THE EFFECT OF LEPTIN ADMINISTRATION AND RECOVERY ON ENERGY METABOLISM, UNCOUPLING PROTEINS, AND ADIPOSE TISSUE APOPTOSIS

by

P. SCOTT GULLICKSEN

(Under the direction of CLIFTON A. BAILE)

ABSTRACT

Administering leptin, an adipocyte-derived hormone related to fat mass, to rodents dramatically reduces food intake and body weight while enhancing energy expenditure. After treatment withdrawal, body weight recovery was retarded compared to pair-fed rodents that lost less weight and exhibited a faster regain. The changes induced by leptin and the processes behind a slower recovery were investigated here. The first objective was to determine the period of leptin influence on food intake, body weight, and energy expenditure. Rats were injected ICV with either leptin or the vehicle for 4 days before being killed or placed in calorimetry chambers for 21 days. Leptin-treated rats maintained a lower body weight up to 6 days after treatment ended. An overshoot of food intake and respiratory quotient during recovery was offset by a greater heat production (HP) throughout recovery. These responses paralleled an increase in uncoupling protein (UCP) expression in brown (BAT) and white adipose tissues. BAT was the most responsive to leptin by dramatically changing UCP1 & UCP3 mRNA levels. The second objective was to determine the sustained effect of leptin on adipose tissue cellularity, measured using the Coulter counter, and apoptosis. Leptin significantly reduced the masses of all white fat pads [retroperitoneal (RP) > inguinal (ING) > epididymal (EPI)] but not BAT. Cell volume was significantly reduced in EPI and ING. Only ING had a significantly reduced cell number and exhibited apoptosis by increased DNA fragmentation and DNA laddering, plus upregulation of pro-apoptosis Bax protein. The other fat pads exhibited a general increase in the Bcl-2/Bax ratio. Recovery allowed for normalization of white fat pad mass, cell number and cell volume; however, BAT mass increased dramatically. After recovery, apoptosis was not detected, Bcl-2 protein increased in ING, and the Bcl-2/Bax ratio had risen overall. These results indicate that leptin induces apoptosis, at least in ING, and has lasting effects on energy metabolism that contribute to a slower body weight recovery. A secondary effect that opposed adipocyte loss during recovery supported near full normalization of body weight and adiposity. This demonstrated a resiliency with respect to energy homeostasis after leptin treatment.

INDEX WORDS: Adipose tissue, Apoptosis, Energy metabolism, Leptin, Obesity, Thermogenesis, Uncoupling proteins

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P. SCOTT GULLICKSEN B.S., The University of Vermont, 1992

M.S., The University of Vermont, 1996

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P. SCOTT GULLICKSEN

Approved:

Major Professor: Clifton A. Baile

Committee: Michael J. Azain Carolyn D. Berdanier Roger G. Dean William P. Flatt

Electronic Version Approved:

Gordhan L. Patel Dean of the Graduate School The University of Georgia August 2002

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	iv
LIST OF TABLES	viii
LIST OF FIGURES	x
CHAPTER	
1 INTRODUCTION	1
2 REVIEW OF THE LITERATURE	4
Obesity and Health Risk	4
The <i>ob</i> Gene and Obesity	9
The Leptin Receptor and Leptin Signaling	15
Body Weight Regulation and Leptin	
Thermogenesis and Uncoupling Proteins	
Leptin Induces UCPs & Thermogenesis	47
Apoptosis, Programmed Cell Death	50
Adipose Tisue Apoptosis	61
Leptin and Adipocyte Apoptosis	67
3 ENERGY METABOLISM AND EXPRESSION OF UNCOUPLING	
PROTEINS 1, 2, AND 3 AFTER 21 DAYS OF RECOVERY FROM	
INTRACEREBROVENTRICULAR MOUSE LEPTIN IN RATS	
Abstract	
Introduction	

	Materials and methods	. 76
	Results	. 82
	Discussion	. 88
4	ADIPOSE TISSUE CELLULARITY AND APOPTOSIS AFTER	
	INTRACEREBROVENTRICULAR INJECTIONS OF LEPTIN AND 21	
	DAYS OF RECOVERY IN RATS	. 96
	Abstract	. 97
	Introduction	. 99
	Materials and Methods	102
	Results	107
	Discussion	115
5	DETECION OF DNA FRAGMENTATION, APOPTOTIC PROTEINS, AND	
	THE QUANTIFICATION OF UNCOUPLING PROTEIN EXPRESSION BY	
	REAL-TIME RT-PCR	125
	Detection of DNA Fragmentation in White Adipose Tissue	126
	Subcellular Fractionation of Adipose Tissue and the Detection of Bcl-2	and
	Bax Apoptotic Proteins	133
	Quantification of Uncoupling Proteins in Adipose Tissue using Real-Ti	ime
	RT-PCR	139
6	SUMMARY	144
REFE	RENCES	148
APPE	ENDICIES	
A	Adipose Tissue Cellularity Using Osmium Tetroxide	187

В	Artificial Cerebrospinal Fluid (aCSF) Preparation	. 193
С	Coomassie [®] Plus Protein Quantitation Microplate Assay	. 194
D	DNA Fragmentation in Adipose Tissue Using Centrifugation	. 195
E	DNA Laddering Using Fluorescent End-Labeling	. 197
F	DNA Laddering Using Radioactive End-Labeling	. 198
G	Intracerebroventricular (ICV) Cannulation of Rats	. 201
Η	PicoGreen [®] DNA Quantitation Microplate Assay	. 203
Ι	Real-Time RT-PCR Using the ABI Prism 7700 Sequence Detection System	. 205
J	RiboGreen [®] RNA Quantitation Microplate Assay	. 208
K	RNA, DNA & Protein Isolation Using TRIzol® Reagent	. 209
L	Subcellular Fractionation of Adipose Tissue for the Isolation of Mitochondria	1
	Protein	. 214
Μ	Surgical Denervation of the Rodent Inguinal White Adipose Tissue	. 216

LIST OF TABLES

Page
Table 2.1. Obesity-associated diseases and conditions
Table 2.2. Classification of overweight and obesity by BMI, waist circumference,
and associated disease risk
Table 2.3. Single-gene mutations in rodent obesity
Table 2.4. Abnormalities of leptin deficiency/resistance
Table 2.5. Modulators of food intake and energy homeostasis 19
Table 2.6. Characteristics of leptin administration and food restriction
Table 2.7. Location for the expression of the long form of the leptin receptor, Ob-Rb 32
Table 2.8. Tissue expression of the UCPs 37
Table 2.9. UCP amino acid homology
Table 2.10. Differential characteristics of the types of cell death
Table 2.11. Potential regulators of adipocyte number 63
Table 3.1. Primer and probe sequences used for real-time RT-PCR of UCPs
Table 3.2. UCP transcripts in adipose tissues after 4 days of leptin injections and
after 21 days of recovery
Table 4.1. Growth performance and adipose tissue characteristics after 4 days of 10
μ g of leptin injected ICV and after 21 days of recovery in Sprague-Dawley rats 109
Table 5.1. Quantification of fragmented DNA in inguinal white adipose tissue from
rats injected ICV with leptin or the vehicle
Table 5.2. Primer and probe sequences used for real-time RT-PCR of UCPs 140

LIST OF FIGURES

Page

Figure 2.18. DNA fragmentation and the appearance of the "DNA ladder" 58
Figure 2.19. DNA ladder from cells undergoing apoptosis and stained by SYBR
Green I nucleic acid stain
Figure 2.20. Phagocytes (including dendritic cells and macrophages) engulf and
degrade both apoptotic and necrotic cells
Figure 2.21. Mitochondrial pathway in apoptosis
Figure 2.22. Creatine kinase pathway in apoptosis and inhibition by $Bcl-x_L$
Figure 3.1. Daily food intake and body weight of Sprague-Dawley rats injected ICV
with 2.5 or 10 µg of mouse leptin or 10 µl aCSF (control) for 4 days 82
Figure 3.2. Daily food intake (g) of rats injected ICV with leptin or vehicle
Figure 3.3. Daily body weight (g) of rats injected ICV with leptin or vehicle
Figure 3.4. Daily respiratory quotient (RQ) of rats injected ICV with leptin or vehicle. 84
Figure 3.5. Daily heat production per metabolic body size (kcal/kg ^{0.75}) of rats
injected ICV with leptin or vehicle
Figure 3.6. Daily retained energy (RE), an estimate of energy balance, of rats
injected ICV with leptin or vehicle
Figure 4.1. Cell number counted after osmium tetroxide fixation within the inguinal
and epididymal fat pads after 4 days of 10 μ g leptin administration ICV or after
21 days of recovery
Figure 4.2. DNA fragmentation (small DNA/total DNA, %) in adipose tissues after 4
days of 10 µg leptin administration ICV or after 21 days of recovery 112
Figure 4.3. Agarose gel of DNA from inguinal white adipose tissue separated into
two fractions of small (soluble) and large (insoluble) then run in separate lanes 113

Figure	4.4.	Bcl-2/Bax	protein	ratio	in	white	adipose	tissue	after	subcellular	
fra	ctionat	ion into cyte	osolic an	d mitc	ochc	ondrial	fractions.				114

CHAPTER 1

INTRODUCTION

The prevalence of overweight and obesity has been increasing consistently in the United States and western countries. The cause of obesity is a combination of environmental and genetic factors that is continuing to be investigated. Obesity is associated with a significant increase in morbidity and mortality, and weight loss is recommended to improve the health of these individuals but many treatment regimes have little long-term success. Most weight-control programs involve a low-calorie diet that is difficult to sustain and can have adverse side effects. For those with morbid obesity, it is especially important to discover the underlying metabolic and genetic basis. Researchers hunting for obesity genes have had some success but the application of discoveries to the treatment of human obesity has been less fruitful.

Several obesity-related genes have been found. The discovery of mutations in obese rodents, particularly the obese (*ob*) and diabetes (*db*) mice, have led to the identification of an adipocyte-derived hormone called leptin that maintains body fat content in a narrow range by modulating energy intake and expenditure. Serum leptin levels correlate strongly with the amount of body fat. As body fat increases, adipocytes produce and secrete leptin into the blood stream where it travels to the brain, binds to leptin receptors in the hypothalamus, and signals satiety and to increase energy expenditure. Conversely, food restriction or fasting, which also lowers fat mass, causes both leptin levels and energy expenditure to decrease. Other factors, such as the neuropeptides NPY and melanocortin, are involved in the leptin pathway for controlling energy balance.

Unfortunately, most human obesity is characterized by high leptin levels and unresponsiveness to exogenous leptin. A similar condition exists in *db* mice, which have a mutation in the gene encoding the leptin receptor. Administering leptin to these mice produces no effect.

In addition to reducing food intake, leptin can dramatically raise energy expenditure when administered centrally or peripherally, whereas food restriction alone lowers metabolic rate. Energy expenditure is increased, in part, by stimulating thermogenic mechanisms such as uncoupling proteins (UCPs) in the mitochondria of brown adipose tissue of animals. UCPs function to dissipate heat at the expense of fatty acids or assist in the transport of fatty acids during β -oxidation. The result is a preferential use of fat as a fuel source and a specific reduction of body fat without a loss of valuable lean tissue.

Leptin also causes specific changes to adipose tissue cellularity by dramatically reducing the average cell size in all fat tissues, and in some cases, cell number is also reduced. There is evidence that adipocyte number is reduced by the process of apoptosis, or programmed cell death, the cell-directed process of an elimination of individual cells without an inflammatory response. This has very important implications as it was thought that once fat cells were created they could not be eliminated without surgical removal.

The effects of leptin are not due to reduced food intake alone. Food restriction results in a smaller loss of body weight and fat mass and includes lean tissue. Food-restricted animals also recover much faster than leptin-treated animals after treatment withdrawal. The advantages of leptin treatment over food restriction for weight control are obvious but only where leptin sensitivity is sufficient. Data suggest that leptin-induced changes in energy expenditure and adipose tissue cellularity may last for an extended period slowing body weight recovery. The work presented here provides additional insight into the effects of leptin on energy metabolism and adiposity, and examines the slowed body weight recovery after leptin administration. Chapter 3 focuses on energy metabolism and enhanced thermogenic processes during recovery from leptin administration in rats. Chapter 4 focuses on the changes in adipose tissue cellularity and the loss of fat cells through leptin-induced adipose tissue apoptosis. Adipose is often a difficult tissue to assay, so Chapter 5 extensively details protocols used for assaying DNA, RNA, and protein in adipose tissues.

Leptin administration resulted in a combination of prolonged enhancement of energy expenditure and a loss in adipocyte number, at least in the inguinal fat pad, which contributed to a slowed body weight recovery. These results revealed more questions and may provide eventual targets for anti-obesity therapies. A complete analysis of the effects of leptin on the changes in energy metabolism, uncoupling proteins, adipose tissue cellularity and apoptosis is discussed. The discovery of leptin and other molecules in recent years has dramatically advanced our knowledge about the pathogenesis of weight control. Continued study of the leptin pathway and its regulation may lead to new targets for drug development in the treatment of obesity and related disorders.

CHAPTER 2

REVIEW OF THE LITERATURE

Obesity and Health Risk

In 1995, there were an estimated 200 million obese adults worldwide and another 18 million under-five year-old children classified as overweight, defined as having a Body Mass Index (BMI) of 30 or more (WHO, 1998). As of 2000, the number of obese adults has increased to over 300 million (WHO, 2002). In the United Stated, more than half of all adults are considered overweight and approximately 20% are extremely overweight or obese (Flegal et al., 1998). The prevalence of overweight among U.S. adults increased by 61% from 1991 to 2000 (CDC, 2002). Public health recommendations call for weight loss in those who are overweight with associated medical conditions or who are obese (National Institute of Diabetes and Digestive and Kidney Diseases, 2000). A large body of evidence indicates that higher levels of body fat are associated with an increased risk for the development of numerous adverse health consequences (Visscher & Seidell, 2001). These are listed in Table 2.1.

Table 2.1. Obesity-associated diseases and conditions

- Type 2 diabetes mellitus
- Hypertension
- Cardiovascular disease (CVD)
- Stroke
- Gallbladder disease
- Liver disease
- Musculoskeletal disorders (osteoarthritis, low back pain)
- Sleep apnea and pulmonary dysfunction
- Reproductive dysfunction
- Cancer (endometrium, breast, kidney, gallbladder, colon)

Obesity is defined as an excess of body fat (Bray, Bouchard, & James, 1998). Since the precise measurement of an individual's body fat is time consuming, expensive, and difficult to do on large populations, adiposity and health risk associated with obesity is commonly predicted using the BMI and waist circumference or waist-to-hip ratio (an indicator of body fat distribution). BMI, which describes relative weight for height, is significantly correlated with total body fat content (Kraemer, Berkowitz, & Hammer,

$$BMI = \frac{weight_{kg}}{height_{m^2}}$$

1990). The Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults (National Heart Lung and Blood Institute, 1998) define *overweight* as a BMI of 25 to 29.9 and *obesity* as a BMI of at least 30 (Table 2.2). Using these criteria, the third U.S. National Health and Nutrition

	BMI	Disease Risk* (Relative to Normal Weight and Waist Circumference)			
	(kg/m ⁻)	Men ≤ 40 in (102 cm) Women ≤ 35 in (88 cm)	> 40 in (102 cm) > 35 in (88 cm)		
Underweight	< 18.5	_	_		
Normal	18.5-24.9	_	-		
Overweight	25.0-29.9	Increased	High		
Obesity (Class I)	30.0-34.9	High	Very High		
Obesity (Class II)	35.0-39.9	Very High	Very High		
Extreme obesity (Class III)	\geq 40	Extremely High	Extremely High		

 Table 2.2. Classification of overweight and obesity by BMI, waist circumference, and associated disease risk (National Heart Lung and Blood Institute, 1998)

*Disease risk for type 2 diabetes, hypertension, and CVD.

Examination Survey (1988-94) shows that approximately 25% of US adult women and 20% of US adult men are clinically obese (Flegal et al., 1998) and that the prevalence of obesity has increased more than 50% within the past 10 to 15 years (from 14.5% to 22.5%). Individuals with a BMI of at least 30 have a 50% to 100% increased risk for death due to all causes, compared with individuals at a BMI of 20 to 25 (Manson et al., 1987; Troiano et al., 1996; National Heart Lung and Blood Institute, 1998). In addition, the early onset of obesity leads to an increased likelihood of obesity in later life as well as an increased prevalence of obesity-related disorders (Dietz, 1994; Kotani, 1997).

The economic costs of obesity are estimated to be \sim 7% of total health care costs in the United States (Colditz, 1999) and around 1 to 5% in Europe (Seidell, 1996). It has been calculated that approximately 10% of the total costs of loss of productivity due to sick leave and work disability could be attributable to obesity-related diseases (Narbro et al., 1996). In addition, obesity can have negative consequences on the quality of life and physical, social, and mental functioning (Visscher et al., 2001).

A combination of diet modification, increased physical activity, and behavior therapy can be effective for many individuals. Intentional weight loss (not due to illness) in obese individuals reduces risk factors for and improves symptoms of obesity-related conditions, including heart disease, type 2 diabetes mellitus, osteoarthritis, and others (Allison & Pi-Sunyer, 1995; National Heart Lung and Blood Institute, 1998). Weight loss reduces blood pressure in both overweight hypertensive and nonhypertensive individuals; reduces serum triglycerides and increases high-density lipoprotein (HDL)cholesterol; and generally produces some reduction in total serum cholesterol and lowdensity lipoprotein (LDL)-cholesterol. It also reduces blood glucose levels in overweight and obese persons without diabetes; and reduces blood glucose levels and HbA1c in some patients with type 2 diabetes. Based on calculations for relative risk, a weight loss of about 10% would reduce the number of life years with hypertension by 1.2-2.9 years and type 2 diabetes mellitus by 0.5-1.7 years, and life expectancy would increase by 2-7 months (Oster et al., 1999). Although reducing body weight improves the health of obese and overweight individuals, the remedies provided by the \$50 billion/year diet industry have failed to keep weight off in the vast majority of cases (Wadden, 1993). More than 90% of individuals who lose weight by dieting return to their original weight within 2 to 5 years. Successful treatment of obesity will require a better understanding of its causes. Most traditional treatments are targeted at symptoms not the causative factors.

In 1953, G.C. Kennedy suggested that body fat is physiologically controlled and that deviations in weight in either direction elicit a potent counter-response that resists that change. This implies that biological factors determine each individual's body mass, be it lean or obese, and that this state is defended. Thus, obesity is not simply a result of a lack of self-control but is a complex disorder of appetite regulation and energy metabolism controlled by specific biological factors (Campbell & Dhand, 2000). In addition, obesity is not a single disorder but a heterogeneous group of conditions with multiple causes (Figure 2.1) such as a combination of genetic susceptibility, increased availability of high-energy foods and decreased requirement for physical activity in modern society (Kopelman, 2000).

Twin studies, analyses of familial aggregation, and adoption studies all indicate that obesity is largely the result of genetic factors (Stunkard et al., 1990; Barsh, Farooqi, & O'Rahilly, 2000). Genes have been identified that predispose humans and animals to



Environmental Factors

Figure 2.1. Factors influencing the development of obesity (Kopelman, 2000).

obesity, signifying the importance of genetic factors in its development. "Susceptibility genes" increase the risk of developing a characteristic but are not essential for the expression or, by themselves, sufficient to explain the development of a disease (Kopelman, 2000). Obesity-associated monogenic syndromes are rare but genes have been identified with human obesity or its metabolic complications. Some of these genes are involved in appetite regulation and/or mechanisms of thermogenesis. Monogenic rodent models of obesity are characterized by early onset of obesity, hyperinsulinemia, and insulin resistance. It has been proposed that signals reflecting nutritional state are sensed by the brain, which in turn, modulates food intake and energy expenditure (Hetherington & Ranson, 1942; Kennedy, 1953). These signals have been identified using monogenic rodent models of obesity, such as *ob* and *db*, and have provided excellent resources for the study of energy balance and obesity.

The *ob* Gene and Obesity

The genetically obese (ob) mouse, one of five recessive single-gene obesity mutations, was described by Ingalls et al. in 1950. These mice are characterized by hyperphagia, obesity, type II diabetes, insulin resistance, and lower core temperature (Friedman & Leibel, 1992). Hypertrophy of white adipose tissue occurs well before the onset of hyperphagia (Lin, Romsos, & Leveille, 1977; Thurlby & Trayhurn, 1978), even when pair-fed with lean littermates (Trayhurn, 1984) indicating substantial energy conservation by the *ob* mouse. In 1978, D.L. Coleman used parabiosis (crosscirculation) experiments between mutant and wild-type mice to prove that ob mice are deficient for a blood-borne factor that regulates nutrient intake and metabolism (Figure 2.2). A factor produced by normal mice caused ob mice to lose fat mass. db mice, which are resistant to the factor, have an over production of it that caused normal mice to become exceedingly lean and eventually die from starvation. The overexpression of the factor in db mice also causes ob mice to lose fat and eventually starve to death. The identity of that factor was not known until the product of the *ob* gene was positionally cloned by Friedman's group in 1994 (Zhang et al., 1994). Other parabiotic experiments that lesioned the ventral medial hypothalamus (VMH) or simulated the lateral hypothalamus (LH) have demonstrated the importance of this area of the brain in food intake and body weight regulation (Figure 2.2).

The *ob* gene was positioned on chromosome 6 and encodes a 4.5 kilobase (kb) mRNA that translates into a 167 amino acid peptide, including a 21 amino acid signal sequence. This peptide, called 'leptin' (derived from Greek leptos, meaning thin), is normally secreted by adipocytes into the blood stream as a 16 kDa, 146 amino acid



Figure 2.2. Summary of experiments using parabiosis (Casanueva & Dieguez, 1999)

peptide and circulates in free and bound forms (Hamilton et al., 1995; Masuzaki et al., 1995). Human leptin is 84% homologous to mouse and 83% homologous to rat. It has a four-helix bundle tertiary structure (Figure 2.3) that is similar to the tertiary structure of cytokines, protein hormones involved in cell-cell communication, that include interleukin-6 (IL-6), IL-11, IL-12, leukemia inhibitory factor (LIF), granulocyte colonystimulating factor (G-CSF), ciliary neurotrophic factor (CNTF), and oncostatin M (Prolo, Wong, & Licinio, 1998). In circulation, leptin has a half-life of 25 minutes in humans, and is the same in obese and normal-weight individuals (Klein et al., 1996). The short half-life is mainly determined by renal clearance. Leptin is principally expressed in adipocytes of normal animals (Maffei et al., 1995a) but is also synthesized in the placenta (Masuzaki et al., 1997), gastric epithelium (Bado et al., 1998) and bone (Friedman & Halaas, 1998). Several other physiological functions have also been described for leptin: as a signal of energy reserves to the reproductive system (Chehab, Lim, & Lu, 1996; Barash et al., 1996; Spicer & Francisco, 1997; Masuzaki et al., 1997; Magni, Motta, & Martini, 2000), a permissive factor for puberty, and as a factor in the immune system (Finck et al., 1998; Takahashi, Waelput, &

Guisez, 1999), bone formation (Fleet, 2000), and haematopoiesis.

Normally, as lipid accumulates in adipocytes, leptin is produced and released into the bloodstream in proportion to amount of fat mass. Leptin levels in the blood communicate the status of triacylglycerol



Figure 2.3. Protein structure of leptin.

levels in the adjpocytes to the central nervous system (CNS), modulating functions that regulate food intake and energy expenditure. This hypothesis was confirmed by experiments replacing leptin in leptin-deficient ob/ob mice by intraperitoneal (IP) or intracerebroventricular (ICV) injections, which led to significant reductions in food intake, body weight, body fat, and serum insulin (Pelleymounter et al., 1995). The potent effect of injecting leptin ICV indicates a central action of leptin; however, it is not clear whether leptin acts solely through the CNS or peripherally via functional receptors detected in non-neuronal tissues (Table 2.6) (the leptin receptor is discussed in the next section). Campfield et al. (1995) found that IP injections of leptin in ob/ob and dietinduced obese mice resulted in a lowering of body weight within days and by 33 days, 40% of the original weight was lost. A dose-dependent decrease in food intake and body weight occurs in normal rodents as well, but to a lesser extent than in ob mice (Halaas et al., 1995; Pelleymounter et al., 1995; Seeley et al., 1996; Flatt et al., 1997; Halaas et al., 1997; Baile et al., 1997b; Muzzin et al., 2000). Reduced food intake cannot account entirely for the effects of leptin as demonstrated by pair-feeding studies in obese and lean mice (Levin et al., 1996). Leptin-injected mice lose more body weight and adipose tissue mass than their pair-fed counterparts, illustrating an important component of leptin's action is stimulating and regulating energy expenditure independent of food intake (Halaas et al., 1995; Pelleymounter et al., 1995; Levin et al., 1996; Lowell & Spiegelman, 2000).

The *db/db* mouse, which is phenotypically identical to the *ob/ob* mouse, has a mutation in the leptin receptor (Ob-R) gene that gives rise to a defective leptin receptor (Chua, Jr. et al., 1996), and has, by contrast, high circulating levels of leptin.

Administration of leptin to this mouse has no effect on appetite, body weight, or body fat. The obese Zucker fatty rat (fa/fa) also has a mutant leptin receptor. A missense mutation (an A to C conversion at nucleotide position 806) was found in the extracellular domain of all the Ob-R isoforms in fa/fa rats, which resulted in an amino acid change from Gln to Pro at + 269 (the Gln269Pro mutation) (Iida et al., 1996; Takaya et al., 1996; Phillips et al., 1996). Obese Zucker rats pair-fed to lean littermates gained more body fat on the same intake indicating greater energy efficiency (Deb & Martin, 1975). These rats also have a very limited response to leptin administration (da Silva et al., 1998). Single-gene mutations have dramatically advanced the discovery of pathways and the factors involved in the regulation of food intake and body weight. A summary of single-gene mutations is listed in Table 2.3.

Name	Allele	Gene product	Where	Chromosome/ Inheritance	Degree/Onset
Mice					
• Obese	ob	Leptin	Adipocytes	6/Recessive	Extreme/early
• Diabetes	db	Leptin receptor	Hypothalamus; choroid plexus; elsewhere	4/Recessive	Extreme/early
• Agouti	A^{y}	Agouti protein	Ubiquitously in mutants, skin in normal mice	2/Dominant	Moderate/adult
• Fat	fa	Carboxypeptidase E	Endocrine/neuro-endocrine tissues	8/Recessive	Moderate/adult
 Tubby 	tub	Tubby protein	Hypothalamus	7/Recessive	Moderate/adult
Rats					
• Corpulent	La/nf/f	Leptin receptor	Hypothalamus	5/Recessive	Extreme/early
 Zucker 	fa	Leptin receptor	Hypothalamus	5/Recessive	Extreme/early

Table 2.3. Single-gene mutations in rodent obesity (Bray, 1997; Leibel, Chung, & Chua, Jr., 1997; Comuzzie & Allison, 1998; Barsh et al., 2000)

Mutations that result in leptin deficiency lead to massive obesity and other abnormalities (Table 2.4). In humans, it was considered that obesity might be due to a relative or absolute deficiency of leptin. However, most obese humans have a normal gene encoding for leptin (Considine et al., 1995; Maffei et al., 1996; Considine et al., 1996b; Shigemoto et al., 1997) and have high circulating levels of leptin in proportion to fat mass (Pelleymounter et al., 1995; Hamilton et al., 1995; Lönnqvist et al., 1995; Maffei et al., 1995b; Considine et al., 1996c; Friedman et al., 1998). These individuals are leptin-resistant; the high level of leptin does not induce an appropriate reduction in food intake. Leptin resistance may be due to a lack of transport to the hypothalamus, a lack of binding to/activation of the receptor, a defective (post-receptor) signal transduction, or a combination of factors (Caro et al., 1996b).

Table 2.4. Abnormalities of leptin deficiency/resistance

- Hyperphagia
- Obesity
- Preferential storage of calories as adipose tissue
- Susceptibility to diabetes & insulin resistance
- Growth retardation*
- Hypothyroidism*
- Decreased body temperature
- Decreased energy expenditure, including activity
- Decreased immune function
- Infertility
- Elevated blood glucocorticoid concentrations^R
- Impaired growth of bone and lean tissue^R

^{*}Humans with defective receptors but not ligand. ^RRodents but not humans.

Only a handful of individuals with severe obesity have been identified with either a congenital deficiency of leptin (Montague et al., 1997; Strobel et al., 1998; Ozata, Ozdemir, & Licinio, 1999) or a mutation in the leptin receptor gene where the receptor is truncated before the transmembrane domain (Considine et al., 1996a; Clément et al., 1998). Family members homozygous for the mutation exhibited phenotypes of morbid obesity, increased appetite and hyperphagia, and hypogonadotropic hypogonadism (Montague et al., 1997; Strobel et al., 1998). Of those found with a leptin deficiency,

treatment for more than a year with daily subcutaneous injections of recombinant human leptin experienced a marked reduction in body weight, adiposity, appetite, and agerelated regression of gonadotropic function (Farooqi et al., 1999). Humans with a mutation in the receptor also have significant growth retardation and central hypothyroidism, which is not seen in those with a defective ligand (Considine et al., 1996a; Clément et al., 1998). By contrast, mice deficient for leptin or the receptor display very similar endocrine abnormalities; both exhibit reduced levels of growth hormone and retarded linear growth (Bray & York, 1971; Barsh et al., 2000). When treating obese humans without a leptin deficiency, clinical trials using recombinant leptin have shown only modest and variable amounts of weight loss, but those receiving the greatest dose of leptin lost the most weight (Heymsfield et al., 1999). Leptin must be given in doses that raise serum leptin concentrations to 20 to 30 times the normal concentration in order to promote weight loss in normal lean mice or obese adults.

The Leptin Receptor and Leptin Signaling

The region of the lower brain involved in the regulation of appetite, food intake, and body weight is called the hypothalamus, and is the chief target of leptin (Schwartz et al., 1996c). Five different forms of the leptin receptor (Ob-R) have been identified in the hypothalamus and peripheral locations such as lung, kidney, pancreas, liver, gonads, and adipose tissue (Tartaglia et al., 1995). All share identical extracellular, ligand-binding domains but they differ at the C terminus due to alternative mRNA splicing (Figure 2.4). Four of the five have transmembrane domains, but only Ob-Rb, the "long form", encodes all protein motifs capable of activating the JAK-STAT signal transduction pathway. ObRa is expressed ubiquitously, whereas Ob-Rc, -Rd, and -Re RNAs are only detectable using very sensitive methods (i.e., PCR). Ob-Rb is present in the arcuate, ventromedial, dorsomedial, and lateral hypothalamic nuclei but is not detectable in other brain regions (Fei et al., 1997). Ob-Re is truncated before the membrane-spanning domain and is the only secreted isoform, which may act as a soluble binding-protein for leptin in the blood (Lollmann et al., 1997). *db/db* mice have a mutation that prevents Rb but allows Ra expression, demonstrating that the cytoplasmic domain is required for signal transduction and normal energy homeostasis (Chua, Jr. et al., 1996). The Zucker fatty rat has the *fa* mutation, which is a missense mutation in the extracellular domain on the two major isoforms of the leptin receptor, Ob-Ra and -Rb (da Silva et al., 1998).



Figure 2.4. Leptin receptor isoforms and receptor mutations in rodent models of obesity (Friedman et al., 1998)

The leptin receptor is a member of the class 1 cytokine receptor family and does not have intrinsic tyrosine kinase activity (Porras et al., 1997; Schwartz et al., 2000). However, they do have docking sites for janus kinases (JAK), a family of tyrosine kinases involved in intracellular cytokine signaling (Tartaglia, 1997). Activated JAK phosphorylates members of the signal transduction and transcription (STAT) family of intracellular proteins (Figure 2.5). STAT proteins, in turn, stimulate transcription of target genes. The mutant *fa* receptor causes defective JAK-STAT signaling and constitutive activation of egr-1 transcription, a common target of several cytokines and growth factors, possibly leading to downregulation of signaling pathways and contribution to leptin resistance (White et al., 1997; da Silva et al., 1998).



Figure 2.5. Leptin receptor signaling (Schwartz et al., 2000)

Leptin also affects neuronal firing rate independently of its transcriptional effects. For example, a subset of 'glucose-responsive' neurons in the hypothalamus become hyperpolarized (decrease firing rate) within minutes of leptin stimulation (Spanswick et al., 1997). Glucose indirectly increases neuronal firing rate through its oxidation to generate ATP (Dunn-Meynell, Rawson, & Levin, 1998).

The neurotransmitters that coordinate behavioral and metabolic responses and CNS pathways that lie downstream of leptin are continuing to be elucidated. Several key molecules in the neural network related to leptin signaling include the brain peptides neuropeptide Y (NPY) and agouti-related protein (AGRP), which stimulate food intake, and α -melanocyte-stimulating hormone (α -MSH) and cocaine- and amphetamineregulated transcript (CART), which decrease food intake (Erickson, Hollopeter, & Palmiter, 1996; Fan et al., 1997; Schwartz et al., 2000). (A list of factors involved in food intake and energy homeostasis can be found in Table 2.5.) During the active depletion of body fat stores, NPY gene expression and secretion of NPY peptide in the hypothalamus are increased (White et al., 1990; Kalra et al., 1991), which reduces leptin and insulin signaling in the brain (Wilding et al., 1993) leading to the stimulation of food intake. Considering the function of NPY, one may reason that an absence of NPY would lead to a lower food intake and body weight. However, NPY-deficient (NPY^{-/-} knockout) mice have normal body weight and food intake, revealing that other factors are involved (Erickson, Clegg, & Palmiter, 1996). Leptin inhibits NPY expression and secretion (Stephens et al., 1995; Schwartz et al., 1996a). Interestingly, NPY^{-/-} ob/ob mice have reduced hyperphagia and obesity (Erickson et al., 1996), "indicating that the full response to leptin deficiency requires NPY signaling" (Schwartz et al., 2000). Leptin also

stimulates the expression of corticotrophin-releasing hormone (CRH), an inhibitor of food intake, in the paraventricular nucleus (PVN) (Schwartz et al., 1996c; Gardner, Rothwell, & Luheshi, 1998).

Table 2.5.	Modulators of food	intake and ener	gy homeostasis
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† Food Intake (Anabolic)	↓ Food Intake (Catabolic)
• AGRP (Agouti-related peptide)*	Anorectin
• Galanin	Bombesin
• GHRH (Growth hormone releasing	Caerulein
hormone)	• CART (Cocaine- & amphetamine-regulated
 Glucagon, ICV 	transcript)
 MCH (Melanin-concentrating 	Calcitonin
hormone)	 Calcitonin-gene-related peptide
 Norepinephrine (α2 receptor) 	• CCK (Cholecystokinin)
 NPY (Neuropeptide Y)* 	 CRH (Corticotropin-releasing hormone)*
 Opioids (β-endorphin, dynorphin) 	• Cyclo (His-Pro)
• Orexin A & B	• Enterostatin
• Peptide YY	• Insulin*
	 Glucagon, peripheral
	• GLP-1 (Glucagon-like peptide 1)
	 IL-1β (Interleukin-1β)
	• IGF (Insulin-like growth factor)
	• Leptin*
	 α-MSH (α-melanocyte stimulating
	hormone)*
	• Neurotensin
	• Oxytocin
	• Serotonin
	Somatostatin
	• TRH (Thyrotropin-releasing hormone)
	• Urocortin
	 Vasoactive intestinal peptide
	Vasopressin

*These molecules are particularly important in the regulation of adiposity.

Proopiomelanocortin (POMC) is an important second messenger in the leptin pathway with POMC neurons in the arcuate nucleus that express the long form of the leptin receptor. Leptin modulates POMC gene expression and POMC cleavage to form α-MSH and adrenocorticotrophic hormone (ACTH) (Thornton et al., 1997; Mountjoy & Wong, 1997). α-MSH acts via the melanocortin-4 receptor (MC4-R) to reduce food intake and increase energy expenditure (Fan et al., 1997). MC4-R knockout mice are hyperphagic and very obese (Huszar et al., 1997), while humans with MC4-R mutations show similar characteristics (Vaisse et al., 1998; Yeo et al., 1998). AGRP blocks MC4-R leading to the obese phenotype (Lu et al., 1994). Agouti, normally synthesized in hair follicles, is overexpressed in the hypothalamus of the agouti ($A^{y/a}$) yellow obese mouse. In addition, agouti-related transcript (ART) or agouti-related peptide (AGRP) is an endogenous antagonist of MC3 and MC4 receptors. Overexpression of AGRP results in obesity (Ollmann et al., 1997). AGRP is upregulated by fasting (Hahn et al., 1998; Broberger et al., 1998) and leptin deficiency (Shutter et al., 1997). These data demonstrate the importance of central melanocortin signaling in body weight regulation (Figure 2.6).

Leptin resistance is common in obesity and the mechanisms responsible may include mutations of the leptin receptor, leptin transport, or one or more of the genes involved in leptin signal transduction. A mutation in the hypothalamic leptin receptor is associated with leptin resistance and extreme obesity (Clément et al., 1998). There are also cases that suggest defective transport of leptin across the blood-brain barrier results in leptin resistance (Schwartz et al., 1996b; Friedman et al., 1998). Obese individuals have higher plasma-to-cerebrospinal fluid (CSF) ratios of leptin in comparison to lean controls (Caro et al., 1996a; Schwartz et al., 1996b), suggesting a potential source of leptin resistance is due to defective (or impaired) transport into the CSF. Leptin enters the CNS via a specific and saturable transport system in proportion to plasma level (Baura et al., 1993;

Banks et al., 1996; Caro et al., 1996a; Schwartz et al., 1996b; Banks, 2001), but one report indicates that transport is actually increased at lower leptin concentrations (Mantzoros et al., 1997). It may be mediated through the short form of the leptin receptor (Ob-Ra) present in high density in the choroid plexus (Tartaglia et al., 1995; Schwartz et al., 1996b) and in brain microvessels at the blood-brain barrier (Bjørbæk et al., 1998b). A rodent model for leptin resistance due to a decreased transport into the CSF is the New Zealand Obese (NZO) mouse, which does not respond to peripheral leptin but is normally responsive to leptin when administered ICV (Van Heek et al., 1997).

Another form of resistance results from impaired downstream leptin signaling. Following the activation of the leptin receptor, a protein called "suppressor of cytokine signaling 3" (SOCS-3) is expressed that functions to inhibit further leptin signaling (Bjørbæk et al., 1998a; Baskin, Breininger, & Schwartz, 2000; Ziotopoulou et al., 2000). Increased expression or decreased degradation of this peptide could result in leptin resistance. Details of this and other possible mechanisms are actively being investigated.

Body Weight Regulation and Leptin

Energy homeostasis, or energy balance, is the equilibrium between energy intake (caloric intake) and energy expenditure. Where energy intake and expenditure are not equal, body weight is gained or lost. Typically, our daily meal-to-meal energy intake is so varied that it does not match our daily energy expenditure. However, when measured over many meals, cumulative energy intake closely matches energy expenditure (Edholm, 1977). When there is a drastic change in energy intake, such as during fasting or caloric restriction (a period of no or low caloric intake), a period of overeating (hyperphagia) is triggered when food becomes available, followed by a gradual return to normal levels

(Harris, Kasser, & Martin, 1986). This seems to be an effort to recover lost body weight and return to baseline values. To explain this phenomenon, Kennedy (1953) proposed that body fat is physiologically controlled and that deviations in weight in either direction elicit a potent counter-response that resists that change. The long-term regulatory process to promote stability of energy stored in the form of fat is called the 'lipostat theory' (Schwartz et al., 2000).

The stability of fat stores is directly related to leptin production and plasma concentration with leptin being a strong afferent signal indicating the size of fat stores. Plasma leptin levels correlate with absolute fat mass better than BMI or percent body fat, and the rate of leptin production is directly related to fat mass (Pelleymounter et al., 1995; Friedman et al., 1998). An increase in body fat is associated with an increase in leptin production and subsequent reduction in food intake. Decreasing body fat leads to lower leptin levels, which in turn stimulates food intake. By such a mechanism, body weight is maintained within a relatively narrow range in normal individuals or animals. Females actually secrete up to twice as much leptin as males, given the same amount of body fat (Frederich et al., 1995a; Couillard et al., 2000).

Acute changes in energy flux (drastic changes in food intake) can dissociate leptin secretion from body fat levels. For example, overfeeding dramatically increases plasma leptin concentration, whereas food deprivation lowers leptin levels much more rapidly and to a greater extent than the relative changes in body fat content, indicating that leptin release is regulated by factors other than changes in body fat mass (Boden et al., 1996). "This exaggerated early decline of leptin levels would enable compensatory responses to be activated before energy stores are substantially depleted" (Schwartz et al., 2000). After a meal, leptin does not increase significantly nor does it, by itself, lead to meal termination (Maffei et al., 1995b; Considine et al., 1996c). It appears to function largely in the long-term control of energy balance and influences the quantity of food consumed relative to the amount of energy expended. In addition, in the human, leptin is secreted in a circadian and pulsatile fashion with a nocturnal rise and an organized pattern of pulsatility, with an average of 32 pulses per day, each lasting an average of 33 minutes (Licinio et al., 1997; Licinio et al., 1998a; Licinio et al., 1998b).

Plasma leptin concentrations will actually change more rapidly during weight loss than during weight gain (Maffei et al., 1995b). Rather than acting to suppress body fat, Rosenbaum and Leibel (1999) suggest that the chief function of leptin may be to defend fat stores. In this model, depletion of adipose tissue lowers the rate of leptin synthesis and leptin actions in the CNS, and thus evokes compensatory changes in energy intake to favor a return to the usual body weight. When adequate energy intake and energy stores are restored, an individualized threshold concentration of leptin is exceeded and the behavioral, metabolic, and endocrine conditions of energy restriction are relieved. A further elevation in serum leptin concentration would have little or no effect unless supraphysiological concentrations are attained (Rosenbaum & Leibel, 1999). A neurohumoral system that functions to conserve (rather than to reduce) body energy stores presents clear evolutionary advantages, especially during times of undernutrition.

The neuroendocrine abnormalities seen during starvation are similarly found in totally leptin-deficient *ob/ob* mice (Ahima et al., 1996) and conditions that decrease leptin levels, independent of food intake, activate a hormonal response that is characteristic of the starved state (Boden et al., 1996; Hardie et al., 1996; Ahrén et al., 1997; Weigle et al.,
1997). During starvation, thyroid hormone and growth hormone are reduced to help conserve energy but glucocorticoid hormone release is increased. Fertility is lessened so that reproduction only takes place when energy demands of pregnancy and lactation can be met. Associated with this is the low levels of leptin found in individuals with anorexia nervosa and amenorrhea (Rothwell, Saville, & Stock, 1984; Ahima et al., 1996). Using exogenous leptin to prevent a starvation-induced fall in leptin substantially blunts the changes in gonadal, adrenal and thyroid axis in male mice, and prevents the starvationinduced delay in ovulation in female mice (Ahima et al., 1996). In contrast, leptin repletion during a period of starvation has little or no effect on body weight, blood glucose or ketones (Ahima et al., 1996). Exogenous replacement of leptin in a fasted animal reverses many of the physiological responses to starvation or fasting, such as reversing the suppression of the thyroid axis and release of glucocorticoid. Therefore, it has been suggested that the main role of leptin might be to respond to energy deprivation and not energy excess. This function could have evolved from an ancestral situation of human famine and scarcity of food rather than excess. The most important action of leptin may result from a fall in concentration with a major role to improve survival when food is scarce (Flier, 1998). Another adverse result of fasting or food restriction is the loss of valuable lean tissue, which has high metabolic activity (Leibel, Rosenbaum, & Hirsch, 1995). For these reasons, drastic food restriction is not a successful method for long-term maintenance of weight loss. Leptin maintains lean tissue mass and selectively targets adipose tissue for elimination. These data suggest that leptin may be a good alternative to food restriction, such as during a low-calorie diet; however, most human obesity is characterized by resistance to, rather than a lack of leptin. We must continue to

investigate the mechanism and pathways involved with leptin. Characteristic effects of leptin versus food restriction are listed in Table 2.6.

Leptin administration	Food restriction/Starvation
\downarrow Food intake	\downarrow Food intake
↑ Energy expenditure	\downarrow Energy expenditure
\downarrow Fat mass	↓ Fat mass
n/c lean mass	\downarrow Lean mass
↑ UCP1, 2, 3 (BAT/WAT)	\downarrow UCP1, 3 (BAT); \uparrow UCP2, 3 (SKM)
n/c plasma fatty acids or ketones	\uparrow plasma fatty acids and ketones
Weight regain slow	Weight regain rapid
Adipose tissue apoptosis pos (+)	Adipose tissue apoptosis neg (-)
Difference wated in held test	

 Table 2.6. Characteristics of leptin administration and food restriction

Differences noted in **bold** text.

Several molecules have been implicated in the control of short-term and long-term food intake and energy homeostasis (Table 2.5). Insulin is one of these important factors and has an intricate relationship with leptin. Both leptin and insulin circulate in the blood in concentrations proportional to body fat content (Bagdade, Bierman, & Porte, Jr., 1967; Maffei et al., 1995b; Considine et al., 1996c). The receptors for both are expressed in brain neurons involved in energy intake (Baskin et al., 1988; Cheung, Clifton, & Steiner, 1997; Baskin, Breininger, & Schwartz, 1999). Administration of either peptide directly into the brain reduces food intake, whereas deficiency of either has the opposite effect (Figure 2.6). Under conditions of leptin/insulin deficiency, the NPY/AgRP neurons are activated and the POMC neurons are inhibited in the arcuate nucleus, activating/deactivating mechanisms to increase body fat. Increased leptin/insulin action inhibits the NPY/AgRP anabolic pathway and stimulates the POMC catabolic pathway,

activating/deactivating mechanisms to reduce body fat. The absence of leptin causes severe obesity with hyperphagia despite high insulin levels. In contrast, obesity is not induced by insulin deficiency; fat synthesis and deposition actually requires insulin.



Figure 2.6. Role of arcuate nucleus neurons in adipose signaling and pathways of leptin/insulin action (Schwartz et al., 2000).

The rate of insulin-stimulated glucose utilization in adipocytes is a key factor linking leptin secretion to body fat mass (Mueller et al., 1998). Insulin upregulates leptin expression by fat cells *in vitro* (Rentsch & Chiesi, 1996; Leroy et al., 1996) and in rodents *in vivo* (Cusin et al., 1995), but it does not acutely regulate plasma leptin levels in humans (Kolaczynski et al., 1996; Dagogo-Jack et al., 1996). However, when insulin and

glucose levels were clamped at basal levels during a 72-hour fast in four normal subjects, the typical fall in leptin concentration did not occur, suggesting that insulin and/or glucose does play a role in the regulation of leptin release in humans (Boden et al., 1996). Additional evidence of this lies with the hexosamine biosynthetic pathway, an intracellular 'sensor' of energy (carbohydrate, amino acids, and fat) availability within the cell that mediates the effects of glucose on the expression of several gene products (McClain & Crook, 1996). Leptin transcription has been found to be stimulated in both fat and muscle cells by hexosamines (Wang et al., 1998a).

The central administration of leptin enhances insulin-stimulated glucose metabolism in all tissues, including BAT and skeletal muscle, but not WAT (Kamohara et al., 1997; Cusin et al., 1998). This also occurs under fasting conditions and in the presence of hyperinsulinemia at clamped glucose after an acute intravenous injection of leptin in normal rodents (Sivitz et al., 1997).

Leptin ultimately targets its source, the adipocyte, by stimulating fat oxidation. This is demonstrated by the typically low RQ measured after the administration of leptin (Hwa et al., 1996; Hwa et al., 1997) and reflects the preferential depletion of fat stores (Halaas et al., 1995; Pelleymounter et al., 1995; Campfield et al., 1995; Halaas et al., 1997; Sarmiento et al., 1997; Kaibara et al., 1998). Despite extensive lipolysis that occurs in all fat depots, leptin-treated normal mice maintain generally normal levels of substrates (such as glucose, ketone bodies, and nonesterified FFA) and hormones (including insulin, glucagons, thyroxine, and glucocorticoids) (Sarmiento et al., 1997; van Dijk et al., 1999). In addition, if normal rats are made hyperleptinemic by infusion of (adenovirus) AdCMV-leptin, they show rapid nonketotic fat loss (-87%) within six days, which

persists for over two weeks (Zhou et al., 1999). This effect was mediated through downregulation of lipogenic enzymes and their transcription factor, PPAR γ , and upregulation of enzymes of fatty acid oxidation and their transcription factor, PPAR α , and uncoupling proteins, UCP1 & UCP2. This was also accompanied by the loss of adipocyte markers, aP2, TNF α and leptin, and by the appearance of preadipocyte marker Pref-1.

A highly significant inverse correlation exists between adipocyte PPAR γ expression and BMI (*r*=-0.7) (Montague et al., 1998). The PPARs (peroxisome proliferatoractivated receptors) are members of the nuclear receptor superfamily. They server to activate the expression of genes that encode enzymes of fatty acid metabolism and are required for the differentiation of adipose tissue *in vivo* and *in vitro* (Chawla et al., 1994; Rosen et al., 1999). PPAR γ_2 is expressed exclusively in adipocytes. It plays a role in their differentiation (Tontonoz et al., 1994a; Tontonoz, Hu, & Spiegelman, 1994b) and in the upregulation of lipogenic enzyme gene expression (Schoonjans, Staels, & Auwerx, 1996). TNF α , produced by preadipocytes and adipocytes, is elevated in obesity with up to 3-fold increases in mRNA, protein, and circulating levels (Hotamisligil et al., 1995; Dandona et al., 1998). It stimulates lipolysis by inducing insulin resistance through reduced insulin signaling, downregulation of lipoprotein lipase activity (suppression of lipogenesis), and upregulation of hormone-sensitive lipase activity and leptin production (Hotamisligil et al., 1994; Hauner et al., 1995; Hotamisligil et al., 1996).

Adipose tissue is a target organ for glucocorticoids. These hormones are known to play very active role in the expression of many genes related to lipogenesis and fat storage. Obese rodents, particularly *db/db* and *ob/ob* mice, exhibit markedly increased

glucocorticoid production which is not seen in comparable obese human subjects (Bray et al., 1971; Barsh et al., 2000). High circulating levels of exogenous or endogenous glucocorticoids stimulate food intake and inhibit the secretion of growth hormone, thyrotropin, and norepinephrine (Lobo, Remesar, & Alemany, 1993; Strack et al., 1995; Rohner-Jeanrenaud & Jeanrenaud, 1997), while adrenalectomy ameliorates the obesity-related phenotypes. The phenotypic differences in leptin deficiency between humans and mice may be related to the high level of glucocorticoids in obese mice (Farooqi et al., 1999).

Increased glucocorticoid levels and decreased sympathetic neural activity may contribute to the elevated *ob* mRNA expression observed in genetically obese, hyperglucocorticoid rodents. Glucocorticoid treatment of isolated rat adipocytes increased both *ob* mRNA and secreted leptin levels (Slieker et al., 1996). The addition of glucocorticoids at physiological concentrations to the medium of cultured visceral adipose tissue from nonobese subjects showed stimulated leptin secretion, but the effect was more pronounced in cultured visceral adipose tissue from obese subjects, suggesting that glucocorticoids and a greater responsiveness of the *ob* gene contributes to the hyperleptinemia seen in human obesity (Halleux et al., 1998).

Although glucocorticoids appear to increase leptin levels, other research suggests that glucocorticoids promote obesity by inducing leptin resistance (Figure 2.7) (Ur, Grossman, & Després, 1996). A single injection (1 μ g ICV) of leptin suppressed food intake of adrenalectomized mice similar to that of sham operated animals, indicating that glucocorticoids are not essential for leptin to suppress food intake (Mistry, Swick, & Romsos, 1997). However, a larger dose of leptin (3 μ g ICV) had stronger and more-

lasting effects in decreasing both food intake and body weight when administered to adrenalectomized rats (Zakrzewska et al., 1997). Physiological glucocorticoid replacement in adrenalectomized mice treated with leptin (0.5 μ g/h infusion) increased food intake but not body weight, and did not reverse leptin inhibition of hypothalamic NPY mRNA levels (Solano & Jacobson, 1999), indicating that normal levels of glucocorticoid can interfere with some leptin actions. Replacement with higher amounts of glucocorticoid caused a greater increase in food intake and an increase in body weight and caused a dose-dependent inhibition of the effects of leptin (Zakrzewska et al., 1997). Conversely, exogenous leptin reduces corticosterone levels in *ob/ob* mice. Leptin directly inhibited glucocorticoid secretion by normal human and rat adrenal gland as demonstrated by preincubating isolated primary cells from adrenal glands with recombinant leptin (Pralong et al., 1998). Basal cortisol secretion was unaffected by



Figure 2.7. Glucocorticoid-leptin pathway in energy metabolism.

leptin, but a significant and dose-dependent inhibition of ACTH-stimulated cortisol secretion was observed. Leptin and cortisol have an inverse circadian rhythm, suggesting that a regulatory feedback mechanism is present (Casanueva et al., 1999).

Under normal conditions, the morphological analyses of white fat depots of lean C57 mice show that the average size of fat cells differs between the depots: epididymal/parametrial > retroperitoneal > inguinal in both male and female mice (Sarmiento et al., 1997). Leptin injected subcutaneously (20 mg/kg/day) for 14 days reduced the size of large unilocular adipocytes (Figure 2.9) to smaller unilocular or multilocular adipocytes. The fat depots were depleted at approximately the same rate by leptin administration with reductions of 65-70% by 8 days and 80-94% by 15 days.

Since the long form of the receptor is found mainly in the hypothalamus, it is thought that leptin exerts its actions primarily through the central nervous system. However, expression of the long form of the leptin receptor, Ob-Rb, has been detected in white and brown adipocytes (Lee et al., 1996; Siegrist-Kaiser et al., 1997; Kielar et al., 1998) and other tissues (Table 2.7), and *in vitro* experiments have demonstrated the direct effect of leptin on peripheral tissues. In isolated white adipocytes, leptin regulated fat metabolism by decreasing insulin-induced glucose uptake, inhibiting fatty acid biosynthesis, and induced the synthesis of lipolytic enzymes and the expression of uncoupling proteins (Sarmiento et al., 1997; Shimabukuro et al., 1997; Muller et al., 1997). In isolated muscle cells (C2C12 myotubes), leptin has insulin-like effects on glucose uptake and glycogen synthesis, likely through the activation of phosphoinositide (PI) 3-kinase, without affecting insulin action (Berti et al., 1997). Leptin was also reported to have a non-significant effect on basal or insulin-stimulated glycogen synthesis in isolated soleus

muscle of wild-type mice, while it inhibited these processes in muscles of *ob/ob* mice (Liu, Emilsson, & Cawthorne, 1997). The full-length leptin receptor (Ob-Rb) was found expressed in soleus muscle of both *ob/ob* and wild-type mice but with no detectable differences in expression level.

 Table 2.7. Location for the expression of the long form of the leptin receptor, Ob-Rb

• • •				
Organ, tissue, or cell type				
• Brain*	• Colon			
• Heart	• White & brown adipocytes			
• Placenta	• Bone marrow			
• Lung	• Marrow stromal cells			
• Liver	• Yolk sac			
• Muscle	• CD34 ⁺ cells			
• Kidney	• CD4 ⁺ and CD8 ⁺ lymphocytes			
• Pancreas	• Platelets			
• Spleen	• Endothelial cells			
• Thymus	• Leydig cells			
• Prostate	• Mammary gland during pregnancy and lactation			
• Testes	 Placental syncytiothrophoblasts 			
• Ovary	• Leukemia cells (1° AML)			
• Small intestine				
*Primary site.				



E2, estrogen; FFA, free fatty acids; GH, growth hormone; GR, glucocorticoid receptor; INS, insulin; RA, retinoic acid

Figure 2.8. Regulation of food intake and energy homeostasis.

Thermogenesis and Uncoupling Proteins

In response to environmental temperature or diet, animals and humans adjust heat production by a mechanism known as adaptive thermogenesis, which serves to protect the organism from cold exposure and unusual changes in diet (Lowell et al., 2000). For example, both acute and chronic cold exposure increases oxygen consumption (an indirect measure of heat production) nearly two- to four-fold in rodents (Davis et al., 1960; White et al., 1990). Humans exhibit a much smaller response to changes in temperature (Dauncey, 1981). The principal site of adaptive thermogenesis is a specialized tissue called brown adipose tissue (BAT), located subcutaneously in the intrascapular region of the back with smaller depots in the axillary, cervico-intramuscular, intercostals, periaortic, and perirenal regions (Jansky, 1973; Foster & Frydman, 1979; Himms-Hagen, 1985). Humans lose most of their BAT capacity soon after birth, a condition that could have arisen by artificially controlling our environment in a narrow range of temperature, thereby limiting our exposure to cold.

BAT is easily distinguished from WAT morphologically. WAT normally consists of 80% to 90% lipid (Bloom & Fawcett, 1972) with large unilocular lipid droplets and very few mitochondria (Figure 2.9). In contrast, BAT is made up of multilocular lipid-storing cells, called brown adipocytes (Figure 2.10), which have a high concentration of mitochondria, the energy producing organelle of the cell, and very active metabolism. It was in 1976 that the membranous protein specifically induced in BAT mitochondria of cold-adapted rats was identified as thermogenin or uncoupling protein (UCP) (Ricquier & Kader, 1976; Desautels, Zarorbehrens, & Himms-Hagen, 1978; Heaton et al., 1978; Ricquier et al., 1979). UCP is a 307 amino acid, 32,000 Da protein

that may be up to 10% of the protein present in BAT mitochondria (Klingenberg & Huang, 1999). Administration of norepinephrine or β_3 -adrenergic receptor (β_3 -AR) agonists produces the same effects seen in BAT after cold exposure (Himms-Hagen, 1990; Cousin et al., 1992; Garruti & Ricquier, 1992).



Figure 2.9. Unilocular white fat cell.



Figure 2.10. Multilocular brown fat cell.

Normally, ATP synthesis is coupled to ATP use where a fixed number of protons (H^+) are pumped across the mitochondrial inner membrane and re-enter the mitochondrial matrix by means of ATP synthase, generating ATP for cellular work (Figure 2.11). UCP, in the inner membrane of mitochondria, provides a path for protons to return to the mitochondrial matrix without passing through the F_0F_1 complex for ADP phosphorylation (Nedergaard & Lindberg, 1982; Nicholls & Locke, 1984; Bouillaud et al., 1985; Jacobsson et al., 1985; Himms-Hagen, 1985; Bouillaud, Weissenbach, & Ricquier, 1986; Klingenberg, 1990; Klaus et al., 1991; Nedergaard & Cannon, 1992; Ricquier & Bouillaud, 1997). This, in turn, dissipates (uncouples) the proton gradient allowing mitochondria to oxidize substrates rapidly and release the oxidation energy as heat. Although uncoupling is a normal occurrence and is limited by the hydrogen input from

substrates (Klingenberg et al., 1999), the unique UCP protein specifically and highly regulates the degree of uncoupling.



Figure 2.11. Energy metabolism of the mitochondria (Lowell et al., 2000).

Two additional uncoupling proteins, homologues of BAT UCP, were identified in the late 1990's. These are UCP2, primarily found in WAT (Fleury et al., 1997; Tu et al., 1999; Silva, 2000), and UCP3, primarily found in skeletal muscle (Gong et al., 1997; Vidal-Puig et al., 1997; Boss et al., 1997b). The original BAT UCP is now commonly known as UCP1. Even more recently, two others have been identified: UCP4 in the brain (Mao et al., 1999) and UCP5 in the brain and testis (Adams, 2000; Yu et al., 2000). The

complete tissue expression and amino acid homology of the UCPs can be found in Tables 2.8 and 2.9. The bulk of what is known about UCP in relation to energy and adipose tissue metabolism largely concerns the first three proteins; therefore, continued discussion will relate to UCP1, UCP2 and UCP3 in brown and white adipose tissues.

UCP1	BAT, WAT?
UCP2	WAT, BAT, skeletal muscle, heart, liver,
	kidney, spleen, thymus, bone marrow, brain
UCP3	Skeletal muscle, BAT, WAT
UCP4	Brain
UCP5	Brain, testis, liver, skeletal muscle
D 110	· • • · · · ·

Table 2.8. Tissue expression of the UCPs

Boldface indicates predominant expression.

	UCP2	UCP3	UCP4	UCP5
UCP1	57-59%	51-57%	29%	34%
UCP2		71-73%	33%	38%
UCP3			34%	39%
UCP4				39%

 Table 2.9. UCP amino acid homology

UCP has a tripartite structure that is typical of other members of the mitochondrial carrier family (Figure 2.12) (Saraste & Walker, 1982; Aquila, Link, & Klingenberg, 1987; Klingenberg et al., 1999). It is divided into three similar domains each containing about 100 amino acid residues and a pair of transmembrane α -helices (Aquila et al., 1987; Jezek, Modriansky, & Garlid, 1997).

UCP1 is generally considered a very simple proton transporter or carrier that does not require ATP or involve cotransportation or exchanging of other molecules. It has been proposed that UCP1 may not function by transporting protons as such, but proton



Figure 2.12. Topology of UCP in the inner mitochondrial membrane (Klingenberg et al., 1999).

transport may be an indirect consequence of anion transport (Garlid et al., 1996; Nedergaard et al., 2001). This occurs by the free movement of protonated fatty acids in the lipid phase of the membrane that undergo de-protonation on the inner side of the membrane (Bouillaud et al., 2001). The anionic form of the fatty acid is then driven back to the outer side by the mitochondrial carrier, where it can be protonated starting a new cycle.

At least four parameters influence H^+ transport activity by UCP1: free fatty acid (FFA) concentration (substrate), ATP4- concentration, pH and membrane potential. At thermoneutrality (28°C), mitochondria are largely coupled by ATP inhibition of UCP1. Free fatty acids can directly interact with UCP1 to enhance its H^+ transport activity and deplete ATP for FFA activation (Klingenberg et al., 1999). Very high FFA concentrations (up to 20 mM) can occur in BAT (Bieber, Pettersson, & Lindberg, 1975) as a result of the stimulation of adrenergic receptors by cold exposure or norepinephrine or β -AR agonist administration causing both a rapid increase in cAMP and cAMP-activated lipolysis (Prusiner et al., 1968; Bukowiecki et al., 1981). FFA can also mimic the norepinephrine effects on adipocytes (Rial & Gonzalez-Barroso, 2001). The acute

drop in ATP concentration causes the dissociation of bound ATP from UCP1, yielding more free UCP1 molecules. This unmasking of nucleotide binding sites allow for greater GDP binding, which is used as a measured index of thermogenic capacity (Rial & Nicholls, 1984; Martins et al., 1991). Thus, high FFA and low ATP concentrations allow for high thermogenic activity in contrast to the low basal thermogenic activity at 28°C.

 H^+ transport by UCP1 increases with pH while a maximum pH depends on the chain length of the FFA (Klingenberg & Winkler, 1985; Winkler & Klingenberg, 1992). With short-chain FFA such as C-8 (caprylic acid) and C-10 (decanoic acid) the pH maximum is low (pH 6 to 6.5) but then increases with increasing chain length and reaches a maximum of pH 8.0 with oleic acid (C-18) (Rial, Poustie, & Nicholls, 1983; Winkler & Klingenberg, 1994; Jezek et al., 1997). Thus, H⁺ transport is most active in the presence of medium (C-12) to long chain (C-18) fatty acids (Klingenberg et al., 1999). FFAs that are more soluble are also better activators. Saturated fatty acids (e.g., palmitic acid, C-16) have lower activity due to the poor aqueous solubility. Membrane potential $(\Delta \psi)$ also drives H⁺ transport. GDP acts to inhibit UCP1 activity by binding to a site on the cytosolic side of the protein, on the outer face of the mitochondrial inner membrane (Rafael, Ludolph, & Hohorst, 1969). The inhibition by GDP can be overridden by a high $\Delta \psi$ in cooperation with FFA (Klingenberg et al., 1999). The UCP2 and UCP3 isoforms share similar basic structure and pH control of nucleotide binding as UCP1 (Klingenberg et al., 1999) but lack the same response to increasing FFA (Rial et al., 2001). A more defined purpose and regulation of UCP2 and UCP3 are currently being investigated.

Prolonged cold exposure, which increases norepinephrine release, causes hyperplasia and hypertrophy of BAT during which dormant brown adipocytes and precursor cells are converted into mature brown adipocytes with many mitochondria and a high UCP1 content (Ricquier et al., 1997). Norepinephrine increases thermogenic activity of BAT by stimulating both the activity of UCP and the synthesis of new UCP. This is achieved through the release of norepinephrine from sympathetic nerves and a β_3 -AR and cAMP-dependent mechanism (Figure 2.13) (Bouillaud et al., 1984; Astrup, 1986; Himms-Hagen, 1989; Borst et al., 1994). UCP synthesis under noradrenergic control is regulated at the level of transcription (Ricquier et al., 1986).



Figure 2.13. Pathway of β -receptor stimulation in lipolysis, fatty acid release and UCP activation (Nedergaard et al., 2001).

Studies also report that UCP2 and UCP3 levels are increased by cold exposure. UCP2 mRNA in BAT, heart and skeletal muscle were increased after forty-eight hours of cold exposure (Boss et al., 1997a). UCP3 expression was increased 3-fold in skeletal muscle after 24-h cold exposure (5°C) (Simonyan et al., 2001) but not after 48 hours (6°C) (Boss et al., 1998). The authors suggest that in skeletal muscle with increased UCP3 levels, cold exposure is required for sensitizing the membrane potential to the uncoupling action of increasing FFAs (Simonyan et al., 2001). UCP2 expression was upregulated in white adipose tissue of young rats that were exposed to cold for 72 hours (Yamashita, Sato, & Mori, 1999) but other reports stated that cold exposure or β_3 -AR agonists did not alter UCP2 expression in WAT (Yoshitomi et al., 1998).

Regular feeding acutely raises metabolic rate by 25-40% in humans and rodents, a phenomenon referred to as the thermic effect of food (TEF) or diet-induced thermogenesis (DIT) (Sims & Danforth E Jr, 1987; Shibata & Bukowiecki, 1987). Although TEF constitutes approximately 10% of daily energy expenditure, it is variable between individuals (Poehlman & Horton, 1992; Reed & Hill, 1996) and, in the long term, may affect whether some individuals gain more weight than others do. TEF lasts 4 to 8 hours depending on the quantity and type of macronutrient ingested (i.e., protein, fat, or carbohydrate). Overfeeding, as demonstrated in rodents induced to overeat by being fed a varied and palatable diet (the 'cafeteria diet'), causes a further marked increase in thermogenesis which serves to reduce, or prevent, the development of obesity (Stock & Rothwell, 1983; Levine, Eberhardt, & Jensen, 1999), while starvation or restricted feeding reduces thermogenesis in an apparent means to conserve energy (Ma & Foster, 1986; Shibata et al., 1987). Overfeeding in lean rats, more specifically, leads to enhanced BAT thermogenic capacity and decreased energetic efficiency (Glick, Teague, & Bray, 1981; Stock et al., 1983; Marchington et al., 1986; Specter et al., 1995). Thus, BAT may function to prevent obesity during periods of high caloric intake. A low-protein diet will also significantly increase thermogenesis similar to overfeeding (Rothwell, Stock, &

42

Tyzbir, 1983). Unlike rodents, humans do not have large distinct depots of BAT; therefore, the metabolic changes seen in rodents are less likely in humans. Skeletal muscle is also being investigated for its contribution to adaptive thermogenesis because it represents up to 40% of total body weight in humans and has significant mitochondrial capacity.

BAT activity is reduced in some genetically obese animals whereas physiological or pharmacological activation of BAT boosts energy expenditure and decreases body fat (Rothwell & Stock, 1979; Himms-Hagen, 1989; Ricquier et al., 1997). For example, obese fa/fa rats have decreased UCP gene expression (Ricquier et al., 1986). Injections of β_3 -AR agonists turn on full and rapid expression of UCP1 in these rats. The same type of stimulation reduces the body fat content of normal rodents and dogs (Champigny et al., 1991). Similarly, increasing UCP1 expression directed by the fat-specific aP2 promoter in WAT of A^{vv} transgenic obese mice result in decreased adiposity and body weight compared to wild-type mice (Kopecky et al., 1995). The concept that BAT functions to prevent obesity (Rothwell & Stock, 1984) is supported by evidence that mice made BATdeficient using a transgenic toxigene have 96% less UCP and develop obesity, which is exacerbated by a high fat diet (Lowell et al., 1993; Hamann, Flier, & Lowell, 1996). Similarly, UCP1-deficient (UCP1^{-/-}) mice consume less oxygen after treatment with a β_3 -AR agonist, compared to normal mice, and are sensitive to cold, indicating that their thermoregulation is defective (Enerbäck et al., 1997). However, this deficiency caused neither hyperphagia nor obesity in mice fed on either a standard or a high-fat diet. The authors suggest that the loss of UCP1 may have been compensated by an increase in UCP2 expression in different adipose tissues possibly to help maintain thermogenic

capacity (Enerbäck et al., 1997; Thomas & Palmiter, 1997). However, it should be noted that higher environmental temperatures would lower UCP1 expression in wild-type mice and minimize its expected contribution to differences in energetic efficiency between wild-type and transgenic mice (Enerbäck et al., 1997; Thomas et al., 1997; Commins et al., 1999b). Rearing UCP1 knockouts (Lowell et al., 1993) at thermoneutrality minimizes the difference in fat deposition between control and transgenic mice (Melnyk, Harper, & Himms-Hagen, 1997). These studies support a role for BAT and UCP1 to function in the prevention of obesity.

Starvation can reduce resting metabolic rate by as much as 40% (Blaxter, 1989). Food restriction sufficient to maintain a 10% reduction in body weight is also associated with decreased energy expenditure (Leibel et al., 1995). This adaptation is valuable when food is scarce but is counter-productive during dieting for intentional weight loss. BAT atrophy caused by food deprivation is characterized by reductions in tissue weight, lipid content, mitochondrial proteins and UCP1, without a change in tissue DNA (Muralidhara & Desautels, 1989; Champigny & Ricquier, 1990; Matamala et al., 1996), and an ultimate decline in thermogenesis. Expression of UCP2 and UCP3, as well as UCP1, in BAT was decreased in lean rats after food restriction for four days (Gong et al., 1997; Cusin et al., 1998), whereas no effect on UCP2 expression in BAT or heart tissue was found after short-term fasting (48 h) (Boss et al., 1997a). Diet-induced obese rats lacked a response of UCP1 to food deprivation (Matamala et al., 1996).

In tissues other than BAT, the responses of UCP2 and UCP3 to food restriction and starvation are different. Short-term fasting (48-72 h) unexpectedly increased UCP2 mRNA about 2-fold in skeletal muscle (Boss et al., 1997a; Weigle et al., 1998; Bezaire et

al., 2001) and a 24-hour fast increased UCP3 expression 4 to 10-fold in skeletal muscle (Gong et al., 1997; Weigle et al., 1998). In lean and obese humans, calorie restriction induced a similar 2-2¹/₂-fold increase in UCP2 and UCP3 mRNA levels in adipose tissue and skeletal muscle (Millet et al., 1997). To determine whether glucocorticoid was the signaling molecule responsible for increased UCP3 during fasting, it was injected into normal mice to mimic fasting levels (Weigle et al., 1998). UCP3 expression did not increase; thus, glucocorticoid is not responsible for this effect during fasting.

UCP3 expression in skeletal muscle is related to increasing FFA as a fuel source during fasting. This was demonstrated by artificially increasing circulating FFA levels in fed animals by an infusion of Intralipid plus heparin, which increased UCP3 mRNA (Weigle et al., 1998). Under fasting conditions, UCP3-knockout mice (UCP3^{-/-}) have an increased rate of ATP synthesis at the cellular level in skeletal muscle, suggesting that UCP3 has uncoupling activity (Cline et al., 2001). However, a different study reported that fasted mice did not have a significant change in mitochondrial proton leak with increased UCP2 and UCP3 (Bezaire et al., 2001). UCP3^{-/-} mice showed no change in whole body energy expenditure (Cline et al., 2001) but had higher respiratory quotients than wild-type mice in the fed resting state, indicating impaired fatty acid oxidation and suggesting that UCP3 does play a role in fatty acid oxidation (Bezaire et al., 2001), probably in fatty acid transport. Overexpression of UCP3 in cultured human muscle resulted in a lower mitochondrial membrane potential, greater ATP efficiency, and a favoring of fatty acid vs. glucose oxidation (Garcia-Martinez et al., 2001). These and other studies have lead Himms-Hagen (2001) to propose the hypothesis that UCP2 and UCP3 function as transporter proteins, not as uncouplers. According to their hypothesis,

these UCPs help to export FFA anions to the cytosol, thereby supplying CoASH to the mitochondria and permitting rapid FFA oxidation when FFA are the predominant substrate in muscle during fasting or in BAT during exposure to cold.

A great deal of the knowledge regarding BAT metabolism and UCPs has been gained from the use of rodents with highly responsive adaptive thermogenesis and rodents with specific gene knock-outs. Determining the role of BAT or UCPs in humans has been difficult because of low BAT content and invasive procedures. However, research on several candidate genes, including the UCP1 gene, is ongoing in the search for genetic defects contributing to human obesity. A DNA polymorphism in the human UCP1 gene has been detected but with less than significant correlation to obesity (Oppert et al., 1994). An 8.3 kb allele of the UCP1 gene was found at a higher frequency in a group of high fat gainers when compared to low fat gainers for percent body fat during a 12-year period. When the UCP1 gene polymorphism was investigated in moderate and morbidly obese subjects, it was found that this allele was a predictive factor associated with high weight gain and, in a second study, a small decrease in body mass index during food restriction (Ricquier et al., 1997). A common G/A polymorphism in the UCP2 promoter region is associated with enhanced adipose tissue mRNA expression in vivo (Commins et al., 1999a), which lead to the finding of a modest but significant reduction in obesity prevalence associated with the less-common allele in never-obese vs. obese middle-aged subjects. The presence of UCP2 in several tissues (WAT, lung, kidney, and liver) and the presence of UCP3 in muscles, make UCP2 and UCP3 potential candidates for regulating body weight in humans, which lack UCP1-enriched BAT; however, as noted earlier,

UCP2 and UCP3 may not function as uncouplers. Another possibility would be to figure out how to express and activate UCP1.

It was previously thought that UCP1 was only expressed in brown adipocytes and that brown adipocytes were only located in BAT. Studies using more advanced, highly sensitive techniques indicate that this is not true. WAT from cold-exposed rodents or β_3 -AR agonist-treated rodents have been shown to have an increased number of adipocytes with morphological characteristics of brown adipocytes (Loncar, Afzelius, & Cannon, 1988; Cousin et al., 1992; Morroni et al., 1995; Ghorbani & Himms-Hagen, 1997; Ghorbani, Claus, & Himms-Hagen, 1997). Although the origin of these cells has not been established, it is possible that brown preadipocytes were induced to differentiate or that transdifferentiation of pluripotent preadipocytes that reside in the WAT occurred (Commins et al., 1999b). In addition, UCP1 was also found to be expressed in WAT depots of rats after cold exposure or β_3 -AR agonist treatment although in small quantity (Himms-Hagen et al., 1994; Nagase et al., 1996; Collins et al., 1997; Picó, Bonet, & Palou, 1998). It is not known whether the increase in UCP1 in WAT translates to an increase in thermogenesis, but WAT is becoming recognized as more important in energy metabolism rather than a tissue of low energetic significance. An example of this was demonstrated by creating genetically engineered mice in which β_3 -ARs were expressed exclusively in white and brown adipocytes, or in brown adipocytes only (Grujic et al., 1997). Re-expression of β_3 -ARs in both BAT and WAT was necessary to restore the increase in oxygen consumption seen in animals treated with a β_3 -AR agonist, whereas re-expression of the β_3 -AR in BAT only failed to restore this effect on oxygen

consumption. This illustrates that WAT is an obligatory participant in the thermogenic process.

Leptin Induces UCPs & Thermogenesis

One of the most important peripheral effects of leptin is increased thermogenesis in brown adipose tissue that is presumably mediated by increased sympathetic activity and norepinephrine turnover in BAT and in WAT (Collins et al., 1996; Haynes et al., 1997b). Leptin administration causes striking morphological changes in BAT, which gains features of metabolically activated fat cells with numerous mitochondria, and WAT, which becomes grossly and histologically atrophied. BAT changes from a tan color to an intensely dark red, sharply demarcated mass, which is markedly reduced in size and completely devoid of any overlying white fat tissue (Sarmiento et al., 1997). The multilocular brown adjocytes with abundant cytoplasm are transformed into markedly smaller cells with cytoplasm depleted of most or all visible fat droplets. BAT is normally highly innervated and more vascularized than WAT, but WAT depots also become highly vascularized after leptin administration. In WAT, mature, unilocular fat cells with a single, large lipid vacuole were transformed into progressively smaller, multilocular fat cells with multiple, smaller lipid vacuoles, all of which virtually disappeared in the fully depleted adipocyte. In addition, leptin administration, as well as cold exposure and β_3 -AR agonists, appeared to induce recruitment of brown adipocytes in the inguinal and retroperitoneal fat depots of mice (Sarmiento et al., 1997). A negative feedback loop exists to reduce leptin expression by WAT after the stimulation of β_3 -adrenergic receptors $(\beta_3$ -AR) on adjocytes through the sympathetic nervous system (Mantzoros et al., 1996).

Leptin administration to lean rodents, either centrally or peripherally, increases the expression of UCP1, UCP2 and UCP3 in BAT (Sarmiento et al., 1997; Scarpace et al., 1997; Satoh et al., 1998; Cusin et al., 1998), and the expression of UCP2 and UCP3 in WAT, muscle, and liver (Sarmiento et al., 1997; Zhou et al., 1997; Cusin et al., 1998; Zhou et al., 1999). Using a rat leptin cDNA-containing adenovirus (AdCMV-leptin) to induce hyperleptinemia, Zhou et al. (1997) showed that leptin increased UCP2 mRNA in WAT of lean Zucker rats, but not in fatty Zucker rats. Some studies report depot-specific responses to leptin administration. For example, the epididymal fat pad in both lean and *ob/ob* mice but not the retroperitoneal fat pad had increased UCP2 mRNA after IP injections of leptin (Commins et al., 1999b). The magnitudes of the increases are generally greater in males than in females.

The effects of starvation on BAT metabolism and UCP regulation are nearly the opposite of leptin administration. Starvation causes leptin synthesis in adipose tissue and sympathetic nerve activity to BAT to decrease (Landsberg, Saville, & Young, 1984), and this is associated with lower UCP1 expression (Champigny et al., 1990) and a fall in whole body metabolic rate (Rothwell et al., 1984). Leptin-deficient *ob/ob* mice have reduced sympathetic nerve activity to brown adipose tissue and show many of the abnormalities seen in starved animals, including decreased core temperature (Himms-Hagen, 1989), decreased energy expenditure (including activity), decreased immune function, and infertility (Coleman, 1978). Leptin replacement corrects the abnormalities associate with "perceived starvation" (*ob/ob*), in part by increasing sympathetic nerve activity to BAT, WAT, kidney, hindlimb, and adrenal gland (Collins et al., 1996; Haynes et al., 1997a; Haynes et al., 1997b). Other effects include the combination of increased

fat oxidation, oxygen consumption, serum FFAs, and core temperature (Pelleymounter et al., 1995).

UCP1, previously believed to be present only in BAT has now been detected in WAT. After six days of infusion of AdCMV-leptin in lean mice, increases in UCP1 and UCP2 mRNA and UCP2 protein were found in the epididymal fat pad (Zhou et al., 1999). Another study also found increased UCP1 mRNA in WAT, especially in the epididymal fat pads, of lean mice after subcutaneous injections (20 mg/kg BW/day) (Sarmiento et al., 1997). Three days of leptin administration (20 μ g/g BW/day) to *ob/ob* mice produced a 5- to 10-fold increase in UCP1 mRNA and protein in both BAT and retroperitoneal WAT (Commins et al., 1999b). This revealed that leptin could increase the thermogenic capacity of WAT, in addition to BAT, given that thermogenic capacity is directly related to the amount of UCP1 expressed. It is unclear if the increased UCP1 in retroperitoneal WAT allows significant fat oxidation to occur in this tissue (Commins et al., 1999b). It was found that cold exposure or β_3 -AR agonists did not alter UCP2 expression in WAT but produced a robust increase in UCP1 (Commins et al., 1999b). This indicates that leptin does not regulate UCP1 and UCP2 through a common mechanism and suggests that if leptin does influence UCP2 expression, it does so through an indirect mechanism not involving modulation of sympathetic tone or cAMP.

The neural circuits targeted by leptin and cold exposure also regulate energy expenditure by means of effects on several key molecules including UCPs and PPAR γ co-activator-1 (PGC-1), a key regulator of the genes that control thermogenesis from sympathetic nervous system input through β -ARs and cAMP action (Puigserver et al., 1998; Lowell et al., 2000).

Apoptosis, Programmed Cell Death

The death of cells occurs by necrosis, upon injury, or by apoptosis – the regulated destruction of a cell or 'programmed cell death' (Kerr, Wyllie, & Currie, 1972). The word, apoptosis, comes from two Greek words, apo- and ptosis-, and the second 'p' is silent (Funder, 1994). "Apo" means "separate from" and "ptosis" means "fall from" - a description of cells that naturally, and without any inflammatory response, die as part of normal development. Apoptosis allows the organism to control tightly cell number and tissue size, and to protect itself from rogue cells that threaten homeostasis. It plays a very important role in many physiological and pathological conditions such as embryo and organ development, immune responses, tumor development and growth. Generally, there are diseases with too little apoptosis (e.g., cancer) and diseases with too much apoptosis (e.g., AIDS). It is characterized by many biological and morphological changes; such as, change of mitochondrial membrane potential, activation of caspases, membrane inversion, exposure of phosphatidylserine residues, blebbing, DNA fragmentation, chromatin condensation, and formation of apoptotic bodies. Apoptosis is initiated by a variety of stimuli including injury, growth factor withdrawal ("death by neglect"), UV- or γ -irradiation, chemotherapeutic drugs, and activation of the so-called 'death receptors' expressed on the cell surface. The process of apoptosis involves a large web of integrating pathways and factors that continue to be discovered. Described here are only the basic pathways and machinery. A comparison between apoptosis and necrosis is found in Figure 2.14 and Table 2.10.

Protein-cleaving enzymes, or proteases, are instrumental in the sequential signaling pathway of apoptosis. A family of intracellular cysteinyl-aspartate-specific proteases,

called caspases, plays a central and critical role in the execution phase of apoptosis. Over a dozen caspases have been identified in humans, about two-thirds of which have been suggested to function in apoptosis (Thornberry & Lazebnik, 1998; Earnshaw, Martins, & Kaufmann, 1999). Caspases are synthesized as inactive pro-enzymes (zymogens) or procaspases. There are three possible mechanisms of caspase activation: proteolytic cleavage by an upstream caspase, recruitment or aggregation of multiple caspase molecules into close proximity, and holoenzyme formation (cytochrome c and ATPdependent oligomerization of Apar-1) (Hengartner, 2000). A proteolytic caspase cascade exists that activates or deactivates substrates, amplifying and integrating pro-apoptotic



Figure 2.14. Morphological features of necrosis and apoptosis (Roche Diagnostics Corporation, 1998).

signals. Important targets of caspases include cytoplasmic and nuclear proteins such as keratin 18, poly ADP ribose polymerase (PARP) and lamins. Blocking caspases can slow down or even prevent apoptosis (Earnshaw et al., 1999), or overexpression of caspase-3, for example, induces apoptosis.

Table 2.10. Differential characteristics of the types of cell death

Necrosis	Apoptosis
Morphological features	
Loss of membrane integrityNo alteration of membrane asymmetry	 Membrane blebbing, but no loss of integrity Alteration of membrane asymmetry (translocation of phosphatidylserine from cytoplasmic to extracellular side)
• Lysosomal leakage	Lysosomes intact
• Clumpy, poorly-defined aggregation of chromatin	 Chromatin compact into uniformly dense masses
• Cells swell and lyse	 Cells shrink, ultimately forming apoptotic bodies
Biochemical features	
• Loss of regulation of ion homeostasis	• Tightly regulated process involving activation and enzymatic steps
• No energy requirement (passive process)	• Energy (ATP)-dependent (active process)
 No requirements for protein or nucleic acid synthesis 	• Requires macromolecular synthesis and de novo gene transcription
Random digestion of DNA	• Nonrandom oligonucleosomal-length fragmentation of DNA
Physiological significance	
 Death of groups of contiguous cells Evoked by non-physiological disturbances (complement attack, lytic viruses, hypothermia, hypoxia, ischemia, metabolic poisons) Phagocytosis by macrophages 	 Deletion of single cells Induced by physiological stimuli (lack of growth factors, changes in hormonal environment) Phagocytosis by adjacent normal cells or macrophages
 Significant inflammatory response 	 No inflammatory response

The Bcl-2 family is a set of apoptotic regulators comprised of over a dozen proteins, which have been classified into three functional groups, based on structural similarities and functional criteria (Adams & Cory, 1998; Green & Reed, 1998; Antonsson & Martinou, 2000; Ranger, Malynn, & Korsmeyer, 2001). Members of group I possess

anti-apoptotic activity, such as Bcl-2 and Bcl-xL, whereas members of groups II (multidomain) and III (BH3 domain-only) promote cell death (Figure 2.15). They possess up to four Bcl-2 homology domains (BH1-4) corresponding to α -helical segments (denoted by colored boxes). Many members also possess a carboxy-terminal hydrophobic transmembrane (TM) domain (denoted by a red box). Structure/function studies suggest that anti- versus pro-apoptotic activity is determined by relatively large regions of the protein; including two large α -helices that have been proposed to participate in membrane insertion. For example, the Bcl-2 protein can dimerize with itself or with the product of the Bax gene. Several Bcl-2 family proteins reside in the outer membrane of the mitochondrion, nucleus and endoplasmic reticulum, or shuttle between the cytosol and organelles. The control of cell death depends on overall levels of pro- and anti-apoptotic



Figure 2.15. The Bcl-2 family (Ranger et al., 2001).

family members: cells with more pro-death proteins are sensitive to death; cells with an excess of protective family members are usually resistant.

The key function of Bcl-2 family members seems to be to regulate, by alteration of mitochondrial permeability, the release of pro-apoptotic factors, in particular cytochrome c, from the mitochondrial intermembrane compartment into the cytosol followed by subsequent depolarization of the inner mitochondrial membrane (Adams et al., 1998; Hengartner, 2000; Antonsson et al., 2000). In other words, pro- and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria where they compete to regulate cytochrome c release. Mitochondria are used as "amplifiers" to initiate the executionary apoptosis caspase cascade (Kroemer, Dallaporta, & Resche-Rigon, 1998). (The basic concept of activation and inhibition of apoptosis signaling cascades can be found in Figure 2.16.) In the cytosol, cytochrome c (Apaf-2) promotes caspase activation by binding to Apaf-1, which then recruits Apaf-3 (caspase 9) into an activated complex called the apoptosome (Li et al., 1997). At the apoptosome, pro-caspase-9 is autocatalytically processed to the mature enzyme and initiates a caspase cascade downstream of the mitochondrion (Zou et al., 1999). Caspase-9 is an integral factor in mitochondrial-dependent apoptosis pathway in C. elegans and mammals (Figure 2.16). In caspase-9 knockout mice, Fas-mediated apoptosis still takes place in hepatocytes bypassing mitochondrial involvement (Zheng et al., 2000), but dexamethasone-induced cell death of immature T cells is inhibited (Daniel, 2000; Zheng & Flavell, 2000). This emphasizes the central role of mitochondrial activation in steroid-triggered apoptosis. Other factors are also released into the cytosol from the mitochondria upon activation,

such as AIF (apoptosis inducing factor), which has proteolytic activity and is by itself sufficient to induce apoptosis (Nicotera & Leist, 1997; Kroemer & Reed, 2000).



Figure 2.16. Basic apoptosis signaling pathways (Daniel, 2000).

The mechanism by which the Bcl-2 family members regulate cytochrome *c* release is debated between three different hypotheses: Bcl-2 members form channels that facilitate protein transport, Bcl-2 members interact with other proteins to form channels, and Bcl-2 members induce rupture of the outer mitochondrial membrane (Hengartner, 2000). One example is the activation of the mitochondria by the pro-apoptotic Bcl-2 family member Bid. Bid is a substrate of caspase-8, which is activated in low amounts (Gross et al., 1999). Truncated Bid translocates to the mitochondria and induces loss of $\Delta \psi_m$ and release of apoptogenic factors. Cytochrome *c* release from mitochondria is an almost universal feature of apoptotic cell death and the point of no return in most cases. Inhibitors of cytochrome *c* release prevent apoptosis (Loeffler & Kroemer, 2000). This signifies the importance of mitochondria in the apoptosis process.

Additional apoptotic signaling pathways exist that bypass the mitochondria and are independent of mitochondrial activity. A family of death receptors was discovered that trigger apoptosis specifically and independently. There are at least nine known death receptors but the best characterized death receptors are CD95 (also called Fas or Apo1) and TNFR1 (also called p55 or CD120a) (Figure 2.17) (Scaffidi et al., 1998; Krammer, 1999). These death receptors belong to the TNF/NGF-receptor superfamily and are characterized by extracellular cysteine-rich domains (Schulze-Osthoff et al., 1998). They have an intracellular 'death domain' that couples the receptor to the apoptosis-inducing machinery after receptor-ligand binding. The ligands also display striking structural homologies, which exhibit similar mechanisms of receptor recognition and triggering. Bid provides crosstalk and integration between the death-receptor and mitochondrial pathways (Li et al., 1998; Luo et al., 1998; Gross et al., 1999). TNF, and possibly the activation of other death receptors (i.e., Fas-mediated), activates Bid cleavage, which in turn mediates a conformational change in (activating) Bax (Daniel, 2000) in the mitochondrial apoptosis pathway. Under most conditions, this crosstalk is minimal and the two pathways operate largely independent of each other.

Internucleosomal DNA fragmentation is considered a hallmark of apoptosis. As an irreversible event that commits the cell to die, it occurs late in the apoptotic cycle but before changes in plasma membrane permeability (prelytic DNA fragmentation). During apoptosis, an activated endogenous Ca^{2+} and Mg^{2+} -dependent nuclear endonuclease, specifically known as caspase-activated DNase (CAD) (Nagata, 2000), cuts the genomic



Figure 2.17. Death factor and receptor (TNF and TNFR) family members (Schneider & Tschopp, 2000). Trimeric ligands are shown at the top and receptors at the bottom of the Positions of cysteine residues in the extracellular portion of receptors are figure. indicated by horizontal bars. When present, death domains are represented by black boxes. Interactions that have been reported in the literature are indicated by arrows. Some of the ligands and receptors have several names: FasL/Apo-1L/CD95L, VEGI/TL-TRAIL/Apo-2L, RANK/OPGL/TRANCE, TWEAK/Apo-3L, GITRL/AITRL, 1. BAFF/TALL-1/THANK/BlyS, DcR3/TR6, Fas/Apo-1/CD95, TRAILR1/DR4, TRAILR2/DR5/Killer/Apo-2, TRAILR4/DcR2, TRAILR3/DcR1, TARMP/Apo-3/DR3/WSL-1/LARD, HVEM/ATAR, GITR/AITR.

DNA between nucleosomes to generate DNA fragments with lengths corresponding to multiple integers of approximately 180 base pairs (Bortner, Oldenburg, & Cidlowski, 1995). These fragments can be extracted from tissue or cells and result in the appearance

of "DNA laddering" when the DNA is analyzed by agarose gel electrophoresis (Figures 2.18 & 2.19). The DNA of non-apoptotic cells, which remains largely intact does not display this "laddering".



Figure 2.18. DNA fragmentation and the appearance of the "DNA ladder" (Roche Diagnostics Corporation, 1998).

Another important feature of apoptosis includes an alteration at the cell surface and plasma membrane. In the early stages of apoptosis, the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer causes PS to be exposed at the external surface of the cell (Green & Beere, 2000). These cells are then considered 'marked' for removal (phagocytosis) by nearby phagocytes through interaction with the phosphatidylserine receptor (PSR) on the phagocyte. The balance between apoptosis and necrosis determines the biological response (immunity or

tolerance) of the phagocyte (Figure 2.20). The extent of PS exposure and binding of a fluorescent probe, such as Annexin-V FITC, can distinguish apoptotic cells from non-apoptotic cells.



Figure 2.19. DNA ladder from cells undergoing apoptosis and stained by SYBR Green I nucleic acid stain (Molecular Probes, 2002).



Figure 2.20. Phagocytes (including dendritic cells and macrophages) engulf and degrade both apoptotic and necrotic cells (Green et al., 2000).


Figure 2.21. Mitochondrial pathway in apoptosis.

Adipose Tissue Apoptosis

Once fat cells achieve a maximum size, further increases in the mass of an adipose tissue must involve an increase in cell number (Hirsch & Batchelor, 1976; Faust & Miller, Jr., 1983). It was believed that adipocyte acquisition is permanent (Ailhaud et al., 1991) and weight loss causes a decrease in cell size only and not in cell number (Ginsberg-Fellner & Knittle, 1981). Those with hyperplastic obesity who lose weight maintain the weight loss for a shorter time and experience a higher relapse rate than those who are hypertrophically obese. A complete removal of adipocytes would be advantageous to maintaining weight loss, especially in hyperplastic obese individuals. Based on more recent evidence, Prins and O'Rahilly (1997) contend that adipocyte number is quite variable and co-regulates with cell size. They state that since fat mass is relatively stable over time and if adipocyte acquisition does occur, it must be balanced by a process including cell deletion.

Evidence has been emerging to support the endogenous elimination of adipocytes within adipose tissue. Geloen (1989b) demonstrated that the loss of adipocytes (based on histology and the quantification of DNA) occurs in rats rendered catabolic by streptozotocin-induced diabetes. In several patients with malignancy-associated weight loss, apoptosis was detected in abdominal subcutaneous and omental fat (Prins et al., 1994b). Adipocyte apoptosis occurred in subcutaneous fat (lipodystrophy) of patients with exposure to HIV-1 protease inhibitors assessed by the TUNEL method (Domingo et al., 1999). When tumor-bearing and diet-restricted rabbits were compared, both had lost significant body weight but only the tumor-bearing rabbits had adipose tissue apoptosis with an associated increase in Bax protein (Ishiko et al., 2001). Cyclic plasma perfusion

(CPP) causes tumor-bearing rabbits to regain lost body fat; however it did not suppress adipocyte apoptosis, indicating that CPP removed a humoral factor that reduces body fat by lipolysis but a second factor must be present for the persistent apoptosis (Nishimura et al., 2000). Evidence also exists that dietary conjugated linoleic acid (CLA) induces adipose tissue apoptosis (Azain et al., 2000; Tsuboyama-Kasaoka et al., 2000; Miner et al., 2001; Roche et al., 2001; Lin et al., 2002).

Several studies also report that fat cell number is not reduced, but the conditions under which weight loss is induced should be noted. Rats fasted for a week were shown to have a 99% reduction in fat mass and a significant loss of total adipose tissue DNA content but no evidence of fat cell loss (Miller, Jr. et al., 1983). The number of fat cells in the right epididymal depot of the food-deprived rats equaled both the number seen in the left depot after refeeding and the number seen in corresponding depot of nonfasted controls. Analysis of the tissue indicated a loss (and recovery after refeeding) of stromalvascular cells. This report also demonstrates that total DNA content alone is not an accurate method of determining the specific loss of adipocytes. Food restriction of male rats over a 12-week period was effective in reducing body weight and epididymal fat pad weight; however, adipocyte number was not significantly affected (Askew, Hecker, & Stifel, 1976). The starving of rats with diet-induced obesity for 8, 15, and 25 days lost fat mass in proportion to the duration of starvation (Yang, Presta, & Bjorntorp, 1990). Fat cell size decreased but not fat cell number. Food restriction in combination with exercise, but not by itself, reduced fat cell number of the inguinal fat pad, but not epididymal or perirenal depots, in male Wistar rats (Bailey, Barker, & Beauchene, 1993). In addition, adult rats showed an increase in fat cell number, in addition to increased fat cell size,

when induced to be obese from a highly palatable diet. Fat cell number was maintained after a return to normal caloric intake (Faust et al., 1978). Collectively, these studies suggest that it is the conditions (e.g., fasting vs. malignancy) for weight loss that determines whether the loss of fat mass is due to a reduction in cell size only (lipolysis) or both cell size and cell number (apoptosis). It appears that unknown factors produced in malignancy but not during fasting contribute to cell death of adipocytes. This underscores the poor performance of weight maintenance from low-calorie diets.

Several regulators of adipocyte number have been identified that may encourage fat cell acquisition or promote fat cell removal (Table 2.11). A combination of factors often must be met for either effect to occur. For example, during apoptosis, factors encouraging fat cell acquisition should be low while those promoting cell removal should be relatively increased. A lack of insulin, such as with diabetes mellitus, leads to a loss of proliferative activity in adipose tissue. The restoration of circulating insulin levels

	Increase (+)/
	Decrease (-)
 Insulin 	+
 PPARγ 	+/_
• Retinoids	_
• Galectin-12	_
• Bax	-
 Corticosteroids 	+
 TNFα 	-
• GLP-1	-
• 5-hydroxytryptamine	_
• NPY	+
• Leptin	_

Table 2.11. Potential regulators ofadipocyte number

restores fat mass by increasing cell size and, secondarily, by cell proliferation (Geloen et al., 1989a). Interestingly, this occurred in the parametrial white adipose tissue but not in brown adipose tissue. Geloen et al. (1989b) showed that the regression of white adipose tissue in streptozotocin-induced diabetic rats was due to a loss of adipocytes (using quantitative cellular frequency) and not just cell size. However, the mechanism by which cells were lost was not determined.

As discussed previously, $TNF\alpha$ has a role involved in limiting fat mass through the induction of insulin resistance and leptin production, stimulation of lipolysis, suppression of lipogenesis, induction of adipocyte dedifferentiation, and impairment of preadipocyte differentiation in vitro. TNFa has also been found to induce apoptosis of human preadipocytes and adipocytes in vitro, with indexes between 5 and 25% [assessed using morphological (histology, nuclear morphology following acridine orange staining, electron microscopy) and biochemical criteria (demonstration of DNA laddering and of annexin V staining by immunocytochemistry)] (Prins et al., 1997). In another study, intra-abdominal omental preadipocytes in culture were more likely to undergo apoptosis in response to TNF α treatment or serum starvation than those obtained from the abdominal subcutaneous depot (Niesler, Siddle, & Prins, 1998). This suggested that the regulation of adipose tissue distribution in humans could involve depot-specific differences in rates of preadipocyte apoptosis. *ob/ob* mice with targeted null mutations in the genes encoding the two TNF receptors (absence of both TNF receptors) resulted in a significant reduction in brown adipocyte apoptosis (Nisoli et al., 2000).

PPARγ triggers adipocyte differentiation by inducing the expression of several genes critical for adipogenesis (Tontonoz et al., 1994a; Tontonoz et al., 1994b). PPARγ

expression is downregulated by fasting and insulin-deficient diabetes. A high fat diet increases expression of PPARy in adipose tissue of normal mice and in liver of obese mice. PPARy is present normally at low levels in 3T3-L1 preadipocytes but is increased dramatically during adjpocyte conversion using normal differentiating conditions (fetal calf serum, dexamethasone, isobutyl-methylxanthine, and insulin). Prostaglandin J2 (PGJ2) derivatives function as activating ligands for PPARy but phosphorylation of PPARy by PGF2a will block adipogenesis (Reginato et al., 1998). An antiproliferative effect of PPAR γ activation was demonstrated by the treatment of human gastric cancer cells with the PPARy agonist troglitazone, which caused the induction of apoptosis (a significant increase of annexin V-positive cells) and G1 cell cycle arrest (Sato et al., 2000). The effect was augmented by the simultaneous addition of 9-cis retinoic acid, a ligand of RXR α . Troglitazone also induced the expression of galectin-12, a newly identified protein related to the family of galectin proteins that bind to β-galactoside residues and have diverse physiological functions, which paralleled an increase in the number of apoptotic cells in adipose tissue (Hotta et al., 2001). Collectively, these studies indicate that PPAR γ plays a role in both the proliferation and apoptosis of adipocytes.

The absence or removal of growth factors can promote apoptosis. Fat tissues extracted from patients undergoing surgery were subjected to either growth factor deprivation or mild heat to induce apoptosis (Prins et al., 1994a). Both treatments induced characteristic features of apoptosis including morphological changes and DNA laddering, compared to those cultured in a healthy medium. 3T3-L1 preadipocytes underwent apoptosis after growth factor withdrawal as assessed by Hoescht staining, DNA fragmentation, and TUNEL (Magun et al., 1998a). Mature cells did not show apoptosis, suggesting that expression of cell survival genes (e.g., Bcl-2) is modulated during 3T3-L1 adipocyte differentiation, potentially contributing to a state of cell death resistance. Growth factors indirectly function to maintain mitochondrial membrane integrity by allowing the activation of the PI3-kinase/protein kinase B pathway that leads to the phosphorylation of Bad, which does not associate with Bcl-2 (Raff, 1998). In the absence of growth factors, Bad is no longer phosphorylated allowing for heterodimerization with Bcl-2 and, consequently, permits cytochrome c release and the triggering of the caspase cascade.

When an IL-3-dependent pro-B cell line is induced for apoptosis by IL-3 withdrawal, an increase in creatine kinase (CK) activity was observed (Daniel, 2000; Khaled et al., 2001). CK catalyzes the transfer of the high-energy phosphate to creatine causing creatine phosphate to become sequestered in the mitochondrial inner membrane space (Figure 2.22). This led to the depletion of ATP and an increased electron transfer rate in



Figure 2.22. Creatine kinase pathway in apoptosis and inhibition by Bcl-x_L.

the oxidative chain that could account for the increased lipid peroxidation observed during apoptosis (Daniel, 2000). Overexpression of Bcl- x_L , a Bcl-2-related gene with anti-apoptotic properties (Boise et al., 1993), prevented the increase in creatine phosphate allowing the release of creatine phosphate into the cytosol, which resulted in the immediate restoration of the ATP pool (Gottlieb, Vander Heiden, & Thompson, 2000; Heiden et al., 2001).

Neuronal apoptosis inhibitor protein (NAIP) appears to suppress apoptosis through inhibition of caspases (Mercer et al., 2000). Low levels of NAIP are expressed in 3T3-L1, 3T3-F442A, and human preadipocytes, and increase dramatically after differentiation (Magun et al., 1998b; Gagnon, Rabie, & Sorisky, 1999) contributing to apoptosis resistance in mature adipocytes. NAIP is also increased in preadipocytes from subcutaneous abdominal fat biopsies obtained from patients after weight reduction (Sorisky et al., 1999), suggesting that weight loss improves resistance to apoptosis. This compliments results from an earlier study showing a lack of adipocyte apoptosis after fasting in rats (Miller, Jr. et al., 1983). These rats also regained weight faster over time.

Leptin and Adipocyte Apoptosis

Leptin exerts proliferative and anti-apoptotic activities in a variety of cell types, including T lymphocytes, leukemia cells, and endothelial cells (Sierra-Honigmann et al., 1998; Takeda et al., 1998; Lord et al., 1998; Bouloumie et al., 1998; Konopleva et al., 1999). However, the opposite effect occurs in adipose tissue. As a potent regulator of fat mass, leptin specifically targets adipose tissue for reduction via the sympathetic nervous system and possibly by other mechanisms. In addition to a reduction in cell size by lipolysis, recent data suggests that leptin may also reduce fat mass by stimulating adipocyte apoptosis. Adipose tissue can be completely ablated upon overexpression of leptin in rats (Chen et al., 1996; Flier, 1997; Wang et al., 1999c). Apoptotic features, such as DNA laddering and TUNEL, were exhibited in normal rats injected ICV with leptin (2.5 µg UGA recombinant rat leptin/day for 4 days) (Qian et al., 1998a). This was in addition to an 80-85% reduction in white adipose tissue mass and a significant decrease in adipose tissue DNA content (-64%). TUNEL and periodic acid-Schiff Reagent *in situ* staining determined that the cells undergoing apoptosis were adipocytes. In contrast, rats that were pair-fed to the leptin-treated rats had smaller decreases in fat pad weight and no significant change in total DNA content or evidence of apoptosis in the adipose tissues. In another study in which leptin was injected ICV (5 μ g UGA recombinant rat leptin/day for 5 days) into young and mature rats, the results suggested that normal rats become resistant to leptin as they age (Qian et al., 1998b). The young rats lost 52% of body fat and increased protein content. The weights of epididymal and retroperitoneal fat pads were reduced by 75% and 85%, respectively, and there was a reduction in total adipocyte number, cellular volume, and DNA content, although no specific apoptotic measurements were made.

Some studies have reported data that conflicts with the hypothesis of leptin-induced apoptosis but it is worth noting the methods of administration, design, and source of leptin. Injecting leptin subcutaneously (20 mg/kg/d) for two weeks in lean C57BL/6 mice resulted in a significant loss of fat mass but not of fat cell number (assessed by the normal BrdU-labeling index) and no evidence of apoptosis or necrosis was observed (Sarmiento et al., 1997). After six days of adenovirus-induced hyperleptinemia (infusion

of AdCMV-leptin), normal rats exhibited rapid nonketotic fat loss (-87%) with a 95% decrease in total triglyceride content of the epididymal fat pads, but total DNA content had decreased by only 16%, which was not significantly different from control level (Zhou et al., 1999). These results suggest that the method of administration and dose may affect the induction or degree of apoptosis. When injecting leptin ICV, a much smaller dose is required to achieve the same effect as peripheral injections. Leptin stimulation may also last longer when injected ICV since the half-life of leptin is primarily due to renal clearance. Furthermore, a larger dose of leptin may induce effects not seen with smaller doses. The source of leptin and animal model should also be considered. Evidence of leptin-induced adipocyte apoptosis exists but the mechanism has yet to be exposed.

The mechanism of leptin-induced apoptosis was investigated by Qian (1998c) using the experimental design as previously described (Qian et al., 1998b). Leptin caused a 70-80% increase in PPAR γ expression in the epididymal fat pad but a decrease (-40%) in TNF α in the young rats only. It was concluded that PPAR γ might mediate leptin-induced apoptosis whereas TNF α may play a role in lipolysis, although, lower TNF α levels are probably a secondary effect of reduced fat mass. A positive correlation between PPAR γ and apoptosis has been found in cancer cells treated with troglitazone (Sato et al., 2000) and in white adipose tissue from rodents treated with TZD (Yamauchi et al., 2001). However, this increase in PPAR γ conflicts with another report showing a dramatic decrease (<1% of control) in rats made hyperleptinemic by AdCMV-leptin infusion (Zhou et al., 1999). This is a more plausible effect because a reduction in PPAR γ activity leads to downregulation of lipogenic enzymes and increased fatty acid oxidation and leptin levels (Schoonjans et al., 1996; Yamauchi et al., 2001). In addition, fasting typically reduces the expression of PPAR γ (Vidal-Puig et al., 1996). Supplementing mature adipocytes in culture with leptin induced the expression of angiopoietin-2 (Ang-2), an angiogenic factor that is a receptor antagonist whose expression is limited to sites of vascular remodeling, with initiation of apoptosis, suggesting an autocrine mechanism of leptin-induced loss of adipocytes (Cohen et al., 2001). Although no direct apoptosis-inducing factor has emerged, experiments are going forward to examine the long-term effects of leptin.

Rodents administered leptin have a greater amount of weight loss and regain weight at a slower rate during recovery compared to pair-fed rodents even though food intake returns to normal or overshoots control level (Chen et al., 1996; Azain et al., 1999). What accounts for this apparent extended effect of leptin has not been investigated. In addition to an increase in heat production from UCP stimulation, this effect could be due to a loss of adipocytes within the fat stores. During recovery, energy intake is high and the available adipose mass may not be adequate to absorb the incoming calories. The animal may also provide signals to return to a set fat level. Not only would a recovery of lost adipocytes require more time, additional energy would be required for adipogenesis. Leptin-induced apoptosis could help explain the effect of slower recovery; however, a secondary effect of leptin may limit the loss of cells actually to enhance recovery of the tissue when the environment changes (adequate caloric intake). For example, after an injection of a large, acute dose of leptin, apoptosis may precede an upregulation of cellular defensive mechanisms (e.g., anti-apoptotic Bcl-2 proteins, UCP, ?). Apoptosis would therefore be an immediate but temporary response. Defensive mechanisms would

help prevent further cell death and possibly create a resistance to leptin-induced apoptosis, thereby reducing the effectiveness of subsequent leptin injections. Experimentation shows that the greatest weight loss occurs after the first or first two injections of leptin and each injection thereafter is less effective (Sarmiento et al., 1997). Decreased endogenous production may also play a part.

Leptin-induced adipocyte apoptosis is a relatively new concept as adipocytes were thought to be extremely stable. Most studies have only reported a positive or negative result for the presence of apoptosis. Neither the duration nor the mechanism of leptininduced apoptosis has been uncovered. It is mediated through the CNS but a blood factor has not been ruled out. Answering these questions could provide specific targets or strategies for anti-obesity treatments. The next two chapters will examine the effect of leptin on energy metabolism, adipose tissue cellularity and apoptosis and the changes that occur during recovery. A third chapter details experimental protocols for adipose tissue.

CHAPTER 3

ENERGY METABOLISM AND EXPRESSION OF UNCOUPLING PROTEINS 1, 2, AND 3 AFTER 21 DAYS OF RECOVERY FROM INTRACEREBROVENTRICULAR MOUSE LEPTIN IN RATS¹

¹ Gullicksen, P.S., W.P. Flatt, R.G. Dean, D.L. Hartzell, and C.A. Baile. 2002. *Physiology & Behavior* 75:473-482. Reprinted here with permission from Elsevier Science.

Abstract

Animals tend to maintain a lower body weight for an extended period after leptin administration has ended. This may be due to an enhancement of metabolic rate that persists after treatment withdrawal. Our objectives were to determine the period of leptin influence, when injected intracerebroventricularly (icv), on food intake, body weight, and energy expenditure. Additionally, the relationship between expressions of UCP1, UCP2, and UCP3 in different adipose tissues and heat production (HP) was assessed. Twentyfour adult male Sprague-Dawley rats were injected intracerebroventricularly with either 10 mg mouse leptin or 10 ml vehicle once per day for 4 days. At 24 h after the last injection, one group was killed while the other was placed in calorimetry chambers and monitored for 21 days of recovery. Leptin-injected rats exhibited an overshoot of food intake and respiratory quotient (RQ) during recovery, but body weight remained significantly lower up to 6 days. HP decreased in both groups over time but remained higher in the leptin group through recovery. However, retained energy (RE) was significantly greater than control for about 8 days. Overall, UCP expression was reduced at the end of recovery in parallel with the decline in HP. Brown adipose tissue (BAT) was the most responsive to leptin administration by dramatically changing UCP1 and UCP3 mRNA levels. Our data show that leptin has extended effects on energy expenditure but relieves control on food intake and RQ after treatment withdrawal. This translated into a reduced positive energy balance that slowed body weight recovery.

Keywords: *ob* protein, Food intake, Body weight, Energy metabolism, Uncoupling proteins

Introduction

The hormone leptin, product of the *ob* gene, is a potent regulator of food intake and energy expenditure. Mice with a mutation in the ob gene do not produce leptin and become hyperphagic, overweight and obese. Exogenous replacement of leptin restores their metabolic status to normal by decreasing food intake, body weight, and body fat; normalizing serum levels of glucose and insulin; and increasing metabolic rate, body temperature, and activity to levels similar to those of lean littermates (Pelleymounter et al., 1995). In normal rodents, administration of leptin also causes a dramatic decrease in food intake and loss in body weight (Halaas et al., 1995; Seeley et al., 1996; Flatt et al., 1997; Baile et al., 1997b; Muzzin et al., 2000). The metabolic response to reduced food intake is markedly different from the response to leptin though. Food restriction leads to the loss of lean body mass as well as adipose tissue mass (Halaas et al., 1995; Halaas et al., 1997), and a lower metabolic rate (Even & Nicolaidis, 1993) and level of plasma leptin (Frederich et al., 1995b; Ahima et al., 1996). Refeeding after food restriction results in hyperphagia; this, combined with a lower metabolic rate contributes to a faster recovery of body weight compared to those administered leptin (Chen et al., 1996; Halaas et al., 1997; Wang et al., 1999b). Leptin prevents the reduced energy expenditure normally associated with decreased food intake (Baile et al., 1997a; Doring et al., 1998), which could help explain the difference in recovery. Azain et al. (1999) found that normal, lean rats injected ICV with 2.5 µg per day for five days of recombinant rat leptin fail to return to control weight after eight days. Even fasted lean rats given ICV leptin maintain weight loss for a longer period after refeeding (Cusin et al., 1996). In addition, a single ICV injection of a recombinant adeno-associated virus vector encoding rat leptin

cDNA suppressed weight-gain without a dramatic change in food intake (Dhillon et al., 2001). Leptin appears to stimulate metabolic pathways of higher energy expenditure that are maintained after the leptin stimulus is withdrawn.

The prolonged effect of leptin on energy expenditure could be linked to its effect on heat production. Leptin acts via the sympathetic nervous system to activate and stimulate synthesis of UCP1 in brown adipose tissue (BAT), which uncouples ATP synthesis from oxygen consumption in mitochondria, producing heat at the expense of fatty acids (Hwa et al., 1996; Sarmiento et al., 1997; Scarpace & Matheny, 1998; Commins et al., 1999b). UCP2 & UCP3, isoforms of UCP1, are also upregulated in BAT and white adipose tissue (WAT) by over-expression or chronic ICV infusion of leptin (Trayhurn & Rayner, 1996; Zhou et al., 1997; Scarpace, Nicolson, & Matheny, 1998). Biochemical studies indicate that UCP2 and UCP3, like UCP1, have uncoupling activity but cannot substitute for UCP1 (Nedergaard et al., 2001). UCP2 and UCP3 also appear to be regulated differently than UCP1 and may play a role other than in adaptive thermogenesis, such as in fatty acid mobilization (Himms-Hagen & Harper, 2001) or the control of reactive oxygen species (Boss, Hagen, & Lowell, 2000). Conversely, food restriction causes a reduction of leptin gene expression and UCP1 mRNA levels in BAT. It also results in either reduced or no change in the levels of UCP2 and UCP3 mRNA in BAT or WAT (Zhou et al., 1997; Boss et al., 1997a; Scarpace et al., 1998), but increases UCP2 and UCP3 in skeletal muscle (Gong et al., 1997; Boss et al., 1997a; Weigle et al., 1998; Cadenas et al., 1999). Leptin administration also increases UCP3 in BAT and skeletal muscle (Gong et al., 1997; Vidal-Puig et al., 1997). The upregulation of UCPs may contribute to a higher metabolic rate or assist in the modulation body weight during recovery from leptin administration.

Research show that body weight recovery is slower after leptin withdrawal; however, the duration and processes involved during recovery have not been the focus. Slower recovery may be explained by an extended effect on energy metabolism induced by leptin, causing a prolonged relative energy deficit. This study expands on the limited data published on metabolic changes that occur during an extended period after leptin administration. Parameters of energy metabolism (food intake, RQ, heat production, etc.) were measured daily for 21 days of recovery with measurements of UCP expression at the start and end of recovery. It was hypothesized that leptin-induced heat production will be maintained at a higher level for an extended time and would be mimicked by an increase in UCP expression. Here, we report an overshoot of food intake and RQ offset by a prolonged enhancement in energy expenditure and associated UCP expression that slows the rate of body weight recovery.

Materials and methods

Animals and Housing

Male Sprague-Dawley rats (225-250 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed individually in suspended stainless steel cages for at least one week of acclimation in the animal care facility. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals (NAS, 1996). Rats had access to ground PMI Rat Chow 5012 (Purina, Richmond, IN) and tap water *ad libitum*. Ambient room temperature was maintained at 25°C.

Intracerebroventricular (ICV) Cannulation

Lateral cerebroventricular cannulations were performed under aseptic conditions. The rats were anesthetized with 1 ml/kg of a 3:2:1 v/v/v mixture of ketamine HCl (Ketaset[®], Fort Dodge Laboratories, Inc., Fort Dodge, IA, 100 mg/ml), acepromazine macerate (PromAce[®], Fort Dodge Laboratories, Inc., 10 mg/ml), and xylazine (Rompun[®], Miles Inc., Mission, KS, 20 mg/ml) IP, and when necessary, methoxyflurane (Metafane[®], Pittman-Moore, NJ) by inhalation. The dorsum of the head was shaved and vacuumed. The rat was placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA) and the skin disinfected with chlorhexidine (Nolvasan[®], Fort Dodge Laboratories, Inc.). A 22gauge guide cannula (C313G, Plastics One, Roanoke, VA) cut to a length of 12.80 mm was implanted into the right lateral cerebroventricle. The stereotaxic coordinates used were AP, 0.8 mm, ML, 1.4 mm with respect to bregma, and DV, -3.5 mm from the skull surface. The cannula was held in place with three stainless steel machine screws (Small Parts Inc., Miami Lakes, FL) and cranioplastic cement (Plastics One) attached to the skull. A 28-gauge stylet (C313DC, Plastics One) was installed into the guide cannula when the rat was not receiving an injection.

Rats were allowed to recover following surgery for at least one week before being subjected to an angiotensin II (ANG II) drinking test. ANG II (Sigma, St. Louis, MO) was dissolved in sterile artificial cerebrospinal fluid (aCSF) (Durect Corporation, 1998) at a concentration of 10 ng/ μ l. An increased drinking response of at least 1.5 ml of water in the 30 minutes following an ICV injection of 100 ng ANG II confirmed correct cannula placement. Cannula placement was verified again at the completion of each experiment after the rats had been killed. A 0.14% methylene blue solution (10 μ l) was injected into the ventricle and the brain was extracted from the skull. Cannula placement was confirmed by the appearance of dye in the ventricular system.

Injections

The rats were transferred to the calorimetry lab one day prior to the start of injections (day 0). Ten μ l of leptin (10 μ g recombinant mouse leptin, R&D Systems, Minneapolis, MN) or vehicle (aCSF) were injected into the lateral ventricle over one minute, approximately one hour prior to the onset of the dark period. Rats received one injection per day for four days. Mouse leptin, rather than rat leptin, was used because rat leptin was not commercially available at the time of this experiment. This dose of leptin has been shown to induce significant weight loss by five days as determined by a preliminary study using 0, 2.5 or 10 μ g of mouse leptin in Sprague-Dawley rats (Figure 3.1). The injections were made using an injector cannula (C313I, Plastics One) that extends 1.5 mm below the tip of the guide cannula and was connected to a 92-cm length of polyethylene tubing (PE 20). Following the injection, the injector was left in place for about 30 seconds to allow for diffusion of the test article; the injector was then removed and the stylet replaced into the guide cannula.

Rats were randomly assigned to treatments after being blocked into four groups of six by weight. One-half of the rats were killed 24 hours after the last injection (Day 4) and the others were monitored for 21 days in small animal calorimetry chambers before being killed (Day 25). Body weight, food and water intakes were recorded each day. Twentyone days were chosen for the recovery period as full recovery was expected to have occurred by this time.

Indirect Calorimetry

A computer-controlled, open circuit calorimetry system for small animals (Oxymax, Columbus Instruments Co., Columbus, OH) was used in an environmentally-controlled room (25°C, 40% humidity) to measure oxygen consumption, carbon dioxide production, respiratory quotient (RQ) and heat production (HP) for each rat at 16.5-min intervals during the recovery period. Gas analyzers were calibrated daily using cylinders of primary gas standard mixtures with known concentrations of CO₂, O₂, and N₂. The calculation for RQ is CO₂ production (liters)/O₂ consumption (liters). The percentage of heat production (HP) resulting from fat and carbohydrates oxidized are based on the RQ (Brody, 1945). Heat production calculations were based on the Brouwer equation (Brouwer, 1965): HP = 3.820 O_2 consumption (liters) + 1.150 CO_2 production (liters). The notation for metabolic body size (MBS) is body weight in kilograms raised to the 0.75 power (kg^{0.75}). HP was subtracted from metabolizable energy (raw food intake \times dry matter $\% \times 3.643$ kcal/g) to calculate retained energy (RE) as an estimate of energy balance (National Research Council, 1981). Calorimetry measurements were taken every 16.5 minutes, 24 hours per day except during a short period (approx. 45 min.) of daily weighing of rats, food, water, and fecal mass. The data for RQ, HP, and RE were averaged over 24 hours.

Tissue Collection

Rats were killed with CO_2 in a small chamber. Inguinal (ING), epididymal (EPI), and retroperitoneal (RP) white fat pads and the intrascapular brown fat pad (BAT) were

removed, weighed, immediately frozen in liquid nitrogen, and stored at -80°C for later analysis.

Real-time RT-PCR

Total RNA was extracted from frozen tissue with TRIzol Reagent (Life Technologies, Rockville, MD) as directed. RNA yield was determined by a highly sensitive, high precision fluorescent assay using RiboGreen (Molecular Probes, Eugene, OR). Fluorescent measurement of RNA alleviates many of the problems associated with absorbance measurements at 260 nm. The sensitivity exceeds that achieved with ethidium bromide-based assays by 200-fold or absorbance determination by 1000-fold (Molecular Probes, 2001). The very accurate method was chosen because each mRNA target would be related to total RNA. A one-step real-time RT-PCR was performed using the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) using the software SDS 1.6.3 following the guidelines of the TaqMan Gold RT-PCR Kit (Applied Biosystems). Reaction conditions were: RT (30 min, 48°C; 10 min, 95°C) and 40 cycles of a two-phase PCR (15 s, 95°C; 60 s, 60°C) with 1× TaqMan PCR reaction buffer; MgCl₂, 5.5 mM; dATP, dCTP, and dGTP (300 μ M each); dUTP, 600 μ M; each primer, 100 nM; probe, 100 nM; AmpliTaq Gold, 0.025 u/µl; and MuLV Reverse Transcriptase, 0.25 u/µl. The primer and probe sequences (Table 3.1) were designed using Primer Express 1.0A (Applied Biosystems) and purchased from Applied Biosystems. Each mRNA was measured against a standard curve of cDNA that was generated by RT-PCR, purified on an agarose gel, extracted using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and yield determined by PicoGreen (Molecular

Probes, Eugene, OR). The optimum amount of total RNA added to the reaction was determined for each target in each tissue by running dilution curves. The efficiency of the reaction was determined by the slope of the exponential phase of amplification. Samples with aberrant slopes were rerun. Standards and sample values were determined in triplicate. Copy number was calculated as $602,300,000 / (base pairs \times 0.6498 / 1000)$.

Table 3.1. Primer and probe sequences used for real-time RT-PCR of UCPs

	Sense	Antisense	Probe			
UCP1	GTGTACCCAGCTGTGCAATGA	CGCAAACCCTTTGAAAAAGG	TGCCGGTCCTTCCTTGGTGTACATG			
UCP2	CCTGAAAGCCAACCTCATGAC	CAATGACGGTGGTGCAGAAG	ACGACCTCCCTTGCCACTTCACTTCTG			
UCP3	TGCAGCCTGTTTTGCTGATCT	GGGTTCTCCCCTTGGATCTG	TTCCCCCTGGACACCGCCAA			

Statistical Analysis

The overall effects of leptin administration and time on daily measurements (food intake, water intake, body weight, RQ, and heat production) were analyzed by two-way analysis of variance (ANOVA) using a General Linear Model Repeated Measures procedure. UCP data were analyzed by one-way ANOVA to test means between groups within each day (2 groups) and by two-way ANOVA to test treatment-by-day (4 groups, overall). If UCP sample variances were unequal (significantly different based on the Levene test), logarithmic (log) transformation of the data was performed to determine significant differences. Data were expressed as means \pm standard error of the mean (SEM). Values of $P \le 0.05$ were accepted as being statistically significant. All statistical analyses were run using SPSS 10.0.7 for Windows.

Results

An objective of the present study was to produce a maximal effect from leptin so that the responses during the recovery period would be evident. In a preliminary study, the dose response of 2.5 and 10 μ g of mouse leptin produced similar results during the injection period (Figure 3.1). The data do not suggest how the different doses affect recovery since, by design, there was no recovery period. The higher dose did not produce any observed behavioral or adverse effects; therefore, 10 μ g was used.

The effects of leptin administration on daily food consumption are detailed in Figure 3.2. During the injection period, total

food intake averaged 10.4 g/day in the leptin group and 17.8 g/day in the control group, which translates into a 42% decrease (-30 g) in food intake due to leptin [F(1,22)=196.65,P<0.001]. Adjusting for body weight (BW), food intake decreased by 22.7 mg/g BW/day or 38% [F(1,22)=75.55, P < 0.001]. There was a rebound effect following the end of leptin administration whereby food intake increased two days after the last injection and was significantly greater than control for eight days (P < 0.05).



Figure 3.1. Daily food intake and body weight of Sprague-Dawley rats injected ICV with 2.5 or 10 μ g of mouse leptin or 10 μ l aCSF (control) for 4 days. n=6 per group. Data expressed as mean \pm SEM. **P*<0.05, ***P*<0.01 vs. control.

The leptin-treated rats at significantly more than the control rats for the last 20 days of recovery [F(1,10)=8.80, P<0.02].



Figure 3.2. Daily food intake (g) of rats injected ICV with leptin (10 μ g/day) or control (10 μ l vehicle). For each group, n=12 during injection period and n=6 during recovery. Data expressed as mean \pm SEM. **P*<0.05, ***P*<0.01 vs. control.



Figure 3.3. Daily body weight (g) of rats injected ICV with leptin (10 μ g/day) or control (10 μ l vehicle). For each group, n=12 during injection period and n=6 during recovery. Data expressed as mean \pm SEM. **P*<0.05, ***P*<0.01 vs. control.

Calorimetry measurements were started 24 hours after the last set of injections, making the first data point two days into recovery (Figures 3.4 & 3.5). On this first day of measurement (day 5), the RQ of leptin-treated rats was still lower than control (0.95 \pm 0.007, leptin; 0.99 \pm 0.008, control; *P*<0.01). Prior research indicates that leptin can reduce RQ to 0.86 (Wang et al., 1999b). RQ became significantly higher than the control group two days later (day 7) and reached a maximum of 1.02 five days after the last injection (day 9; 1.02 \pm 0.005 vs. 0.99 \pm 0.004; *P*<0.01). RQ was maintained higher in the leptin-treated rats for a total of eleven days during recovery, and a repeated measures ANOVA analysis indicated a significant difference for the last 19 days of recovery [*F*(1/10)=28.96, *P*<0.01].



Figure 3.4. Daily respiratory quotient (RQ) of rats injected ICV with leptin (10 μ g/day, n=4) or control (10 μ l vehicle, n=4). Data expressed as mean \pm SEM. **P*<0.05, ***P*<0.01 vs. control. 2nd order polynomial trend lines were added to the control group, R²=0.22, and to the leptin group during early recovery (first 3 days), R²=0.99. A 3rd order polynomial trend line was added to later recovery (last 19 days), R²=0.79.



Figure 3.5. Daily heat production per metabolic body size (kcal/kg^{0.75}) of rats injected ICV with leptin (10 μ g/day, n=4) or control (10 μ l vehicle, n=4). Data expressed as mean \pm SEM. Logarithmic trend lines added: Control, y = -9.1113Ln(x) + 125.51, R²=0.70; Leptin, y = -11.024Ln(x) + 137.24, R²=0.71. **P*<0.05, ***P*<0.01 vs. control. Different from vehicle-injected rats (repeated measures ANOVA): *P*<0.05.

Heat production adjusted for metabolic body size (HP) was 6.9% higher in the leptininjected rats two days after the end of injections (118 ± 1 vs. 111 ± 2 kcal/kg^{0.75}/24 hrs; P<0.01. Figure 3.5). By the end of recovery, it declined to a difference of only 5.4% (103 ± 2 vs. 98 ± 1 kcal/kg^{0.75}/24 hrs; P<0.05). A repeated measures ANOVA indicated that the leptin group had a greater HP throughout the 21 days of recovery with an average difference of 6% or 6.0 kcal/kg^{0.75}/24 hrs [F(1,6)=8.15, P<0.05]. The difference between metabolizable energy and HP calculated as retained energy (RE) and plotted with separate trend lines to indicate the two phases of recovery in the leptin group (Figure 3.6). The control rats averaged 15.8 kcal/day RE throughout the study. The leptintreated rats were in positive RE (positive energy balance) two days after the last injection but were still significantly lower than control (4.7 ± 3.1 vs. 14.8 ± 1.6 kcal, P<0.05). It was not until four days into recovery that the leptin-treated rats had an RE significantly greater than control, and reached a maximum of 27.5 kcal on day 10, seven days after injections ended. There were eight days that the RE of leptin-treated rats was significantly greater than control, and a repeated measures ANOVA analysis indicated that the last 19 days of recovery were significantly different [F(1,6)=28.27, P<0.01].



Figure 3.6. Daily retained energy (RE), an estimate of energy balance, of rats injected ICV with leptin (10 μ g/day, n=4) or control (10 μ l vehicle, n=4). Data expressed as mean \pm SEM. **P*<0.05, ***P*<0.01 vs. control. A 3rd order polynomial trend line was added to the control group, R²=0.06. A 2nd order polynomial trend line was added to the leptin group during early recovery (first 3 days), R²=0.99, and a 4th order polynomial trend line was added to later recovery (last 19 days), R²=0.75.

Expression (mRNA) of UCP1, UCP2 and UCP3 was measured in the intrascapular brown fat pad (BAT) and the retroperitoneal (RP), epididymal (EPI), and inguinal (ING) white fat pads. Initial traditional (not "real-time") RT-PCR products using the real-time RT-PCR primers produced single bands on agarose gels at 67, 82, and 77 bases corresponding to UCP1, UCP2, and UCP3, respectively. These bands were extracted from the gel and the DNA quantitated for use as an external standard. The results from real-time RT-PCR experiments are detailed in Table 3.2. Leptin promoted a 90% increase in overall (1, 2, & 3) UCP expression in BAT, RP and EPI, but had almost no effect in ING, as measured at 24 hours after the last injection (Day 4). At the end of the recovery period, overall UCP expression decreased by 76% in the leptin group and 67% in the control group, equating to a 40% greater amount of UCP expression in the leptin group but most of the individual statistical differences that were seen earlier were gone.

One day after the injection period, UCP1 and UCP3 mRNA levels were 2-fold higher in BAT from leptin-injected rats compared to the vehicle-injected rats ($P \le 0.01$ and P < 0.05, respectively). In the white fat pads, UCP2 mRNA made up the greatest fraction of UCP transcripts while UCP1 mRNA was barely detectible. The RP pad had 2- to 3fold increases in all UCP mRNA; however, due to high variability, only UCP3 was significant (161%, P=0.05). EPI had large increases in both UCP2 and UCP3 mRNA (122%, P=0.06 and 44%, P<0.05, respectively). ING had the least response to leptin administration but was the only tissue that exhibited a slight decrease in UCP2 mRNA after leptin injections (-39%; P NS). After recovery, ING UCP2 mRNA was significantly greater in the leptin-treated rats by 2.5-fold (P<0.05) and was similar to levels measured one day after the injection period (level did not change in the leptin The effect of recovery was examined by comparing the difference in copy group). numbers between Day 25 and Day 4 within each group. BAT showed the greatest reductions in UCP expression after recovery in both leptin and control groups (UCP1, -84% and -71%, P < 0.01; UCP3, -93% and -86%, P < 0.01; respectively), but still had a greater expression of all UCPs than WAT, principally due to high UCP1 expression.

	Day 4			Day 25			Overall ^a		
	Control	Leptin	P^{b}	Control	Leptin	P^{b}	Trt P	Day P	$Trt \times Day$ P
BAT									
UCP1	$23,\!678 \pm 2090$	$50,\!796\pm5822$.01 ^c	6911 ± 850	7891 ± 954	.46	.01 ^c	.01 ^c	.01 ^c
UCP2	61.0 ± 6.8	84.4 ± 19.1	.31	45.7 ± 8.4	71.4 ± 14.5	.18 ^c	.11 ^c	.19 ^c	.64 ^c
UCP3	36.4 ± 5.8	67.5 ± 12.1	.04	5.00 ± 1.82	4.52 ± 1.24	.83	.25°	.01°	.45°
RP									
UCP1	0.82 ± 0.26	2.29 ± 1.07	.25	0.34 ± 0.10	2.31 ± 1.04	.02 ^c	.01 ^c	.38 ^c	.31 ^c
UCP2	150 ± 40	305 ± 85	.13	228 ± 78	611 ± 238	.23 ^c	.07 ^c	.43°	.93°
UCP3	24.3 ± 7.8	63.5 ± 16.6	.05	22.8 ± 4.5	22.4 ± 4.7	.96	.09 ^c	.10 ^c	.07 ^c
EPI									
UCP1	3.02 ± 0.20	3.07 ± 0.21	.86	2.96 ± 0.20	3.34 ± 0.26	.26	.33	.63	.46
UCP2	1374 ± 239	3050 ± 755	.06 ^c	1081 ± 44	1458 ± 271	.35 ^c	.04 ^c	.06 ^c	.27 ^c
UCP3	50.8 ± 7.1	73.3 ± 5.2	.03	40.3 ± 6.2	32.4 ± 8.5	.46	.29	.01	.04
ING									
UCP1	2.79 ± 0.12	2.77 ± 0.21	.91	2.67 ± 0.09	2.57 ± 0.09	.45	.65	.26	.79
UCP2	4837 ± 1428	2960 ± 1224	.34	1475 ± 538	3627 ± 715	.03	.89	.21	.06
UCP3	50.1 ± 6.5	42.0 ± 7.0	.41	45.5 ± 14.5	60.8 ± 12.7	.44	.75	.53	.30

Table 3.2. UCP transcripts (copies/ng total RNA) in adipose tissues after 4 days of leptin injections (Day 4) and after 21 days of recovery (Day 25)

Data represent mean \pm SEM for five to six male Sprague-Dawley rats/treatment/day.

BAT, brown adipose tissue; RP, retroperitoneal; EPI, epididymal; and ING, inguinal white fat pads.

^a One-way ANOVA comparing leptin vs. control within each day.

^b Two-way ANOVA.

^c Log transformation was performed due to heterogeneity of variances.

Discussion

Research has shown that administration of leptin causes a greater amount of weight loss than pair-fed rodents, and the rate of weight (re)gain after treatment withdrawal is slower after leptin resulting in a lower body weight for a longer period (Chen et al., 1996; Azain et al., 1999). The leptin-induced enhancement of metabolic rate may continue after treatment withdrawal preventing a fast regain of weight that is seen after foodrestriction. The increase in energy expenditure during leptin administration results from an increase in heat production and UCP activity in BAT (Collins et al., 1996; Scarpace et al., 1997), which apparently extends into recovery as suggested by the data in this study. The changes in energy metabolism to modulate body weight during 21 days of recovery from ICV leptin injections are examined here.

During four days of leptin injections, we observed the characteristic reduction in food intake and body weight that were similar to previous reports using mouse leptin (Flatt et al., 1997) and rat leptin (Baile et al., 1997a; Wang et al., 1999b) in Sprague-Dawley rats. A rebound effect occurred in food intake during early recovery resulting in hyperphagia for nine days but this consequence is related to the reduced food intake and not directly to leptin. This is demonstrated by pair-feeding a group of rodents to a group that is leptintreated. Although a pair-fed group was not part of this experimental paradigm, pair-fed rodents show a similar hyperphagic response after unlimited food becomes available (Wang et al., 1999b).

Indicated by a decreased RQ, leptin stimulates preferential use of fat as a fuel source (Hwa et al., 1996; Wang et al., 1998b) while sparing body protein (Kaibara et al., 1998). The present study found the same effect in lowering RQ plus, for an additional two days following the last leptin injection, it continued to stay lower than control. RQ rebounded during recovery similar to the pattern of food intake. It increased rapidly and leveled off significantly above control level (RQ>1) then slowly declined. Separate trend lines were added to the RQ plots (Figure 3.4) to indicate this. A strong anabolic state lasted over eleven days during recovery with total energy derived primarily from carbohydrate while promoting fat accretion/repletion and weight gain. Earlier studies have shown that food-

restricted rats have similar responses in RQ, especially the time of the rise and level achieved (Wang et al., 1999b).

Energy expenditure and oxygen consumption have been demonstrated to be increased by leptin during the treatment period (Hwa et al., 1996; Scarpace et al., 1997; Azain et al., 1999), whereas they are reduced by food restriction. This study examines the duration of enhanced metabolic rate and potential contributing factors, specifically UCPs. Leptin administration raised heat production 7%. While both groups exhibited a similar decline over time of about -13%, leptin instilled an average 6% increase in heat production throughout recovery. This result conflicts with a previous report where mice infused ICV with recombinant human leptin for 30 days (8 ng/hr) showed no difference in energy expenditure on a one-day measurement, 10 days into recovery (Halaas et al., 1997). However, the reduced weight was maintained for the duration of the infusion while food intake returned to control level within eight days from the start of infusion. This suggests leptin induced a lower retained energy as found in this study, and a possible separate mechanism to maintain energy expenditure. The sustained increase in heat production observed could also be partly due to the increase in food intake but is not likely because evidence suggests that responses to leptin administration during short-term recovery are different from those seen after food restriction. Pair-fed rats also become hyperphagic during three days of refeeding but heat production only returns to control level (Wang et al., 1999b).

The leptin-stimulated increase in energy expenditure is tied to the activation of uncoupling proteins and thermogenesis, especially by UCP1 in BAT (Astrup, 1986; Scarpace et al., 1997). Using two groups of rats, UCP1, UCP2, and UCP3 expression

was examined at 24 hours and 21 days after leptin administration. The relative change in transcript numbers indicated that ICV leptin administration increased the overall expression of the uncoupling proteins in the fat pads: BAT > RP > EPI >> ING. Both UCP1 and UCP3 mRNA were increased two-fold in BAT but UCP1 was predominantly expressed, making up greater than 99% of the total UCP transcripts in BAT (Table 3.2). In WAT, UCP2 mRNA was predominant, making up 97% of the total. Although there was a dramatic change in overall UCP expression, the majority of transcripts were of UCP1 in BAT, highlighting the recognized function of BAT as the thermogenic tissue. The physiological significance of the increased UCP3 mRNA in BAT to thermogenesis is probably minimal since it made up less than 1% of the total copies but may reflect a different role as in providing fatty acid substrates. In retroperitoneal and epididymal white fat pads, leptin administration increased UCP3 expression and provided a trend to increase UCP2 expression. Leptin administration had the least effect in the inguinal fat pad on expression of UCPs but there was a trend to lower UCP2 mRNA levels in this A similar observation was reported where reduced UCP2 expression was tissue. observed in periovian WAT after a 5-hour ICV infusion of leptin in C57BL/6J mice (Combatsiaris & Charron, 1999). This suggested a lower energy demand by the inguinal fat pad but increased demand by BAT after acute leptin administration. Other studies have shown leptin increases UCP2 in different white adipose tissues (Sarmiento et al., 1997; Zhou et al., 1997). The differences between the fat pad depots in UCP expression may relate to their level of innervation, metabolic activity, and vascularization; for example, the visceral fat pads (retroperitoneal and epididymal) are more metabolically active and have a faster turnover than the inguinal fat pad. UCP2 mRNA levels

diminished in the inguinal pad of the control rats, while it appeared to be maintained in the leptin-treated rats; in other words, UCP2 mRNA was unchanged in the leptin-treated rats over recovery.

The relationship between UCP expression and heat production was assessed. Measurement of heat production can be done continually but UCP levels can be measured only once per animal since it requires the removal of the fat pad, and therefore limits the strength of association. Although the true contribution of UCP to heat production is difficult to assess, it was expected that as heat production decreased through recovery, UCP mRNA levels would decline likewise. An initial greater expression of uncoupling proteins supports increased heat production during early days of recovery. On the first day of measurement (early recovery), a 7% increase in heat production was associated with a 90% increase in overall UCP expression. The decline in heat production during recovery was similar between the leptin and control group (-13% and -12%, respectively), while the decline was also similar in overall UCP expression (-76%) and -67%, respectively) or in BAT-specific UCP expression (-64%, -61%, respectively). However, even after recovery, the leptin group had a 40% greater transcript number of UCPs, which may have contributed to the sustained metabolic rate during of recovery. At this point, the transcript numbers suggest that BAT and UCP1 contributes the most to thermogenesis, although the RP pad had maintained a greater percent of UCP expression, mostly UCP2. These data suggest the acknowledged relationship between thermogenesis and uncoupling proteins. However, the robust and definitive upregulation of UCP1 over UCP2 & UCP3 suggests a more specific regulation of UCP1 by leptin whereas UCP2 and UCP3 may be less directly regulated and fulfill a different role other than producing heat.

If heat production had decreased to the level of control, it would have been expected that UCP levels might have as well by 21 days. Rather, both heat production and UCP levels were raised in the leptin group. However, this does not exclude other energy wasting mechanisms that could contribute to an increased metabolic rate.

The apparent delay in the decline of both thermogenesis and UCP mirrors the reported delay in their rise during leptin stimulation. A prior study found that there is a delay in the rise of energy expenditure until several days after the initiation of leptin administration (Wang et al., 1999b), which reflects a requirement for the synthesis of new UCP (Scarpace et al., 1997). The current study suggests that the newly synthesized UCP may be stable enough to provide a lasting effect that slowly declines after treatment withdrawal. This determination is limited because the actual heat contribution of UCP to adaptive thermogenesis has not been determined and the daily changes in UCPs between the two time points are unknown. Furthermore, the protein content of each UCP and their activities were not measured but they would provide important contributing data. Protein measurements might show even more dramatic sustained changes. This lingering of increased UCPs could contribute to the usefulness to leptin-like anti-obesity agents.

Although heat production was sustained at a greater level during recovery, it was opposed by a substantial energy intake. To estimate energy balance, retained energy (RE) was calculated as the difference between metabolizable energy (energy intake) and heat production (energy expenditure). An RE of zero would imply no change in body mass. During leptin administration and up until three days after withdrawal, RE was either negative or lower than control, which states that they were losing weight or at least not gaining faster than control. After this point, the dramatic overshoot of food intake caused RE to be significantly greater than control for eight days. Clearly, the rats recovering from leptin administration had no energy deficit beyond the first three days of recovery and weight gain was thus favored. However, without the leptin-induced increase in energy expenditure, RE would have attained a greater level and would be maintained for a longer period resulting in a faster weight gain. This helps explain why food-restricted rats recover faster, especially when combined with the loss of high metabolically active lean tissues. RE normalized 12 days after treatment withdrawal. At this point, both groups should continue to gain weight at a similar rate and this is what was observed. The body weight difference between the groups changed very little between this point and the end of recovery (-3% and -2%, respectively). Compared to the injection period, leptin had caused a 10% loss in body weight.

Research has shown that the body weight of rats administered ICV of recombinant rat leptin (2.5 μ g per day for four days) was significantly lower than control even after eight days of recovery (Azain et al., 1997; Azain et al., 1999). The commercial mouse leptin used in the present study at 10 μ g per day induced similar changes in food intake and body weight during the injection period. Differences appeared during the recovery period where the rats injected with mouse leptin had, surprisingly, a much greater rate of weight gain than previously seen and a statistical difference in body weight up to only six days. This was, though, a longer recovery compared to food restriction; pair-fed rats normalize within a couple days after unlimited feeding (Wang et al., 1999b). If leptin is injected ICV into fasted rats at the start of refeeding, weight loss is maintained up to six days (Cusin et al., 1996). It is uncertain why recovery from the mouse leptin was different from the rat leptin even though the effects during injections were similar. The

dose of leptin used in this study was relatively high and the responses may be pharmacological (Spiegelman & Flier, 1996; Flier & Elmquist, 1997). The use of a physiological dose of leptin may produce different results during recovery. Continued work focusing on several time points while including a pair-feeding component would provide further insight.

In summary, leptin prevented the decrease in energy expenditure that typically occurs during food restriction. UCP expression does not appear to be turned off when exogenous leptin is no longer provided, but may continue to sustain higher energy expenditure through recovery. The trend in heat production correlated with overall UCP expression in the adipose tissues but UCP1 exhibited the greatest expression owing to BAT as the primary contributor to thermogenesis. Although a rebound in food intake occurred, the result was a lower retained energy and slower body weight recovery after treatment termination compared to that achieved with food restriction as previously reported. These data suggest that leptin injected ICV, working through the central nervous system, has extended effects on sustained energy expenditure but relinquishes control on food intake within a couple days after discontinuation. By 21 days of recovery, no differences were found in retained energy or growth rate while body weight was only modestly lower. These results also demonstrate a strong tendency for the return of food intake and body weight to the levels of control.
CHAPTER 4

ADIPOSE TISSUE CELLULARITY AND APOPTOSIS AFTER INTRACEREBROVENTRICULAR INJECTIONS OF LEPTIN AND 21 DAYS OF RECOVERY IN RATS¹

¹ Gullicksen, P.S., D.B. Hausman, R.G. Dean, D.L. Hartzell, and C.A. Baile. Submitted to *International Journal of Obesity*, 5/9/2002.

Abstract

OBJECTIVE: To determine the effect of leptin and post-treatment recovery on adipose tissue cellularity and apoptosis. In addition, to investigate whether Bcl-2 and/or Bax is involved in the mechanism of leptin-induced adipose tissue apoptosis.

DESIGN: Twenty-four adult male Sprague-Dawley rats were injected ICV with either 10 μ g mouse leptin or 10 μ l vehicle once per day for 4 days. At 24 hours after the last injection, one group was killed while the other was monitored for 21 days.

MEASUREMENTS: DNA fragmentation and Bcl-2 & Bax protein levels were determined in inguinal (ING), epididymal (EPI) and retroperitoneal (RP) white adipose tissues and the intrascapular brown adipose tissue (BAT). Cellularity was determined in ING and EPI.

RESULTS: Leptin significantly reduced the masses of all white fat pads [RP > ING > EPI] but not BAT. Cell volume was significantly reduced in EPI and ING. Only ING had a significantly reduced cell number from leptin treatment plus exhibited apoptosis by increased DNA fragmentation and DNA laddering, and upregulation of pro-apoptosis Bax protein. The other fat pads exhibited a general trend to increase the Bcl-2/Bax ratio. Recovery allowed for normalization of white fat pad mass, cell number and cell volume; however, BAT increased 42% over control. After recovery, apoptosis was not detected, Bcl-2 protein had increased in ING, and the Bcl-2/Bax ratio had risen overall.

CONCLUSIONS: Central administration of mouse leptin in the rat targets white fat depots individually to reduce mass by a reduction in cell volume plus adipocyte deletion in, at least, the inguinal fat pad by Bax-mediated apoptosis. Even after a dramatic loss in

adipose tissue mass and change in cellularity, the rat demonstrates a resilient return to control levels together with an increase in factors that prevent adipocyte loss.

Introduction

Leptin, a major regulator of food intake and energy expenditure, is increased during the accretion of body fat and circulates in the blood in concentrations proportional to body fat content (Maffei et al., 1995b; Considine et al., 1996c). Central and peripheral administration of recombinant leptin reduces food intake, body weight, and body fat content in rodents without functional leptin (Pelleymounter et al., 1995) and with functional leptin (Halaas et al., 1995; Seeley et al., 1996; Flatt et al., 1997; Halaas et al., 1997; Baile et al., 1997b; Muzzin et al., 2000). Normal rodents treated with leptin lose more weight than rodents fed the same amount of food and the rate of regain is slower after treatment withdrawal than food-restricted rodents (Chen et al., 1996; Azain et al., 1999). The result is a weight loss primarily composed of fat (Halaas et al., 1995; Kaibara et al., 1998). Decreased food intake does not completely account for adiposity reduction after leptin infusion (Levin et al., 1996). Following leptin treatment, body weight and fat mass were dramatically reduced while repletion of fat depots during an eight-day recovery period was markedly delayed when compared to pair-fed rats (Azain et al., 1997). The delay in repletion after treatment withdrawal is due, in part, to an enhanced metabolic rate and thermogenic processes maintained after treatment withdrawal (Gullicksen et al., 2002). In addition to increasing lipolysis, it is also possible that a loss of adipocytes (Qian et al., 1998a; Sorisky, Magun, & Gagnon, 2000) would result in a deficit of cells for lipid esterification as the body recovers. Available adjpocytes may not be able to absorb excess substrates coming from a rebound in food intake. Generating new cells would require extra energy that would contribute to the prolonged metabolic enhancement seen after leptin administration. Previous studies found that leptin

administration reduces epididymal and retroperitoneal cell number (Qian et al., 1998b) and induces apoptosis within retroperitoneal and parametrial fat tissues (Qian et al., 1998a), and this was not due to food restriction. Data on the duration of the impact on cell number or the mechanism of leptin-induced apoptosis is limited.

Apoptosis, the regulated destruction of a cell, is a complicated process involving many factors that is continuing to be discovered (Hengartner, 2000). Key features of apoptosis involve the mitochondria and the apoptotic proteins Bcl-2 and Bax. Overexpression of Bax accelerates apoptotic death by acting on mitochondria to disrupt the electron transport chain and promote the release of cytochrome c into the cytoplasm (Oltvai, Milliman, & Korsmeyer, 1993; Green et al., 1998). This, in turn, activates proteolytic molecules known as caspases that are crucial for the execution of apoptosis. Bcl-2, localized to mitochondria, endoplasmic reticulum, and nuclear membranes, interferes with the activation of the caspases by preventing the release of cytochrome c (Korsmeyer et al., 1993). Bcl-2 inhibits Bax-induced apoptosis and other mitochondrial disrupters (Rosse et al., 1998; Murphy et al., 2000); however, overexpression of Bax can counter the death repressor activity of Bcl-2 (Oltvai et al., 1993). The Bcl-2/Bax ratio appears to determine the susceptibility to apoptosis by regulating mitochondrial function following an apoptotic stimulus (Korsmeyer et al., 1993).

Several studies have also linked these proteins to energy metabolism and obesity. A loss of Bcl-2 expression is associated with apoptotic death of brown adipocytes while upregulation is associated with prevention of apoptosis (Navarro et al., 1999). Brown adipocytes and pancreatic β -cells of obese (*fa/fa*) rats were found to have lower Bcl-2 or Bcl-2/Bax mRNA and protein ratios than those of their lean littermates (Briscini et al.,

1998; Shimabukuro et al., 1998b). Fatty acid can induce apoptosis of β -cells in culture, but a leptin-dependent maintenance of Bcl-2 expression has been found to protect against this (Shimabukuro et al., 1998b). In addition, daily injections of recombinant leptin to 21-day-old female rats caused a significant increase in Bcl-2 expression and Bcl-2/Bax ratio in granulosa cells (Almog et al., 2001). Evidence of leptin-induced white adipose tissue apoptosis (Qian et al., 1998a; Qian et al., 1998b) suggests that leptin is not acting to increase the Bcl-2/Bax ratio in these tissues but no data are available in the literature to describe in detail the effect of leptin on this process in adipose tissue.

The delay in body weight recovery after leptin administration may be related to a loss in adipocytes. The study of this phenomenon provides further incite to the metabolic control of body fat by leptin. Therefore, we hypothesize that leptin administration causes a reduction in cell size and number within adipose tissues by apoptosis. Tissues exhibiting apoptosis (DNA fragmentation) may also have a lowering of the Bcl-2/Bax ratio. Lastly, recovery should allow for the normalization of adipose tissue mass, favoring an increase in volume over cell number and an absence of apoptosis. The experimental results indicate that although all white fat pads lose a significant amount of mass, only loss in the inguinal fat pad includes apoptosis influenced by the upregulation of Bax protein. An increase of the Bcl-2/Bax ratio in the fat pads during recovery may act to prevent cell loss as the tissues show a tendency to return to control levels of adipose tissue cellularity by 21 days.

Materials and Methods

Animals and housing

Male Sprague-Dawley rats (225-250 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed individually in suspended stainless steel cages for at least one week of acclimation in the animal care facility before intracerebroventricular cannulation surgery. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals (NAS, 1996) and the experimental procedures were approved by the Animal Care and Use Committee for The University of Georgia prior to initiating the study. Rats had access to ground PMI Rat Chow 5012 (Purina, Richmond, IN) and tap water *ad libitum*. Ambient room temperature was maintained at 25°C.

Experimental design

Lateral cerebroventricular cannulations and injections were performed as previously described (Gullicksen et al., 2002). Ten μ l of leptin (10 μ g recombinant mouse leptin, R&D Systems, Minneapolis, MN, USA) or vehicle [artificial cerebrospinal fluid (Durect Corporation, 1998)] were injected into the lateral ventricle approximately one hour prior to the onset of the dark period. Rats received one injection per day for four days. This dose of leptin has been shown to induce significant weight loss by five days (Gullicksen et al., 2002). Rats were randomly assigned to treatments after being blocked into four groups of six by weight. One-half of the rats were killed 24 hours after the last injection (Day 4) and the others were monitored for 21 days before being killed (Day 25). Body weight and food and water intakes were recorded each day. Twenty-one days were

chosen for the recovery period as full recovery was expected to have occurred by this time. Rats were killed with CO_2 in a small chamber. Inguinal (ING), epididymal (EPI) and retroperitoneal (RP) white fat pads and the intrascapular brown fat pad (BAT) were removed, weighed, immediately frozen in liquid nitrogen, and stored at -80°C for later analysis.

Cellularity

Fat cell size and number were determined through electronic quantification using the method of Hirsch and Gallian (1968) as modified by Cartwright (1987). Duplicate adipose tissue samples (50-70 mg) were fixed in a solution containing 0.2 M osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA, USA) in 50 mM collidine (2,4,6-trimethylpyridine) buffer. Samples were fixed for at least 1 wk at room temperature, rinsed with 0.9% NaCl, and then placed in 8 M urea for several days to facilitate separation of cells in the tissue. Fixed adipocytes were rinsed with 0.9% NaCl through a 240-µm nylon screen and then collected on a 20-µm nylon screen (Tetko, Briarcliff Manor, NY, USA). Samples of cells that met quality standards during the fixation process were analyzed on a Coulter electronic particle counter (MultiSizer Model ZM, Coulter Electronics, Hialeah, FL, USA). Counts in all size ranges were performed in duplicate and are reported as percent distribution. Number of adipocytes per pad was calculated by multiplying the mean number of cells per milligram of sample by the total weight of the corresponding depot.

DNA fragmentation assay

DNA fragmentation was assayed by a modification of the method of Shimabukuro et al. (1998a) and Duke and Sellins (1989). WAT (180-200 mg) or BAT (40-50 mg) was homogenized in 1 ml of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, and 0.5% Triton X-100, pH 8.0) in a glass-Teflon homogenizer and incubated on ice for 20 min. After centrifugation at 14,000 \times g for 15 min at 4°C, the fat cake was removed and the supernatant containing fragmented (soluble) DNA was transferred to a new tube. DNAzol (Molecular Research Center, Cincinnati, OH, USA) (0.5 ml) was added to the pellet containing insoluble DNA. Both tubes were treated with RNase A (0.5 mg/ml) for 15-30 min at 37°C. To the soluble DNA fraction, an equal volume of phenol/chloroform/isoamyl alcohol was added, vortexed briefly and centrifuged at 16,000 \times g for 10 min at RT. The aqueous phase was transferred to a new tube and the organic phase reextracted with 100 µl TE buffer, pH 8.0 with centrifugation at $10,000 \times g$ for 2-3 min. To the combined aqueous phases, polyacryl carrier (Molecular Research Center), MgCl₂ (< 10 mM final conc.), and $\frac{1}{2}$ vol 7.5 M ammonium acetate were added, and mixed by inversion. An equal volume of isopropyl alcohol was added, mixed by inversion, and allowed to precipitate for at least 20 min. The DNA precipitate was pelleted by centrifugation at $10,000 \times g$ for 30 min, and washed twice with 0.8-1 ml 70% ethanol and centrifuged at $10,000 \times g$ for 2-3 min.

The large DNA fraction containing DNAzol was centrifuged at $16,000 \times g$ for 2-3 min to sediment insoluble material. The supernatant was transferred to a new tube, polyacryl carrier added and the solution mixed by inversion. The DNA was precipitated by the addition of $\frac{1}{2}$ vol of ethanol and mixed at RT. After 3 min, the DNA precipitate

was sedimented by centrifugation at $10,000 \times g$ for 5 min, washed twice with 0.8-1 ml of 70% ethanol and centrifuged at $5,000 \times g$ for 2-3 min. DNA pellets from both fractions were air-dried for several minutes and resolubilized in a minimal volume of TE buffer (pH 8): 20-25 µl added to the small (soluble) DNA fraction and 50-100 µl added to the large (insoluble) DNA fraction.

DNA concentration was measured using the fluorescent PicoGreen assay (Molecular Probes, Eugene, OR, USA). DNA fragmentation was calculated as soluble DNA/(soluble + insoluble DNA) × 100%. The soluble DNA fractions were loaded onto a 2% agarose gel and run at 128V (8V/cm) or on 6% TBE-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA). After electrophoresis, the gel was stained with SYBR Gold (Molecular Probes) and visualized with UV light using the FluorChem Imager 8000 (Alpha Innotech, San Leandro, CA, USA) with a SYBR Gold filter (520 nm).

Western blot of Bcl-2 and Bax

The Bcl-2 and Bax proteins were detected by means of immunoblotting after the extraction of total protein from mitochondrial and cytosolic fractions of adipose tissue. White adipose tissue (250 mg) or brown adipose tissue (50 mg) were homogenized in a glass-Teflon homogenizer with 2 ml isolation medium [250 mM Sucrose, 10 mM HEPES, 0.2 mM potassium EDTA, ddH₂O, protease inhibitors (Roche Complete[®]), 4°C] (5:1-10:1 vol to tissue weight). The homogenate was poured into a clean centrifuge tube and centrifuged at 700 × g for 10 min at 4°C. The supernatant (the fat cake is included) was carefully decanted into a new tube. Mitochondria were collected by centrifugation at 15,000 × g for 10 min at 4°C. The supernatant was transferred to a new tube for

cytosolic protein isolation. The mitochondrial pellet was suspended in protein sample buffer (2% SDS, 40 mM tris base, ddH₂O; 50-100 µl for WAT, 250-500 µl for BAT) and briefly sonicated. Protein was precipitated out of the cytosolic fraction by the addition of at least 3 volumes of 10% TCA in acetone, 20 mM DTT and stored at -20°C for 45 min – 2 hours. The solution was centrifuged at 7,500 × g for 30 min at 4°C. The supernatant was discarded and the pellet washed twice with 1 ml of cold 90% acetone with centrifugation at 7,500 × g for 3 minutes at 4°C. Residual acetone was removed by airdrying for 5-10 minutes. The pellet was dissolved in protein sample buffer (above) and sonicated briefly.

Protein content in each fraction was determined using Coomassie Plus protein assay (Pierce, Rockford, IL, USA). 20-50 µg of total protein was resolved by reducing SDS-PAGE in Xcell II mini cell on 10% Bis-Tris NuPAGE gels (Invitrogen) using MOPS running buffer as recommended by the manufacturer. In addition, ECL protein molecular weight markers (RPN 2107, Amersham Biosciences, Piscataway, NJ, USA) and a Jurkat lysate as positive control (sc-2204, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were run in separate lanes, plus dotted-on peptides for Bax and Bcl-2 (#PP51 and #PP52, Oncogene, Boston, MA, USA). After electrophoresis, proteins from the gel were electroblotted onto 0.2 µm PVDF membranes following manufacturer's directions. Each blot was stained with SYPRO Ruby (Molecular Probes) and imaged on a FluorChem Imager 8000 (Alpha Innotech, San Leandro, CA, USA) and densitometrically analyzed using ImageQuant 5.1 software (Molecular Dynamics, Sunnyvale, CA, USA) for total protein transferred. The amounts of the Bcl-2 and Bax proteins were determined by means of immunoblotting for 2 h at RT using specific monoclonal anti-Bax (sc-7480,

Santa Cruz Biotechnology) at 1:250 dilution and polyclonal anti-Bcl-2 (#610538, BD Biosciences, San Diego, CA, USA) at 1:500 dilution. The immunostaining was detected using horseradish peroxidase-conjugated anti-mouse immunoglobulin (#210-4302, Rockland Immunochemicals, Gilbertsville, PA, USA) at 1:26,000 dilution for 1 h at RT and ECL-Plus (RPN 2132, Amersham Biosciences). The membrane was stripped according to the ECL-Plus instructions between detections of each protein. Chemiluminescence FluorChem imaged using the Imager 8000 was and densitometrically analyzed using ImageQuant 5.1 software. The relative amounts of each protein was determined by adjusting for total protein transferred to the blot as measured by SYPRO Ruby protein blot stain rather than a single protein (e.g., actin).

Statistical analysis

All data are presented as the mean \pm standard error of the mean (SEM) and analyzed by one-way analysis of variance (ANOVA) with *post hoc* differences determined by the least significant difference (LSD) method. Growth performance and adipose tissue characteristics were also analyzed by two-way Factorial ANOVA. Analyses were run within each fraction of each protein and not between fractions or between proteins. Differences were considered statistically significant at *P*<0.05. All statistical analyses were run using Statistica 6.0 for Windows (StatSoft, Tulsa, OK, USA).

Results

Four days of ICV leptin administration in normal rats caused food intake to decrease by 42% (-7.4 g/d, *P*<0.001) and a loss in body weight of 6% (-15.8 g) compared with pre-

treatment weight $(273 \pm 2 \text{ g vs. } 288 \pm 2 \text{ g}, P < 0.001)$. After the withdrawal of treatment, the leptin group rebounded by eating 7% more (+1.2 g/d, P < 0.05) than the control group during recovery. This contributed to a 38% greater weight gain (+0.93 g/d, P < 0.01). By the end of recovery, the body weight of the leptin group was 2% (-7.8 g, P > 0.05) lower than controls. Leptin caused significant reductions in all the white fat pad masses but not in the brown fat pad (Table 4.1). Weights of the RP, EPI and ING white fat pads were reduced by 67%, 20% and 40%, respectively (all P < 0.05), while BAT weight was reduced by 12% (P > 0.05). There was a 31% loss in relative total white adipose tissue adjusted for body weight (g/100 g BW, P < 0.01).

Because the amount of tissue required for the assays exceeded the amount available, only two fat pads with the greatest mass were chosen to determine cellularity: the inguinal and epididymal white fat pads. Cell number was reduced 46% in the inguinal fat pad by leptin (P<0.01; Figure 4.1) while it was reduced 18% in the epididymal pad (P>0.05). Average cell diameter was reduced similarly in both fat pads (inguinal, -9%, P=.06; epididymal, -11%, P<0.01; Table 4.1). Average cell volume, calculated from size distribution data, was reduced 24% and 30% in the inguinal and epididymal fat pads by leptin (P=.05 and P<0.01, respectively; Figure 4.1). Cell size distribution measurements were grouped into two size ranges of small (30-69 µm) and large (70-240 µm) cells. Leptin administration caused a significant reduction in the number of large fat cells in both the inguinal and epididymal fat pads while increasing the number of small fat cells in both pads. This was an apparent shift of cell size from large to small due to shrinking. It is also likely that more cells were moving into the small cell group than were detected.

Cells smaller than 30 μ m were not counted, as they cannot be reliably identified and separated from non-cell particles or static (Mersmann & MacNeil, 1986).

	Day 4			Day 25			Overall ^a		
	Control	Leptin	P^{b}	Control	Leptin	P^{b}	Trt P	Day P	Trt×Day P
Food intake, ^c g/d	17.8 ± 0.1	10.4 ± 0.5	.01	18.3 ± 0.4	19.5 ± 0.3	.03	.01	.01	.01
Weight gain, ^c g/d	2.61 ± 0.46	-3.94 ± 2.01	.01	2.44 ± 0.50	3.37 ± 0.35	.01	.01	.58	.16
ING									
Mass, g	2.18 ± 0.07	1.31 ± 0.12	.01	3.16 ± 0.26	2.84 ± 0.19	.33	.01	.01	.13
Cells/pad, n×10 ⁶	5.15 ± 0.50	2.77 ± 0.57	.01	9.35 ± 1.72	8.35 ± 1.17	.64	.12	.01	.44
Cell diameter, µm	52.3 ± 1.3	47.4 ± 2.0	.06	50.8 ± 1.4	50.9 ± 1.9	.96	.16	.58	.14
70-240 µm, %	18.7 ± 2.2	9.9 ± 2.3	.02	24.8 ± 2.7	22.0 ± 2.6	.46	.02	.01	.23
30-69 µm, %	81.3 ± 2.2	90.1 ± 2.3	.02	75.2 ± 2.7	78.0 ± 2.6	.46	.02	.01	.23
Cell volume, pl	75.8 ± 5.5	57.3 ± 6.7	.05	69.2 ± 5.5	70.4 ± 8.2	.90	.19	.62	.14
EPI									
Mass, g	1.98 ± 0.10	1.58 ± 0.12	.03	3.33 ± 0.12	3.10 ± 0.28	.42	.05	.01	.60
Cells/pad, n×10 ⁶	3.20 ± 0.46	2.61 ± 0.17	.34	8.77 ± 0.51	10.12 ± 1.09	.26	.57	.01	.16
Cell diameter, µm	61.5 ± 1.2	54.5 ± 1.5	.01	66.5 ± 0.7	65.6 ± 2.0	.67	.01	.01	.02
70-240 μm, %	40.5 ± 4.2	29.5 ± 4.3	.09	51.3 ± 1.6	48.8 ± 5.4	.63	.22	.01	.54
30-69 µm, %	59.5 ± 4.2	70.5 ± 4.3	.09	48.7 ± 1.6	51.2 ± 5.4	.63	.22	.01	.54
Cell volume, pl	123 ± 7	86 ± 7	.01	154 ± 5	149 ± 13	.74	.01	.01	.05
RP									
Mass, g	0.90 ± 0.05	0.30 ± 0.08	.01	2.05 ± 0.24	1.81 ± 0.30	.54	.01	.01	.01
Total WAT									
Mass. g	5.06 ± 0.14	3.19 ± 0.30	.01	8.54 ± 0.54	7.80 ± 0.75	.43	.01	.01	.01
Relative, g/100 g BW	1.71 ± 0.05	1.18 ± 0.11	.01	2.41 ± 0.13	2.25 ± 0.23	.54	.01	.01	.05
BAT									
Mass, g	0.17 ± 0.01	0.15 ± 0.01	.19	0.24 ± 0.01	0.34 ± 0.02	.01	.01	.01	.01

Table 4.1. Growth performance and adipose tissue characteristics after 4 days of 10 µg of leptin injected ICV (Day 4) and after 21 days of recovery (Day 25) in Sprague-Dawley rats

Data represent mean \pm SEM for 6 rats/group/day except for body weight and intake on Day 4, which had 12 rats/group.

RP, retroperitoneal; EPI, epididymal; and ING, inguinal white fat pads; BAT, brown adipose tissue;

^a Two-way Factorial ANOVA.

^b One-way ANOVA comparing leptin vs. control within each day.

^c Cummulative over 4 days (Day 4) or 21 days (Day 25).



Figure 4.1. Cell number counted after osmium tetroxide fixation within the inguinal (ING) and epididymal (EPI) fat pads after 4 days of 10 μ g leptin administration ICV (Day 4) or after 21 days of recovery (Day 25). Data are presented as the mean \pm SEM and analyzed by one-way ANOVA with *post hoc* differences determined by the least significant difference (LSD) method within each tissue. Different letters indicate significant difference (*P*<0.05) within each pad.

Following the termination of injections, every fat pad in both groups increased in mass over the 21 days of recovery but the leptin-treated rats showed a larger increase than the control rats, and by Day 25, there were no statistically significant differences in any white fat pad between groups (Table 4.1). The combined total white adipose tissue mass in the leptin group was 9% smaller than control at this time (P>0.05). All cellularity attributes, cell number, diameter and volume, within the inguinal and

epididymal white fat pads recovered to where there were no statistically significant differences between the groups. The growth of cells over time (over 21 days of recovery) was apparent in the change in cell size distribution. In both groups, the number of small cells (30-69 μ m) was reduced by Day 25 with a concomitant rise in large cells (70-240 μ m). There were no statistically significant differences in the number of small or large cells at Day 25 between leptin treated and control groups. In contrast to the white fat pads, the brown fat pad mass, rather than normalizing to control level, increased by 127% over 21 days in those that were leptin-treated, whereas the control increased by 41% (Table 4.1). The result was a brown fat mass of formally leptin-treated rats that was 42% greater than that of control rats on Day 25 (P<0.001) and was superficially indistinguishable from white adipose tissue.

Indicators of apoptosis, DNA fragmentation and DNA laddering, and proteins associated with apoptosis, Bcl-2 and Bax, were analyzed in each adipose tissue. DNA was extracted and separated into small (soluble/fragmented) and large (insoluble) DNA fractions, and a percent fragmentation calculated. The inguinal fat pad was the only one to have an increase in fragmentation due to leptin treatment (2.4-fold compared to control, P<0.02; Figure 4.2). Only this tissue showed an apoptotic laddering when the small DNA fraction was run on a gel (Figure 4.3). This pattern of DNA degradation was absent in both groups of rats on Day 25. DNA from the other adipose tissues only appeared as faint streaking and did not contain the 180-multiple base pair ladder. The combination of increased percent fragmentation and a DNA ladder strongly suggests apoptosis occurred in the inguinal fat pad. The same DNA fragmentation technique was used for all the fat pads. The epididymal adipose tissue produced much less fragmentation (<1%) in both

groups that was significantly less than what was found in the other fat tissues. Increased fragmentation in the inguinal fat pad occurred without a significant change in total DNA (Figure 4.2). Leptin treatment had little impact on total DNA in any fat pad on Day 4, although by Day 25, total DNA in the epididymal fat pad of the leptin group was significantly increased from Day 4 (P<0.05). A similar trend was seen in the retroperitoneal fat pad.



Figure 4.2. DNA fragmentation (small DNA/total DNA, %) in adipose tissues after 4 days of Four bars on fragmentation and four bars on right side represent total DNA per tissue. Data are expressed as means \pm SEM. Different letters indicate significant 10 μg leptin administration ICV (Day 4) or after 21 days of recovery (Day 25). left side on each chart represent % DNA difference (P<0.05) between groups



Figure 4.3. Agarose gel of DNA from inguinal white adipose tissue separated into two fractions of small (soluble) and large (insoluble) then run in separate lanes. DNA laddering is apparent in the small DNA fraction of inguinal white adipose tissue from leptin-treated rats (L4 Sm). DNA from control (C4) and leptin at Day 25 (L25) rats showed no laddering. L, leptin; C, control; 4, Day 4; 25, Day 25; Lg, large insoluble DNA; Sm, small soluble DNA; M, 100-bp DNA marker.

Immunodetection of Bcl-2 and Bax proteins was carried out separately by stripping the membrane before detection of the second protein. Therefore, results for each protein should not be compared between each other but relative changes within each protein data set were examined. The only significant change in Bcl-2 protein levels due to leptin treatment was a 58% increase within the cytosolic fraction of the epididymal fat pad on Day 4 (P<0.05; Figure 4.4), although, there was a trend to increase mitochondrial Bcl-2 levels in all fat pads. For Bax protein, the only change was a 2-fold increase within the cytosolic fraction of the inguinal pad (P<0.05). The other fat pads showed a trend of

decreasing Bax or no change. A combination of these changes caused a rise (EPI) or fall (ING) of the Bcl-2/Bax ratio in the cytosol. The mitochondrial Bcl-2/Bax ratio showed a trend toward an increase in all fat pads. By Day 25, previously leptin-treated rats had cytosolic Bcl-2 levels reduced to 41% of control in the epididymal fat pad (P>0.05) but



within the protein fraction.

were increased in the inguinal fat pad by 67% (P < 0.01) and in the brown fat pad by 69% (P < 0.01). Cytosolic Bax in the inguinal fat pad of previously leptin-treated rats was still significantly increase of 76% (P < 0.05). Overall, leptin treatment tended to increase the Bcl-2/Bax ratio within the cytosolic fraction of the epididymal and brown fat pads, and within the mitochondrial fraction of all fat pads at Day 4 and 25. Bcl-2 is typically associated with the mitochondrial membrane and other organelles whereas Bax is found in its inactive form in the cytosol. Incomplete fractionation, damaged mitochondria, or an increase in other organelles may skew the results by allowing for more Bcl-2 or Bax in the cytosolic fraction.

Discussion

Research has shown that rodents administered leptin (re)gain weight at a much slower rate after treatment withdrawal compared to pair-fed rodents (Chen et al., 1996; Azain et al., 1999). In other words, animals treated with leptin maintain a lower body weight for a longer period than after food restriction. The composition of the loss in body weight has been found to be primarily fat mass and not lean tissue (Halaas et al., 1995; Pelleymounter et al., 1995; Sarmiento et al., 1997; Kaibara et al., 1998). Considerably more body fat is lost after leptin administration than after pair-feeding, indicating that decreased food intake does not completely account for the reduction in adiposity (Levin et al., 1996). Specific targeting of adipose tissue by leptin also upregulates the activity and gene expression of lipolytic enzymes (Siegrist-Kaiser et al., 1997; Wang, Lee, & Unger, 1999a). Since adipose tissue mass is determined by both the number and volume of adipose cells (cellularity), it is plausible that the duration of weight loss may be

affected by a loss of adipocytes (Qian et al., 1998a) in addition to reduced cell size by lipolysis. Fewer adipocytes would delay the repletion of fat mass and slow weight gain since new cells must be recruited and energy expended for replication. We have previously found that metabolic rate is enhanced during recovery from leptin administration (Gullicksen et al., 2002). This study expands upon the prior report by adding adipose tissue apoptosis as another contributing factor preventing a speedy weight recovery after leptin administration. Examination of adipose tissue cellularity reveals leptin-induced changes are not permanent but provides data relevant to the physiological control of body fat.

In the present study, 4 days of mouse leptin injections caused significant reductions in food intake and body weight similar to previous reports (Flatt et al., 1997; Baile et al., 1997a; Wang et al., 1999b). In addition, these findings agree with previous reports of fat-specific losses during leptin-induced weight-loss (Halaas et al., 1995; Kaibara et al., 1998). All white adipose tissue depots, but not BAT, had lost a significant amount of mass. The amount of mass lost was depot-specific: the retroperitoneal fat pad was reduced 67%, the inguinal fat pad was reduced 40%, and the epididymal fat pad was reduced 20%. Cellularity was investigated in the epididymal and inguinal fat pads. Although these fat pads were reduced in weight by leptin, they were affected differently on a cellular level. The change in epididymal fat mass was due to a greater change in volume (-30%, P<0.01) rather than number (-18%, P=0.34), whereas both cell number and cell volume were significantly reduced in the inguinal fat pad (-46%, P<0.001 and -24%, P=.05, respectively). Although the reduction in cell number was not statistically significant in the epididymal pad, it was previously reported that after 5 days of

recombinant rat leptin (5 μ g per day ICV) in Sprague-Dawley rats, cell number was significantly reduced within the epididymal and retroperitoneal fat pads (Qian et al., 1998b).

In pair-feeding studies it was indicated that the reduction in cell number by leptin, determined using a Coulter counter, was not the result of reduced food intake (Qian et al., 1998a; Qian et al., 1998b). In addition, fasting of rats for a week resulted in a 99% reduction in fat mass and a significant loss of total adipose tissue DNA content, but it did not reduce adjocyte number; rather, there was a loss of stromal-vascular cells (Miller, Jr. et al., 1983). Thus, leptin and fasting/restricted feeding provide very different signals. The lack of a loss of adjocytes during starvation has evolutionary advantages. For example, during times of undernutrition, fat cells are maintained as available storage units, essentially ready to store a valuable resource (energy as triglyceride). This observation underscores the poor performance of maintaining body weight using lowcalorie diets. Exogenous leptin, on the other hand, essentially signals a large body fat store, which can be reduced by lipolysis or a combination of lipolysis plus apoptosis depending on the tissue. Examination of the distribution of cell size shows that the number of large fat cells (70-240 μ m) was reduced by leptin treatment in both pads with a concomitant increase in the number of small fat cells (30-69 µm). Simply put, large cells were shrinking into the small category, while some small cells either became smaller than 30 µm or were deleted by apoptosis. Similar changes in distribution were previously found using recombinant rat leptin (5 µg per day ICV) for 5 days in 3-monthold Sprague-Dawley rats (Qian et al., 1998b). However, these studies did not consider changes during recovery.

The inability to accurately count cells smaller than 30 μ m in size is a limitation of the cellularity assay using particle counters (Mersmann et al., 1986). Although the number of cells measured by the particle counter was reduced by leptin administration, it is possible that the cells could have just become smaller than the minimum measured size. If fat cells were actually being removed, there should be evidence of apoptosis. In the later stage of apoptosis, activated nucleases cause internucleosomal cleavage of DNA into small fragments resulting in an increasing amount of small (<1500 bp) soluble DNA that can be measured and related to total DNA. We found that leptin administration increased DNA fragmentation in the inguinal fat pad only (Figure 4.2). The inguinal fat pad was also the only one to show an apoptotic ladder when its DNA was run on a gel (Figure 4.3). No other fat pad had a significant increase in DNA fragmentation or displayed a distinct DNA ladder. These quantitative (percent fragmentation) and visual (ladder) data provide evidence that apoptosis is occurring in, at least, the inguinal adipose tissue in association with leptin treatment. Although not seen in this study, DNA laddering in the retroperitoneal and parametrial fat pads of normal rats has been found (Qian et al., 1998a). However, the rats in the prior study were treated with a recombinant rat leptin from a different source which also appeared to have a much stronger response in most parameters, such as total body weight and adipose tissue mass (Azain et al., In the present study, the inguinal fat pad had both an increase in DNA 1997). fragmentation and a reduced cell number suggesting that the deletion of adipocytes by apoptosis contributed to the reduction in fat mass. Using TdT-mediated dUTP nick endlabeling and periodic acid-Schiff Reagent in situ staining, Qian et al. (1998a) determined that the cells undergoing apoptosis were adipocytes. The epididymal fat pads of this

study, which had the smallest reduction in mass but a significant reduction in volume, had neither an increase in fragmentation nor a significant loss of cells. This suggests that the loss of mass in the epididymal pads was due to a primary loss of lipid through lipolysis. The retroperitoneal fat pad, which had the largest decrease in mass during leptin injections and largest increase during recovery, likely also atrophied primarily through lipolysis rather than apoptosis.

Twenty-one days of recovery allowed for normalization all white fat pad weights in the leptin group (-9% overall, P>0.05) with no significant differences in cellularity or DNA fragmentation compared to that of the control group. Cell number increased similarly in both the inguinal and epididymal fat pads. This resulted in the epididymal fat pads of the leptin-treated rats having a slightly larger number of cells than that of the control rats after recovery (15%, P>0.05), whereas the inguinal fat pad of the leptintreated rats had a slightly smaller number of cells after recovery (-11%, P>0.05). It appears as if the epididymal fat pad made up for the loss of cells in the inguinal fat pad. This observation compliments the results of increased protective factors against apoptosis in the epididymal fat pad and an increase in the pro-apoptotic factor, Bax, the inguinal fat pad; discussed next.

As a possible mechanism of leptin-induced adipose tissue apoptosis, we investigated the apoptotic proteins, Bcl-2 and Bax. The ratio of anti-apoptotic proteins (i.e., Bcl-2) to pro-apoptotic proteins (i.e., Bax) appears to determine whether the cell undergoes apoptosis (Korsmeyer et al., 1993). The measurements of these two proteins in the cytosolic and mitochondrial fractions from adipose tissue revealed two major leptininduced changes: an increase in cytosolic Bcl-2 in the epididymal fat pad and an increase in cytosolic Bax in the inguinal fat pad. The increase in Bcl-2, which raised the Bcl-2/Bax ratio, is consistent with an anti-apoptotic state like the one found in the epididymal fat pad with the smallest decrease in mass and without a decrease in cell number or an increase in DNA fragmentation. There was also a general trend of higher mitochondrial Bcl-2 levels and Bcl-2/Bax ratios in all fat pads. However, the sharp increase in cytosolic Bax in the inguinal fat pad, lowering the Bcl-2/Bax ratio, suggests that Bax is involved in leptin-induced apoptosis in this fat pad. After 21 days of recovery, there were greater anti-apoptotic signals than there were after 4 days of leptin treatment, especially in the inguinal, epididymal, and brown fat pads. The cytosolic Bax level in the inguinal pad was still high but was now opposed by an increased Bcl-2 level, improving the Bcl-2/Bax ratio. This anti-apoptotic trend may prevent the loss of cells for the support of tissue recovery and growth. The retroperitoneal fat pad was the most responsive to leptin administration in reducing mass and gaining mass during recovery (5-fold increase in mass), yet there were only slight trends to increase Bcl-2/Bax ratio at Day 4 and 25. This pad is likely to be more involved with lipolytic responses while maintaining cellularity for a maximal response during recovery. Since the apoptotic cycle is estimated to be between 12 and 24 hours (Saraste, 1999), and tissue sampling was at 24 hours after the last leptin injection, the detection of both DNA fragmentation and an increase in antiapoptotic factors is possible. Also worth noting is that body weight had reached its minimum at the time of sampling and weight gain was at its greatest after this point (Gullicksen et al., 2002). Thus, the animal was switching to an anabolic mode and apoptosis would not be induced. Measurement of DNA fragmentation and apoptotic protein levels closer to the time of injection may yield different results.

The lack of apoptosis in the other adipose tissue depots versus the inguinal white fat pad may relate to the greater response of these tissues to increase uncoupling proteins (Gullicksen et al., 2002). Reactive oxygen species (ROS) are directly involved with the induction of apoptosis (Simon, Haj-Yehia, & Levi-Schaffer, 2000). Leptin has been found to increase ROS in endothelial cells by increasing fatty acid oxidation via protein kinase A activation (Yamagishi et al., 2001) or by activating the NH₂-terminal c-Jun kinase/stress-activated protein kinase pathway as demonstrated through enhanced JNK activity and AP-1 DNA binding (Bouloumie et al., 1999). Leptin also increased ROS production by stimulated polymorphonuclear neutrophils in response to infection (Caldefie-Chezet et al., 2001). The presence or increased activity of uncoupling proteins may prevent apoptosis by dissipating the mitochondrial proton gradient in defense against the generation of ROS (Jezek & Garlid, 1998). The lack of a response by the inguinal fat pad to increase uncoupling proteins may not have provided an adequate defense against a large, acute apoptotic signal generated by leptin injections. Other reports provide evidence of an anti-apoptotic role for leptin. For example, normal islets cultured in fatty acids show a decline in Bcl-2 mRNA and apoptosis of beta cells, an effect completely blocked by leptin (Shimabukuro et al., 1998b). Increasing sympathetic output to BAT by exposure to low temperature increased the Bcl-2/Bax protein ratio (Briscini et al., 1998) and reduced apoptotic cell death of brown adipocytes (Nechad, Nedergaard, & Cannon, 1987; Porras, Fernández, & Benito, 1989; Lindquist & Rehnmark, 1998). This was also mimicked by the addition of noradrenaline to brown adipocytes in culture (Briscini et al., 1998; Lindquist et al., 1998) The present study similarly suggests that leptin administration increases signals, such as sympathetic outflow, to the retroperitoneal and

epididymal white fat pads and BAT to upregulate UCP and/or the Bcl-2/Bax ratio in these tissues. Bcl-2 may function to maintain mitochondrial stability allowing uncoupling proteins to defend against leptin-induced ROS production.

In contrast to the white fat pads after leptin administration, BAT did not significantly lose mass but changed from a tan color to an intensely dark brown-red, sharply demarcated mass, completely devoid of any overlying white adipose tissue. Leptin administration stimulates brown fat metabolism by increasing the number of mitochondria and content of lipogenic and thermogenic proteins (Sarmiento et al., 1997; Siegrist-Kaiser et al., 1997). The rise in protein content likely offset the loss of lipid. Twenty-one days of recovery allowed for the normalization of WAT mass, cell number and volume but the brown fat pad significantly gained mass over that of control during recovery, which may be due to the priming of the tissue for the uptake of fatty acids by increasing vascularization, lipoprotein lipase (LPL) activity, and fatty acid transporters (Siegrist-Kaiser et al., 1997; Picard et al., 1998). An increased vascularization and LPL content and activity also enhances the blood flow and uptake of fatty acids by BAT during leptin-induced thermogenesis (Cinti, 2001). The influx of fatty acids would be maintained after the leptin stimulus is removed. This, in combination with decreasing UCP activity and energy expenditure and hyperphagia (Gullicksen et al., 2002), favors lipid storage as the animal switches to an anabolic mode. In addition, apoptosis was not detected in BAT in this study or in previous studies that increased the noradrenergic output to BAT (Briscini et al., 1998). Therefore, BAT likely had no loss of cells but increased vascularization, which would enable the tissue to easily take-up and store lipid. Similarly, the epididymal fat pad, which also lacked positive indicators of apoptosis (i.e.,

an increase in DNA fragmentation), showed a larger increase in cell number and average volume after recovery compared to the inguinal fat pad, which had apoptosis. These conditions allowed BAT to increase mass to a level greater than control and turn into what superficially looked like white adipose tissue without a well-defined border.

In summary, the individual response by each fat pad makes it impossible to group them together when describing the effects of leptin on adipose tissue. White adipose tissue mass is lost through leptin-induced lipolysis (Siegrist-Kaiser et al., 1997; Wang et al., 1999a), but the inguinal fat pad also loses cells through apoptosis, in part, from a Bax-related pathway and a lack of a defensive mechanism due to low UCP induction. The result of apoptosis is that extra time and energy is required during recovery for the synthesis of new cells, which could contribute to a delayed body weight recovery seen after the termination of leptin administration. However, this only occurred in one fat tissue in this study. Using a different source of leptin, previous studies have indicated apoptosis in several white fat pads coupled with a longer delay in body weight recovery. The return of adipose tissue mass and cellularity to control levels after 21 days of recovery, supported by a higher Bcl-2/Bax ratio, demonstrates the resilient nature of energy homeostasis. Future experiments using shorter recovery periods and multiple measurements would be helpful to assess the processes behind leptin-induced apoptosis and changes in adipose tissue cellularity. We conclude that leptin has two actions related to apoptosis when administered ICV as an acute high dose. First, leptin provides a quick, short-acting induction of apoptosis in, at least, the inguinal fat pad; and secondly, in a more gradual effect to induce protective factors (i.e., Bcl-2, UCP, ?) against further apoptosis. These likely act through different mechanisms and, if identified, could create

new opportunities for anti-obesity treatments. Considering the growing number of apoptotic factors, other pathways may be involved and more research needs to be done.

CHAPTER 5

DETECTION OF DNA FRAGMENTATION, APOPTOTIC PROTEINS, AND THE QUANTIFICATION OF UNCOUPLING PROTEIN EXPRESSION BY REAL-TIME RT-PCR IN ADIPOSE TISSUE¹

¹ Gullicksen, P.S., R.G. Dean, and C.A. Baile. To be submitted to *Journal of Biochemical and Biophysical Methods*..

Abstract

Analyzing adipose tissue can be difficult for the analysis of DNA, RNA, and protein. The first method is a simple one for the separation of fragmented DNA and the nonisotopic visualization of apoptotic DNA laddering. This technique also allows for the estimation of the amount of DNA fragmentation. The second method details subcellular fractionation of adipose tissue for the extraction of protein in the mitochondrial and cytosol fractions and the measurement of apoptotic protein (Bcl-2 and Bax) levels. The last method involves extraction of total RNA and the measurement of uncoupling protein mRNA using real-time RT-PCR.

Detection of DNA Fragmentation in White Adipose Tissue

Apoptosis, or programmed cell death, is a normal physiological process in which a cell actively participates in its own destruction without triggering an inflammatory response (Kroemer et al., 1995; Heemels, 2000). This process is characterized by a decrease in mitochondrial membrane potential, activation of caspases, loss of plasma membrane asymmetry, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. The formation of distinct DNA fragments of oligonucleosomal size (180-200 bp lengths) is a biochemical hallmark of apoptosis (Bortner et al., 1995) that can be visualized on a gel as a distinct ladder of bands at multiples of approximately 180 base pairs. The ability to visualize a DNA ladder is dependent on the amount and quality of fragmented DNA. This can be partly controlled by preventing DNA degradation, which can occur within thawed tissues by nucleases or during the extraction process from shearing and/or nucleases. Techniques have been developed to improve the extraction of

DNA and sensitivity of DNA ladder detection. These include the isotopic end-labeling of DNA with ³²P-dNTP using Klenow polymerase (Rösl, 1992) and other methods (Blanco et al., 1998). The source of DNA can also affect the quality of a visualized DNA ladder. For example, bands are often more discrete when using DNA extracted from cells rather than from tissues. With its high lipid content and small amount of DNA per mass unit, DNA laddering in white adipose tissue (WAT) is particularly difficult. Staining methods may not provide enough sensitivity to visualize fragmented DNA from adipose tissue. This may be due to a low number of apoptotic cells (not enough fragmented DNA to create distinct multiple bands) or excessive DNA degradation that can overshadow a ladder with streaking. In this case, a more sensitive technique could be employed, such as end-labeling with ³²P-dNTP.

To avoid the use of radioactive isotopes while trying to achieve sensitive detection, we present here a rapid nonisotopic method for the detection of a DNA ladder and a quantitative estimation of DNA fragmentation. This protocol is a modification of Duke and Sellins (1989) and Shimabukuro et al. (1998b), that we developed specifically for use with WAT. It employs high-speed centrifugation to separate fragmented (small, soluble) DNA from non-fragmented (large, insoluble) DNA. DNA in each fraction is quantified and a ratio of fragmentation calculated. The DNA is then run on a gel, stained and visualized under UV light. The extraction process takes about 8 hours per 8 samples while the quantification, electrophoresis and staining takes about 3 hours. A flow diagram of this protocol is presented in Figure 5.1.

Since the amount of soluble/fragmented DNA is very small (<1-15%), more tissue is required than in assays using total DNA. This is complicated by the fact that apoptosis-

induced WAT results in a smaller fat pad size (less available tissue) and the amount of DNA per mg of tissue is small. Approximately 0.5 µg of DNA is recommended to run on a gel for DNA laddering. To achieve a goal of 0.5 µg soluble DNA, 3-50 µg of total DNA is required. 180-200 mg of WAT or 40-50 mg of BAT will provide this much DNA. Tissues are pre-minced with fine scissors in a microfuge tube with a small amount of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, and 0.5% Triton X-100, pH 8.0) before adding to a 5 ml glass-Teflon homogenizer (Wheaton Potter-Elvehjem). Tissues are homogenized gently at low speed (e.g., setting 2, Wheaton overhead stirrer) for 15-30 seconds in a total of 1 ml lysis buffer. To reduce sticking on the walls of the homogenizer, the homogenate is immediately transferred to a centrifuge tube and the homogenizer rinsed with of a minimal volume of lysis buffer. After 20 min in an ice bath, the homogenate is centrifuged at $14,000 \times g$ for ~15 minutes at 4°C in a swingbucket rotor. After centrifugation, the fat cake is lifted or removed and the supernatant containing soluble DNA is transferred to a new tube. At this point, there are two tubes, one with fragmented DNA in solution and one with an insoluble DNA-containing pellet. The insoluble DNA can be extracted from the pellet using standard methods or the DNAzol method (Molecular Research Center, Cincinnati, OH USA). DNAzol (0.5 ml) is added to the pellet and the pellet dispersed using a micropestle. Both tubes are then treated with RNase A (0.5 mg/ml) for 15-30 min at 37°C. The use of proteinase K does not significantly improve the results for DNA quantification or ladder visualization. The procedure continues below with the extraction of fragmented DNA and later returns to the isolation of large, insoluble DNA.

DNA in the soluble fraction (supernatant) is extracted by adding an equal volume of phenol/chloroform/isoamyl alcohol. The solution is vortexed briefly and centrifuged at $16,000 \times g$ for 10 min at room temperature (RT). The aqueous phase is transferred to a new tube and the organic phase reextracted with 100 µl TE buffer (pH 8.0). The aqueous phase is combined with the first followed by extraction with another equal volume of phenol/chloroform/isoamyl alcohol but with centrifugation at $10,000 \times g$ for 2-3 min. The aqueous phase is carefully removed, transferred to a clean tube, and 8 μ l of polyacryl carrier (Molecular Research Center), 5 μ l of MgCl₂ (< 10 mM final conc.), and $\frac{1}{2}$ vol of 7.5 M ammonium acetate are added. The contents are mixed by inversion. DNA is precipitated by the addition of an equal volume of isopropyl alcohol. The samples are mixed by inverting the tubes several times and stored for at least 20 min at -20°C. The DNA precipitate is sedimented by centrifugation at $10,000 \times g$ for 30 min, and the pellet washed twice with 0.8-1 ml of 70% ethanol and centrifuged at 5,000 \times g for 2-3 min. The DNA pellet will not stick well to the walls of the tube after the ethanol wash and care must be taken to avoid aspirating the pellet out of the tube.

The insoluble DNA fraction with added DNAzol is centrifuged at 16,000 × g for 2-3 min to sediment insoluble material and the supernatant is transferred to a new tube. In some cases, floating fragments in the supernatant are seen but are poured-out after DNA precipitation. Three µl of polyacryl carrier is added to the supernatant and the solution mixed by inversion. DNA is precipitated by the addition of $\frac{1}{2}$ vol of ethanol then mixed and stored for 1-3 min at RT. The DNA precipitate is sedimented by centrifugation at 10,000 × g for 5 min and the pellet washed twice with 0.8-1 ml of 70% ethanol and centrifuged at 5,000 × g for 2-3 min. DNA pellets from both fractions are air-dried for

several minutes then solubilized in a minimal volume of TE buffer (pH 8.0): 20-25 μ l added to the soluble DNA fraction and 50-100 μ l added to the insoluble DNA fraction.

DNA concentration is measured using the fluorescent PicoGreen assay (Molecular Probes, Eugene, OR USA). DNA fragmentation is calculated as soluble DNA / (soluble + insoluble DNA) × 100%. The soluble DNA fraction, or both fractions, is loaded onto a 2% agarose gel and run at 128V (8V/cm) or on 6% TBE-polyacrylamide gels (Invitrogen, Carlsbad, CA USA) and run per manufacturer's recommendation. After electrophoresis, the gel is stained with SYBR Gold (Molecular Probes) and visualized under UV light using a FluorChem Imager 8000 (Alpha Innotech, San Leandro, CA USA) with a SYBR Gold filter (520 nm). Figure 5.2 shows the results of this method using inguinal adipose tissue from leptin-treated and control rats. Leptin administration increased the amount of fragmented DNA by 2.4-fold (Table 5.1). In addition, using 0.5 μ g of soluble DNA rather than 0.5 μ g of total DNA produces a brighter image with more distinct bands in the lower bp region.






Figure 5.2. SYBR Gold-stained agarose gel of electrophoresed DNA extracted from inguinal white adipose tissue from rats injected ICV with leptin to induce apoptosis. Lane 1 is a 100 bp marker and lane 2 is total genomic DNA showing a faint ladder. Total DNA is separated into insoluble/large DNA (lane 3) and soluble/fragmented DNA (lane 4).

 Table 5.1. Quantification of fragmented DNA in inguinal white adipose

 tissue from rats injected ICV with leptin (Treatment) or the vehicle (Control)

	Tissue (g)	Soluble DNA (ng/mg tissue)	Insoluble DNA (ng/mg tissue)	Fragmentation, %
Control	0.24 ± 0.01	0.55 ± 0.17	28.1 ± 3.9	1.7 ± 0.5
Treatment	0.21 ± 0.01	2.46 ± 0.57	50.8 ± 2.7	4.1 ± 0.7

Values presented as mean \pm SEM of 6 rats per group.

Subcellular Fractionation of Adipose Tissue and the Detection of Bcl-2 and Bax Apoptotic Proteins

Many proteins are involved in the complicated process of apoptosis but Bcl-2 and Bax have been regularly considered major factors. Bax accelerates apoptotic death by acting on mitochondria (Oltvai et al., 1993; Green et al., 1998) whereas Bcl-2, localized to mitochondria, endoplasmic reticulum, and nuclear membranes, can interfere with apoptosis activation by Bax and other mitochondrial disrupters (Korsmeyer et al., 1993; Rosse et al., 1998; Murphy et al., 2000). The ratio of these two proteins (Bcl-2/Bax) appears to determine the susceptibility to apoptosis by regulating mitochondrial function following an apoptotic stimulus (Korsmeyer et al., 1993).

The method described here involves the detection of Bcl-2 and Bax proteins by means of immunoblotting after the extraction of total protein from mitochondrial and cytosolic fractions of adipose tissue (Figure 5.4). This protocol is based on several methods (Cannon & Lindberg, 1979; Murphy et al., 2000; Mikhailov et al., 2001) but does not have a goal of isolating live mitochondria. The recommended tissue weight for white adipose tissue is 250 mg and for brown adipose tissue is 50 mg. Tissues are minced with fine scissors in a microfuge tube with a small volume of isolation medium [250 mM sucrose, 10 mM HEPES, 0.2 mM potassium EDTA, ddH₂O, protease inhibitors (Roche Complete[®]), 4°C] before being transferred to a 5 ml glass-Teflon homogenizer. Tissues are homogenized briefly in a total of 2 ml of isolation medium (5:1-10:1 vol to tissue weight). Excessive homogenization should be avoided, as it can cause damage to the mitochondrial membrane and release mitochondrial components. The homogenate is immediately poured into a clean centrifuge tube and placed on ice until the other samples

are processed. The homogenate is centrifuged at 700 \times g for 10 min at 4°C. The supernatant, including the fat cake, is carefully decanted into a new tube without disturbing the soft pellet (nuclei). Mitochondria are collected by centrifugation of the supernatant at $15,000 \times g$ for 10 min at 4°C. (Some tissue types, such as epididymal fat, may require up to 30 minutes for a satisfactory pellet.) The supernatant (cytosol + microsomes) is transferred without the fat layer to a new tube (compatible RCF > $7500 \times$ g) for cytosolic protein isolation. The mitochondrial pellet is suspended in protein sample buffer (2% SDS, 40 mM tris base, pH 8.0, ddH₂O; 50-100 μ l for WAT, 250-500 ul for BAT) and briefly sonicated while kept on ice. Protein in the cytosolic fraction is precipitated by the addition of at least 3 volumes of 10% TCA in acetone, 20 mM DTT and stored at -20°C for 1-2 hours. The protein is sedimented by centrifugation at 7,500 \times g for 30 min at 4°C. The supernatant is discarded and the pellet washed at least twice with 1 ml of ice-cold 90% acetone with centrifugation at 7,500 \times g for 3 min at 4°C. The pellet should be dispersed and washed very well to remove TCA; else, it will be more difficult to dissolve the pellet in sample buffer. Residual acetone is removed by airdrying for 5-10 minutes (do not over-dry or it will be nearly impossible to dissolve). The pellet is dissolved in protein sample buffer (above), sonicated briefly then kept on ice.

Protein content in each fraction is determined using the Coomassie Plus protein assay (Pierce, Rockford, IL USA). Insoluble material is spun down at $16,000 \times g$ for 2-3 min prior to analysis. 20-50 µg of total protein is resolved by reducing SDS-PAGE in Xcell II mini cell on 10% Bis-Tris NuPAGE gels (Invitrogen) using MOPS running buffer as recommended by the manufacturer. In addition, separate lanes are used for ECL protein molecular weight markers (RPN 2107, Amersham Biosciences, Piscataway, NJ USA) and Jurkat lysate as a positive control (sc-2204, Santa Cruz Biotechnology, Santa Cruz, CA USA), plus peptides for Bax and Bcl-2 are dotted onto the membrane (#PP51 and #PP52, Oncogene, Boston, MA USA). After electrophoresis, proteins from the gel are electroblotted onto 0.2 µm PVDF membranes following manufacturer's directions. Each blot is stained with SYPRO Ruby (Molecular Probes) and imaged on a FluorChem Imager 8000 (Alpha Innotech) adjusting exposure time as to not overexpose (saturate) the image. Each lane is densitometrically analyzed for total protein using ImageQuant 5.1 software (Molecular Dynamics, Sunnyvale, CA USA) by drawing identical large vertical rectangles that best represent the protein in each lane. The amounts of the Bcl-2 and Bax proteins are determined separately by means of immunoblotting for 2 h at RT using specific monoclonal anti-Bax (sc-7480, Santa Cruz Biotechnology) at 1:250 dilution and polyclonal anti-Bcl-2 (610538, BD Biosciences, San Diego, CA USA) at 1:500 dilution. The immunostaining is detected using horseradish peroxidase-conjugated anti-mouse immunoglobulin (#210-4302, Rockland Immunochemicals, Gilbertsville, PA USA) at 1:26,000 dilution for 1 h at RT and ECL-Plus (RPN 2132, Amersham Biosciences). The membrane is stripped according to ECL-Plus instructions between detections of each protein. The chemiluminescence is exposed up to 50 minutes using the FluorChem Imager 8000 and densitometrically analyzed using ImageQuant 5.1 by drawing identical horizontal rectangles surrounding specific bands.

The relative amount of each protein is determined by adjusting for total protein as measured by SYPRO Ruby staining rather than a single protein (e.g., actin). The areas (rectangles) used within each blot for density analysis should be identical since they are copied from lane to lane or band to band. However, the area is likely to be different from blot to blot and the staining or immunodetection may not be consistent, therefore, the density values should be normalized across blots. This is done by finding the blot with the highest average density value and raising the average density value of all other blots up to the highest value. This is done for total protein (UV) and individual protein (chemiluminescence) images. This normalization procedure does not change the relationship between samples/treatments within each blot. In addition, it may not be proper to compare directly the amounts between the two proteins since they are detected separately, at different times and without knowing the true affinity of each antibody. This method is applied to adipose tissues from rats injected ICV with leptin or the vehicle. Normalized values are subjected to statistical analysis with results presented in Figure 5.3.



Figure 5.3. Bcl-2 and Bax protein levels in cytosolic (cyto) and mitochondrial (mito) fractions of inguinal (ING) and epididymal (EPI) white adipose tissues from rats injected with either leptin or the vehicle for 4 days. Data expressed as mean \pm SEM.



Figure 5.4. Flowchart for the subcellular fractionation of adipose tissue and extraction of protein from mitochondrial and cytosolic fractions.

Real-time RT-PCR provides accurate and reproducible quantification of RNA over a wide dynamic range by detecting PCR products during amplification. Results are provided in less time and with fewer materials than traditional RT-PCR using gel electrophoresis. Our desire is to apply this technology to the measurement of uncoupling protein (UCP) mRNA in adipose tissues. The protocol described here is a one-step (single-tube) RT-PCR reaction that proceeds without the addition of reagents between the RT and PCR steps, thus providing a convenient and timesaving technique. Assay design and procedure is developed with help from Applied Biosystems (Foster City, CA) (2000; 2001a; 2001b). It contains primer and probe design using Primer Express[®] software (Applied Biosystems), reagent configuration, and thermal cycling parameters for the ABI Prism[®] 7700 Sequence Detection System (Applied Biosystems). For more complete guidelines on assay optimization, refer to the documents provided by Applied Biosystems. The standard procedure takes approximately 2.5 hours for preparation of samples to fill a whole plate plus another 2 hours of run-time on the 7700 Sequence Detector.

Complete coding sequences for UCP1 (Accession M11814), UCP2 (Accession AF039033), and UCP3 (Accession AB006614) are entered into Primer Express 1.0A as recommended (Applied Biosystems, 2000; Applied Biosystems, 2001a) to identify suitable primers. The sequences chosen (Table 5.2) from the list generated by Primer Express are purchased from Applied Biosystems. The reporter dye used for each probe is 6-FAM but a different one could be used.

Table 5.2. Frimer and probe sequences used for real-time KT-PCK of UCFS				
	Sense	Antisense	Probe	
UCP1	GTGTACCCAGCTGTGCAATGA	CGCAAACCCTTTGAAAAAGG	TGCCGGTCCTTCCTTGGTGTACATG	
UCP2	CCTGAAAGCCAACCTCATGAC	CAATGACGGTGGTGCAGAAG	ACGACCTCCCTTGCCACTTCACTTCTG	

TTCCCCCTGGACACCGCCAA

average used for real time DT DCD of UCDs Table 5.2. Primer and pro

GGGTTCTCCCCTTGGATCTG

UCP3

TGCAGCCTGTTTTGCTGATCT

Next, a standard and reference (control) signal is considered. It is possible to amplify and detect the target amplicon and the endogenous control amplicon in the same tube (multiplex PCR); however, the endogenous control amplicon requires a second set of primers+probe. Plasmid DNA or *in vitro* transcribed RNA are commonly used to prepare absolute standards. An absolute RNA standard would be the most appropriate but the acquisition and stability of an RNA standard is often problematic. Rather, we reverse transcribed RNA, which is extracted from a tissue sample known to have the expressed UCP, to generate a DNA sequence equal to the amplicon that would be used as a standard. The DNA sequence is generated by traditional RT-PCR using the primer set that was used for the real-time process. The over-amplified DNA sequence is purified on an agarose gel and extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA USA). The yield is determined by the highly sensitive, DNA-specific fluorescent PicoGreen assay (Molecular Probes). A 5-point standard curve is constructed by running a large range of DNA standard using the conditions set below. A threshold above the noise close to the baseline but still in the linear region of the semi-log plot is selected and DNA amounts are chosen that had a cycle threshold (C_T) in a relative range of 20-30 cycles. The copy numbers used are 400, 2000, 10000, 50000, and 250000. This curve is remarkably reproducible over several plates. Copy number is calculated as 602,300,000 / (base pairs \times 0.6498 / 1000).

Total RNA is extracted from WAT or BAT samples using TRIzol Reagent (Life Technologies, Rockville, MD) as directed. Accurate measurement of RNA is required because each mRNA target would be related to total RNA. Therefore, RNA yield is determined by the highly sensitive, fluorescent RiboGreen assay (Molecular Probes). Fluorescent measurement of RNA alleviates many of the problems associated with absorbance measurements at 260 nm (Molecular Probes, 2001). The sensitivity exceeds that achieved with ethidium bromide-based assays by 200-fold or absorbance determined by 1000-fold. The optimum amount of total RNA added to the reaction is determined for each target in each tissue by running dilution curves. These values (Table 5.3) should be used as a guide and may require adjusting under different circumstances.

Table 5.3. Average total RNA (ng)determined for optimum amplification

	UCP1	UCP2	UCP3
BAT	10	25	35
IWAT	10	20	50
EWAT	10	30	20
RPWAT	30	30	50

BAT, brown adipose tissue; IWAT, inguinal; EWAT, epididymal; RPWAT, retroperitoneal white adipose tissue.

The ingredients of a 20-µl reaction, one-step reaction mix are listed in Table 5.4. Enough master mix is prepared to run samples in triplicate plus one reaction without the reverse transcriptase (-RT, NAC, no amplification control) (4 reactions per sample) and one reaction in triplicate for the no template control (NTC). The -RT control is important for determining the presence of genomic DNA contamination and the NTC tells if the contaminating DNA is coming from the sample. To prevent cross-contamination, only the plate column of wells that is currently being added to is exposed. After a column is completed, it is capped with optical caps. After the plate is complete, it is centrifuged briefly to spin down the contents and eliminate any air bubbles. Thermal cycle parameters for one-step RT-PCR are listed in Table 5.5. The efficiency of the reaction is determined by the slope in the exponential phase of amplification; samples with aberrant slopes are run again (this rarely occurs). This method is used to measure the expression of UCP1, UCP2, and UCP in adipose tissue samples from rats injected ICV with leptin or the vehicle. Results for BAT and EPI can be found in Figure 5.5.

Component	(µl)	Concentration
RNase-free water	See below ^a	
10× TaqMan Buffer A	2.0	$1 \times$
25 mM Magnesium chloride	4.4	5.5 mM
dNTP mix (2.5 mM each dNTP)	2.4	300 µM
10 μM Forward Primer	0.2	100 nM
10 µM Reverse Primer	0.2	100 nM
10 μM Probe	0.2	100 nM
AmpliTaq DNA Polymerase (5 u/µl)	0.1	0.025 u/µl
Reverse Transcriptase (50 u/µl) ^b	0.1	0.25 u/µl
RNase Inhibitor (20 u/µl)	0.4	0.4 u/µl

 Table 5.4. One-step RT-PCR reaction mix components

^aVolume of RNase-free water (μ l) = 10-RNA sample volume.

^bRT will be left out in the no-RT control, instead use RNase-free water.

Step	Reverse Transcription	AmpliTaq Activation		PCR	
				CYCLE (40 cycles)	
	HOLD	HOLD	Denature	Anneal/Extend	
Time	30 min	10 min	15 sec	1 min	
Temperature	48°C	95°C	95°C	60°C	

 Table 5.5. Cycle parameters for one-step RT-PCR



Figure 5.5. Uncoupling protein expression (copies/ng total RNA) in brown adipose tissue (BAT) and epididymal white adipose tissue (EPI) from rats injected ICV with either leptin or the vehicle for 4 days. Data expressed as mean \pm SEM.

CHAPTER 6

SUMMARY

A review of the scientific literature presented in Chapter 2 provided three important aspects of leptin regarding the control of body weight. 1) Leptin increases energy expenditure while lowering food intake, 2) leptin produces a greater weight loss than an equivalent degree of food restriction alone, and 3) leptin slows body weight recovery after treatment termination. These effects occur without the negative aspects of food restriction. Studies were conducted to examine the reasons behind the prolonged weight loss after leptin treatment. Energy metabolism was the focus of investigation in Chapter 3 using indirect calorimetry and the quantification of UCP expression by real-time RT-PCR. In Chapter 4, adipose tissue cellularity, using a Coulter cell counter, and apoptosis, by DNA fragmentation and apoptotic proteins, were analyzed. The nature of adipose tissue, compared to other tissues, makes molecular techniques more difficult. Therefore, detailed descriptions of adipose tissue protocols were prepared and presented in Chapter 5.

The experimental design integrated a leptin-induced weight loss with a recovery period lasting 21 days. Changes to energy metabolism included a reduced food intake and increased energy expenditure, which produced a significant body weight loss and decrease in white adipose tissue mass. The leptin-treated rats maintained a lower body weight for 6 days post injection, whereas pair-fed rats typically recovered within 2-3 days. A greater heat production throughout recovery offset the rebound in food intake, contributing to lower retained energy and less calories available for anabolic processes.

Brown adipose tissue (BAT) thermogenesis was one of the mechanisms involved in wasting calories. The increased heat production in the leptin-treated rats was associated with an upregulation of UCP expression, particularly of UCP1 in BAT. Analysis after recovery suggests that greater UCP expression continues to sustain higher thermogenesis after exogenous leptin was discontinued. Overall UCP expression changed in a similar way as heat production but UCP1 exhibited the greatest expression, owing to BAT as the primary contributor to thermogenesis. The upregulation of UCP2 and UCP3 may have assisted in the transport of fatty acids during leptin-stimulated fat mobilization and lipolysis. The overall upregulation of UCPs in adipose tissues contributed to the catabolism of stored lipid leading to a specific loss of fat mass. However, BAT mass was unchanged. The leptin-induced changes in this tissue, which included an increase in protein content for enhanced fatty acid uptake and mitochondrial metabolism, setup conditions that would allow it transform into what appears as white fat.

Another potential reason for slow body weight recovery could be a loss of adipocytes. Additional time and energy would be required to recover any lost cells. Although fat mass is lost during food restriction, cell number is maintained. In fact, food restriction is more likely to increased resistance to apoptosis and favor weight gain during refeeding. Several aspects of leptin are different from food restriction including changes to adipose tissue cellularity. Leptin treatment was found to reduce cell number in the inguinal fat pad with evidence of apoptosis as increased DNA fragmentation. Thus, a loss of adipocytes contributed to a delayed body weight recovery. An increase in Bax, a proapoptotic protein, was associated with increased DNA fragmentation and a lower cell number in the inguinal fat pad, suggesting a leptin-induced, Bax-mediated induction of apoptosis in this tissue. However, the anti-apoptotic Bcl-2 protein and Bcl-2/Bax ratio were generally increased in the other tissues and was even more pronounced after recovery suggesting a greater resistance to apoptosis.

The combination of increased Bax and low UCP expression in inguinal adipose tissue after leptin treatment favors apoptosis. UCP provides a defensive mechanism against increased ROS after leptin administration. The combination of greater UCP content and higher Bcl-2/Bax ratios in BAT and retroperitoneal and epididymal adipose tissues affords protection against apoptosis. A greater defense against apoptosis means maintenance of cell number and an enhanced ability for these tissues to recover. BAT may have been afforded the greatest protection, which, in combination with tissue priming (increased vascularization and uptake of fatty acids) by leptin, would contribute to the great increase in mass during recovery, compared to the white fat pads, which only recovered to control levels.

By the end of recovery, most parameters had normalized, no evidence of increased apoptosis was found, and body weight was only modestly lower. The apparent return of body weight and adipose tissue characteristics to control levels after 21 days demonstrates the resilient nature of energy homeostasis. The low level of plasma leptin, created after the abrupt termination of treatment, may potentiate factors that promote food intake, lipogenesis, and weight gain. A gradual reduction of dose may result in a different outcome, possibly absent hyperphagia and a greater delay of body weight recovery. Because of the differences in metabolism and response to treatment between humans and rodents, we should remain cautious about extrapolating these results to human. The results of these experiments suggest that leptin, working through the central nervous system, induces changes in thermogenic mechanisms and adipose tissue cellularity that delay body weight recovery but also provide for normalization. The study and experimentation involving leptin and body weight regulation has generated an interesting story but even more questions. Continued investigation of the processes behind leptin-induced changes to adipose tissue cellularity and apoptosis is desired. This includes using shorter recovery periods, multiple measurements, and different treatment regimes. Identifying the factors and mechanism involved in the leptin pathway could create opportunities for the treatment of obesity.

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APPENDICIES

APPENDIX A

Adipose Tissue Cellularity Using Osmium Tetroxide

(Procedure of A.L. Cartwright)

Adipocyte tissue cellularity is investigated by using osmium tetroxide fixation and electronic counting and sizing methods (Hirsch & Galian, 1968; Etherton et al., 1977). This procedure allows adipocyte fixation in adipose tissue matrix, thereby minimizing cell rupture. Electronic apparatus permits rapid counting and sizing of large numbers of fixed adipocytes.

Day 1

- **1.** Prepare collidine-HCl buffer and osmium tetroxide (OsO₄) solutions under a hood at least one day before adding samples.
- 2. Excise whole tissue from animal and weigh in tarred plastic boat.
- 3. Weigh out 50-60 mg adipose tissue samples in duplicate and place in 20 ml vials with 0.154 M (0.9%) NaCl at 37°C. [Oil droplets are removed from the tissue surface by two brief rinsings in 0.154 M NaCl.]

Day 2

- **4.** Prepare scintillation vials with **1 ml collidine-HCl buffer and 1.5 ml OsO₄ solution** under a hood. (For isolated cells, use 0.6 ml collidine-HCl buffer and 1.0 ml OsO₄ soln.)
- Weigh out 40-60 mg samples and add to the scintillation vials (use a minimum of ~25 mg sample and not more than 60 mg). Try to get the sample into the solution without getting the forceps exposed to OsO₄.
- 6. Cover the vials and store at room temperature for 4 to 7 days. Longer than 7 days may be needed if the tissue does not break-up well. [Fixed adipocytes are separated from tissue debris and connective tissue is solubilized.]

Day 3

- 7. Filter sample through a 20-µm nylon screen using aspirator flask apparatus, small funnel, and 0.154 M NaCl. Use a funnel that has a short spout, is smooth and without burrs. Remove the sample from the aspirator promptly to not dry out the sample. Return the sample to the same vial by washing the sample off the screen with 0.154 M NaCl, trying not to use more than 20 ml that the vial can hold. You may need to rub the screen between your fingers to unstick some cells. This should be performed under a hood because of the OsO4 and waste poured into a hazardous waste container. [OsO4 solution and tissue debris are separated from fixed tissue by filtration.]
- **8.** Cover the vials with aluminum foil and let sit for 24 hours.

Day 4

- Recollect cells on a 20 µm nylon screen, rinsing with 0.154 M NaCl. Return the sample to the vial with 8 M urea in 0.154 M NaCl. Waste NaCl solution can be dumped down the drain. [Urea breaks-up the tissue.]
- **10.** Cover vials and leave at room temperature for 4 to 7 days or longer. Occasionally agitate the samples and/or place on shaker.

Days 5, 6...

- **11.** Prepare (six) identical 400 ml disposable beakers by making sure they are clean and dust-free. Number them (1-6) then place upside-down in a small plastic tub. Cover the whole tub with a sheet of foil after the samples have been added to keep dust and pollen from contaminating your samples.
- 12. Filter the sample through 240 µm & 20 µm nylon screens using a funnel and aspirator apparatus. Rinse well the vial and cap with 4.0% or 0.9% NaCl into the funnel. Fixed oil droplets and tissue debris which collect on the 240 µm screen are discarded. Adipocytes are collected on the 20 µm screen. Remove the top tube and rinse onto the 240 µm screen. Rub the 240 µm screen between your thumb and forefingers this will dislodge some cells onto your fingers rinse your fingers with 4.0% NaCl into a 400 ml disposable beaker then rinse your thumb over the 240 µm screen. Again, rinse the 240 µm screen over the aspirator and repeat the rub a second time. Remove the tube holding the 20 µm screen and rinse the screen into the beaker. Rinse the tube into the beaker using strong short bursts to unstick any cells.
- 13. Add several milliliters of 0.1% Triton X-100 in 4.0% NaCl to prevent cell aggregation.
- 14. Add a total volume of ~400 ml 4.0% NaCl to each beaker (about ½ inch from beaker rim) and weigh. Make sure you have tarred an empty <u>identical</u> 400 ml beaker and recorded the empty weight beforehand on the Coulter Counter Record sheet.
- **15.** With the Coulter Counter, use multiple 2000 µl aliquots of continually stirring cell suspensions. (See Coulter Counter instructions below.)

Total volume of cell suspension is determined gravimetrically. Adipocyte number and size distribution are measured electronically using the Dual Threshold Analysis Technique with Coulter Counter model ZM and 400 μ m aperture.

Adipocyte cell distributions are determined in approximate 10 µm increments. Total cell number of the fat pad is determined using tissue weight and adjustments for suspension volume.

Solutions *•* Prepare solutions under a hood!

Collidine-HCI Buffer, 50 mM (500 ml)			
Amount			
Collidine	3.5 ml		
dH ₂ O	71.5 ml		
0.1N HCI	67.5 ml		
0.154M NaCl	qs to 500 ml		
pH to 7.4			

Collidine-HCI Buffer, 50 mM (1000 ml)		
	Amount	
Collidine	7.0 ml	
dH₂O	143 ml	
0.1N HCI	135 ml	
0.154 M NaCl	qs to 1 L	
pH to 7.4		

OsO ₄ solution (for fixation, 1g/20 ml)		
	Amount	
Osmium tetroxide* 50 mM collidine-HCl buffer	1 g 20 ml	
Use a bottle or flask that has been marked for osmium use. Empty the 1 g units into bottle and add collidine buffer. Stir overnight.		

* Electron Microscopy Sciences (FW 254.20)

Urea, 8 M in 0.154 NaCl (2 L)		
	Amount	
Urea	960.96 g	
NaCl	18 g	
dH₂O	2 L	
Use 4 L beaker, add urea & NaCl to ~1000 ml dH_2O , let stir for hours or overnight, qs to 2 L with dH_2O . Takes a long time to go into solution. Urea (MW 60.06), Fisher #415-3.		

○ NaCl, 0.154 M (0.9%), filtered (≥202 ml/sample)

• NaCl, 4.0%, filtered

Triton X-100, 0.1% in filtered 4.0% NaCI

Coulter Multisizer

- **1.** Turn-on sampling stand (switch on left rear), Multisizer (button on lower left front), and computer.
- 2. Check settings on Multisizer. Use <SET UP> key to move to the next setup screen. Use "MENU" keys to move cursor.

ORIFICE DIAMETER:	400 µm
ORIFICE LENGTH:	300 µm
SET UP:	Manual
ANALYSIS:	Sample
CALIBRATION:	Kd
KD:	4045.3
SIZE:	42.59E
SIZE UNITS:	μm

ANALYSIS SETUP-1	COULTER MULTISIZER II 02/25/99
CURRENT and GAIN:	Manual
APERATURE CURRENT:	3200 µA
GAIN:	1
POLARITY:	Alternate +/-
INSTRUEMENT CONTROL: TIME:	Siphon 30 secs
CHANNEL COUNT:	10000
TOTAL COUNT:	200E2

	COULTER MULTISIZER II 02/25/99
128	
On	
Off	
Off	
2000 µl	
1	
<u>0</u>	
On	
	128 On Off 2000 µl 1 % On

COMMUNICATIONS SETUP			COULTER MULTISIZER II 02/25/99
	RC232C SERIAL	OUTPUT	
	BAUD RATE: DEVICE: AUTO OUTPUT:	19200 Computer No	
	PRINT/PLOT OP	TIONS	
	CHANNEL DATA: ANALOG PLOT: SCREEN DUMP: OVERLAY MODE: FORMAT:	Yes No No No Standard	
	COMPUTER OPT	IONS	
EI END J	SEND STX/ETX: ND FIELD CHAR: LINE CHAR(S): LEADING ZEROS:	Yes 59 CR No	

Press <FULL> key on Multisizer. Move cursor arrows to 'Sample' and change ## if desired.

```
SAMPLE: 001 128 CHANNEL FULL RANGE COULTER
MULTISIZER II
02/25/99
X-AXIS (ACC LAW): Lin Diam.
Y-AXIS (DISPLAY): Number Diff
BLANK SUBSTRACT: No (or Yes, if desired)
125=100% (scaling factor)
```

Lower Threshold

To check: Move left cursor all the way to the left (using left LEFT CURSOR arrow) until it matches up with the Y-axis line. Check the number given below it (make sure that it is in 'um').

To change: Move left cursor (using left & right arrows and monitoring number on the lower left of screen) to desired lower threshold cut-off (i.e., \sim 25 or 30 um). When desired number is obtained, with Y-axis line "blinking", hit the right MENU arrow – this will move the Y-axis to the new lower threshold.

<u>Check aperture tube for cleaning</u>. You may want to clean it before using to get better results (greater repeatablity). To clean the aperture tube, remove the elastics holding the tube then pull down easily. Place a beaker underneath so that excess liquid will fall into it. Clean the tube using a small amount of gentle detergent and rinse well. Cover ¾ of tube lip with vacuum grease. Refill to the lip with 4% saline and reinsert to sampling stand. Press up while replacing elastics. Back-flush the tube to fill completely. Run a blank to make sure you get low counts.

On the sampling stand, empty the waste container if needed. Check the MANOMETER SEL°CT control – should be set at 2000 μ l.

Keep samples covered at all times to prevent dust contamination.

- 3. Record the weight of the filled beaker.
- **4.** Place sample on sampling stand platform. Raise platform such that electrode is submerged and aperture and stirring rod are ~¼ inch from beaker bottom.
- **5.** Turn STIRRER CONTROL to suspend cells. Avoid excessive stirring as this will lead to bubbling and false counts. For a full beaker, use stir setting '6'.
- 6. Turn COUNT knob (top-right) to RESET (top or bottom position). ALWAYS MOVE CLOCKWISE! Sample will be drawn up, light will come on in sampling stand, wait for pulse pattern to be displayed on MULTISIZER MONITOR.

7. Turn knob to COUNT. Distribution profile will appear on screen. Two numbers on the left will change:

0.00s = sample time (seconds) [∞] WATCH: Should be 3.5-3.7 N = total counts in sample [∞] WATCH: Should get good repeatability

Number at bottom:

G = counts in designated window

8. MULTISIZER AccuComp software for Windows (version 1.19, Jun 29, 1995)

Click Acquire... New Sample...

Group ID: <Enter the sample ID/no.> Sample ID: <Enter the study no.> Make any other desired changes. Click OK.

Click Aquire... Collect from MULTISIZER...

On MULTISIZER, press <PRINT> key to send data to computer. Wait for data transmission to complete and "Save as" screen. (Click 'Directory' to change to your user directory, click OK.)

Click Save.

Close this window (titled with your sample ID).

Repeat at "Click Acquire... Collect from MULTISIZER..." to run next replicate.

After all replicates are collected, click File... Average... Select the files you just collected. Click OK.

Click RunFile... Export... Enter a filename (your sample ID). Use the extension "txt". Place a check mark next to "Sample info" and "Listing". Export format: "Comma delimited". Click Export...

Close this window (titled "untitled"). You don't need to "save before proceeding", you just exported the data.

For a new sample, repeat steps above starting with "Click Acquire... New Sample...".

- **9.** When data transfer is complete ("SENDING DATA" message disappears) press <RESET> key to clear the data for a new run.
- **10.** Repeat at step 6 to take another reading. Repeat again until all aliquots are taken (six readings per sample are suggested).
- 11. When finished with sample: shut-off stirrer, remove sample beaker from sampling stand, clean aperture tube by removing or back-flush. For back-flush, open COUNT knob then FILL knob for ~5 sec, close first FILL knob then Count knob repeat for several cycles.
- 12. When finished with session: carefully remove and rinse-out the aperture tube, refill aperture tube with Isolyzer solution, put aperture tube and electrode in small beaker of Isolyzer solution, and wipe-up any spilled saline. Save your data to disk. Power-off all three units.

Beckman Coulter, Inc. Technical Support, (800) 327-6531. <u>http://www.beckmancoulter.com/Coulter/PC/</u>

APPENDIX B

Artificial Cerebrospinal Fluid (aCSF) Preparation

Preparation of artificial CSF. Durect Corporation, Cupertino, CA. http://www.alzet.com/faq/proto2.htm (5/27/2000)

Prepare aCSF by combing Solution A and Solution B in a 1:1 ratio.

Solution A	Amount
Pyrogen-free, sterile water	500 ml
NaCl	8.66 g
KCI	0.224 g
CaCl ₂ -2H ₂ O	0.206 g
MgCl ₂ -6H ₂ O	0.163 g

Solution B	Amount
Pyrogen-free, sterile water Na ₂ HPO ₄ -7H ₂ O	500 ml 0.214 g
NaH ₂ PO ₄ -H ₂ O	0.027 g

Materials & equipment

- Pyrogen-free, sterile water
- NaCl (MW 58.44, Fisher Biotech BP358-212)
 KCl (FW 74.55, J.T. Baker 3040-01)
- CaCl₂-2H₂O (FW 110.99, J.T. Baker 1313-01)
- MgCl₂-6H₂O (FW 203.30, J.T. Baker 2444-01)
- Na₂HPO₄-7H₂O (FW 268.07, EM Science SX0715-1)
- NaH₂PO₄-H₂O (FW 137.99, J.T. Baker 3818-01)

APPENDIX C

Coomassie[®] Plus Protein Quantitation Microplate Assay

Pierce Cat. No. 23236. This method has been modified for the 96-well microplate assay. Use these notes in conjunction with the Pierce assay instructions.

Materials & equipment

- PBS, 10x (Bio-Rad)
- BSA stock (Pierce)
- o dH₂O (NanoPure)
- 96-well, all-clear microplate (Greiner, Costar)
- 1.7 ml or greater microcentrifuge tubes
- 1.5 ml microcentrifuge tubes

Prepare diluent (PBS)

	Standard curve in triplicate	Samples dilution (separate tube)	Sample dilution (in-well)
10x PBS dH ₂ O		<example></example>	<example></example>
Total volume	3025 µl	600 µl × # samples	150 × 3 × # samples

Standard curve

Make up 50 µg/ml standard for triplicate measure		
BSA stock, 2.0 mg/ml	20 µl	
Diluent	780 µl	
50 µg/ml standard	800 µl	

	Volume (µl) of diluent	Volume (µl) of 50 µg/ml standard	Final concentration
Α	450	0	blank
В	475	25	2.5 µg/ml
С	450	50	5 µg/ml
D	400	100	10 µg/ml
Е	300	200	20 µg/ml
F	150	350	35 µg/ml
	2225	725	

- 1. Add 150 µl of each standard to standard wells.
- **2.** Add 148 μl of <u>diluent</u> per sample well and 2 μl of sample per sample well, OR add 150 μl of diluted sample (e.g., 1:200) per sample well.
- 3. Add 150 µl of Coomassie Plus Reagent per well using multipipetter.
- **4.** Take an endpoint measurement at 595 nm.

3. Carefully aspirate fat layer and transfer (decant or use needle & syringe) supernatant containing fragmented (soluble) DNA to a new 2 ml microfuge tube (~920 µl). Remove adhering fat from the wall

SUPERNATANT

volume.)

of the tube.

(Fragmented DNA)

- 4. Add RNase A (1 µl of 100 mg/ml, final conc. 50-500 µg/ml), mix by inverting tube, and incubate at 37°C for 15-30 min.
- 5. Add ≥ equal volume of phenol/chloroform/isoamyl alcohol (warmed to room temperature and pH to 8.0 with 1 M tris).

CAUTION: Phenol is extremely caustic. Cap and vortex vigorously for 10 sec. Centrifuge at 16,000 $\times q$ for 10 min at room temperature (RT).

- 6. Transfer all of the top (aqueous) phase to a new tube (~860 µl). Reextract the organic phase with 100 µl TE buffer, pH 8.0, and pool the aqueous phase with that from the first extraction. Repeat step 5 but spin at $10,000 \times q$ for 2-3 min. Carefully remove the aqueous phase containing the DNA and transfer to a new tube (~900 µl).
- 7. Add carrier (polyacryl at 8 µl/ml or glycogen), 5 µl of 1 M MgCl₂ (<10 mM final conc.), and ¹/₂ vol of 7.5 M ammonium acetate, and mix. Precipitate DNA by the addition of an equal volume (calculated after salt addition) of isopropanol. Mix by inverting tube several times and let precipitate for at least 20 min at 4 to 25°C.
- 8. Sediment the precipitated DNA by centrifugation at 10,000 × g for 30 min at 4 to 25°C. Discard the ethanol supernatant using a pipette.
- 9. Wash the DNA precipitate with 0.8-1 ml of 70% ethanol. Invert the tube several times and centrifuge at $10,000 \times g$ for 2-3 min at 4 to 25°C. Discard the supernatant.

PELLET (Insoluble DNA)

Below describes the Molecular Research Center protocol. You may substitute a different DNA isolation procedure such as phenol/chloroform or DNeasv.

- 4. Add a small amount (0.1-0.25 ml) of DNAzol to the pellet. Transfer to a microfuge tube using a plastic Pasture pipette. Add DNAzol up to 0.5 ml to rinse. Disperse the pellet using a micropestle. Store for 5-10 min at RT.
- 5. Add RNase A (1 µl of 100 mg/ml, final conc. 50-500 µg/ml), mix, and incubate at 37°C for 15-30 min.
- 6. Pellet the insoluble material at 16,000 × g for 2-3 min at 4 to 25°C. Transfer (pour) the supernatant to a new microfuge tube. Estimate the volume transferred (~440 µl).
- 7. Add carrier (3 µl polyacryl carrier or glycogen) and mix. Precipitate DNA by the addition of 1/2 vol of ethanol. Mix by inverting tubes 5-8 times and store at RT for 1-3 min.
- 8. Sediment the precipitated DNA by centrifugation at $10,000 \times g$ for 5 min at 4 to 25°C. Discard the supernatant (hazardous waste), being careful not to disturb the pellet.
- 9. Wash the DNA precipitate twice with 0.8-1 ml of 70% ethanol. Invert the tube several times and centrifuge at $5,000 \times g$ for 2-3 min at 4 to 25° C. Discard the supernatant.

Rev. 5-Feb-2002

DNA Fragmentation in Adipose Tissue Using Centrifugation

APPENDIX D

- 1. Keep samples and buffer on ice. Homogenize 180-200 mg white adipose tissue or 40-50 mg brown adipose tissue (record tissue weight used) in 0.75 ml lysis buffer and transfer homogenate to a centrifuge tube. Use another 0.25 ml to rinse homogenizer (1 ml lysis buffer/200 mg tissue). Incubate at 4°C for 20 min.
- 2. Centrifuge at 14,000 \times g for 15 min* at 4°C. (*Time may be adjusted based on tissue type, cells, and

- 10. Let residual ethanol evaporate for 5-10 min or dry (<u>DO NOT OVER-DRY</u>) the pellet in a Speedvac evaporator (1-5 min). Dissolve the large DNA fraction in 50 to 100 μl (80 μl) 8 mM NaOH (<1 month old) or TE buffer; and dissolve the small DNA fraction in 20 to 25 μl (25 μl). Store for at least 1 hour at room temperature before quantification.</p>
- 11. DNA quantification of each fraction

Suggestions: Spin-down insoluble material at $16,000 \times g$ (full speed Eppendorf 5415C) for 2-3 min. For quantitation, if the large DNA fraction was dissolved in 80 µl, make a 1:100 dilution (min 4/400) in separate tubes (1:200 final inwell dilution). Use the small DNA fraction straight (do no dilute) by adding 2 µl per well with 98 µl TE (1:100 final inwell dilution). The small DNA fraction may require concentration if the total volume is larger than the volume of the gel well.

12. Gel electrophoresis

Suggestions: Add **≤0.5 µg DNA** per lane in a 2% agarose (4-5 mm, 0.5 g agarose/25 ml 0.5X-1X TBE) or 6% TBEpolyacrylamide gel, and run fast at high voltage (e.g., for 16 cm agarose minigel use 128V ~45 min). Use a gel with wells of at least 25 µl volume. Stain gel with SYBR Gold, SYBR Green I, or ethidium bromide.

Materials

- Lysis buffer (below)
- DNAzol (Molecular Research Center # DN127), or other DNA isolation method materials
- Polyacryl carrier (Molecular Research Center # PC152)
- RNase A, DNase-free
- 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (Amresco # 0883-100ML)
- 7.5 M ammonium acetate soln. (Sigma # A2706)
- Magnesium chloride, 1 M soln.
- Isopropanol
- Ethanol: 100% & 70%
- 8 mM NaOH or TE buffer, pH 8.0
- 4 ml tubes compatible for swing-bucket rotor (Sorvall # 3105 or equiv.)
- 1.5 ml, 2 ml microfuge tubes, and 4-5 ml tubes when using a swing-bucket rotor
- Centrifuge with swing-bucket rotor (preferred)
- SYBR Gold or SYBR Green I (Molecular Probes), or ethidium bromide

DNA extraction: 6 hrs/12 samples Quantitation, electrophoresis & staining: 3 hrs

Allow 2 days to complete.

Lysis buffer for DNA separation			
	Final conc.	Amount	
Tris base (MW 121.14) EDTA (MW 372.24) Triton X-100 dH ₂ O	10 mM 10 mM 0.5%	0.242 g 0.744 g 1 ml to 200 ml	

Dissolve EDTA in ~160 ml (~pH 7.1), add tris, adjust to pH 8.0 with NaOH, add Triton X-100 then bring to volume. Store at 4°C.

References

- 1. R. C. Duke and C. B. Sellins. Progress in leukocyte biology. 9:311-314, 1989.
- 2. M. Shimabukuro, et al. J Biol Chem 273 (49):32487-32490, 1998.
- Current Protocols on CD-ROM. Molecular Biology. Basic protocol: phenol extraction and ethanol precipitation of DNA. 2001.

APPENDIX E

DNA Laddering Using Fluorescent End-Labeling

DNA is isolated and end-labeled with fluorescein-12-dUTP using terminal transferase then run on an agarose gel. Refer to the fluorescein and terminal transferase product information guides for further information.

- Fluorescein-12-2'-deoxy-uridine-5'-triphosphate (Roche/Boehringer Mannheim # 1373 242)
- Terminal transferase (Roche # 220 582)
- Polyacryl carrier (Molecular Research Center # PC 152) or glycogen
- 0.2 M EDTA, pH 8.0
- Ethanol, 100% and 75%
- TE buffer, pH 8.0
- 1. It is recommended to purify the DNA by phenol/chloroform extraction and ethanol precipitation, or use DNAzol.

250 mg fat tissue \approx 1 µg small DNA fraction (4-6 µg DNA/100 mg tissue)

2. Add the following to a 0.6 ml microfuge tube with a good-sealing cap on ice:

	Final conc.	Amount
dH ₂ O, sterile		up to 11 µl
5× Reaction buffer	1×	4 µl
25 mM CoCl ₂	2.5	2 µl
dNTP labeling mix w/fluorescein-dUTP		2 µl
DNA		[]µl(1µg)
Terminal transferase		1 µl (25 u)
Total		20 µl

- **3.** Centrifuge briefly and incubate for at least 37°C for 60 minutes. Longer incubation (up to 20 h) can increase the amount of labeled DNA.
- 4. Stop the reaction by adding 2 µl 0.2 M EDTA, pH 8.0 solution.
- 5. Precipitate the labeled DNA with 1 µl polyacryl carrier or 0.5 µl of 20 mg/ml glycogen (100 µg/ml final conc.) and 75 µl ethanol. Mix well.
- 6. Leave for at least 30 min.
- 7. Centrifuge at 12,000 × g for 5 minutes, wash the pellet with 75% ethanol, spin again, discard supernatant, and dissolve in 20 μ l TE buffer.
- Run the labeled soluble fraction of DNA on a 2% agarose (4-5 mm, 0.5 g agarose/25 ml 0.5X-1X TBE) or 6% TBE-polyacrylamide gel, and run fast at high voltage (e.g., for 16 cm agarose minigel use 128V ~45 min).

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APPENDIX F

DNA Laddering Using Radioactive End-Labeling

DNA laddering is used as an indicator of apoptosis. Total time: ~8.5 hours + exposure time (4+ hours).

Isotopic labeling

1. Prepare a reaction with 1 μg DNA sample or 5 μl marker (see product information)

Nuclease-free water	χ μΙ
10× Klenow buffer	5 μΙ
DNA*	χ μΙ (1 μg)
Klenow enzyme	1 μΙ (5 υ)
$[\alpha^{-32}P]dCTP$	χ μΙ (30 μCi)
Total volume	50 µl

*For Promega 100 bp marker, use 5 µl (650 ng).

Note: Return the enzyme to the freezer immediately. Do not keep the enzyme on ice.

- a. Incubate for 15 min at 30°C.
- b. Stop the reaction by adding 1 µl of 0.5 M EDTA (20 mM final concentration) to the mixture, or by heating the mixture for 10 min at 75°C.

Phase separation

 Add an equal volume (50 μl) of phenol/chloroform/isoamyl alcohol. Mix gently for 10 sec. Centrifuge at 10,000 × g for 5 min. Transfer top, aqueous phase containing DNA (~50 μl) to a new tube.

DNA precipitation

 Add ½ volume (20 µl) of 7.5 M ammonium acetate. Add 1 µl of 20 mg/ml glycogen to facilitate DNA precipitation. Mix well but gently. (You may make up a solution contain these two and pipette only once.) Add 2 to 3 volumes (180 µl) of 100% ethanol. Mix gently. Store at -20°C for ≥30 min, or -70°C for ≥15 min. 4. Centrifuge at $10,000 \times g$ for 15+ min. Remove the supernatant and place in radioactive B waste container.

DNA wash & 2nd precipitation

- 5. Add 100 µl of 1 M ammonium acetate. Add 1 µl of 20 mg/ml glycogen. Dissolve and mix well by drawing up into pipette tip with a wide opening. Add 2 to 3 volumes (300 µl) of 100% ethanol. Mix gently. Store at -20°C for ≥30 min, or -70°C for ≥15 min.

Redissolve the DNA

- Dissolve the pellet in 20 µl of a 5:1 solution of TE buffer/6× loading dye. Mix well by drawing up into pipette tip with a <u>wide</u> <u>opening</u>. Heat mixture at 65°C for 5 min. Spin briefly.
- Measure and record the radioactivity of each sample by placing the bottom of the tube near the Geiger counter opening/sensor. Calculate an equalized amount of radioactivity to be added to each lane.

Gel electrophoresis

- Prepare enough 0.5× TBE buffer or 1× TAE buffer for both casting and running of the gel.
 Prepare a 2% agarose gel. (For a 5½ × 4½ inch, 14 × 11.5 cm gel, use 100 ml.)
- **10. Load 20 µl or equalized amount of DNA** to each lane.
- **11.** Run fast at high voltage (e.g., for 16 cm agarose minigel use 128V ~45 min).
- Check pipettes, tube racks, and area for contamination. Small hot spots may be cleaned with 500 mM EDTA or decontamination solution.

Gel drying

12. Phase 1. Place Whatman paper (cut to a size just larger than the gel) on top of ~15 layers of paper towels cut in half. Remove the gel and place on Whatman paper. Cut off any excess gel and dispose in radioactive waste container. Center gel on Whatman paper. Place another piece of Whatman paper on top of gel and stack on the other half of paper towels. Place a flat, heavy item on top. Allow to sit for 1 hour. Place waste paper towels and paper into radioactive waste container.

Turn on refrigerated condensation trap of gel dryer one hour before using.

13. Phase 2. Place gel on a new sheet of Whatman paper and then onto gel dryer. Cut Mylar membrane to ~2 cm larger than the gel all around. <u>Wet the membrane completely</u> with the buffer used to make the gel and roll over gel removing any creases or bubbles. Cover with the gel dryer's flexible plastic cover. Turn on vacuum pump, verify vacuum and adherence of membrane all around. Close cover and set heater at 60°C for 1-1½ hours or until gel is dry (feels like the same temperature as rest of the slab and is very thin). Shut off heater and vacuum pump and release vacuum in tubes if necessary. Peal off paper from gel-membrane; leave gel stuck to membrane.

If storing overnight, wrap gel in plastic wrap, place flat in x-ray cassette, and place in freezer.

Exposing film

- 14. Take gel, x-ray cassette and film to dark room. Tape corners of film to cassette. Tape corners of gel-membrane to film. Lay a second film on top. Snap-close the cassette before turning on the lights. Place cassette in -70°C or -20°C and expose for 4 or more hours.
- 15. Let cassette come to room temperature before opening. In dark room, remove top film. Close the cassette to continue exposing 2nd film for another 3 or more hours. Develop film using film processor (Kodak X-OMAT) in dark room.

Reagents & Solutions

TE buffer, 20×			
	Final conc.	Amount	
Tris base ¹ (FW 121.14) EDTA ² (FW 372.24) dH ₂ O, nuclease-free*	10 mM 1 mM	1.2114 g 0.3722 g to 50 ml	
pH 7.5 or 8.0 with concentrated HCl. Store at 4°C or RT. *Do not use DEPC-treated H ₂ O since this is an enzymatic reaction. ¹ Bio-Rad 161-0719. ² Bio-Rad 161-0729.			

TBE electrophoresis buffer, 0.5×				
	Final conc.	Amount		
$10 \times TBE$ stock soln. ¹ dH ₂ O	0.5×	50 ml to 1 L		
<i>Make-up 1 or 2 L. Do not pH. Store at RT.</i> ¹ Bio-Rad 161-0733 or 161-0741.				

TAE electrophoresis buffer, 1×				
	Final conc.	Amount		
TAE buffer stock soln. ¹ dH ₂ O	1×	20 ml to 1 L		
Make-up 1 or 2 L. Do not pH. pH ~8.5. ¹ Sigma-Aldrich 93297 or 50× stock soln.				

TAE electrophoresis buffer, 50×			
	Final conc.	Amount	
Tris base (FW 121.1) Glacial acetic acid EDTA (FW 372.24) dH_2O	0.5 M 10 M	242 g 57.1 ml 37.2 g to 1 L	

Materials & Equipment	Per Sampl or Exp	Source
 [α-³²P]dCTP (3000 Ci/mmol @ 250 µCi/25 µl) DNA Polymerase I Large (Klenow) Fragment 10× Klenow Buffer Phenol/chloroform/isoamyl alcohol (25:24:1) 7.5 M Ammonium acetate Glycogen (20 mg/ml), Type II Oyster TE buffer TBE or TAE buffer Agarose, high strength analytical grade 100 bp DNA ladder/marker Blue/Orange 6× loading dye Ethanol, 100% ice-cold 	30 µCi 5 u 5 µl 20 µl 20 µl 100 µl 2 µl 16.67 µl 1.8 g 7.69 µl 3.33 µl 480 µl	Amersham Pharmacia Biotech PB 10205 Promega M220 included in Promega kit M220 Amresco 0883-100ML Sigma A2706 Sigma G8751 see Reagents & solutions see Reagents & solutions Bio-Rad 162-0126 Promega G210 Promega G188
 Whatman paper 3MM CHR 3030-347 or equiv. Mylar membrane Paper towels Gel blot stand or bowl 250 ml bottle or flask Plastic wrap Gel dryer Imaging film Film developer 		Fisher Scientific 05-716-6A Idea Scientific Company Kodak X-OMAT AR Film, Fuji, or equiv. Kodak X-OMAT Processor
O Pipette tips, nuclease-tree O Microcentrifuge		

References

- Qian, Hao. Personal communication. University of Georgia (1998).
 Qian, Hao, et al. Brain administration of leptin causes deletion of adipocytes by apoptosis. *Endocrinology* 139(2):791-794 (1998).
 Rösl, Frank. A simple and rapid method for detection of apoptosis in human cells. *Nucleic Acids Research* 20(19):5243 (1992).

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APPENDIX G

Intracerebroventricular (ICV) Cannulation of Rats

Time: ~45 minutes/animal.

Prepare for surgery

- Administer anesthetic IP: 1 ml/kg of 3:2:1 v/v/v ketamine HCL/acepromazine maleate/xylazine. Record amount, time, and placement.
- **2.** Shave rat and vacuum fur gently. Lubricate eyes with Artificial Tears. Wipe surgery site with sterile solution 4 times.
- **3.** Place animal on stereotaxic instrument. Adjust and tighten ear bars. Place teeth over bar and clamp nose.
- 4. Affix cannula to stereotaxic apparatus.

Incision

- 5. Disinfect the skin with chlorhexidine.
- 6. Make incision from slightly lateral of midline from just behind eyes to back of skull. Scrape exposed skull with back of scalpel and wipe with sterile gauze. Use retractor to hold open incision.
- 7. Level skull using bubble level.

Coordinates: A-P, 0.8 mm and M-L, 1.4 mm with respect to bregma

- 8. Drill 2 or 3 holes for screws: one directly behind cannula, one aft and lateral, and one fore and lateral. Remove bone fragments and insert screws.
- **9.** Re-check level and center cannula over bregma. Take measurement: anterior-posterior, medial-lateral, and then move to correct coordinate.

- **10.** Verify cannula is vertical, or zero on stereotaxic device. Mark skull at intended cannula insertion point.
- **11.** Drill hole for cannula. Make sure cannula will not touch bone upon insertion. Remove any bone fragments with tweezers.
- **12.** Re-check coordinates and position. Place cannula just touching the cortex. Take reading and calculate proper depth.

Coordinate: D-V, -3.5 mm from the skull surface

13. Lower cannula to proper depth.

Cementing cannula

- **14.** Apply cranioplastic cement by measuring out equal parts of powder and liquid. Mix together–not over 30 seconds–and apply.
- **15.** When material is in position, allow 20-30 minutes for hardening.
- **16.** Detach the cannula from the stereotaxic device.
- **17.** Insert a 28-gauge stylet into the guide cannula. This will remain inserted when the rat is not receiving an injection.
- **18.** Suture loose skin, if necessary, using loose square knots 3 times.

Recovery

19. Let the animal recover in a shoe-box cage with soft bedding. Place a warmed isothermal pad under the cage.

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APPENDIX H

PicoGreen[®] DNA Quantitation Microplate Assay

The PicoGreen assay is 400-fold more sensitive than the Hoechst 33258-based assay. This short protocol has been adjusted for 200 µl volume. Use in conjunction with Molecular Probes' protocol (MP 07581).

- Caution: no data are available addressing the mutagenicity or toxicity of PicoGreen DNA quantitation reagent. Because this reagent binds to nucleic acids, it should be treated as a potential mutagen and handled with appropriate care.
- All solutions should be prepared in sterile disposable, polypropylene plasticware rather than glassware, as the reagent may adsorb to glass surfaces.
- Protect the PicoGreen working solution from light by covering them with foil or placing them in the dark, as the RiboGreen reagent is susceptible to photodegradation.

General instructions: Prepare the sample dilutions first, the DNA standards, pipette them all to the plate, and then add the PicoGreen working solution.

1. 1X TE buffer

Prepare enough 1X TE buffer for making up PicoGreen working solution, DNA standard, DNA standard curve, and sample dilution.

2. DNA standard curve

 Prepare a 2 μg/ml DNA stock solution by diluting 100 μg/ml DNA standard 1:50 with 1X TE buffer.

	Single	Triplicate
1X TE buffer	157.78 µl	539 µl
DNA stock	3.22 µl	11 µl
Total volume (2 µg/ml)	161 µl	550 µl

b. For the high-range standard curve, dilute the 2 μ g/ml DNA stock solution into small tubes as shown in the 3× table below.

	Volume (µl) of TE	Volume (µl) of 2 µg/ml DNA stock	Dilution in tube
А	300	0	0
В	396	4	1:100
С	279	31	1:10
D	155	155	1:2
Е	0	300	1:1
	1130	490	3×

c. Pipette 100 μl of each standard into the corresponding well.

	Volume (µl) of standard	Volume (µl) of 200-fold diluted PG reagent	Final DNA concentration in the assay
Α	100	100	blank
В	100	100	10 ng/ml
С	100	100	100 ng/ml
D	100	100	500 ng/ml
Е	100	100	1000 ng/ml

Sample analysis
 Add 2-4 µl DNA sample in well with 96-98 µl
 TE buffer or dilute in TE buffer before
 adding to well (e.g., 4 µl/800 µl; 1:200 dilution).
 Change the dilution of the sample if the
 measured concentration is not within the
 standard curve limits. Pipette 100 µl of
 diluted sample into the corresponding well.

4. PicoGreen working solution (1/200) *Prepare in opaque plastic bottle.*

	Std.curv. in triplicate	Per sample in triplicate
1X TE buffer	1492.5 µl	298.5 µl
PicoGreen stock	7.5 µl	1.5 µl
Total volume	1500 µl	300 µl

+additional 100 μ l to final volume or, when using a multipipettor, add 800 μ l (8 rows × 100 μ l) for a whole plate.

For best results, this solution should be used within a few hours of its preparation.

Add 100 µl of the PicoGreen working solution to each well. (A multipipetter works

well for this.) Mix well and incubate for 2-5 minutes at RT, protected from light.

5. Measure fluorescence

Molecular Devices *SpectraMax Gemini* settings: Ex/Em: 488/528 nm; Cutoff: 515 nm.
Materials & equipment

- 1. TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5)
- 2. Opaque plastic bottle (~25 ml)
- 3. Microcentrifuge tubes
- 4. 96-well all-black plates (Greiner #655076, Costar #3915, or equiv.)

References

- Molecular Probes. PicoGreen[™] DNA Quantitation Reagent and Kit, P-7589 & P-7581. 22 Jun 1999 <<u>http://www.probes.com/lit/product/summary/PicoGreen.html</u>>
- Molecular Probes. Optimizing excitation and emission wavelengths for narrow Stokes' shift fluorophores using the SPECTRAmax GEMINI and SOFTmax Pro. Application Note 31. (0120-1162A).

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APPENDIX I

Real-Time RT-PCR Using the ABI Prism 7700 Sequence Detection System

This protocol is base on the One-Step RT-PCR using the TaqMan Gold RT-PCR Kit. Use this short protocol in conjunction with Applied Biosystems "User Bulletin #2" and "TaqMan Gold RT-PCR Kit" protocol for relative quantitation of gene expression.

The ingredients for a 20-µl RT-PCR reaction mix using the TaqMan Gold RT-PCR Kit are listed in the table on the next page. The amounts used have been optimized to minimize waste, though, they can be adjusted to suit your needs. Prepare enough master mix to run samples in triplicate plus one reaction without the reverse transcriptase (-RT, NAC, no amplification control) (4 reactions per sample), and one reaction in triplicate for the no template control (NTC). The -RT control is important for assessing how much contaminating genomic DNA is present in each RNA sample. The NTC will tell you if the contaminating DNA is coming from your sample. Follow the flow diagram on the page 3 as a guide to setup the reaction tubes.

Time: 2.5h preparation/96-well plate + ~2.5h run.

Sample dilutions

 Prepare a dilution of each RNA sample so that the total RNA concentration is equal among all samples and the volume of sample to be added is 10 μl (e.g., 4 ng/μl = 40 ng/10 μl).

Start with enough copies of the RNA template to be sure of obtaining a signal by 25-30 cycles. Preferably, begin with 10,000 copies of template and less than 40 ng of total RNA per 20 µl.

Master mix

 Prepare a master mix without the RT (-RT) including at least one extra reaction volume (or ~10%) to accommodate reagent losses during pipetting. Mix by gentle inversion and centrifuge the tube(s) to spin down the contents and eliminate any air bubbles from the solution(s).

This -RT step is optional but is good to run at least once to check for contamination.

3. Transfer the -RT reaction mixture (31.68 μl for triplicates) to one corresponding tube per sample and add water (0.32 μl) up to total volume.

- **4.** Add **RT** to the remaining mix. Mix by gentle inversion and centrifuge.
- **5. Transfer the reaction mixture** (32 µl for triplicates) to a set of new tubes.
- **6. To the NTC tube, add H₂O** (32 μl for triplicates). Cap the NTC wells to prevent contamination.

Transfer the diluted total RNA sample (32 µl) to its corresponding tube with reaction mix.

- 7. Transfer 20 μl of the reaction mix + RNA into each well.
- 8. Cap the plate/tubes with MicroAmp optical caps. Centrifuge the plate/tubes to spin down the contents and eliminate any air bubbles from the solutions.

Sequence Detector setup

- **9.** Place the plate in the Sequence Detector and setup the template in the software (example on page 3).
- **10.** Run the reactions using the standard thermal cycling parameters listed on the next page

RT- Com	PCR Master mix	Volume/ tube (µl)	Final conc.
1.	RNase-free water	0.0	_
2.	10× TaqMan Buffer A	2.0	1×
3.	25 mM MgCl2	4.4	5.5 mM
4.	dNTP mix (2.5 mM each dNTP)	2.4	300 µM
5.	RNase Inhibitor (20 u/µl)	0.4	0.4 u/µl
6.	10 µM Forward Primer	0.2	100 nM
7.	10 µM Reverse Primer	0.2	100 nM
8.	10 µM Probe ^a	0.2	100 nM
9.	AmpliTaq DNA Polymerase (5 u/µl)	0.1	0.025 u/µl
10.	Reverse Transcriptase (50 u/µl) ^b	0.1	0.25 u/µl
11.	RNA Sample (10 pg-100 ng)	10.0	_
	Tatal values (ul)	20 0	

Total volume (µl) 20.0

^aIt is recommended to start from a 1:10 dilution of (100 µM) stock probe or primer solution. ^bOnly 4 of 5 tubes will have RT.

Note: To run a control sample, dilute the Control RNA 1:50 using RNase-free water. Use 1 µl of the diluted RNA in the reaction or an amount previously determined.

Thermal Cycling Parameters for One-Step RT-PCR

The following thermal cycling parameters are optimized for several systems by PE Biosystems. See thermal cycler manuals for details on operation.

Step	RT	AmpliTaq Gold activation	PCR	
			CYCLE (40 cycles)	
	HOLD	HOLD	Denature	Anneal/Extend
Time	30 min	10 min	15 sec	1 min
Temperature	48°C	95°C	95°C	60°C

Notes

- Resolubilize oligos in TE buffer or nuclease-free water, and store aliquoted at -20°C. Freeze/thaw cycles should NOT exceed 5 per aliquot.
- All fluorescent-labeled oligos and TaqMan probes should be stored away from daylight. TaqMan probes are supplied in TE buffer.
- Reverse Transcriptase and RNase Inhibitor are sensitive to air oxidation. Keep these reagents at -20°C until just prior to use.



Typical process of samples in triplicate

	98-011 eWAT UCP1											
Sa	Sample Type: UNKN - Unknown Thermal Cycler Conditions 7700 Single Reporter											
Sa	Sample Name: 74 eWAT 10ng											
	Replicat	e: 74							Comm	nent :		
	Oursetit								98-01	11 eWAT RI	NA for UCP	1 mRNA.
	Quantity	y.							psg			
s	how Analy:	sis Dye	Layer: 📃	FAM 🔻]							
	1	2	3	4	5	6	7	8	9	10	11	12
	STND	STND	STND	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
٠	UCP1 cD 2 5e+05	UCP1 cD 2 5e+05	. UCP1 cD 2 5e+05	.78 eV AT	.78 eV AT	.78 eWAT	.90 eWAT	.90 eWAT	.90 eWAT	.99 eWAT	.99 eWAT	.99 eWAT
	STND	STND	STND	LINKN	LINKN	LINKN			LINKN	LINKN	LINKN	LINKN
в	UCP1 cD	UCP1 cD	UCP1 cD	81 eWAT	.81 eWAT	.81 eWAT	.91 eWAT	.91 eWAT	.91 eWAT	.100 eV A	.100 eWA	100 eWA
_	5.0e+04	5.0e+04	5.0e+04									
	STND UCR1 oD	STND UCB1 oD	STND UCR1 oD				UNKN 92 ow AT	UNKN 92 ovr AT	UNKN 92 ov AT	UNKN 104 AWA	UNKN 104 ovra	UNKN 104 AWA
С	1.0e+04	1.0e+04	1.0e+04	00 en M1	.00 61 41	.00 61 MT						104 61 0.
	STND	STND	STND	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
D	UCP1 cD	UCP1 cD 2.0o+07	. UCP1 cD	84 eWAT	.84 eWAT	.84 eWAT	.93 eWAT	.93 eWAT	.93 eWAT	.105 eWA	.105 eWA	105 eWA
	STND	STND	STND				LINKN					l
E	UCP1 cD	UCP1 cD	UCP1 cD	85 eWAT	.85 eWAT	.85 eWAT	.94 eWAT	.94 eWAT	.94 eWAT			
_	4.0e+02	4.0e+02	4.0e+02									
	UNKN 24 oW AT	UNKN 74 ov AT	UNKN 74 ov AT	UNKN 97 ov AT	UNKN 97 ov AT	UNKN 97 ov AT	UNKN 95 ow at	UNKN 95 ow at	UNKN 95 ov AT			
F	7 C II AL.				.01 61 41	.01 68 41						
	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN			
G	76 eWAT	.76 eWAT	.76 eWAT	.88 eWAT	.88 eWAT	.88 eWAT	.97 eWAT	.97 eWAT	.97 eVAT			
			LINIZA	LINIZAL		LINIKA	LINKA	LINKM		NTC	NTC	NTC
н	77 eVAT	77 eVAT.	.77 eWAT	.89 eWAT	.89 eWAT	.89 eWAT	.98 eWAT	.98 eWAT	.98 eVAT	H10	H11	H12

Sample plate setup

APPENDIX J

RiboGreen[®] RNA Quantitation Microplate Assay

Review the Molecular Probes RiboGreen protocol (R-11490 & R-11491) before using this short protocol. This short protocol has been adjusted for use in 200 µl volume, black 96-well plates (Greiner #655076, Costar, or compatible). The RiboGreen assay is 200-fold more sensitive than ethidium bromide and 1000-fold more sensitive than ultraviolet absorbance determination (260 nm).

- Caution: no data are available addressing the mutagenicity or toxicity of RiboGreen RNA quantitation reagent. Because this reagent binds to nucleic acids, it should be treated as a potential mutagen and handled with appropriate care.
- All solutions should be prepared in sterile disposable, polypropylene plasticware rather than glassware, as the reagent may adsorb to glass surfaces.
- Protect the RiboGreen working solution from light by covering them with foil or placing them in the dark, as the RiboGreen reagent is susceptible to photodegradation.

1X TE assay buffer

Prepare from a 20X stock solution (200 mM Tris-HCl, 20 mM EDTA, pH 7.5) or use the kit's 20X TE.

RNA standard solution (2 µg/ml)

	Single	Triplicate			
1X TE buffer	181.3 µl	588 µl			
RNA stock (100 µg/ml)	3.7 µl	12 µļ			
Total volume	185.0 µl	600 µl [†]			
[†] Includes an extra 45 μl (8%).					

RiboGreen high-range working solution (WS) (1/200)

	Per well
1X TE buffer	99.5 µl
RiboGreen stock	0.5 µl
Total volume	100 µl

Add another 300 µl when using a repeat pipetter.

Add the RiboGreen working solution to the wells before adding the standard or sample.

For best results, the RiboGreen assay solution should be used within a few hours of preparation.

RNA standard curve, high-range

-			
Volume	Volume(µl)	Volume (µl)	Final RNA
(µI)	of 2 µl/ml	of 200-fold dil	concentration
of TE	RNA stock	RG reagent	in RG assay
100	0	100	Blank
90	10	100	100 ng/ml
75	25	100	250 ng/ml
50	50	100	500 ng/ml
0	100	100	1000 ng/ml
315	945	185	1x
555	500	1500	3x

Sample analysis

Dilute RNA sample in TE buffer so that the measured concentration is within the standard curve limits (OD 500~2400).

Add 100 μ I RiboGreen working solution to each well. Add 100 μ I of the diluted RNA sample.

Volume (ul)	Volume (µI) of 200-fold diluted	
of diluted	RiboGreen	
RNA sample	Reagent	Dilution
100	100	1/2

Mix well and incubate for 2 to 5 minutes at room temperature, protected from light.

Read OD using fluorescence

Molecular Devices SpectraMax Gemini settings: Ex/Em: 482/530 nm; Cutoff: 495 nm

pss

APPENDIX K

RNA, DNA & Protein Isolation Using TRIzol[®] Reagent

This is a modified method of the Life Technologies TRIzol Reagent protocol for extraction of RNA, DNA, and protein (Form 3796). Review the Life Technologies protocol, which contains more precautions and isolation notes, before using this short protocol.

CAUTION: When working with TRIzol Reagent use gloves and eye protection (shield, safety goggles). Avoid contact with skin or clothing. Use in a chemical fume hood. Avoid breathing vapor.

Note: **Volumes used are per 1 ml denaturing solution (TRIzol Reagent).** Unless otherwise stated, the procedure is carried out at 15 to 30°C and reagents are at 15 to 30°C.

A. RNA EXTRACTION

Materials & Equipment	Per sample or exp.	Source
O TRIzol Reagent O Chloroform (MW 1219.38) O Isopropyl alcohol O Ethanol, 70-75% in DEPC-treated water O RNase-free water or 0.5% SDS solution	1 ml 0.2 ml 1 ml 1 ml 50 µl	Life Technologies 15596 J.T. Baker 9180-01, Amresco 0757-950ML J.T. Baker 9095-03
 O 1.5 ml Microcentrifuge tubes O Disposable blade or scalpel O Homogenizer, glass-Teflon or equiv. 	3	Wheaton Potter-Elvehjem Tissue Grinder, 5 ml

1. Homogenization of tissues

- a. Tissues should be rapidly removed from the animal, pieces cut of 2 g, and quick-frozen in liquid nitrogen.
- b. Homogenize tissue samples in 1 ml of TRIzol Reagent per 50-100 mg of tissue using a glass-Teflon homogenizer or power homogenizer by applying as few strokes as possible. (Wheaton overhead stirrer setting 2.6-2.8). The sample volume should not exceed 10% of the volume of TRIzol Reagent used for homogenization.
- c. Transfer homogenate to a 1.5 microcentrifuge tube using a large orifice pipette tip.
- Incubate the homogenized samples at 15 to 30°C for ≥5 min to permit the complete dissociation of nucleoprotein complexes.

2. Remove insoluble material

This optional isolation step may be required for samples with high content of proteins, fat, polysaccharides or extracellular material such as muscles, fat tissue, and tuberous parts of plants. In samples from fat tissue, an excess of fat collects as a top layer that should be removed.

Sediment the homogenate by centrifugation at 500 × g for 15 min at 2 to 8°C. Transfer (pour) the cleared homogenate solution to a new tube.

3. Phase separation

 Add 0.2 ml of chloroform. Cap sample tubes securely. Shake tubes vigorously by hand for 15 sec. Store for 2-3 minutes at 15 to 30°C. Centrifuge the samples at 12,000 × g for 15 min at 2 to 8°C.

Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase, whereas the DNA and proteins are in the interphase and lower organic phase. The volume of the aqueous phase is about 60% of the volume of TRIzol Reagent used for homogenization.

b. Transfer the aqueous phase overlying the interphase (~590 μl) to a new tube for isolation of RNA. Save the organic phase for DNA and/or protein isolation (place in 4°C, do not freeze).

Careful removal of the aqueous phase is critical for the quality of the isolated RNA.

4. RNA precipitation

- a. Precipitate the RNA from the aqueous phase by adding 1 ml (1 vol) of 100% isopropyl alcohol. Mix by inversion. Store for 5-10 min at 15 to 30°C.
- b. Sediment RNA by centrifugation at 10,000 × g for 15 min at 2 to 8°C (up to 30 min for adipose tissue). Discard (pour-off) the supernatant.
 The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

5. RNA wash

- Add at least 1 ml of 70-75% ethanol. Mix RNA pellet by vortexing and/or passing the solution through a pipette tip. Store for 10 min at 15 to 30°C with periodic mixing. Centrifuge at 7,500 × g for 10 min at 2 to 8°C. Carefully discard (pour-off) supernatant.
- b. Dry (air-dry or vacuum-dry) the RNA pellet for 5-10 min. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. <u>Do not dry the RNA by</u> <u>centrifugation under vacuum.</u>

6. Solubilize the RNA

a. Dissolve the RNA pellet in 50 to 200 µl RNasefree water (DEPC-H₂O), TE buffer (in DEPC- $H_2O),\,0.5\%$ SDS solution (in DEPC- $H_2O)$, or freshly deionized formamide by passing the solution a few times through a pipette tip. [For 100 mg fat tissue, dissolve in 200 μ l.] (Avoid SDS when RNA will be used in subsequent enzymatic reactions.) Incubate for 10-15 min at 55 to 60°C.

 Store RNA at -70°C for long-term storage or -20°C for short-term storage either as an aqueous solution or as an ethanol precipitate.

Expected yields of RNA per mg of tissue

- White adipose tissue, 0.2-2 µg
- Brown adipose tissue, 1-3 µg

B. DNA EXTRACTION

Materials & equipment	Per sample or exp.	Source
O RNase A, DNase-free (10 mg/ml)	1 µl	Promega A7973, CSS 668500
O Proteinase K (20 mg/ml)	300 µl	Amresco E195-25ML
O DNA extraction buffer	0.5 ml	See Solutions
O TE buffer, pH 8.0	70 µl	

•••Method A•••

1. DNA phase separation

- a. Remove any remaining aqueous phase (RNA) overlaying the interphase.
- Add 0.5 ml DNA extraction buffer per 1.0 ml TRIzol used. Vigorously mix by inversion for 15 seconds and store for 10 min at room temperature (RT).
- c. Centrifuge at 12,000 × g for 15 min at 4°C.
- d. Transfer the upper aqueous phase containing DNA to a new tube and save the interphase and organic phase at 4°C for subsequent protein isolation.

2. DNA precipitation

- a. Precipitate the DNA with ethanol by **adding 0.4 ml of isopropanol** per ml of TRIzol used. The isopropanol volume is 0.8 volumes of the aqueous phase. If the expected DNA yield is less than 20 µg, add a microcarrier (i.e., glycogen) to the aqueous phase prior to isopropanol addition and mix.
- b. Mix the tube contents by inversion and store for 5 min at RT.
- Sediment DNA by centrifugation at 12,000 x g for 5 min at 4-25°C and remove the supernatant. Wash the DNA pellet with 1.0 ml of 75% ethanol.

d. Proceed with DNA solubilization as described in Method B, step 8.

•••Method B•••

1. DNA precipitation

 Precipitate the DNA from the interphase and organic phase with ethanol by adding 0.3 ml of 100% ethanol and mix by inversion. Store for at least 30 min to overnight at 4 to 25°C.

The protein-containing phenol-ethanol solution can be stored at RT for a week, 4°C for a month, and -20°C for 3 months without apparent protein degradation.¹²

- Sediment DNA by centrifugation at 12,000 × g for 10-30 min at 4 to 25°C.
- c. Remove (pour-off) the phenol-ethanol supernatant into a new tube and save for protein isolation.

2. DNA wash

- a. Add 1-2 ml of 75% ethanol. Store for 10 min at 4 to 25°C (with periodic mixing).
- b. Centrifuge at 12,000 × g for 10-30 min at 4 to 25°C. Carefully discard (pour-off) the supernatant.
- Remove the ethanol by air-drying the pellet: store open tubes for 10-15 min at RT. (Do not dry under centrifugation, it will be more difficult to dissolve.)

3. DNA solubilization

a. Dissolve the DNA pellet in **200 µl TES solution**, TE buffer, or 8 mM NaOH to approach DNA concentration of <1 µg/µl.

Resuspending in weak base is highly recommended since isolated DNA does not resuspend well in water or in Tris buffers.

b. Mix the solution periodically by gently tapping the tube or place on shaker.

4. RNase & proteinase treatment

- **RNase treatment** is generally not necessary if the aqueous phase was completely removed. If needed, add 1 μl of 10 mg/ml RNase A (final conc. of 10-20 μg/ml). Mix the sample by inverting the tube 25 times. Incubate for 15 min at 37°C.
- Add 1.5 μl of 20 mg/ml Proteinase K (final conc. of 50-100 μg/ml). Mix and incubate for 2 hours at 37°C. Cool to RT for 5 min.

Promega recommends that Proteinase K be used in 10 mM Tris-HCl (pH 7.8), 5 mM EDTA, and 0.5% SDS.

5. Phase separation

- a. Add an equal volume (300 µl) of phenol/chloroform/isoamyl alcohol (prepared with 1M Tris, pH 8) to DNA solution (0.1 to 0.4 ml). Shake tubes vigorously by hand for 15 sec.
- b. Centrifuge at 1,700 × g for 10 min.
- c. Transfer top (aqueous) phase containing DNA to a new tube.

High salt concentrations can cause inversion of the aqueous and organic (yellow) phases. Repeat step 5a on aqueous phase if thick white precipitate is present at interface.

For large amounts of DNA (>50-100 µg/ml), precipitation is essentially instantaneous at RT. Recovery of <1 µg DNA can be improved by reextracting organic phase with 100 µl TE buffer.

6. DNA precipitation 2

- a. Add ½ vol (~150 μl) of 7.5 M ammonium acetate to facilitate ethanol precipitation. Mix.
- Add 2 to 2.5 volumes (1 ml) of 100% ethanol. Mix by inverting tube several times. Store for at least 30 min to overnight at 15 to 30°C.
- c. Centrifuge at 12,000 × g for 30 min at 15-30°C. Aspirate the supernatant. For large pellets, pour off the supernatant.

The pellet may not be visible at this point.

7. DNA wash 2

- a. Add 1 ml of 75% ethanol. Mix by inverting the tube several times.
- Centrifuge at 12,000 × g for 15-30 min at 15-30°C. Aspirate the supernatant.
- c. Air-dry the pellet by storing open tubes for 15-30 min at RT.

8. DNA solubilization

- a. Dissolve DNA in 25 to 50 µl TE buffer (pH 8.0) or 8 mM NaOH to approach DNA concentration of ≤1 µg/µl (note amount used).
- <u>Gentle</u> vortexing should dissolve <25 µg DNA; larger quantities may require 10 to 60 min at 65°C. Periodically mix the solution by gently tapping the tube or place on shaker.

Alternatively, rehydrate the DNA by incubating the solution overnight at RT or at 4°C. High molecular weight DNA may require one to several days to dissolve and should be shaken gently (not vortexed) to avoid shearing.

C. PROTEIN EXTRACTION

Materials & equipment	Amount	Source
O Trichloroacetic acid (TCA, FW 163.39)		Fisher Scientific A322
O Acetone	3 ml	Fisher Scientific A18
O Dithiothreitol (DTT, MW 154.25)		Fisher Biotech BP172
O Complete [®] protease inhibitor or PMSF		Roche 1697498
O Protein sample buffer		See Reagents & solutions
O 5 ml round-bottom centrifuge tubes		Falcon 352063
O Microcentrifuge tubes		

1. Protein precipitation

- a. Add 3 volumes (~3 ml) of 10% TCA in acetone, 20 mM DTT. Precipitate proteins for at least 45 minutes. Overnight precipitation may help samples with low protein.
- b. Centrifuge at 7,500 × g for 5-30 minutes (depending on amount of ppt and volume) at 2 to 8°C. Discard supernatant. Be careful not to pour out the soft pellet.

2. Wash pellet

- a. Wash with **1 ml cold 90% acetone**. Mix by inversion or vortex. Sonicate pellets that do not disperse well.
- b. Centrifuge at 7,500 × *g* for 3 minutes at 2 to 8°C. Discard supernatant by pipette.
- c. Wash with 1 ml cold 90% acetone. Mix by inversion, vortex, or sonication. Transfer volume to microcentrifuge tube. Centrifuge at 7,500 × g for 3 minutes at 2 to 8°C. Discard supernatant by pipette.

An additional wash may be necessary to remove residual TCA. When adding sample to Laemmli buffer, a color change to yellow indicates an acidic solution caused by residual TCA. Remove residual acetone by air-drying for 5-10 minutes. **Do not over-dry**

3. Dissolve the protein pellet

a. Add sample buffer (i.e., sds/urea/tris). Disperse protein pellet manually or by sonication.

Suggested sample buffer per 100 mg tissue:

- WAT, 50 90 µl
- BAT, 400 500 μl

Samples dissolved in a buffer containing urea should remain <u>at room temperature</u> for at least 1 hour for full denaturation and solubilization prior to centrifugation and subsequent sample application. When using an SDS buffer, complete dissolution of the protein pellet may require incubating the sample at 50°C, 5 min.

Note: Heating of the sample should only be done prior to the addition of urea, as heating in the presence of urea will cause urea to hydrolyze to isocyanate, which modifies proteins by carbamylation.

When you have added enough sample buffer, the solution should look transparent. However, some fat samples may look cloudy even though the protein has dissolved. Be careful not to over-dilute the sample.

- b. Sediment any insoluble material by centrifugation at $10,000 \times g$ for 10 minutes. Transfer the supernatant to a fresh tube.
- c. Store solubilized protein at -20°C until use or at -80°C for long-term storage.

Solutions

DNA extraction bu	uffer		
		Final conc.	Amount
Guanidine thiocyanate Sodium citrate (FW 29 1 M Tris, pH 8.0	e (MW 118.16) 94.10)	4 M 50 mM 1 M	47.264 g 1.471 g <100 ml
Protein sample			
	Final conc.	Amount	
SDS, 10% solution 1 M Tris-Cl, pH 8.0*	2.0% 40 mM	5.0 ml 1.0 ml	

Complete [®] protease inhibitor stock, 25×						
	Final conc.	Amount				
Complete [®] dH₂O	25×	1 tablet to 2 ml				
The stock solution is stable for 1-2 weeks, stored at 2° to 8°C, or at least for 12 weeks at -15° to -25°C. (Roche Cat. No. 1 697 498 or 1 836 145)						
PMSF protease in	hibitor stock, 200	mM				
	Final conc.	Amount				
PMSF (FW 174.2)	200 mM	34.8 mg				
H ₂ O (NanoPure)		1 ml				
Store 200 mM stock in 100% ethanol up to 6 months at -20°C. Add PMSF just prior to use from a 200 mM stock by diluting 1/100 (i.e., 2 µl into 200 µl of sample buffer/lysis solution); final						

References

 ddH_2O

Store at 4°C.

*Final solution pH 6.8-8.8.

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conc. 0.2 mM.

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to 25 ml

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APPENDIX L

Subcellular Fractionation of Adipose Tissue for the Isolation of Mitochondrial Protein

Rev. 4-Apr-2002

All steps are carried out at 0° to 4°C.

Homogenization

Note: Suggested minimum tissue weights: WAT ~250 mg, BAT ~50 mg. These amounts allow for enough apoptotic proteins in the mitochondrial fraction for RIA or Western blot analysis.

Homogenize tissue briefly in isolation medium (5:1-10:1 vol to weight of tissue; ~2 ml/250 mg, ~0.5 ml/50 mg) with protease inhibitors (80 μl Roche Complete/2 ml) using a glass-Teflon homogenizer or other gentle method. Transfer (pour) the supernatant into a centrifuge tube. Excessive homogenization should be avoided, as it can cause damage to the mitochondrial membrane and release mitochondrial components.

Differential centrifugation

- **2.** Centrifuge at 700 × g for 10 min at 4°C. Very carefully, transfer the supernatant, including fat cake, into a new tube without disturbing the soft pellet. Discard the pellet (nuclear fraction).
- **3.** Collect the mitochondria by centrifugation at $15,000 \times g$ for 10 min at 4°C. Transfer the supernatant (cytosolic-microsomal fraction) without the fat layer to a new tube (size = vol + vol×3; compatible with acetone and RCF ≥ 7,500 × g) for protein isolation if desired by decanting, pipet, or needle & syringe; aspirate the fat on the tube wall.

Mitochondrial protein

4. Disperse/mix (sonicate briefly and keep cold) the mitochondrial pellet in **50-100 μl protein sample buffer for WAT, 200-500 μl for BAT**. Allow protein to solubilize at room temperature (RT) for a minimum of 1 hour. Continue with protein quantification (below).

Cytosolic protein isolation

- 5. Precipitate protein in the cytosolic fraction by the addition of at least 3 volumes of 10% TCA in acetone, 20 mM DTT. Let precipitate for at least 45 min at -20°C. Overnight precipitation should help samples with low protein.
- **6.** Sediment the precipitated protein by centrifugation at 7,500 \times *g* for 30 min (depending on amount of precipitate and volume) at 4°C. Discard supernatant by pouring or aspiration, being careful not to disturb the pellet. Aspirate any remaining fat on the tube wall.
- 7. Wash the pellet with 1 ml of cold 90% acetone and transfer to a 1.5 ml microfuge tube. Mix by inversion. Centrifuge at 7,500 × g for 2-3 min at 4°C. Aspirate the supernatant by pipet. Repeat wash at least twice.

An additional wash may be necessary to remove residual TCA. When adding sample to Laemmli buffer, a color change to yellow indicates an acidic solution caused by residual TCA and, in this case, additional wash steps are needed.

8. Remove residual acetone by air-drying 5-10 min. <u>DO NOT OVER-DRY</u>. Dissolve the pellet in **protein sample buffer*** and disperse manually or by sonication briefly without heating (keep on ice). Store at RT for a minimum of 1 hour.

*Suggested volume of **sample buffer is 25-75 \muI for WAT, or 400-500 \muI for BAT. When enough sample buffer has been added, the solution should look transparent but straw-colored. However, some fat tissue samples may look cloudy even though the protein has dissolved. Be careful not to over-dilute the sample.**

Store solubilized protein at -20°C until use or at -80°C for long-term storage.

Protein quantification

Suggestion: Spin-down insoluble material at 16,000 × g (full speed Eppendorf 5415C) for 2-3 minutes and transfer the supernatant to a new tube. Use $Pierce Coomassie^{®}$ Plus or other method. For 250 mg sample solubilized in 40-50 µl protein sample buffer, dilute samples **1:250** before adding to plate. Also use a sample buffer blank.

Materials & equipment

- Isolation medium (below)
- Protein sample buffer (below or other)
- Protease inhibitors (Roche Complete[®] Roche # 1697498 or 1836145)
- Dithiothreitol (DTT, MW 154.25)
- o 4 ml round-bottom centrifuge tubes (Sorvall # 03105 (11×75 ml) or equiv.)
- \circ 10-15 ml centrifuge tubes rated for ≥ 7500 × g (e.g., Corning # 430052)
- 1.5 & 0.6 ml microcentrifuge tubes
- o Glass-Teflon homogenizer (5 ml Wheaton Potter-Elvehjem Tissue Grinder)
- Disposable blade or scalpel
- o High-speed centrifuge with swing-bucket rotor
- o Sterile filter system, 500 ml, 0.45 μm (Corning # 25943-500)

Subcellular fractionation medium, 1×			Protein sample buffer, 1×		
	Final conc.	Amount		Final conc.	Amount
Sucrose (FW 342.30) HEPES (MW 238.2) Potassium EDTA*	250 mM 10 mM 0.2 mM	21.39 g 0.596 g 0.020 g	SDS, 10% solution 1 M Tris-Cl, pH 8.0* ddH_2O	2.0% 40 mM	5.0 ml 1.0 ml to 25 ml
ddH2O to 250 ml pH to 7.2-7.4 with 1 N KOH then make to volume and		*Final solution pH 6.8-8.8. Store at 4°C.			
filter twice (0.8 µM then again after 2 weeks. *Dipotassium salt, MW	0.2 µM). Store 400.47 (ACROS	at 4°C. Filter AC20974).			

References

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 Murphy, K. M., et al. Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. *Cell Death Differ* 7 (1):102-111, 2000.

 Mikhailov, V., M. et al. Bcl-2 prevents bax oligomerization in the mitochondrial outer membrane. J Biol Chem 276 (21):18361-18374, 2001.

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APPENDIX M

Surgical Denervation of the Rodent Inguinal White Adipose Tissue

The technique of denervating the inguinal white fat pad in the rodent was demonstrated by Prof. Tim Bartness at Georgia State University (August 1999). Because each WAT pad is unilaterally innervated, axotomy of one of a pair of WAT pads can be done, with the contralateral WAT pad serving as a within-animal intact neural control.² It is impossible to selectively sever only the SNS innervation of the tissue; thus SNS, sensory, and parasympathetic nerves all are cut, although the latter does not seem to exist. This procedure typically does not produce 100% denervation of the fat pad but is permanent. The best result is considered around 80% denervation. One hundred percent denervation is only achieved by cutting the blood supply, which will cause some or all of the tissue to undergo necrosis. Take care not to cut any of the major blood supply to the tissue. Even a sham surgery that cuts around the fat pad leaving all apparent blood supply and nerves intact can cause a 20% denervation; sham surgeries are no longer done for comparison. The amount of denervation is determined by analyzing the tissue for catecholamines one or two weeks after surgery but can be measured at a minimum of two days after surgery. The growth of the fat pad after surgery indicates that it is receiving adequate blood supply and nutrients—a favorable outcome.

The rodent strain chosen for denervation will affect the time involved. The procedure was first learned on a Syrian hamster; these seem to have well defined inguinal fat pads. The BHE rat has a slightly less defined fat pad and different vascular system around the pad. In the Sprague-Dawley rat (250-300 g) the inguinal fat pad is not discrete and it is more difficult to isolate the pad while avoiding the blood vessels. (This procedure is not recommended for the Sprague-Dawley rat.).

Time: ~2 hours per rodent

A right-handed person should denervate the <u>left</u> <u>side</u> of the animal (the animal's left).

Prep the animal

- Anesthetize the animal with 3:2:1 v/v/v ketamine/acepromazine maleate/xylazine (1 ml/kg).
- 2. Remove (shave) the fur from the selected hindquarter. The animal will be positioned on its anterior (stomach).
- **3.** Clean the shaved area with a chlorhexidine-soaked gauze sponge.
- **4.** Place a warmed isothermal pad under the surgical platform.

Metafane should be used if the animal starts to come out of the anesthesia during surgery.

Incision

- 5. Make a ~2 cm incision dorsally on th skin from a point over the flank region and lateral to the spinal column, about 1.5 cm from the spine. The depth of the incision should avoid the underlying blood vessels and musculature. Spread apart the opening to expose the area with the inguinal fat pad by tearing the skin. (Tearing the skin allows better wound recovery than cutting with a blade.)
- 6. Separate the pad from the abdominal wall and overlying skin by blunt dissection, keeping intact the major blood vessels leading into or through the pad. At the same time, nerves identified at ×4 magnification as terminating in the pad are cut in two or more locations by pulling on the nerve to make a loop and cutting that loop (they look like white fibers).

Throughout the surgery keep the pad moist with <u>cold saline solution</u>. The use of two bottles of saline solution makes it easy to switch one out of the refrigerator for the next animal.

NOTE: Do not cut any of the major blood vessels. If you do, you may as well start with a new animal. This would cause all or some of the pad to die. There should not be a lot of bleeding. If there is, you probably cut a major blood vessel.

7. Cut the nerves terminating in the pad. You will see nerves below the pad that go to the muscles; these do not have to be cut. Most nerves will follow the vascular system with nerves on both sides of a blood vessel. Pull the nerves away from the blood vessel and avoid cutting or nicking the vessel. You may

cut the very small blood vessels or capillaries if they get in the way.

8. Continue around the whole pad: around one side to the bottom, then the other side to the bottom. You should be able to almost lift the pad and be able to get all the way around. The lateral part of the pad, close to the skin (bottom), is difficult to separate but most nerves come from the medial side closer to the spine. You should not have to completely separate the lateral part of the pad.

Finishing

- **9.** When you believe all or most of the nerves to the fat pad have been cut, place the pad in its most natural position against the abdominal wall and rinse with cold saline.
- **10.** Overlay the pad with the skin. Gently pinch the skin at one end of the incision with forceps then add VetBond or sutures to seal the wound. Switch to the other end of the incision and work your way toward the middle.
- **11.** Apply betadine ointment to the wound area.

Recovery

- **12.** Place the animal on a recovery cage on top of a warm isothermal pad.
- 13. Let the animal recover for at least 1 week.
- **14.** Place the saline back in the fridge to become cool again.

The fat pad can be removed anywhere from 2 days on to see the effects of denervation on catecholamines.

References

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 Bartness, TJ. and Bamshad, M. Innervation of mammalian white adipose tissue: implications for the regulation of total body fat. *Am J Physiol* 275 (5):R1399-R1411, 1998.
 Youngstrom, TG and Bartness, TJ. White adipose tissue sympathetic nervous system denervation increases fat pad mass and fat cell number. *Am J Physiol* 275 (5 Pt 2):R1488-R1493, 1998.

prs

Material & Equipment		Per animal or exp.	Source
000000000	Dosing form Mask Sterile gloves Clippers Ear notcher & international code for ear notching Autoclave bag Autoclave tape Surgical platform or sterile dressing	1 12 1 1 1 1 roll 1	3M #8511 or equiv. Fisher #11-394-95F (8.5, large) Wahl
000000000000000000000000000000000000000	1-cc syringe 26-gauge 3/8" needle 3:2:1 v/v/v ketamine/acepromazine maleate/xylazine Ketamine HCI (100 mg/ml) Acepromazine maleate (10 mg/ml) Xylazine (20 mg/ml) Methoxyflurane Chlorhexidine Artificial Tears Metafane inhalant device Resuscitation device 0.15 M (0.9%) sterile saline solution, cold Fluid dropper (for metafane)	1 0.250 ml 0.125 ml 0.083 ml 0.042 ml 1 tube 1 1	Monoject #501400 B-D #305110 Ketaject [®] (Phoenix) (Fermenta Animal Health) Rompun [®] (Bayer) Metafane [®] (Pittman-Moore) Nolvasan (Fort Dodge) Butler Co.
0000000	Gauze sponges, sterile (2×2-12 PLY) Swabs, sterile (6 inch) Blade No. 15 Straight forceps Tissue forceps Spring scissors, fine Curved scissors	1 pack 2 packs 1 2-3 1 1 1	Butler Co. #WA161202 Hardwood Products Co. #806-WC Bard-Parker #371263 Dumont No. 5 Roboz RS-8248 Roboz RS-5618 Roboz ?
$\stackrel{\bigcirc}{\circ}$	Microscope Flexible lighting	1 1	ausJENA OPM 212T or equiv.
00000	VetBond (glue) Betadine ointment Isothermal pads Oven or microwave oven Shoe-box cage with soft bedding	1 tube 1 tube 1 1 1	3M Animal Care Products #1469 Purdue Frederick Co.