

2004

Site and Mechanism of Leptin's Antisteatotic and Insulin Sensitizing Actions in Lipodystrophic Liver

Esra Asilmaz

Follow this and additional works at: http://digitalcommons.rockefeller.edu/student_theses_and_dissertations

 Part of the [Life Sciences Commons](#)

Recommended Citation

Asilmaz, Esra, "Site and Mechanism of Leptin's Antisteatotic and Insulin Sensitizing Actions in Lipodystrophic Liver" (2004). *Student Theses and Dissertations*. Paper 31.



**Site and Mechanism of Leptin's Antisteatotic and Insulin Sensitizing Actions in
Lipodystrophic Liver**

A thesis presented to the faculty of
The Rockefeller University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

by
Esra Asilmaz

The Rockefeller University
New York
June, 2004

Dedication

This thesis is dedicated to Anne, Baba, Eda and Anneanne for their endless love and encouragement. I could not have done any of this without their love and support.

Acknowledgements

I would like to acknowledge my thesis advisor Jeffrey Friedman who was the best mentor I could have asked for. For the past four years Dr. Friedman let me pursue the questions that I was truly interested in while making sure that he was always available whenever I needed an advice. I annoyed Jeff over the years by calling him Dr. Friedman but I only did this out of respect for him as a great scientist and a dedicated mentor.

Paul Cohen is not only a very talented scientist but also became a great collaborator and friend over the years. Paul was my second mentor in the Friedman laboratory. Paul's humor made laboratory a very friendly and fun place. Furthermore, I was very lucky to work with four very talented scientists who became very good friends over the years. Silvia Novelli and Dvora Shmulewitz were not only my bay mates but also became very good friends whom I shared a lot of good memories inside and outside of Friedman laboratory. Agnes Viale and Shirly Pinto were the two other wonderful members of our "girls group" in the laboratory whom I always turned for scientific or nonscientific advice. These four friends made the laboratory a very friendly place for me. Bahar Taneri was not an official member of the Friedman laboratory however; she was my apartment mate as well as being a wonderful member of Friedman laboratory's "girls group" Bahar is a very talented scientist and artist who was always there for me whenever I needed help, advice or support. She helped me greatly during these years and I am very lucky to have her as my best friend.

There were several other past and present laboratory members who helped me tremendously including Alena Pithart and Gulnorakhan Fayzikhodjaeva whose help with animal experiments as well as their friendship I truly appreciate. Other members

including Makoto Ishii, Zhu Chen, Jeremy Segal, Jason Montez, Wolfgang Liedtke, Ratnendra Sharma, Jeff DeFalco, Xioli Cai, Florence Massiera, Alex Soukas, Aaron Roseberry, Susan Korres, Martin Wirtz, Javier Torrens and Matt Rodeheffer all helped me throughout the years.

Over the past four years, I had several very talented collaborators. Nicholas Socci helped us tremendously with the analysis of microarray data. Agnes Viale at Genomics Core Laboratory at Memorial Sloan-Kettering Cancer Center helped me with the initial analysis of my microarray data. I was very lucky to collaborate with James Ntambi at the University of Wisconsin as well as his colleagues Makoto Miyazaki, Pawel Dobrzyn and Agnieszka Dobrzyn on the SCD studies in lipodystrophic and β -less mice. I collaborated with Ronald Kahn at Joslin Diabetes Center and his colleague Kohjiro Ueki on insulin signaling studies in lipodystrophic animals. I collaborated with Gerald Shulman at Yale University as well as his colleagues Stefan Bilz and Tony Romanelli for fatty acyl CoA analysis in Chapter 5. β -less mice studies were performed in collaboration with Bradford Lowell and Eric Bachman at Beth Israel Deaconess Medical Center.

I was very lucky to have Markus Stoffel, Jan Breslow and Luciano Rossetti as members of my thesis committee. I have learned a lot from them and I am grateful for all of their advice for my thesis work. I would also like to acknowledge everyone in Dean's Office for all their help throughout my Ph.D.

Last but certainly not the least, I would like to acknowledge all my family and friends for their love, support and encouragement over the years. In particular, I would like to acknowledge Anne, Baba, Eda, Anneanne, Dede, Hatice Hala as well as my friends Ipek and Dilek without whom I would not have enjoyed these years as much.

Table of Contents

Dedication	<i>iii</i>
Acknowledgements	<i>iv-v</i>
Table of Contents	<i>vi-vii</i>
Directory of Figures	<i>viii</i>
Directory of Tables	<i>ix</i>
List of Publications	<i>x</i>

Abstract 1-2

Chapter 1 3-41 **Introduction**

3	Diabetes as a medical problem
7	Causes of diabetes
10	Glucose homeostasis
17	Mechanisms for fatty acid induced insulin resistance
24	Leptin's metabolic effects
34	Role of leptin in glucose homeostasis in humans
39	Molecular targets for glucose homeostasis regulation
40	Specific Aims

Chapter 2 42-50 **Materials and Methods**

Chapter 3 51-63 **Site of leptin action in a rodent model of lipodystrophy**

51	Introduction
52	Results
52	Central leptin administration in aP2-nSREBP-1c mice
53	Comparison of gene expression in icv and subcutaneous leptin treated aP2-nSREBP-1c livers
58	Computational analysis of hepatic and muscle transcription profiles
61	Transcription profiles in skeletal muscle
62	Discussion

Chapter 4 65-78 **Role of leptin mediated genes in hepatic steatosis and diabetes: SCD-1 and hepatic steatosis**

65	Introduction
66	Results
66	Role of SCD-1 in lipodystrophic fatty liver
67	Mechanism for SCD-1 deficiency's metabolic effects

	72	Role of SCD-1 deficiency in glucose metabolism
	75	Discussion
Chapter 5	79-92	Leptin-mediated correction of insulin resistance in lipodystrophic mice
	79	Introduction
	80	Results
	80	Subcutaneous leptin dose response study
	81	Insulin signaling at a low dose that improves glucose levels
	86	Cluster analysis of hepatic transcription profiles with several subcutaneous leptin doses
	89	Discussion
Chapter 6	93-102	Efferent signals mediating leptin's metabolic effects
	93	Introduction
	95	Results
	95	Attenuated leptin response in β -less mice
	98	Gene expression analysis in leptin treated β -less mice
	101	Discussion
Chapter 7	103	Conclusion
	103	Summary of findings
	110	Directions for future research
	117	Conclusion
References	118	
Appendix A	136	Figure 5.4a
Appendix B	137	Figure 5.4b
Appendix C	138	Figure 5.4c
Appendix D	139	Figure 5.4d
Appendix E	140	Figure 5.4e
Appendix F	141	Figure 5.4f

Directory of Figures

Figure 1.1	4	Percentage of diabetics and nondiabetics surviving heart disease
Figure 1.2	14	Insulin signaling molecules
Figure 1.3	28	Hepatic steatosis, reduction of WAT and undifferentiated BAT in aP2-nSREBP-1c transgenic mice
Figure 3.1	54	Effects of central and peripheral leptin in rodent lipodystrophy
Figure 3.2	55	Correction of fatty liver with central leptin treatment
Figure 3.3	59	Computational analyses of the hepatic transcription profile after icv and subcutaneous leptin treatment
Figure 3.4	63	Computational analyses of the muscle transcription profile after icv and subcutaneous leptin treatment
Figure 4.1	68	Regulation of SCD-1 by icv leptin
Figure 4.2	69	Correction of fatty liver in <i>ab^J/ab^J</i> ;aP2-nSREBP-1c transgenic mice
Figure 4.3	71	Mechanism for the correction of fatty liver in <i>ab^J/ab^J</i> ;aP2-nSREBP-1c mice
Figure 4.4	73	Aggravation of diabetes in <i>ab^J/ab^J</i> ;aP2-nSREBP-1c transgenic mice
Figure 4.5	74	Gene expression analysis in the livers of aP2-nSREBP-1c mice and <i>ab^J/ab^J</i> ;aP2-nSREBP-1c mice
Figure 5.1	82	Dose-response curve for leptin treatment
Figure 5.2	84	Insulin signal transduction after leptin treatment
Figure 5.3	85	Fatty acyl CoA analysis in the muscle and liver of aP2-nSREBP-1c mice treated with PBS, 50ng/hr and untreated wild type controls (WT)
Figure 5.4	136-141	Cluster analysis of hepatic gene expression in aP2-nSREBP-1c mice treated with PBS, 12ng/hr, 25ng/hr, 50ng/hr, 100ng/hr and 200ng/hr subcutaneous leptin
Figure 5.5	88	Hepatic gene expression analysis in aP2-nSREBP-1c mice treated with PBS, 25ng/hr, 50ng/hr, 100ng/hr and 200ng/hr subcutaneous leptin
Figure 6.1	97	Attenuation in the leptin response in β -less mice
Figure 6.2	99	Plasma glucose and insulin levels in β -less mice and wild type mice treated with leptin or PBS for either 2 days or 12 days
Figure 6.3	100	Gene expression analysis in the liver of β -less and WT mice treated with leptin for 3 days and in the BAT of β -less and WT mice treated with leptin for 2 days
Figure 7.1	109	A proposed mechanism for leptin mediated correction of hepatic steatosis in lipodystrophic aP2-nSREBP-1c transgenic mice

Directory of Tables

Table 1.1	5	Definition of diabetes and hyperglycaemia
Table 3.1	57	Hepatic gene expression comparison between icv leptin and subcutaneous leptin treatment

Publications

M. Miyazaki, M.J. Jacobson, W.C. Man, P. Cohen, E. Asilmaz, J.M. Friedman, and J.M. Ntambi. 2003. Identification and characterization of murine SCD4: a novel heart-specific stearoyl-CoA desaturase isoform regulated by leptin and dietary factors. *J Biol Chem* **278**: 33904-33911.

E. Asilmaz, P. Cohen, M. Miyazaki, P. Dobrzyn, K. Ueki, G. Fayzikhodjaeva, A.A. Soukas, C.R. Kahn, J.M. Ntambi, N.D. Socci, and J.M. Friedman. 2004. Site and mechanism of leptin action in a rodent form of congenital lipodystrophy. *J Clin Invest* **113**: 414-424.

P. Dobrzyn, A. Dobrzyn, M. Miyazaki, P. Cohen, E. Asilmaz, D.G. Hardie, J.M. Friedman, and J.M. Ntambi. 2004. Stearoyl-CoA desaturase 1 deficiency increases fatty acid oxidation by activating AMP-activated protein kinase in liver. *Proc Natl Acad Sci USA* **101**: 6409-6414.

C. Wolfrum, E. Asilmaz, E. Luca, J.M. Friedman and M. Stoffel. Foxa2 regulates glucose and fatty acid metabolism in the liver during starvation and in diabetes. *Nature. In Press*.

J.A. Sennello, E. Asilmaz, J.M. Friedman, and G. Fanzuzzi. Thymocyte alterations in lipodystrophic aP2-nSREBP-1c transgenic mice. *Submitted*.

A. Dobrzyn, P. Dobrzyn, S. Lee, M. Miyazaki, P. Cohen, E. Asilmaz, D.G. Hardie, J.M. Friedman and J.M. Ntambi. Stearoyl-CoA desaturase 1 deficiency reduces ceramide synthesis by down-regulating serine palmitoyltransferase and by increasing fatty acid oxidation in skeletal muscle. *Submitted*.

J.A. Sennello, R.A. Fayad, A.M. Morris, R.H. Eckel, E. Asilmaz, J. Montez, J.M. Friedman, C.A. Dinarello and G. Fantuzzi. Interplay between adiponectin and leptin in the regulation of T-cell mediated hepatic inflammation: a study in mice with generalized lipodystrophy. *In preparation*.

Abstract

Leptin, an adipocyte hormone which functions as the afferent signal in a negative feedback loop regulating energy balance, has profound effects on glucose and lipid metabolisms. Lipodystrophic humans and mice have a complete or partial absence of adipose tissue and a secondary reduction of leptin. This is associated with insulin resistance and hepatic steatosis, which are corrected by leptin administration. This thesis aimed to define the site of leptin action and understand the molecular basis of leptin's insulin sensitizing and antisteatotic effects in lipodystrophy.

To determine the site of leptin action, we treated lipodystrophic aP2-nSREBP-1c mice with a low dose of intracerebroventricular (icv) leptin. These studies showed that leptin acts on the brain mediating its peripheral effects indirectly via the CNS. The efferent pathways mediating leptin's peripheral effects are currently unknown. Using mice with a knockout of all three β -adrenergic receptors, we showed that sympathetic nervous system, specifically β -adrenergic receptors are in part responsible for mediating leptin's peripheral effects.

Using microarray analysis in livers of lipodystrophic animals treated with either icv or subcutaneous leptin, we determined that central leptin administration repressed stearoyl-CoA desaturase-1 (SCD-1) mRNA levels and enzymatic activity. We then studied SCD-1's role in leptin-mediated correction of hepatic steatosis and insulin resistance by generating aP2-nSREBP-1c mice homozygous for SCD-1 deletion. These animals had reduced hepatic steatosis. We showed that the mechanism by which SCD-1 deficiency leads to improved hepatic steatosis involved increased AMPK phosphorylation in parallel with decreased ACC activity. The latter resulted in decreased

malonyl CoA levels and increased CPT-1 activity. SCD-1 deficient lipodystrophic animals remained diabetic.

To understand the mechanism of leptin's insulin sensitizing effects, we performed a leptin dose-response curve. We identified suboptimal leptin doses which improved hyperglycemia and hyperinsulinemia in aP2-nSREBP-1c mice but did not substantially alter hepatic steatosis. One of these leptin doses improved insulin-stimulated insulin receptor and insulin receptor substrate 2 (IRS-2) phosphorylation, IRS-2-associated PI3K and Akt activity in liver suggesting that leptin improved insulin resistance via an insulin-dependent pathway. To identify leptin-regulated genes important in leptin's insulin sensitizing effects, we used cluster analysis of hepatic gene expression with several peripheral leptin doses.

Chapter 1: Introduction

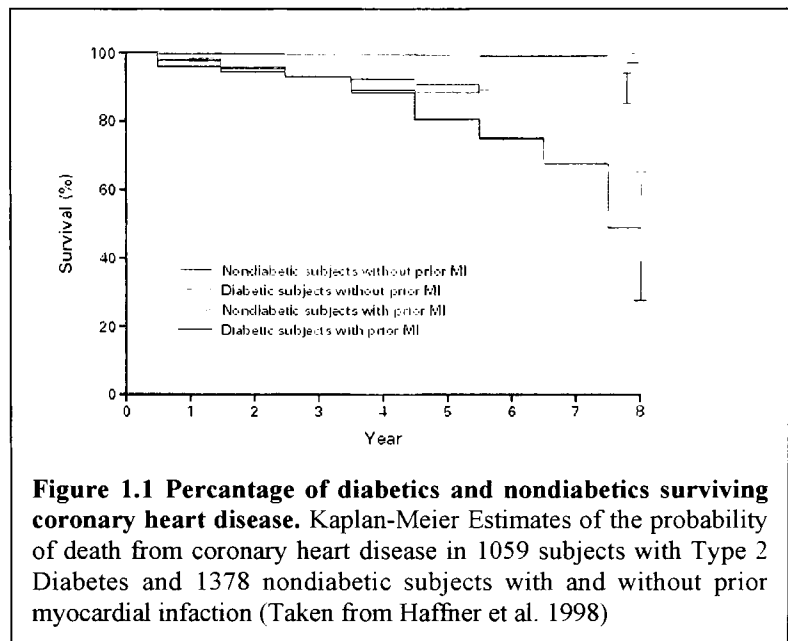
Diabetes as a medical problem

Consequences of diabetes

Diabetes, formerly considered a minor health issue, has become a major health problem over the past years (Zimmet 2000). Worldwide, there is an increasing number of people diagnosed with diabetes every year. Furthermore, diabetes together with obesity has now become the epidemic of the 21st century (Zimmet et al. 2001). It is expected that by year 2020, there will be approximately 250 million people worldwide diagnosed with non-insulin dependent diabetes mellitus (O'Rahilly 1997). This is a 46% increase from the current number of diabetics worldwide (Zimmet et al. 2001). Furthermore, there is an alarming increase in the number of cases of children and young people affected by non-insulin dependent diabetes mellitus (Zimmet et al. 2001). With this epidemic increase in diabetes, this disease is now a huge burden on the healthcare systems worldwide. It is estimated that more than \$100 billion are spent on diabetes treatment in the United States each year (Ross et al. 2004).

Diabetes is associated with a number of diseases that increase the morbidity and mortality of the affected patients. These include an increased risk for macrovascular complications such as coronary artery disease, peripheral arterial disease and cerebrovascular disease as well as microvascular complications such as retinopathy, nephropathy and neuropathy. In the United States, diabetes is the major cause of limb amputations, renal failure and blindness. Atherosclerosis is the primary cause of

mortality in patients with non-insulin dependent diabetes mellitus (Ross et al. 2004)(Figure 1.1)(Haffner et al. 1998).



Type I and Type II diabetes definitions

There are two main types of diabetes, type I and type II. Type I diabetes is also known as juvenile diabetes or insulin-dependent diabetes mellitus (IDDM). Type I diabetes is caused by total insulin deficiency due to the destruction of pancreatic islet β -cells. The destruction of β -cells is caused by an autoimmune reaction against the islet cells of the pancreas (Devendra et al. 2004). Type I diabetics are dependent on insulin therapy for their survival. Even though, the incidence of type I diabetes is also increasing, less than 10% of the 16 million diabetics in the United States have type I diabetes (Ross et al. 2004).

Type II diabetes, non-insulin dependent diabetes mellitus (NIDDM), is the more common form of diabetes. Type II diabetes is a complex metabolic disease, which is characterized by relative insulin deficiency, decreased insulin action and insulin resistance (Ross et al. 2004). Type II diabetes has been increasingly associated with a complex number of conditions such as hyperinsulinemia, dyslipidemia, hypertension, and visceral obesity (Zimmet et al. 2001). For the rest of this thesis, diabetes will be used in order to refer to type II diabetes unless stated otherwise.

Other types of diabetes include diabetes associated with monogenic defects in β -cell function, which usually results in early-onset mild hyperglycemia. These defects include mutations in MODY (Maturity Onset Diabetes of the Young) genes such as HNF1 α , PDX-1, NEURO-D1, glucokinase and HNF4 α (Stoffel et al. 1992b; Vionnet et al. 1992; Stoffel and Duncan 1997; WHO 1999).

Diabetes mellitus is defined by hyperglycemia: fasting plasma venous glucose concentrations of ≥ 7.0 mmol l⁻¹ or plasma venous glucose concentrations ≥ 11.1 mmol l⁻¹ after a 2-h post-glucose load (Zimmet et al. 2001). People with impaired glucose tolerance (IGT) are at a high risk of developing diabetes. According to WHO definitions, IGT is described as hyperglycemia where glucose levels are between normal and diabetic, which is fasting glucose plasma venous concentrations < 7.0 mmol l⁻¹ and between 7.8 mmol l⁻¹ and 11.0 mmol l⁻¹ after a 2-h post-glucose load (Zimmet et al. 2001) (Table 1.1).

Impaired fasting glucose (IFG) was defined by American Diabetes Association as another way of measuring glucose metabolism for the likelihood of

Table 1 Values for diagnosis* of diabetes and other types of hyperglycaemia

	Glucose concentration (mmol l ⁻¹)			
	Plasma Venous	Capillary	Whole blood Venous	Capillary
Diabetes mellitus				
Fasting	≥ 7.0	≥ 7.0	≥ 6.1	≥ 6.1
2-h post-glucose load	≥ 11.1	≥ 12.2	≥ 10.0	≥ 11.1
Impaired glucose tolerance				
Fasting concentration	< 7.0	< 7.0	< 6.1	< 6.1
2-h post-glucose load	7.8–11.0	8.9–12.1	6.7–9.9	7.8–11.0
Impaired fasting glucose				
Fasting	6.1–6.9	6.1–6.9	5.6–6.0	5.6–6.0
2-h post-glucose load	< 7.8	< 8.9	< 6.7	< 7.8

*Note that diabetes can be diagnosed in an individual only when these diagnostic values are confirmed on another day. Data from ref. 24.

Ranges of values are inclusive; that is, 6.1–6.9 means ≥ 6.1 and < 7.0 .

Table 1.1 Definitions of diabetes and hyperglycaemia (Taken from Zimmet et al. 2001)

developing future diabetes (WHO 2003).

The Diabetes Epidemic

Type II diabetes is one of the leading metabolic disorders which affects about 5 to 10% of most populations and its prevalence is rapidly growing among developing nations (Goldstein 2003). The incidence of type II diabetes is rapidly expanding especially in certain populations. Pacific island of Nauru is one of these populations where diabetes was a rare disease until recently whereas currently, 40% of adults are diabetic (Zimmet et al. 1990). There was a similar increase in the prevalence of diabetes, obesity and heart disease among the inhabitants of the Micronesian island of Kosrae (Shmulewitz et al. 2001). In the Indian island of Mauritius whose population includes Asian Indian, Chinese and Black (Creole) descent people, the prevalence of obesity increased two folds and now about 20% of the population above 30 years old are affected (Zimmet 2000). Similar trends are also seen among the Singaporean Chinese and Taiwanese (Chang et al. 1998; Tan et al. 1999).

The rising incidence of diabetes is linked closely to the worldwide obesity epidemic. According to WHO, an 18-year old lean woman who does not gain weight into her adulthood is 80 times less likely to develop diabetes compared to an overweight woman who continues to gain weight. With the increasing number of children and young people developing obesity, diabetes is now a significant health issue among children and youth (Zimmet et al. 2001). There are several reports of diabetic children from Japan, US, Pacific Islands, Hong Kong, Australia and the UK. For example, in Japan, type II diabetes in children increased considerably, currently in this country, 80% of children with diabetes are type II diabetics (Zimmet et al. 2001).

Causes of diabetes

An epidemic due to nature or nurture?

Diabetes is a disease of glucose metabolism in which insulin action on insulin sensitive peripheral tissues is impaired due to insulin resistance and defective insulin secretion by pancreatic β -cells. Diabetes is a complex disorder, which involves an interplay between genetic and environmental factors. Diabetes is likely to be the result of several genes interacting with each other and with several environmental factors thus resulting in this complex disease.

Emergence of obesity and hence the diabetes epidemics is largely blamed on the “western” diet and lifestyle which is also adapted by many third world countries. The reason for some ethnic populations to have such a high prevalence of these epidemics is thought to be due to their genetic susceptibilities. These already existing susceptibility genes when combined with the new environmental factors result in these high disease incidences (Zimmet 2000). The increase in childhood and youth obesity and diabetes epidemics are also largely blamed on the changes in children’s and youths’s habits and diets. Nowadays, children are spending less time doing physical activities and more time sitting in front of television and consuming high fat, high calorie food products. For example, studies among young Japanese people showed that they are participating less and less in exercise and sports. Similar studies in the US showed that 27% of high school girls and 43% of boys do not engage in sufficient amount of physical activity (Bloomgarden 2004).

“Thrifty genotype” hypothesis aims to explain the apparent differential susceptibility of certain ethnic groups or populations for developing obesity and diabetes.

According to this hypothesis, during evolution, certain populations and ethnic groups, especially the ones, which went through periods of famine or natural disasters were selected for these susceptibility genes that enabled them to store and conserve more fat as an energy resource. However, in this century where high fat food is plentiful and easy to obtain, these individuals have a much higher risk of developing obesity and diabetes (Neel 1962).

“Thrifty phenotype” hypothesis on the other hand aims to explain the association between low birth weight with the increased likelihood of developing obesity, diabetes and hypertension as an adult. According to this hypothesis, a decreased birth size indicates malnutrition in the uterus, which causes permanent structural and functional changes that predispose such individuals to metabolic diseases such as obesity and diabetes (Hales and Barker 1992).

Even though environmental factors are clearly very important for the pathogenesis of diabetes, genetic factors are as important. For example, in two genetic models of obesity and diabetes, *ob/ob* and *db/db* mice, early experiments suggested the presence of a soluble factor, which was missing in *ob/ob* mice and its receptor that was missing in *db/db* mice as the reason for the obesity and diabetes of these animals (Coleman 1973). This soluble factor, an adipocyte hormone named leptin and its receptor, leptin receptor were cloned in 1994 further confirming that both obesity and diabetes have a strong genetic basis (Zhang et al. 1994; Tartaglia et al. 1995).

Genetic causes of diabetes

While environmental factors clearly influence the pathogenesis of diabetes, genetic factors are as important for the development of this disease. Monogenic forms of diabetes as well as twin and sibling studies clearly show the importance of genetic factors in the development of diabetes. Family history has been shown to be an important determinant in the pathogenesis of this disease. Siblings are 30-40% more likely to develop diabetes relative to the 7% likelihood in the general population. Furthermore, monozygotic twins are much more likely to develop diabetes compared to dizygotic twins (Florez et al. 2003).

A number of single gene defects have been identified that cause β -cell diabetes and insulin resistance. For example, single gene mutations that cause β -cell diabetes have been identified in patients with MODY. MODY genes include hepatocyte nuclear factor 4 α (HNF4 α), glucokinase, hepatocyte nuclear factor 1 α (HNF1 α), insulin promoter factor 1, hepatocyte nuclear factor 1 β (HNF1 β) and neurogenic differentiation 1 (NEUROD1).

A number of single gene mutations that cause insulin resistance were also identified in other genes such as seipin, 1-acylglycerol-3-phosphate-O-acyltransferase, lamin A/C which cause congenital lipodystrophies in humans with metabolic abnormalities such as diabetes and insulin resistance. Mutations in the insulin and insulin receptor genes themselves were also identified in diabetic humans. Furthermore, a rare maternally inherited form of diabetes is caused by a mutation in the mitochondrial gene for transfer RNA for leucine. This gene is important for the transcription of genes important in oxidative phosphorylation in mitochondria (Florez et al. 2003).

A number of susceptibility genes for diabetes were also identified. One of these genes is calpain 10 gene, which was identified in diabetic Mexican Americans. Polymorphisms in other genes such as peroxisome proliferator-activated receptor γ (PPAR γ), sulfonylurea receptor, glucagon receptor, glucokinase, glucose transporter GLUT1 and potassium inward rectifier channel K_{ir}6.2 were also associated with diabetes (Florez et al. 2003; Bloomgarden 2004). Recently, a mutation in the protein kinase Akt2/PKB β gene was identified in a family, which has a dominant inheritance of severe insulin resistance and diabetes mellitus (George et al. 2004).

Even though mutations in several genes were identified in rare monogenic forms of diabetes, these mutations only explain a very small proportion of the common diabetes seen in rest of the population; therefore, genes that cause the common form of diabetes still remain to be identified. It is therefore critical to define the molecular basis for glucose metabolism.

Glucose metabolism

Role of insulin in glucose metabolism

Banting and Best discovered insulin as the glucose-lowering hormone about 80 years ago. Since its discovery, insulin has not only saved many patients with diabetes but also opened the doors for researchers who have been trying to understand the molecular mechanisms for glucose homeostasis.

We now know that insulin is secreted from pancreatic islet β -cells upon carbohydrate intake and it acts on insulin sensitive tissues such as muscle and adipose

tissue in order to promote glucose uptake into these tissues. Insulin signaling was found to activate the translocation of glucose transporters, mainly isoform 4, GLUT4, onto the cell membrane thereby facilitating glucose uptake into these tissues where it gets metabolized for ATP, NADPH, ribose phosphate, hexosamine production and for glycogen synthesis (Cushman and Wardzala 1980; Suzuki and Kono 1980; Ziel et al. 1988; Ren et al. 1993; Ross et al. 2004). This is a key action of insulin since this step was shown to be defective in diabetic states where insulin stimulated Glut4 translocation in muscle and adipose tissue is impaired.

Animal models of Glut4 deficiency or overexpression also confirmed the important role of this molecule in glucose homeostasis. Although a global knockout of Glut4 did not have a diabetic phenotype, these mice were somewhat insulin resistant (Katz et al. 1995). It is likely that other glucose transporter isoforms compensate for the loss of Glut4 in this model. When Glut4 gene was knocked out in a tissue-specific manner using the cre-lox system, both the muscle-specific and the adipose tissue specific knockout mice were severely insulin resistant (Zisman et al. 2000; Kim et al. 2001) (Abel et al. 2001). Furthermore, mice overexpressing Glut4 in muscle have increased glucose transport and metabolism in muscle (Liu et al. 1993; Hansen et al. 1995). When these transgenic mice were crossed to the leptin receptor mutant *db/db* mice, which exert both obesity and diabetes, the diabetic phenotype of the *db/db* mice was improved. These animals showed lower plasma glucose levels and increased insulin sensitivity (Brozinick et al. 2001). These studies established the importance of insulin mediated Glut4 translocation in glucose homeostasis; however, the exact mechanisms by which insulin stimulates this translocation are currently under investigation.

Once glucose is taken into the cell, it is converted to glucose-6-phosphate either by hexokinase or glucokinase enzymes. At this step, some of the glucose is oxidized to produce energy for the cell and the rest is converted to glycogen by glycogen synthase. Insulin activates protein phosphatase 1 (PP1) which dephosphorylates and hence activates glycogen synthase. At the same time, insulin inhibits two other enzymes, protein kinase A (PKA) and glycogen synthase kinase-3 (GSK-3), which phosphorylate and hence inactivate glycogen synthase (Ross et al. 2004).

In addition to stimulating glucose uptake into muscle and adipose tissue and increasing glycogen synthesis, insulin also acts on liver to affect glucose metabolism. Glucose is an essential energy source for many organs such as the brain. Plasma glucose levels are kept constant in between meals by glucose release from the liver (hepatic glucose output). There are two pathways by which liver releases glucose: Gluconeogenesis and glycogenolysis. Gluconeogenesis is the de novo synthesis of glucose from smaller precursors such as glycerol, lactate, alanine and pyruvate. Glycogenolysis is the release of glucose from glycogen stores. Upon acting on liver, insulin inhibits hepatic glucose output. Insulin controls gluconeogenesis through transcriptional regulation of gluconeogenic genes in liver. The expression of two key enzymes in gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are transcriptionally controlled. Peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) and forkhead transcription factors are implicated to have a role in this regulation (Nakae et al. 1999; Yoon et al. 2001). In liver, insulin also enhances glucose oxidation via transcriptional activation of glycolytic genes such as pyruvate kinase and glucokinase.

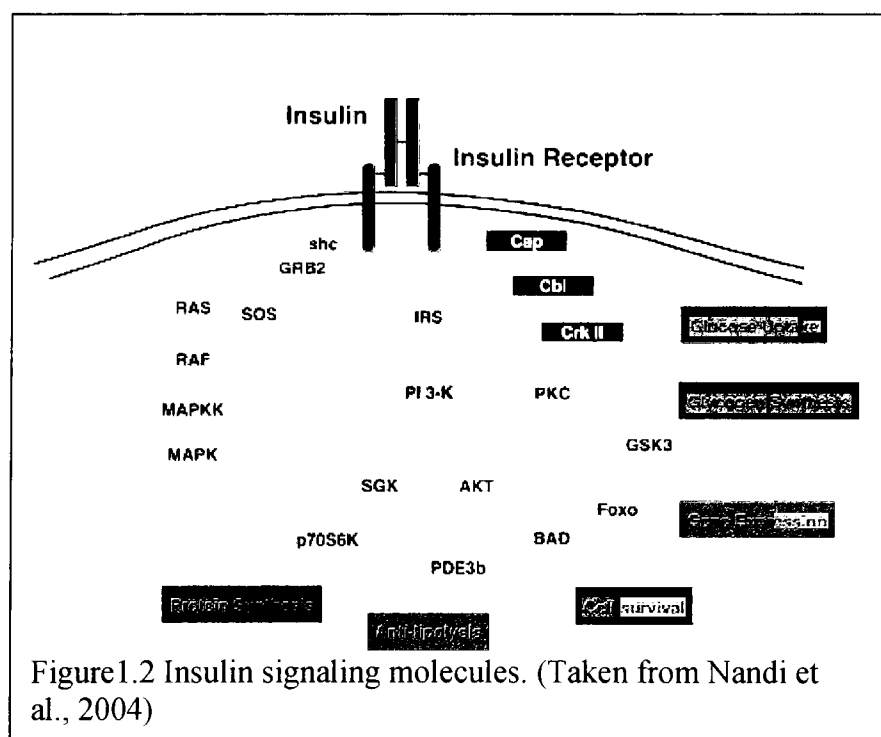
In diabetes, glucose uptake into muscle and adipose tissue, as well as inhibition of hepatic glucose output upon insulin stimulation are impaired. Both of these defects contribute to hyperglycemia observed in diabetes. Furthermore, insulin secretion from the pancreatic islet β -cells also becomes defective in this disease.

Insulin signal transduction pathway

Insulin receptor (IR) is a receptor tyrosine kinase that forms a heterotetramer of two α and two β subunits. Upon insulin binding, a conformational change occurs which enables the α subunit, which under basal conditions allosterically inhibits the β subunit; relieve its inhibition on the β subunit. Specific tyrosine residues on the β subunit then get transphosphorylated, which leads to the activation of the tyrosine kinase activity of the receptor that may now phosphorylate substrate molecules. Three tyrosine residues in the β subunit, Tyr-1146, Tyr-1162 and Tyr-1163, were identified as being critical for insulin signaling (Wilden et al. 1990). Insulin receptor is essential for a number of insulin mediated biological events. Humans have been identified with mutations in insulin receptor gene, which abolishes the kinase domain of this receptor. These individuals develop diabetes as well as a number of other cellular defects (Taira et al. 1989). Moreover, even though global IR knockout mice develop normally in uterus, they die shortly after birth due to severe hyperglycemia and hyperketonaemia (Accili et al. 1996; Kadowaki 2000). Tissue specific knockouts of IR were also made in order to study the relative importance of IR signaling in various tissues. Muscle-specific IR knockout mice have increased adipose tissue, elevated levels of serum triglycerides and free fatty acids as well as insulin resistance similar to metabolic syndrome. These mice have normal

plasma glucose and insulin levels and have normal glucose tolerance. It should however be noted that these animals do not have a complete reduction of the IR (Bruning et al. 1998). Adipose tissue specific IR knockout mice on the other hand, have decreased adiposity, increased lipolysis and they do not have a diabetic phenotype (Bluher et al. 2002). Liver-specific IR knockout mice develop a severe insulin resistance, hyperglycemia and hyperinsulinemia. In these mice, insulin also fails to suppress gluconeogenesis (Michael et al. 2000). Neuron specific IR knockout mice on the other hand, develop obesity with mild insulin resistance. These animals also have impaired fertility (Bruning et al. 2000). Pancreatic β -cell specific insulin receptor knockout mice surprisingly only develops a mild diabetic phenotype (Kadowaki 2000; Nandi et al. 2004).

The first insulin receptor substrate identified was insulin receptor substrate-1 (IRS-1) (Keller et al. 1991; Sun et al. 1991; Keller et al. 1993). Later, several other insulin receptor substrates including IRS1-4, Dok, Gab-1, Cbl, APS and Shc isoforms, were also identified. IRS-1 is



a 160kDa docking protein, which gets phosphorylated on several tyrosine residues upon insulin binding to its receptor. There are a number of proteins with src homology 2 (SH2) domain which then dock on these phosphorylated tyrosine residues on insulin substrate molecules to further relay insulin signaling. Some of these docking proteins include PI3K, Nck, Grb2 and CrkII, which might either act as adapter molecules or carry out enzymatic functions themselves (Ross et al. 2004).

Animal knockout studies further indicated the importance of the IRS molecules in insulin mediated signaling and that these molecules are probably not redundant but complementary in nature. IRS-1 knockout mice have peripheral insulin resistance as well as impaired glucose tolerance and pre- and postnatal growth retardation (Araki et al. 1994; Tamemoto et al. 1994). IRS-2 knockout mice show more of a type II diabetic phenotype with peripheral insulin resistance, hepatic insulin resistance and diminished β -cell mass (Withers et al. 1998). IRS-3 and IRS-4 knockout mice on the other hand do not show a diabetic phenotype (Liu et al. 1999; Fantin et al. 2000). The exact roles of these two molecules are not well understood. Since diabetes is a polygenetic disease, in order to study the contribution of several genes in insulin signaling and diabetes, researchers generated double heterozygote mutants of IR, IRS-1 and IRS-2. Global double heterozygous knockouts of IR and IRS-1 mice had significantly higher insulin levels and they developed late onset diabetes. The fact that these animals had 5-50 times higher insulin levels when each protein was half of normal suggested that these proteins act in synergy (Bruning et al. 1997). When double knockouts of IR/IRS-1 and IR/IRS-2 were generated, both of these mouse models had muscle insulin resistance, albeit more severe in IR/IRS-1 knockout mice (Bruning et al. 1997). These studies suggested that IRS-2

isoform is particularly important for hepatic insulin signaling whereas IRS-1 isoform is more important in muscle. Mice lacking both IRS-1 and IRS-2 molecules are embryonic lethal (Nandi et al. 2004) whereas mice lacking both IRS-1 and IRS-3 molecules develop lipodystrophy with insulin resistance but they do not develop hepatic steatosis (Laustsen et al. 2002) and mice lacking both IRS-1 and IRS-4 molecules have almost the same phenotype as IRS1 knockout mice (Nandi et al. 2004).

One of the critical downstream mediators of insulin action is phosphatidylinositol 3-kinase (PI3K). PI3K has two protein subunits 85 and 110kDa, p85 and p110. p85 has an SH2 domain, which associates with tyrosine phosphorylated IRS molecules. This association then activates the catalytic p110 domain. Catalytic p110 then phosphorylates phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate forming phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (Ross et al. 2004). Several studies suggested the importance of the above mechanism in insulin stimulated glucose transport via Glut4 translocation. In cell culture studies, PI3 inhibitors, wortmannin and LY-294002, as well as dominant negative PI3 mutants were shown to completely block insulin stimulated Glut4 translocation and glucose transport (Cheatham et al. 1994; Okada et al. 1994; Haruta et al. 1995; Kotani et al. 1995; Katagiri et al. 1997). Other studies where PI3K was either overexpressed or a constitutively active form was used, showed increased Glut4 vesicle translocation and glucose uptake (Frevert and Kahn 1997) (Martin et al. 1996). The molecular link between PI3K activation and Glut4 translocation still remains unknown.

A number of signaling molecules act downstream of PI3K in order to relay insulin signal transduction. One of these molecules is Akt or protein kinase B, which is a serine/

threonine kinase. Insulin activates Akt by phosphorylating its serine threonine residues (Kohn et al. 1995), which can be blocked by PI3K inhibitors (Franke et al. 1995; Kohn et al. 1996b). In cell culture experiments, it was shown that constitutively active Akt1 may promote Glut4 translocation with no insulin stimulation (Kohn et al. 1996a). Mice knockout studies also revealed the importance of Akt molecules in insulin signal transduction. Although Akt1 knockout mice do not show a diabetic phenotype, Akt2 knockout mice have increased blood glucose and insulin levels, hepatic insulin resistance and modest muscle insulin resistance (Cho et al. 2001a; Cho et al. 2001b). Although the link between Akt activation and Glut4 translocation remains to be determined, a Rab-GAP containing Akt substrate could possibly be important since this protein family is important in membrane trafficking (Kane et al. 2002) (Zerial and McBride 2001). The role of other PI3K substrates such as atypical protein kinase C (PKC), PKC λ , ζ or δ , still remains to be determined (Braiman et al. 1999; Standaert et al. 1999).

Mechanisms for fatty acid induced insulin resistance

Insulin and Nutrient Sensing

The epidemic increases in obesity and type II diabetes draw a lot of attention to nutrient sensing pathways and to the regulation of food intake and energy expenditure. It is evident that increased food intake and weight gain is correlated with insulin resistance. In addition to increasing glucose uptake into insulin sensitive tissues and inhibiting hepatic glucose output, insulin also exerts other metabolic effects on several tissues.

Before the cloning of the adipocyte hormone leptin, insulin was suggested to be the peripheral hormone, which can relay information about the peripheral energy stores to the CNS. First of all, there is a correlation between adiposity and plasma insulin levels (Woods et al. 1998). Furthermore, plasma insulin levels correlate well with acute energy status, in the order of minutes to hours. During a positive energy balance, there is a subsequent increase in plasma insulin levels, which decreases with a negative energy balance such as fasting (Benoit et al. 2004). Insulin is very important in both glucose and lipid usage as well as glucose storage by peripheral tissues. In insulin resistant states, plasma insulin levels increase which result in increased fat accumulation in adipocytes. Secondly, when insulin is administered into the brain, this decreases food intake while increasing energy expenditure (Woods et al. 1996b; Air et al. 2002). Peripheral administration of insulin was also shown to decrease food intake (Nicolaidis and Rowland 1976; VanderWeele et al. 1982). Furthermore, when insulin antibodies were infused in rat brains, these animals ate more and gained weight (McGowan et al. 1992). Insulin is suggested to act through its receptors in the arcuate nucleus of the hypothalamus in order to exert its effects on food intake and energy expenditure (Benoit et al. 2004). When insulin was injected into the third ventricle, there was a decrease in neuropeptide Y (NPY) expression (Schwartz et al. 1992; Sipols et al. 1995), and an increase in corticotrophin (ACTH)-releasing hormone expression (Sipols et al. 1995; Schwartz et al. 1996).

Central insulin action was also shown to affect peripheral insulin sensitivity. When insulin receptor was knocked out specifically in the brain, these mice developed mild insulin resistance as well as hypertriglyceridemia. These mice also showed

increased food intake and adiposity (Bruning et al. 2000). In other studies where insulin receptor was downregulated in the arcuate nucleus of rats using antisense oligonucleotides, these animals became hyperphagic and gained weight (Obici et al. 2002a). Under hyperinsulinemic-euglycemic clamp conditions, these animals showed impaired hepatic insulin sensitivity with a reduced response to insulin-mediated suppression of gluconeogenesis (Obici et al. 2002c).

Nutrient sensing is crucial in body weight homeostasis and insulin sensitivity. Increased body weight is strongly associated with insulin resistance. One pathway by which the organism senses nutrient fluxes is by malonyl coenzyme A (CoA)/long chain fatty acid (LCFA)-CoA pathway (Obici and Rossetti 2003). Malonyl CoA was initially suggested to be a fuel sensor that operates as a switch between fatty acid oxidation and glucose oxidation (McGarry et al. 1977). In the presence of high levels of glucose and insulin, malonyl CoA levels increase which act as a negative inhibitor of carnitine palmitoyltransferase (CPT)1. CPT1 is the rate-limiting enzyme for fatty acid oxidation. This is the transporter for LCFA-CoAs into the mitochondria where fatty acid oxidation takes place. This way when glucose is available, the cell favors glucose oxidation in place of fatty acid oxidation and lipid is stored as triglycerides in order to be used in the absence of glucose (Obici and Rossetti 2003).

In addition to cellular nutrient sensing in the peripheral tissues, it was recently shown that hypothalamic neurons may also be important in nutrient sensing. When oleic acid, a LCFA-CoA, was administered directly into the brain, there was a decrease in food intake and glucose production (Obici et al. 2002b). This suggests that LCFA-CoAs may act in the hypothalamus to signal increased nutrient availability thus inhibiting excess

nutrient intake and glucose production. This was also supported by studies where CPT1 expression was inhibited in the hypothalamus. In these studies, there was also a decrease in food intake and glucose production (Obici et al. 2003).

Insulin resistance and fatty acid metabolism

Nutrient sensing pathways are crucial for body weight and energy homeostasis and changes in these pathways might lead to an increase in adiposity and insulin resistance. There is a positive correlation between body fat content, diabetes and insulin resistance (Colditz et al. 1990). It is also suggested that regional fat depots are better indicators of diabetes and insulin resistance. For example, intra-abdominal fat depots are much better indicators of diabetes compared to subcutaneous fat depots (Kissebah and Krakower 1994).

A number of different mechanisms are implicated in the pathogenesis of insulin resistance and diabetes associated with obesity. One of these mechanisms is reduced insulin signaling in obesity due to increased phosphatase activity. Phosphatases dephosphorylate insulin signaling molecules hence impairing insulin signal transduction as well as the following insulin mediated events. In obese humans and rodents, there is an increase in protein tyrosine phosphatase (PTP) expression and/or activity (Goldstein et al. 1998). Furthermore, PTP1b knockout mice have increased energy expenditure, are resistant to diet-induced obesity and show increased insulin sensitivity in muscle and liver (Elchebly et al. 1999; Klamann et al. 2000).

A second mechanism is the role of fatty acids which accumulate ectopically in peripheral tissues such as liver, muscle and β -cells that cause an impairment of insulin

signaling and hence insulin resistance in these tissues. In humans, a strong correlation has been shown between insulin resistance and increased circulating triglycerides and free fatty acids as well as increased intracellular fat in several tissues (Schalch and Kipnis 1965; Reaven 1995; McGarry 2002). A number of studies in both rodents and humans showed that whole-body insulin sensitivity is inversely proportional to muscle triglyceride content (McGarry 2002). A number of studies reported that intramyocellular lipid (IMCL) has a very strong positive correlation with insulin resistance (Forouhi et al. 1999; Krssak et al. 1999; Perseghin et al. 1999; Levin et al. 2001). Furthermore, when healthy individuals were infused with heparin/lipid over a period of several hours under hyperinsulinemic-euglycemic clamp conditions, insulin-mediated muscle glucose uptake was significantly suppressed (Boden et al. 1995; Roden et al. 1996; Brechtel et al. 2001).

One of these studies showed that opposite to Randle's hypothesis, there was a decrease in glucose-6-phosphate levels in the muscle following lipid infusion (Roden et al. 1996). This observation suggested that fatty acids impaired insulin sensitivity in muscle either through impaired glucose uptake or phosphorylation. Randle and his colleagues performed a number of studies about 40 years ago, which lead to their proposal of the fatty acid-glucose cycle. These studies showed that in isolated rat heart muscle and rat diaphragm muscle, there was a competition between glucose and fatty acid oxidation and further speculated that fatty acid oxidation leads to obesity associated insulin resistance (Randle et al. 1963; Randle et al. 1964; Randle et al. 1965). Randle proposed that with elevated intramyocellular fatty acids, ratios of intramitochondrial acetyl CoA/CoA and NADH/NAD⁺ both increased. These increases in turn inactivate pyruvate dehydrogenase and increase citrate levels. Increased citrate in turn inactivates

phosphofructokinase, which in turn would increase glucose-6-phosphate levels in the cells. This would in turn inhibit hexokinase II activity hence causing glucose to accumulate in the cell and eventually inhibiting glucose uptake (Shulman 2000). Studies by Shulman and colleagues however, suggest that this mechanism is not entirely accurate. In a number of studies, they have shown that increased fatty acyl CoAs, diacylglycerol and ceramides activate protein kinase C θ (PKC θ), which in turn activates a serine/threonine kinase cascade. Activation of this cascade increases serine/threonine phosphorylation of IRS-1 and IRS-2 molecules and hence decreases insulin-stimulated tyrosine phosphorylation. This results in a subsequent insensitivity to insulin in the cell, where PI 3 kinase activity and Glut4 translocation to the cell membrane decreases as well as a subsequent decrease in glucose uptake (Rothman et al. 1992; Petersen et al. 1998; Dresner et al. 1999; Griffin et al. 1999).

Other mediators of insulin resistance

There are several possible reasons for lipid accumulation in peripheral tissues. One of these reasons might be failure of new fat cell formation. Adipocytes from insulin resistant individuals are larger and suggest that there might be defects in adipocyte differentiation and proliferation (Ravussin and Smith 2002). A second reason might be an impairment in fatty acid oxidation, which in turn leads to intracellular fat accumulation. It is possible that fatty acid oxidation may not be able to keep up with increased food intake or there might be a defect in fatty acid oxidation which in turn would increase intracellular lipid accumulation (Ravussin and Smith 2002). A third reason might be due to defective regulation of one or more of several adipocyte-secreted

factors. Adipose tissue is now considered an endocrine organ. It secretes several hormones and factors that are important in glucose homeostasis. These factors include leptin, adiponectin, TNF- α , IL-6 and resistin. Several of these factors have been associated with insulin resistance (Pittas et al. 2004).

Lately, adiponectin has been increasingly associated with insulin resistance. Unlike other adipocytokines, obese and diabetic rodents and humans have reduced adiponectin mRNA and protein levels (Hu et al. 1996; Statnick et al. 2000). Adiponectin treatment partially improves hyperglycemia and hyperinsulinemia associated with lipodystrophy and obesity in rodents. When lipodystrophic mice were simultaneously treated with adiponectin and leptin, insulin resistance was completely corrected (Yamauchi et al. 2001). Furthermore, adiponectin overexpressing *ob/ob* mice have improved insulin sensitivity (Yamauchi et al. 2003). Adiponectin knockout mice on the other hand have moderate insulin resistance and glucose intolerance (Kubota et al. 2002). Adiponectin was also shown to increase hepatic insulin action and thus improve glucose homeostasis (Berg et al. 2001). More recently, adiponectin was also shown to act in the brain to exert its effects on body weight and glucose homeostasis by activating energy expenditure (Qi et al. 2004).

Resistin is another polypeptide secreted by adipose tissue. In both *ob/ob* and *db/db* mice, resistin levels are elevated (Steppan and Lazar 2002). When resistin is administered to wild type mice, this has adverse effects on insulin sensitivity whereas when resistin is immunoneutralized in insulin resistant mice, their insulin sensitivity improves (Steppan et al. 2001). Recently, resistin was suggested to inhibit insulin mediated suppression of hepatic glucose output (Rajala et al. 2003).

TNF- α is a proinflammatory cytokine, which is also elevated in obese and insulin resistant animals and humans (Fried et al. 1998). In obese rats, immunoneutralization of TNF- α improved their insulin sensitivity (Hotamisligil et al. 1993), however, in humans these results have not yet been confirmed (Ofei et al. 1996). IL-6 is another cytokine, whose levels are elevated in obesity and insulin resistance (Kern et al. 2001; Vozarova et al. 2001). When IL-6 was administered to healthy individuals, blood glucose levels were elevated in these individuals (Tsigos et al. 1997).

Leptin is the most intensely studied adipocyte hormone, which has several metabolic effects and was shown to be important in insulin sensitivity. The metabolic effects of leptin are discussed in more detail in the section below. Other factors such as glucocorticoids, growth factors, IGF-1 and sex steroids might be important factors that modulate adipocyte size and have an effect on glucose homeostasis (Ravussin and Smith 2002).

Leptin's Metabolic Effects

Leptin as a satiety hormone

Kennedy proposed the lipostatic hypothesis which stated that circulating factors from body fat depots act on the brain in order to control body fat mass by changing food intake and energy expenditure (Kennedy 1953). Later in 1970s, Coleman's parabiosis experiments, which used two monogenic rodent forms of obesity and insulin resistance, *ob/ob* mice and *db/db* mice as well as lean mice, suggested the presence of a factor in *db/db* mice which was missing in the *ob/ob* mice and the receptor for this factor was

missing in *db/db* mice but was present in *ob/ob* mice (Coleman 1973). About 30 years after these observations, Dr. Friedman and his colleagues cloned the *ob* gene, leptin, and subsequently shortly after, leptin receptor, ObR, was also cloned (Zhang et al. 1994; Tartaglia et al. 1995).

Leptin is mainly expressed by white adipose tissue. It is a 146 amino acid and 16kDa hormone that regulates energy balance (Friedman and Halaas 1998; Schwartz et al. 2000; Bjorbaek and Kahn 2004). *Ob/ob* mice has a mutation in the leptin gene and hence make a defective protein that does not get secreted (Zhang et al. 1994). Administration of leptin into *ob/ob* mice, which are obese, diabetic, sterile with decreased energy expenditure, body temperature and activity, was able to correct these metabolic abnormalities (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). In contrast to *ob/ob* mice, which make no leptin, it was shown in both humans and animals that there is a direct correlation between plasma leptin levels and body fat content. Obese individuals and animals have higher plasma leptin levels compared their lean counterparts (Frederich et al. 1995; Maffei et al. 1995). Leptin has been shown to have a number of direct and indirect effects on food intake, energy expenditure as well as other metabolic functions such as glucose and fat metabolism, reproductive function and immune function (Friedman and Halaas 1998; Baile et al. 2000).

Leptin receptors belong to the class I cytokine receptor family. This receptor family acts through janus activating kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway. Leptin receptors are expressed on several peripheral tissues such as liver, muscle, pancreatic β -cells and adipose tissue as well as in chroid plexus and hypothalamus. There are 5 splice variants of the *ob* receptor gene,

ObRa-e with various C-terminal lengths (Lee et al. 1996). ObRb is the long form of the leptin receptor which contains the full cytoplasmic tail and is thought to be the only form that can signal. *db/db* mice have a mutation in the leptin receptor gene, which replaces ObRb isoform with ObRa isoform, which lacks the full cytoplasmic tail, and these animals do not respond to leptin (Chen et al. 1996). The shorter forms of leptin receptor might be important for leptin binding such as the soluble ObRe form (Lee et al. 1996; Li et al. 1998) or for leptin transport across the blood-brain barrier such as the ObRa isoform that is highly expressed in choroid plexus and brain microvessels (Fei et al. 1997; Bjorbaek et al. 1998).

Initial studies of leptin focused on the central action of leptin to control food intake and energy expenditure. When leptin was administered directly into the brain in rodents, it was shown to reduce food intake and body weight (Campfield et al. 1995; Stephens et al. 1995). More recently, mice with a brain specific knockout of the leptin receptor were generated, which have an obese phenotype further supporting the importance of CNS in leptin's effect on body weight control (Cohen et al. 2001). Hypothalamus has specific nuclei where ObRb isoform of the leptin receptor is highly expressed; these regions include arcuate, dorsomedial, ventromedial and ventral premamillary nuclei. Other regions such as periventricular hypothalamic nucleus, lateral hypothalamic area and paraventricular nucleus also express this receptor albeit at lower levels (Mercer et al. 1996; Fei et al. 1997; Elmquist et al. 1998). These hypothalamic nuclei were shown to express several neuropeptides that are important regulators of leptin's effects on food intake and energy homeostasis (Friedman 2002b). These neuropeptides include the anorectic peptides such as alpha melanocyte-stimulating

hormone (α -MSH) secreted by POMC neurons in the arcuate nucleus and cocaine and amphetamine-regulated transcript (CART); both of these neuropeptides are activated by leptin. Orexigenic peptides such as melanocortin receptor antagonist agouti-related protein (AgRP) and neuropeptide Y (NPY), on the other hand, are inhibited by leptin (Schwartz et al. 2000; Friedman 2002b).

Leptin and Energy Metabolism

In addition to its effects on food intake and body weight regulation via the CNS, leptin was also shown to have a number of metabolic effects on lipid and glucose metabolisms in peripheral tissues indirectly via the CNS and directly on some other tissues. There is evidence suggesting that leptin's metabolic effects are independent of its effect on food intake (Friedman 2002b). Food restriction causes a decrease in both lean and fat body mass whereas leptin treatment is selective for fat mass reduction (Halaas et al. 1995; Levin et al. 1996; Halaas et al. 1997). Moreover, food restriction causes a reduction in energy expenditure whereas energy expenditure does not decrease with leptin treatment (Halaas et al. 1997). The reason for this might be that leptin treatment increases uncoupling protein (UCP). There is data suggesting that UCP-1 and UCP-2 expression increases in WAT and BAT and UCP-3 expression increases in BAT and muscle following subcutaneous leptin treatment in both leptin deficient *ob/ob* mice and in wild type mice (Sarmiento et al. 1997; Scarpace and Matheny 1998; Commins et al. 1999).

Central leptin treatment also causes an increase in UCP-1 expression suggesting that leptin mediates its actions via the sympathetic nervous system (SNS) acting on beta3

adrenergic receptors (β 3AR) on peripheral tissues (Collins et al. 1996). There are three beta-adrenergic receptors. β 3AR knockout mice do not have an obesity phenotype presumably due to compensation from the other two adrenergic receptors (Susulic et al. 1995). However, when all three beta adrenergic receptors were knocked out in mice, these animals were slightly obese on a chow diet but they developed diet induced obesity suggesting the importance of beta adrenergic activity in diet induced thermogenesis (Bachman et al. 2002).

Leptin and Lipid Metabolism

In addition to decreasing triacylglycerol storage in adipose tissue and hence decreasing adipose tissue mass and changing gene expression in this adipose tissue (Soukas et al. 2000), leptin has unique effects on lipid metabolism in nonadipose tissues such as liver, muscle, heart and pancreatic β -cells. This effect of leptin is very important in peripheral insulin sensitivity.

In obesity, where there is too much adipose mass and in lipodystrophy, where there is not enough adipose mass, lipids accumulate in peripheral tissues such as liver, muscle, heart and pancreatic β -cells and this accumulation is suggested to have a “toxic” effect on these tissues. Leptin is suggested to have

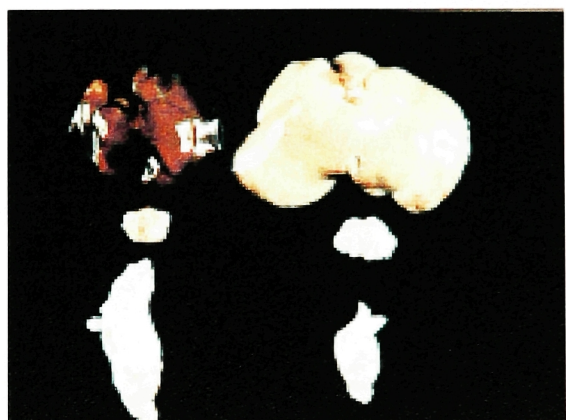


Figure 1.3 Hepatic Steatosis , reduction of WAT and undifferentiated BAT in aP2-nSREBP-1c transgenic mice (Taken from Shimomura et al. 1998)

a protective role against this “lipotoxicity” (Friedman 2002a; Unger 2003). This role of

leptin in lipid metabolism is perhaps most evident in *ob/ob* or *db/db* mice where in the absence of the leptin or its receptors, in addition to increased body fat mass, there is increased accumulation of triglycerides in peripheral tissues such as muscle, heart, pancreatic β -cells and liver which results in the enlarged fatty livers in these mice (Lee et al. 2001). Hepatic steatosis is also evident in leptin deficient lipodystrophic mice, which lack adipose tissue (Shimomura et al. 1998) (Figure 1.3).

Hepatic steatosis is a disease, which is highly associated with obesity and insulin resistance. This condition is caused by the accumulation of triglycerides into hepatocytes which might be caused by a number of defects. Hepatic steatosis may progress via steatohepatitis to fibrosis and cirrhosis of the liver, which might be fatal (Clark and Diehl 2002). Nonalcoholic fatty liver disease (NAFLD) is the general name given to all of these liver conditions and this disease affects about 30 million people in the United States of America (Browning and Horton 2004).

Although the exact mechanisms by which leptin exerts its antisteatotic effects are still under investigation, a number of studies suggest that leptin increases fatty acid oxidation and blocks fatty acid synthesis (lipogenesis) in peripheral tissues. In studies where cultured pancreatic islets were used, leptin decreased triglyceride synthesis and increased fatty acid oxidation hence decreasing triglyceride content of these cells (Shimabukuro et al. 1997). Normal rats treated with a leptin adenovirus also had decreased triglyceride content in tissues such as liver, muscle and pancreas with no increase in plasma ketones or free fatty acids (Shimabukuro et al. 1997).

In another study, leptin was shown to act via PPAR α in order to exert its effects on fatty acid oxidation. PPAR α knockout mice treated with a leptin adenovirus lost less

adipose tissue with no change in liver triacylglycerol compared to leptin treated wild-type mice (Lee et al. 2002). Another study suggested that, leptin was able to activate fatty acid oxidation and decrease triglyceride content of isolated rat hearts (Atkinson et al. 2002). Another mechanism by which leptin prevents lipotoxicity is via activating AMP-activated protein kinase (AMPK) (Chehab et al. 2004). In one study, leptin was shown to phosphorylate and activate AMPK in muscle directly and indirectly via the CNS (Minokoshi et al. 2002). AMPK is suggested to be a “fuel sensor” in cells (Hardie and Carling 1997). AMPK phosphorylates and inactivates acetyl-coA carboxylase (ACC) and activates malonyl CoA decarboxylase (MCD). Both of these has an end result of lowering malonyl CoA levels which in turn releases the inhibition on the mitochondrial fatty acid transporter carnitine palmitoyl transferase 1 (CPT-1) (McGarry et al. 1977; McGarry 2002). This leads to increased fatty acid import into the mitochondria where fatty acid oxidation takes place (Winder and Hardie 1999). In one study, where rats were treated with subcutaneous leptin, both AMPK expression and phosphorylation and ACC phosphorylation in soleus and gastrocnemius muscles were increased (Steinberg et al. 2003). These data are in accordance with the ACC2 knockout mice which have increased fatty acid oxidation and decreased adiposity (Abu-Elheiga et al. 2001). Moreover, these mice are resistant to diet induced obesity and diabetes (Abu-Elheiga et al. 2003).

Another important enzyme in leptin mediated fatty acid oxidation is stearoyl-CoA desaturase-1 (SCD-1) (Cohen et al. 2002). This is a rate-limiting enzyme in the triglyceride synthesis pathway and it generates monounsaturated fatty acids from saturated fatty acids (Ntambi and Miyazaki 2003). This enzyme is upregulated in the livers of leptin-deficient *ob/ob* mice and leptin downregulates its expression and activity.

When asebia mice, a natural knockout for SCD-1 gene, were crossed to *ob/ob* mice, these mice had reduced body mass, increased metabolic rate and improved hepatic steatosis (Cohen et al. 2002). Similarly, SCD-1 knockout mice were leaner and resistant to diet induced obesity compared to their littermate controls. They also had increased metabolic rate and increased muscle insulin sensitivity (Ntambi et al. 2002; Rahman et al. 2003).

Leptin and Carbohydrate Metabolism

Leptin has unique effects on glucose metabolism that are independent of its effects on food intake and body fat reduction. Initially, leptin was shown to correct hyperinsulinemia and hyperglycemia associated with *ob/ob* mice even at doses that has no effect on body weight (Pelleymounter et al. 1995). Leptin corrected both hepatic steatosis and insulin resistance in aP2-nSREBP-1c lipodystrophic mice whereas food restriction had no effect on insulin resistance even though it improved hepatic steatosis (Shimomura et al. 1999). In insulin-deficient rats, leptin also normalized glucose levels and glucose turnover (Chinookoswong et al. 1999). Moreover, mice which overexpress leptin were shown to have improved insulin sensitivity (Ogawa et al. 1999; Qiu et al. 2001).

Several studies suggest that leptin acts on pancreatic β -cells affecting insulin secretion but it should be noted that there are conflicting results regarding these effects (Kieffer and Habener 2000). Many of these studies suggest that leptin inhibits insulin secretion. When pancreatic islets from *ob/ob* mice were incubated with leptin or when pancreas from *ob/ob* mice was perfused with leptin, both of these treatments inhibited insulin secretion (Emilsson et al. 1997; Kieffer et al. 1997). In these studies, leptin is

suggested to act via the ATP-dependent K^+ (K_{ATP}) channels, hyperpolarizing and hence opening and activating these channels and inhibiting voltage-dependent Ca^{2+} channels, thus inhibiting insulin secretion (Kieffer et al. 1997). Several studies also suggest that leptin suppresses preproinsulin gene expression in pancreatic β -cells (Kulkarni et al. 1997; Seufert et al. 1999).

Several other reports also suggest that insulin can change leptin gene expression and secretion. In rats, it was shown that leptin gene expression has diurnal regulation, expression levels increasing at night when rats are feeding and plasma insulin levels are high. Food deprivation inhibits leptin gene expression, which may be restored with refeeding as well as with insulin administration in the fasted state (Saladin et al. 1995). In insulin-deficient rats, insulin treatment but not food restriction increases leptin gene expression.

In a number of acute leptin treatment studies, leptin was shown to have effects on gluconeogenesis and hepatic glycogen synthesis. In wild-type mice, both central and peripheral leptin administration were shown to stimulate whole-body glucose turnover, causing increased muscle and brown fat glucose uptake, a decrease in liver glycogen content and an increase in glycolysis (Kamohara et al. 1997). In other studies, both central and peripheral leptin treatments were shown to inhibit hepatic glycogenolysis while increasing gluconeogenesis through PEPCK activation, therefore promoting fatty acid oxidation. In these studies, leptin had no effect on insulin-stimulated glucose clearance or glycolysis (Rossetti et al. 1997; Liu et al. 1998). In *ob/ob* mice on the other hand, leptin was shown to decrease PEPCK expression in liver whereas glucose-6-phosphatase activity and hepatic glucose output were increased. Glucose turnover and

glucose uptake in several tissues such as brain, BAT and heart were also increased (Burcelin et al. 1999).

In other studies, where leptin was administered for a long period rather than acutely, leptin's effects were rather different. In one such study where rats were treated with subcutaneous leptin for eight days, there was a decrease in adiposity and increase in insulin sensitivity compared to food-restricted rats. In liver of these animals, leptin decreased hepatic glucose production by inhibiting glycogenolysis even though PEPCK expression and gluconeogenesis were induced. In these rats, there was an increase in whole-body and liver glycogen synthesis as well as an increase in hepatic glycolysis (Barzilai et al. 1997). In another study in rats, chronic leptin treatment also increased glycolysis, PEPCK expression and gluconeogenesis (Barzilai et al. 1999). Both acute and chronic leptin were also shown to increase hepatic glycogen synthesis in both *ob/ob* and wild type mice using ^{13}C NMR spectroscopy (Cohen et al. 1998).

Even though there is evidence from cell culture experiments showing that leptin can directly act on and hence effect carbohydrate and lipid metabolism in hepatocytes (Cohen et al. 1996; Wang et al. 1997), mice with a liver-specific knockout of the leptin receptor are normal with no abnormalities in lipid or glucose metabolism whereas brain-specific knockout of the leptin receptor are obese, diabetic and have hepatic steatosis (Cohen et al. 2001) suggesting that leptin acts via the CNS to exert its actions on liver.

For a long time, too much fat was considered to be bad for both lipid and glucose metabolisms, however, the importance of adipose tissue was once again understood when lipodystrophic mice and humans were studied. These studies suggested that too much fat is definitely bad however, too little fat and specially in the wrong places is similarly bad

(Friedman 2002a). Leptin was shown to improve insulin resistance and hepatic steatosis in a number of lipodystrophic mouse models such as A-ZIP/F1 mice, aP2-nSREBP-1c transgenic mice and IRS-1/IRS-3 double knockout mice (Shimomura et al. 1999; Ebihara et al. 2001; Laustsen et al. 2002). Crossing leptin overexpressing mice to the lipodystrophic A-ZIP/F1 mice was able to correct insulin resistance in these lipodystrophic animals (Ebihara et al. 2001). Furthermore, adipose tissue transplantation from wildtype mice but not from *ob/ob* mice corrects insulin resistance in A-ZIP/F1 mice (Gavrilova et al. 2000; Colombo et al. 2002). Similar effects of leptin treatment were also shown in lipodystrophic humans (Oral et al. 2002; Petersen et al. 2002).

Role of leptin in glucose homeostasis in humans

Mutations identified in human diabetes and insulin resistance

Type II diabetes is a complex disorder where both insulin sensitivity and β -cell function are impaired. Obesity and type II diabetes are closely associated, more than 85% of type II diabetics are also obese (O'Rahilly 2002). Type II diabetes is a complex disorder where genetic factors interact with each other and with environmental factors to manifest the disease. Rare single gene mutations that cause defects in β -cell function and hence cause early onset diabetes have been identified in humans. Some of these single gene mutations cause early onset diabetes called maturity onset diabetes of the young (MODY). Mutations in glucokinase gene cause MODY2 (Stoffel et al. 1992a; Stoffel et al. 1992b; Vionnet et al. 1992). It was later shown that mutations in hepatocyte nuclear factor-4 α gene cause MODY1 (Yamagata et al. 1996). Mutations in hepatocyte

nuclear factor-1alpha cause MODY3, insulin promoter factor-1 mutations cause MODY4 and hepatocyte nuclear factor-1beta mutations cause MODY5 (Shih and Stoffel 2002).

Mutations in insulin receptor gene were also shown to cause extreme insulin resistance syndromes in humans such as Donahue's syndrome, Rabson-Mendenhall syndrome and Type A insulin resistance. Individuals with these mutations usually do not have major developmental defects, however, they have severe insulin resistance, acanthosis nigricans, hyperandrogenization and women usually also have polycystic ovarian disease (Kahn et al. 1996).

Individuals with severe hyperinsulinaemia were also found to have mutations in PPAR γ gene, which is an important regulator of lipogenesis and insulin sensitivity, as well as in PPPIR3 A gene which encodes for the regulatory subunit of phosphatase 1. This enzyme is important in glycogen synthesis in striated muscle (O'Rahilly 2002).

Lipoatrophy and lipodystrophy syndromes are also associated with severe insulin resistance. Lipoatrophy is the characteristic loss of fat and lipodystrophy is the selective loss and abnormal distribution of fat. These syndromes are associated with diabetes, dyslipidemia, hepatic steatosis and acanthosis nigricans as well as polycystic ovary syndrome in women (Oral 2003; Garg 2004). There are two types of lipodystrophies, acquired form and inherited form. Inherited form of lipodystrophy include type 1 and type 2 congenital generalized lipodystrophy. Type 1 congenital generalized lipodystrophy is an autosomal recessive disease, caused by mutations in the 1-acylglycerol-3-phosphate o-acyltransferase (AGPAT)2 gene. This enzyme is expressed in adipose tissue and is involved in triglyceride and phospholipid synthesis. Mutations in this enzyme might cause decreased triglyceride and phospholipid synthesis in adipocytes

and hence cause the extreme lack of adiposity observed in the affected individuals since birth. Type 2 congenital generalized lipodystrophy is also an autosomal recessive disease caused by mutations in the seipin gene. The function of this gene is unknown and individuals who have mutations in this gene have extreme lack of adiposity since birth as well as mild mental retardation and cardiomyopathy. A second form of inherited lipodystrophy is called Familial partial lipodystrophy. There are two types of this form, the first type is called Dunnigan variety and is caused by autosomal dominant mutations in LMNA gene. LMNA gene encodes for nuclear lamina proteins lamin A and C. The exact mechanism by which these mutations cause lipodystrophy is not known but these mutations might cause adipocyte apoptosis. Individuals with these mutations have selective loss of subcutaneous adipose tissue at puberty from their arms, legs and trunk. The second type of familial partial lipodystrophy is caused by autosomal dominant mutations in peroxisome-proliferator-activated receptor ($\text{PPAR}\gamma$) gene. This gene encodes for a transcription factor crucial in adipocyte differentiation and dominant negative mutations in this gene might inhibit adipocyte differentiation. Individuals with these mutations lose subcutaneous fat from their arms, legs and face. The third type of inherited lipodystrophy is mandibuloacral dysplasia, which has two types, type A and type B. Type A lipodystrophy is an autosomal recessive mutation in LMNA gene. These individuals have skeletal anomalies as well as subcutaneous fat loss from their arms, legs and trunk. Type B lipodystrophy is an autosomal recessive mutation in ZMPSTE24 gene. This gene encodes for zinc metalloproteinase, which is important for lamin A protein processing from prelamin A protein. These individuals also have skeletal

anomalies as well as a generalized fat loss, premature renal failure and progeroid features (Garg 2004).

Acquired lipodystrophy is associated with HIV patients that are being treated with the highly active antiretroviral therapy (HAART) of protease inhibitors. The mechanism by which these drugs cause lipodystrophy is not known but they might be inhibiting adipocyte differentiation or causing adipocyte apoptosis. Other acquired forms of lipodystrophy include acquired partial or acquired generalized lipodystrophy, which might be caused by an autoimmune attack on adipose tissue. A localized acquired lipodystrophy is also seen in some patients and a number of mechanisms might be involved (Garg 2004).

Role of leptin in the pathogenesis of diabetes

So far, leptin was shown to be efficient in treating two rare human conditions, one of these is obesity associated with leptin deficiency and the other is lipodystrophy associated with very low leptin levels (Gorden and Gavrilova 2003). Initially, leptin was shown to correct insulin resistance, hyperinsulinemia and hyperglycemia in *ob/ob* mice in addition to reducing food intake and body weight while increasing energy expenditure in these animals (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). Secondly, leptin was shown to correct metabolic abnormalities including insulin resistance, hyperglycemia, hyperinsulinemia and hepatic steatosis associated with aP2-nSREBP-1c transgenic mice, which is a rodent form of congenital lipodystrophy. These mice express a truncated version of the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c), which stays in the nucleus and is constitutively active

(Shimomura et al. 1998; Shimomura et al. 1999). Later, leptin was also shown to correct insulin resistance and hepatic steatosis associated with another form of lipoatrophy. A-ZIP/F-1 mice, which was generated by expressing a dominant negative protein to A-ZIP transcription factor (Moitra et al. 1998; Ebihara et al. 2001). Furthermore, in these mice transplanting adipose tissue from wild-type mice, but not from leptin-deficient *ob/ob* mice, corrected insulin resistance (Gavrilova et al. 2000; Colombo et al. 2002). More recently, leptin was also shown to correct the metabolic abnormalities including insulin resistance in two other models of lipoatrophy (Yamauchi et al. 2001; Laustsen et al. 2002). Leptin's effects in both *ob/ob* and lipodystrophic mice are not due merely of its effects on food intake. Food restriction in these animal models causes reduction in body weight and improvement in hepatic steatosis but does not improve insulin resistance (Shimomura et al. 1999; Ebihara et al. 2001).

Similar to its effects in mouse models of lipodystrophy, leptin was also shown to improve hyperglycemia, hyperinsulinemia, insulin resistance, hypertriglyceridemia and hepatic steatosis in human lipodystrophic patients (Oral et al. 2002; Petersen et al. 2002). Euglycemic-hyperinsulinemic clamp studies of leptin treated lipodystrophic patients showed that leptin increased insulin sensitivity in these patients as well as lowering blood, liver and muscle triglyceride content (Petersen et al. 2002).

Recently, recombinant methionyl human leptin therapy was also shown to improve severe insulin resistance, hyperglycemia, hyperinsulinemia as well as glucose and insulin tolerance in two patients with Rabson-Mendenhall Syndrome. These patients presumably had insulin receptor mutations with extreme insulin resistance and low serum triglyceride levels (Cochran et al. 2004).

Molecular targets for glucose homeostasis regulation

Current and future treatments for diabetes

The increasing epidemic of type II diabetes is becoming a huge burden on the healthcare systems worldwide therefore, prevention and efficient treatments of this disease are extremely important. Currently, the first suggested treatment for type II diabetics is diet and exercise however, if and when these fail to control blood glucose levels, there are five classes of oral antidiabetic drugs that may be used. These drugs include metformin, a biguanide, which might activate AMPK and hence suppress hepatic glucose output and increase muscle glucose uptake (Zhou et al. 2001), pioglitazone and rosiglitazone, which are thiazolidinediones that act through activating PPAR γ , α -glucosidase inhibitors which act by delaying intestinal carbohydrate absorption and finally sulfonylureas (SU) and non-sulfonylurea (non-SU) that stimulate pancreatic β -cell insulin secretion. In more severe forms of diabetes, insulin is also prescribed (Ross et al. 2004). Besides these drugs, which are currently being used, there are a number of other drug targets that are currently under trials. These include, insulin receptor mimetics, PTP1B inhibitors, adiponectin, inhibitors for IKK β and JNK1 and AMPK activators (Ross et al. 2004). However, none of the available therapies can prevent or treat all of the different aspects of this disease. Therefore, it is of great interest to understand the exact molecular mechanisms that underlie insulin resistance in order to be able to prevent the development of type II diabetes and also to identify better drug targets for treatment. Since, the recent obesity and diabetes epidemics are linked, it is important to understand

the molecular mechanisms underlying insulin resistance that is associated with obesity and other lipid disorders.

Specific Aims

This thesis aims to further elucidate the molecular mechanisms of leptin's antidiabetic effects. The site of leptin's antidiabetic effects was studied by peripheral and central administrations of leptin into lipodystrophic aP2-nSREBP-1c transgenic mice, which are insulin resistant (Chapter 3). These studies showed that leptin acts via the central nervous system in order to correct insulin resistance in these mice. Furthermore, we showed that leptin also acts via the CNS to regulate liver gene expression. We then identified stearoyl-CoA desaturase-1 (SCD-1) as an important leptin regulated enzyme in the livers of aP2-nSREBP-1c mice and showed that leptin corrects hepatic steatosis by downregulating this enzyme. We also showed that leptin mediated SCD-1 downregulation corrects hepatic steatosis by activating the AMPK pathway and hence increasing fatty acid oxidation in the livers of aP2-nSREBP-1c mice. However, correcting hepatic steatosis by downregulating SCD-1 was not enough to correct the diabetes of ab^J/ab^J ;aP2-nSREBP-1c mice (Chapter 4). In order to understand the molecular mechanisms by which leptin corrects insulin resistance, we then performed a subcutaneous leptin dose response study. We identified two suboptimal leptin doses, which improved insulin resistance without correcting hepatic steatosis. We then performed microarray studies to identify leptin-regulated genes important in insulin sensitivity (Chapter 5). Finally, in order to understand the efferent signals from the CNS

that mediate leptin's effects, we studied leptin's effects in mice lacking all three β -adrenergic receptors (Chapter 6). Finally, the last part of this thesis is a discussion of all of our findings and future directions for this research (Chapter 7).

Chapter 2: Materials and Methods

Materials and Methods from Chapter 3

Mouse breeding and maintenance. aP2-nSREBP-1c mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). aP2-nSREBP-1c males (C57BL/6J X SJL background) were crossed to C57BL/6J females. aP2-nSREBP-1c males and littermate controls generated from this cross were used in the following studies. All animals were housed on a 12 hour light:dark cycle at 23°C with free access to water and food. All procedures were in accordance with the guidelines of The Rockefeller University Laboratory Animal Research Center.

Metabolic assays and procedures. Upon sacrificing the mice, plasma was obtained following centrifugation of blood collected on EDTA. We measured plasma leptin and insulin levels using mouse Elisa kits (R&D Systems Inc., Minneapolis, Minnesota and Alpco Diagnostics, Windham, New Hampshire, respectively). We determined plasma glucose, triglyceride and cholesterol levels using Glucose Trinder reagent, Infinity Triglycerides reagent and Infinity Cholesterol reagent, respectively (Sigma Chemical Co., St. Louis, Missouri). We measured liver triglycerides as previously described (Cohen et al. 2002). Histology was performed on livers fixed in 10% formalin and stained with hematoxylin and eosin.

Intracerebroventricular (ICV) leptin treatment. Mice were studied between 14-18 weeks of age. Two weeks before leptin treatment, mice were individually caged. Baseline weight and food intake were measured every other day in the mid-light cycle. At the beginning of treatment, mice were divided into two weight-matched groups. One group

was treated with 12 ng/hr of recombinant mouse leptin (Amgen, Thousand Oaks, California) and the other was treated with PBS for 12 days. Mice were anesthetized using ketamine/xylazine, and a cannula connected to an Alzet miniosmotic pump (Alza Co., Palo Alto, California) was placed into their third ventricle as previously described (Halaas et al. 1997). During the treatment, food intake and body weight were recorded daily, and on day 12, mice were sacrificed during the mid-light cycle.

Subcutaneous leptin treatment. aP2-nSREBP-1c mice were treated with 12ng/hr, 25ng/hr, 50ng/hr, 100ng/hr, and 200ng/hr of recombinant mouse leptin or PBS for 12 days using subcutaneously placed Alzet miniosmotic pumps, as previously described (Halaas et al. 1997). Food intake and weight were monitored as described above.

Microarrays. RNA isolated from four individual liver samples was pooled, labeled and hybridized once to Affymetrix Murine MG-U74 Av2 chips according to Affymetrix protocols (Affymetrix Inc., Santa Clara, California). In specific cases, Affymetrix results were reconfirmed using real-time PCR Taqman assay. Initial analysis and comparisons were performed using a fold change cut-off of 1.5, change p-value<0.0025 and detection p-value of 0.05 in the experiment or baseline depending on the sign of the fold change.

Cluster and correlation analysis. Cluster analysis was performed using standard hierarchical clustering with the average linkage method. The distance measure was $(1-p)/2$, where p is the Pearson correlation function.

Correlation analysis: For gene selection, a filter of 1.5 fold change and the standard Affymetrix values for change and detection p-values were used. A gene had to have a significant fold change in at least 5 samples to pass the filter. Correlation for genes in the intersecting set of pairs was calculated using an absolute log base 2 fold change cutoff

greater than 0.6, change p-value<0.0025 and detection p-value<0.04 (these p-values are the default α_1 and γ_1 values for MAS 5.0 (Affymetrix Inc., Santa Clara, California). The criteria used are quite stringent for gene selection, and for most randomly matched pairs, a very small number of genes meet these criteria. This is critical because the distribution of random correlations is highly dependent on the number of points, with a small number of points having a much greater chance of being highly correlated than a large number of points. To minimize this problem, we changed the selection criteria for computing background statistics. This should not affect our results provided that the same statistic is used for all computations. We calculated the background correlation using the top 250 genes ranked by absolute log fold change in the union set. Note that 250 is an arbitrary cutoff and we tested cases from 50-500 genes. However, we chose to report the 250 case since it was the most conservative one (i.e. highest p-value). We calculated the Pearson correlation coefficient:

$$r^2 \equiv \frac{\sum_i (X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum_i (X_i - \bar{X})^2 \sum_i (Y_i - \bar{Y})^2}}$$

Where \bar{X} is the mean of X and similarly for Y , and X and Y are the logs of the fold changes. To assess the significance of this correlation, we calculated the distribution of correlations for a large number of quasi-randomly paired samples. This model was “random” in the sense that 30 pairs of comparisons were paired arbitrarily (we did not mix samples from different tissue types) to create 95 fold-change files. We then exhaustively paired all 95 of these samples, ensuring that we were always looking at four

different samples at each time. This resulted in 3,692 sets for which we computed correlation coefficients. We looked at the distribution of the absolute value of the correlation coefficient. To calculate the p-value, we used the formula: $p\text{-value} = [(N_{\#} > 0.738) + 1] / [(N_{\text{TOTAL}}) + 1]$ where $N_{\#}$ is the number of pairs with $\rho > 0.738$ (4 in our case), and N_{TOTAL} is the total number of pairs.

Statistical analysis. All data are expressed as means \pm SE. An unpaired Student's t-test was used to determine significance.

Materials and Methods from Chapter 4

Mouse breeding and maintenance. Asebia (ab^J/ab^J) mice were purchased from the Jackson Laboratory. ab^J/ab^J ;aP2-nSREBP-1c mice were generated by first crossing ab^J/ab^J (background information is available at <http://www.informatics.jax.org/external/festing/mouse/docs/ABJ.shtml>) to aP2-nSREBP-1c mice to generate, $ab^J/+$;aP2-nSREBP-1c males. These males were crossed with $ab^J/+$ females to generate ab^J/ab^J ;aP2-nSREBP-1c mice and littermate controls. Genotypes were determined by genomic PCR and Southern blotting.

SCD enzymatic activity. Conversion of $[1\text{-}^{14}\text{C}]$ stearoyl-CoA to $[1\text{-}^{14}\text{C}]$ oleate was used to measure SCD enzyme activity from individual liver extracts as previously described (Cohen et al. 2002).

Taqman. SCD-1 mRNA levels were quantitated using Taqman real-time PCR from individual livers with probes and primers designed for SCD-1. SCD-1 mRNA levels were measured in duplicate in aP2-nSREBP-1c mice, wild-type littermates and aP2-

nSREBP-1c mice treated with ICV leptin, ICV PBS and several doses of peripheral leptin. A cyclophilin probe and primers were used in the same assay as the baseline.

Fatty acid analysis. Total lipids were extracted from tissues according to the method of Bligh and Dyer as previously described (Bligh 1959). The fatty acids were quantitated by GLC as previously described (Miyazaki et al. 2001). Pentadecanoic acid (Sigma, St Louis, MO) was added as an internal standard for the quantitation of fatty acids.

Extraction and measurement of ACC activity. Isolation of ACC was performed as previously described (Kudo et al. 1995). ACC activity in the 6% PEG 8000 fraction was determined using the [14 C]bicarbonate fixation assay (Witters and Kemp 1992).

Malonyl-CoA content determination. Malonyl-CoA levels in liver were measured using highly purified fatty acid synthetase as previously described (McGarry et al. 1978).

Western blot analysis. 50 μ g of purified protein was loaded onto a 9% SDS-PAGE gel. The separated proteins were transferred to nitrocellulose membranes which were blotted using antibodies against phosphopeptides based on the amino acid sequence surrounding Thr172 of the α subunit of human AMPK and Ser79 of rat ACC (Upstate, Waltham, MA). Protein levels of α 1 and α 2 AMPK, and ACC1, ACC2, and CPT1 were determined using specific antibodies (Alpha Diagnostic Int., San Antonio, TX). Anti-AMPK α 1 and α 2 and anti-AMPK-pT172 antibodies were obtained as described previously (Woods et al. 1996a; Sugden et al. 1999). The proteins were visualized using ECL as described by the manufacturer.

Carnitine palmitoyltransferase 1 (CPT1) activity assay. CPT1 activity was measured in isolated mitochondria as previously described (Bremer 1981). Mitochondria were isolated essentially as described by Vance (Vance 1990). Briefly, mitochondrial protein

was added to assay medium containing 20 mM HEPES, pH 7.3, 75 mM KCL, 2 mM KCN, 1% fat free BSA, 70 μ M palmitoyl-CoA and 0.25 mM L-[3H]-carnitine. Samples were incubated in 37°C for 3 minutes. The reaction was stopped by adding 0.5 ml 4 M ice-cold PCA. Mitochondria were centrifuged at 13,000 g for 10 minutes. The pellet was washed with 500 μ l 2 mM PCA and the centrifugation repeated. The resulting pellet was washed then resuspended in 800 μ l ddH₂O and extracted with 600 μ l butanol. 300 μ l of butanol phase was counted by liquid scintillation.

Statistical analysis. All data are expressed as means \pm SE. An unpaired Student's t-test was used to determine significance.

Materials and Methods from Chapter 5

Animal studies. Animals used in these studies were generated and studied the same way as previously described in Materials and Methods from Chapter 3. Metabolic parameters and assays as well as subcutaneous leptin treatment studies were performed as previously described in Materials and Methods from Chapter 3.

Liver glycogen content. Liver glycogen was quantitated with the amyloglucosidase method as previously described (Keppler 1974; Soukas 2001).

In vivo insulin stimulation and analysis of insulin signaling proteins. Male aP2-nSREBP-1c mice were treated with either 50ng/hr of subcutaneous leptin or PBS for 12 days. On day 12, mice were anesthetized with pentobarbital after an overnight fast, and injected with 5 units of regular human insulin (Lilly Research Laboratories) into the inferior vena cava. Livers and muscles were excised after 5 minutes and freeze-clamped

in liquid nitrogen. Insulin signaling molecules were analyzed as previously described (Ueki et al. 2002).

PI3K Assay and AKT kinase assay. Liver and muscle homogenates from the in vivo insulin stimulation were used to assay PI3K and AKT kinase activities as previously described (Ueki et al. 2002).

Fatty acyl CoA analysis. Tissue fatty acyl CoA concentrations were measured from tissues, which were freeze-clamped in liquid nitrogen as previously described (Yu et al. 2002).

Microarrays and Taqman gene expression. Microarray studies and real-time RT-PCR Taqman analysis were performed as previously described in Materials and Methods from Chapter 3. Sak, HNF6, G-6-Pase, FB Pase and PEPCK mRNA levels were quantitated using Taqman real-time PCR from individual livers with probes and primers designed for sak, HNF-6, G-6-Pase, FB Pase and PEPCK respectively. Cyclophilin primers and probe were used as an internal control.

Cluster analysis. Before clustering, the genes were filtered to include those that had a fold change of 1.5 (up or down) and a change p-value of 0.001 (as scored by the MAS 5.0 software from Affymetrix). The genes were then clustered using an extension of the *k*-means clustering algorithm (Hartigan 1975; Kohonen 1997). The basic procedure for *k*-means clustering is to first specify the starting *k* cluster centers. Then the following two steps are repeated: (1) For each point in the data, find the cluster center it is closest to and assign it to that group, (2) for each group determined in step 1, find the mean of that group and set the new value of the cluster center to it. These steps were iterated until the amount by which the cluster center moved drops below 1 part in 10^6 . To prevent the

algorithm from getting trapped in a local minimum, we used a recursive technique (Duda 1973) to begin clustering. In brief, we started by finding the mean of the entire dataset. We then iterated from 1 to k each time adding one more center to the problem. To go from any $j - 1$ to j means, the mean associated with the cluster with the largest variance was split into two closely separated points and then the standard k -means steps were performed. Finally, to further prevent the algorithm from being trapped, we ran the algorithm for 1,000 independent runs each seeded with a different random number so that the splitting procedure would be different for each run. To determine the best clustering trial for the final result, we computed the trace of the scattering matrices $\text{Tr}(S_b S_w^{-1})$, as previously described by Duda and Hart (Duda 1973) which measures the “average” separation between clusters divided by the “average” width of the clusters. We determined the optimal number of clusters by using a standard measure of cluster fit as a function of cluster number, the Davies-Bouldin index (Jain 1988).

Statistical analysis. All data are expressed as means \pm SE. An unpaired Student’s t-test was used to determine significance.

Materials and Methods from Chapter 6

Animal studies. β -less mice and their littermate controls were generated as previously described (Bachman et al. 2002).

Subcutaneous leptin treatment. β -less mice and their littermate controls were treated with subcutaneous recombinant mouse leptin or PBS for either 2 or 3 days or for 12 days using subcutaneously placed Alzet miniosmotic pumps as previously described (Halaas et

al. 1997). Food intake and weight were monitored as described in Materials and Methods from Chapter 3.

Body composition analysis. Body composition was analyzed as previously described (Halaas et al. 1995). Carcasses were first weighed and then dried until the weight stabilized in a 90°C oven. The difference in weight was calculated as the total body water and then the carcass was homogenized and the total body fat was calculated by extracting it using a 3:1 mixture of chloroform:methanol using a soxhlet apparatus. The remaining weight was calculated as the total lean mass.

Metabolic parameters. Metabolic parameters were measured as previously described in Material and Methods from Chapter 3.

SCD mRNA and activity. SCD-1 mRNA and SCD activity were measured as previously described in Materials and Methods from Chapter 4.

Gene expression analysis. Real-time Taqman multiplex RT-PCR analysis was used to measure the mRNA levels of HSL and UCP-1 from BAT mRNA using primers and probe specific for HSL and UCP-1 respectively. Primers and probe specific for 18S mRNA were used as an internal control.

Statistical analysis. All data are expressed as means \pm SE. An unpaired Student's t-test was used to determine significance.

Chapter 3: Site of leptin action in a rodent model of lipodystrophy

Introduction

Leptin is an adipocyte hormone that modulates energy balance, metabolism, the hypothalamic-pituitary axis and immune function (Zhang et al. 1994; Friedman 2000). Leptin also enhances insulin action, although the underlying mechanism has not been fully elucidated (Kamohara et al. 1997; Chinookoswong et al. 1999; Ogawa et al. 1999). Recently leptin was found to be highly effective for treating the diabetes, insulin resistance and hepatic steatosis associated with human lipodystrophy (Oral et al. 2002; Petersen et al. 2002). Leptin has similar effects in lipodystrophic rodents, most notably in aP2-nSREBP-1c transgenic mice. These animals express a truncated, constitutively active form of the SREBP-1c transcription factor under the control of the adipose-specific aP2 promoter and develop lipodystrophy with very low plasma leptin levels as well as hyperphagia, massive fat accumulation in peripheral tissues, hyperglycemia and hyperinsulinemia (Shimomura et al. 1998). In these mice, peripheral leptin administration corrects the metabolic abnormalities associated with lipodystrophy (Shimomura et al. 1999). In A-ZIP/F1 mice, a second form of rodent lipodystrophy, leptin also corrects insulin resistance and hepatic steatosis, albeit at higher doses (Moitra et al. 1998; Ebihara et al. 2001). In these animals, fat transplants from wild-type, but not *ob/ob* mice, also ameliorate this condition confirming that leptin is necessary for the anti-diabetic effects of fat transplants (Gavrilova et al. 2000; Colombo et al. 2002). Recently, leptin was shown to reverse the hyperglycemia and hyperinsulinemia associated with *Irs1*^{-/-}; *Irs3*^{-/-} lipodystrophic mice (Laustsen et al. 2002).

Leptin has been reported to act directly on a number of tissues including brain, skeletal muscle, heart and pancreatic β -cells (Kieffer et al. 1997; Cohen et al. 2001; Atkinson et al. 2002; Minokoshi et al. 2002). However, at present, neither the site nor the mechanism by which leptin corrects the abnormalities associated with lipodystrophy have been established. Here, we show that low dose intracerebroventricular (ICV) leptin corrected the metabolic abnormalities associated with lipodystrophy. ICV leptin treatment was able to correct both diabetes and hepatic steatosis associated with lipodystrophy as well as subcutaneous leptin treatment does. Moreover, comparison of hepatic gene expression following ICV and subcutaneous leptin treatment reveals that these gene expression profiles are highly correlated suggesting that leptin changes hepatic gene expression via the CNS. On the other hand, muscle gene expression following ICV and subcutaneous leptin treatment reveals that these gene expression profiles have a low correlation suggesting that leptin might have direct effects on muscle. In summary, our data suggests that leptin acts on the CNS in order to exert its metabolic effects in lipodystrophy. Furthermore, leptin regulates liver gene expression through its actions on the CNS.

Results

Central leptin administration in aP2-nSREBP-1c mice

We administered recombinant murine leptin (12 ng/hr) or phosphate buffered saline (PBS) intracerebroventricularly to aP2-nSREBP-1c mice for 12 days (Halaas et al. 1997) (Figure 3.1). In separate studies, lipodystrophic animals were treated with a higher

dose of subcutaneous leptin (200 ng/hr for 12 days) or PBS. This subcutaneous dose has previously been shown to fully correct the metabolic defects in lipodystrophic mice (Shimomura et al. 1999). ICV leptin (12ng/hr) did not change plasma leptin levels in lipodystrophic mice (Figure 3.1a). In animals treated with ICV leptin, food intake was reduced by 26.2% (Figure 3.1b) and body weight was reduced by 17.3% (Figure 3.1c) relative to PBS treated controls. This dose of ICV leptin treatment markedly improved insulin sensitivity, as both plasma glucose and insulin levels were significantly lowered (Figure 3.1d and e). Finally, ICV leptin treatment decreased liver triglyceride levels seven-fold (Figure 3.2a) and reduced liver mass by 67.5% relative to PBS treated controls (Figure 3.2b). Histological analysis confirmed that ICV treatment corrected the fatty liver of aP2-nSREBP-1c mice (Figure 3.2c). As an additional control, we treated a separate group of aP2-nSREBP-1c mice with 12 ng/hr of peripheral leptin, the same dose used in the ICV treatment. This dose had no effect on any of the parameters shown in Figures 3.1 and 3.2 (data not shown). In all cases, ICV leptin was as or more potent than a much higher dose of peripheral leptin in correcting the metabolic abnormalities associated with lipodystrophy. These data suggest that leptin's effects on insulin action, diabetes, and hepatic steatosis in lipodystrophy can be mediated exclusively via the CNS.

Comparison of gene expression in icv and subcutaneous leptin treated aP2-nSREBP-1c livers

We next compared liver gene expression profiles in aP2-nSREBP-1c and wild type mice with and without peripheral or central leptin treatment. mRNAs for several enzymes involved in fatty acid synthesis, glycolysis and gluconeogenesis have been

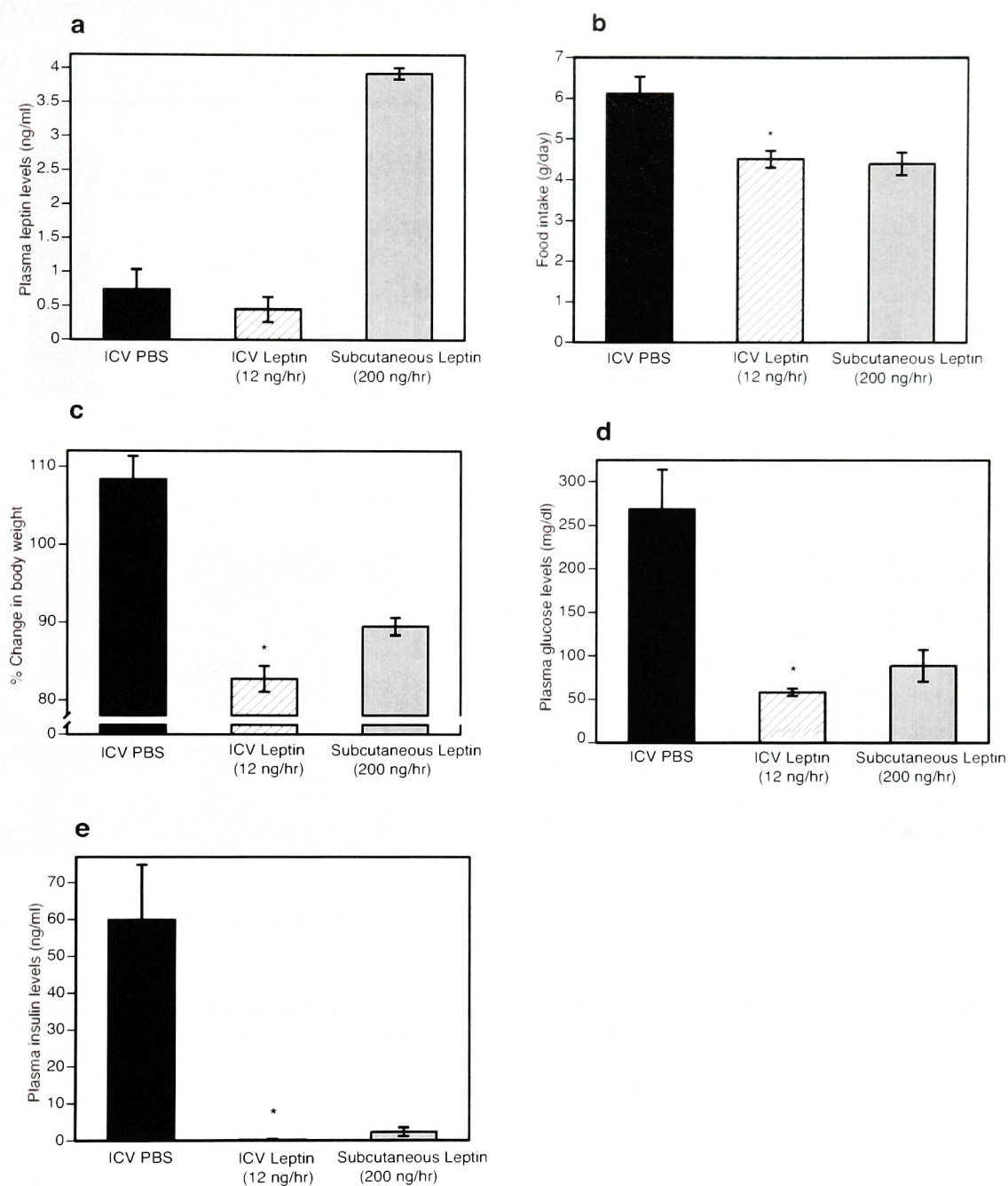


Figure 3.1 Effects of central and peripheral leptin in rodent lipodystrophy.

Several metabolic parameters in aP2-nSREBP-1c transgenic mice treated with icv PBS, icv leptin (12 ng/h), and subcutaneous leptin (200ng/h) are shown. (a, d, and e) Plasma levels of leptin, glucose, and insulin respectively. (b) Average daily food intake for the 12-day treatment. (c) Percentage change in body weight. One hundred percent change indicates the weight of the mice on the day of pump insertion. Error bars indicate the SE for four mice per group for each treatment. *P < 0.05, icv leptin vs. icv PBS.

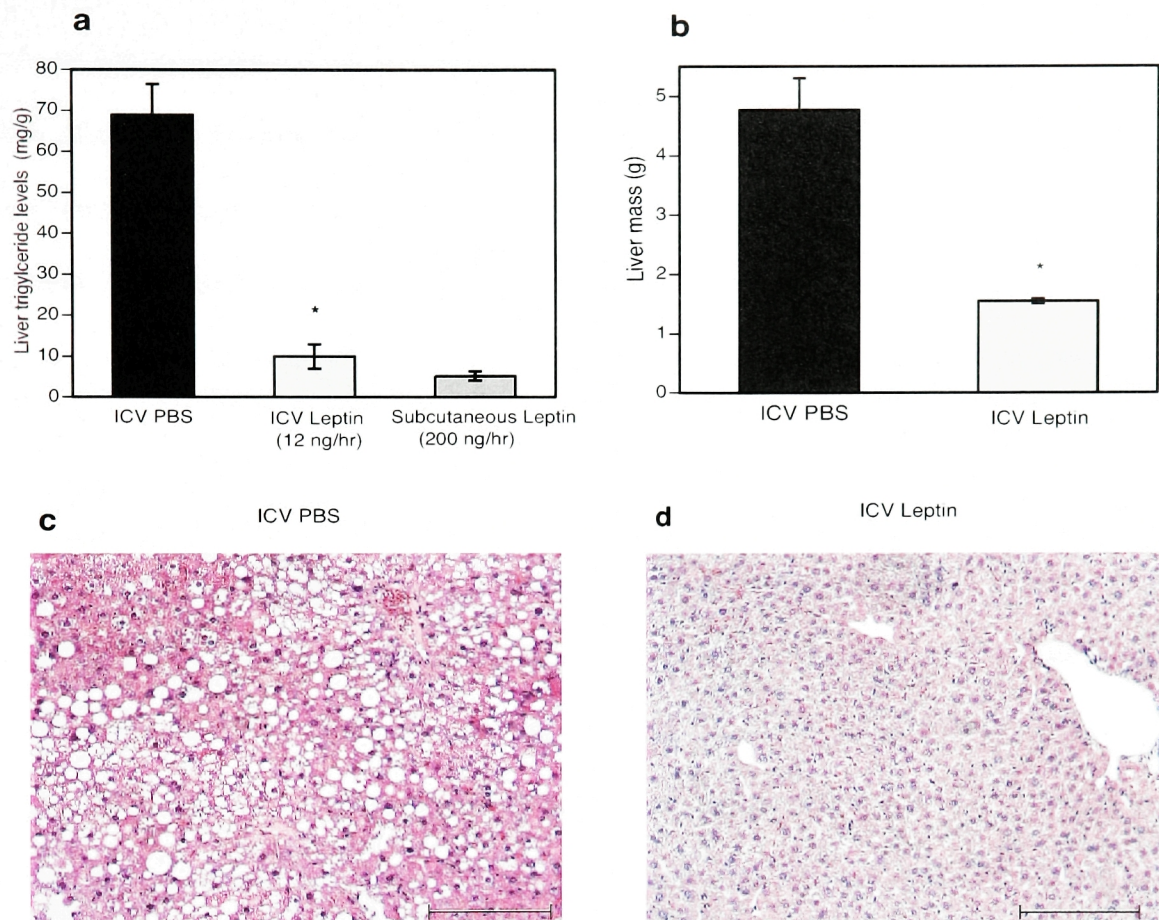


Figure 3.2 Correction of fatty liver with central leptin treatment.

(a) Liver triglyceride content. (b) Liver mass in grams. Error bars indicate the SE for four mice per group for each treatment. * $P < 0.05$, icv leptin vs. icv PBS. (c and d) Representative liver sections from mice treated with icv PBS and icv leptin. Original magnification, X200 ; scale bars: 100 μm .

found to be differentially expressed in livers of aP2-nSREBP-1c mice and normalized by peripheral leptin treatment (Shimomura et al. 2000). We examined the expression profiles of a group of genes known to regulate lipid and glucose metabolism following either central or peripheral leptin administration (Table 3.1). For each of these genes, an equivalent or greater response was evident after ICV leptin treatment. For example, malic enzyme and fatty acid synthase, enzymes involved in fatty acid synthesis, were upregulated 3.0 and 3.7-fold respectively in aP2-nSREBP-1c relative to wild type liver. These two genes were downregulated 7.0 and 4.3-fold respectively after ICV leptin administration versus 2.6 and 2-fold respectively after peripheral leptin treatment (relative to PBS treated transgenic livers). In aP2-nSREBP-1c livers, pyruvate kinase, a glycolytic enzyme, and glucose-6-phosphatase, a gluconeogenic enzyme, were upregulated 3.0 and 3.3-fold respectively relative to wild type livers. ICV leptin treatment downregulated these genes 7.0 and 4.3-fold respectively, and peripheral leptin administration downregulated them 2.5 and 1.5-fold respectively (relative to PBS treated transgenic livers). Expression level of these genes was normalized to wild type levels following ICV leptin treatment. Several other genes were similarly regulated by both central and peripheral leptin, with most genes more strongly regulated by ICV than by peripheral leptin (see Supplementary Tables 1 and 2 online; <http://www.jci.org/cgi/content/full/113/3/414/DC1>). Furthermore, many of the genes that were altered between lipodystrophic and wild type livers were also differentially expressed in livers of *ob/ob* mice compared to wild type mice, indicating that the alterations in liver gene expression in aP2-nSREBP-1c mice resemble those of congenital leptin deficiency (Cohen et al. 2002).

Accession number	Name	Function	Fold change	
			icv leptin	Subcutaneous leptin
J02652	Malic enzyme	Lipid metabolism	-6.96	-2.64
D63764	Pyruvate kinase	Glucose metabolism	-6.96	-2.46
X13135	Fatty acid synthase	Lipid metabolism	-4.29	-2.00
U00445	Glucose-6-phosphatase	Glucose metabolism	-4.29	-1.52
AW122523	L-chain fattyacyl elongase	Lipid metabolism	-6.96	-3.25
U10374	PPAR γ	Transcription factor	-6.96	-1.52
X85983	Carnitine acetyltransferase	Lipid metabolism	-2.14	-1.62
X58426	Hepatic lipase	Lipid metabolism	2.14	1.62
U95945	HNF6	Transcription factor	3.03	2.83
X74938	HNF3 γ	Transcription factor	1.74	2.14
AB033887	Fatty acyl-CoA ligase	Lipid metabolism	2.30	1.87

The line space separates the genes downregulated by leptin treatment (above the line space) from the genes upregulated by leptin treatment (below the line space)

Table 3.1 Hepatic gene expression comparison between icv leptin and subcutaneous leptin treatment .
Comparison of gene expression regulated by icv leptin versus subcutaneous leptin in livers of aP2-nSREBP-1c transgenic mice.

Computational analysis of hepatic and muscle transcription profiles

In order to extend the analysis of gene expression beyond the comparison of a few key genes, we used standard hierarchical clustering to assess the similarity of central versus peripheral leptin treatment (Figure 3.3a). In the resulting dendrogram, the transcriptional profiles in liver after subcutaneous and ICV leptin treatments clustered together, indicating that the gene expression pattern of ICV leptin treatment was more similar to the 200 ng/hr dose of peripheral leptin treatment than to any others tested.

In order to provide more robust quantitative information on how significant the similarities were, we developed a novel approach to determine the significance of the similarity (correlation) between the transcriptional profiles in liver following ICV and subcutaneous leptin administration. We compared the global gene expression profiles of ICV and subcutaneous leptin treatment using two methods: 1) correlation analysis between genes that are similarly regulated in both experiments, in what is referred to as the intersection set, and 2) correlation analysis among genes that are regulated in either or both experiments, in what is referred to as the union set. For both methods, we first calculated the ratio of gene expression between ICV leptin versus ICV PBS treatment and then subcutaneous leptin versus subcutaneous PBS treatment. It was necessary to establish the ratio of gene expression in the ICV leptin and subcutaneous leptin treated samples relative to their own controls because the insertion of ICV and subcutaneous pumps each have distinct background effects. In the first method, the standard “detection p-values” and “change p-values” from the Affymetrix software were used to identify leptin-regulated genes with an absolute fold change cutoff of 1.5 relative to controls.

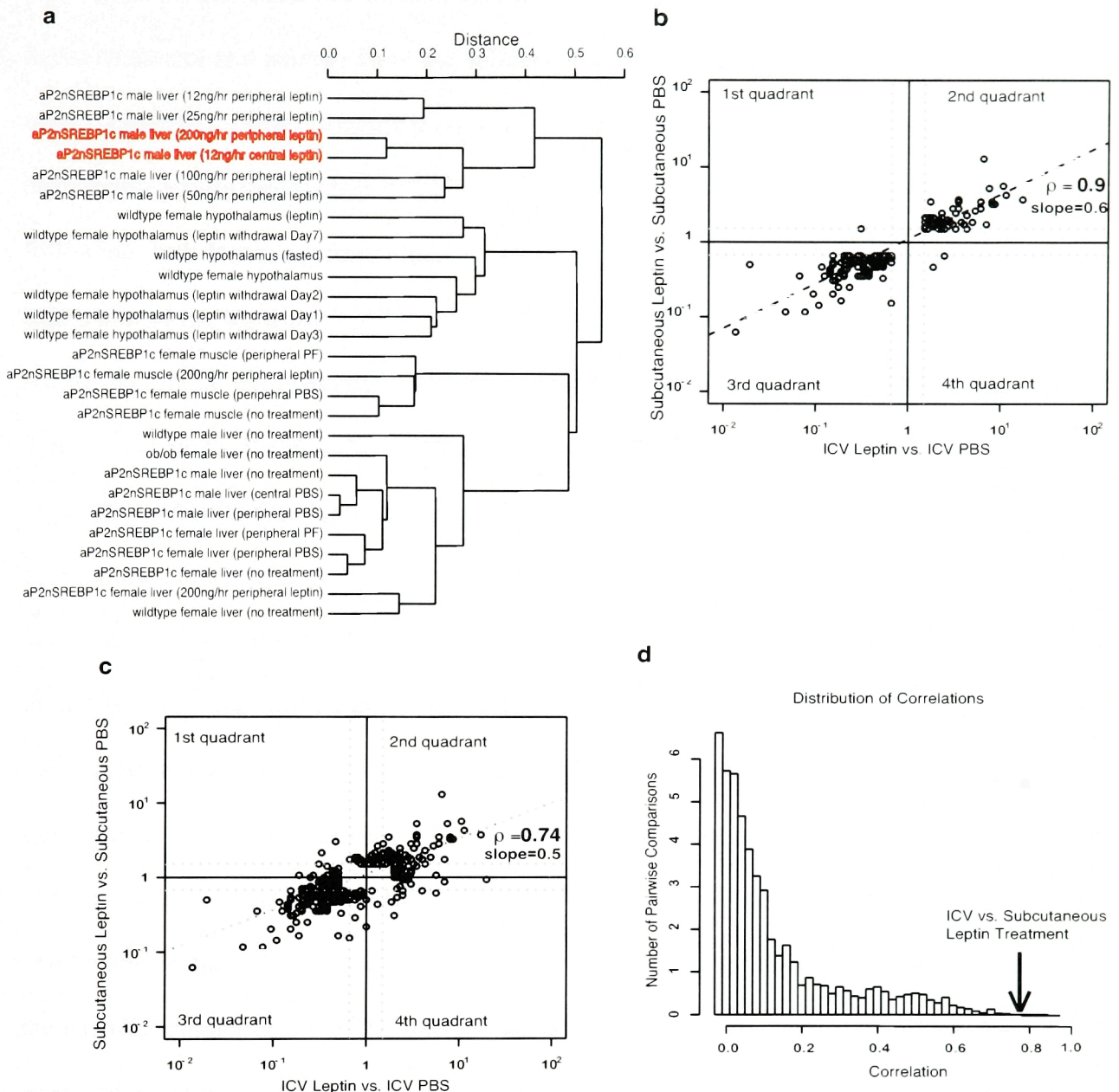


Figure 3.3 Computational analyses of the hepatic transcription profile after icv and subcutaneous leptin treatment. Cluster analysis, correlation analysis, and the distribution of correlations of liver gene expression from mice treated with icv leptin versus icv PBS and subcutaneous leptin versus subcutaneous PBS are shown. (a) Standard hierarchical clustering of 30 microarray experiments, including icv leptin and subcutaneous leptin treatments, show that the transcription profiles of icv and subcutaneous leptin are more similar to each other than to any other sample. (b) A pairwise comparison of icv and subcutaneous leptin gene expression is shown for genes regulated by both treatments (the intersection set). In the first quadrant, gene expression is increased in subcutaneous treatment and decreased in icv treatment; in the second quadrant, gene expression is increased in both treatments; in the third quadrant, gene expression is decreased in both treatments; and in the fourth quadrant, gene expression is decreased in subcutaneous treatment and increased in icv treatment. In almost all cases, gene expression is similarly regulated by both treatments. r denotes the correlation value. (c) The logarithm of fold changes of subcutaneous leptin versus PBS and icv leptin versus PBS for genes regulated by either treatment (the union set) is shown. (d) The distribution of correlations of 3,962 pairwise comparisons from our database is shown. The arrow shows the correlation of the icv leptin versus PBS treatment and the subcutaneous leptin versus PBS treatment. This shows that gene expression after icv and subcutaneous leptin is more highly correlated than for the other comparisons ($P < 0.002$).

From this, we compiled two distinct lists of leptin-regulated genes: one from the ICV leptin treatment and another from the subcutaneous leptin treatment (each relative to its own control; see Supplementary Tables 3, 4, 5 and 6 online; <http://www.jci.org/cgi/content/full/113/3/414/DC1>). Expression of 185 genes changed following both leptin administration protocols (i.e. ICV and subcutaneous). A linear regression of the logarithms of the fold changes in the intersection set showed a slope of 0.6 for peripheral leptin versus ICV leptin and a correlation coefficient of 0.897 (Figure 3.3b). The slope of 0.6 indicated that, in general, ICV leptin treatment had a greater effect than subcutaneous leptin administration. Even though 0.897 is an intrinsically high correlation coefficient, we further evaluated the significance of this correlation by analyzing the patterns of all genes that changed in abundance after either treatment modality. In order to establish the statistical significance of this correlation, we calculated a background correlation from 30 microarray experiments previously performed in our laboratory (see Supplementary Table 7 online; <http://www.jci.org/cgi/content/full/113/3/414/DC1>) and matched them into a total of 3692 pairs. Initially, we looked for genes that were similarly regulated in both samples in the pairwise comparison (i.e. the intersection set) but found that only ~ 10% of the 3692 pairs had a number of genes comparable to the 185 identified in ICV versus subcutaneous leptin treatment. Because there were an insufficient number of genes in the intersection set for most of the 3692 pairs, we modified the analysis by computing the correlation of genes in the union set for the lists of regulated genes. There were 379 genes in the union set of ICV leptin versus PBS and subcutaneous leptin versus PBS treatment. In this analysis, the correlation of ICV versus subcutaneous leptin treatment was 0.738 (Figure

3.3c), with a calculated p-value of approximately 0.0014. This correlation was among the highest correlations observed among the 3692 pairs of samples that were studied, indicating that the transcriptional response to ICV and subcutaneous leptin is highly similar (Figure 3.3d). A list of the RNA sources from the top 25 pairwise comparisons can be found in Supplementary Table 8 online at <http://www.jci.org/cgi/content/full/113/3/414/DC1>. The comparisons producing equivalent correlations to that of ICV versus subcutaneous leptin were invariably between very similar samples, such as between a wild type liver and liver from a leptin treated aP2-nSREBP-1c mouse. Leptin is known to normalize the liver phenotype of these animals (see above) (Shimomura et al. 1999). Another strong similarity was between *ob/ob* liver and PBS treated aP2-nSREBP-1c liver. The livers from these animals are both steatotic and this comparison is essentially between two leptin deficient livers (see above). Other pairwise analysis with high correlations included comparisons between untreated or PBS treated tissue or treatment with subtherapeutic doses of leptin (i.e.; low doses that have no biologic effect).

Transcription profiles in skeletal muscle.

As muscle is the primary site of insulin stimulated glucose disposal, we also analyzed gene expression profiles in skeletal muscle of aP2-nSREBP-1c transgenic mice following ICV and subcutaneous leptin administration. In contrast to the effects of leptin on gene expression in liver, gene expression in skeletal muscle was largely unchanged by central and peripheral leptin. The genes that were leptin regulated included major urinary protein group 1, acetyl CoA dehydrogenase, dienoyl-CoA isomerase ECH1p and fatty

acid binding protein 3. Consistent with other findings, the correlation between central and peripheral leptin was relatively low (Figure 3.4), suggesting that leptin might directly influence gene expression in muscle but not in liver (Minokoshi et al. 2002).

Discussion

Leptin has potent antidiabetic and antisteatotic effects in human and rodent lipodystrophy (Shimomura et al. 1999; Oral et al. 2002). Our findings strongly suggest that leptin's metabolic effects and its actions on liver gene expression in lipodystrophy are indirect via the CNS. This conclusion is consistent with data from ICV treatment of leptin deficient *ob/ob* and wild type mice (Halaas et al. 1995). Furthermore, mice with a neuron-specific knockout of the leptin receptor are obese and develop hepatic steatosis, while mice with a liver-specific knockout do not (Cohen et al. 2001). This conclusion is also consistent with previous studies showing that the CNS plays an important role in regulating glucose homeostasis (Miles et al. 1991). Several recent reports suggest that leptin can exert direct effects on peripheral tissues. Leptin increases fatty acid oxidation in skeletal muscle by activating AMP-activated protein kinase (AMPK), which in turn phosphorylates and inactivates ACC (Minokoshi et al. 2002). Leptin has also been suggested to activate fatty acid oxidation in heart independent of AMPK activation (Atkinson et al. 2002). In liver, leptin is suggested to increase fatty acid oxidation by a mechanism dependent on PPAR α (Lee et al. 2002). While our results do not exclude the possibility that direct actions of leptin on peripheral tissues such as muscle may contribute to its effects in lipodystrophy, these actions are not required for the metabolic

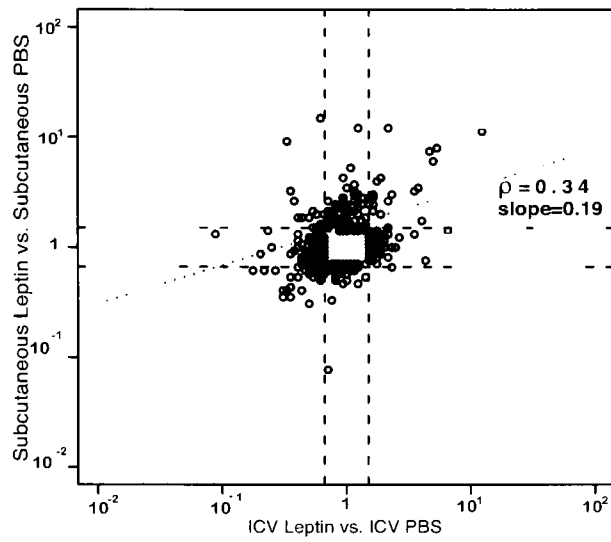


Figure 3.4 Computational analyses of the muscle transcription profile after icv and subcutaneous leptin treatment. A pairwise comparison of icv and subcutaneous leptin gene expression is shown for genes regulated by either treatment (the union set). r denotes the correlation value.

improvement observed following leptin treatment. This is because the abnormalities in aP2-nSREBP-1c mice were corrected by central leptin administration at doses that had no effect on plasma leptin levels.

Finally, we utilized a novel approach that quantifies the extent of similarity between two pairs of samples and computes the significance of the correlation to compare the effects of central and peripheral leptin. This analysis revealed a nearly identical set of effects of ICV and subcutaneous leptin on liver gene expression. This type of analysis is potentially relevant in other settings, including clinical pathology and could be used to determine the significance of the similarity of two tumor samples. However, the analyses of microarray data do suggest that peripheral leptin has distinct effects on gene expression in skeletal muscle compared to central leptin. While the data from the CNS leptin infusions suggest that these effects are not essential for leptin's actions, the physiological relevance of these direct effects can be further investigated using mice with a muscle specific knockout of the leptin receptor (Bruning et al. 1998; Cohen et al. 2001). These results showed that for the vast majority of leptin regulated genes ICV leptin is even more potent than a much higher dose of peripheral leptin. This includes the RNAs for several SREBP-1c regulated genes, which are generally repressed by leptin. However, in this case, we did not observe a change in the RNA levels of SREBP-1c itself. It is possible however that SREBP-1c is regulated at the post-transcriptional level and that, as previously shown in adipose tissue, leptin influences the amount of SREBP cleavage in liver (Soukas et al. 2000).

Chapter 4: Role of Leptin regulated genes in Hepatic Steatosis and Diabetes:

SCD-1 and Hepatic Steatosis in Lipodystrophy

Introduction

Recently, SCD-1 was shown to be an important mediator of leptin's weight reducing and antisteatotic effects (Cohen et al. 2002). SCD is a rate-limiting enzyme in the triglyceride biosynthesis pathway and it converts saturated fatty acids into monounsaturated fatty acids by introducing a double bond between carbons 9 and 10 (Ntambi and Miyazaki 2003). SCD converts its preferred substrates palmitoyl-CoA and stearoyl-CoA into palmitoleoyl-CoA and stearoyl-CoA (Ntambi 1999). There are several SCD isoforms, SCD-1 is mainly expressed in adipose tissue and liver, SCD-2 is mainly expressed in brain, SCD-3 is abundant in Harderian gland and SCD-4 was recently cloned in heart (Miyazaki et al. 2003). There is only one SCD isoform in humans, which is highly homologous to mouse SCD-1 isoform (Cohen et al. 2003).

Recently, a number of studies in leptin-deficient *ob/ob* mice and in SCD-1 knockout mice showed the importance of this enzyme in lipid metabolism. SCD-1 knockout mice were shown to have reduced adiposity and these mice are resistant to diet-induced obesity on high fat diet. There is an increase in energy expenditure and oxygen consumption in these mice. Moreover, these animals have reduced plasma leptin and insulin levels and were suggested to have increased insulin sensitivity (Ntambi et al. 2002; Rahman et al. 2003). In the livers of leptin-deficient *ob/ob* mice, both SCD-1 mRNA levels and activity were increased and leptin treatment reduced it back to wild-

type levels or lower. When asebia mice (*ab^J/ab^J*), which have a deletion in the SCD-1 gene, were crossed to *ob/ob* mice, there was a significant reduction in their obesity and a significant increase in their energy expenditure. Hepatic steatosis associated with leptin-deficiency in *ob/ob* mice was also significantly improved (Cohen et al. 2002). The mechanism by which SCD deficiency causes its metabolic effects and its role in insulin sensitivity still remains unknown.

In these studies, we studied the role of SCD-1 in leptin-mediated correction of hepatic steatosis and insulin resistance in lipodystrophy. First, we studied SCD-1 mRNA expression and activity in the livers of aP2-nSREBP-1c mice and showed that both mRNA expression and SCD enzymatic activity were significantly increased in the livers of aP2-nSREBP-1c mice compared to wild type mice. We then showed that ICV leptin treatment was able to repress SCD-1 mRNA levels and activity in the livers of lipodystrophic animals. The contribution of SCD-1 to the diabetic and steatotic phenotype of lipodystrophic mice was then assessed in crosses to SCD-1 deficient (asebia) mice. These studies showed that leptin acts via the CNS to reduce SCD-1 mRNA levels and activity in liver, thereby improving hepatic steatosis.

Results

Role of SCD-1 in lipodystrophic fatty liver

The microarray studies of hepatic gene expression in aP2-nSREBP-1c mice, wild type mice, ICV leptin treated aP2-nSREBP-1c mice and subcutaneous leptin treated aP2-nSREBP-1c mice showed that the levels of hepatic SCD-1 mRNA were elevated in lipodystrophic livers and reduced by both central and peripheral leptin treatments. Real-

time RT-PCR (Taqman) analysis showed that mRNA level for SCD-1 was elevated 2.3 fold in the liver of aP2-nSREBP-1c mice relative to wild type mice and its enzymatic activity was increased 3.8-folds. ICV leptin administration reduced SCD-1 mRNA levels 5.2-fold versus a 3.2-fold decrease for peripheral leptin treatment (Figure 4.1a). SCD enzyme activity was reduced 87.4% with ICV leptin infusion and 68.2% with subcutaneous leptin administration relative to PBS treated controls (Figure 4.1b). Both SCD-1 mRNA levels and SCD enzymatic activity were corrected to wild type levels following either treatment.

To determine the extent to which high levels of SCD contribute to the fatty liver and insulin resistance of aP2-nSREBP-1c mice, we generated aP2-nSREBP-1c transgenic mice lacking SCD-1. The resulting *ab^J/ab^J*;aP2-nSREBP-1c mice remained lipodystrophic, possessing virtually no white adipose tissue. However, in *ab^J/ab^J*;aP2-nSREBP-1c mice, the gross liver appearance was markedly improved (Figure 4.2a). Histological sections of the livers showed a marked reduction in the number of lipid droplets (Figure 4.2b) and liver triglyceride levels were significantly reduced (Figure 4.2c). Furthermore, in aP2-nSREBP1c mice, the hepatic levels of 16:1 and 18:1 monounsaturated fats were increased compared to livers of wild-type mice. SCD-1 deficiency in *ab^J/ab^J*;aP2-nSREBP-1c mice, decreased the relative levels of monounsaturated fats to wild type levels (Figure 4.2d).

Mechanism for SCD-1 deficiency's metabolic effects

Leptin was shown to increase fatty acid oxidation in muscle by phosphorylating and activating AMPK pathway, which in turn activates fatty acid oxidation through ACC

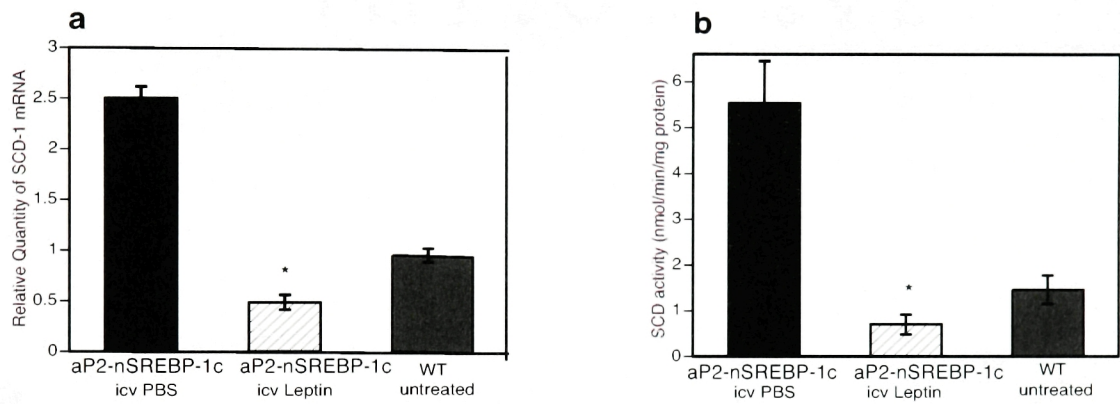


Figure 4.1 Regulation of SCD-1 by icv leptin. SCD-1 mRNA levels and enzyme activity are shown for aP2-nSREBP-1c transgenic mice treated with icv PBS, icv leptin, and WT littermate controls. (a) TaqMan real-time PCR of liver RNA samples using primers and probe specific for SCD-1. (b) Enzymatic activity measured in liver extracts. Error bars indicate the SE; $n = 4$ for icv PBS and icv leptin, and $n = 6$ for the WT group. * $P < 0.05$, icv leptin vs. icv PBS.

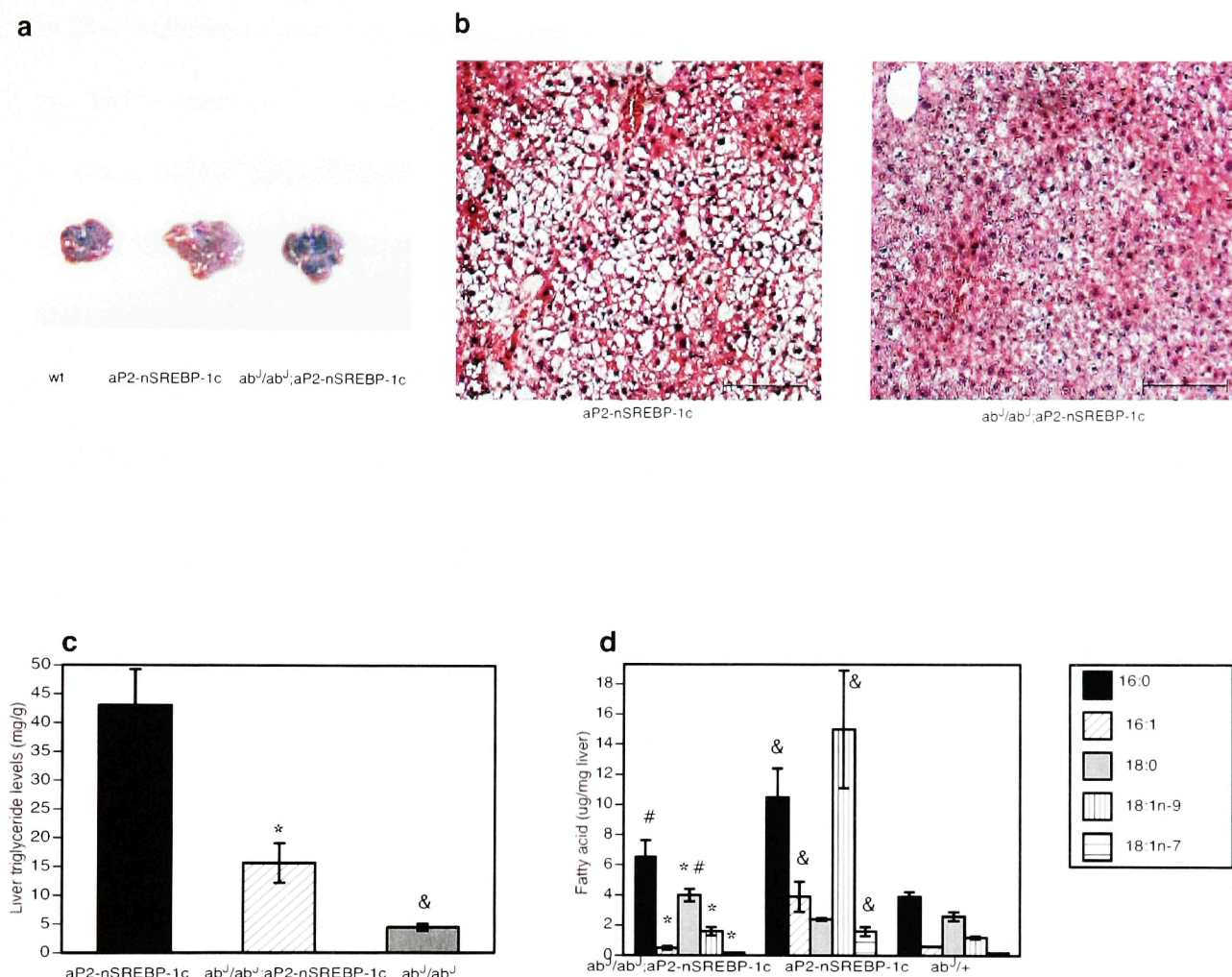


Figure 4.2 Correction of fatty liver in ab^J/ab^J;aP2-nSREBP-1c transgenic mice. (a) Gross liver appearance in ab^J/ab^J;aP2-nSREBP-1c transgenic mice, aP2-nSREBP-1c transgenic mice, and WT mice. (b) Representative liver sections from aP2-nSREBP-1c and ab^J/ab^J;aP2-nSREBP-1c mice. Original magnification, X200; scale bars: 100 μ m. (c) Liver triglyceride levels. (d) Fatty acid content in liver. Error bars indicate the SE; n = 7 for the aP2-nSREBP-1c group, n = 6 for the ab^J/ab^J;aP2-nSREBP-1c group, and n = 3 for the ab^J/ab^J group. *P < 0.05, ab^J/ab^J;aP2-nSREBP-1c vs. aP2-nSREBP-1c mice; †P < 0.05, aP2-nSREBP-1c vs. ab^J/ab^J or ab^J/+; #P < 0.05 ab^J/ab^J;aP2-nSREBP-1c vs. ab^J/ab^J or ab^J/+.

inactivation and malonyl CoA downregulation (Minokoshi et al. 2002). To determine if SCD-1 deficiency improves hepatic steatosis in lipodystrophy through downregulating the AMPK pathway, we studied this pathway's components in the livers of aP2-nSREBP-1c mice, *ab^J/ab^J*;aP2-nSREBP-1c mice and wild-type mice. There was no difference in AMPK phosphorylation between wild type and aP2-nSREBP-1c mice however, AMPK phosphorylation was increased in *ab^J/ab^J*;aP2-nSREBP-1c mice relative to aP2-nSREBP-1c mice suggesting that AMPK activity will also be increased in *ab^J/ab^J*;aP2-nSREBP-1c mice relative to aP2-nSREBP-1c mice (Figure 4.3a). AMPK specifically phosphorylates Ser79 on ACC. There was no difference in ACC Ser79 phosphorylation in aP2-nSREBP-1c mice relative to wild type mice. However, consistent with increased AMPK phosphorylation, there was an increase in ACC Ser79 phosphorylation in *ab^J/ab^J*;aP2-nSREBP-1c mice relative to aP2-nSREBP-1c mice. Furthermore, ACC1 and ACC2 protein levels were higher in aP2-nSREBP-1c transgenic mice compared to wild-type mice (Figure 4.3b). ACC protein levels were decreased in *ab^J/ab^J*;aP2-nSREBP-1c mice relative to aP2-nSREBP-1c mice. Consistent with the decreased protein levels and increased phosphorylation, ACC activity was also significantly decreased in *ab^J/ab^J*;aP2-nSREBP-1c mice (Figure 4.3b). Decreased ACC activity also led to a significant decrease in malonyl CoA levels in *ab^J/ab^J*;aP2-nSREBP-1c mice which were significantly elevated in aP2-nSREBP-1c mice compared to wild type levels (Figures 4.3c). A decrease in malonyl CoA levels would be expected to result in increased mitochondrial CPT-1 activity. When we measured CPT-1 activity, there was a significant increase in CPT-1 activity in *ab^J/ab^J*;aP2-nSREBP-1c mice compared to aP2-

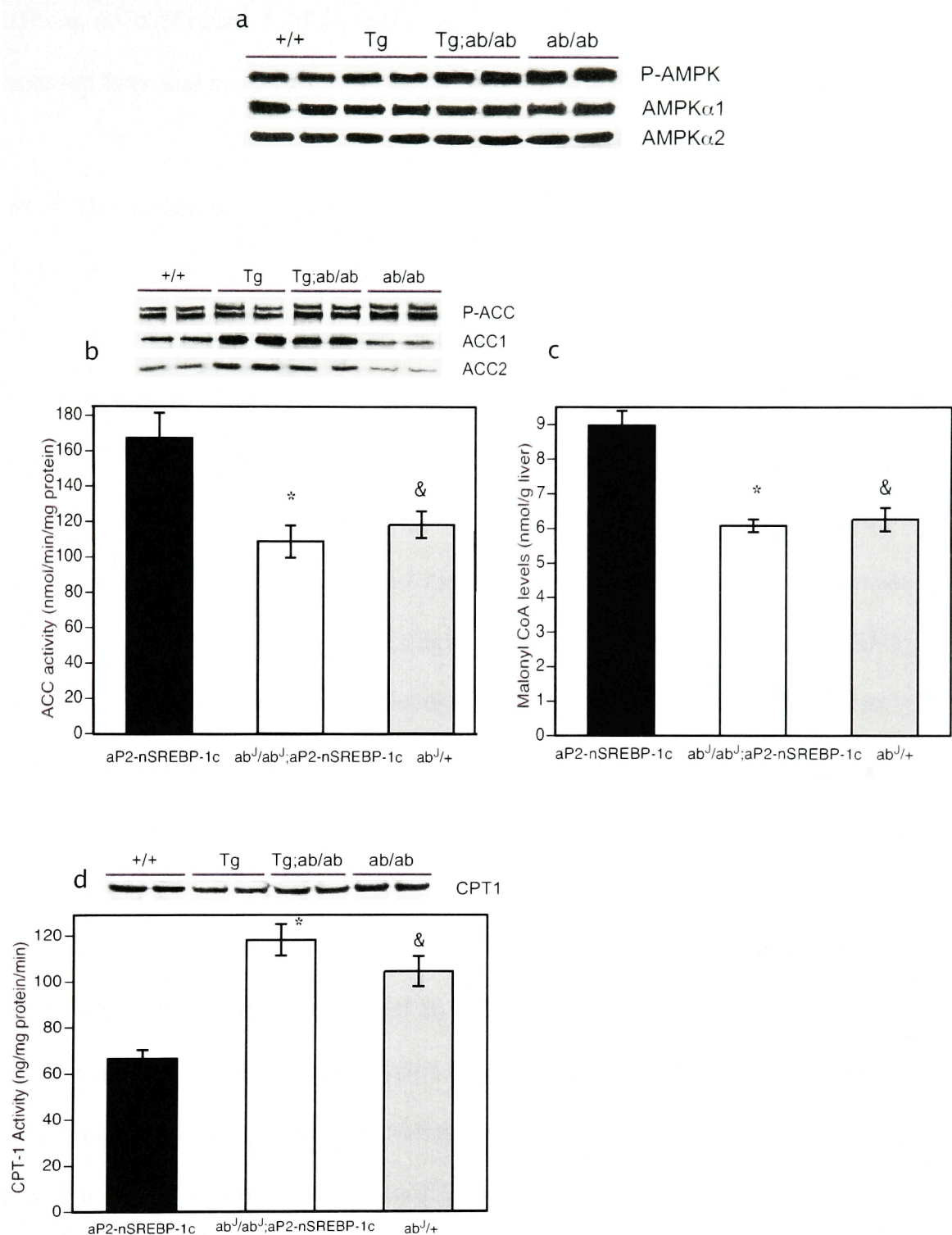


Figure 4.3 Mechanism for the correction of fatty liver in ab^J/ab^J ;aP2-nSREBP-1c mice. a. AMPK phosphorylation in liver. b. ACC phosphorylation and activity in liver. c. Malonyl CoA levels in liver. d. CPT-1 phosphorylation and activity in liver. Error bars indicate the SE, n=7 for aP2-nSREBP-1c (Tg) group, n=6 for ab^J/ab^J ;aP2-nSREBP-1c (Tg;ab/ab) group and n=3 for ab^J/ab^J group. * $P < 0.05$ ab^J/ab^J ;aP2-nSREBP-1c vs. aP2-nSREBP-1c mice, & $P < 0.05$ aP2-nSREBP-1c vs. ab^J/ab^J or $ab^J/+$, # $P < 0.05$ ab^J/ab^J ;aP2-nSREBP-1c vs. ab^J/ab^J or $ab^J/+$.

nSREBP-1c mice (Figure 4.3d). Increased CPT-1 activity would then increase mitochondrial fatty acid import and fatty acid oxidation in ab^J/ab^J ;aP2-nSREBP-1c mice.

Role of SCD-1 deficiency in glucose metabolism

Despite improved hepatic steatosis, ab^J/ab^J ;aP2-nSREBP-1c animals were hyperglycemic with glucose levels as high or higher than those of standard aP2-nSREBP-1c transgenic mice (Figure 4.4a). ab^J/ab^J ;aP2-nSREBP-1c mice had lower levels of insulin than aP2-nSREBP-1c transgenic mice (Figure 4.4b), which coupled with the higher glucose levels, suggested that there was β -cell failure. It has been previously suggested that accumulation of saturated fatty acids in a cell can lead to apoptosis (Listenberger et al. 2003). These data might suggest that lipid content and SCD-1 activity might also influence β -cell function or viability. Plasma triglycerides, cholesterol and free fatty acids (FFA) were unchanged in ab^J/ab^J ;aP2-nSREBP-1c mice versus aP2-nSREBP-1c controls (data not shown).

Real-time PCR (Taqman) analysis of hepatic gene expression showed that gene expression of three key enzymes in the gluconeogenesis pathway were upregulated in ab^J/ab^J ;aP2-nSREBP-1c mice compared to aP2-nSREBP-1c mice. PECK gene expression was upregulated 1.3 folds (Figure 4.5a), G-6-Pase expression was increased 1.7 folds (Figure 4.5b) and F,16BPase expression was 1.2 folds higher (Figure 4.5c) in ab^J/ab^J ;aP2-nSREBP-1c mice relative to aP2-nSREBP-1c mice. Furthermore, gene expression of peroxisome proliferator-activated coactivator-1 (PGC-1) which is suggested to activate gluconeogenesis and fatty acid oxidation was also increased significantly in the livers of ab^J/ab^J ;aP2-nSREBP-1c mice (Barthel and Schmoll 2003).

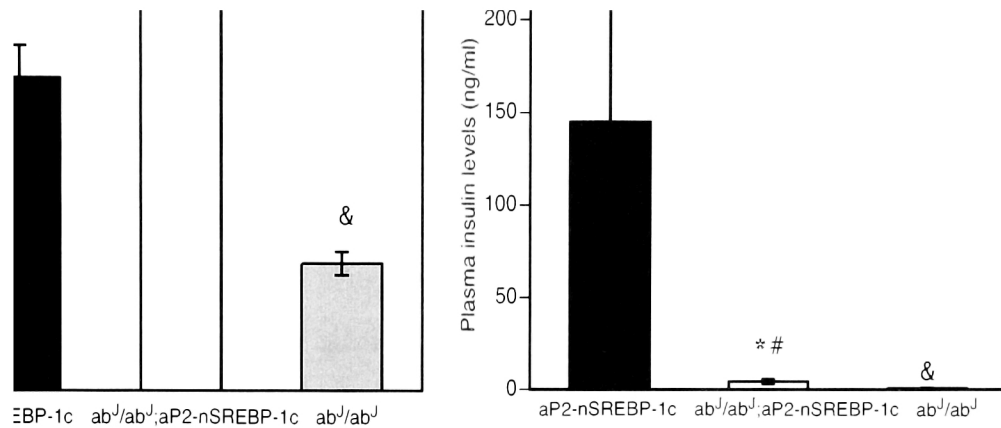


Figure 4.4 Aggravation of diabetes in ab^J/ab^J;aP2-nSREBP-1c transgenic mice. (a) Plasma glucose and (b) plasma insulin levels, respectively. Error bars indicate standard deviation; n = 7 for the aP2-nSREBP-1c group, n = 6 for the ab^J/ab^J;aP2-nSREBP-1c group, and n = 3 for the ab^J/ab^J group. *P < 0.05, ab^J/ab^J;aP2-nSREBP-1c vs. aP2-nSREBP-1c mice; †P < 0.05, aP2-nSREBP-1c vs. ab^J/ab^J or ab^J/+; #P < 0.05, ab^J/ab^J;aP2-nSREBP-1c vs. ab^J/ab^J or ab^J/+.

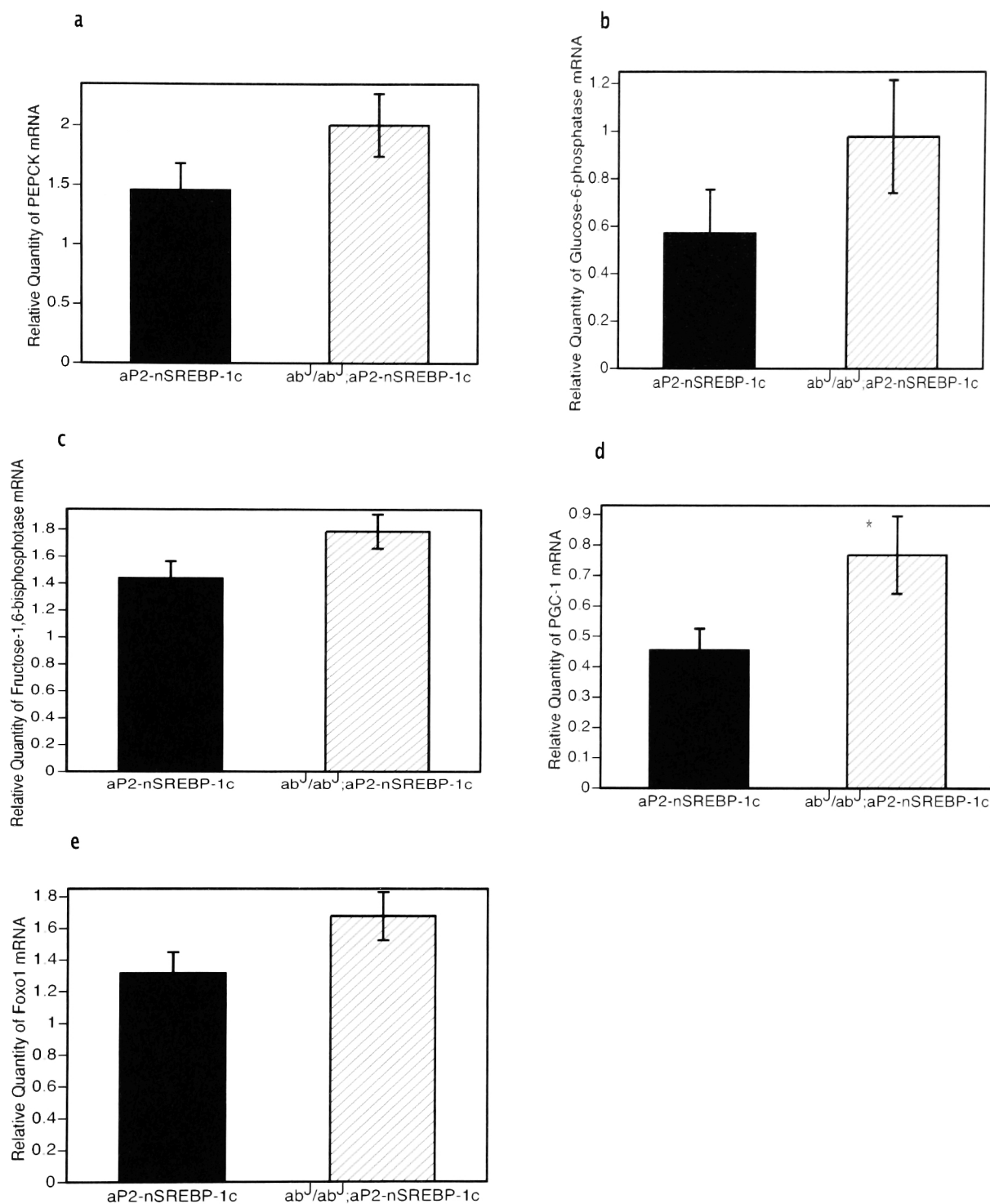


Figure 4.5 Gene expression analysis in the livers of aP2-nSREBP-1c mice and ab^J/ab^J;aP2-nSREBP-1c mice. a. Taqman real-time PCR using primers and probes specific for PEPCCK. b. Taqman real-time PCR using primers and probes specific for Glucose-6-Phosphatase. c. Taqman real-time PCR using primers and probes specific for Fructose-1,6-bisphosphatase. d. Taqman real-time PCR using primers and probes specific for PGC-1. e. Taqman real-time PCR using primers and probes specific for Foxo1. Error bars indicate the SE, n=7 for aP2-nSREBP-1c and n=6 for ab^J/ab^J;aP2-nSREBP-1c. *P<0.05, aP2-nSREBP-1c vs. ab^J/ab^J;aP2-nSREBP-1c.

PGC-1 mRNA levels were 1.6 folds higher in *ab^f/ab^f*;aP2-nSREBP-1c mice relative to aP2-nSREBP-1c mice (Figure 4.5d). Expression of Foxo1 was also increased 1.3 folds in *ab^f/ab^f*;aP2-nSREBP-1c mice (Figure 4.5e).

Discussion

Recent data suggested that leptin reduces adiposity and liver triglyceride content, in part by reducing SCD-1 mRNA and enzymatic activity (Cohen et al. 2002; Ntambi et al. 2002). Our data is consistent with the possibility that leptin's effects to correct hepatic steatosis in lipodystrophy are also mediated, at least in part, by inhibiting SCD-1. Thus SCD-1 deficiency markedly improves hepatic steatosis in lipodystrophic animals. In aP2-nSREBP-1c transgenic mice, both SCD-1 mRNA levels and enzymatic activity were increased relative to wild type controls. ICV leptin treatment significantly reduced SCD-1 mRNA levels and enzymatic activity. This suggests that efferent signals from the CNS are capable of repressing this enzyme. It was previously shown that saturated fatty acyl CoAs can inhibit ACC, an effect which would decrease cellular malonyl CoA levels (Volpe and Vagelos 1976; Lunzer et al. 1977). A decrease in malonyl CoA would be expected to de-repress the activity of carnitine palmitoyltransferase 1 (CPT-1), the rate-limiting enzyme for the mitochondrial import and oxidation of fatty acids (McGarry et al. 1977). The data reported here further suggest that repression of SCD-1 is associated with increased AMPK phosphorylation, which in turn would increase its activity. In muscle, leptin was shown to phosphorylate and hence increase AMPK activity, which in turn regulates leptin's fatty acid oxidation effects in this tissue (Minokoshi et al. 2002). Our

data further showed that increased AMPK activity was also associated with increased Ser79 phosphorylation and hence with reduced levels of ACC enzymatic activity. Decreased ACC activity then resulted in decreased malonyl CoA levels and increased mitochondrial CPT-1 activity. An increase in CPT-1 activity is expected to increase fatty acid import and β -oxidation in mitochondria. In the livers of SCD-1 knockout mice and *ab^J/ab^J;ob/ob* mice, SCD-1 deficiency is suggested to act through a similar mechanism of AMPK activation resulting in increased mitochondrial oxidation of palmitoyl-CoA (Dobrzyn et al. 2004). Other mechanisms might also contribute to the anti-steatotic effects of a deficiency of this enzyme.

While the CNS pathways and efferent signals responsible for SCD-1 repression by leptin are unknown, some of leptin's effects might be mediated by repression of neuropeptide Y (NPY). Leptin is known to repress NPY gene expression in hypothalamus and NPY infusions induce liver ACC activity as well as other lipogenic genes (Zarjevski et al. 1993). SCD-1 gene expression was recently shown to be reduced in *MCH^{-/-};ob/ob* mice suggesting that leptin's effects on SCD-1 expression may also be mediated by regulation of melanin-concentrating hormone (MCH) in the lateral hypothalamus (Segal-Lieberman et al. 2003). Recently ICV MTII injections were shown to decrease SCD-1 expression suggesting that leptin represses SCD-1 through melanocortin receptors (Lin et al. 2003).

Previous data suggested that SCD-1 knockout mice have increased insulin sensitivity compared to wild type mice (Ntambi et al. 2002). Furthermore, SCD-1 knockout mice had increased insulin signaling in muscle. In the muscle of SCD-1 knockout mice, glucose uptake, as well as glycogen content, were increased. There was

also decreased PTP1B activity in the muscles of SCD-1 knockout mice (Rahman et al. 2003). While SCD-1 deficiency reduced hepatic steatosis in lipodystrophic aP2-nSREBP-1c mice, these animals remained severely diabetic. This suggests that leptin's anti-diabetic effects are not simply a result of its ability to reduce triglyceride levels in peripheral tissues. It is still possible however that other lipid moieties that modulate insulin sensitivity remain unchanged in the absence of SCD-1 (Shulman 2000). It is also possible that the decreased plasma levels indicate improved insulin sensitivity rather than β -cell failure. The residual diabetes of *ab^J/ab^J*;aP2-nSREBP-1c mice is consistent with the finding that pair-feeding aP2-nSREBP-1c transgenic mice significantly reduces liver triglycerides to near normal levels, while the mice remain diabetic (Shimomura et al. 1999). Similarly *ob/ob* mice lacking PPAR γ in liver have a significant reduction in liver triglyceride levels while remaining hyperglycemic and insulin resistant (Matsusue et al. 2003). Thus, reducing the liver triglyceride content of lipodystrophic mice is by itself insufficient to correct their diabetes.

Our data suggests that SCD-1 deficiency in liver activated fatty acid oxidation in this tissue. It is suggested that fatty acid oxidation increases plasma free fatty acids, which in turn activate gluconeogenic enzymes thus increasing hepatic glucose output. Furthermore, fatty acid oxidation is suggested to provide precursors for gluconeogenesis, which in turn also increase hepatic glucose output (Elks 1990; Lam et al. 2003). Plasma insulin levels were lower in *ab^J/ab^J*;aP2-nSREBP-1c mice compared to aP2-nSREBP-1c mice and it is known that insulin inhibits gluconeogenesis by suppressing gluconeogenic gene expression (Barthel and Schmolli 2003). Gene expression of key gluconeogenic enzymes were upregulated in livers of *ab^J/ab^J*;aP2-nSREBP-1c mice compared to aP2-

nSREBP-1c mice. PEPCK, G-6-Pase and F1,6BPase gene expression were all moderately upregulated in *ab^J/ab^J*;aP2-nSREBP-1c mice compared to aP2-nSREBP-1c mice which already had higher expression levels of these enzymes compared to wild type mice (data not shown) (Shimomura et al. 2000). The further upregulation of gluconeogenic enzymes might be through PGC-1 upregulation. PGC-1 α is known to activate gluconeogenesis through HNF4 α and fatty acid oxidation via PPAR α (Koo et al. 2004). PGC-1 expression was significantly increased in the livers of *ab^J.ab^J*;aP2-nSREBP-1c mice. Forkhead transcription factor Foxo1, which is also implicated in the activation of gluconeogenic enzymes through PGC-1 (Puigserver et al. 2003) was also modestly increased in the livers of *ab^J/ab^J*;aP2-nSREBP-1c mice. Even if there is increased muscle insulin sensitivity in the muscles of *ab^J/ab^J*;aP2-nSREBP-1c mice, it is possible that increased gluconeogenesis and hence increased hepatic glucose output might confound skeletal muscle insulin sensitivity resulting in the aggravated plasma glucose levels observed in these animals. It is also possible that decreased plasma insulin levels in *ab^J/ab^J*;aP2-nSREBP-1c mice result in improved insulin sensitivity rather than β -cell failure.

Chapter 5: Leptin-mediated correction of insulin resistance in lipodystrophic mice

Introduction

Fat accumulation in peripheral insulin sensitive tissues such as liver, muscle and pancreatic β -cells has been implicated in inducing insulin resistance in these tissues. Fat accumulation is suggested to interfere with insulin signaling in these tissues thus impairing insulin sensitivity (Shulman 2000; Unger 2003). aP2-nSREBP-1c mice are very insulin resistant as indicated by their glucose and insulin levels and their insulin unresponsiveness by insulin tolerance test. When muscle insulin sensitivity was studied in these animals, these animals had normal insulin receptor, IRS-1, IRS-2 and Glut4 mRNA expression in this tissue. Moreover, they had a normal response to insulin-stimulated glucose uptake in muscle (Shimomura et al. 1998). A physiological dose of peripheral leptin was shown to correct insulin resistance, diabetes and hepatic steatosis in these mice independent of food intake effects. Food restricted aP2-nSREBP-1c mice had decreased body weight and ameliorated hepatic steatosis with no improvement in insulin resistance (Shimomura et al. 1999). In both leptin-deficient *ob/ob* mice and in lipodystrophic aP2-nSREBP-1c mice, glycolytic enzymes such as glucokinase and pyruvate kinase as well as gluconeogenic enzymes are upregulated in liver. These genes are no longer sensitive to insulin's lowering effects however, leptin treatment lowers the expression of these genes to wild-type levels (Shimomura et al. 2000). Furthermore, IRS-2 mRNA and protein levels were decreased in aP2-nSREBP-1c lipodystrophic mice and insulin-stimulated Akt phosphorylation was impaired in these animals (Shimomura et

al. 2000). Leptin was also shown to improve insulin resistance and diabetes in lipodystrophic human patients (Oral et al. 2002). In these lipodystrophic patients, leptin was shown to increase both hepatic and muscle insulin sensitivity as well as decreasing triglyceride content in both tissues (Petersen et al. 2002).

The mechanism by which leptin improves insulin resistance is not well understood. Leptin could improve insulin resistance by clearing all the lipids from peripheral tissues such as liver and muscle and hence making them insulin sensitive again or leptin could improve insulin sensitivity through other mechanisms independent of its lipid clearing effects. In these studies, we identified two suboptimal subcutaneous leptin doses, which improved hyperglycemia and hyperinsulinemia without correcting hepatic steatosis and with mild effects on SCD activity. These studies suggest that leptin may improve diabetes in lipodystrophic mice via an insulin-dependent pathway, which appears to be independent of its effects on SCD-1 and steatosis.

Results

Subcutaneous leptin dose response study

The results from studies of *ab^J/ab^J;aP2-nSREBP-1c* mice (Chapter 4) suggest that leptin's anti-diabetic effects are independent of its ability to clear lipid in liver and other tissues. In order to further explore this possibility and the underlying mechanism, we generated a dose response curve for subcutaneous leptin in aP2-nSREBP-1c mice. The objective of this study was to assess whether leptin's ability to improve diabetes was evident at the same or a different dose as its ability to reduce steatosis. We infused leptin at 12ng/hr, 25ng/hr, 50ng/hr and 100ng/hr with the highest dose being 200ng/hr, a dose

which has previously been shown to both improve diabetes and reduce steatosis (Shimomura et al. 1999). Body weight decreased 1.5% with the 50ng/hr leptin dose, 6.7% with the 100ng/hr leptin dose and 10.5% with the 200ng/hr leptin dose (Figure 5.1a). There was a significant decrease in food intake only with the 200ng/hr leptin dose (Figure 5.1b). Plasma leptin levels were raised to physiological levels only with the 200ng/hr dose with intermediate increases following other doses (Figure 5.1c). Liver triglyceride levels were corrected to wild type levels at the 200ng/hr leptin dose with modest reductions at the lower infusion rates (Figure 5.1d). SCD activity was corrected to wild type levels only with the 200ng/hr leptin infusion (Figure 5.1e).

While SCD activity and liver triglyceride levels were normalized only at the highest dose, there was a significant reduction in both plasma glucose and plasma insulin levels at the 50ng/hr, 100ng/hr and 200ng/hr leptin doses (Figure 5.1f-g). There was also a significant reduction in liver glycogen content at the 50ng/hr, 100ng/hr and 200ng/hr leptin doses (Figure 5.1h). Thus, leptin improves diabetes at doses that do not normalize hepatic steatosis or reduce SCD-1 activity to wild type levels. This suggests the possibility that leptin's antidiabetic effects are independent of its ability to repress SCD-1 and reduce steatosis.

Insulin signaling at a low dose that improves glucose levels

In principal, leptin could improve diabetes by increasing glucose uptake or decreasing hepatic glucose output independent of insulin or by improving insulin signal transduction. To distinguish between these possibilities, we studied the effects of insulin in aP2-nSREBP-1c mice following treatment with a dose of peripheral leptin (50ng/hr)

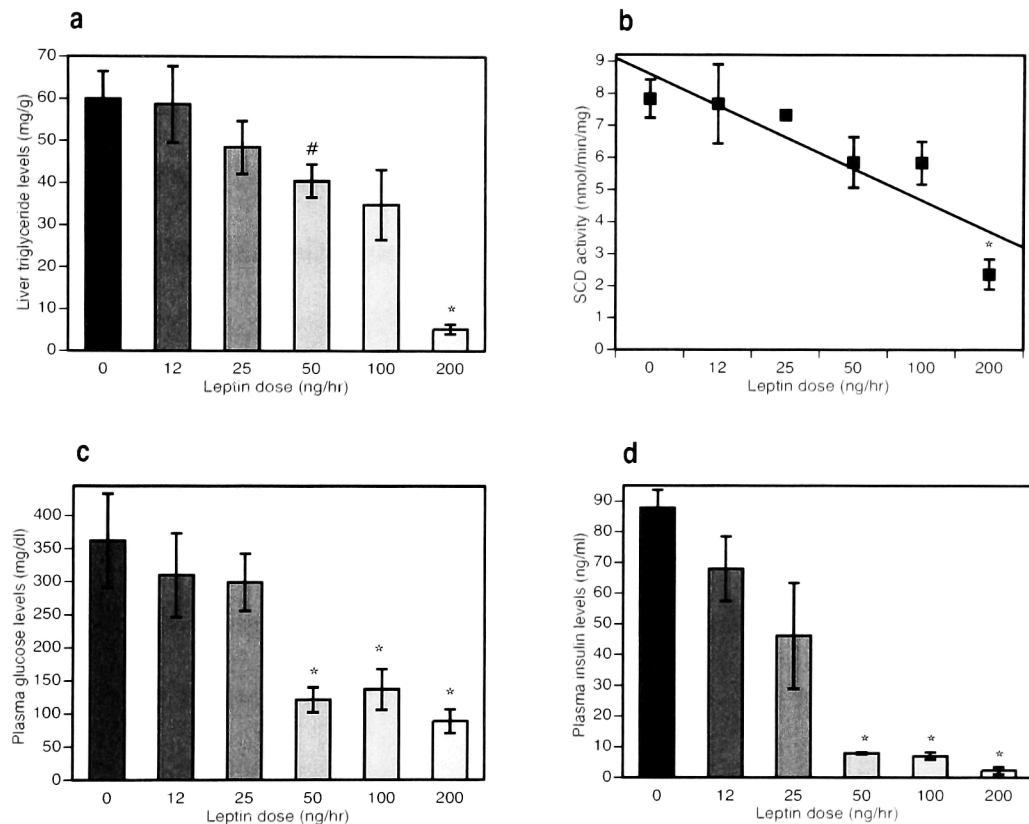


Figure 5.1 Dose-response curve for leptin treatment. Leptin corrects hyperinsulinemia and hyperglycemia at leptin doses that have no effect on SCD activity and gross liver appearance (50 ng/h and 100 ng/h). (a) Liver triglyceride content. (b) SCD enzymatic activity measured in liver extracts of aP2-nSREBP-1c mice treated with several doses of subcutaneous leptin. (c and d) Plasma glucose and plasma insulin levels, respectively. Error bars indicate the SE; $n = 3$ for 12 ng/h and 25 ng/h leptin, and $n = 4$ for 0 ng/h, 50 ng/h, 100 ng/h, and 200 ng/h leptin. * $P < 0.005$, 200 ng/h leptin dose vs. 0 ng/h dose. # $P < 0.05$, 50 ng/h leptin dose vs. 0 ng/h dose.

that improves diabetes but does not correct steatosis or reduce SCD-1 activity to wild type levels (Figure 5.1).

In the livers from lipodystrophic mice, there was a marked reduction in insulin-stimulated insulin receptor substrate 2 (IRS-2) phosphorylation and a decrease in both IRS-2 associated phosphatidylinositol 3-kinase (PI 3-Kinase) activity and Akt activity compared to the livers from wild type animals (Figures 5.2a-c). Leptin treatment (50ng/hr) normalized insulin stimulated IRS-2 phosphorylation and IRS-2 associated PI 3-Kinase activity and Akt activity in livers of lipodystrophic mice (Figure 5.2a-c). In skeletal muscles of the lipodystrophic mice, there was also a decrease in IRS-1 associated PI 3-Kinase activity and Akt activity. However in this tissue leptin treatment did not fully correct the attenuated response to insulin (Figure 5.2d-e).

The improved insulin signaling in the livers of aP2-nSREBP-1c mice treated with 50ng/hr leptin dose does not seem to be associated with liver fatty acyl CoA levels. Liver fatty acyl CoA levels in aP2-nSREBP-1c mice treated with 50ng/hr leptin dose were not significantly different from the levels in aP2-nSREBP-1c mice treated with PBS (Figure 5.3a). Consistent with this data, muscle fatty acyl CoA levels were also not significantly different (Figure 5.3b). This suggests that 50ng/hr leptin dose improves insulin sensitivity in these animals independent of liver and muscle fatty acyl CoA levels and leptin's effects on insulin sensitivity are not merely through its lipid clearing effects in these insulin sensitive tissues. It is possible however that leptin changes the level of another lipid moiety, which might be important in insulin sensitivity. It is also possible that there is a threshold effect for plasma leptin levels as well as liver triglyceride content and hepatic SCD activity, which would suggest that plasma leptin levels above and

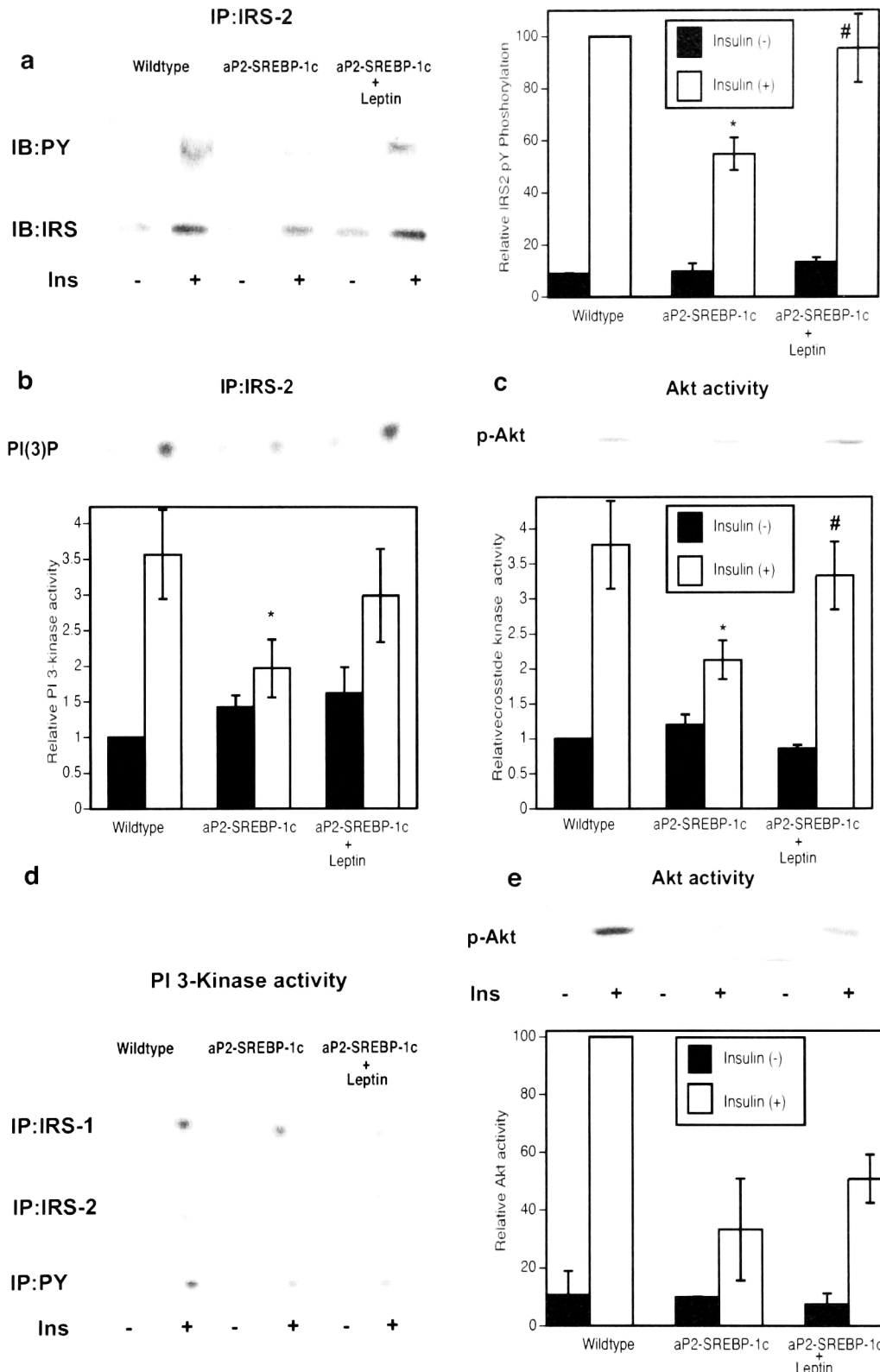


Figure 5.2 Insulin signal transduction after leptin treatment. Subcutaneous leptin leads to an improvement of insulin signaling in liver but not in muscle of aP2-SREBP-1c mice treated with 50 ng/h of subcutaneous leptin. (a) Insulin-induced tyrosine phosphorylation of IRS molecules in liver. (b) PI3K activities associated with tyrosine-phosphorylated proteins in liver. (c) In vitro AKT kinase activity in liver. The upper panels in b and c show representative results of relative immunoblot analysis, and in the lower panels, error bars indicate the SE; $n = 4$. * $P < 0.05$, WT vs. aP2-nSREBP-1c; # $P < 0.05$, aP2-nSREBP-1c vs. aP2-nSREBP-1c plus leptin. (d) PI3K activities associated with tyrosine-phosphorylated proteins in muscle. (e) In vitro AKT kinase activity in muscle. The upper panel shows representative results of immunoblot analysis, and in the lower panel, each bar represents the mean calculated from two independent experiments. IB, immunoblot; IP, immunoprecipitation; PY, phospho tyrosine; PI(3)P, PI(3) phosphate.

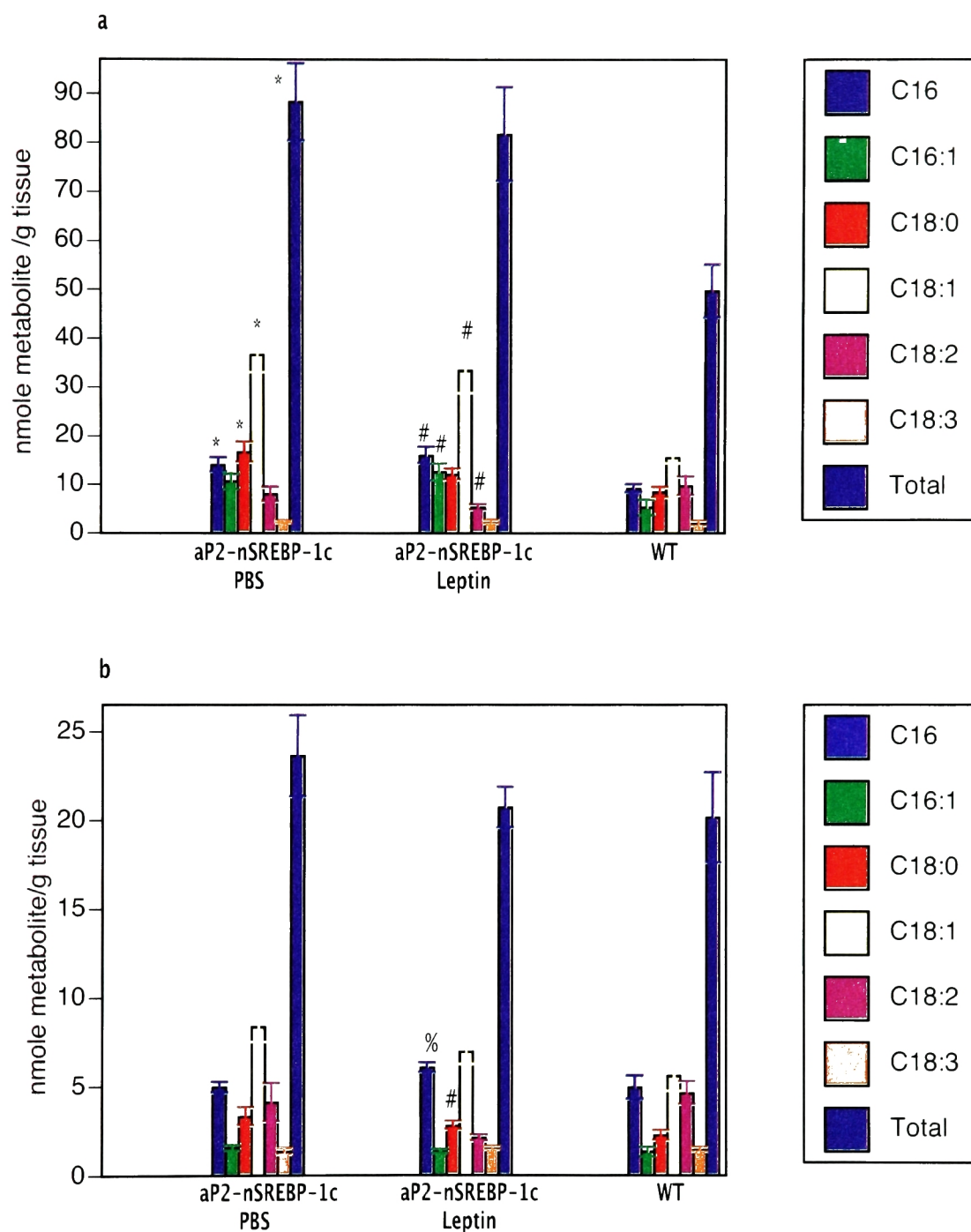


Figure 5.3 Fatty acyl CoA analysis in the muscle and liver of aP2-nSREBP-1c mice treated with PBS (aP2-nSREBP-1c PBS), 50ng/hr leptin (aP2-nSREBP-1c Leptin) and untreated wild type controls (WT). a. Fatty acyl CoA levels in liver. b. Fatty acyl CoA levels in muscle.

Error bars indicated the SE; n=3 for WT, n=3 for aP2-nSREBP-1c PBS, n=6 for aP2-nSREBP-1c Leptin in (a) and n=4 for aP2-nSREBP-1c Leptin in (b). *P<0.05, WT vs aP2-nSREBP-1c PBS, #P<0.05, WT vs aP2-nSREBP-1c Leptin, %P<0.05, aP2-nSREBP-1c Leptin vs aP2-nSREBP-1c PBS.

plasma triglyceride content and hepatic SCD activity below a certain threshold have an improving effect on insulin action.

Cluster analysis of hepatic transcription profiles with several subcutaneous leptin doses

In the leptin dose response study, we identified two leptin doses, which improve hyperglycemia and hyperinsulinemia without correcting hepatic steatosis. In order to identify genes important in leptin's insulin sensitizing effects, we then performed cluster analysis of hepatic gene expression in the leptin dose response study. This cluster analysis was similar to the cluster analysis previously described in WAT of leptin treated wild type mice over a period of time (Soukas et al. 2000).

From this cluster analysis, we chose the analysis that has given us six clusters, three clusters of leptin induced genes and three clusters of leptin repressed genes. One of these clusters included genes that were induced at 50ng/hr leptin dose or higher (Appendix A; Figure 5.4a). This clustered contained several ESTs and some known genes such as angiogenin and hepatocyte nuclear factor 3 γ . The second cluster included genes that were mostly induced at 200ng/hr leptin dose (Appendix B; Figure 5.4b). This cluster included genes such as insulin-like growth factor binding protein-2 (IGFBP-2) and hepatic nuclear factor 6 (HNF6). The last cluster of induced genes included genes that were induced at all leptin doses (Appendix C; Figure 5.4c). This cluster also contained mostly ESTs, however, there were some known genes such as eukaryotic translation initiation factor 1A. The first cluster of leptin-repressed genes included genes that were repressed at 50ng/hr leptin dose or higher (Appendix D; Figure 5.4d). This

cluster included genes such as glutathione s-transferase and monocarboxylate transporter 1. The second cluster contained genes that were mostly repressed at 200ng/hr leptin dose (Appendix E; Figure 5.4e). This cluster included genes such as cell death activator CIDE-A and malic enzyme. The last cluster contained genes that were repressed at all leptin doses (Appendix F; Figure 5.4f). This cluster contained genes such as a serine/threonine kinase sak-b.

We then performed real time RT-PCR (Taqman) analysis to verify some of the genes that were present in our cluster analysis as well as other genes important in glucose metabolism. One of the genes that were downregulated at all leptin doses was a serine/threonine kinase, sak-b. Taqman analysis of this gene showed that sak mRNA levels were significantly lower in animals treated with 100ng/hr and 200ng/hr leptin dose compared to PBS treated animals (Figure 5.5a). HNF-6 was another leptin regulated gene, which was in a cluster of genes that were upregulated mainly at the 200ng/hr leptin dose. Taqman analysis of HNF-6 showed that there was a trend for this gene to be increased by leptin however this was not significant (Figure 5.5b). In order to determine if suboptimal leptin doses improved hyperglycemia and hyperinsulinemia in aP2-nSREBP-1c mice by decreasing hepatic glucose output and suppressing gluconeogenesis, we measured mRNA levels of three key gluconeogenic enzymes. There was a significant reduction in glucose-6-phosphatase mRNA levels following both 50ng/hr leptin dose and 200ng/hr leptin dose (Figure 5.5c). There was also a reduction in F1,6BPase mRNA levels with 50ng/hr and 200ng/hr leptin doses however this was not significant (Figure 5.5d). PEPCK mRNA levels on the other hand showed an increasing trend at the 50ng/hr

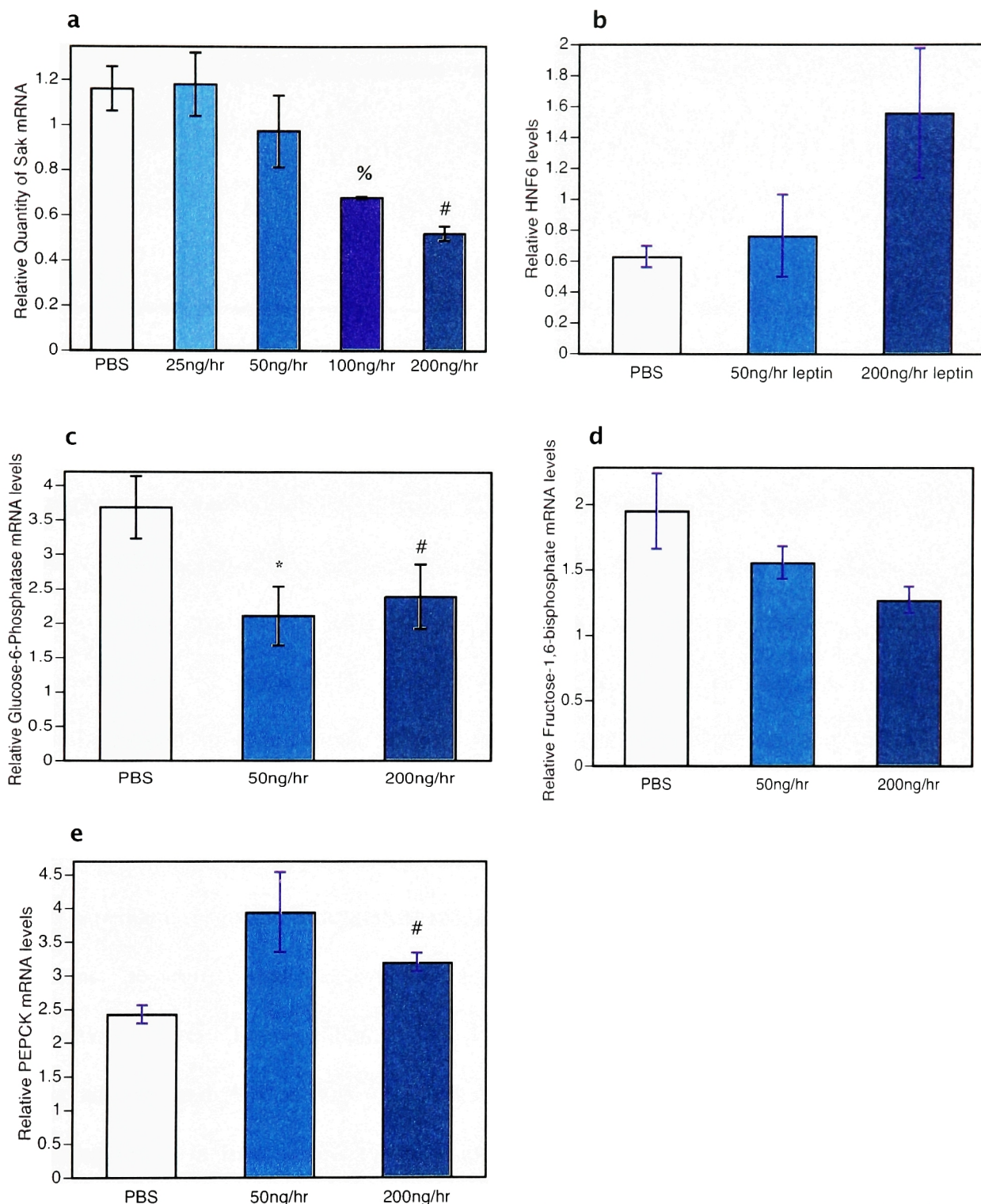


Figure 5.5 Hepatic gene expression analysis in aP2-nSREBP-1c mice treated with PBS, 25ng/hr, 50ng/hr, 100ng/hr and 200ng/hr subcutaneous leptin. a. Taqman real-time PCR of RNA samples using primers and probes specific for Sak. b. Taqman real-time PCR of RNA samples using primers and probes specific for HNF6. c. Taqman real-time PCR of RNA samples using primers and probes specific for Glucose-6-Phosphatase. d. Taqman real-time PCR of RNA samples using primers and probes specific for Fructose-1,6-bisphosphatase. e. Taqman real-time PCR of RNA samples using primers and probes specific for PEPCCK. Error bars indicate the SE, n=4 for PBS, n=3 for 25ng/hr, n=4 for 50ng/hr, n=4 for 100ng/hr and n=4 for 200ng/hr leptin dose. *P<0.05, PBS vs. 50ng/hr, %P<0.05, PBS vs 100ng/hr and #P<0.05, PBS vs 200ng/hr.

and 200ng/hr leptin doses but this increase was also not statistically significant (Figure 5.5e).

Discussion

While SCD-1 deficiency reduced hepatic steatosis in lipodystrophic mice, these animals remained severely diabetic (Chapter 4) (Asilmaz et al. 2004). Thus, reducing the liver triglyceride content of lipodystrophic mice was by itself insufficient to correct their diabetes. This suggested that leptin's antidiabetic effects are not simply a result of its ability to reduce triglyceride levels in peripheral tissues. This possibility is also suggested by our observation that leptin can correct diabetes and improve hepatic insulin sensitivity in lipodystrophic animals at doses that do not reduce SCD activity and correct hepatic steatosis.

These suboptimal leptin doses could improve diabetes in lipodystrophy by an insulin-dependent or insulin-independent mechanism. The data presented here supports the former possibility and suggests that leptin treatment improves insulin signal transduction in liver. Leptin enhances IRS-2 phosphorylation, IRS-2 associated PI 3-kinase activity and Akt activity in livers of aP2-nSREBP-1c mice. This insulin sensitizing effect in liver seems to be SCD independent, since leptin can improve hyperglycemia and hyperinsulinemia at doses that do not correct SCD-1 expression and enzymatic activity to wild type levels. This suggests that other leptin regulated genes or pathways are necessary for leptin's insulin sensitizing effects. It is also possible that

there is a threshold effect of SCD activity on insulin action such that SCD activity below a certain threshold improves insulin action.

In order to determine other leptin regulated genes and pathways that are important for leptin's insulin sensitizing effects, we used a clustering algorithm to cluster genes that responded to several leptin doses with a certain expression pattern (Soukas et al. 2000). This enabled us to identify pathway of genes regulated similarly by leptin or identify individual genes important in leptin's effects in lipodystrophy. In this clustering analysis, we analyzed genes in six clusters that might be important for leptin's effects. There were six clusters with three unique expression patterns since each pattern was both induced and repressed. In two of the clusters, expression of these genes were either induced or repressed at all doses of leptin suggesting that genes in these clusters might be very sensitive to leptin and hence might be important for leptin's actions. Sak-b, a serine/threonine kinase with an unknown function was in the cluster of genes whose expression was repressed at all leptin doses. Since activation of serine/threonine kinases are implicated in fatty acid induced insulin resistance in peripheral tissues (Shulman 2000; Perseghin et al. 2003), leptin might be improving insulin sensitivity by downregulating serine/threonine kinases. In two other clusters, expression of genes was either induced or repressed at 50ng/hr or higher leptin doses. These clusters might contain genes that are important in leptin's insulin sensitizing effects since at the 50ng/hr and 100ng/hr doses, leptin was able to improve hyperinsulinemia and hyperglycemia. HNF3 γ was one of the genes whose expression was induced at 50ng/hr and higher leptin doses. This is a transcription factor which is implicated in glucose metabolism (Shen et al. 2001) therefore, it might be important in leptin's insulin sensitizing effects. In two

other clusters, gene expression was either induced or repressed mainly at 200ng/hr leptin dose. This leptin dose was able to correct both insulin resistance and hepatic steatosis of lipodystrophic animals therefore, these genes might be important for both leptin's insulin sensitizing and antisteatotic effects (Shimomura et al. 1999) (Chapter 3) (Asilmaz et al. 2004). HNF6 is a transcription factor, which was induced mainly with 200ng/hr leptin dose. HNF6 might be important in glucose metabolism (Lannoy et al. 2002). Insulin-like binding protein 2 (IGFBP-2) is another gene whose expression was induced at 200ng/hr leptin dose. The role of this protein in leptin's effects remains unknown. In the cluster of genes repressed at 200ng/hr, there were genes that are already known to be important in obesity and glucose metabolism. These genes included cide-a, malic enzyme, glycerol-3-phosphate acyltransferase and glycerol-3-phosphate dehydrogenase. Cide-a knockout mice were shown to be resistant to diet induced obesity (Zhou et al. 2003; Li 2004). Malic enzyme, glycerol-3-phosphate acyltransferase and glycerol-3-phosphate dehydrogenase are important in glucose metabolism.

Real-time RT-PCR (Taqman) analysis of two key gluconeogenic genes, glucose-6-phosphatase and F1,6-bisPase showed that the mRNA levels for these genes were reduced at both 50ng/hr and 200ng/hr leptin dose, on the other hand, PEPCK levels were slightly elevated at 50ng/hr leptin dose but this increase was not significant. This might suggest that suboptimal leptin doses could improve insulin signaling and repress gluconeogenesis in liver and hence improve hyperglycemia and hyperinsulinemia with no significant improvement of hepatic steatosis. It is also known that a number of hormonal and nonhormonal factors regulate the transcription of these gluconeogenic enzymes which may also be regulated by nontranscriptional mechanisms. High insulin levels

downregulate glucose-6-phosphatase mRNA and PEPCK mRNA levels by PI-3K activation (Barthel and Schmoll 2003). High glucose on the other hand, is known to increase glucose-6-phosphatase mRNA and decrease PEPCK mRNA levels (Pilkis and Granner 1992). The observation that insulin action is improved at a dose of leptin as low as 50 ng/hr may simplify further efforts to elucidate the biochemical mechanism, since potentially confounding effects of this hormone on triglyceride levels and perhaps lipid metabolism would not be evident at this dose.

The effects of chronic leptin treatment reported here are somewhat different from the acute effects of leptin. In previous studies, leptin was shown to acutely stimulate glucose metabolism in wild type mice by an insulin-independent mechanism and to acutely increase glucose turnover in *ob/ob* mice (Kamohara et al. 1997; Burcelin et al. 1999). The basis for this difference is unknown but could relate to the differential effects of this hormone on liver before and after glycogen stores are depleted.

Chapter 6: Efferent signals mediating leptin's effects

Introduction

There is data suggesting that leptin has direct effects on peripheral tissues such as liver, muscle, heart and pancreatic β -cells (Kieffer et al. 1997; Cohen et al. 2001; Atkinson et al. 2002; Minokoshi et al. 2002). However, several different lines of evidence suggest that the main site of leptin action is the brain (Halaas et al. 1997; Cohen et al. 2001; Asilmaz et al. 2004). Leptin is an afferent signal between peripheral tissues and brain. Leptin is suggested to act through the CNS in order to exert its effects on food intake, energy expenditure and other peripheral metabolic effects such as its effects on glucose and lipid metabolism. The efferent signals mediating leptin's peripheral effects are not very well understood. Leptin may affect glucose and lipid metabolism through hormonal effects or via neural effects. Sympathetic nervous system is suggested to be one of the efferent pathways mediating leptin's peripheral effects. Leptin was shown to increase sympathetic outflow and this effect of leptin was independent of its effects on food intake. Sympathetic nerve activity to peripheral tissues such as BAT, kidney, hindlimb and adrenal medulla increased following intravenous leptin treatment (Haynes et al. 1997a). It has been shown that leptin can no longer promote glucose uptake into peripheral tissues in denervated animals or in animals treated with β -AR blockers (Haque et al. 1999). In denervated animals, leptin can no longer upregulate UCP-1 expression in BAT (Scarpace and Matheny 1998). A possible mechanism is suggested as follows: Leptin is secreted mainly from the WAT and it crosses the blood-brain barrier and acts on its receptors in the hypothalamus affecting neurotransmitters such as NPY, AGRP, MSH

and MCH. AGRP and MSH then act on melanocortin 4 (MC4) receptors in the hypothalamus hence stimulating sympathetic nerve activity on peripheral tissues (Lowell and Bachman 2003).

Sympathetic nervous system was shown to affect glucose and lipid metabolism and these effects are probably mediated by hormones such as glucagon and epinephrine as well as direct innervation of peripheral tissues such as liver, adipose tissue and skeletal muscle (Nonogaki 2000). Sympathetic nervous system was shown to increase hepatic glucose production as well as glucose uptake into skeletal muscle, WAT and BAT. Moreover, sympathetic nervous system was also suggested to have effects on lipid metabolism such as increasing lipolysis in WAT and stimulating thermogenesis in BAT (Nonogaki 2000). Sympathetic nervous system may act either through the α -adrenergic receptors or β -adrenergic receptors on peripheral tissues. β -adrenergic receptors are implicated in regulating peripheral metabolism.

There are three subtypes of β -adrenergic receptors (β -ARs), which are G-protein coupled receptors. These include β_1 AR, β_2 AR and β_3 AR. β_1 ARs and β_2 ARs are widely expressed throughout different tissues of the body whereas β_3 ARs are almost exclusively expressed on adipocytes (Collins and Surwit 2001). All of these receptors are located on both WAT and BAT, the relative importance of each of these three receptors is not well established (Robidoux et al. 2004). In order to understand the function of these receptors in metabolism, knockouts of individual isoforms have been performed, however, these knockout mice did not have a significant metabolic or obese phenotype probably due to compensation by the other isoforms (Susulic et al. 1995; Rohrer et al. 1996; Revelli et al. 1997; Chruscinski et al. 1999). β_3 -AR is mostly implicated in the regulation of energy

homeostasis, glucose and lipid metabolism as well as leptin signaling (Nonogaki 2000). When mice with a knockout of all three isoforms were generated (β -less mice), these animals were slightly heavier than their littermate controls on a regular chow diet however, they developed a more severe diet induced obesity on high fat diet compared with their littermate controls (Bachman et al. 2002).

Here, we investigated the role of β -adrenergic receptors in leptin mediated body mass reduction and in mediating leptin's metabolic effects. We first treated β -less mice and their littermate controls with subcutaneous leptin either for 2-3 days or for 12 days. In these studies, we showed that β -adrenergic receptors are necessary for leptin mediated fat mass reduction. Moreover, we studied leptin regulated gene expression in peripheral tissues such as WAT, BAT, liver and muscle and showed that leptin acts through β -adrenergic receptors in order to change the expression of certain genes in peripheral tissues. Our data suggested that sympathetic nervous system is one of the efferent pathways mediating leptin's peripheral effects.

Results

Attenuated leptin response in β -less mice

In order to study the role of sympathetic nervous system in leptin's peripheral actions, we treated β -less mice and their littermate controls with subcutaneous leptin, subcutaneous PBS and a separate group was treated with subcutaneous PBS and at the same time was food restricted "pair-fed" to the leptin group as an extra control to differentiate between leptin's effects on food intake from its effects that are independent

of food intake (Halaas et al. 1997). There was a significant reduction in body weight and food intake in both β -less mice and their littermate controls treated with leptin. A similar decrease in both weight and food intake was also observed in the pair-fed groups. The decrease in body weight and food intake was lower in β -less mice compared to their littermate controls (Figure 6.1a). After 12 days of leptin treatment, even though there was a comparable weight loss between the β -less mice and their littermate controls, littermate control mice had almost no WAT left, whereas, β -less mice still had significantly more WAT (Figure 6.1b). This observation was further confirmed by body composition analysis, which showed that there was no difference in percent fat mass in β -less mice treated with either leptin, PBS or pair-fed whereas there was a significant reduction in percent fat mass of littermate controls treated with leptin compared to PBS or pairfed controls (Figure 6.1c). Baseline leptin levels were slightly higher in β -less mice compared to their littermate controls and plasma leptin levels of wild type mice were higher than those of β -less mice following leptin treatment (data not shown).

Sympathetic nervous system was also previously implicated in glucose homeostasis. We measured plasma insulin and glucose levels of β -less mice and their littermate controls following leptin and PBS treatment. After three days of leptin treatment, there was no significant difference in plasma glucose between leptin and PBS treatments either in β -less mice or their littermate controls. Plasma insulin levels were lower in both groups following leptin treatment however these differences were not significant. On the other hand, both plasma insulin and glucose levels were significantly reduced in wild type mice treated with leptin for 12 days compared to PBS treated mice

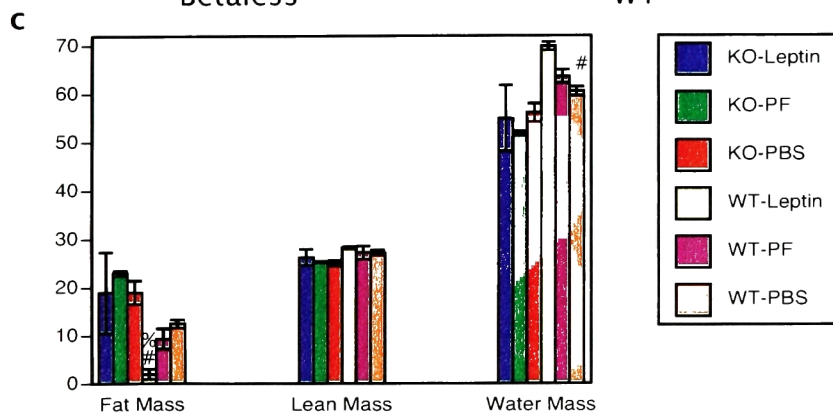
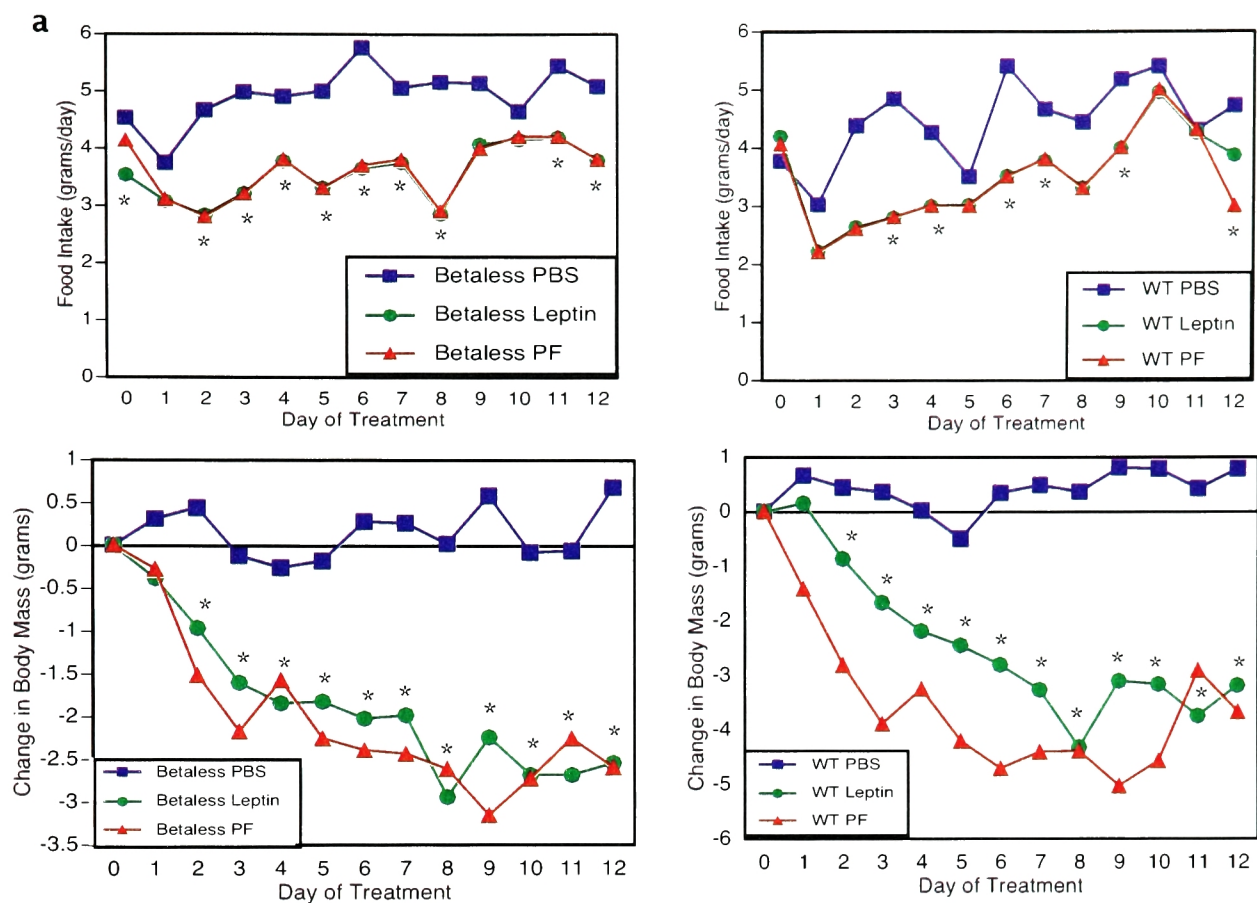


Figure 6.1 Attenuation in the leptin response in betaless mice. a. Weight curves and Food intake measurements of betaless and wild type (WT) mice treated with leptin, PBS or pairfed (PF) for 12 days. b. Gross anatomy of betaless and WT mice following 12 days of leptin treatment. c. Body composition analysis of betaless (KO) and WT mice following 12 days of leptin treatment. Error bars indicate the SE, n=5 for both betaless and wild type groups. *P=0.05, betaless leptin vs betaless pbs and WT leptin vs. WT PBS. #P<0.00001 WT leptin vs WT PBS. %P=0.05 betaless leptin vs. WT leptin.

whereas there was no significant difference in plasma insulin and glucose levels of β -less mice treated with either leptin or PBS (Figure 6.2a-b).

Gene expression analysis in leptin treated β -less mice

We then studied gene expression of a few genes in peripheral tissues, such as BAT and liver in β -less mice and their littermate controls in response to 2-3 days of leptin treatment. In liver, we studied the expression and activity of SCD-1, which is a leptin-regulated gene (Cohen et al. 2002). Both SCD-1 mRNA levels and SCD activity were higher in the livers of β -less mice compared to their littermate controls. There was a significant reduction in both SCD-1 mRNA levels and SCD activity following leptin treatment in both β -less mice and their littermate controls (Figure 6.3a-b). In BAT, we studied the expression of two genes, uncoupling protein 1 (UCP-1), which is part of the electron transport chain and is important for thermogenesis, and hormone sensitive lipase (HSL), which is involved in lipolysis. UCP-1 expression was significantly elevated in wild type mice treated with leptin compared to PBS treated controls whereas there was no increase in UCP-1 mRNA levels in β -less mice treated with leptin (Figure 6.3c). Similarly, HSL expression was also upregulated in wild type mice treated with leptin and there was no induction of HSL levels in leptin treated β -less mice (Figure 6.3d).

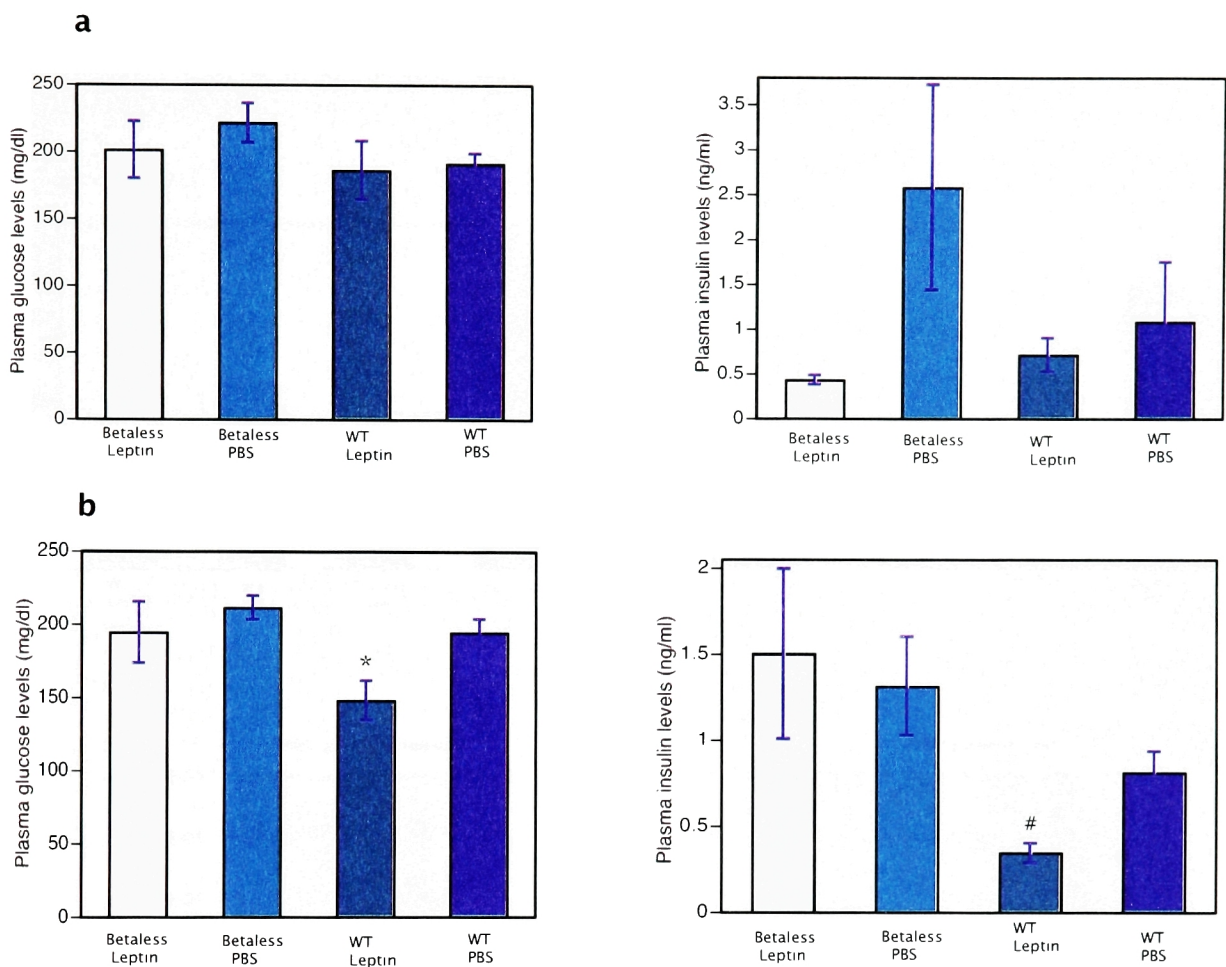


Figure 6.2 Plasma glucose and insulin levels in betaless mice and wild type mice treated with leptin or PBS for either 3 days or 12 days. a. Plasma glucose and insulin levels in betaless and WT mice treated with leptin or PBS for 3 days. b. Plasma glucose and insulin levels in betaless and WT mice treated with leptin or PBS for 12 days. Error bars indicate the SE; $n=5$ for each group. * $P<0.05$, WT leptin vs. WT PBS, # $P<0.01$, WT leptin vs. WT PBS.

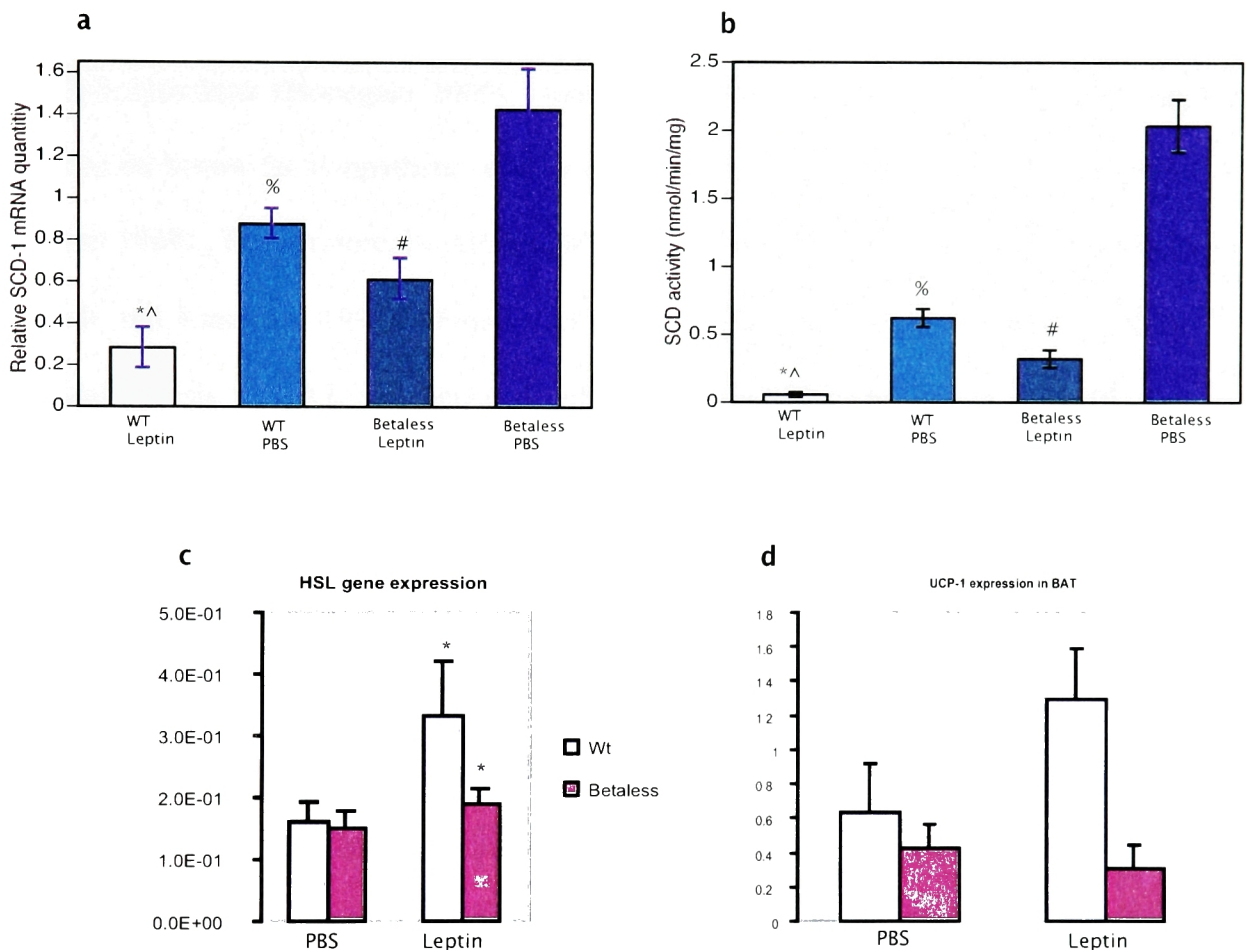


Figure 6.3 Gene expression analysis in the liver of betaless and WT mice treated with leptin for 3 days and in the BAT of betaless and WT mice treated with leptin for 2 days. a. Taqman real-time PCR of RNA samples using primers and probe specific for SCD-1. b. Enzymatic activity measured in liver extracts. c. Taqman real-time PCR of RNA samples using primers and probe specific for HSL. d. Taqman real-time PCR of RNA samples using primers and probe specific for UCP-1. Error bars indicate the SE, n=5 for each group. *P<0.05, WT leptin vs WT PBS. ^P<0.05, WT leptin vs. betaless leptin. %P<0.05, WT PBS vs betaless PBS. #P<0.005, betaless leptin vs. betaless PBS.

Discussion

Sympathetic nervous system has been implicated in the regulation of energy homeostasis as well as in the regulation of other metabolic pathways such as glucose and lipid homeostasis (Nonogaki 2000). Genetically obese animals were shown to have decreased brown fat sympathetic activity as well as decreased thermogenesis (Himms-Hagen 1989). Furthermore, β_3 -AR agonists were shown to increase energy expenditure (Arch and Kaumann 1993). Finally, mice lacking all three β -ARs failed to induce thermogenesis in BAT and hence developed diet-induced obesity on high fat diet (Bachman et al. 2002). Here, we investigated the role of sympathetic nervous system, specifically the role of β -ARs, in mediating leptin's peripheral effects. Our data suggests that sympathetic nervous system is necessary for leptin's effects on weight reduction and possibly on glucose metabolism. This conclusion is consistent with the data showing that leptin treatment increases sympathetic nerve activity to BAT, kidney, hind limb and adrenal gland in wild type animals. In addition, in leptin-deficient *ob/ob* mice, leptin administration increased energy expenditure partly through sympathetic nerve activity in BAT (Collins et al. 1996). Other studies also suggested that defective leptin signaling decreased sympathetic nerve activity while increasing parasympathetic nerve activity (Haynes et al. 1997b).

Our data suggests that leptin reduces the expression of genes such as UCP-1 and HSL in BAT through β -adrenergic signaling thus regulating lipolysis and thermogenesis in this tissue. Previously, leptin was suggested to decrease food intake and at the same time increase energy expenditure and regulate glucose and lipid metabolisms through

autonomic nervous system (Friedman 2002b). β -ARs are thought to be important mediators of leptin's effects since, leptin may no longer induce glucose uptake into BAT either in sympathetic nerve denervated animals or in animals treated with β -AR blockers (Haque et al. 1999). Furthermore, leptin induced UCP-1 expression in BAT was also blunted in denervated animals suggesting that leptin mediated activation of the sympathetic nervous system induces UCP-1 activity in BAT hence increasing thermogenesis in this tissue (Scarpace and Matheny 1998).

Our data also suggests that leptin might be influencing glucose metabolism through β -adrenergic stimulation. In wild type mice, leptin treatment for 12 days reduced both plasma insulin and glucose levels whereas it failed to do so in β -less mice. In general, obese humans and rodents have high plasma leptin levels, however, they are "resistant" to leptin's actions. In these individuals, decreased sympathetic outflow is suggested to impair glucose tolerance and lead to obesity. For example, in Pima Indians, Finns and in patients with morbid obesity, a missense mutation in β_3 -AR was associated with abdominal obesity and Type II diabetes (Clement et al. 1995; Walston et al. 1995; Widen et al. 1995). This suggests that impairment in leptin and/or in sympathetic nervous system signaling may predispose individuals to obesity and diabetes. Therefore, it is important to fully understand the efferent signals mediating leptin's peripheral actions in order to be able to develop better therapeutics for obesity and type II diabetes.

Chapter 7: Conclusion

Summary of findings

The studies in this thesis aim to understand the molecular mechanisms by which, leptin corrects insulin resistance and hepatic steatosis. We specifically studied leptin's effects mainly on liver as well as on muscle in lipodystrophic aP2-nSREBP-1c transgenic mice. These animals have metabolic abnormalities such as insulin resistance and hepatic steatosis associated with lipodystrophy (Shimomura et al. 1998). Peripheral leptin administration was shown to correct both of these metabolic abnormalities and leptin's effects are independent of its effects on food intake (Shimomura et al. 1999). Furthermore, leptin also corrects insulin resistance associated with lipodystrophy in humans (Oral et al. 2002; Petersen et al. 2002). However, neither the site nor the mechanism of leptin action in lipodystrophy is currently fully understood. In the absence of adipose tissue and/or adipokines such as leptin, triglycerides and fatty acids are accumulate in peripheral tissues such as liver, muscle, heart and pancreatic β -cells. Accumulation of triglycerides and fat in non-adipose tissues is suggested to lead to "lipotoxicity" hence interfering with the normal function of these tissues (Friedman 2002a; Unger 2003). Fat accumulation in insulin sensitive peripheral tissues such as liver and muscle is also suggested to interfere with insulin signaling hence leading to insulin resistance (Shulman 2000). aP2-nSREBP-1c mice were shown to have impaired insulin signaling and an induction of gluconeogenic gene expression in liver (Shimomura et al. 2000). Leptin's improvement of insulin action was also shown in other settings however, the basis for these effects are not fully understood (Kamohara et al. 1997;

Chinookoswong et al. 1999; Ogawa et al. 1999). The primary goal of this thesis was to understand the molecular basis for leptin-mediated correction of insulin resistance and hepatic steatosis.

Initially, we were interested in understanding the site of leptin action in lipodystrophy. We wanted to know if leptin corrects hepatic steatosis and insulin resistance associated with lipodystrophy indirectly via the CNS or through direct effects on peripheral tissues (Chapter 3). Although a large number of reports suggested that the main site of leptin action is the brain and leptin exerts its metabolic effects indirectly via the CNS, there are other reports suggesting that leptin acts directly on peripheral tissues to exert its metabolic effects (Campfield et al. 1995; Halaas et al. 1997; Kieffer et al. 1997; Cohen et al. 2001; Atkinson et al. 2002; Minokoshi et al. 2002). In order to determine the site of leptin action in lipodystrophy, we first treated aP2-nSREBP-1c mice with a low dose of ICV leptin. In this study, we showed that this dose of central leptin treatment was sufficient to correct the metabolic abnormalities associated with lipodystrophy as well as a much higher peripheral dose, suggesting that in lipodystrophy, leptin acts indirectly via the CNS (Chapter 3)(Asilmaz et al. 2004). In order to further determine whether leptin changes peripheral gene expression indirectly via the CNS or by direct effects on peripheral tissues, we compared hepatic and muscular gene expression from aP2-nSREBP-1c mice treated with ICV leptin and with subcutaneous leptin. This analysis revealed that there was a very strong correlation in the hepatic gene expression between ICV leptin and subcutaneous leptin treatments suggesting that leptin changes hepatic gene expression indirectly via the CNS. However, there was a low correlation in muscle gene expression between ICV leptin and subcutaneous leptin treatments, which

suggests that leptin might act directly on muscle to change gene expression in this tissue. However, our data also suggests that any direct peripheral leptin effects are not essential since ICV leptin treatment was able to completely correct the metabolic abnormalities in lipodystrophy.

Our previous studies suggested that leptin acts directly on the brain to exert its metabolic effects via the CNS; however, the molecular mechanisms for leptin's actions in lipodystrophy were still not known. One of the leptin-regulated genes in the livers of aP2-nSREBP-1c mice following ICV and subcutaneous leptin treatments was SCD-1. Recently, SCD-1 was shown to be an important mediator of leptin's metabolic effects. Deficiency of this enzyme was shown to improve both obesity and hepatic steatosis of *ob/ob* mice (Cohen et al. 2002). Furthermore, SCD-1 knockout mice were leaner than their littermate controls and they were shown to be resistant to diet-induced obesity (Ntambi et al. 2002). In order to inquire the role of SCD-1 in leptin-mediated correction of hepatic steatosis and insulin resistance in lipodystrophy, we studied this enzyme in the livers of aP2-nSREBP-1c mice (Chapter 4). We first showed that similar to *ob/ob* mice, in the livers of aP2-nSREBP-1c transgenic mice, both SCD-1 mRNA levels and activity were upregulated compared to their littermate controls and reduced to wild type levels following both ICV and subcutaneous leptin treatments. This suggests that leptin acts via the CNS in order to reduce SCD-1 gene expression in liver. In order to determine the contribution of SCD-1 upregulation to diabetes and hepatic steatosis associated with lipodystrophy, we generated aP2-nSREBP-1c mice lacking SCD-1, *ab^J/ab^J*;aP2-nSREBP-1c mice. Even though these animals were still lipodystrophic, they had improved hepatic steatosis. We further investigated the mechanism by which SCD-1

deficiency improves hepatic steatosis and showed that in the absence of SCD-1, in the livers of *ab^J/ab^J*;aP2-nSREBP-1c mice, AMPK activity is increased which in turn inactivates ACC, reducing malonyl CoA levels thus increasing CPT-1 activity and mitochondrial β -oxidation. Despite improved hepatic steatosis, *ab^J/ab^J*;aP2-nSREBP-1c mice had increased plasma glucose levels, however, plasma insulin levels were lower in these animals. Gene expression analysis in the livers of these animals suggested that gluconeogenesis was higher in these animals compared to aP2-nSREBP-1c mice, which might explain the aggravated diabetes.

SCD-1 was previously implicated in improving insulin sensitivity by increasing insulin signaling in muscles of non-diabetic animals (Ntambi et al. 2002; Rahman et al. 2003). Fat accumulation was suggested to impair insulin signaling in insulin sensitive peripheral tissues hence causing insulin resistance (Shulman 2000; Unger 2003). Despite the improved hepatic steatosis in *ab^J/ab^J*;aP2-nSREBP-1c mice, these animals remained severely diabetic. These data suggested that leptin corrected insulin resistance in lipodystrophy independent of its effects on SCD-1 and possibly independent of its antisteatotic effects. In order to determine the mechanism of leptin-mediated correction of insulin resistance in lipodystrophic animals, we performed a peripheral dose response study in aP2-nSREBP-1c mice and studied insulin sensitivity and hepatic gene expression (Chapter 5). In this study, we identified two sub-optimal subcutaneous leptin doses, which improved hyperinsulinemia and hyperglycemia but did not correct hepatic steatosis. This observation further suggested that leptin might improve hyperinsulinemia and hyperglycemia independent of its antisteatotic effects. We further studied the mechanism of leptin mediated insulin sensitivity using one of these sub-optimal leptin

doses. We studied insulin signaling in liver and muscle of aP2-nSREBP-1c mice following leptin treatment and showed that leptin improved hepatic insulin signaling but did not have a significant effect on muscle insulin signaling. To identify genes that were important for leptin's insulin sensitizing effects, we then performed cluster analysis of hepatic gene expression in aP2-nSREBP-1c mice treated with several peripheral leptin doses. In addition to other genes, we also identified two leptin regulated hepatocyte nuclear factors in this cluster analysis whose roles in leptin mediated correction of insulin resistance and hepatic steatosis are currently under study.

Our results from Chapter 3 and several other reports suggest that leptin acts directly on the brain and it exerts its peripheral metabolic effects indirectly via the CNS (Halaas et al. 1997; Cohen et al. 2001; Asilmaz et al. 2004). The efferent pathways mediating leptin's peripheral effects however, are poorly understood. We examined the sympathetic nervous system, specifically β -AR activation as part of the efferent pathways mediating leptin's effects (Chapter 6). We first studied the response of β -less mice to leptin and showed that these animals had an attenuated response to leptin. We then studied leptin regulated gene expression in peripheral tissues of β -less mice and showed that leptin can no longer upregulate lipogenic genes such as HSL and thermogenic genes such as UCP-1 in BAT of these animals. These studies further suggested that sympathetic nervous system and β -AR activation is partly responsible for leptin's peripheral effects.

In summary, in these studies, we have shown that leptin mediates its insulin sensitizing and antisteatotic effects indirectly via the CNS. SCD-1 is an important mediator of leptin's antisteatotic effects. Our data suggests that SCD-1 downregulation

improves hepatic steatosis by activating AMPK pathway, which activates fatty acid β -oxidation although other mechanisms are also possible. The mechanism by which AMPK activation inhibits SCD activity is currently unknown. SCD-1 inhibition does not correct diabetes in the lipodystrophic aP2-nSREBP-1c. Therefore, it is likely that other genes are involved in leptin mediated correction of insulin resistance (Figure 7.1).

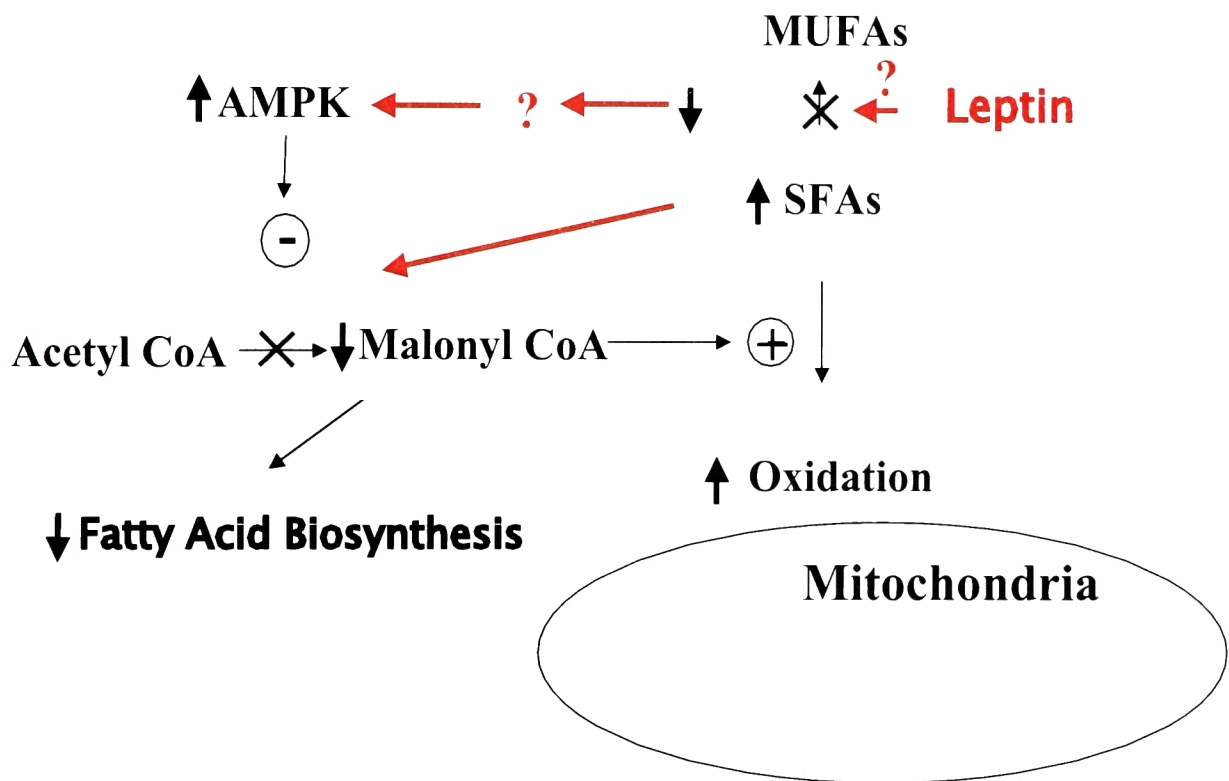


Figure 7.1 A proposed mechanism for leptin mediated correction of hepatic steatosis in lipodystrophic aP2-nSREBP-1c transgenic mice. SCD-1 deficiency results in increased AMPK phosphorylation and activity, ACC phosphorylation and inactivation, decreased malonyl-CoA levels and hence de-inhibition of CPT-1 and an increase in fatty acyl CoA transport and β -oxidation in mitochondria (Taken from Dobryzn et al. 2004)(Straight arrows do not necessarily suggest direct effects).

Directions for future research

The findings in this thesis generated many questions regarding leptin's effects in insulin sensitivity and hepatic steatosis, which remain to be answered. In regard to the site of leptin action in lipodystrophy, we have shown that brain is the main site of leptin action. Leptin mediates its peripheral actions indirectly via the CNS. Gene expression analysis of ICV leptin and peripheral leptin treated aP2-nSREBP-1c transgenic mice also revealed that hepatic gene expression is mediated via the CNS. On the other hand, gene expression analysis in muscle suggested that leptin might have direct effects in muscle. In order to test this possibility, we are currently generating muscle-specific knockout of leptin receptor using the Cre-lox technology (Cohen et al. 2001). Recently, leptin was suggested to directly activate AMPK in muscle (Minokoshi et al. 2002). Muscle-specific leptin receptor knockout mice will enable us to determine any direct leptin effects on this tissue.

The efferent pathways mediating leptin's peripheral effects are currently unknown. As an attempt to further define the efferent pathways mediating leptin's effects, we studied β -less mice (Bachman et al. 2002). These studies suggested that leptin's effects are in part mediated by sympathetic nervous system and β -AR activation. These studies could further be extended to generating aP2-nSREBP-1c lipodystrophic mice with no β -ARs by crossing β -less mice to aP2-nSREBP-1c transgenic mice and studying the response of leptin in these animals. Gene expression analysis in peripheral tissues in these settings would also reveal leptin-regulated genes whose expression is regulated via the sympathetic nerve activation. Furthermore, parabiosis studies using

aP2-nSREBP-1c animals and β -less animals may be used to differentiate between the relative importance of humoral factors versus the autonomic nervous system. Parabiosis studies between two aP2-nSREBP-1c transgenic mice where one is treated with ICV leptin might determine if a humoral efferent signal exists that controls leptin's insulin sensitizing and antisteatotic effects in these animals. Parabiosis between a β -less mouse that is treated with ICV leptin and its littermate control might also be important in determining the existence of a humoral factor or the role of the sympathetic nervous system. Gene expression analysis in liver and other tissues would be useful in order to determine the efficacy of leptin action in these settings. Correlation analysis and cluster analysis used in this thesis may also be used in order to compare the relative efficacy of leptin treatment in each setting and to identify gene clusters that might be regulated via a specific efferent pathway. Since it would be very time consuming to breed aP2-nSREBP-1c mice to β -less mice, lipodystrophy might be induced in the latter animals using a CLA diet, essentially generating lipodystrophic β -less mice which should exhibit the metabolic abnormalities, insulin resistance and hepatic steatosis, associated with lipodystrophy (Tsuboyama-Kasaoka et al. 2000). Leptin treatment of these animals would then reveal the significance of β -AR activation in leptin's effects.

SCD-1 is an important mediator of leptin's weight reducing and antisteatotic effects (Cohen et al. 2002). Our data showed that leptin acts via the CNS to inhibit SCD-1 activity and hence in part mediates its antisteatotic effects by inhibiting this enzyme. Currently, the exact mechanism by which leptin inhibits SCD-1 is not known. However, we have shown that leptin treatment of β -less mice also have decreased SCD activity in liver suggesting that the efferent pathways regulating this enzyme are independent of β -

AR activation. Previously suggested parabiosis experiments might provide evidence for a humoral signal for SCD-1 regulation. In these experiments, leptin-regulated genes such as SCD-1 and others might be used as markers for leptin action.

Our data suggested that SCD-1 is one of the mediators of leptin's antisteatotic effects in lipodystrophy. Our data further suggested that SCD-1 deficiency acts by increasing AMPK phosphorylation, which has an end result of increasing fatty acid oxidation in liver. This is also consistent with the data from SCD-1 knockout mice and *ob/ob* mice lacking SCD-1, *ab^J/ab^J;ob/ob* mice (Dobrzyn et al. 2004). It has previously been shown that AMPK phosphorylation increases its enzymatic activity however, we have not yet measured AMPK activity in the livers of *ab^J/ab^J;aP2-nSREBP-1c* mice therefore, AMPK activity needs to be measured in the livers of these animals. Moreover, fatty acid β -oxidation rate also needs to be determined in the livers of these mice. We would like to measure the palmitic acid incorporation into lipids in order to show that this is downregulated in the livers of *ab^J/ab^J;aP2-nSREBP-1c* mice and palmitoyl-CoA oxidation in mitochondria to show that this is upregulated in the livers of these mice as our data predicts. The mechanism by which SCD-1 deficiency leads to increased AMPK phosphorylation and activation in liver is currently not known. Leptin was shown to directly and indirectly increase AMPK phosphorylation and fatty acid oxidation in muscle (Minokoshi et al. 2002). However in heart, leptin was shown to increase fatty acid oxidation independent of AMPK (Atkinson et al. 2002). The fact that AMPK phosphorylation is increased in the absence of leptin in *ab^J/ab^J;aP2-nSREBP-1c* mice suggests that AMPK activation is independent of leptin. Moreover, SCD-1 deficiency's effects on steatosis in other tissues such as heart and skeletal muscle of *ab^J/ab^J;aP2-*

nSREBP-1c mice is still unknown and these are currently under investigation. Based on our previous data, we expect to observe increased fatty acid β -oxidation and reduced steatosis in these tissues in the absence of SCD-1.

Despite the improved hepatic steatosis in ab^J/ab^J ;aP2-nSREBP-1c mice, these animals were still severely diabetic, they had higher plasma glucose levels compared to aP2-nSREBP-1c mice. Initial gene expression analysis in liver suggested that these animals might have increased gluconeogenesis, which would explain the aggravated diabetes. In order to prove this, it is necessary to study these animals under euglycemic-hyperinsulinemic clamp conditions. These clamp studies would determine if there is increased hepatic glucose output in these animals. Fat accumulation in peripheral tissues is suggested to impair insulin signaling and hence lead to insulin resistance (Shulman 2000). If this hypothesis is correct, since SCD-1 deficiency improves steatosis, this implicates that it would also improve insulin sensitivity. SCD-1 deficiency was shown to improve insulin sensitivity and increase insulin signaling in the muscles of nondiabetic animals (Ntambi et al. 2002; Rahman et al. 2003). It is therefore necessary to study insulin signaling in the muscles of ab^J/ab^J ;aP2-nSREBP-1c mice in order to determine if insulin signaling in muscle is also improved in these diabetic animals. It is possible that insulin signaling and sensitivity is improved in the muscles of ab^J/ab^J ;aP2-nSREBP-1c mice however, due to impaired insulin sensitivity and increased gluconeogenesis in liver, these animals are still diabetic. The euglycemic-hyperinsulinemic clamp studies in these animals, will also determine skeletal muscle insulin sensitivity by measuring glucose uptake into this tissue. In the livers of ab^J/ab^J ;aP2-nSREBP-1c mice, we have shown that fatty acid content is lower and saturated fatty acids are increased compared to

monounsaturated fatty acids, however, we have not performed detailed analysis of fatty acyl CoAs or other lipid moieties either in livers or muscles of these animals in order to determine if any of these were elevated compared to wild type levels which could possibly lead to insulin resistance.

Even though plasma glucose levels were severely elevated in *ab^J/ab^J;aP2-nSREBP-1c* mice, plasma insulin levels were significantly lower than their aP2-nSREBP-1c littermate controls. Since these animals are severely diabetic, the decreased insulin levels suggest that there is possibly β -cell failure in these animals. The role of SCD-1 in pancreatic β -cells is currently not known. It has been previously suggested that accumulation of saturated fatty acids in a cell can lead to apoptosis (Listenberger et al. 2003). In order to prove this, we will study the pancreas of *ab^J/ab^J;aP2-nSREBP-1c* mice with immunofluorescence for insulin and glucagon and compare the number of β -cells in these animals to their littermate controls. It is however possible that there is no β -cell failure in these animals but the decreased plasma insulin levels are suggestive of improved insulin resistance. This possibility will also be answered by the studies suggested above.

Humans have a single SCD gene, which has highest homology to the murine SCD-1 gene (Ntambi and Miyazaki 2003). It is necessary to study the role of SCD downregulation by leptin in lipodystrophic patients. SCD mRNA levels and activity needs to be measured in lipodystrophic patients before and after leptin treatment. Furthermore, global gene expression profiling using microarray analysis of liver and skeletal muscle biopsies of lipodystrophic patients before and after leptin treatment would reveal all of the leptin regulated genes in humans which are essential for leptin's

insulin sensitizing and antisteatotic effects in these patients. The comparison of these human leptin-regulated genes to murine leptin-regulated genes would be of great interest in terms of translating this research to humans.

Our data from the peripheral leptin dose response study suggested that in aP2-nSREBP-1c mice, leptin could improve liver insulin signaling at a dose, which did not correct hepatic steatosis. Liver gene expression analysis further suggested that this leptin dose decreased gluconeogenic gene expression. In order to be able to understand the insulin sensitizing effects of this dose of leptin, it is necessary to study aP2-nSREBP-1c animals treated with this leptin dose under euglycemic-hyperinsulinemic clamp conditions. This will provide us a better understanding of leptin's effects on muscle glucose uptake versus its effects on hepatic glucose output. Furthermore, cluster analysis of gene expression in the livers of aP2-nSREBP-1c transgenic mice treated with several different doses of peripheral leptin, which have different physiological effects, would be useful in identifying genes in different pathways, which mediate leptin's metabolic effects. Some of the genes in these clusters are already known to be important in obesity and diabetes however, there are several other genes whose functions are either unknown or their role in obesity and diabetes have not been studied. For example, one of the leptin-regulated genes in these clusters was a serine/threonine kinase, sak-b. This gene was upregulated in the livers of aP2-nSREBP-1c mice and it was downregulated by leptin treatment. The function of this gene in leptin's metabolic effects is currently under investigation. Fat accumulation in insulin sensitive peripheral tissues such as liver and muscle were shown to impair insulin signaling by activating serine/threonine kinases that phosphorylate IRS molecules on serine/threonine residues hence impairing insulin

signaling and leading to insulin resistance in these tissues (Shulman 2000). It is possible that leptin inactivates these serine/threonine kinases hence improving insulin signaling in these tissues independent of its antisteatotic effects. It is also possible that leptin might activate an inhibitor of serine/threonine kinases or activate a phosphatase, which would explain its insulin sensitizing effects despite intracellular fat accumulation. Two other interesting genes in two separate clusters were HNF3 γ and HNF6. Both of these genes were upregulated by leptin in the livers of aP2-nSREBP-1c mice. The function of these genes is currently under investigation by crossing HNF3 γ and HNF6 knockout mice to aP2-nSREBP-1c transgenic mice and studying the response of these transgenic-knockout mice to leptin.

A possible mechanism for the antidiabetic effects of leptin has been provided with the identification of the Foxa2 transcription factor as a component of the signal transduction pathway activated by insulin. In recent studies, Foxa2 was shown to be an important regulator of adipocyte differentiation and metabolism (Wolfrum et al. 2003b). Furthermore, it was shown that insulin phosphorylates Foxa2, which leads to its exclusion out of the nucleus where it can no longer activate its target genes (Wolfrum et al. 2003a). Studies of Foxa2 localization showed that Foxa2 is localized in the cytoplasm in lipodystrophic aP2-nSREBP-1c transgenic mice (C. Wolfrum and M. Stoffel unpublished results). Furthermore, a constitutively active Foxa2 was shown to improve both hepatic steatosis and insulin sensitivity in aP2-nSREBP-1c transgenic mice (C. Wolfrum and M. Stoffel unpublished results). It will therefore be relevant to determine Foxa2 localization in *ab^f/ab^f*;aP2-SREBP-1c and treat these mice with the constitutively active Foxa2 adenovirus to determine if constitutively active Foxa2 would also improve

the diabetic phenotype of *ab^J/ab^J*;aP2-SREBP-1c mice. Furthermore, it would be of interest to compare gene expression profiles between aP2-nSREBP-1c mice treated with leptin, treated with constitutively active Foxa2 and aP2-nSREBP-1c mice with SCD-1 deficiency in order to identify genes specifically important for insulin resistance, hepatic steatosis or both.

Conclusion

Type II diabetes mellitus which is often associated with obesity has become a worldwide epidemic in the past few decades. Type II diabetes is a complex disease where several genes interact with each other and with environmental factors to mediate the pathogenesis of the disease. Leptin was shown to ameliorate Type II diabetes associated with rare leptin deficiency syndromes of obesity and lipodystrophy in humans. However, the majority of individuals with obesity-associated Type II diabetes, is not leptin deficient, but have high plasma leptin levels due to increased adiposity. The molecular basis for leptin's metabolic effects is not well understood. Defining the molecular basis for leptin's antidiabetic effects in rare forms of leptin deficiency syndromes associated with Type II diabetes would be helpful for identifying genes that are important in glucose and lipid metabolism. These genes might then be used as drug targets in order to treat common forms of Type II diabetes.

References

- Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, Minnemann T, Shulman GI, Kahn BB (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature* 409:729-33
- Abu-Elheiga L, Matzuk MM, Abo-Hashema KA, Wakil SJ (2001) Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science* 291:2613-6
- Abu-Elheiga L, Oh W, Kordari P, Wakil SJ (2003) Acetyl-CoA carboxylase 2 mutant mice are protected against obesity and diabetes induced by high-fat/high-carbohydrate diets. *Proc Natl Acad Sci U S A* 100:10207-12
- Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI, Westphal H (1996) Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nat Genet* 12:106-9
- Air EL, Benoit SC, Blake Smith KA, Clegg DJ, Woods SC (2002) Acute third ventricular administration of insulin decreases food intake in two paradigms. *Pharmacol Biochem Behav* 72:423-9
- Araki E, Lipes MA, Patti ME, Bruning JC, Haag B, 3rd, Johnson RS, Kahn CR (1994) Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* 372:186-90
- Arch JR, Kaumann AJ (1993) Beta 3 and atypical beta-adrenoceptors. *Med Res Rev* 13:663-729
- Asilmaz E, Cohen P, Miyazaki M, Dobrzyn P, Ueki K, Fayzikhodjaeva G, Soukas AA, Kahn CR, Ntambi JM, Socci ND, Friedman JM (2004) Site and mechanism of leptin action in a rodent form of congenital lipodystrophy. *J Clin Invest* 113:414-24
- Atkinson LL, Fischer MA, Lopaschuk GD (2002) Leptin activates cardiac fatty acid oxidation independent of changes in the AMP-activated protein kinase-acetyl-CoA carboxylase-malonyl-CoA axis. *J Biol Chem* 277:29424-30
- Bachman ES, Dhillon H, Zhang CY, Cinti S, Bianco AC, Kobilka BK, Lowell BB (2002) betaAR signaling required for diet-induced thermogenesis and obesity resistance. *Science* 297:843-5
- Baile CA, Della-Fera MA, Martin RJ (2000) Regulation of metabolism and body fat mass by leptin. *Annu Rev Nutr* 20:105-27
- Barthel A, Schmoll D (2003) Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol Endocrinol Metab* 285:E685-92
- Barzilai N, She L, Liu L, Wang J, Hu M, Vuguin P, Rossetti L (1999) Decreased visceral adiposity accounts for leptin effect on hepatic but not peripheral insulin action. *Am J Physiol* 277:E291-8
- Barzilai N, Wang J, Massilon D, Vuguin P, Hawkins M, Rossetti L (1997) Leptin selectively decreases visceral adiposity and enhances insulin action. *J Clin Invest* 100:3105-10
- Benoit SC, Clegg DJ, Seeley RJ, Woods SC (2004) Insulin and leptin as adiposity signals. *Recent Prog Horm Res* 59:267-85

- Berg AH, Combs TP, Du X, Brownlee M, Scherer PE (2001) The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med* 7:947-53
- Bjorbaek C, Elmquist JK, Michl P, Ahima RS, van Bueren A, McCall AL, Flier JS (1998) Expression of leptin receptor isoforms in rat brain microvessels. *Endocrinology* 139:3485-91
- Bjorbaek C, Kahn BB (2004) Leptin signaling in the central nervous system and the periphery. *Recent Prog Horm Res* 59:305-31
- Bligh EG, and Dyer, W.J (1959) A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Physiol* 37:911-917
- Bloomgarden ZT (2004) Type 2 diabetes in the young: the evolving epidemic. *Diabetes Care* 27:998-1010
- Bluher M, Michael MD, Peroni OD, Ueki K, Carter N, Kahn BB, Kahn CR (2002) Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. *Dev Cell* 3:25-38
- Boden G, Chen X, Rosner J, Barton M (1995) Effects of a 48-h fat infusion on insulin secretion and glucose utilization. *Diabetes* 44:1239-42
- Braiman L, Alt A, Kuroki T, Ohba M, Bak A, Tennenbaum T, Sampson SR (1999) Protein kinase Cdelta mediates insulin-induced glucose transport in primary cultures of rat skeletal muscle. *Mol Endocrinol* 13:2002-12
- Brechtel K, Dahl DB, Machann J, Bachmann OP, Wenzel I, Maier T, Claussen CD, Haring HU, Jacob S, Schick F (2001) Fast elevation of the intramyocellular lipid content in the presence of circulating free fatty acids and hyperinsulinemia: a dynamic ¹H-MRS study. *Magn Reson Med* 45:179-83
- Bremer J (1981) The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA. *Biochim Biophys Acta* 665:628-31
- Browning JD, Horton JD (2004) Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest* 114:147-52
- Brozinick JT, Jr., McCoid SC, Reynolds TH, Nardone NA, Hargrove DM, Stevenson RW, Cushman SW, Gibbs EM (2001) GLUT4 overexpression in db/db mice dose-dependently ameliorates diabetes but is not a lifelong cure. *Diabetes* 50:593-600
- Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Muller-Wieland D, Kahn CR (2000) Role of brain insulin receptor in control of body weight and reproduction. *Science* 289:2122-5
- Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ, Kahn CR (1998) A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell* 2:559-69
- Bruning JC, Winnay J, Bonner-Weir S, Taylor SI, Accili D, Kahn CR (1997) Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. *Cell* 88:561-72
- Burcelin R, Kamohara S, Li J, Tannenbaum GS, Charron MJ, Friedman JM (1999) Acute intravenous leptin infusion increases glucose turnover but not skeletal muscle glucose uptake in ob/ob mice. *Diabetes* 48:1264-9

- Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P (1995) Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 269:546-9
- Chang CJ, Wu JS, Lu FH, Lee HL, Yang YC, Wen MJ (1998) Fasting plasma glucose in screening for diabetes in the Taiwanese population. *Diabetes Care* 21:1856-60
- Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR (1994) Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol* 14:4902-11
- Chehab FF, Qiu J, Ogus S (2004) The use of animal models to dissect the biology of leptin. *Recent Prog Horm Res* 59:245-66
- Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, Morgenstern JP (1996) Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* 84:491-5
- Chinookoswong N, Wang JL, Shi ZQ (1999) Leptin restores euglycemia and normalizes glucose turnover in insulin-deficient diabetes in the rat. *Diabetes* 48:1487-92
- Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, 3rd, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ (2001a) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 292:1728-31
- Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ (2001b) Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* 276:38349-52
- Chruscinski AJ, Rohrer DK, Schauble E, Desai KH, Bernstein D, Kobilka BK (1999) Targeted disruption of the beta2 adrenergic receptor gene. *J Biol Chem* 274:16694-700
- Clark JM, Diehl AM (2002) Hepatic steatosis and type 2 diabetes mellitus. *Curr Diab Rep* 2:210-5
- Clement K, Vaisse C, Manning BS, Basdevant A, Guy-Grand B, Ruiz J, Silver KD, Shuldiner AR, Froguel P, Strosberg AD (1995) Genetic variation in the beta 3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *N Engl J Med* 333:352-4
- Cochran E, Young JR, Sebring N, DePaoli A, Oral EA, Gorden P (2004) Efficacy of recombinant methionyl human leptin therapy for the extreme insulin resistance of the Rabson-Mendenhall syndrome. *J Clin Endocrinol Metab* 89:1548-54
- Cohen B, Novick D, Rubinstein M (1996) Modulation of insulin activities by leptin. *Science* 274:1185-8
- Cohen P, Miyazaki M, Socci ND, Hagge-Greenberg A, Liedtke W, Soukas AA, Sharma R, Hudgins LC, Ntambi JM, Friedman JM (2002) Role for stearoyl-CoA desaturase-1 in leptin-mediated weight loss. *Science* 297:240-3
- Cohen P, Ntambi JM, Friedman JM (2003) Stearoyl-CoA desaturase-1 and the metabolic syndrome. *Curr Drug Targets Immune Endocr Metabol Disord* 3:271-80
- Cohen P, Zhao C, Cai X, Montez JM, Rohani SC, Feinstein P, Mombaerts P, Friedman JM (2001) Selective deletion of leptin receptor in neurons leads to obesity. *J Clin Invest* 108:1113-21

- Cohen SM, Werrmann JG, Tota MR (1998) ^{13}C NMR study of the effects of leptin treatment on kinetics of hepatic intermediary metabolism. *Proc Natl Acad Sci U S A* 95:7385-90
- Colditz GA, Willett WC, Stampfer MJ, Manson JE, Hennekens CH, Arky RA, Speizer FE (1990) Weight as a risk factor for clinical diabetes in women. *Am J Epidemiol* 132:501-13
- Coleman DL (1973) Effects of parabiosis of obese with diabetes and normal mice. *Diabetologia* 9:294-8
- Collins S, Kuhn CM, Petro AE, Swick AG, Chrnyk BA, Surwit RS (1996) Role of leptin in fat regulation. *Nature* 380:677
- Collins S, Surwit RS (2001) The beta-adrenergic receptors and the control of adipose tissue metabolism and thermogenesis. *Recent Prog Horm Res* 56:309-28
- Colombo C, Cutson JJ, Yamauchi T, Vinson C, Kadowaki T, Gavrilova O, Reitman ML (2002) Transplantation of adipose tissue lacking leptin is unable to reverse the metabolic abnormalities associated with lipoatrophy. *Diabetes* 51:2727-33
- Commings SP, Watson PM, Padgett MA, Dudley A, Argyropoulos G, Gettys TW (1999) Induction of uncoupling protein expression in brown and white adipose tissue by leptin. *Endocrinology* 140:292-300
- Cushman SW, Wardzala LJ (1980) Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. *J Biol Chem* 255:4758-62
- Devendra D, Liu E, Eisenbarth GS (2004) Type 1 diabetes: recent developments. *Bmj* 328:750-4
- Dobrzyn P, Dobrzyn A, Miyazaki M, Cohen P, Asilmaz E, Hardie DG, Friedman JM, Ntambi JM (2004) Stearoyl-CoA desaturase 1 deficiency increases fatty acid oxidation by activating AMP-activated protein kinase in liver. *Proc Natl Acad Sci U S A* 101:6409-14
- Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI (1999) Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 103:253-9
- Duda ROaH, P.E. (1973) Pattern classification and scene analysis. Wiley&Sons, New York
- Ebihara K, Ogawa Y, Masuzaki H, Shintani M, Miyanaga F, Aizawa-Abe M, Hayashi T, Hosoda K, Inoue G, Yoshimasa Y, Gavrilova O, Reitman ML, Nakao K (2001) Transgenic overexpression of leptin rescues insulin resistance and diabetes in a mouse model of lipoatrophic diabetes. *Diabetes* 50:1440-8
- Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP (1999) Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* 283:1544-8
- Elks ML (1990) Fat oxidation and diabetes of obesity: the Randle hypothesis revisited. *Med Hypotheses* 33:257-60
- Elmqvist JK, Bjorbaek C, Ahima RS, Flier JS, Saper CB (1998) Distributions of leptin receptor mRNA isoforms in the rat brain. *J Comp Neurol* 395:535-47

- Emilsson V, Liu YL, Cawthorne MA, Morton NM, Davenport M (1997) Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory action of leptin on insulin secretion. *Diabetes* 46:313-6
- Fantin VR, Wang Q, Lienhard GE, Keller SR (2000) Mice lacking insulin receptor substrate 4 exhibit mild defects in growth, reproduction, and glucose homeostasis. *Am J Physiol Endocrinol Metab* 278:E127-33
- Fei H, Okano HJ, Li C, Lee GH, Zhao C, Darnell R, Friedman JM (1997) Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. *Proc Natl Acad Sci U S A* 94:7001-5
- Florez JC, Hirschhorn J, Altshuler D (2003) The inherited basis of diabetes mellitus: implications for the genetic analysis of complex traits. *Annu Rev Genomics Hum Genet* 4:257-91
- Forouhi NG, Jenkinson G, Thomas EL, Mullick S, Mierisova S, Bhonsle U, McKeigue PM, Bell JD (1999) Relation of triglyceride stores in skeletal muscle cells to central obesity and insulin sensitivity in European and South Asian men. *Diabetologia* 42:932-5
- Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81:727-36
- Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS (1995) Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med* 1:1311-4
- Frevert EU, Kahn BB (1997) Differential effects of constitutively active phosphatidylinositol 3-kinase on glucose transport, glycogen synthase activity, and DNA synthesis in 3T3-L1 adipocytes. *Mol Cell Biol* 17:190-8
- Fried SK, Bunkin DA, Greenberg AS (1998) Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab* 83:847-50
- Friedman J (2002a) Fat in all the wrong places. *Nature* 415:268-9
- Friedman J (2002b) Leptin and the Neural Circuit Regulating body Weight and Metabolism. In: al. Ke (ed) *Brain Somatic Cross-Talk and the Central Control of Metabolism*. Springer-Verlag Berlin Heidelberg, pp 15-35
- Friedman JM (2000) Obesity in the new millennium. *Nature* 404:632-4
- Friedman JM, Halaas JL (1998) Leptin and the regulation of body weight in mammals. *Nature* 395:763-70
- Garg A (2004) Acquired and inherited lipodystrophies. *N Engl J Med* 350:1220-34
- Gavrilova O, Marcus-Samuels B, Graham D, Kim JK, Shulman GI, Castle AL, Vinson C, Eckhaus M, Reitman ML (2000) Surgical implantation of adipose tissue reverses diabetes in lipodystrophic mice. *J Clin Invest* 105:271-8
- George S, Rochford JJ, Wolfrum C, Gray SL, Schinner S, Wilson JC, Soos MA, Murgatroyd PR, Williams RM, Acerini CL, Dunger DB, Barford D, Umpleby AM, Wareham NJ, Davies HA, Schafer AJ, Stoffel M, O'Rahilly S, Barroso I (2004) A family with severe insulin resistance and diabetes due to a mutation in AKT2. *Science* 304:1325-8
- Goldstein BJ (2003) Insulin resistance: from benign to type 2 diabetes mellitus. *Rev Cardiovasc Med* 4 Suppl 6:S3-10

- Goldstein BJ, Ahmad F, Ding W, Li PM, Zhang WR (1998) Regulation of the insulin signalling pathway by cellular protein-tyrosine phosphatases. *Mol Cell Biochem* 182:91-9
- Gorden P, Gavrilova O (2003) The clinical uses of leptin. *Curr Opin Pharmacol* 3:655-9
- Griffin ME, Marcucci MJ, Cline GW, Bell K, Barucci N, Lee D, Goodyear LJ, Kraegen EW, White MF, Shulman GI (1999) Free fatty acid-induced insulin resistance is associated with activation of protein kinase C θ and alterations in the insulin signaling cascade. *Diabetes* 48:1270-4
- Haffner SM, Lehto S, Ronnema T, Pyorala K, Laakso M (1998) Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 339:229-34
- Halaas JL, Boozer C, Blair-West J, Fidathusein N, Denton DA, Friedman JM (1997) Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc Natl Acad Sci U S A* 94:8878-83
- Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 269:543-6
- Hales CN, Barker DJ (1992) Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* 35:595-601
- Hansen PA, Gulve EA, Marshall BA, Gao J, Pessin JE, Holloszy JO, Mueckler M (1995) Skeletal muscle glucose transport and metabolism are enhanced in transgenic mice overexpressing the Glut4 glucose transporter. *J Biol Chem* 270:1679-84
- Haque MS, Minokoshi Y, Hamai M, Iwai M, Horiuchi M, Shimazu T (1999) Role of the sympathetic nervous system and insulin in enhancing glucose uptake in peripheral tissues after intrahypothalamic injection of leptin in rats. *Diabetes* 48:1706-12
- Hardie DG, Carling D (1997) The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* 246:259-73
- Hartigan JA (1975) *Clustering Algorithms*. Wiley&Sons, New York
- Haruta T, Morris AJ, Rose DW, Nelson JG, Mueckler M, Olefsky JM (1995) Insulin-stimulated GLUT4 translocation is mediated by a divergent intracellular signaling pathway. *J Biol Chem* 270:27991-4
- Haynes WG, Morgan DA, Walsh SA, Mark AL, Sivitz WI (1997a) Receptor-mediated regional sympathetic nerve activation by leptin. *J Clin Invest* 100:270-8
- Haynes WG, Sivitz WI, Morgan DA, Walsh SA, Mark AL (1997b) Sympathetic and cardiorenal actions of leptin. *Hypertension* 30:619-23
- Himms-Hagen J (1989) Brown adipose tissue thermogenesis and obesity. *Prog Lipid Res* 28:67-115
- Hotamisligil GS, Shargill NS, Spiegelman BM (1993) Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 259:87-91
- Hu E, Liang P, Spiegelman BM (1996) AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 271:10697-703
- Jain AKaD, R.C. (1988) *Algorithms for Clustering Data*. Prentice Hall, Englewood Cliffs, NJ
- Kadowaki T (2000) Insights into insulin resistance and type 2 diabetes from knockout mouse models. *J Clin Invest* 106:459-65

- Kahn CR, Vicent D, Doria A (1996) Genetics of non-insulin-dependent (type-II) diabetes mellitus. *Annu Rev Med* 47:509-31
- Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ (1997) Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* 389:374-7
- Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, Lienhard GE (2002) A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J Biol Chem* 277:22115-8
- Katagiri H, Asano T, Inukai K, Ogihara T, Ishihara H, Shibasaki Y, Murata T, Terasaki J, Kikuchi M, Yazaki Y, Oka Y (1997) Roles of PI 3-kinase and Ras on insulin-stimulated glucose transport in 3T3-L1 adipocytes. *Am J Physiol* 272:E326-31
- Katz EB, Stenbit AE, Hatton K, DePinho R, Charron MJ (1995) Cardiac and adipose tissue abnormalities but not diabetes in mice deficient in GLUT4. *Nature* 377:151-5
- Keller SR, Aebersold R, Garner CW, Lienhard GE (1993) The insulin-elicited 160 kDa phosphotyrosine protein in mouse adipocytes is an insulin receptor substrate 1: identification by cloning. *Biochim Biophys Acta* 1172:323-6
- Keller SR, Kitagawa K, Aebersold R, Lienhard GE, Garner CW (1991) Isolation and characterization of the 160,000-Da phosphotyrosyl protein, a putative participant in insulin signaling. *J Biol Chem* 266:12817-20
- Kennedy GC (1953) The role of depot fat in the hypothalamic control of food intake in the rat. *Proc R Soc Lond B Biol Sci* 140:578-96
- Keppler DaKD (1974) Glycogen determination with amyloglucosidase. In: Bergmeyer HU (ed) *Methods of Enzymatic Analysis*. Academic Press, New York, pp 1127-31
- Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G (2001) Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 280:E745-51
- Kieffer TJ, Habener JF (2000) The adipoinsular axis: effects of leptin on pancreatic beta-cells. *Am J Physiol Endocrinol Metab* 278:E1-E14
- Kieffer TJ, Heller RS, Leech CA, Holz GG, Habener JF (1997) Leptin suppression of insulin secretion by the activation of ATP-sensitive K⁺ channels in pancreatic beta-cells. *Diabetes* 46:1087-93
- Kim JK, Zisman A, Fillmore JJ, Peroni OD, Kotani K, Perret P, Zong H, Dong J, Kahn CR, Kahn BB, Shulman GI (2001) Glucose toxicity and the development of diabetes in mice with muscle-specific inactivation of GLUT4. *J Clin Invest* 108:153-60
- Kissebah AH, Krakower GR (1994) Regional adiposity and morbidity. *Physiol Rev* 74:761-811
- Klaman LD, Boss O, Peroni OD, Kim JK, Martino JL, Zabolotny JM, Moghal N, Lubkin M, Kim YB, Sharpe AH, Stricker-Krongrad A, Shulman GI, Neel BG, Kahn BB (2000) Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. *Mol Cell Biol* 20:5479-89
- Kohn AD, Kovacina KS, Roth RA (1995) Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase. *Embo J* 14:4288-95

- Kohn AD, Summers SA, Birnbaum MJ, Roth RA (1996a) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem* 271:31372-8
- Kohn AD, Takeuchi F, Roth RA (1996b) Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J Biol Chem* 271:21920-6
- Kohonen T (1997) Self organizing maps. Vol 426. Springer, Berlin
- Koo SH, Satoh H, Herzig S, Lee CH, Hedrick S, Kulkarni R, Evans RM, Olefsky J, Montminy M (2004) PGC-1 promotes insulin resistance in liver through PPAR- α -dependent induction of TRB-3. *Nat Med* 10:530-4
- Kotani K, Carozzi AJ, Sakaue H, Hara K, Robinson LJ, Clark SF, Yonezawa K, James DE, Kasuga M (1995) Requirement for phosphoinositide 3-kinase in insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 209:343-8
- Krssak M, Falk Petersen K, Dresner A, DiPietro L, Vogel SM, Rothman DL, Roden M, Shulman GI (1999) Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a ^1H NMR spectroscopy study. *Diabetologia* 42:113-6
- Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, Yano W, Froguel P, Nagai R, Kimura S, Kadowaki T, Noda T (2002) Disruption of adiponectin causes insulin resistance and neointimal formation. *J Biol Chem* 277:25863-6
- Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD (1995) High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem* 270:17513-20
- Kulkarni RN, Wang ZL, Wang RM, Hurley JD, Smith DM, Ghatei MA, Withers DJ, Gardiner JV, Bailey CJ, Bloom SR (1997) Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, in vivo, in mice. *J Clin Invest* 100:2729-36
- Lam TK, Carpentier A, Lewis GF, van de Werve G, Fantus IG, Giacca A (2003) Mechanisms of the free fatty acid-induced increase in hepatic glucose production. *Am J Physiol Endocrinol Metab* 284:E863-73
- Lannoy VJ, Decaux JF, Pierreux CE, Lemaigre FP, Rousseau GG (2002) Liver glucokinase gene expression is controlled by the onecut transcription factor hepatocyte nuclear factor-6. *Diabetologia* 45:1136-41
- Laustsen PG, Michael MD, Crute BE, Cohen SE, Ueki K, Kulkarni RN, Keller SR, Lienhard GE, Kahn CR (2002) Lipoatrophic diabetes in *Irs1*($-/-$)/*Irs3*($-/-$) double knockout mice. *Genes Dev* 16:3213-22
- Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM (1996) Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379:632-5
- Lee Y, Wang MY, Kakuma T, Wang ZW, Babcock E, McCorkle K, Higa M, Zhou YT, Unger RH (2001) Liporegulation in diet-induced obesity. The antisteatotic role of hyperleptinemia. *J Biol Chem* 276:5629-35

- Lee Y, Yu X, Gonzales F, Mangelsdorf DJ, Wang MY, Richardson C, Witters LA, Unger RH (2002) PPAR alpha is necessary for the lipopenic action of hyperleptinemia on white adipose and liver tissue. *Proc Natl Acad Sci U S A* 99:11848-53
- Levin K, Daa Schroeder H, Alford FP, Beck-Nielsen H (2001) Morphometric documentation of abnormal intramyocellular fat storage and reduced glycogen in obese patients with Type II diabetes. *Diabetologia* 44:824-33
- Levin N, Nelson C, Gurney A, Vandlen R, de Sauvage F (1996) Decreased food intake does not completely account for adiposity reduction after ob protein infusion. *Proc Natl Acad Sci U S A* 93:1726-30
- Li C, Ioffe E, Fidahusein N, Connolly E, Friedman JM (1998) Absence of soluble leptin receptor in plasma from dbPas/dbPas and other db/db mice. *J Biol Chem* 273:10078-82
- Li P (2004) Cidea, brown fat and obesity. *Mech Ageing Dev* 125:337-8
- Lin J, Choi YH, Hartzell DL, Li C, Della-Fera MA, Baile CA (2003) CNS melanocortin and leptin effects on stearoyl-CoA desaturase-1 and resistin expression. *Biochem Biophys Res Commun* 311:324-8
- Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Jr., Ory DS, Schaffer JE (2003) Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci U S A* 100:3077-82
- Liu L, Karkanias GB, Morales JC, Hawkins M, Barzilai N, Wang J, Rossetti L (1998) Intracerebroventricular leptin regulates hepatic but not peripheral glucose fluxes. *J Biol Chem* 273:31160-7
- Liu ML, Gibbs EM, McCoid SC, Milici AJ, Stukenbrok HA, McPherson RK, Treadway JL, Pessin JE (1993) Transgenic mice expressing the human GLUT4/muscle-fat facilitative glucose transporter protein exhibit efficient glycemic control. *Proc Natl Acad Sci U S A* 90:11346-50
- Liu SC, Wang Q, Lienhard GE, Keller SR (1999) Insulin receptor substrate 3 is not essential for growth or glucose homeostasis. *J Biol Chem* 274:18093-9
- Lowell BB, Bachman ES (2003) Beta-Adrenergic receptors, diet-induced thermogenesis, and obesity. *J Biol Chem* 278:29385-8
- Lunzer MA, Manning JA, Ockner RK (1977) Inhibition of rat liver acetyl coenzyme A carboxylase by long chain acyl coenzyme A and fatty acid. Modulation by fatty acid-binding protein. *J Biol Chem* 252:5483-7
- Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, et al. (1995) Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1:1155-61
- Martin SS, Haruta T, Morris AJ, Klippel A, Williams LT, Olefsky JM (1996) Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3-L1 adipocytes. *J Biol Chem* 271:17605-8
- Matusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, Brewer B, Jr., Reitman ML, Gonzalez FJ (2003) Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. *J Clin Invest* 111:737-47
- McGarry JD (2002) Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* 51:7-18

- McGarry JD, Mannaerts GP, Foster DW (1977) A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J Clin Invest* 60:265-70
- McGarry JD, Stark MJ, Foster DW (1978) Hepatic malonyl-CoA levels of fed, fasted and diabetic rats as measured using a simple radioisotopic assay. *J Biol Chem* 253:8291-3
- McGowan MK, Andrews KM, Grossman SP (1992) Chronic intrahypothalamic infusions of insulin or insulin antibodies alter body weight and food intake in the rat. *Physiol Behav* 51:753-66
- Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Trayhurn P (1996) Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Lett* 387:113-6
- Michael MD, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA, Kahn CR (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* 6:87-97
- Miles PD, Yamatani K, Lickley HL, Vranic M (1991) Mechanism of glucoregulatory responses to stress and their deficiency in diabetes. *Proc Natl Acad Sci U S A* 88:1296-300
- Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, Kahn BB (2002) Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415:339-43
- Miyazaki M, Jacobson MJ, Man WC, Cohen P, Asilmaz E, Friedman JM, Ntambi JM (2003) Identification and characterization of murine SCD4, a novel heart-specific stearoyl-CoA desaturase isoform regulated by leptin and dietary factors. *J Biol Chem* 278:33904-11
- Miyazaki M, Kim HJ, Man WC, Ntambi JM (2001) Oleoyl-CoA is the major de novo product of stearoyl-CoA desaturase 1 gene isoform and substrate for the biosynthesis of the Harderian gland 1-alkyl-2,3-diacylglycerol. *J Biol Chem* 276:39455-61
- Moitra J, Mason MM, Olive M, Krylov D, Gavrilova O, Marcus-Samuels B, Feigenbaum L, Lee E, Aoyama T, Eckhaus M, Reitman ML, Vinson C (1998) Life without white fat: a transgenic mouse. *Genes Dev* 12:3168-81
- Nakae J, Park BC, Accili D (1999) Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. *J Biol Chem* 274:15982-5
- Nandi A, Kitamura Y, Kahn CR, Accili D (2004) Mouse models of insulin resistance. *Physiol Rev* 84:623-47
- Neel JV (1962) Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? *Am J Hum Genet* 14:353-62
- Nicolaidis S, Rowland N (1976) Metering of intravenous versus oral nutrients and regulation of energy balance. *Am J Physiol* 231:661-8
- Nonogaki K (2000) New insights into sympathetic regulation of glucose and fat metabolism. *Diabetologia* 43:533-49
- Ntambi JM (1999) Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. *J Lipid Res* 40:1549-58

- Ntambi JM, Miyazaki M (2003) Recent insights into stearoyl-CoA desaturase-1. *Curr Opin Lipidol* 14:255-61
- Ntambi JM, Miyazaki M, Stoeckl JP, Lan H, Kendziora CM, Yandell BS, Song Y, Cohen P, Friedman JM, Attie AD (2002) Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. *Proc Natl Acad Sci U S A* 99:11482-6
- O'Rahilly S (1997) Science, medicine, and the future. Non-insulin dependent diabetes mellitus: the gathering storm. *Bmj* 314:955-9
- O'Rahilly S (2002) Insights into obesity and insulin resistance from the study of extreme human phenotypes. *Eur J Endocrinol* 147:435-41
- Obici S, Feng Z, Arduini A, Conti R, Rossetti L (2003) Inhibition of hypothalamic carnitine palmitoyltransferase-1 decreases food intake and glucose production. *Nat Med* 9:756-61
- Obici S, Feng Z, Karkanas G, Baskin DG, Rossetti L (2002a) Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. *Nat Neurosci* 5:566-72
- Obici S, Feng Z, Morgan K, Stein D, Karkanas G, Rossetti L (2002b) Central administration of oleic acid inhibits glucose production and food intake. *Diabetes* 51:271-5
- Obici S, Rossetti L (2003) Minireview: nutrient sensing and the regulation of insulin action and energy balance. *Endocrinology* 144:5172-8
- Obici S, Zhang BB, Karkanas G, Rossetti L (2002c) Hypothalamic insulin signaling is required for inhibition of glucose production. *Nat Med* 8:1376-82
- Ofei F, Hurel S, Newkirk J, Sopwith M, Taylor R (1996) Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* 45:881-5
- Ogawa Y, Masuzaki H, Hosoda K, Aizawa-Abe M, Suga J, Suda M, Ebihara K, Iwai H, Matsuoka N, Satoh N, Odaka H, Kasuga H, Fujisawa Y, Inoue G, Nishimura H, Yoshimasa Y, Nakao K (1999) Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. *Diabetes* 48:1822-9
- Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *J Biol Chem* 269:3568-73
- Oral EA (2003) Lipoatrophic diabetes and other related syndromes. *Rev Endocr Metab Disord* 4:61-77
- Oral EA, Simha V, Ruiz E, Andewelt A, Premkumar A, Snell P, Wagner AJ, DePaoli AM, Reitman ML, Taylor SI, Gorden P, Garg A (2002) Leptin-replacement therapy for lipodystrophy. *N Engl J Med* 346:570-8
- Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F (1995) Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 269:540-3
- Perseghin G, Petersen K, Shulman GI (2003) Cellular mechanism of insulin resistance: potential links with inflammation. *Int J Obes Relat Metab Disord* 27 Suppl 3:S6-11
- Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, Vanzulli A, Testolin G, Pozza G, Del Maschio A, Luzi L (1999) Intramyocellular triglyceride

- content is a determinant of in vivo insulin resistance in humans: a 1H - ^{13}C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* 48:1600-6
- Petersen KF, Hendler R, Price T, Perseghin G, Rothman DL, Held N, Amatruda JM, Shulman GI (1998) $^{13}\text{C}/^{31}\text{P}$ NMR studies on the mechanism of insulin resistance in obesity. *Diabetes* 47:381-6
- Petersen KF, Oral EA, Dufour S, Befroy D, Ariyan C, Yu C, Cline GW, DePaoli AM, Taylor SI, Gorden P, Shulman GI (2002) Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy. *J Clin Invest* 109:1345-50
- Pilkis SJ, Granner DK (1992) Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. *Annu Rev Physiol* 54:885-909
- Pittas AG, Joseph NA, Greenberg AS (2004) Adipocytokines and insulin resistance. *J Clin Endocrinol Metab* 89:447-52
- Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D, Spiegelman BM (2003) Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1 α interaction. *Nature* 423:550-5
- Qi Y, Takahashi N, Hileman SM, Patel HR, Berg AH, Pajvani UB, Scherer PE, Ahima RS (2004) Adiponectin acts in the brain to decrease body weight. *Nat Med* 10:524-9
- Qiu J, Ogus S, Lu R, Chehab FF (2001) Transgenic mice overexpressing leptin accumulate adipose mass at an older, but not younger, age. *Endocrinology* 142:348-58
- Rahman SM, Dobrzyn A, Dobrzyn P, Lee SH, Miyazaki M, Ntambi JM (2003) Stearoyl-CoA desaturase 1 deficiency elevates insulin-signaling components and down-regulates protein-tyrosine phosphatase 1B in muscle. *Proc Natl Acad Sci U S A* 100:11110-5
- Rajala MW, Obici S, Scherer PE, Rossetti L (2003) Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. *J Clin Invest* 111:225-30
- Randle PJ, Garland PB, Hales CN, Newsholme EA (1963) The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1:785-9
- Randle PJ, Garland PB, Newsholme EA, Hales CN (1965) The glucose fatty acid cycle in obesity and maturity onset diabetes mellitus. *Ann N Y Acad Sci* 131:324-33
- Randle PJ, Newsholme EA, Garland PB (1964) Regulation of glucose uptake by muscle. 8. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles. *Biochem J* 93:652-65
- Ravussin E, Smith SR (2002) Increased fat intake, impaired fat oxidation, and failure of fat cell proliferation result in ectopic fat storage, insulin resistance, and type 2 diabetes mellitus. *Ann N Y Acad Sci* 967:363-78
- Reaven GM (1995) The fourth musketeer--from Alexandre Dumas to Claude Bernard. *Diabetologia* 38:3-13
- Ren JM, Marshall BA, Gulve EA, Gao J, Johnson DW, Holloszy JO, Mueckler M (1993) Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. *J Biol Chem* 268:16113-5

- Revelli JP, Preitner F, Samec S, Muniesa P, Kuehne F, Boss O, Vassalli JD, Dulloo A, Seydoux J, Giacobino JP, Huarte J, Ody C (1997) Targeted gene disruption reveals a leptin-independent role for the mouse beta3-adrenoceptor in the regulation of body composition. *J Clin Invest* 100:1098-106
- Robidoux J, Martin TL, Collins S (2004) Beta-adrenergic receptors and regulation of energy expenditure: a family affair. *Annu Rev Pharmacol Toxicol* 44:297-323
- Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, Shulman GI (1996) Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest* 97:2859-65
- Rohrer DK, Desai KH, Jasper JR, Stevens ME, Regula DP, Jr., Barsh GS, Bernstein D, Kobilka BK (1996) Targeted disruption of the mouse beta1-adrenergic receptor gene: developmental and cardiovascular effects. *Proc Natl Acad Sci U S A* 93:7375-80
- Ross SA, Gulve EA, Wang M (2004) Chemistry and biochemistry of type 2 diabetes. *Chem Rev* 104:1255-82
- Rossetti L, Massillon D, Barzilai N, Vuguin P, Chen W, Hawkins M, Wu J, Wang J (1997) Short term effects of leptin on hepatic gluconeogenesis and in vivo insulin action. *J Biol Chem* 272:27758-63
- Rothman DL, Shulman RG, Shulman GI (1992) ³¹P nuclear magnetic resonance measurements of muscle glucose-6-phosphate. Evidence for reduced insulin-dependent muscle glucose transport or phosphorylation activity in non-insulin-dependent diabetes mellitus. *J Clin Invest* 89:1069-75
- Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B, Auwerx J (1995) Transient increase in obese gene expression after food intake or insulin administration. *Nature* 377:527-9
- Sarmiento U, Benson B, Kaufman S, Ross L, Qi M, Scully S, DiPalma C (1997) Morphologic and molecular changes induced by recombinant human leptin in the white and brown adipose tissues of C57BL/6 mice. *Lab Invest* 77:243-56
- Scarpace PJ, Matheny M (1998) Leptin induction of UCP1 gene expression is dependent on sympathetic innervation. *Am J Physiol* 275:E259-64
- Schalch DS, Kipnis DM (1965) Abnormalities in carbohydrate tolerance associated with elevated plasma nonesterified fatty acids. *J Clin Invest* 44:2010-20
- Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG (1996) Identification of targets of leptin action in rat hypothalamus. *J Clin Invest* 98:1101-6
- Schwartz MW, Sipols AJ, Marks JL, Sanacora G, White JD, Scheurink A, Kahn SE, Baskin DG, Woods SC, Figlewicz DP, et al. (1992) Inhibition of hypothalamic neuropeptide Y gene expression by insulin. *Endocrinology* 130:3608-16
- Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. *Nature* 404:661-71
- Segal-Lieberman G, Bradley RL, Kokkotou E, Carlson M, Trombly DJ, Wang X, Bates S, Myers MG, Jr., Flier JS, Maratos-Flier E (2003) Melanin-concentrating hormone is a critical mediator of the leptin-deficient phenotype. *Proc Natl Acad Sci U S A* 100:10085-90
- Seufert J, Kieffer TJ, Habener JF (1999) Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice. *Proc Natl Acad Sci U S A* 96:674-9

- Shen W, Searce LM, Brestelli JE, Sund NJ, Kaestner KH (2001) Foxa3 (hepatocyte nuclear factor 3gamma) is required for the regulation of hepatic GLUT2 expression and the maintenance of glucose homeostasis during a prolonged fast. *J Biol Chem* 276:42812-7
- Shih DQ, Stoffel M (2002) Molecular etiologies of MODY and other early-onset forms of diabetes. *Curr Diab Rep* 2:125-34
- Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, Newgard CB, Unger RH (1997) Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci U S A* 94:4637-41
- Shimomura I, Hammer RE, Ikemoto S, Brown MS, Goldstein JL (1999) Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* 401:73-6
- Shimomura I, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL, Brown MS (1998) Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev* 12:3182-94
- Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS, Goldstein JL (2000) Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. *Mol Cell* 6:77-86
- Shmulewitz D, Auerbach SB, Lehner T, Blundell ML, Winick JD, Youngman LD, Skilling V, Heath SC, Ott J, Stoffel M, Breslow JL, Friedman JM (2001) Epidemiology and factor analysis of obesity, type II diabetes, hypertension, and dyslipidemia (syndrome X) on the Island of Kosrae, Federated States of Micronesia. *Hum Hered* 51:8-19
- Shulman GI (2000) Cellular mechanisms of insulin resistance. *J Clin Invest* 106:171-6
- Sipols AJ, Baskin DG, Schwartz MW (1995) Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression. *Diabetes* 44:147-51
- Soukas A, Cohen P, Socci ND, Friedman JM (2000) Leptin-specific patterns of gene expression in white adipose tissue. *Genes Dev* 14:963-80
- Soukas AA (2001) Leptin Regulated Programs of Gene Expression in White Adipose Tissue. The Rockefeller University, New York
- Standaert ML, Bandyopadhyay G, Perez L, Price D, Galloway L, Poklepovic A, Sajan MP, Cenni V, Sirri A, Moscat J, Toker A, Farese RV (1999) Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. *J Biol Chem* 274:25308-16
- Statnick MA, Beavers LS, Conner LJ, Corominola H, Johnson D, Hammond CD, Rafaeloff-Phail R, Seng T, Suter TM, Sluka JP, Ravussin E, Gadski RA, Caro JF (2000) Decreased expression of apM1 in omental and subcutaneous adipose tissue of humans with type 2 diabetes. *Int J Exp Diabetes Res* 1:81-8
- Steinberg GR, Rush JW, Dyck DJ (2003) AMPK expression and phosphorylation are increased in rodent muscle after chronic leptin treatment. *Am J Physiol Endocrinol Metab* 284:E648-54

- Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, et al. (1995) The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* 377:530-2
- Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA (2001) The hormone resistin links obesity to diabetes. *Nature* 409:307-12
- Steppan CM, Lazar MA (2002) Resistin and obesity-associated insulin resistance. *Trends Endocrinol Metab* 13:18-23
- Stoffel M, Duncan SA (1997) The maturity-onset diabetes of the young (MODY1) transcription factor HNF4 α regulates expression of genes required for glucose transport and metabolism. *Proc Natl Acad Sci U S A* 94:13209-14
- Stoffel M, Froguel P, Takeda J, Zouali H, Vionnet N, Nishi S, Weber IT, Harrison RW, Pilkis SJ, Lesage S, et al. (1992a) Human glucokinase gene: isolation, characterization, and identification of two missense mutations linked to early-onset non-insulin-dependent (type 2) diabetes mellitus. *Proc Natl Acad Sci U S A* 89:7698-702
- Stoffel M, Patel P, Lo YM, Hattersley AT, Lucassen AM, Page R, Bell JI, Bell GI, Turner RC, Wainscoat JS (1992b) Missense glucokinase mutation in maturity-onset diabetes of the young and mutation screening in late-onset diabetes. *Nat Genet* 2:153-6
- Sugden C, Crawford RM, Halford NG, Hardie DG (1999) Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5'-AMP. *Plant J* 19:433-9
- Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF (1991) Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* 352:73-7
- Susulic VS, Frederick RC, Lawitts J, Tozzo E, Kahn BB, Harper ME, Himms-Hagen J, Flier JS, Lowell BB (1995) Targeted disruption of the beta 3-adrenergic receptor gene. *J Biol Chem* 270:29483-92
- Suzuki K, Kono T (1980) Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc Natl Acad Sci U S A* 77:2542-5
- Taira M, Hashimoto N, Shimada F, Suzuki Y, Kanatsuka A, Nakamura F, Ebina Y, Tatibana M, Makino H, et al. (1989) Human diabetes associated with a deletion of the tyrosine kinase domain of the insulin receptor. *Science* 245:63-6
- Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, et al. (1994) Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182-6
- Tan CE, Emmanuel SC, Tan BY, Jacob E (1999) Prevalence of diabetes and ethnic differences in cardiovascular risk factors. The 1992 Singapore National Health Survey. *Diabetes Care* 22:241-7
- Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, et al. (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83:1263-71

- Tsigos C, Papanicolaou DA, Kyrou I, Defensor R, Mitsiadis CS, Chrousos GP (1997) Dose-dependent effects of recombinant human interleukin-6 on glucose regulation. *J Clin Endocrinol Metab* 82:4167-70
- Tsuboyama-Kasaoka N, Takahashi M, Tanemura K, Kim HJ, Tange T, Okuyama H, Kasai M, Ikemoto S, Ezaki O (2000) Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes* 49:1534-42
- Ueki K, Yballe CM, Brachmann SM, Vicent D, Watt JM, Kahn CR, Cantley LC (2002) Increased insulin sensitivity in mice lacking p85beta subunit of phosphoinositide 3-kinase. *Proc Natl Acad Sci U S A* 99:419-24
- Unger RH (2003) Minireview: weapons of lean body mass destruction: the role of ectopic lipids in the metabolic syndrome. *Endocrinology* 144:5159-65
- Vance JE (1990) Phospholipid synthesis in a membrane fraction associated with mitochondria. *J Biol Chem* 265:7248-56
- VanderWeele DA, Harackiewicz E, Van Itallie TB (1982) Elevated insulin and satiety in obese and normal-weight rats. *Appetite* 3:99-109
- Vionnet N, Stoffel M, Takeda J, Yasuda K, Bell GI, Zouali H, Lesage S, Velho G, Iris F, Passa P, et al. (1992) Nonsense mutation in the glucokinase gene causes early-onset non-insulin-dependent diabetes mellitus. *Nature* 356:721-2
- Volpe JJ, Vagelos PR (1976) Mechanisms and regulation of biosynthesis of saturated fatty acids. *Physiol Rev* 56:339-417
- Vozarova B, Weyer C, Hanson K, Tataranni PA, Bogardus C, Pratley RE (2001) Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. *Obes Res* 9:414-7
- Walston J, Silver K, Bogardus C, Knowler WC, Celi FS, Austin S, Manning B, Strosberg AD, Stern MP, Raben N, et al. (1995) Time of onset of non-insulin-dependent diabetes mellitus and genetic variation in the beta 3-adrenergic-receptor gene. *N Engl J Med* 333:343-7
- Wang Y, Kuropatwinski KK, White DW, Hawley TS, Hawley RG, Tartaglia LA, Baumann H (1997) Leptin receptor action in hepatic cells. *J Biol Chem* 272:16216-23
- WHO (1999) Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications. World Health Organization, Geneva, pp 1-59
- WHO (2003) Screening for Type 2 Diabetes. World Health Organization and International Diabetes Federation Meeting, Geneva, pp 1-48
- Widen E, Lehto M, Kanninen T, Walston J, Shuldiner AR, Groop LC (1995) Association of a polymorphism in the beta 3-adrenergic-receptor gene with features of the insulin resistance syndrome in Finns. *N Engl J Med* 333:348-51
- Wilden PA, Backer JM, Kahn CR, Cahill DA, Schroeder GJ, White MF (1990) The insulin receptor with phenylalanine replacing tyrosine-1146 provides evidence for separate signals regulating cellular metabolism and growth. *Proc Natl Acad Sci U S A* 87:3358-62
- Winder WW, Hardie DG (1999) AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* 277:E1-10

- Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF (1998) Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900-4
- Witters LA, Kemp BE (1992) Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J Biol Chem* 267:2864-7
- Wolfrum C, Besser D, Luca E, Stoffel M (2003a) Insulin regulates the activity of forkhead transcription factor Hnf-3beta/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization. *Proc Natl Acad Sci U S A* 100:11624-9
- Wolfrum C, Shih DQ, Kuwajima S, Norris AW, Kahn CR, Stoffel M (2003b) Role of Foxa-2 in adipocyte metabolism and differentiation. *J Clin Invest* 112:345-56
- Woods A, Salt I, Scott J, Hardie DG, Carling D (1996a) The alpha1 and alpha2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity in vitro. *FEBS Lett* 397:347-51
- Woods SC, Chavez M, Park CR, Riedy C, Kaiyala K, Richardson RD, Figlewicz DP, Schwartz MW, Porte D, Jr., Seeley RJ (1996b) The evaluation of insulin as a metabolic signal influencing behavior via the brain. *Neurosci Biobehav Rev* 20:139-44
- Woods SC, Seeley RJ, Porte D, Jr., Schwartz MW (1998) Signals that regulate food intake and energy homeostasis. *Science* 280:1378-83
- Yamagata K, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ, Fajans SS, Signorini S, Stoffel M, Bell GI (1996) Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature* 384:458-60
- Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, Ito Y, Takakuwa K, Matsui J, Takata M, Eto K, Terauchi Y, Komeda K, Tsunoda M, Murakami K, Ohnishi Y, Naitoh T, Yamamura K, Ueyama Y, Froguel P, Kimura S, Nagai R, Kadowaki T (2003) Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. *J Biol Chem* 278:2461-8
- Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7:941-6
- Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB, Spiegelman BM (2001) Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 413:131-8
- Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI (2002) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 277:50230-6
- Zarjevski N, Cusin I, Vettor R, Rohner-Jeanrenaud F, Jeanrenaud B (1993) Chronic intracerebroventricular neuropeptide-Y administration to normal rats mimics hormonal and metabolic changes of obesity. *Endocrinology* 133:1753-8
- Zerial M, McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* 2:107-17

- Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425-32
- Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108:1167-74
- Zhou Z, Yon Toh S, Chen Z, Guo K, Ng CP, Ponniah S, Lin SC, Hong W, Li P (2003) Cidea-deficient mice have lean phenotype and are resistant to obesity. *Nat Genet* 35:49-56
- Ziel FH, Venkatesan N, Davidson MB (1988) Glucose transport is rate limiting for skeletal muscle glucose metabolism in normal and STZ-induced diabetic rats. *Diabetes* 37:885-90
- Zimmet P (2000) Globalization, coca-colonization and the chronic disease epidemic: can the Doomsday scenario be averted? *J Intern Med* 247:301-10
- Zimmet P, Alberti KG, Shaw J (2001) Global and societal implications of the diabetes epidemic. *Nature* 414:782-7
- Zimmet P, Dowse G, Finch C, Serjeantson S, King H (1990) The epidemiology and natural history of NIDDM--lessons from the South Pacific. *Diabetes Metab Rev* 6:91-124
- Zisman A, Peroni OD, Abel ED, Michael MD, Mauvais-Jarvis F, Lowell BB, Wojtaszewski JF, Hirshman MF, Virkamaki A, Goodyear LJ, Kahn CR, Kahn BB (2000) Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat Med* 6:924-8

Appendix A

a

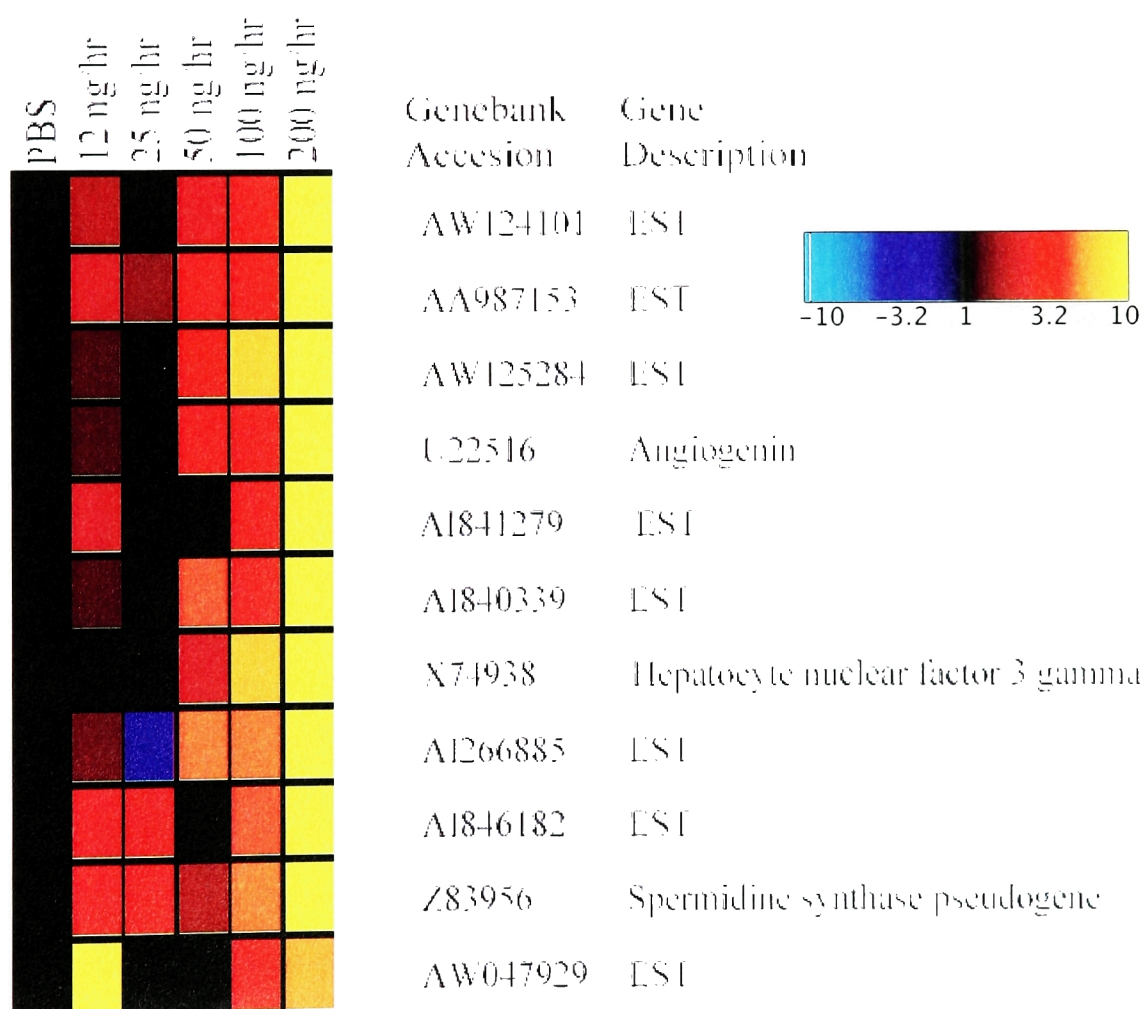
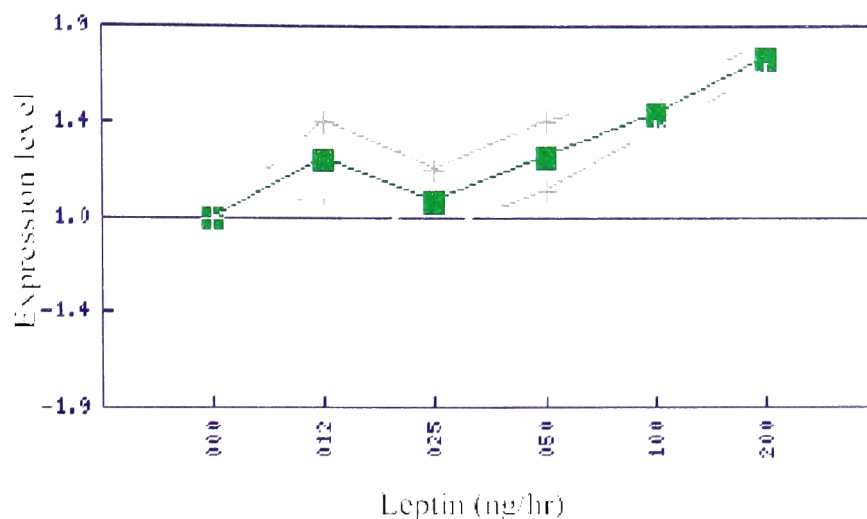


Figure 5.4 Cluster analysis of hepatic gene expression in aP2-nSREBP-1c mice treated with PBS, 12ng/hr, 25ng/hr, 50ng/hr, 100ng/hr and 200ng/hr subcutaneous leptin. a. Genes induced at 50ng/hr or higher leptin doses.

Appendix B

b

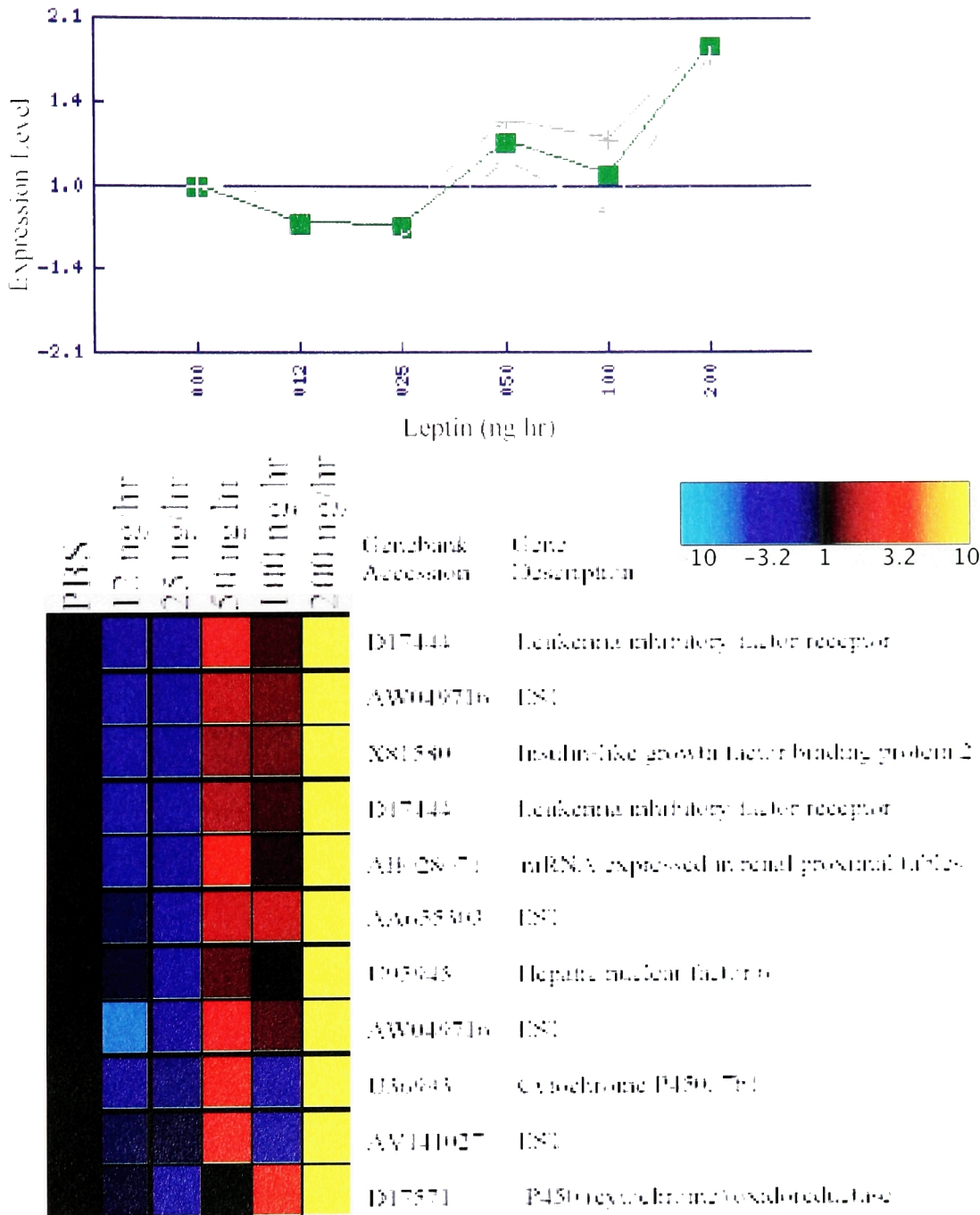


Figure 5.4 Cluster analysis of hepatic gene expression in aP2-nSREBP-1c mice treated with PBS, 12ng/hr, 25ng/hr, 50ng/hr, 100ng/hr and 200ng/hr subcutaneous leptin. b. Genes induced mostly at 200ng/hr leptin dose.

Appendix C

c

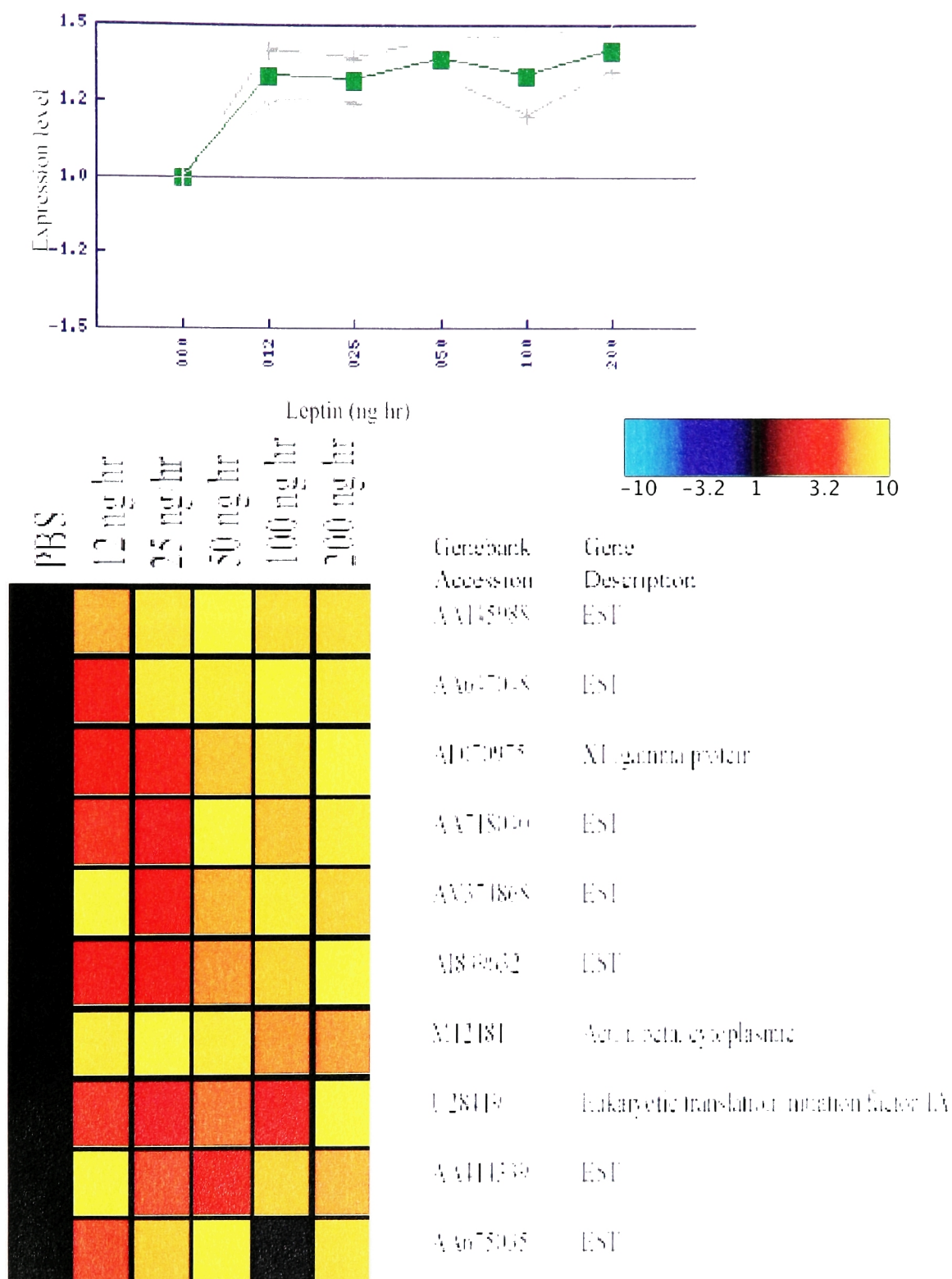


Figure 5.4 Cluster analysis of hepatic gene expression in aP2-nSREBP-1c mice treated with PBS, 12ng/hr, 25ng/hr, 50ng/hr, 100ng/hr and 200ng/hr subcutaneous leptin. c. Genes induced at all of the leptin doses.

Appendix D

d

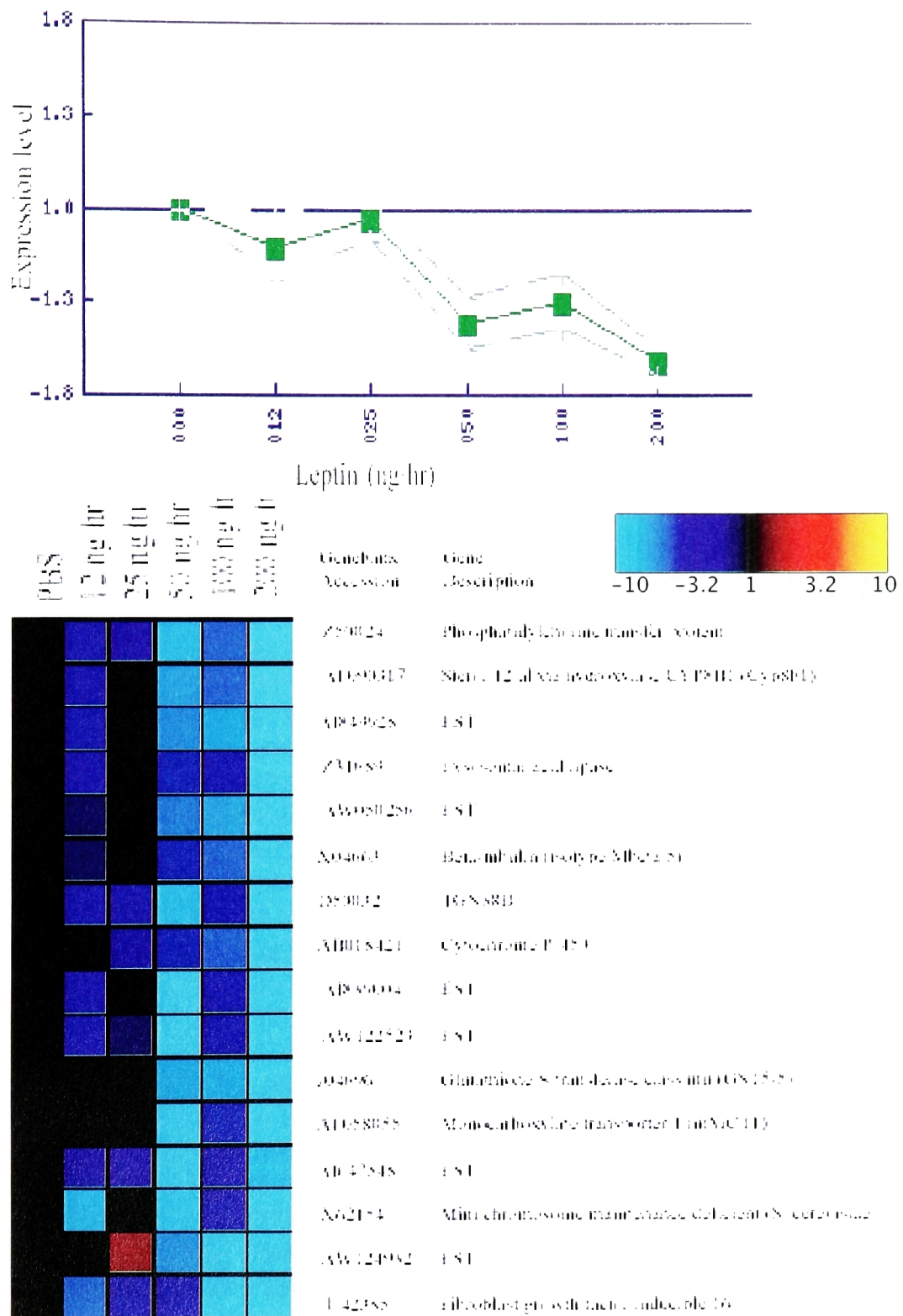


Figure 5.4 Cluster analysis of hepatic gene expression in aP2-nSREBP-1c mice treated with PBS, 12ng/hr, 25ng/hr, 50ng/hr, 100ng/hr and 200ng/hr subcutaneous leptin. d. Genes repressed at 50ng/hr or higher leptin doses.

Appendix E

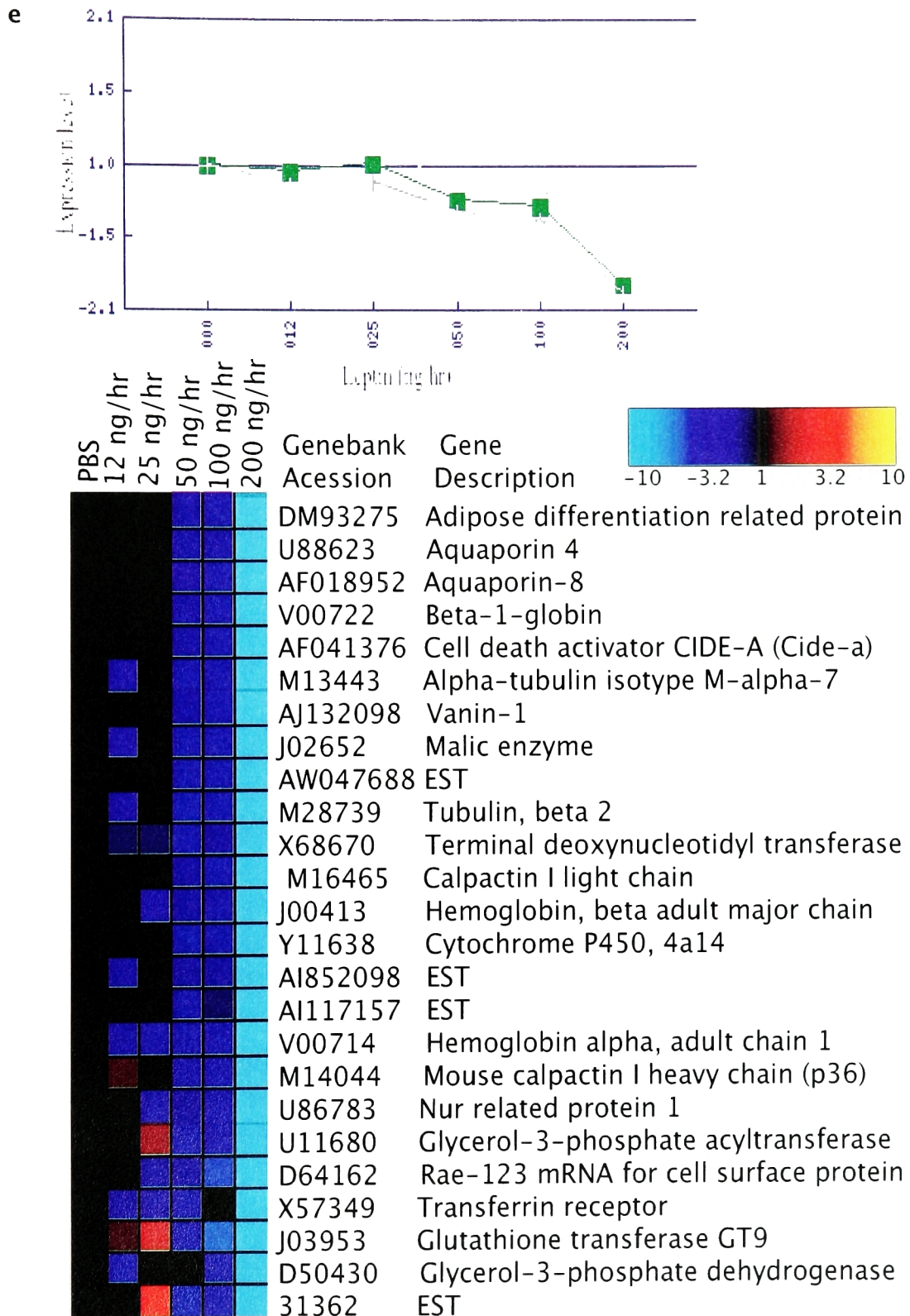


Figure 5.4 Cluster analysis of hepatic gene expression in aP2-nSREBP-1c mice treated with PBS, 12ng/hr, 25ng/hr, 50ng/hr, 100ng/hr and 200ng/hr subcutaneous leptin. e. Genes repressed mostly at 200ng/hr leptin dose.

Appendix F

f

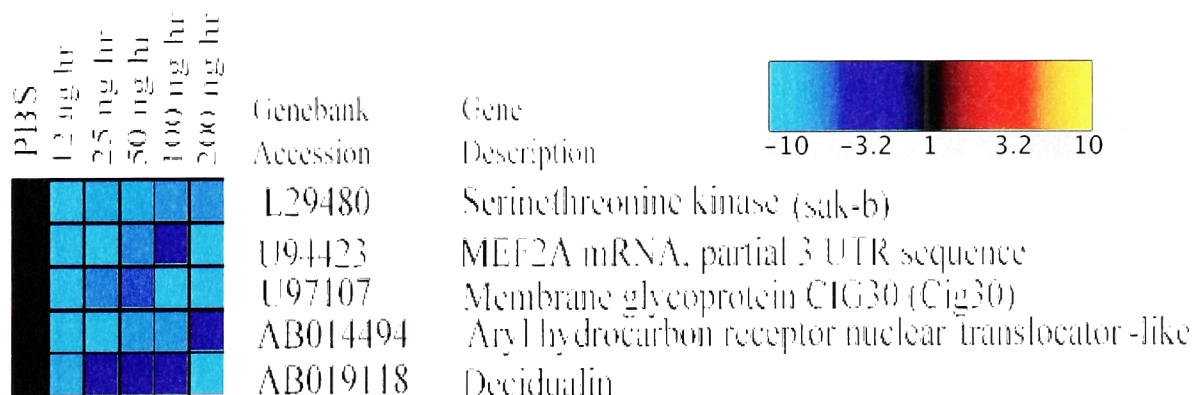
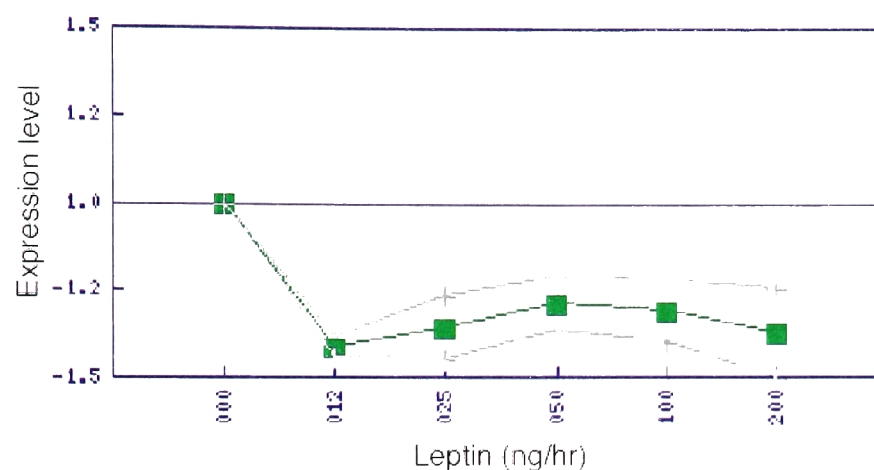


Figure 5.4 Cluster analysis of hepatic gene expression in aP2-nSREBP-1c mice treated with PBS, 12ng/hr, 25ng/hr, 50ng/hr, 100ng/hr and 200ng/hr subcutaneous leptin. f. Genes repressed at all leptin doses.