core temperature in agouti-overexpressing A^{vy} mice treated with L-type Ca^{2+} channel antagonist nifendipine (11). Although this

action was inexplicable at the time, our present data in this study suggest that up-regulation of UCP2 expression may be responsible. However, further study is required to address the precise mechanism whereby dietary calcium regulates UCP2 expression. Although this up-regulation of UCP2 expression may result directly from inhibition of $[Ca^{2+}]_i$, it is also possible that it is merely a result of increased substrate (fatty acid) flux secondary to increased lipolysis.

Ad lib feeding of the basal low calcium diet caused sustained hyperinsulinemia in the aP2-agouti transgenic mice. We previously found that increasing the level of calcium intake resulted in reductions in circulating insulin in both obese humans and aP2 transgenic mice (8), suggesting that suppression of insulin might contribute to the anti-obesity effect of dietary calcium. However, data from the present study demonstrate that energy restriction *per se* reduced the plasma insulin levels and that increasing dietary calcium did not further reduce insulin levels but nonetheless exerted potent effects on body weight loss and fat pad mass reduction. Thus, the effects of dietary calcium on accelerating weight lost are not due to insulin suppression and instead are likely to result from the observed suppression of adipocyte $[Ca^{2+}]_i$ and modulation of adipocyte lipid metabolism.

Limited data suggest that these findings are relevant to humans. Data from our earlier clinical trial investigating the anti-hypertensive effect of calcium in obese African Americans demonstrated that increasing daily calcium intake from approximately 400-1,000 mg/day for one-year resulted in a 4.9 Kg reduction in body fat (8). Consistent with

this, a recent randomized clinical trial demonstrated a greater weight loss in patients maintained on a milk-based diet for 16 weeks, compared to those on conventional hypocaloric diet at the same energy intake (43). These studies are further supported by our population-based observation in NHANES III, which demonstrate a profound reduction in the odds of being in the highest quartile of adiposity associated with increases in calcium and dairy product intake (8).

In summary, high calcium diets exert potent effects in enhancing reduction of body weight and fat pad mass in energy-restricted aP2-agouti transgenic mice. High calcium diets suppressed adipocyte $[Ca^{2+}]_i$, stimulated lipolysis, inhibited lipogenesis, and caused an increased white adipose tissue uncoupling protein 2 (UCP2) expression and a corresponding increase in core temperature. Consequently, dietary calcium facilitates reduction of fat tissue mass and body weight in Kcal restriction by modulating energy metabolism, serving to reduce energy storage and increase thermogenesis. These data may provide a useful framework for further development of effective dietary intervention in obesity.

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PART 7

1α,25-DIHYDROXYVITAMIN D_3 MODULATES HUMAN ADIPOCYTE METABOLISM VIA NONGENOMIC ACTION

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I. ABSTRACT

We recently reported that suppression of the renal 1α,25-dihyroxyvitamin D_3 (1α,25-$(OH)_2$-D_3) production in aP2-agouti transgenic mice by increasing dietary calcium decreases adipocyte intracellular Ca^{2+} ($[Ca^{2+}]_i$), stimulates lipolysis, inhibits lipogenesis, and reduces adiposity. However, it was not clear whether this modulation of adipocyte metabolism by dietary calcium is a direct effect of inhibition of 1α,25-$(OH)_2$-D_3 induced $[Ca^{2+}]_i$. Accordingly, we have now evaluated the direct role of 1α,25-$(OH)_2$-D_3. Human adipocytes exhibited a 1α,25-$(OH)_2$-D_3 dose-responsive (1-50 nM) increase in $[Ca^{2+}]_i$ ($p<0.01$). This action was mimicked by 1α,25-dihyroxylumisterol$_3$ (1α,25-$(OH)_2$-lumisterol$_3$) ($p<0.001$), a specific agonist for a putative membrane vitamin D receptor (mVDR), and completely prevented by 1β,25-dihydroxyvitamin D_3 (1β,25-$(OH)_2$-D_3), a specific antagonist for the mVDR. Similarly, 1α,25-$(OH)_2$-D_3 (5 nM) caused 50-100% increases in adipocyte fatty acid synthase (FAS) expression and activity ($p<0.02$), a 61% increase in glycerol-3-phosphate dehydrogenase (GPDH) activity ($p<0.01$), an 80% inhibition of isoproterenol-stimulated lipolysis ($p<0.001$), while 1β,25-$(OH)_2$-D_3 completely blocked all these effects. Notably, 1α,25-$(OH)_2$-lumisterol$_3$ exerted more potent effects in modulating adipocyte lipid metabolism, with 2.5-3.0 fold increases in FAS expression and activity ($p<0.001$), a 3 fold increase in GPDH activity ($p<0.001$), and was approximately twice as potent in inhibiting basal lipolysis ($p<0.025$), whereas 1β,25-$(OH)_2$-D_3 completely blocked all these effects. These data suggest that 1α,25-$(OH)_2$-D_3 modulates adipocyte Ca^{2+} signaling and, consequently, exerts a coordinated control over lipogenesis and lipolysis. Thus, a direct inhibition of 1α,25-$(OH)_2$-D_3

induced $[Ca^{2+}]_i$ may contribute to an anti-obesity effect of dietary calcium, and the mVDR may represent an important target for obesity.

II. INTRODUCTION

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays a key role in metabolic disorders associated with obesity and insulin resistance (1-3). We previously reported that increasing $[Ca^{2+}]_i$ via stimulation of either receptor or voltage-mediated calcium channels 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 (4-9). Therefore, increasing $[Ca^{2+}]_i$ appears to promote triglyceride accumulation in adipocytes by exerting a coordinated control over lipogenesis and lipolysis, serving to simultaneously stimulate the former and inhibit the latter.

1α,25-dihyroxyvitamin D_3 (1α,25-$(OH)_2$-D_3) was originally believed to solely function via a nuclear vitamin D receptor (nVDR) to generate genomic action (10). However, this concept was challenged by recent studies revealing that it also generates rapid, nongenomic signal transductions including stimulation of $[Ca^{2+}]_i$ via a putative membrane vitamin D receptor (mVDR) in a wide variety of cells (11-15).

1α,25-$(OH)_2$-D_3 is a highly conformationally flexible molecule and is able to rotate around its 6, 7 carbon-carbon bond of B ring. This rotation allows generation of a continuum of potential ligand shapes ranging from the steroid like 6-s-cis conformation to the extended 6-s-trans conformation (16, 17). Thus, this conformational flexibility of 1α,25-$(OH)_2$-D_3 in a structure and function context underlies the mechanism whereby 1α,25-$(OH)_2$-D_3 generates both genomic and non-genomic cellular responses. It is also suggested that analogs of 1α,25-$(OH)_2$-D_3 with different conformations differ in their

abilities to mediate biological responses (16, 17). Indeed, analogs of 1α,25-$(OH)_2$-D_3 have been extensively examined for their ability to generate genomic or non genomic biological responses (16, 17). 1α,25-dihydroxylumisterol$_3$, a 6-s-cis-locked analog of 1α,25-$(OH)_2$-D_3, preferentially exerts a non-genomic action, including stimulation of Ca^{2+} influx, via the putative mVDR (18, 19). Another A-ring diastereomer analog, 1β,25-dihyroxyvitamin D_3, has been demonstrated to antagonize this action but has no effects on the nVDR (20, 21). Therefore, 1α,25-dihydroxylumisterol$_3$ is a specific agonist of non-genomic action, while 1β,25-dihyroxyvitamin D_3 is a specific antagonist of non-genomic action.

We recently demonstrated that increasing dietary calcium decreases adipocyte intracellular Ca^{2+}, stimulates lipolysis, inhibits lipogenesis, and thereby reduces adiposity in aP2-agouti transgenic mice (22, 23). However, although high calcium diets are associated with suppression of circulating 1α,25-$(OH)_2$-D_3, it is not clear whether this modulation of adipocyte metabolism by dietary calcium is a direct effect of inhibition of 1α,25-$(OH)_2$-D_3 induced $[Ca^{2+}]_i$. Accordingly, the present study was designed to determine the direct role of 1α,25-$(OH)_2$-D_3 in modulating adipocyte Ca^{2+} signaling and lipid metabolism.

III. MATERIALS AND METHODS

A. CULTURE OF HUMAN ADIPOCYTES

A human adipocyte cell line and primary-cultured human adipocytes were both used as cell models in this study. The human adipocyte cell line was differentiated from human preadipocytes obtained from Zen-Bio Inc (Research Triangle Park, NC). Human preadipocytes were inoculated in Dulbecco's Modified Eagle' Medium (DMEM) / Ham' F-10 medium (F-10) (1:1, v/v) containing 10% fetal bovine serum (FBS), 15mM Hepes and antibiotics at a density of 30,000 cells/cm^2. Confluent monolayers of preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM/F-10 (1:1, v/v) medium supplemented with 15 mM Hepes, 3% FBS, 33 μM biotin, 17 μM pantothenate, 100 nM insulin, 0.25 mM methylisobutylxanthine (MIX), 1 μM dexamethasome, 1 μM BRL49653 and antibiotics. Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium in which BRL49653 and MIX were withdrawn. Cultures were refed every 2-3 days (24).

Primary-cultured human adipocytes were derived from human subcutaneous adipose tissue obtained from patients undergoing abdominal plastic surgery with no known history of metabolic disorders. This protocol was approved by the Institutional Review Board for Human Subjects and the Committee for Research Participation of the University of Tennessee. Adipocytes were isolated as previously described (25). Briefly, adipose tissue was first washed several times with Hank's Balanced Salt Solution, minced into small pieces and digested with 1 mg/ml type1 collagenase in a shaking water bath at 37 ^{0}C for 30 min. Adipocytes were then filtered through a sterile 500 μm nylon

mesh and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% fetal bovine serum (FBS), 100 UI/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin. Cells were cultured in suspension and maintained in a thin layer at the top of culture media, which were changed every day. Cells were maintained viable and metabolically responsive under this culture condition for 7 days.

B. HUMAN ADIPOCYTE INTRACELLULAR Ca^{2+} MEASUREMENT

$[Ca^{2+}]_i$ in human preadipocytes was measured using a fura-2 dual wavelength fluorescence imaging system (24). Preadipocytes were plated and differentiated in 35 mm dishes with glass coverslips (P35G-0-14-C, MatTek Corporation). Prior to $[Ca^{2+}]_i$ measurement, cells were preincubated in serum-free medium overnight and rinsed with Hepes Balanced Salt Solution (HBSS) containing the following components (in mM): 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%. Cells were loaded with fura-2 acetoxymethyl ester (AM) (10 μM) in the same buffer for 2 hr at 37 ^{0}C in a dark incubator with 5% CO_2. To remove extracellular dye, cells were rinsed with HBSS 3 times and then postincubated at room temperature for an additional 1 hr 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 4915 CCD camera. Fluorescent images were captured alternatively at excitation wavelength of 340 and 380nM with an emission wavelength of 520 nM. After establishment of stable image baseline, the response to 1α,25-$(OH)_2$-D_3 and its membrane receptor (mVDR) agonist 1α,25-dihydroxylumisterol$_3$ (1α,25-$(OH)_2$-lumisterol$_3$), was determined. $[Ca^{2+}]_i$ was calculated

using a ratio equation (26). Each analysis evaluated responses of 8-10 representative whole cells. Images were analyzed with InCytIm2 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 $[Ca^{2+}]_i$ levels.

C. FATTY ACID SYNTHASE AND GLYCEROL-3-PHOSPHATE DEHYDROGENASE ACTIVITY ASSAY

FAS and GPDH activity was determined spectrophotometrically in crude cytosolic extracts of human adipocytes as previously described (25). Human adipocytes were homogenized in 250 mmol/L sucrose solution containing 1 mmol/L ethylenediamine-tetraacticacid (EDTA), 1 mmol/L dithiothreitol (DTT), and 100 μmol/L phenylmethylsulfonyl fluoride (PMSF) (pH 7.4). The homogenate was centrifuged at 18,500 X g for 1 hr and the infranatant was used for measuring oxidation rate of NADPH or NADH, respectively.

D. LIPOLYSIS ASSAY

Glycerol released into the culture medium was determined as an indicator of lipolysis, using a one-step enzymatic fluorometric method as previously described (8, 27).

E. NORTHERN BLOT ANALYSIS

Northern blot analysis was conducted as previously described (25). Total RNA from human adipose tissue was extracted using $CsCl_2$ density centrifugation, run in 1% agarose gel and transferred to nylon membranes (New England Nuclear, Boston, MA). The membrane was hybridized with FAS cDNA probes that were radiolabeled 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 ^{0}C. Finally, the membrane was exposed to X-ray film (New England Nuclear, Boston, MA) at –80 ^{0}C. All membranes were stripped and reprobed with β-actin as loading control. The blot image was captured using DigiPix 1260^{TM} imager system with a CCD camera and a electronic transilluminator (ULTRA-LUM, Inc. Paramount, CA). A ZERO-Dscan software (Scanalytics, Inc, Fairfax, VA) was used to quantitatively analyze these images.

F. STATISTICAL ANALYSIS

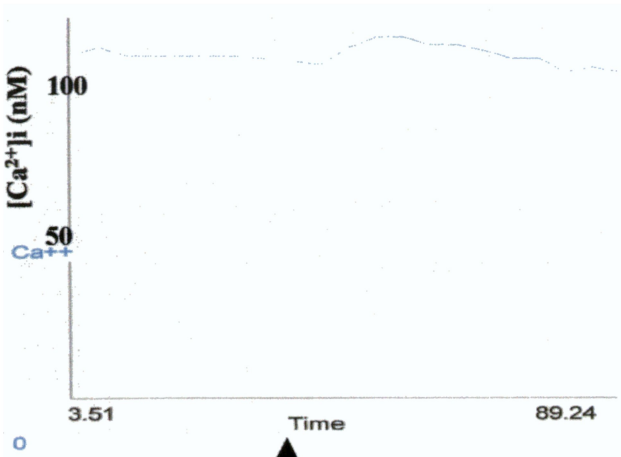
All data are expressed as mean ± SE. Data are evaluated for statistical significance by one way Analysis of Variance (ANOVA), and significantly different group means were then separated by the least Significant Difference (LSD) test using SPSS (SPSS Inc, Chicago, IL). A p value < 0.05 is considered significant.

IV. RESULTS

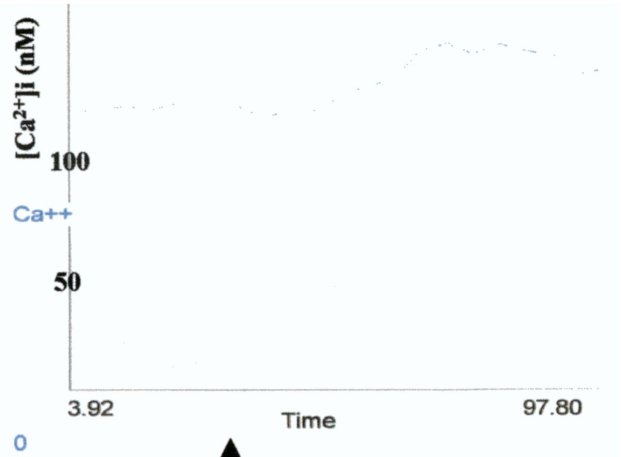
To determine the direct effect of 1α,25-$(OH)_2$-D_3 on adipocyte Ca^{2+} signaling, the $[Ca^{2+}]_i$ response to 1α,25-$(OH)_2$-D_3 was evaluated. 1α,25-$(OH)_2$-D_3 induced a significant increase of $[Ca^{2+}]_i$ in human adipocyte in a dose-dependent manner, with 13.33±1.22 nM vs. 23.33±2.81 nM increases over baseline (5 nM vs.10 nM 1α,25-$(OH)_2$-D_3 treatment, $p<0.02$), respectively (**Fig. 29** upper left). This action was mimicked by 1α,25-dihydroxylumisterol$_3$ (1α,25-$(OH)_2$-lumisterol$_3$), a specific agonist for membrane vitamin D receptor (mVDR). 1α,25-$(OH)_2$-lumisterol$_3$ caused a marked dose-responsive increases in human adipocyte $[Ca^{2+}]_i$, with 27.75±7.82 nM and 131.00±11.00 nM increases over baseline ($p<0.001$), respectively (**Fig. 29** upper right), while these effects were completely prevented by pre-treatment of human adipocytes with 1β,25-dihydroxyvitamin D_3 (1β,25- $(OH)_2$-D_3), a specific antagonist for mVDR (**Fig. 29** lower panel).

To investigate the role of 1α,25-$(OH)_2$-D_3 in regulating lipid metabolism, we treated human adipocytes with 1α,25-$(OH)_2$-D_3 and its mVDR agonist and antagonist, using FAS and GPDH as lipogenic markers and glycerol release as a lipolytic indicator. 1α,25-$(OH)_2$-D_3 (5 nM) caused a 40% increase in adipocyte FAS activity over 48 hrs ($p<0.05$, **Fig. 30** lower panel), while 1α,25-$(OH)_2$-lumisterol$_3$ exerted a more potent effect, with a 2.5 fold increase in FAS activity ($p<0.001$, **Fig. 30** lower panel). However, pretreatment of human adipocytes with 1β,25- $(OH)_2$-D_3 completely prevented this stimulation of FAS (**Fig. 30** lower panel). A similar stimulation was observed on FAS mRNA expression,

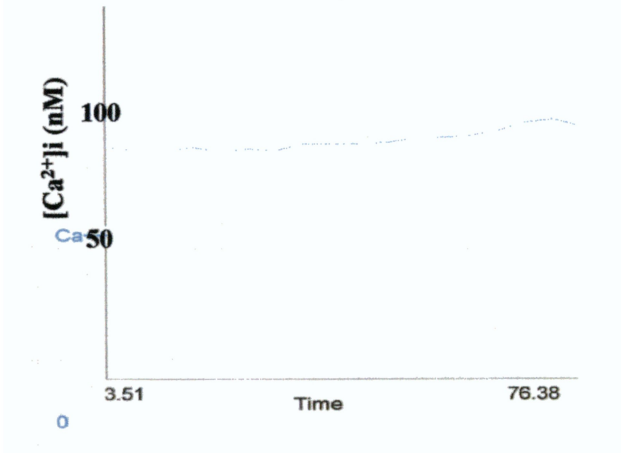
Fig 29. The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on stimulation of human adipocyte $[Ca^{2+}]i$. A fura-2 dual wavelength fluorescent imaging system was used to measure $[Ca^{2+}]i$ as described in Materials and Methods. 4~6 independent experiments were conducted for each treatment. The arrows under each $[Ca^{2+}]i$ tracing figure indicate the time of addition of either 1α,25-$(OH)_2$-D_3 or 1α,25-$(OH)_2$-lumisterol$_3$. Upper left: 1α,25-$(OH)_2$-D_3 stimulated a dose-responsive increase in human adipocyte $[Ca^{2+}]i$, with 13.33 ± 1.22 nM and 23.33 ± 2.81 nM increases over baseline ($p<0.05$), respectively. Upper right: 1α,25-$(OH)_2$-lumisterol$_3$ caused a similar but more sustained dose-responsive increase in human adipocyte $[Ca^{2+}]i$, with 27.75 ± 7.82 nM and 131.0 ± 11.0 nM increases over baseline ($p<0.05$). Lower panel: Pre-treatment of human adipocytes with 1β,25- $(OH)_2$-D_3 completely prevented either 1α,25-$(OH)_2$-D_3 (right) or 1α,25-$(OH)_2$-lumisterol$_3$-induced (left) increases in human adipocyte $[Ca^{2+}]i$.



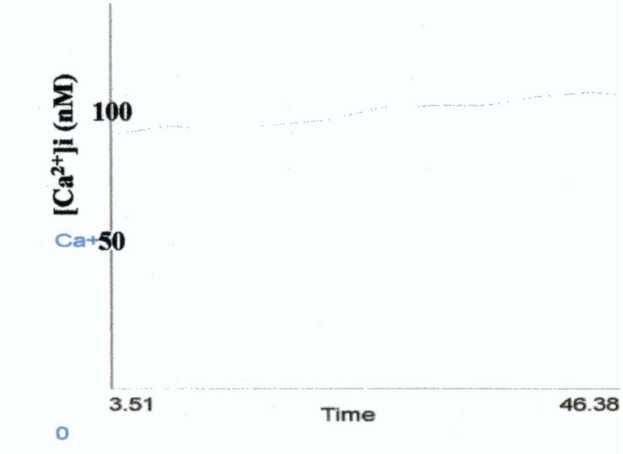
$1\alpha,25\text{-}(OH)_2\text{-}D_3$ 5 nM



$1\alpha,25\text{-}(OH)_2\text{-lumisterol}_3$ 5 nM

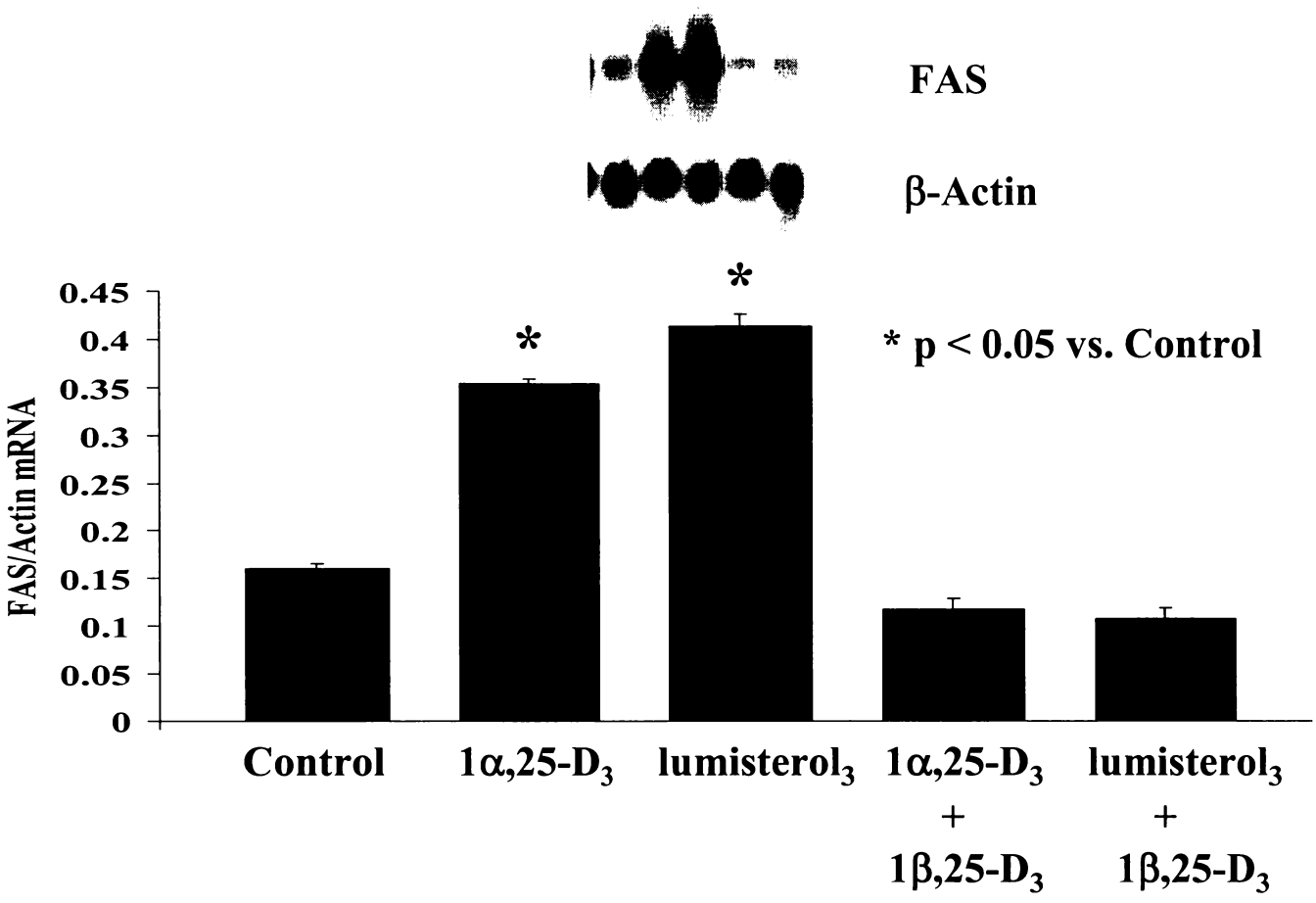


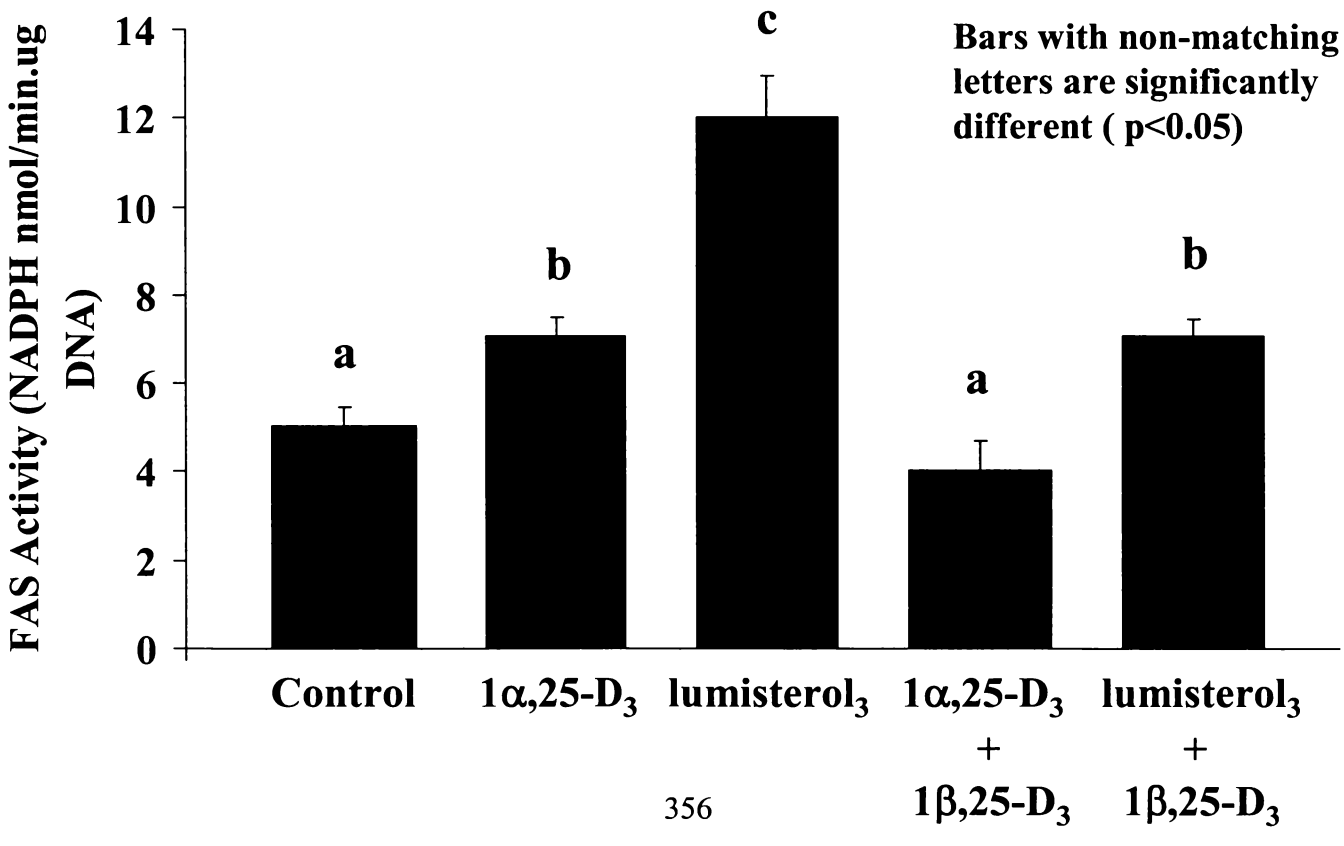
$1\beta,25\text{-}D_3$ + $1\alpha,25\text{-}D_3$



$1\beta,25\text{-}D_3$ + $1\alpha,25\text{-lumisterol}_3$

Fig 30. The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on FAS activity and expression in human adipocytes. Human adipocytes were treated with 1α,25-$(OH)_2$-D_3 (5 nM) alone or 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) alone, 1α,25-$(OH)_2$-D_3 (5 nM) plus 1β,25-$(OH)_2$-D_3 or 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) plus 1β,25- $(OH)_2$-D_3 for 48 hours. FAS activity measurement and expression analysis were conducted as described in Materials and Methods. Lower panel: The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on stimulation of human adipocyte FAS activity. Bars with non-matching letters are significantly different (*$p<0.05$); n=8. Upper panel: The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on stimulation of human adipocyte FAS expression. Blot shown is representative of 3 similar experiments. *$p<0.01$ vs. control.


FAS
β-Actin
FAS/Actin mRNA
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
* p < 0.05 vs. Control
Control
1α,25-D3
lumisterol3
1α,25-D3 + 1β,25-D3
lumisterol3 + 1β,25-D3


FAS Activity (NADPH nmol/min.ug DNA)
14
12
10
8
6
4
2
0
a
b
c
a
b
Bars with non-matching letters are significantly different (p<0.05)
Control
1α,25-D3
lumisterol3
1α,25-D3 + 1β,25-D3
lumisterol3 + 1β,25-D3

with 2 and 2.5 fold increases on 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ treatment ($p<0.001$, **Fig. 30** upper), respectively, while this stimulation was completely blocked by 1β,25- $(OH)_2$-D_3. Consistent with this, 1α,25-$(OH)_2$-D_3 (5 nM) stimulated a 50% increase in human adipocyte GPDH activity ($p<0.05$, **Fig 31**), while a markedly greater stimulation of 2.8 fold was found with 1α,25-$(OH)_2$-lumisterol$_3$ treatment ($p<0.001$, **Fig 31**). Although 1β,25- $(OH)_2$-D_3 exerted little effect on 1α,25-$(OH)_2$-D_3 stimulated GPDH activity, it markedly inhibited 1α,25-$(OH)_2$-lumisterol$_3$ stimulated GPDH activity (**Fig 31**).

Adipocyte lipolysis responded to 1α,25-$(OH)_2$-D_3 and its agonist in an inverse manner to the lipogenesis. **Fig. 32** (upper) illustrates that 1α,25-$(OH)_2$-D_3 exerted a inhibitory effect on adipocyte basal lipolysis, with a 35% reduction ($p<0.05$). A greater inhibition of 50% was found with 1α,25-$(OH)_2$-lumisterol$_3$ treatment ($p<0.01$, **Fig. 32** upper). Conversely, this inhibition was completely prevented by pretreatment with 1β,25- $(OH)_2$-D_3. Similarly, treatment of human adipocytes with isoproterenol resulted in a 3.2 fold increase in lipolysis ($p<0.001$, **Fig. 32** lower panel), while 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ inhibited isoproterenol-stimulated lipolysis by 56% and 53% ($p<0.001$, **Fig. 32** lower panel), respectively. Pretreatment with 1β,25- $(OH)_2$-D_3 prevented this inhibitory effect of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on isoproterenol-stimulated lipolysis (**Fig. 32** lower panel).

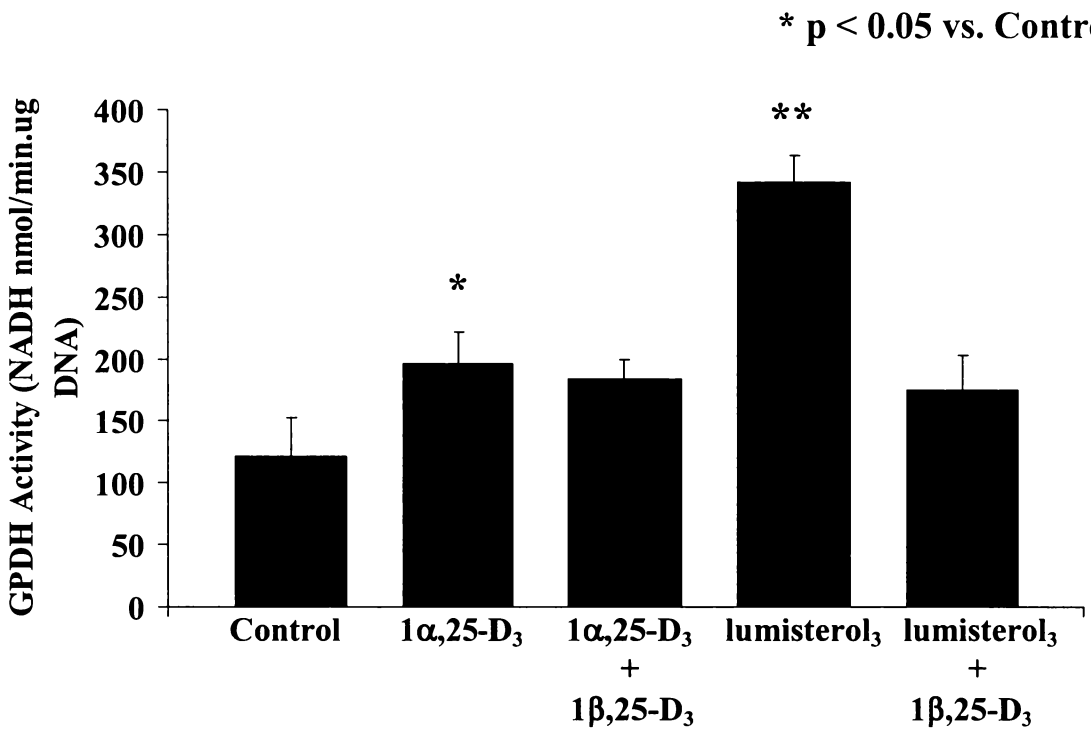
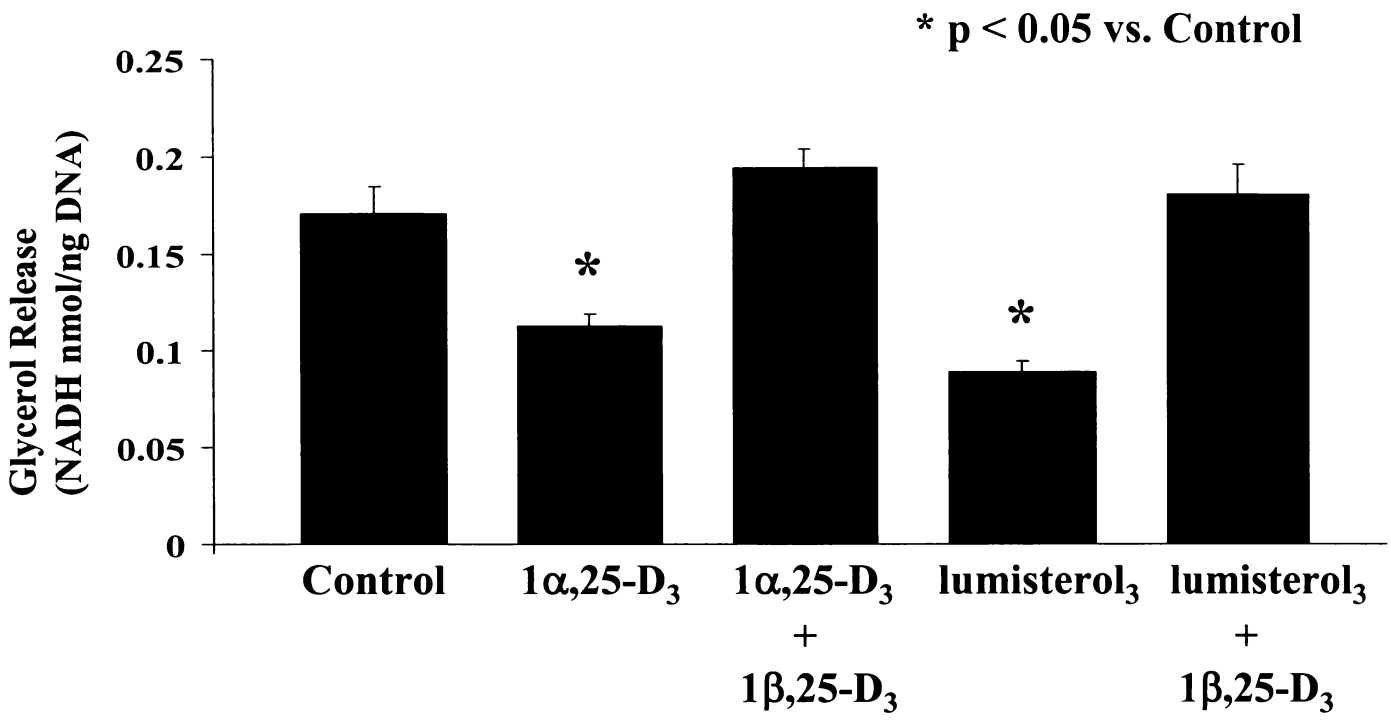
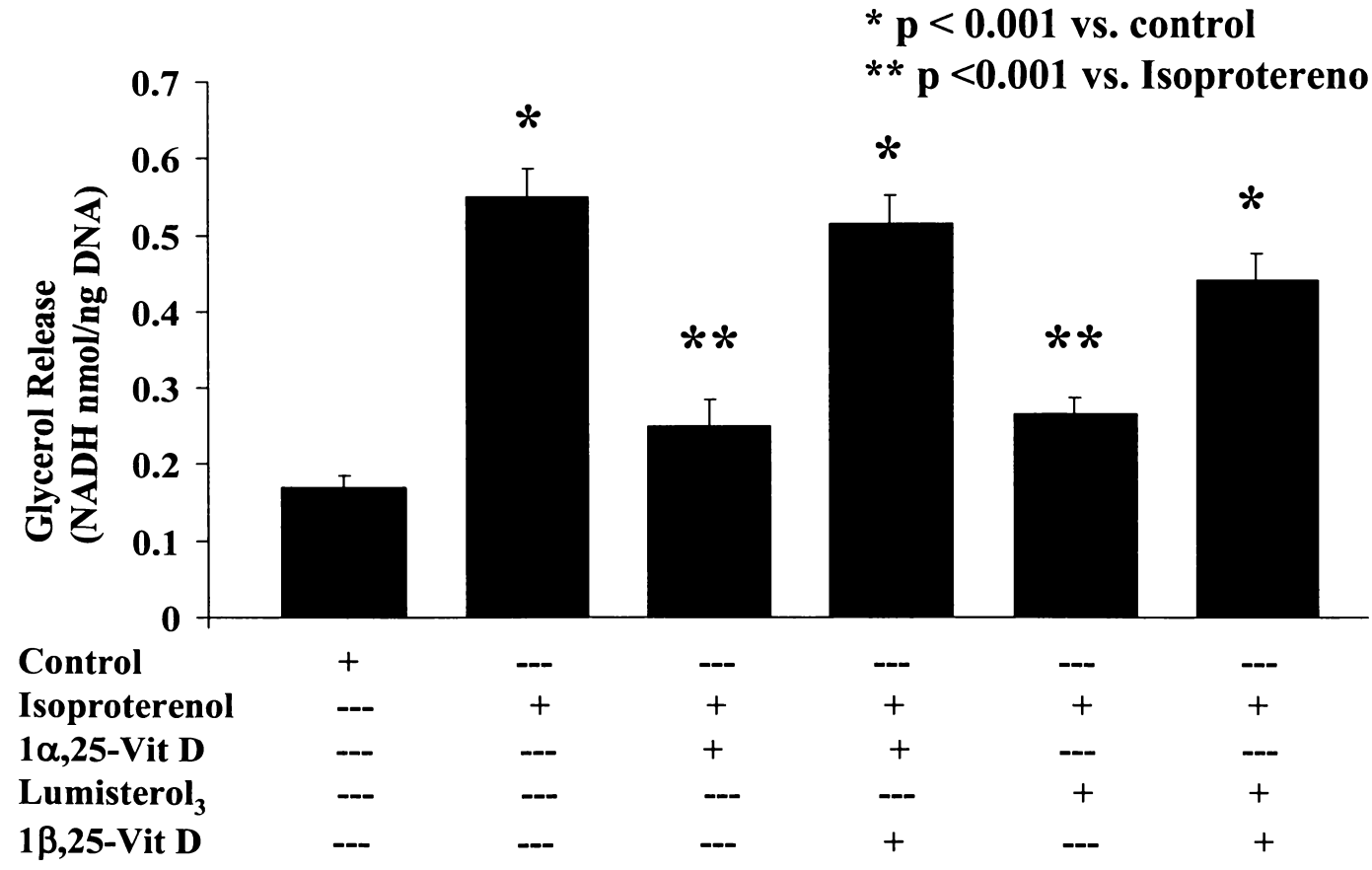


Fig 31. The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-$lumisterol_3$ on GPDH activity in human adipocytes. Human adipocytes were treated with 1α,25-$(OH)_2$-D_3 (5 nM) alone or 1α,25-$(OH)_2$-$lumisterol_3$ (5 nM) alone, 1α,25-$(OH)_2$-D_3 (5 nM) plus 1β,25- $(OH)_2$-D_3 or 1α,25-$(OH)_2$-$lumisterol_3$ (5 nM) plus 1β,25- $(OH)_2$-D_3 for 48 hours. GPDH activity measurement was performed as described in Materials and Methods. *$p<0.05$ vs. control; **$p<0.001$ vs. control; n=8.

Fig 32. The effects of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ on human adipocyte lipolysis. In basal lipolysis study, human adipocytes were treated with 1α,25-$(OH)_2$-D_3 (5 nM) alone or 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) alone for 2 hours, or pretreated with 1β,25-$(OH)_2$-D_3 for half hour and then treated with 1α,25-$(OH)_2$-D_3 (5 nM) plus 1β,25- $(OH)_2$-D_3 or 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) plus 1β,25- $(OH)_2$-D_3 for 2 hours. In isoproterenol-stimulated lipolysis study, human adipocytes were first pre-treated with or without 1β,25- $(OH)_2$-D_3 for half hour, and then treated with or without 1α,25-$(OH)_2$-D_3 (5 nM) alone or 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) alone for half hour, or 1α,25-$(OH)_2$-D_3 (5 nM) plus 1β,25- $(OH)_2$-D_3 or 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) plus 1β,25- $(OH)_2$-D_3 for half hour, and finally treated with isoproterenol alone, or isoproterenol plus previous combinations. Glycerol release was determined as described in Materials and Methods. Upper panel: 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) inhibited adipocyte basal lipolysis, with a 35% or 50% reduction, respectively. *$p<0.05$ vs. control; **<0.01 vs. control; n=8. Lower panel: 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) inhibited isoproterenol-stimulated lipolysis. *$p<0.001$ vs. control; **$p<0.001$ vs. isoproterenol; n=8.


* p < 0.05 vs. Control
Glycerol Release
(NADH nmol/ng DNA)
0.25
0.2
0.15
0.1
0.05
0
*
*
Control
1α,25-D3
1α,25-D3 + 1β,25-D3
lumisterol3
lumisterol3 + 1β,25-D3


* p < 0.001 vs. control
** p <0.001 vs. Isoprotereno
Glycerol Release
(NADH nmol/ng DNA)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
*
**
*
Control + --- --- --- --- ---
Isoproterenol --- + + + + +
1α,25-Vit D --- --- + + --- ---
Lumisterol3 --- --- --- --- + +
1β,25-Vit D --- --- --- + --- +

V. DISCUSSION

We previously reported that *agouti*, an obesity gene expressed in human adipose tissue, stimulates Ca^{2+} influx (4, 5) and promotes triglyceride accumulation in adipocytes by simultaneously stimulating the expression and activity of FAS and inhibiting basal and agonist-stimulated lipolysis via a Ca^{2+} dependent mechanism (6, 7). Moreover, we recently demonstrated that agouti expression is highly correlated with *in vivo* FAS expression and activity in human adipose tissue (28), further suggesting that agouti protein, a physiological Ca^{2+} agonist, may play a role in obesity. Therefore, identifying and characterizing hormones that modulate $[Ca^{2+}]_i$ is a logical approach for elucidating novel mechanisms involved in modulating adiposity.

Data presented here show that the calcitrophic hormone 1α,25-$(OH)_2$-D_3 exhibits a similar role in modulating adipocyte Ca^{2+} signaling, resulting in a coordinated control over lipogenesis and lipolysis. These data indicate that 1α,25-$(OH)_2$-D_3 plays a novel role in mediating energy homeostasis in adipose tissue and suggest that the 1α,25-$(OH)_2$-D_3 mediated signaling pathways in regulating adipocyte energy metabolism may represent a suitable target for the development of pharmacological and/or nutritional interventions in obesity. Although it is possible that 1α,25-$(OH)_2$-D_3 may also exert its effects on adipocyte lipid metabolism via other signal transduction pathways, this is unlikely, as we have previously demonstrated similar effects which result from direct activation of adipocyte Ca^{2+} channels (6, 8, 9, 25) and can be reversed by Ca^{2+} channel inhibition (7, 25). Moreover, we recently demonstrated that protein kinase C does not mediate $[Ca^{2+}]_i$ inhibition of lipolysis (9).

1α,25-$(OH)_2$-D_3, the biologically active form of the vitamin D, was originally believed to solely function via a nuclear receptor in a manner similar to the other members of steroid hormone superfamily, resulting in a biological response (10). Briefly, 1α,25-$(OH)_2$-D_3 binds to and activates a specific nuclear hormone receptor, nVDR. The activated nVDR then interacts with another nuclear receptor RXR and forms a heterodimer complex. This complex functions as a transcriptional factor to act on the direct repeat response element named DR-3 in the promoter region of target genes, thereby stimulating or suppressing transcription of those genes encoding for proteins that carry out a variety of functions (10). Indeed, recent studies of nVDR knockout mouse showed that mice lacking nVDR exhibit a vitamin D deficient phenotype, such as impaired bone formation and growth retardation 29, 30), demonstrating that nVDR mediated genomic action exhibits an important biological function. However, a high calcium/phosphorus/lactose diet fed at 16 days of age in nVDR knockout mice allows normal mineral ion homeostasis and thereby rescues the vitamin D deficient phenotype (31), suggesting that an alternative pathway may compensate to preserve intestinal calcium absorption and bone development and modeling in the absence of a functional nVDR. Moreover, several studies demonstrated that 1α,25-$(OH)_2$-D_3 is able to induce rapid non-genomic effects in nVDR-null cells or cells from nVDR knockout mice (32, 33), indicating that the genomic model of 1α,25-$(OH)_2$-D_3 action is not complete. 1α,25-$(OH)_2$-D_3 also generates rapid, nongenomic signal transduction including modulation of calcium channels (12-15) via a putative membrane vitamin D receptor (mVDR) in a wide variety of cells (11). Nemere et al (34, 35) identified a membrane associated protein with a high binding affinity for 1α,25-$(OH)_2$-D_3, which mediated the rapid non-genomic regulation of PKC. Baran et al (36)

recently found another plasma membrane-bound protein with a specific and saturable binding for 1α,25-$(OH)_2$-D_3, which regulates the rapid effects of 1α,25-$(OH)_2$-D_3 on $[Ca^{2+}]_i$. This protein was identified as annexin II by sequence analysis and Western Blot. However, it is not known whether these identified binding proteins for 1α,25-$(OH)_2$-D_3 are the same protein or different receptors mediating similar non-genomic functions.

Our data further extend these observations by demonstrating that 1α,25-$(OH)_2$-D_3 elicits a non-genomic action in adipocytes, resulting in a stimulation of $[Ca^{2+}]_i$ and corresponding modulation of lipid metabolism. Although 1α,25-$(OH)_2$-D_3 does regulate FAS expression, this is attributed to 1α,25-$(OH)_2$-D_3 induced increases in $[Ca^{2+}]_i$ rather than a direct effect on FAS expression, as this effect can be mimicked by the mVDR agonist and blocked by the mVDR antagonist. Further, we have previously demonstrated modulation of $[Ca^{2+}]_i$ to correspondingly regulate FAS expression (4-7).

The analogs of 1α,25-$(OH)_2$-D_3 have been extensively examined in term of their ability to generate genomic or non genomic biological response (16, 17). Several reports (18, 19) demonstrated that 6-s-cis-locked analogs of 1α,25-$(OH)_2$-D_3, such as 1α,25-dihydroxylumisterol$_3$, are specific for non-genomic action, including stimulation of Ca^{2+} influx, via binding to mVDR. In contrast, this analog exhibits low binding affinity to nVDR and fails to activate the genomic pathway. Further, 1β,25-dihyroxyvitamin D_3, blocks rapid physiological response elicited by 1α,25-$(OH)_2$-D_3 or 1α,25-dihydroxylumisterol$_3$ but has no effects on nVDR (20, 21). Therefore, 1α,25-

dihydroxylumisterol$_3$ is a specific agonist of non-genomic action, while 1β,25-dihyroxyvitamin D_3 is a specific antagonist of non-genomic action. It is noteworthy that 1α,25-dihydroxylumisterol$_3$ exerted more potent effects in modulating adipocyte $[Ca^{2+}]_i$ and corresponding lipid metabolism, and that 1β,25-dihyroxyvitamin D_3 completely blocked these effects. This suggests that 1α,25-dihydroxylumisterol$_3$ acts on the mVDR solely to generate non-genomic action in adipocytes, while 1α,25-$(OH)_2$-D_3 may target both mVDR and nVDR to mediate non-genomic and genomic actions which may interact with each other in signal response, thereby compromising the modulation of lipid metabolism. These data further demonstrate that 1α,25-$(OH)_2$-D_3 mediated non-genomic action via mVDR may play a major role in regulating adipocyte lipogenesis and lipolysis.

Regulation of adipocyte metabolism via 1α,25-$(OH)_2$-D_3 signaling pathways may play an important role in the development of obesity *in vivo*. Several lines of evidence demonstrate that circulating 1α,25-$(OH)_2$-D_3 level is elevated in obese humans (37-40). Accordingly, 1α,25-$(OH)_2$-D_3 mediated signaling pathway in regulating adipocyte energy homeostasis may represent a suitable target for the development of pharmacological and/or nutritional interventions in obesity. Previous studies showed that increasing dietary calcium suppressed 1α,25-$(OH)_2$-D_3 levels (41, 42). Accordingly, we tested the hypothesis that dietary calcium suppression of 1α,25-$(OH)_2$-D_3 would reduce adipocyte $[Ca^{2+}]_i$ and thereby inhibit triglyceride accumulation by coordinated control over lipogenesis and lipolysis. Our data demonstrated that suppression of 1α,25-$(OH)_2$-D_3 by increasing dietary calcium decreased adipocyte intracellular Ca^{2+}, stimulated lipolysis,

inhibited lipogenesis, increased adipocyte uncoupling protein 2 (UCP2) expression and core temperature in aP2-agouti transgenic mice and that dietary calcium not only attenuated diet-induced obesity but also accelerated weight loss and fat mass reduction secondary to caloric restriction (22, 23). Moreover, these results were supported by recent clinical observations (22, 43)

In summary, these data suggest that 1α,25-$(OH)_2$-D_3 modulates adipocyte Ca^{2+} signaling and, consequently, exerts a coordinated control over lipogenesis and lipolysis. Thus, a direct inhibition of 1α,25-$(OH)_2$-D_3 induced $[Ca^{2+}]_i$ may contribute to an anti-obesity effect of dietary calcium, and the mVDR mediated non-genomic pathway may represent an important target for development of therapeutic interventions in obesity.

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PART 8

1α,25-DIHYDROXYVITAMIN D_3 INHIBITS UNCOUPLING PROTEIN 2 EXPRESSION IN HUMAN ADIPOCYTES

I. ABSTRACT

We have previously demonstrated a regulatory role for intracellular Ca^{2+} ($[Ca^{2+}]_i$) in regulating adipocyte lipogenesis and lipolysis. Recent data from this laboratory demonstrates that 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3) stimulates human and murine adipocyte Ca^{2+} influx, and that suppressing 1α,25-$(OH)_2$-D_3 by increasing dietary calcium decreases adipocyte $[Ca^{2+}]_i$, stimulates lipolysis and inhibits lipogenesis. Notably, these high calcium diets also increase core temperature and white adipose tissue uncoupling protein 2 (UCP2) expression in aP2-agouti transgenic mice. However, it is not clear that whether this up-regulation of UCP2 is a direct effect of inhibition of 1α,25-$(OH)_2$-D_3' genomic action, inhibition of non-genomic action induced $[Ca^{2+}]_i$, or a result of increased fatty acid secondary to increased lipolysis. Accordingly, we have evaluated the role of 1,25-$(OH)_2$-D in regulating human adipocyte UCP2 mRNA and protein levels. 48 hr treatment of human adipocytes with 1 nM 1α,25-$(OH)_2$-D_3 caused a 20-50% decrease in basal UCP2 expression and protein levels, while direct stimulation of Ca^{2+} influx did not. Further, 1α,25-$(OH)_2$-D_3 completely prevented the isoproterenol-stimulated 2-3 fold increases in UCP2 expression and protein levels, whereas direct stimulation of Ca^{2+} influx exerted only minimal effects. Moreover, 1α,25-$(OH)_2$-D_3 blocked free fatty acid-stimulated UCP2 expression by 40%, while direct stimulation of Ca^{2+} influx was without effect. In contrast, 1α,25-dihydroxylumisterol$_3$ (1α,25-$(OH)_2$-lumisterol$_3$), a specific agonist for a putative membrane vitamin D receptor (mVDR), was unable to inhibit either basal or isoproterenol-stimulated or fatty acid-stimulated UCP2 expression. Consistent with this, 1β,25-dihydroxyvitamin D_3 (1β,25-$(OH)_2$-D_3), a

specific antagonist for the mVDR, was unable to prevent the 1α,25-$(OH)_2$-D_3' inhibition on either basal or isoproterenol-stimulated or fatty acid-stimulated UCP2 expression. Further, nVDR knockout via antisense oligodeoxynucleotide (ODN) prevented the inhibitory effect of 1α,25-$(OH)_2$-D_3 on adipocyte UCP2 expression. These data indicate that 1α,25-$(OH)_2$-D_3 exerts an inhibitory effect on white adipocyte UCP2 expression and that this effect is mediated via a genomic (nVDR) action. Thus, suppression of 1α,25-$(OH)_2$-D_3 and consequent up-regulation of UCP2 may contribute to our previous observation of increased thermogenesis in mice fed with high calcium diet. This effect, coupled with decreased lipogenesis and increased lipolysis secondary to decreased $[Ca^{2+}]_i$ mediated by nongenomic action may contribute to an anti-obesity effect of dietary calcium.

II. INTRODUCTION

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays a key role in metabolic disorders associated with obesity and insulin resistance (1-3). We previously reported that increasing $[Ca^{2+}]_i$ via stimulation of either receptor-mediated or voltage-dependent calcium channels 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 (4-7). Therefore, increasing $[Ca^{2+}]_i$ appears to promote lipid accumulation in adipocytes by exerting a coordinated control over lipogenesis and lipolysis, serving to simultaneously stimulate the former and inhibit the latter.

1α,25-dihyroxyvitamin D_3 (1α,25-$(OH)_2$-D_3) was originally believed to solely function via a nuclear vitamin D receptor (nVDR) to generate genomic action (8). However, this concept was challenged by studies demonstrating rapid nongenomic signal transduction, including stimulation of $[Ca^{2+}]_i$ via a putative membrane vitamin D receptor (mVDR), in a wide variety of cells (9-13). We recently demonstrated that 1α,25-$(OH)_2$-D_3 stimulates adipocyte $[Ca^{2+}]_i$, promotes lipogenesis and inhibits lipolysis via a rapid non-genomic action (14). We further demonstrated that suppressing 1α,25-$(OH)_2$-D_3 by dietary calcium decreases adipocyte $[Ca^{2+}]_i$, stimulates lipolysis, inhibits lipogenesis and thereby reduces adiposity in aP2-agouti transgenic mice (15, 16). Notably, it also increases core temperature and white adipocyte uncoupling protein 2 (UCP2) expression (15, 16). UCP2, a homolog of UCP1, is ubiquitously expressed, with the highest level in white adipose tissue (17, 18). UCP2 has been shown to decrease the mitochondrial potential in

transformed yeast (17), indicating that this protein stimulates mitochondrial proton leak. Several lines of evidence show that UCP2 is involved in regulating metabolic rate and adiposity in vivo. However, it is not clear whether this up-regulation of UCP2 in our animal studies is a direct effect of inhibition of $1\alpha,25\text{-}(OH)_2\text{-}D_3$' genomic action, or inhibition of non-genomic action induced $[Ca^{2+}]_i$, or a result of increased fatty acid flux secondary to increased lipolysis. Accordingly, this study was designed to determine the role of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ in regulating human adipocyte UCP2 mRNA and protein levels.

III. MATERIALS AND METHODS

A. CULTURE OF HUMAN ADIPOCYTES

A human adipocyte cell line and primary-cultured human adipocytes were both used as cell models in this study. The human adipocyte cell line was differentiated from human preadipocytes obtained from Zen-Bio Inc (Research Triangle Park, NC). Human preadipocytes were inoculated in Dulbecco's Modified Eagle' Medium (DMEM) / Ham' F-10 medium (F-10) (1:1, v/v) containing 10% fetal bovine serum (FBS), 15mM Hepes and antibiotics at a density of 30,000 cells/cm^2. Confluent monolayers of preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM/F-10 (1:1, v/v) medium supplemented with 15 mM Hepes, 3% FBS, 33 μM biotin, 17 μM pantothenate, 100 nM insulin, 0.25 mM methylisobutylxanthine (MIX), 1 μM dexamethasome, 1 μM BRL49653 and antibiotics (100 UI/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml Fungizone). Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium in which BRL49653 and MIX were withdrawn. Cultures were refed every 2-3 days (19). Primary-cultured human adipocytes were derived from human subcutaneous adipose tissue obtained from patients undergoing abdominal plastic surgery with no known history of metabolic disorders. This protocol was approved by the Institutional Review Board for Human Subjects and the Committee for Research Participation of the University of Tennessee. Adipocytes were isolated as previously described (20). Briefly, adipose tissue was first washed several times with Hank's Balanced Salt Solution, minced into small pieces and digested with 1 mg/ml type1 collagenase in a shaking water bath at 37 ^{0}C for 30 min. Adipocytes were then filtered through a sterile 500 μm nylon

mesh and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% fetal bovine serum (FBS), 100 UI/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin. Cells were cultured in suspension and maintained in a thin layer at the top of culture media, which were changed every day. Cells were maintained viable and metabolically responsive under this culture condition for 7 days.

B. NORTHERN BLOT ANALYSIS

Northern blot analysis was conducted as previously described (20). Total RNA from human adipose tissue was extracted using $CsCl_2$ density centrifugation, run in 1% agarose gel and transferred to nylon membranes (New England Nuclear, Boston, MA). The membrane was hybridized with UCP2 cDNA probes that were radiolabeled 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 ^{0}C. Finally, the membrane was exposed to X-ray film (New England Nuclear, Boston, MA) at –80 ^{0}C. All membranes were stripped and reprobed with β-actin as loading control. The blot image was captured using DigiPix 1260^{TM} imaging system with a CCD camera and a electronic transilluminator (ULTRA-LUM, Inc. Paramount, CA). ZERO-Dscan software (Scanalytics, Inc, Fairfax, VA) was used to quantitatively analyze these images.

C. WESTERN BLOT ANALYSIS

Adipocytes were harvested and sonicated in a homogenization buffer containing 50 mM Tris-HCl (PH 7.4), 250 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, 1% (v/v) Triton

X, 10% protease cocktail (Sigma, St. Louis, MO). Cell homogenate was incubated on ice for 1 hr to solubilize all proteins and insoluble portion was removed by centrifugation at 12,500 x g at 4 °C for 15 minute. Homogenate intrafranatant protein from equal numbers of cells (determined via DNA quantitation) was boiled in Laemmli sample buffer and subject to 10 % SDS-PAGE (21). Proteins on the gels were transferred to Hyond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The transferred membranes with proteins were blocked, washed, incubated with UCP2 monoclonal antibody and followed by horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Visualization was detected with chemiluminescence reagent using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ).

D. REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA from human adipocytes was extracted using $CsCl_2$ density centrifugation. RT-PCR was performed as previously described (20). Briefly, 1 μg of human adipocyte total RNA was reverse-transcribed to first strand cDNA using random-hexamer and reverse transcriptase (Perkin Elmer, Norwalk, CT) and amplified by PCR (Perkin Elmer, Norwalk, CT). The PCR conditions were as follows: initial denaturation at 94 ^{0}C for 5min, followed by 34 cycles of denaturation at 94 ^{0}C for 45s, annealing at 58 ^{0}C for 1min, and extension at 72 ^{0}C for 1 min, with a final extension step at 72 ^{0}C for 8 min with 0.2 μM 5' primer (5'-GATCTGTGGGGTGTGTGGAGA-3') and 3' primer (5'-CTGGGAGTGTGTCTGGAGTTG-3') which correspond to nucleotide positions 181-

201 and 625-645 in Genbank access NO. J03258, respectively. The amplified PCR products were then visualized by 1.2% agarose gel electrophoresis.

E. ANTISENSE OLIGODEOXYNUCLEOTIDE (ODN) TRANSFECTION

The phosphorothioate antisense ODNs used in this study were synthesized by Life Technologies. The antisense ODN targeting the nuclear vitamin D receptor (nVDR) was designed corresponding to the start codon region of translation initiation in nVDR, with the sequence 5'-CTGGCCGCCATTGCCTCCAT-3'. A random mutant sequence (5'-ATTGACTCAAATTCATTCTT-3') was used as a control.

A lipofection kit, OLIGOFECTAMINE^{TM} Reagent (Life Technologies), was used to transfect the cells with antisense ODNs. Antisense or mutant ODNs were diluted with OPTI-MEM I Reduced Serum Medium (Life Technologies) and mixed with OLIGOFECTAMINE^{TM} Reagent to give the final concentration 200 nM. Cells were incubated in this medium at 37 ^{o}C in a CO_2 incubator for 4 hr and then cultured in medium containing normal serum.

F. STATISTICAL ANALYSIS

All data are expressed as mean ± SE. Data are 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 using SPSS (SPSS Inc, Chicago, IL). A p value < 0.05 is considered significant.

IV. RESULTS

We first determined the effect of 1α,25-$(OH)_2$-D_3 on adipocyte basal UCP2 expression. **Fig. 33** (left panel) shows that a 48 hr treatment of human adipocytes with 1 nM 1α,25-$(OH)_2$-D_3 caused a 40% decrease in UCP2 mRNA ($p<0.003$), while direct stimulation of Ca^{2+} influx with KCl, a cell membrane depolarization agent, exerted no effect. Similarly, 1 nM 1α,25-$(OH)_2$-D_3 reduced UCP2 protein levels by 50% ($p<0.002$, **Fig. 33** right panel), while KCl stimulation of Ca^{2+} influx was without effect. We then assessed the effect of 1α,25-$(OH)_2$-D_3 on isoproterenol-stimulated UCP2 expression. **Fig. 34** (left panel) illustrates that treatment with 10 nM isoproterenol caused a 2 fold increase in adipocyte UCP2 mRNA ($p<0.002$), which was completely blocked by 1 nM 1α,25-$(OH)_2$-D_3, but only 20% by KCl. Although treatment with KCl inhibited isoproterenol-stimulated increases in UCP2 protein ($p<0.006$, **Fig. 34** right panel), 1α,25-$(OH)_2$-D_3 exerted a more potent effect, reducing UCP2 protein below the basal level. We next evaluated the effect of 1α,25-$(OH)_2$-D_3 on free fatty acid-stimulated UCP2 expression. **Fig. 35** shows that free fatty acids (oleic acid and linoleic acid mixture) caused a ~2 fold increase in UCP2 mRNA, which was completely prevented by 1α,25-$(OH)_2$-D_3. These data indicate that 1α,25-$(OH)_2$-D_3 inhibition of UCP2 expression is largely independent of its effects on Ca^{2+} influx or fatty acid flux.

To study whether membrane vitamin D receptor (mVDR) mediates this inhibition of 1α,25-$(OH)_2$-D_3 on adipocyte UCP2 expression, 1α,25-dihydroxylumisterol$_3$ (1α,25-$(OH)_2$-lumisterol$_3$), a specific mVDR agonist, and 1β,25-dihydroxyvitamin D (1β,25-

Fig 33. The effect of 1α,25-$(OH)_2$-D_3 on basal UCP2 mRNA (left panel) and protein level (right panel) in human adipocytes. Human adipocytes were treated with 1α,25-$(OH)_2$-D_3 (1 nM) or KCl (10 mM). UCP2 mRNA and protein level were measured by Northern blot and Western blot analysis, respectively. These measurements were conducted as described in Materials and Methods. Left panel: The effect of 1α,25-$(OH)_2$-D_3 on basal UCP2 mRNA in human adipocytes. Blot shown is representative of 4 similar experiments. *$p<0.003$ vs. control. Right panel: The effect of 1α,25-$(OH)_2$-D_3 on basal UCP2 protein level in human adipocytes. Blot shown is representative of 3 similar experiments. *$p<0.002$ vs. control.

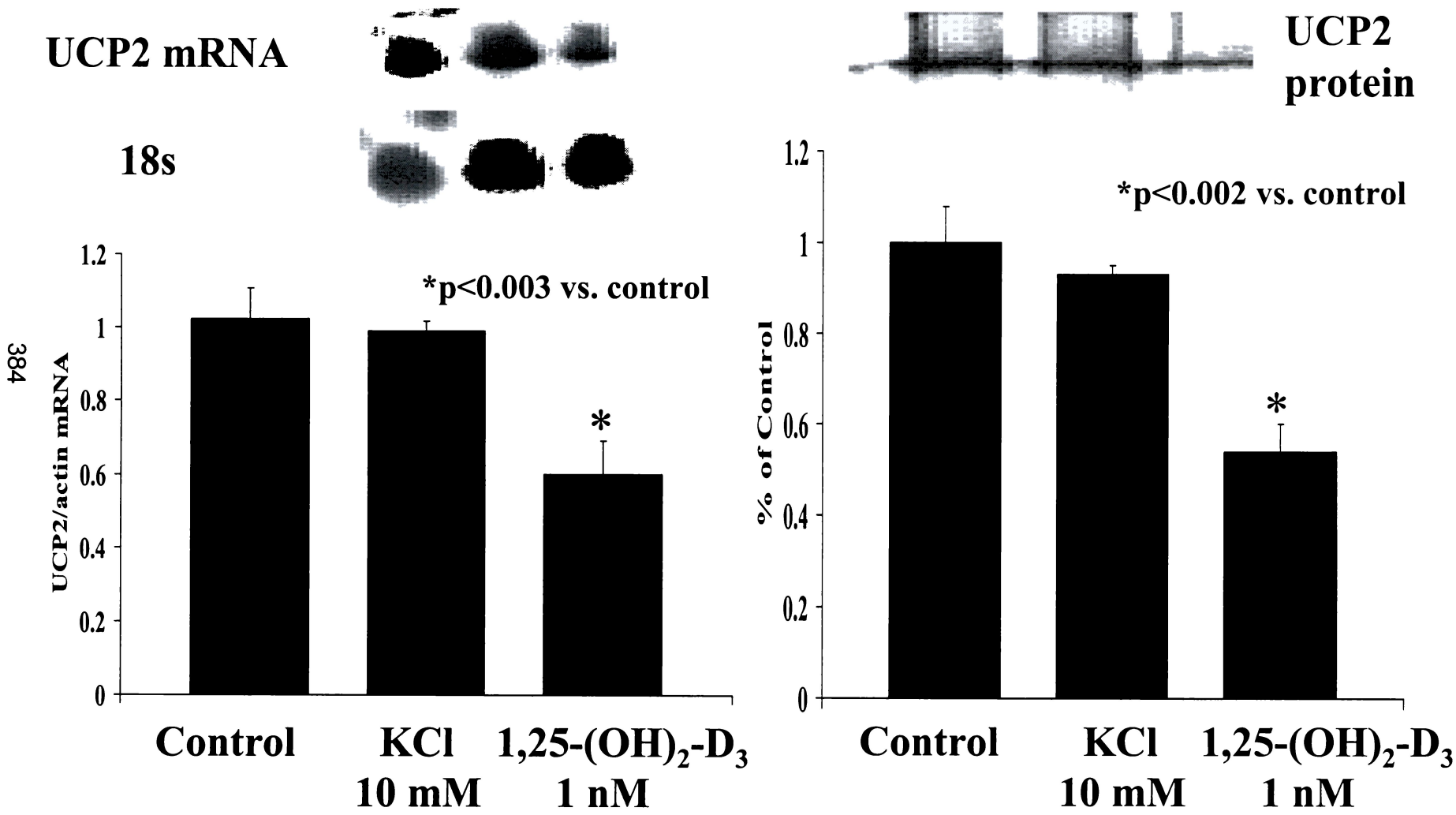
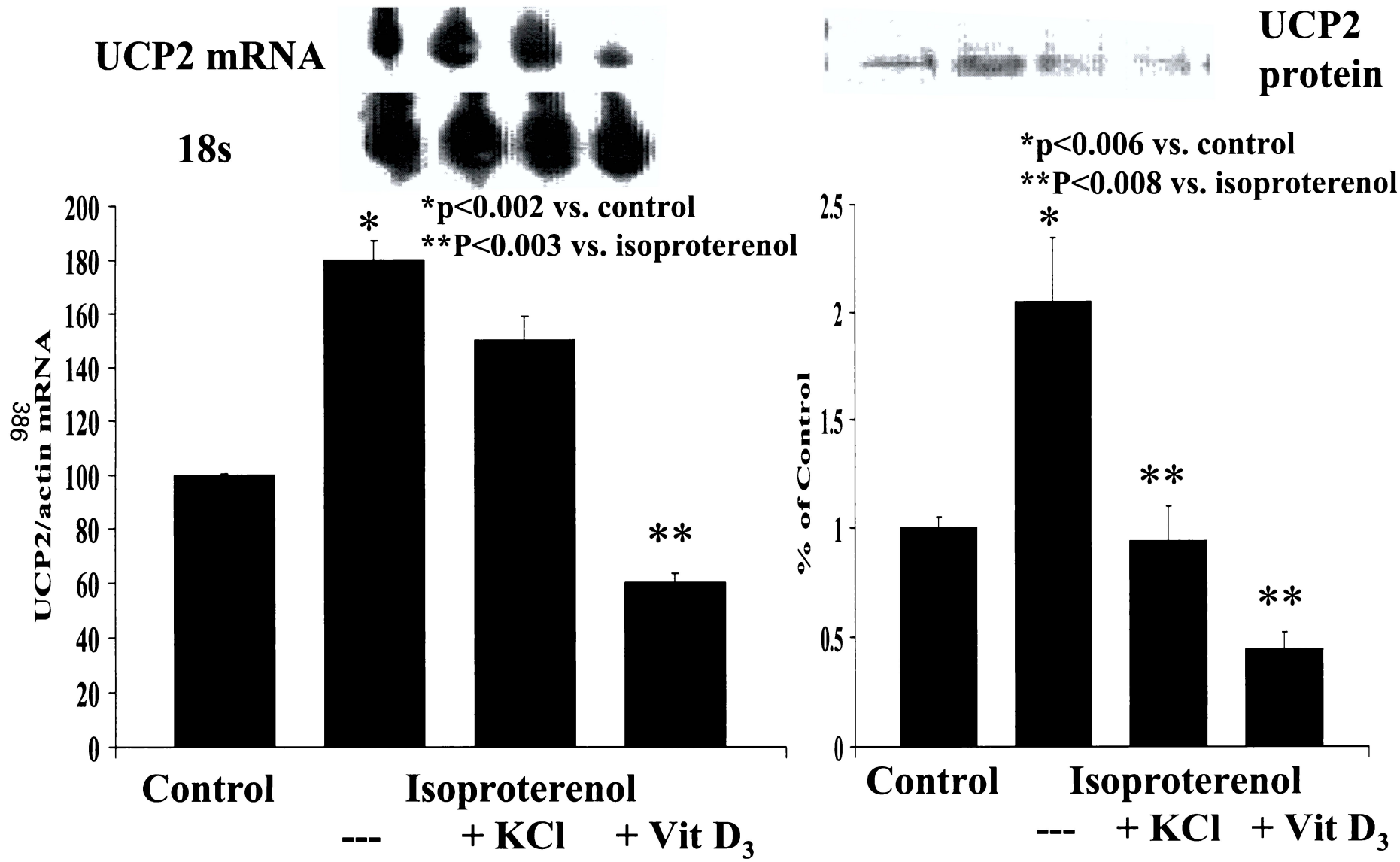

UCP2 mRNA
18s
UCP2
protein
*p<0.003 vs. control
UCP2/actin mRNA
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
KCl
10 mM
1,25-(OH)2-D3
1 nM
*p<0.002 vs. control
% of Control
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
KCl
10 mM
1,25-(OH)2-D3
1 nM

Fig 34. The effect of 1α,25-$(OH)_2$-D_3 on isoproterenol-stimulated UCP2 mRNA (left panel) and protein level (right panel) in human adipocytes. Human adipocytes were treated with or without isoproterenol (10 nM), isoproterenol plus 1α,25-$(OH)_2$-D_3 (1 nM) or isoproterenol plus KCl (10 mM). UCP2 mRNA and protein level were measured by Northern blot and Western blot analysis, respectively. These measurements were conducted as described in Materials and Methods. Left panel: The effect of 1α,25-$(OH)_2$-D_3 on isoproterenol-stimulated UCP2 mRNA in human adipocytes. Blot shown is representative of 4 similar experiments. *$p<0.002$ vs. control, **$p<0.003$ vs. isoproterenol. Right panel: The effect of 1α,25-$(OH)_2$-D_3 on isoproterenol-stimulated UCP2 protein level in human adipocytes. Blot shown is representative of 3 experiments. *$p<0.006$ vs. control, **$p<0.008$ vs. isoproterenol.


UCP2 mRNA
18s
UCP2 protein
*p<0.002 vs. control
**P<0.003 vs. isoproterenol
*p<0.006 vs. control
**P<0.008 vs. isoproterenol
UCP2/actin mRNA
% of Control
Control
Isoproterenol

+ KCl
+ Vit D3

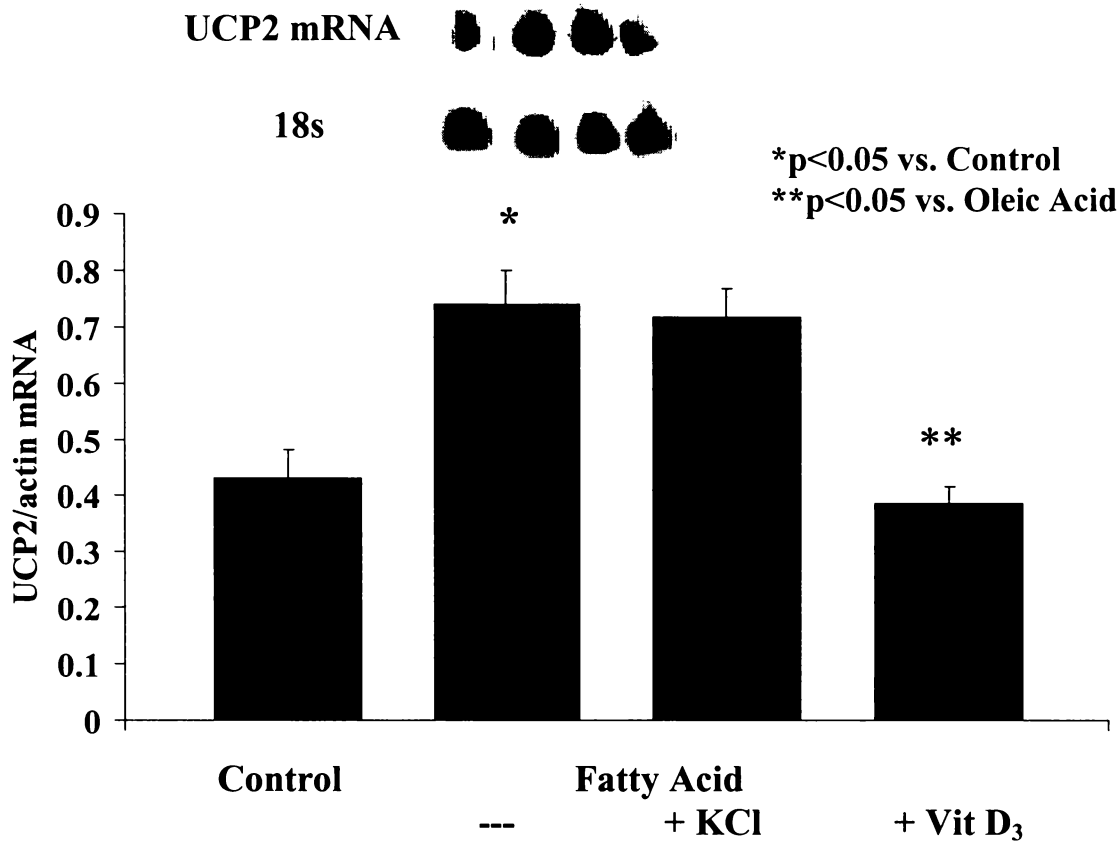


Fig 35. The effect of 1α,25-$(OH)_2$-D_3 on fatty acid-stimulated UCP2 mRNA in human adipocytes. Human adipocytes were treated with or without fatty acid (100 μM), fatty acid plus 1α,25-$(OH)_2$-D_3 (1 nM) or fatty acid plus KCl (10 mM). UCP2 mRNA was measured by Northern blot. This measurement was conducted as described in Materials and Methods. Blot shown is representative of 4 similar experiments. *p<0.05 vs. control, **p<0.05 vs. fatty acid.

$(OH)_2$-D_3), a specific mVDR antagonist, were used to treat human adipocytes. **Fig. 36** demonstrates that 1α,25-$(OH)_2$-lumisterol$_3$ failed to exert an inhibitory effect on UCP2 mRNA, while 1β,25-$(OH)_2$-D_3 was unable to reverse 1α,25-$(OH)_2$-D_3-induced inhibition on UCP2 mRNA. Consistent with this, 1α,25-$(OH)_2$-lumisterol$_3$ failed to prevent isoproterenol-stimulated UCP2 expression, while 1β,25-$(OH)_2$-D_3 was unable to reverse 1α,25-$(OH)_2$-D_3 inhibition of isoproterenol-stimulated UCP2 mRNA (**Fig. 37**). Similarly, the mVDR agonist and antagonist exerted no effect on fatty acid-stimulated UCP2 expression. **Fig. 38** shows that 1α,25-$(OH)_2$-lumisterol$_3$ failed to block fatty acid-stimulated UCP2 expression, while 1β,25-$(OH)_2$-D_3 was unable to prevent 1α,25-$(OH)_2$-D_3 inhibition of fatty acid-stimulated UCP2 mRNA. These data indicate that mVDR does not mediate the inhibitory effect of 1α,25-$(OH)_2$-D_3 on adipocyte UCP2 expression

We next investigated the role of the nuclear vitamin D receptor (nVDR) in mediating the inhibitory effect of 1α,25-$(OH)_2$-D_3 on adipocyte UCP2 expression. Using RT-PCR, we detected a 465 bp nVDR fragment in human adipocytes and preadipocytes, corresponding to the nucleotide sequence region 181-645 in nVDR gene (Genbank access NO. J03258) (**Fig. 39** left panel). This was confirmed by western blot analysis; using an nVDR monoclonal antibody, we detected a ~50 kD protein (**Fig. 39** right panel). We then performed a transient transfection of antisense ODN to knock out the nVDR. A time course study shows that treatment with nVDR antisense ODN inhibited nVDR protein from 48 hr through 96 hr, while the mutant antisense ODN was without effect (**Fig. 40** left panel), demonstrating that the antisense ODN targeting nVDR is specific. We then

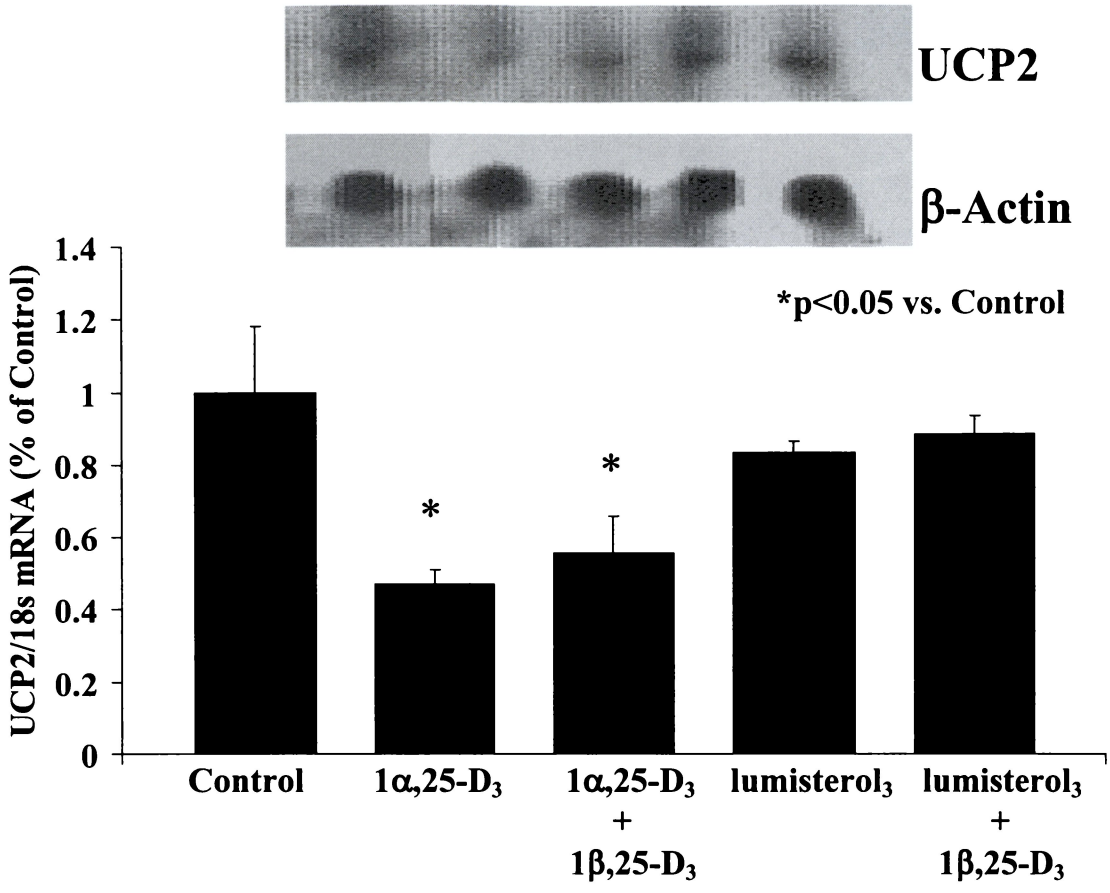


Fig 36. The effects of 1α,25-$(OH)_2$-D_3 and its mVDR agonist (1α,25-dihydroxylumisterol$_3$) and antagonist (1β,25-dihydroxyvitamin D) on basal UCP2 mRNA in human adipocytes. Human adipocytes were treated with 1α,25-$(OH)_2$-D_3 (1 nM) alone or 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) alone, 1α,25-$(OH)_2$-D_3 (1 nM) plus 1β,25- $(OH)_2$-D_3 or 1α,25-$(OH)_2$-lumisterol$_3$ (5 nM) plus 1β,25- $(OH)_2$-D_3. UCP2 mRNA was measured by Northern blot. This measurement was conducted as described in Materials and Methods. *p<0.05 vs. control, n=3.

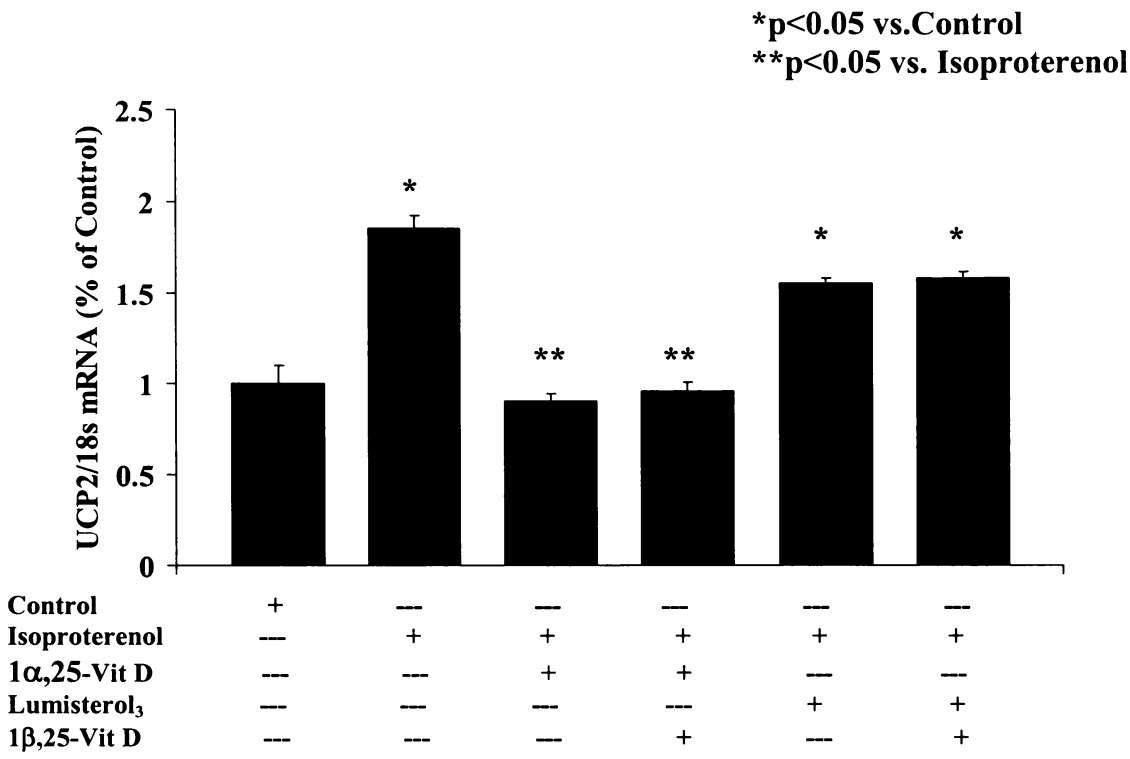


Fig 37. The effects of 1α,25-$(OH)_2$-D_3 and its mVDR agonist (1α,25-dihydroxylumisterol$_3$) and antagonist (1β,25-dihydroxyvitamin D) on isoproterenol-stimulated UCP2 mRNA in human adipocytes. Human adipocytes were treated as indicated in the figure. UCP2 mRNA was measured by Northern blot. This measurement was conducted as described in Materials and Methods. *p<0.05 vs. control, **p<0.05 vs. isoproterenol, n=3.

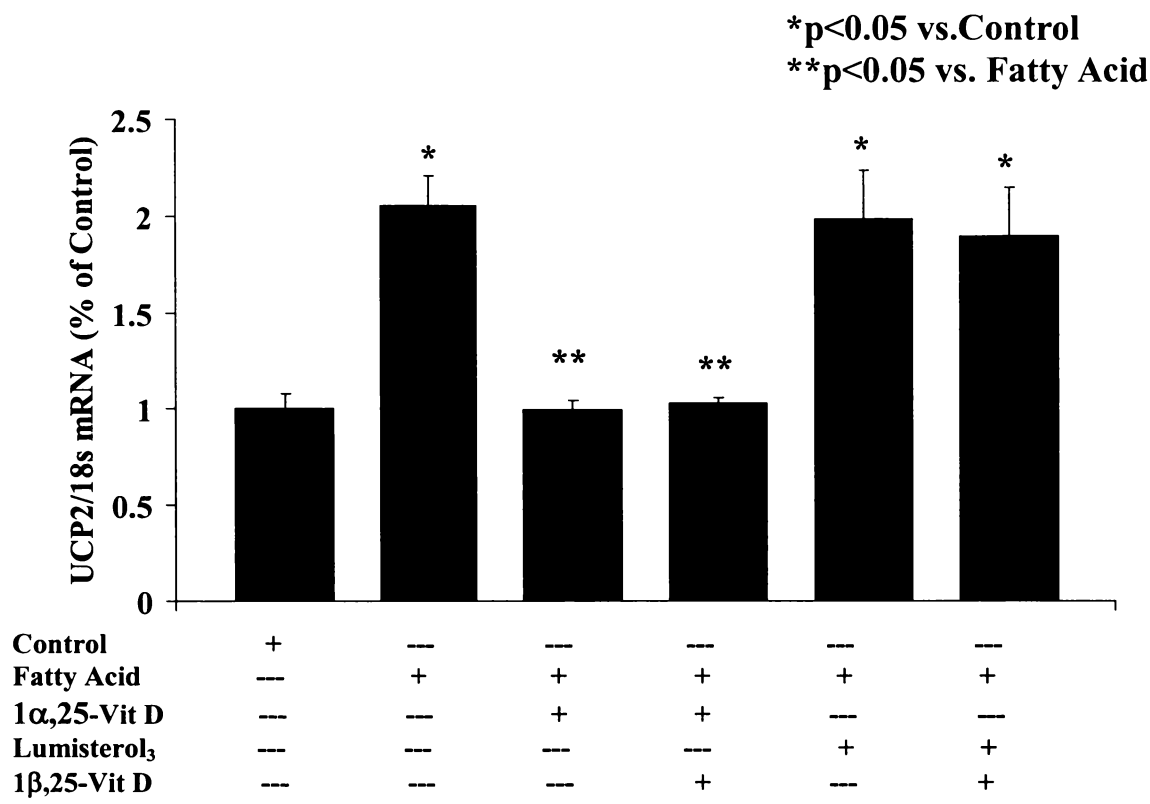


Fig 38. The effects of 1α,25-$(OH)_2$-D_3 and its mVDR agonist (1α,25-dihydroxylumisterol$_3$) and antagonist (1β,25-dihydroxyvitamin D) on fatty acid-stimulated UCP2 mRNA in human adipocytes. Human adipocytes were treated as indicated in the figure. UCP2 mRNA was measured by Northern blot. This measurement was conducted as described in Materials and Methods. *p<0.05 vs. control, **p<0.05 vs. fatty acid, n=3.

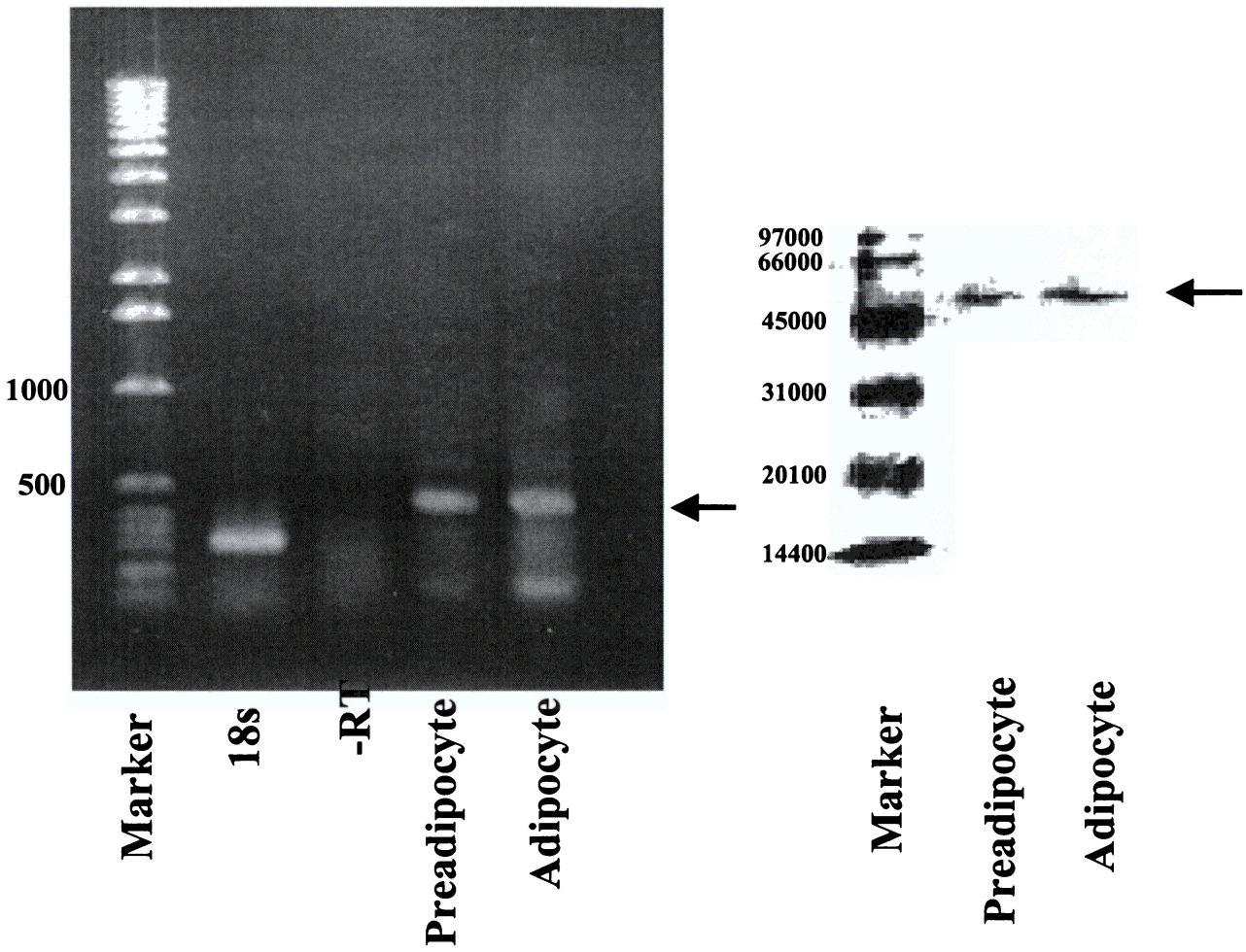


Fig 39. Detection of the expression of nuclear vitamin D receptor (nVDR) in human adipocytes by RT-PCR (left panel) and Western blot (right panel). RT-PCR and Western blot were performed as described in Materials and Methods. Left panel: Detection of the expression of nVDR in human adipocytes by RT-PCR. The PCR product (designated by arrow), a 465 bp nVDR fragment, was visualized by 1.2% agarose gel electrophoresis. Each lane is as indicated in the figure. Right panel: Detection of the expression of nuclear vitamin D receptor (nVDR) in human adipocytes by Western blot. A ~50 Kd protein band (designated by arrow) was detected in the blot. Each lane is as indicated in the figure.

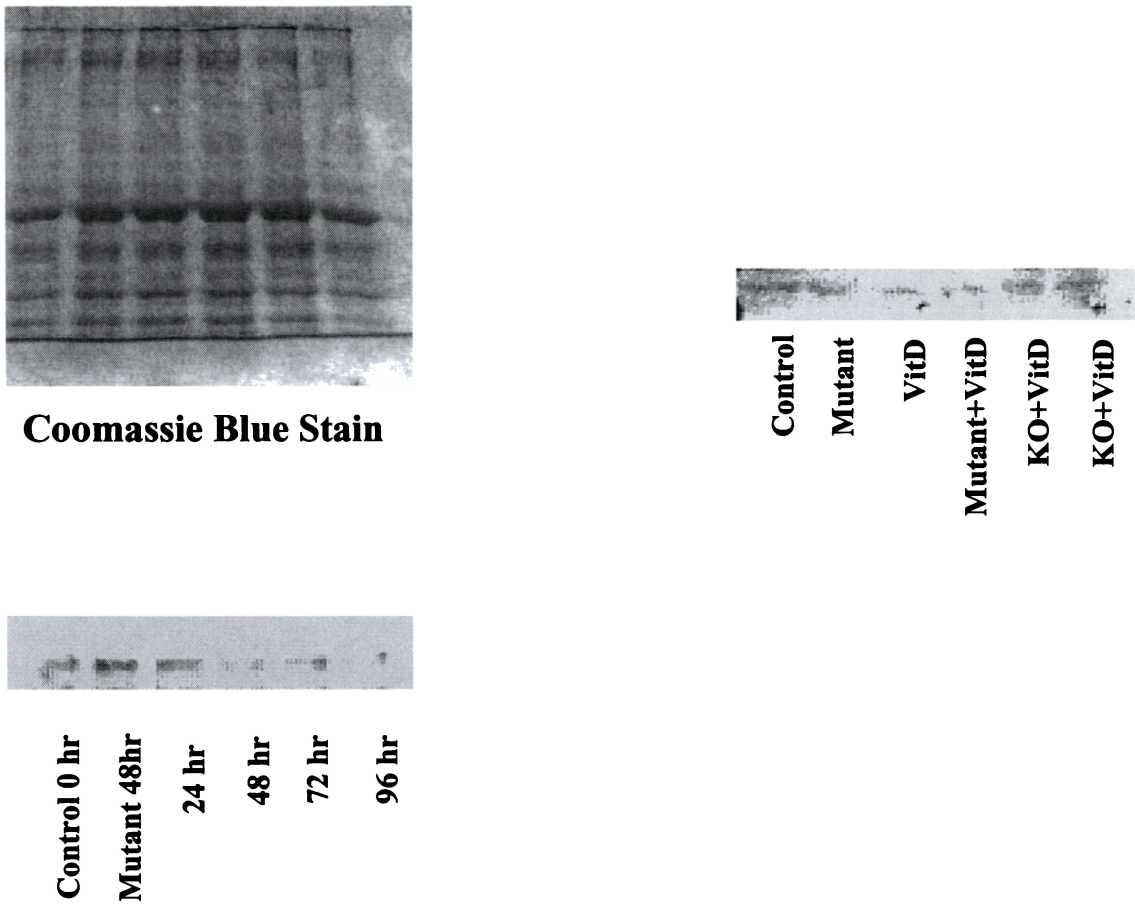


Fig 40. Nuclear vitamin D receptor (nVDR) knockout by antisense oligodeoxynucleotide (ODN) prevented the inhibitory effect of 1α,25-$(OH)_2$-D_3 on adipocyte UCP2 expression. Antisense ODN knockout was conducted as described in Materials and Methods. Left panel: a time course of nVDR knockout by antisense ODN; equal amount of protein loading was achieved by sample DNA measurement and confirmed by SDS-PAGE visualized with coomassie blue stain (upper left). Adipocytes were transfected with nVDR antisense ODN or mutant ODN as indicated in the figure. Right panel: nVDR knockout by antisense ODN prevented the inhibitory effect of 1α,25-$(OH)_2$-D_3 on adipocyte UCP2 expression. nVDR knockout adipocytes or adipocytes transfected with mutant ODN were treated with or without 1α,25-$(OH)_2$-D_3 as indicated in the figure.

treated the nVDR knockout adipocytes with 1α,25-$(OH)_2$-D_3. **Fig. 40** (right panel) shows that 1α,25-$(OH)_2$-D_3 markedly inhibited UCP2 protein in either control adipocytes or adipocytes treated with mutant ODN. However, 1α,25-$(OH)_2$-D_3 was unable to exert the inhibitory effect in nVDR knockout adipocytes, indicating that this effect is mediated by the nVDR.

V. DISCUSSION

We previously reported that 1α,25-$(OH)_2$-D_3 stimulates adipocyte $[Ca^{2+}]i$, promotes lipogenesis and inhibits lipolysis via a rapid non-genomic action (14). Data presented here further demonstrate that 1α,25-$(OH)_2$-D_3 exerts an inhibitory effect on adipocyte UCP2 expression via a genomic action. Therefore, 1α,25-$(OH)_2$-D_3 appears to play an important role in modulating adipocyte lipid metabolism and energy homeostasis and function via both genomic and non-genomic actions.

It is now well recognized that 1α,25-$(OH)_2$-D_3, the biologically active form of the vitamin D, generates biological responses via both genomic and nongenomic pathways. 1α,25-$(OH)_2$-D_3 is a highly conformationally flexible molecule and is able to rotate around its 6, 7 carbon-carbon bond of the B ring. This rotation allows generation of a continuum of potential ligand shapes ranging from the steroid like 6-s-cis conformation to the extended 6-s-trans conformation (22, 23). Thus, this conformational flexibility of 1α,25-$(OH)_2$-D_3 in a structure and function context underlies the mechanism whereby 1α,25-$(OH)_2$-D_3 generates both genomic and non-genomic cellular responses.

1α,25-$(OH)_2$-D_3 generates genomic actions via a nuclear receptor in a manner similar to the other members of steroid hormone superfamily (8). 1α,25-$(OH)_2$-D_3 binds to and activates a specific nuclear hormone receptor, nVDR, and nVDR knockout exhibits a vitamin D deficient phenotype (24, 25). However, several studies demonstrated that 1α,25-$(OH)_2$-D_3 is able to induce rapid non-genomic effects in nVDR-null cells or cells

from nVDR knockout mice (26, 27), indicating that the genomic model of 1α,25-$(OH)_2$-D_3 action is not complete.

Instead, 1α,25-$(OH)_2$-D_3 also generates rapid, nongenomic signal transduction via a putative membrane vitamin D receptor (mVDR) in a wide variety of cells (9). These effects include modulation of calcium channels (10-13, 28-33), production of diacyl glycerol and inositol triphosphate induced by activation of phospholipase (34), activation of protein kinase C (35), and stimulation of MAP kinase (36-38). Most importantly, recent effort of pursuing physical existence of the putative mVDR provides the further evidence to strengthen these findings. Several studies have identified membrane proteins which function as membrane vitamin D receptors (32, 35, 39).

Our previous and present data further extend these observations by demonstrating that 1α,25-$(OH)_2$-D_3 elicits both genomic and non-genomic action in adipocytes, resulting in modulation of lipid and energy metabolism. In this study, we demonstrated that 1α,25-$(OH)_2$-D_3 exerts an inhibitory effect on basal, isoproterenol and fatty acid stimulated UCP2 expression via a genomic action. We confirmed that this inhibition is mediated by nVDR using both pharmacological and molecular approaches. The mVDR agonist and antagonist fail to exert their actions to either mimic or prevent the1α,25-$(OH)_2$-D_3 inhibitory effect on UCP2 expression, while nVDR knockout by antisense ODN prevented the inhibitory effect of 1α,25-$(OH)_2$-D_3 on adipocyte UCP2 expression. Therefore, our data demonstrate a novel role of nVDR in regulating adipocyte energy metabolism. Moreover, a recent report demonstrated that nVDR gene polymorphism is

associated with the susceptibility to obesity in humans with early-onset type II diabetes (40). These findings indicate that nVDR may play a role in energy homeostasis and obesity.

UCP2, a homolog of UCP1, is 56% identical to UCP1 and is ubiquitously expressed, with the highest level found in white adipose tissue (17, 18). UCP2 has been shown to decrease the mitochondrial potential in transformed yeast. Expressing UCP2 in yeast causes a 50% increase in state 4 respiration, a respiration in the absence of ADP (17), demonstrating that this protein may act on proton leaking. This was subsequently confirmed by demonstrating that UCP2 expressed in *Escherichia Coli* inclusion body and then reconstituted into liposomes is able to catalyze electrophoretic proton flux (41). Like UCP1, a well-characterized uncoupling protein, UCP2 requires fatty acid to mediate this proton transport activity (41). Consistent with these findings, a recent study demonstrated that UCP2 reconstituted into vesicles is a highly active proton transporter (42). The UCP2 mediated proton transport activity requires fatty acid and coenzyme Q, an obligatory cofactor for proton transport by UCP1 (43). Moreover, this proton transport process is highly sensitive to purine nucleotides (42). Therefore, all these proton transport properties for UCP2 resemble those for UCP1.

Various physiological states and many nutritional and hormonal factors have been shown to regulate UCP2 expression (see reviews 44-46). Administration of thermogenic hormones such as thyroid hormone (T_3) and leptin to rodents increases UCP2 expression (44-51). Moreover, PPARγ agonist (thiazolidinediones) and retinoic acids are potent

stimulators of UCP2 expression (44-46, 52-56). In addition, fatty acids and β-adrenergic agonists are also able to induce UCP2 expression in vitro and in vivo (44-46, 51, 57-60). Functional characterization of UCP2 promoter region has demonstrated several potent cis-acting regulatory elements including 2 PPARγ responsive elements (PPRE), 3 cAMP responsive elements (CREB-1), 2 putative thyroid hormone responsive elements (TRE) (61-63). These responsive elements could provide mechanisms of the positive regulation of UCP2 expression via PPARγ agonists, fatty acids (ligands of PPARγ), thyroid hormones, β-adrenergic agonists (isoproterenol). However, little is known regarding negative regulatory factors of UCP2 expression. Here we report that $1\alpha,25\text{-}(OH)_2\text{-}D_3$ exerts an inhibitory effect on UCP2 expression. The mechanism of this nVDR mediated inhibition of UCP2 is not known. However, the human UCP2 promoter region does contain several cis-acting negative regulatory elements, which strongly repress promoter activity (61), although it is not clear whether nVDR acts on one of these silencers. Alternatively, nVDR may also compete with other positive transcriptional factors containing similar DNA binding domains on the responsive element binding or similar protein-protein interaction domain (such as PPARγ) on heterodimerization with the same transcriptional factor (RXR). Further studies are required to address the mechanism whereby $1\alpha,25\text{-}(OH)_2\text{-}D_3$ inhibits UCP2 expression.

The proposed physiological functions of UCP2 include control of thermogenesis and energy homeostasis, regulation of insulin secretion, regulation of reactive oxygen species (ROS) and regulation of fatty acid utilization as substrates (see reviews 44-46). UCP2 in different tissues and physiological states may exhibit various functions. A recent study of UCP2 knockout mouse

demonstrated that UCP2-ablated mouse exhibits increased ROS production in macrophage (64). Moreover, studies of UCP2 expression during fasting and re-feeding in rodents have led to a proposal that UCP2 may function in the regulation of lipid as a fuel substrate (65, 66). Most importantly, several lines of evidence show that UCP2 is involved in regulating metabolic rate and adiposity in vivo. A high fat diet increased WAT UCP2 gene expression in obesity-resistant A/J strains but not in obesity-prone C57B6 mice, indicating UCP2 also plays a role in preventing obesity (67). In human study, a positive correlation between UCP2 mRNA expression in WAT and resting metabolic rate (RMR) has been reported (68). In addition, UCP2 expression has also been shown to decrease by 28% in skeletal muscle of obese subjects (69). Consistent with this finding, a reduced UCP2 expression in visceral adipose tissue in morbidly obese subjects has been reported recently (70). A number of genetic linkage studies have also documented association between genetic markers and variants of UCP2 gene and human metabolic rate and/or adiposity (71, 72). A recent study showed that a common G/A polymorphism in the UCP2 promoter region is associated with increased white adipocyte UCP2 mRNA level and reduced adiposity (73). Moreover, this promoter polymorphism results in an increased transcription of a reporter gene in a human adipocyte cell line (73). Recently, many studies have demonstrated that UCP2 is involved in regulating insulin secretion in pancreatic β cells (74-77). Increased UCP2 in pancreatic β cells decrease ATP/ADP ratio and thereby reduce glucose-stimulated insulin secretion (74-77). As $1\alpha,25\text{-}(OH)_2\text{-}D_3$ has been previously shown to induce insulin secretion in pancreatic β cells (78, 79), it is tempting to speculate that $1\alpha,25\text{-}(OH)_2\text{-}D_3$ may induce insulin secretion, in part, by inhibiting UCP2 in pancreatic β cells. Indeed, our previous studies showed that inhibition of circulating $1\alpha,25\text{-}(OH)_2\text{-}D_3$ by increasing dietary calcium reduce insulin level in aP2-agouti transgenic mice (15, 16).

Regulation of adipocyte metabolism via 1α,25-$(OH)_2$-D_3 signaling pathways may play an important role in the development of obesity *in vivo*. Several lines of evidence demonstrate that circulating 1α,25-$(OH)_2$-D_3 level is elevated in obese humans (80-83). Previous studies showed that increasing dietary calcium suppressed 1α,25-$(OH)_2$-D_3 levels (84, 85). Accordingly, we tested the hypothesis that dietary calcium suppression of 1α,25-$(OH)_2$-D_3 would reduce adipocyte $[Ca^{2+}]_i$, increase lipolysis, inhibit lipogenesis, increase UCP2 expression and thereby reduce adiposity in aP2-agouti transgenic mice. Our data demonstrated that suppression of 1α,25-$(OH)_2$-D_3 by increasing dietary calcium decreases adipocyte intracellular Ca^{2+}, stimulated lipolysis, inhibited lipogenesis, increased adipocyte uncoupling protein 2 (UCP2) expression and core temperature in aP2-agouti transgenic mice and that dietary calcium not only attenuated diet-induced obesity but also accelerated weight loss and fat mass reduction secondary to caloric restriction (15, 16). Recent epidemiological data demonstrate comparable effects in humans (15, 86).

In summary, these data indicate that 1α,25-$(OH)_2$-D_3 exerts an inhibitory effect on white adipocyte basal, isoproterenol and fatty acid-stimulated UCP2 expression and that this effect is mediated via a genomic action. Thus, suppression of 1α,25-$(OH)_2$-D_3 and consequent up-regulation of UCP2 may contribute to our previous observation of increased thermogenesis in mice fed with high calcium diet. This effect, coupled with decreased lipogenesis and increased lipolysis secondary to decreased $[Ca^{2+}]_i$ mediated by nongenomic action may contribute to an anti-obesity effect of dietary calcium.

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PART 9

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

In summary, we have evaluated the role of adipocyte $[Ca^{2+}]_i$ in modulation of adiposity.

We demonstrated that increasing $[Ca^{2+}]_i$ exerts a biphasic regulatory role in human adipocyte differentiation, serving to inhibit the early stages of differentiation, while promoting the late stages of differentiation and lipid filling. To antagonize adipocyte Ca^{2+} influx by blocking Ca^{2+} channels, we identified a potential receptor target, adipocyte sulfonylurea receptor (SUR), which mediates adipocyte $[Ca^{2+}]_i$ signaling and corresponding alterations in lipogenesis and lipolysis. Antagonism of this receptor reverses all these actions. We extended this approach to dietary regulation of adipocyte $[Ca^{2+}]_i$. We demonstrated that $1\alpha,25\text{-}(OH)_2\text{-}D_3$, which is regulated by dietary calcium, also stimulates adipocyte $[Ca^{2+}]_i$, promotes lipogenesis, and inhibits lipolysis, thereby resulting in triglyceride accumulation, via a rapid non-genomic action mediated by a putative membrane vitamin D receptor (mVDR). These actions can be mimicked by $1\alpha,25\text{-}(OH)_2\text{-lumisterol}_3$, a specific agonist for mVDR, and completely prevented by $1\beta,25\text{-}(OH)_2\text{-}D_3$, a specific antagonist for the mVDR. Dietary calcium suppression of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ was used as strategy to reduce adipocyte $[Ca^{2+}]_i$ and inhibit triglyceride accumulation in transgenic mice over-expressing agouti specifically in adipose tissue. Our data demonstrated that suppression of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ by increasing dietary calcium decreases adipocyte $[Ca^{2+}]_i$, stimulates lipolysis, inhibits lipogenesis in aP2-agouti transgenic mice and that dietary calcium not only attenuated diet-induced obesity but also accelerated weight loss and fat mass reduction secondary to caloric restriction. Notably, high calcium diets also increase white adipose tissue uncoupling protein 2 (UCP2)

expression and core temperature. We therefore further evaluated the role of 1α,25-$(OH)_2$-D_3 in regulating human adipocyte UCP2 expression. We demonstrated that 1α,25-$(OH)_2$-D_3 exerts an inhibitory effect on white adipocyte UCP2 expression and that this effect is mediated via a genomic action (nuclear vitamin D receptor nVDR). Accordingly, these data demonstrate that SUR-mediated Ca^{2+} signaling and 1α,25-$(OH)_2$-D_3-mediated genomic and non-genomic pathways play important roles in regulation of adipocyte lipid and energy metabolism, thereby contributing to modulation of adiposity, and that antagonism of adipocyte $[Ca^{2+}]_i$ by blocking these pathways may represent an effective approach for development of therapeutic interventions in obesity.

VITA

Hang Shi was born in Jinhua, Zhejiang province, China. In 1986, he entered Shanghai Medical University where he studied for six years and earned his Bachelor of Medicine Degree in 1992. He studied as a graduate student at Biochemistry Department in Shanghai Medical University from 1996-1997. He later studied toward a Ph. D. degree with a major in Human Ecology focusing on obesity research at University of Tennessee, Knoxville from August 1998 to December 2001.

He was awarded the American Society for Nutritional Science/Procter & Gamble Graduate Student Research Award in 1999, 2000, and 2001. He received the Society for Experimental Biology and Medicine Travel Award and College of Human Ecology Outstanding Graduate Student Research Award in 2001. He is a member of American Society for Nutritional Science, Society for Experimental Biology and Medicine, North American Association for the Study of Obesity, and American Association for the Advancement of Science.