

REAL-TIME RT-PCR AND PROTEOMIC ANALYSIS OF ADIPOSE TISSUE FROM OB/OB  
MICE TREATED WITH LEPTIN

by

WEI ZHANG

(Under the Direction of Clifton A. Baile)

ABSTRACT

Leptin, a hormone secreted by adipose tissue, plays a critical role in regulating body weight, energy expenditure, lipid metabolism, and adipocyte apoptosis. In order to elucidate the underlying signaling pathways of leptin on adipocytes, advanced real-time RT-PCR and proteomic technologies were performed to compare mRNA and protein levels from white adipose tissue between control and leptin-treated ob/ob mice. ob/ob mice were treated with either PBS (control), or leptin (2.5 µg/d or 10 µg/d) for 14 days via sc osmotic mini pumps, and three fat depots were collected at the end of the experiment.

In the first study, eighteen genes were found to be the potential targets of leptin in inguinal fat pad of ob/ob mice by real-time RT-PCR and TaqMan<sup>®</sup> low-density arrays (24 genes format). They are classified as genes related to energy expenditure (UCP2, ADRB3, MFN2, SIRT3), genes involved in lipid metabolism (HSL, SCD1, FAS, RBP4), transcription factor SREBF1, genes related to apoptosis (Bcl-2, Bax, Caspase 3), cytokines (TNF α, adiponectin, complement 3), as well as genes taking part in inhibition of angiogenesis (Angiopoietin 2).

In the second study, different detergent combinations were tested, and a modified protocol was described as the basis of extraction buffer, including 8.4M urea, 2.4M thiourea, 2% CHAPS (v/v), 2% ASB14 (v/v), 1% IPG buffer, 15mM Tris and 50 mM DTT.

In the third study, comparative proteomic analysis was performed between control and leptin-treated ob/ob mice in parametrial adipose tissue. Twelve protein entries were differentially expressed, functionally classified as molecular chaperones (CRT, PDIA3, PHB, and Prx-VI), cytoskeleton proteins ( $\beta$ -actin,  $\alpha$ -tubulin and desmin) and other proteins (PC, GDEase, UQCRC, PTRF, and fibrinogen). The mRNA levels of CRT, PDIA3 and PHB were consistent with the fold change in protein expression level by real-time RT-PCR.

In general, our results demonstrated that proteomic analysis offers a promising tool to study the global analysis of proteins, and real-time RT-PCR is a reliable, sensitive and medium-throughput platform to measure mRNA quantitatively in a complex biological system (ob/ob mice) under certain external stimuli (leptin treatment).

INDEX WORDS: Leptin, ob/ob, Real-time RT-PCR, Proteomics, Adipose Tissue

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## DEDICATION

I dedicate my dissertation to my parents and younger brother, whose consistent love, encouragement and support have made it possible for me to fly halfway around the earth to come study for my Ph.D. degree at the University of Georgia.

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## CHAPTER 1 INTRODUCTION

The rapid increase in obesity and associated disorders worldwide has attracted great interest in the study of white adipose tissue, which is now recognized as a major endocrine cell for secreting bioactive peptides and hormones, regulating lipid metabolism. Of them, leptin, the product of *ob* gene, is derived primarily from adipocytes and acts as 'lipostat factor' to regulate body weight through changes in food intake, energy expenditure (1), lipid metabolism (2) and adipose tissue vascularization (3). Although some genes involved in the regulation of appetite, energy expenditure and apoptosis have been identified (such as UCPs (4), TNF  $\alpha$  (5,6), Neuropeptide Y (NPY) (7) and pro-opiomelanocortin (POMC) (8)), molecular mechanisms underlying leptin's effect on weight loss, especially on fat loss, are still unclear. But adipose tissue is an important target organ of leptin, and following leptin treatment.

The genetically obese *ob/ob* mice (9) are characterized by hyperphagia, early-onset obesity, insulin resistance, low core temperature and infertility due to the single *ob* gene mutation, and now are widely used as one of animal models for the research on obesity and diabetes. Leptin, the product of *ob* gene, is the adipocyte-derived hormone as an afferent factor, acting primarily in the hypothalamus to regulate body weight in humans and rodents in a negative-feed back loop (1). The interests in leptin specifically stem from its special properties of weight-reducing effects, which are greatly different from food-restriction from the aspect of metabolism (10). In a recent study, *ob/ob* mice were treated with s.c. leptin at the dose of either 0, 2.5  $\mu\text{g}$  or 10  $\mu\text{g}$  for 14 days. Consistent with previous literature, *ob/ob* mice showed significant reduction in body weight, food intake, weights of all three fat depots (inguinal, parametrial and retroperitoneal)

examined, and fat percentage was also reduced in the leptin-treated group compared to control group (11). In addition, leptin significantly increased body temperature, physical activity and adipose tissue apoptosis in ob/ob mice compared to control ob/ob mice (11). Moreover, insulin sensitivity was increased in ob/ob mice by leptin treatment (11). Adipose tissue from these ob/ob mice provide a unique model for characterizing how leptin-induced alterations in cellular functions are mediated through significant changes in expression of specific genes and/or their protein products.

Therefore, the purpose of the present was to explore several possible cellular pathways at the mRNA and protein level, through which leptin may regulate body weight and fat mass. Chapter 3 focuses on target genes functionally classified as energy expenditure, lipogenesis and lipolysis, adipocyte apoptosis, adipocyte differentiation, glucose transporter, insulin sensitivity and angiogenesis inhibition. Changes in gene expression in adipose tissue following leptin treatment in ob/ob mice were determined by real-time RT-PCR combined with Low Density Card. Chapter 4 and 5 focus on the protein changes in the adipose tissue by leptin treatment in ob/ob mice. Since proteins extracted from adipose tissue are hard to be resolved on two-dimensional (2-D) gel using traditional extraction buffer due to its large amount of lipids, different detergent combinations were varied, tested and assessed by 1-D gel and small size 2-D gel in order to optimize protein separation. Based on the protocol from chapter 4, a global protein analysis was compared in the adipose tissue of leptin-treated as compared with control ob/ob mice (Chapter 5). Twelve different proteins have been identified and functionally classified as molecular chaperones and redox group, cytoskeleton structure proteins and other proteins. Some proteins of interest such as molecular chaperons were further verified by real-time RT-PCR.

Thus, the overall purpose of the reported studies was to identify potential target genes and proteins of leptin in adipose tissue which might be involved in the signaling pathways mediating fat loss in ob/ob mice treated with leptin.



## CHAPTER 2 REVIEW OF THE LITERATURE

### 2.1. White Adipose Tissue and Lipid Metabolism

The prevalence of obesity in the U.S. has recently increased, with almost 65% of adults now overweight [body mass index (BMI)  $\geq 25$ ] and 33% being obese (BMI  $\geq 30$ ). Worldwide, around 250 million people are obese, which is alarming, given that obesity is a major risk factor for many chronic diseases such as type 2 diabetes and cardiovascular diseases (12). The direct cost of obesity is currently estimated at around 7-9% of total health care costs in the U.S. (13). Obesity is characterized not only by excessive deposition of adipose tissue, but also by the generalized steatosis, lipotoxicity, and lipoapoptosis in non-adipose tissues such as pancreatic beta-cells, liver, myocardium, and skeletal muscle due to the over-accumulation of lipids (14). This worldwide increase in incidence of obesity has attracted scientists to the biology of adipose tissue (15).

Adipose tissue generally constitutes 18% of an average person's body weight, and is the main organ providing fuel reserves for the organism. Without the fat stores in adipose tissue, we could not live for more than a few days without eating. Adipose tissue belongs to loose connective tissue, and is composed of unilocular adipocytes (about 90% of mass) filled mainly with triacylglycerol (TAG), blood cells, immune cells and fibroblasts (Figure 2.1) (16). Adipose tissue is richly vascularized and innervated, indicating its high metabolic activity. This tissue may develop almost anywhere, but is mainly located subcutaneously and viscerally.

Although there is no limit for adipocytes to store lipid, animals maintain their body weight fairly constant, and adipose tissue is under tight neural and hormonal control (17). The relatively

constant level of body fat and body weight in the adult mammal is elucidated by the “lipostat” theory. An adiposity signal in the hypothalamus reflects total body fat content by either direct or indirect adipose tissue regulation`. This is supported partially by the experiments with parabiotic animals (18). The successful cloning of the *ob* gene from *ob/ob* mice (1) in 1994 and the *db* gene from *db/db* mice (19) in 1995, as well as the subsequent characterization of leptin and its receptor, has made great progress in understanding the control of energy balance and lipid metabolism in rodents and humans. It is now widely accepted that leptin provides an adiposity signal from body stores to the hypothalamus, binding to receptors to regulate energy balance.

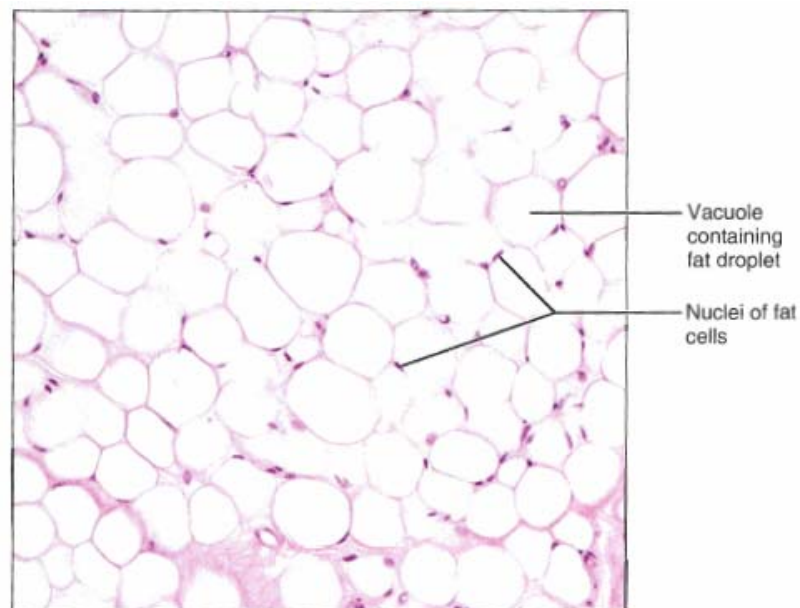


Figure 2.1. Subcutaneous adipose tissue (16)

## 2.2. *Ob* Gene and Leptin

Leptin, the product of the *ob* gene, acts primarily on the brain to regulate energy balance and food intake in a negative-feedback loop regulating the mass of adipose tissue (1) (Figure 2.2). The hormone, which is composed of 167 amino acid residues, is produced almost exclusively

from adipose tissue, and is released into the bloodstream in proportion to the amount of adipose tissue as a signal of adiposity (1). Leptin is primarily secreted from adipose tissue, but is also present in the stomach (20), skeletal muscle (21), mammary gland and placenta (22).

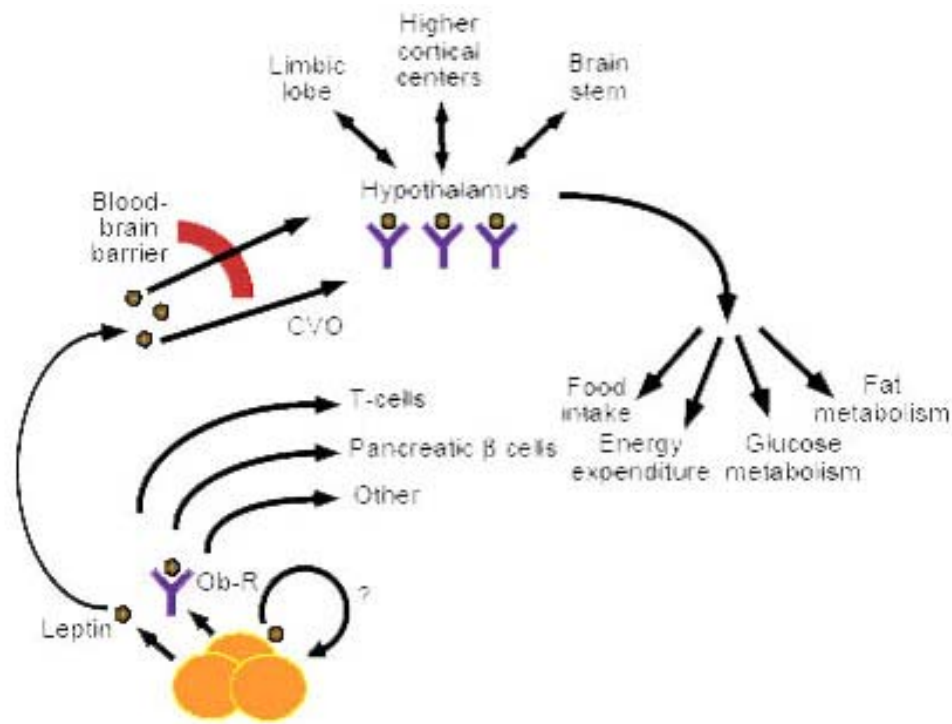


Figure 2.2. Adipocyte leptin and the regulation of adipose tissue mass (10)

There are at least six isoforms of leptin receptors (Ob -Ra, -Rb, -Rc, -Rd, -Re and -Rf) (23), one of which (Ob -Rb) is predominantly localized in the hypothalamus, and is known to be responsible for transmitting and activating the intracellular signal of leptin (24). Within the hypothalamus, different neuronal populations mediate leptin's downstream effects on body weight and energy balance. Leptin decreases expression of orexigenic peptides such as neuropeptide Y (NPY) (25) and agouti-related peptide (AGRP) (26), and increases expression of anorexigenic peptides such as proopiomelanocortin (POMC) (8) and cocaine and amphetamine regulated transcript (CART) (27), leading to an overall decrease in appetite and body weight (28).

Interests in leptin stem from evidence of its special weight-reducing effects in rodents. Central and peripheral administration of leptin results in a dose-dependent decrease in body weight for both ob/ob and wild-type mice (29,30), and leptin-induced weight loss is specifically restricted to adipose tissue while lean body mass (LBM) was spared (29,31). Furthermore, leptin-treated ob/ob mice exhibited a greater decrease in body and fat weights than pair-fed ob/ob mice (32). Following reduced food intake, ob/ob mice treated with leptin maintained high energy expenditures, while food-restricted mice demonstrated a reduction in energy expenditure (30). In addition to regulating food intake and thermogenesis, leptin also protects skeletal muscle (14), pancreatic islets (33), and myocardium (34) from lipotoxicity and lipoapoptosis (14). Although leptin plays a critical role in reproduction (35), immunity (36), blood cell development (37), and bone formation (38), this review focuses specifically on lipid metabolism and its related systems. Although leptin can regulate adiposity indirectly by modulating food intake and energy expenditure (1), accumulating evidence (39,40) indicates that leptin directly acts on adipocytes in regulating cellular lipid balance and promoting  $\beta$ -oxidation.

### **2.3. Leptin: an Essential Regulator of Lipid Metabolism**

#### **Leptin: Lipogenesis and Lipolysis**

The amount of adipose tissue is defined by the size and number of constituent adipocytes. In adult animals, changes in triglycerol content determine changes of fat mass. Numerous studies have shown that leptin has a direct action on the rates of synthesis and degradation of lipids as an autocrine or paracrine signal.

Fruhbeck et al (41) demonstrated that *in vitro* leptin induced lipolysis in a dose-dependent manner in mouse adipocytes from ob/ob mice. Fruhbeck et al (42) also demonstrated that lipolytic rates were increased in adipocytes from ob/ob (no functional leptin), while no changes were observed in db/db mice (no functional leptin receptor) after i.p. injection of leptin. In

addition, ob/ob mice had much more sensitivity to enhanced lipolytic rates of adipocytes by leptin treatment than did the wild type mice (42).

Leptin appears to inhibit lipogenesis by changing the activity of a few key enzymes in lipid metabolism. The procedure of *de novo* lipogenesis in these tissues includes two rate-limiting enzymes: acetyl coenzyme A (CoA) carboxylase (ACC) and the terminal enzyme fatty acid synthase (FAS) (43). For example, leptin suppresses the activity of ACC, therefore inhibiting the subsequent process of triglyceride formation in the fat cells (44). Leptin also decreases the mRNA expression of FAS, and increases the lipolytic enzymes, including lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL) (or Triacylglycerol lipase) (Figure 2.3) in adipose tissue of wild type mice (45). Leptin treatment has been shown to induce lipolysis without a concomitant increase in plasma free fatty acids, as typically occurs during fasting-induced fat mobilization (46).

Although the signaling pathway used by leptin to stimulate lipolysis is not clear, it is suggested that leptin, like catecholamines and glucagon, promotes lipolysis by increasing the concentration of cAMP and stimulating adenylate cyclase protein levels (47).

### **Leptin and Adipocyte Apoptosis**

In addition to regulating lipogenesis and lipolysis in adipocytes, leptin may also reduce adipose mass by inducing adipocyte apoptosis. Apoptosis, or programmed cell death, is a term coined in 1972 by Kerr et al (48), and is a normal physiological process involved in deleting unwanted cells such as aged cells, damaged cells, or precancerous cells, from living tissue during embryonic and adult tissue homeostasis (49). Apoptotic cells are characterized by membrane blebbing, chromatin condensation, fragmentation of nuclear DNA, and formation of apoptotic bodies, which are disposed of without inflammation. Apoptosis is regulated by phylogenetically conserved genetic cascades that rapidly and selectively eliminate unwanted cells, and occurs in

almost all cell types. The process can be initiated through a number of pathways such as intrinsic (or mitochondrial) and extrinsic (death receptor) pathways (50), but ultimately involves the activation of cytoplasmic proteases called caspases that lead to DNA fragmentation, the hallmark of apoptosis (51).

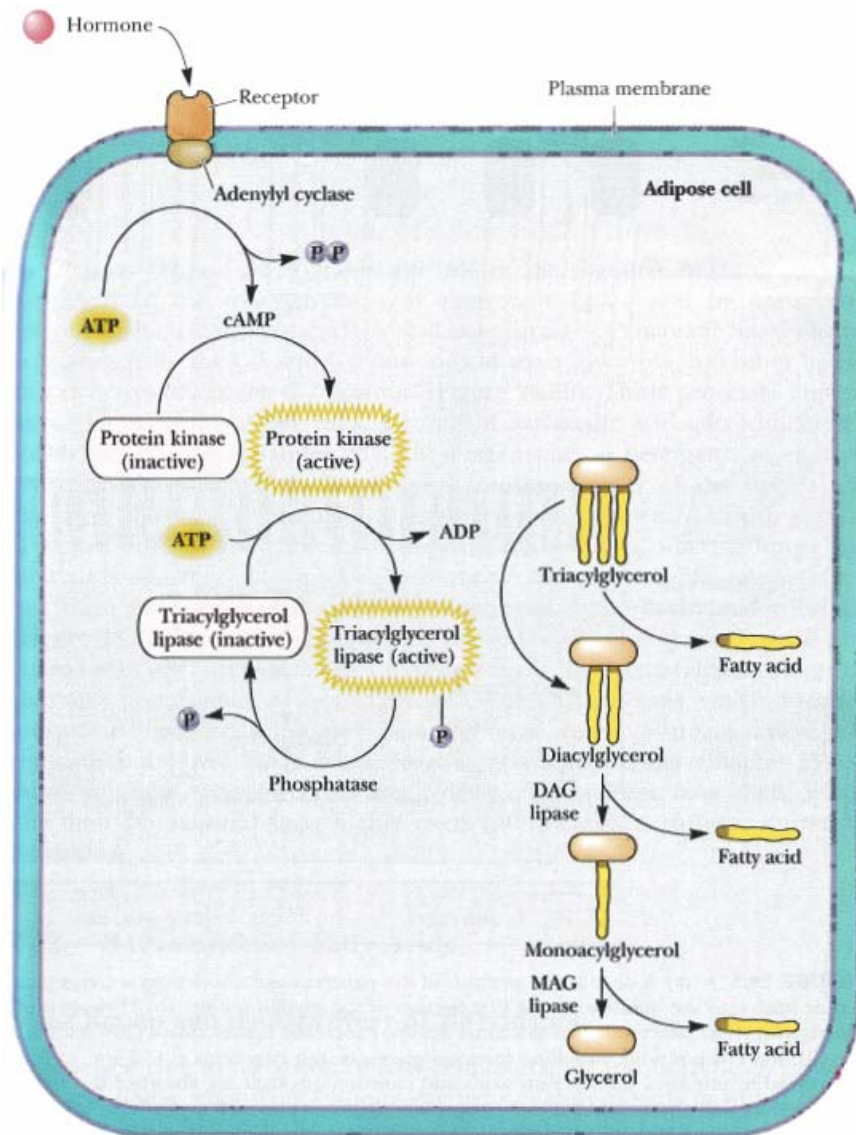


Figure 2.3. Release of fatty acids from triacylglycerol in adipose tissue is hormone-dependent (43)

Although apoptosis has been extensively studied in cancer cells, it has been recognized as a normal part of the adipocyte life cycle only in the past 15 years. Even though adipocytes had been thought to be an extremely stable cell type, Prins (6) contended that fat mass is balanced by cell acquisition and deletion, and that apoptosis plays an important role in the regulation of adipose tissue mass in humans. In addition, adipocyte apoptosis has been described in people with malignant tumors (5), people infected with the human immunodeficiency virus (52) and in diabetic rats (53). There might also be a site-specific difference in the susceptibility of human preadipocytes to apoptosis, with omental preadipocytes being more susceptible to apoptotic stimuli than subcutaneous preadipocytes (54).

Adipose tissue apoptosis induced by leptin was first described by Qian et al in 1998 (55). In his experiment, adipose tissue from leptin-treated rats demonstrated characteristic features of apoptosis as detected by gel electrophoresis, TUNEL *in situ* staining and histological sectioning. Apoptotic DNA fragmentation was not detected in other tissues and organs from the leptin-treated rats, i.e. muscle, brain, liver, kidney, lung, or heart, indicating that leptin-induced apoptosis is an adipocyte-specific response (unpublished). Della-Fera et al (56) have shown that continuous 13-day intraperitoneal infusion of leptin significantly increases adipocyte apoptosis in white adipose tissue in male and female mice, but only male mice developed reduced responsiveness to leptin-induced apoptosis after high-fat (45% fat) feeding for 5 or 15 weeks, thus extending the findings that leptin-induced apoptosis can occur with peripheral infusion in mice. The beta-2 adrenergic agonist, clenbuterol, increased adipose tissue apoptosis *in vivo*, suggesting that activation of adrenergic receptors on adipocytes may be involved in adipocyte apoptosis (57). Gullicksen et al (58) have shown that i.c.v. injection of 10 µg leptin for 4 days in Sprague Dawley rats reduced fat mass by both decreasing adipocyte volume and increasing

apoptosis in fat pads. Moreover, ob/ob mice are more sensitive to leptin-induced adipose tissue apoptosis than lean mice (11).

We have identified a number of agents which can induce adipocyte apoptosis in *in vitro* and *in vivo* studies. Ciliary neurotrophic factor (CNTF) injection centrally leads to adipose tissue apoptosis in rats (59,60). Dietary conjugated linoleic acid (CLA) (60) and genistein (61), have been demonstrated to induce adipocyte apoptosis in mice, while green tea polyphenol epigallocatechin gallate (EGCG) (62), ajoene (63), esculetin (64), and docosahexaenoic acid (65) have been shown to induce adipocyte apoptosis in 3T3-L1 adipocytes.

The cellular and molecular mechanisms of leptin-induced apoptosis are not fully understood. Qian et al (66) found that i.c.v. leptin treatment (5  $\mu$ g/day for 5 days) decreased TNF  $\alpha$  expression in adipose tissue by 40% only in young rats, whereas older rats were resistant to leptin's effects. The expression of peroxisome proliferation-activated receptor- $\gamma$  (PPAR- $\gamma$ ) in adipose tissue was increased by 70-80%, and the expression of uncoupling protein 2 (UCP2) was decreased in young rats, while increased in old rats (66). In addition, down-regulation of CCAAT/ enhancer binding proteins alpha, beta and delta (C/EBP  $\alpha$ ,  $\beta$ ,  $\delta$ ) in adipose tissue occurred in the adipose tissue of leptin-treated rats (67).

#### **Leptin and Adipose Tissue Vascularization**

The growth of both normal tissues and tumors is angiogenesis-dependent (68), and adipose tissue as a highly vascularized tissue is no exception (69). Angiogenesis inhibitors have been shown to decrease body fat in a variety of models of obesity (3). Rupnick et al (3) found that anti-angiogenic agents resulted in a dose-dependent, reversible weight reduction and adipose tissue loss from different obesity models of mice. In adipose tissue sections, vascular remodeling indicates that there is a decreased endothelial proliferation and increased apoptosis in mice treated with angiogenesis inhibitors compared with controls (3). In addition, the preferred



energy substrates shifted from carbohydrate to fatty acid (3). Angiogenic factors such as vascular endothelial growth factor (VEGF), VEGF-C, VEGF-D, soluble VEGF receptor-2 (sVEGFr2), angiopoietin-2, angiogenin and endostatin, are all elevated in overweight and obese individuals (70), indicating the possible link between the increased risk of metastasis rate in obese patients with cancers. Leptin induced the expression of angiopoietin-2 (Angpt-2), an angiogenesis inhibitor, in the adipose tissue in an autocrine manner in ob/ob mice (71). To be noted, this induction of Angpt-2 is associated with the initiation of apoptosis in the endothelial cells of adipose tissue, which is proposed to be one of the reasons leading to adipose tissue regression (71).

#### **2.4. Adipokines**

Since the discovery of leptin in 1994 (1), a wide range of protein factors and signals (over 50) termed adipokines, have been recognized as being released from white adipose tissue at various expression levels (Table 2.1) (72,73). These bioactive factors integrate with other tissues and organs such as the skeletal muscle, brain and sympathetic nervous system, taking part in energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure, lipid metabolism and homeostasis (Figure 2.4) (74). As a result, adipose tissue is now recognized as a major endocrine and secretory organ that acts both locally (paracrine) and distally (endocrine). Although white adipose tissue secretes a variety of factors, only leptin and adiponectin (and possibly resistin) are primarily produced by adipocytes.

#### **Adiponectin**

Adiponectin, (a.k.a. adipocyte complement-related protein (Acrp 30), ACDC) is a complex high molecular protein secreted almost exclusively from adipocytes, with the principal role of protecting against the development of insulin resistance. Adiponectin is an abundant protein

with a serum concentration between 5-10 ng/ml. In contrast to most adipocytokines and specifically leptin, the level of serum adiponectin is reduced in obese humans and rodents (75). The loss of body weight increases its expression, while re-feeding will return adiponectin to its previous low level (76). Furthermore, adiponectin has been shown to act in the brain to decrease body weight (77). Two adiponectin receptors (Adipo R1 and Adipo R2) have been recently identified (78) in muscles and in the liver. The beneficial effect of adiponectin on insulin resistance seems to be the promotion of fatty acid oxidation in muscle cells partly by the activation of 5'-AMP-activated protein kinase (AMPK) (78).

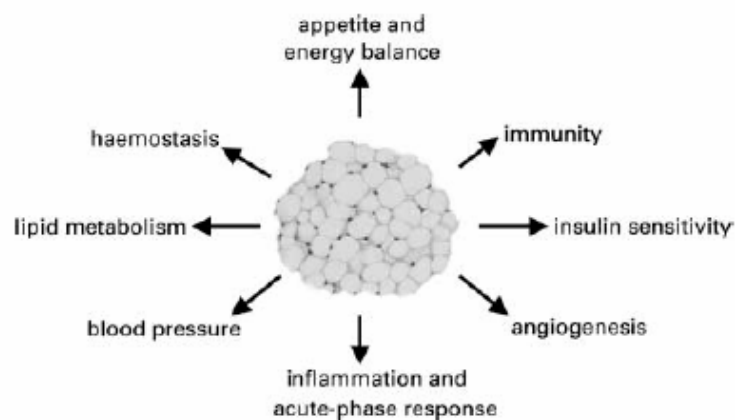


Figure 2.4. Functional groups of adipokines (74)

The expression of adiponectin mRNA was increased in leptin-treated ob/ob mice and 3T3-F442A adipocytes (79). A combination of physiological doses of adiponectin and leptin can completely reverse insulin resistance, while either adiponectin or leptin alone only partially reverse such resistance. In addition, in ob/ob mice transfected with the adiponectin gene, insulin resistance could be partly improved, but not obesity, indicating that adiponectin and leptin appeared to have both distinct and overlapping functions (80). Plasma adiponectin and cytokines

*in vivo* were inversely related, suggesting that interleukin (IL) 6 and TNF- $\alpha$  are potent inhibitors of adiponectin expression (81).

Table 2.1 Adipokines and their main effects (72)

Adipocytokines	Effects on
LPL	Lipid metabolism
HSL	Lipid metabolism
Perilipin	Lipid metabolism
aP2	Lipid metabolism
CETP	Lipid metabolism
RBP	Lipid metabolism
IL-6	Inflammation, atherosclerosis, insulin resistance
TNF- $\alpha$	Inflammation, atherosclerosis, insulin resistance
Adipsin/ASP	Immune-stress response
Metallothionein	Immune-stress response
Angiotensinogen	Vascular homeostasis
PAI-1	Vascular homeostasis
Adiponectin	Inflammation, atherosclerosis, insulin resistance
PPAR- $\gamma$	Lipid metabolism, inflammation, vascular homeostasis
CRP	Inflammation, atherosclerosis, insulin resistance
IGF-1	Lipid metabolism, insulin resistance
TGF- $\beta$	Cell adhesion and migration, growth and differentiation
Monobutylin	Vasodilation of the microvessel
Uncoupling proteins	Energy balance and thermoregulation
Steroid hormones	Lipid metabolism, insulin resistance
Leptin	Food intake, reproduction, angiogenesis, immunity
Resistin	Inflammation, insulin resistance
P450 arom	Lipid metabolism
Apelin	Insulin resistance
Visfatin	Insulin resistance
ZAG	Lipid metabolism, cancer cachexia

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### **Resistin (Retn)**

The name of resistin comes from its association with obesity-related insulin resistance (82). Three groups discovered resistin independently with different goals (82-84). Steppan's group identified resistin when screening potential targets of thiazolidinedione (TZD) in 3T3-L1 adipocytes (82). Kim's group identified resistin as a cytokine factor secreted from adipose tissue by micro-array analysis (83). The Holcomb group found resistin (or FIZZ1) as an expressed sequence tag associated with pulmonary inflammation (84).

The expression of resistin is elevated in ob/ob and db/db mice, as well as in the diet-induced model of diabetes and obesity (82). Resistin mRNA and proteins are down-regulated by antidiabetic TZDs (76) and insulin (77) in 3T3-L1 adipocytes, and is regulated by members of C/EBPs and PPARs family (85), suggesting that resistin expression occurs in response to insulin sensitizing agents. Moreover, resistin mRNA and proteins are both enhanced during 3T3-L1 adipogenesis (73, 74).

### **2.5. Conclusion**

Leptin mediates lipid metabolism by different routes. Its effects are exerted both indirectly through the central nervous system and directly on the peripheral tissues. Both pathways end up with similar outcomes: a decrease in triacylglycerol synthesis, an increase in lipid oxidation and adipocyte apoptosis, and recently, vascular inhibition in adipose tissue. Better understanding of the mechanisms that affect adipose tissue mass are important in developing better treatments for

obesity. Of all peptides studied that affect food intake and body energy balance, leptin is unique in that it acts in the brain to induce loss of adipose tissue by both apoptosis and increased lipid metabolism. Presently, a number of factors (86,87) have been identified that predispose or protect against leptin-induced adipose metabolism/apoptosis, but the complex interaction of these factors makes studies difficult to design and interpret. Therefore, in this project, we used advanced proteomics and real-time RT-PCR technologies to identify specific proteins/genes through which leptin exerts its effects on adipose tissue apoptosis and metabolism.

**CHAPTER 3**

**WHITE ADIPOSE TISSUE GENE EXPRESSION PROFILES IN OB/OB MICE**

**TREATED WITH LEPTIN BY REAL-TIME RT-PCR**

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## ABSTRACT

Leptin, a hormone secreted by adipocytes, has been shown to play a critical role in regulating body weight, lipid metabolism, apoptosis and microvasculature of adipose tissue. Ob/ob mice were treated with either PBS (control) or leptin (2.5 $\mu$ g/d or 10 $\mu$ g/d) for 14 days via subcutaneous (sc) osmotic minipumps. The total RNA from the inguinal (ING) fat pad was extracted, reverse transcribed to cDNA, followed by quantitative PCR (TaqMan®) assays using Low Density Arrays (LDA) via an ABI PRISM 7900 Sequence Detection System (SDS) in order to characterize the potential target genes of leptin related to lipid metabolism and energy expenditure. Eighteen genes were found to be differentially expressed between control ob/ob mice and leptin-treated ob/ob mice. mRNA expression for the leptin receptor, genes involved in lipolysis (hormone sensitive lipase (HSL)), genes related to energy expenditure (uncoupling protein 2 (UCP2), adrenergic receptor  $\beta$  3 (ADRB3), mitofusion 2 (MFN2), sirtuin 3 (SIRT3)), transcription factor sterol regulatory element binding factor 1 (SREBF1), genes related to apoptosis (Bcl-2, Bax, Caspase 3), cytokines (tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ), adiponectin, complement 3 (C3), as well as genes taking part in inhibition of angiogenesis (angiopoietin 2 (ANGPT2)) were increased. Expression of lipogenesis enzymes (stearoyl-coenzyme A desaturase 1 (SCD1), fatty acid synthase (FAS)), and retinol binding protein 4 (RBP4) were repressed. There were no changes in expression of transcription factors involved in adipocyte differentiation (C/EBP $\alpha$ , PPAR $\alpha$ , and PPAR $\gamma$ ). Several cellular mechanisms involved in leptin-mediated adipose tissue loss were identified in ob/ob mice, including the promotion of lipid oxidation and inhibition of lipid synthesis, increase in energy dissipation and apoptosis of adipose tissue. In addition, inhibition of angiogenesis in adipose tissue was another possible pathway by which leptin induced fat loss.

**Key words:** Apoptosis, Lipolysis, Lipogenesis, Angiogenesis



## 1. Introduction

Identification of the *ob* gene product, leptin, in 1994 (1), was a breakthrough for obesity research due to its prominent effects on fat metabolism and energy expenditure. For example, in an experiment conducted in 1995, leptin treatment specifically induced fat loss while maintaining lean body mass (30,32). Furthermore, leptin-treated *ob/ob* mice exhibited a greater decrease in body and fat weights than pair-fed *ob/ob* mice (33). Following reduced food intake, *ob/ob* mice treated with leptin maintained high energy expenditure, while food-restricted mice demonstrated a reduction in energy expenditure (31). The effect of leptin on body weight is mediated primarily through its receptors in the hypothalamus (19), through which it increases expression and triggers the release of anorexigenic peptides and decreases the expression and release of orexigenic molecules (7,8). Leptin receptors are also expressed in peripheral organs such as in adipose tissue, liver, and pancreas (89). Accumulating evidence (40,41) indicates that peripheral leptin administration regulates cellular lipid balance, promoting  $\beta$ -oxidation instead of lipid synthesis.

Leptin's effect on body weight, food intake and white adipose tissue adipocyte apoptosis (11) and loss of bone marrow adipocytes (90) was confirmed by our group. In addition, Duan (91) found that expression of hypothalamic neuropeptide Y (NPY), agouti-related protein (AGRP), and arginine vasopressin (AVP) mRNA were down-regulated, while suppressor of cytokine signaling 3 (SOCS3) was up-regulated (92) in the brain of leptin-treated *ob/ob* mice used for the present study. This suggests that the hypothalamus also takes part in the weight-reducing action of leptin. Both NPY and AGRP are neuropeptides produced in the brain that increase appetite and decrease metabolism and energy expenditure (93). SOCS3 appears to be of particular importance in the development of leptin resistance (94).

Leptin may induce fat loss in rodents in several ways, for instance via increased lipid metabolism, inhibition of adipocyte differentiation, increased mitochondrial proton leakage, and changes in fuel metabolism from carbohydrates to lipids. However, the underlying cellular and molecular mechanism of fat ablation induced by leptin is not well understood. The present study takes advantage of medium-throughput real-time RT-PCR techniques and TaqMan<sup>®</sup> low-density arrays (TLDA) (24-gene format), to delineate the multiple gene clusters simultaneously affected by leptin in ob/ob mice. These findings will help us better understand leptin's target genes and signaling pathways during weight loss.

## **2. Materials and Methods**

### **2.1. Animals**

All experimental and surgical procedures in this study were approved by the Animal Care and Use Committee of The University of Georgia. Fifteen-week-old female obese ob/ob mice (strain B6.V-Lepob, stock no. 000632) (n=12 for each group) were obtained from Jackson Laboratories, Inc. After the mice were acclimated for about one week, they were surgically implanted with osmotic minipumps (model 1002, 0.25  $\mu$ L/h; Alzet Corp., Cupertino, CA) for s.c. infusion of treatment solutions. Leptin was administered at doses of 0 (control), 2.5  $\mu$ g/d and 10  $\mu$ g/d for 14 consecutive days. Inguinal (ING) fat pads were collected, weighed, and then frozen in liquid nitrogen prior to being stored at -80°C. The details of animal studies are further described in Della-Fera et al (11).

### **2.2. RNA extraction**

Total RNA was isolated from the ING fat pads of ob/ob mice, using Trizol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. One hundred mg of frozen adipose tissue from each sample was homogenized by adding 1 ml of Trizol reagent. The

samples were centrifuged at  $12,000 \times g$  for 15 min at  $4^\circ C$  and the aqueous phase was removed and mixed with 0.5 ml of isopropyl alcohol. After centrifugation at  $12,000 \times g$  for 10 min at  $4^\circ$ , the pellet was washed with 1 ml of 75% ethanol and then dissolved in 12  $\mu$ l RNase -free water. 1.0  $\mu$ l of the 12  $\mu$ l sample was used as an integrity check and for quantification by the Agilent 2100 bioanalyzer and RNA 6000 Nano Assay (Agilent Technologies, Foster City, CA).

### **2.3. Reverse Transcription and Real-Time RT-PCR**

All reagents were from Applied Biosystems (ABI) (Foster City, CA). Five hundred nanograms of total RNA in a 20  $\mu$ l reaction was reverse transcribed using a cDNA archive kit (Applied Biosystems Inc., CA, part No.4322171) in accordance with the manufacturer's protocols. Three  $\mu$ l cDNA was mixed well with 47  $\mu$ l water, and 50  $\mu$ l TaqMan<sup>®</sup> Universal PCR Master Mix, and used for PCR reactions. Reactions were incubated initially at  $25^\circ C$  for 10 min and subsequently at  $37^\circ C$  for 120 min. Quantitative PCR (Taqman<sup>®</sup>) assays were performed using 384-well Low Density Arrays on an ABI PRISM 7900 Sequence Detection System. All of the oligonucleotide primers and fluorogenic probe for TaqMan<sup>®</sup> real time PCR were prepared by ABI (Table 3.1). The cycle conditions were:  $94.5^\circ C$  for 15s, followed by 40 cycles of  $97^\circ C$  for 30 s, and  $59.7^\circ C$  for 1 min. Through comparing and analyzing the variability of two housekeeping genes (HKG) (18s and glyceraldehyde-3-phosphate dehydrogenase (GADPH)) from the amplification plot shown in the SDS software, the expression of a target gene was normalized by using GADPH as an endogenous control reference gene, and sample number of 2038 as a calibrator to correct the differences in the amount of total RNA added to each reaction.

### **2.4. Quantification of RT-PCR**

We used a relative quantification method (also known as the  $\Delta\Delta Ct$  method) to calculate the gene expression values as described (User Bulletin No. 2, Applied Biosystems). In brief, the

amplification plot is the plot of fluorescence versus PCR number. The threshold cycle value (Ct) is the fractional PCR cycle number at which the fluorescent signal was accumulating enough to reach detection threshold. Therefore, the input cDNA copy number and Ct are inversely related. Data were analyzed with the Sequence Detector System (SDS) software version 2.1 (ABI) and Ct value is automatically converted to fold change RQ value. The Fold change (RQ) =  $2^{-\Delta\Delta CT}$ , where  $-\Delta\Delta CT = -(\Delta CT_{\text{trt}} - \Delta CT_{\text{control}}) = -[(Ct_{\text{Target}} - Ct_{\text{GADPH}})_{\text{trt}} - (Ct_{\text{Target}} - Ct_{\text{GADPH}})_{\text{control}}]$ . The RQ values from each gene were then used to compare the adipocyte gene expression across all groups (control, 2.5  $\mu\text{g/d}$  and 10  $\mu\text{g/d}$  leptin).

## 2.5. Statistical Analysis

All statistical analyses were conducted with SAS (Version 9.0) (SAS institute, Cary, NC). The whole procedure and SAS codes are in the appendix. The Ct value can be automatically transferred to the RQ value by the software sequence detection system (SDS) (Applied Biosystems). In brief, RQ values in all samples were submitted to logarithm transformation at the base of 2 in order to create a linear range between fluorescent signal versus cycle number (95). The transformed RQ value was then winsorized for dealing with the outliers (96). Statistical significance of treatment ( $p < 0.05$ ) was assessed by a general linear model based on the transformed and winsorized data (tRQ) derived from the original RQ value. The tRQ values were expressed as means  $\pm$  standard error for all genes. Least square means was used to determine significance of differences among means ( $p < 0.05$ ). The correlation between body weight and leptin mRNA expression was examined by means of Spearman's correlation coefficients.

### **3. Results**

#### **3.1. Effect of S.C. Leptin Injection on Leptin and Leptin Receptor mRNA Expression in Adipose Tissue of ob/ob Mice**

Expression of leptin and leptin receptor mRNA in adipose tissue were measured by real time RT-PCR. In the 10 µg/d leptin treated group, leptin mRNA was only 27% of the control group (Figure 3.1A) ( $P<0.05$ ). In contrast, expression of leptin receptor was 6.9- and 5.4-fold higher in the 10 µg/d and 2.5 µg/d leptin treatment groups, respectively, compared to the control group (Figure 3.1A) ( $P<0.05$ ). In spite of the suppression of leptin expression by administration of exogenous leptin, there was still a strong correlation between leptin mRNA and body weight ( $R=0.76$ ,  $p<0.05$ ; Figure 3.1B).

#### **3.2. Energy Expenditure Genes (UCP1, UCP2, UCP3, ADR3, MFN2, and SIRT3) mRNA in Adipose Tissue of ob/ob Mice**

Next we examined the expression of several genes related to energy expenditure. Leptin treatment increased the gene expression of UCP2 and adrenergic receptor  $\beta$  3 (ADRB3) in a dose-dependent manner ( $p<0.05$ ) (Figure 3.2A). 10 µg/d leptin and 2.5 µg/d leptin increased the mRNA expression of UCP2 by 5.85-fold and 1.63-fold, respectively, compared to the control group ( $p<0.05$ ). Similarly, the mRNA level of ADRB3 was increased 4.72-fold ( $p<0.05$ ) and 1.62-fold by 10 µg/d leptin and 2.5 µg/d leptin, respectively, compared to the control group ( $p<0.05$ ). However, leptin injections had no effect on the mRNA expression of UCP1 and UCP3. Leptin treatment also increased mRNA expression of another two genes related to mitochondrial energy metabolism: Mitofusion-2 (MFN2) ( $p<0.05$ ), which is involved in mitochondrial fusion, and sirtuin-3 (SIRT3), which is responsible for increased thermogenesis (97) (Figure 3.2B) ( $p<0.05$ ).

### **3.3. Expression of Key Enzyme Genes (HSL, SCD1, and FAS) in Adipose Tissue of ob/ob Mice**

To further investigate the effects of leptin on adipose metabolism, we examined the mRNA expression of some key enzymes involved in lipid metabolism. Leptin tended to increase the mRNA expression of lipolytic enzymes and decrease the mRNA expression of lipid synthesis enzymes (Figure 3.3). The mRNA levels of hormone sensitive lipase (HSL) in leptin-treated groups were 3.4- and 2.2-fold higher compared to the control group ( $p < 0.05$ ) in adipose tissue. In contrast, 10  $\mu\text{g/d}$  leptin decreased the mRNA level of the lipid synthesis enzyme - stearoyl coenzyme A desaturase 1 (SCD1) ( $p < 0.05$ ), while both doses of leptin decreased the mRNA level of fatty acid synthase (FAS) ( $p < 0.05$ ).

### **3.4. Expression of Genes -Glucose transporter (GLUT 4) and RBP-4 in Adipose Tissue of ob/ob Mice**

There were no mRNA changes in GLUT4 (also known as Slc2a4), a major glucose transporter in muscle and adipose cells (Figure 3.4). The mRNA level of RBP4, a mediator associated with decreasing GLUT 4 transporter and impairing insulin action, was reduced significantly by 10  $\mu\text{g/d}$  leptin in ob/ob mice ( $p < 0.05$ ).

### **3.5. Expression of Adipogenesis-Related Genes- C/EBP $\alpha$ , PPAR- $\alpha$ , PPAR- $\gamma$ , SREBF-1 in Adipose Tissue of ob/ob Mice**

Next, we examined the mRNA expression of transcription factors related to adipogenesis. No mRNA changes were observed for the transcription factors CCAAT/enhancer binding protein alpha (C/EBP $\alpha$ ), peroxisome proliferator activated receptor alpha (PPAR $\alpha$ ) or gamma (PPAR $\gamma$ ) (Figure 3.5). However, leptin increased the expression of sterol regulatory element binding factor 1 (also known as SREBF-1 or SREBP-1) (Figure 3.5).

### **3.6. Expression of Apoptotic Markers Related Genes - Bcl-2, Bax and Caspase-3 in Adipose Tissue of ob/ob Mice**

The mRNA expression of Bcl-2, Bax and caspase-3 was increased by leptin in ob/ob mice ( $p < 0.05$ ) (Figure 3.6).

### **3.7. Expression of Cytokines (TNF $\alpha$ , Adiponectin, Complement 3) in Adipose Tissue of ob/ob Mice**

The mRNA level of TNF $\alpha$ , an adipose tissue cytokine which takes part in apoptosis and the lipolytic process, was increased significantly by 10  $\mu\text{g/d}$  leptin (Figure 3.7) ( $p < 0.05$ ). The mRNA expression of adiponectin (also known as ACDC), a cytokine involved in glucose and lipid metabolism, was also increased by 10  $\mu\text{g/d}$  leptin ( $p < 0.05$ ). The mRNA level of complement 3, a cytokine participating in increased insulin sensitivity, was increased by leptin ( $p < 0.05$ ).

### **3.8. Angiopoietin-2 (ANGPT2) mRNA in Adipose Tissue of ob/ob Mice**

Leptin treatment of ob/ob mice also increased mRNA level of angiopoietin-2, an inhibitor of angiogenesis in adipose tissue ( $p < 0.05$ ) (Figure 3.8).

## **4. Discussion**

Obese ob/ob mice are characterized by adipose tissue hyperplasia, hypothermia, morbid obesity, insulin resistance, and multiple endocrine abnormalities due to the absence of leptin (32). Leptin, as a signal of adiposity, has been widely studied in the regulation of body weight, food intake and body temperature. Consequently, this strain of mouse has been used as a classical model to study obesity and diabetes mellitus. In the present study, s.c. infusion of leptin normalized the phenotype of ob/ob mice, causing a decrease in food intake, body weight and fat pad weights (11). These results are consistent with those of previous studies utilizing i.c.v.

administration of leptin (56). In addition, the mass of brown adipose tissue (BAT) and gastrocnemius (GC) muscle were both significantly increased in leptin-treated groups (Figure 3.9). Furthermore, leptin-treated ob/ob mice exhibited a greater decrease in body and fat weights than pair-fed ob/ob mice (33). This suggests that there are mechanisms other than decreased food intake that are involved in leptin-mediated decrease in body weight; for example, increased energy expenditure.

There are several different ways to increase energy expenditure, including increased core body temperature, increased physical activity, and increased non-shivering thermo-genesis mediated by BAT and GC muscle, which can play an important role in energy expenditure in rodents. In addition, leptin treatment was also shown to favor the use of lipid rather than carbohydrate as the energy substrate, as evidenced by the decrease in respiratory quotient of ob/ob mice treated with leptin (98).

Leptin administration also increased expression of the UCP2 transcript ( $p < 0.05$ ) in adipose tissue. The role of UCP2 and UCP3 is still not as clear as that of its homologue UCP1, which takes part in disrupting the proton gradient in the mitochondria, releasing stored energy as heat without producing ATP. However, an involvement of UCP2 and UCP3 in transporting fatty acids has been hypothesized (99), and it is assumed that the up-regulation of this protein following leptin treatment would increase fatty acid transport across the mitochondrial membrane and enhance lipid  $\beta$ -oxidation. Moreover, leptin only induced the expression of UCP2 in adipose tissue, with no change in level of UCP1 and UCP3 in the present study, which is in agreement with other studies done in normal rats (4,100). One possible explanation is that each subtype of the uncoupling proteins exists in different tissues, UCP 1 is primarily located in BAT, UCP2 is widely expressed in white adipose tissue, heart and muscle; and UCP3 is



expressed mainly in BAT and skeletal muscle (101,102). In the present study, we only measured the expression of UCP homologues in the adipose tissue, but it would be useful to also know their levels in BAT.

The three known subtypes of  $\beta$ -adrenoreceptors ( $\beta$  (1)-AR,  $\beta$  (2)-AR, and  $\beta$  (3)-AR) are differentially expressed in brown and white adipose tissue, and mediate peripheral responses to central modulation of sympathetic outflow by leptin.  $\beta$ -3-AR expression was higher in the ING fat pad of leptin-treated groups in the present study. This subtype of  $\beta$ -adrenoreceptor is a critical signal mediating peripheral responses of leptin to activate sympathetic nervous system (SNS) output from the hypothalamus (103) affecting body temperature and metabolic rate, primarily via BAT. It is also required for leptin-mediated regulation of ob mRNA expression in adipose tissue (104). Therefore, there may be increased numbers of adrenergic receptors in adipose tissue of leptin treated mice (104).  $\beta$ 3-receptors probably mediate the increase in thermogenesis and lipolysis that occurs with adrenergic stimulation.  $\beta$ 3-receptor activation also suppresses leptin expression and secretion (105).

The expression of mitofusion 2 (MFN2) was also increased in the leptin-treated groups ( $p < 0.05$ ). Mitofusion 2, a mitochondria membrane protein of large GTPase, is essential for mitochondrial fusion. Mitochondria are dynamic organelles, and both fission and fusion processes are normal events for mitochondria to regulate apoptosis and production of energy. Its homologue is well studied in *Drosophila fuzzy onion (Fzo)* (106) and yeast (*Fzo 1*) (107), although much less is known about the role of mitofusion 2 in the process of regulating mitochondria morphology in mammals. The activity of MFN 2 was reduced in obesity, and increased following surgery for reducing body weight (108). It is also hypothesized that pro-

fusion factors make cells resistant to apoptosis, while pro-fission factors make cells sensitive to apoptosis (109,110), but this still needs further research.

Sirtuin3 expression was also increased by leptin. Sirtuin3 is the sirtuin homolog of the yeast *sir 2* gene, which is critical for the effect of caloric restriction on extending life span in yeast and *C. elegans*. Sirt3 can be expressed in both white and brown adipose tissues, and it not only promotes the activity of UCP1 and augments thermogenesis, but also increases oxygen consumption (97). Moreover, SIRT3 expression in brown adipose tissue is down-regulated in several strains of genetically obese mice (25). Our finding that leptin treatment increased expression of Sirt3 is consistent with the increased thermogenesis and fat loss that we demonstrated in the *ob/ob* mice treated with leptin (11).

The coordinated regulation between lipid synthesis and oxidation, adipocyte differentiation and de-differentiation determines fat mass. The two opposing biochemical processes, lipolysis and lipid synthesis, are controlled by different enzymes and hormones. Leptin can act as an autocrine or paracrine signal to change the rate of synthesis and degradation of lipids (42,111). Therefore, we hypothesized that leptin regulates gene expression of key enzymes related to lipid metabolism. As expected, the level of expression of hormone sensitive lipase (HSL) (Figure 3) in leptin-treated groups was increased ( $p < 0.05$ ). HSL is the rate-limiting enzyme responsible for the intracellular TG hydrolysis and fatty acid mobilization in adipose tissue. Leptin decreased expression of the lipogenesis enzymes SCD1 and FAS (Figure 3.3) ( $p < 0.05$ ). SCD1 is a lipogenic enzyme catalyzing the synthesis of monounsaturated fatty acids (MUFAs), which are important components of phospholipids, triglycerides and cholesteryl esters, from saturated fatty acids (112). Knockout SCD-1 mice have reduced body fat content and are resistant to diet-induced obesity (113). Mice with disruption of both SCD-1 and *ob/ob* genes are less obese than

ob/ob control mice, and have reduced serum triacylglycerols and very low density lipoproteins in liver (114). Hepatic steatosis and hyperinsulinemia have been improved dramatically in lipoatrophic mice treated with i.c.v. leptin injection, an effect which was due to suppression of SCD1 expression and activity (115). It is proposed that mice lacking SCD-1 have increased energy expenditure and increased oxygen consumption. All findings indicate that down-regulation of SCD-1 is an important component of the metabolic response to leptin. Fatty acid synthase (FAS) is a unique enzyme involved in catalyzing de novo lipid synthesis in the cytosol by converting acetyl-CoA to the long-chain fatty acids palmitate (C16), which is further synthesized into triacylglycerols, and then stored in adipose tissue (116). Our finding of reduced FAS expression in leptin-treated ob/ob mice is consistent with their reduced body weight and fat loss (11).

Retinol binding protein 4 (RBP4) was recently identified as a cytokine from adipose tissue by DNA array analysis (117), and it is associated with decreased activity of the GLUT4 glucose transporter, leading to impaired insulin sensitivity. It was also demonstrated that RBP4 level in adipose tissue was increased in knockout  $Glut^{-/-}$  mice (117). The serum RBP4 levels were elevated in mice and humans with insulin resistance (118), and decreased by rosiglitazone, a drug used for improving insulin sensitivity. The mechanism by which RBP4 affects insulin action and glucose metabolism is not clearly understood. It was shown that RBP4 reduced activity of PI-3 kinase for phosphorylation of insulin receptor substrate-1 and phosphoenol pyruvate carboxykinase (PEPCK) for gluconeogenesis in liver (117). In the present study, leptin reduced the RBP4 level in adipose tissue, which is consistent with leptin-induced enhanced insulin sensitivity in ob/ob mice. It is also consistent with the present results that hyperinsulinemia was partly corrected by leptin (11).

In addition to regulating lipid metabolism, leptin is believed to reduce adipose mass by inducing adipocyte apoptosis. Previous studies showed that leptin treatment caused a rapid loss of fat in rodents, and that body weight remained low even after leptin treatment was terminated (119). Qian, et al., hypothesized that this delayed recovery was due to apoptosis of adipose tissue. In agreement with previous data (56), leptin induced apoptosis in all three fat depots in the present study (11). The genes related to apoptosis (Bcl-2, Bax, and Caspase 3) were correspondingly modified at the level of mRNA (Figure 3.6). The intrinsic pathway of apoptosis is mainly determined by the balance of pro- (Bax, Bak, Bok) and anti-apoptotic (Bcl-2, Bcl-XL) members of Bcl-2 family in the mitochondria (120). Usually, Bax promotes apoptotic death and Bcl2 protein inhibits apoptosis by affecting the electron transport chain and cytochrome c release into the cytoplasm (121,122). To be noted, the ratio of Bcl-2/Bax appears to regulate mitochondrial membrane permeability and therefore modify the susceptibility to apoptosis following an apoptotic stimulus (120). Furthermore, leptin-induced apoptosis was partly mediated by the up-regulation of Bax in adipose tissue (59) and these two proteins, bcl-2 and bax, also took part in the apoptosis observed in BAT (122).

The caspase family of cysteine proteases are primary mediators of apoptosis (120). They are triggered in response to various apoptotic stimuli and mediate proteolytic cleavage of cellular substrates. Caspase-3 is thought of as an effector or executioner caspase and is a crucial mediator for mitochondrial apoptosis (123). Caspase-3 may take part in the process of leptin-induced apoptotic pathways in adipose tissue in rats (124), and inhibition of caspases protects BAT from apoptosis (125).

In the present study, the expression of Bax and caspase-3 were increased significantly by leptin treatments, which is consistent with previous studies (59,124), indicating they may take

part in the apoptosis pathway. However, no change was observed for the ratio of Bcl-2/Bax (data not shown). Considering the role of Bcl-2 as an anti-apoptotic signal, its increased level seems contradictory to the increased apoptosis in adipose tissue observed in the present study and, thus, further study will be required to confirm this observation.

In contrast to leptin, insulin has a lipogenic effect on cultured adipocytes (45). Leptin inhibits the binding of insulin to adipocytes (126) (127), and the lipogenic effect of insulin in skeletal muscle was decreased by leptin treatment (128). In the present study, the reduced level of insulin and its corresponding lipogenic effect may have contributed to the fat loss (11).

There were no leptin-induced changes in expression of C/EBP $\alpha$ , PPAR $\alpha$ , and PPAR $\gamma$ , transcription factors critical for adipogenic regulation, in the current study. PPAR $\alpha$  and  $\gamma$  are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. PPAR $\alpha$  regulates the genes related to fatty acid  $\beta$ -oxidation in liver, whereas PPAR  $\gamma$  promotes adipocyte differentiation and lipid storage preferentially residing in adipose tissue. The expression of PPAR $\gamma$  in normal rats treated with leptin was reported to be up-regulated (129), which may be due to different species genetic background and/or experimental methods, and it need to be further studied. Interestingly, mutant mice with both PPAR $\gamma$  and ob dysfunctional genes had a normal phenotype (130).

There are few studies in the literature about the effect of leptin on the expression of PPAR $\alpha$ , and it was reported (131) that it was up-regulated in myoblasts by leptin. PPAR $\alpha$ -null mice are protected from high-fat diet-induced insulin resistance (132). C/EBP $\alpha$  is another transcription factor which is expressed during late phase of adipogenesis (133). Reports about the effect of leptin on C/EBP $\alpha$  expression are not consistent; one study showed it was down-regulated (134), while another study demonstrated no change in expression by leptin (135).

Sterol regulatory element binding factor 1 (SREBF-1) has been shown to regulate several key genes related to lipid metabolism, particularly FAS in cultured adipocytes and ob mice (136,137). In the present study, leptin increased SREBF-1 expression, which is contrary to a microarray study which showed that leptin decreased SREBF-1 expression at the RNA and protein level in white adipose tissue (138). Recent evidence also shows that SREBF-1 regulated lipid metabolism in the liver and is not involved in obesity in ob/ob mice (139). Further studies are needed to define the relationship between SREBF-1 and lipid metabolism with leptin treatment.

Adipocyte leptin production and secretion are probably maintained through direct autocrine and/or paracrine negative feedback signals (140), as well as regulation by cytokines, such as TNF $\alpha$  (141) and adiponectin (74,142). Expression of both TNF $\alpha$  and adiponectin were increased in the leptin-treated groups in this study. TNF $\alpha$  is a cytokine that acts as a lipolytic factor (143), stimulating lipolysis and inducing apoptosis in adipose tissue (67,144), stimulating VLDL production in the liver, and regulating other adipokines. TNF $\alpha$  promotes lipolysis by inhibiting lipoprotein lipase activity in vivo (145), inhibiting food intake, and promoting insulin resistance. ACDC (adiponectin), is secreted specifically by differentiated adipocytes, and like leptin, it is an abundant adipocytokine primarily produced from subcutaneous fat. Adiponectin has been implicated in improving insulin sensitivity. In contrast to most adipocytokines, and specifically leptin, the level of serum adiponectin is reduced in obese humans and rodents (76), and loss of body weight increases its expression, while refeeding will return adiponectin to its previous low level (77). In the present study, leptin treatment increased the expression of adiponectin in white adipose tissue in ob/ob mice, which is consistent with the study of Delporte et al (80).

There are several potential mechanisms that could explain why leptin treatment up-regulated adiponectin mRNA expression in adipose tissue in ob/ob mice. First, the reduced body weight, especially the reduced fat depots per se, is a critical factor to determine the expression level of adiponectin. Fat mass is usually negatively correlated with the expression of adiponectin (77). Second, enhanced sympathetic nervous activity could be a potential factor. The sympathetic nervous system is a primary pathway through which leptin regulates adipose tissue metabolism (104). The increased weight of BAT and GC muscle, together with the up-regulated expression of ADRB3 and UCP2 are good indicators of enhanced sympathetic tone. It was shown that acute exposure to  $\beta$ -adrenergic agonists decreased adiponectin mRNA levels (146). Third, the reduced hyperinsulinemia or improved insulin sensitivity might play a role in up-regulating adiponectin mRNA level.(147).

C3 was traditionally assumed to be synthesized by the liver (148),, but it is also produced by human adipocytes (149). Acylation-stimulating protein (ASP), a cleavage product of C3, can act as a paracrine signal to be a potent stimulator of triglyceride synthesis (150), and also participates in energy repartitioning (151,152). Plasma C3 was shown to be increased by high fat diet feeding (153), TNF $\alpha$  (154), and in obese animals (155); whereas it was decreased in humans after weight loss. The increased expression of C3 by leptin treatment would tend to increase triglyceride synthesis and decrease energy expenditure, which was contrast to fat loss effect of leptin in the present study. Therefore, further study is needed on the role of C3 in adipose tissue of ob/ob mice.

In addition to the contributions of increasing energy expenditure, lipid oxidation, and adipose cell apoptosis, the increased fat loss caused by leptin treatment could be due to the degeneration of blood vessels in the adipose tissue (72,156). The growth of both normal tissues

and tumors is angiogenesis-dependent, and adipose tissue is no exception (70). Angiogenesis inhibitors have been shown to decrease body fat in a variety of models of obesity (54). ANG-2 was expressed primarily by adipose endothelial cells as a receptor antagonist, taking part in vascular remodeling. Cohen (72,114) showed that the induction of ANG-2 coincided with initiation of apoptosis in endothelial cells in adipose tissue, leading to regression of adipose tissue. In our present study, leptin was shown to induce ANG-2 expression in adipose tissue, suggesting a possible vasculature regression effect. However, other studies reported no mRNA change in ANG2 expression in ob/ob mice following leptin treatment (157). This could be due to differences in age of the ob/ob mice and difference in the dose and length of leptin treatment.

In summary, peripheral leptin is able to increase lipid metabolism via many different routes, including a decrease in lipid synthesis partly mediated by the decreased expression of SCD1 and FAS, and an increase in lipid oxidation partly mediated by the increased expression of HSL. Furthermore, the ability of leptin to reduce lipogenesis can be mediated by insulin, TNF $\alpha$  and adiponectin. Adipocyte apoptosis mediated by the cytokine TNF $\alpha$ , by caspase-3, and mitofusion 2 is another possible way by which leptin may act to reduce adipose tissue mass. Repressed angiogenesis in adipose tissue via increased ANG-2 expression is also a possible mechanism to enhance the capacity of leptin to regulate fat metabolism. In addition, the reduced food intake (energy input) and the increased energy expenditure through increased physical activity, higher core body temperature, and nonshivering thermogenesis all contributed to leptin's weight-loss effects. The increased expression of  $\beta$ -3AR and Sirturin 3, resulting from increased sympathetic tone, play a role in the increased thermogenesis induced by leptin acting via its hypothalamic receptors.



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This work was supported in part by the Georgia Research Alliance Eminent Scholar endowment held by CAB.

Table 3.1. Probes used in real-time PCR I

Gene Symbol	Gene Name	Context Sequence
18S	Eukaryotic 18S rRNA	CCATTGGAGGGCAAGTCTGGTGCCA
ACDC	Adipocyte, C1Q and collagen domain containing	AAGGAGATGCAGGTCTTCTTGGTCC
ADRB3	Adrenergic receptor, beta 3	TCCACCGCTCAACAGTTCCTTCTT
ANGPT2	Angiopoietin 2	ACCCAACCTCCAAGAGCTCGGTTGCT
CACH	Cytosolic acetyl-CoA hydrolase	AGCAGGTTTCAGCGATTCCATTTGTA
CEBP $\alpha$	CCAAT/enhancer binding protein (C/EBP), alpha	AGCCACCGCCGCCACCGCCACCGCC
FAS	Fatty acid synthase	GTGGATGGAGGTATCAACCCAGCCT
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GTGAACGGATTTGGCCGTATTGGGC
GPD1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	TGCCCCCAATGTGGTGGCCATCCC
LEP	Leptin	TTTCACACACGCAGTCGGTATCCGC
LEPR	Leptin receptor	AGCTGAGGTATCACAGGCGCAGCCT
HSL	Lipase, hormone sensitive	GCCCACAGCAGAGTCTGTGCGCCCC
LPL	Lipoprotein lipase	ATCCATGGATGGACGGTAACGGGAA
MFN2	Mitofusin 2	TTTTTTGGCCGGACGAGCAATGGGA
PPAR $\alpha$	Peroxisome proliferator activated receptor alpha	TGTGGAGATCGGCCTGGCCTTCTAA
PPAR $\gamma$	Peroxisome proliferator activated receptor gamma	AGTGGAGACCGCCCAGGCTTGCTGA
SCD1	Stearoyl-Coenzyme A desaturase 1	CACCTGCCTCTTCGGGATTTTCTAC
SIRT3	Sirtuin 3 (silent mating type information regulation 2, homolog) 3 ( <i>S. cerevisiae</i> )	AGGGGAAGACATATGGGCTGATGTG
GLUT4	Solute carrier family 2 (facilitated glucose transporter), member 4	TGGCTCTGCTGCTGCTGGAACGGGT
SREBF1	Sterol regulatory element binding factor 1	ACATCGAAGACATGCTCCAGCTCAT
TNF $\alpha$	Tumor necrosis factor	CCCAAAGGGATGAGAAGTTCCCAAA
UCP1	Uncoupling protein 1, mitochondrial	AAAGTCCGCCTTCAGATCCAAGGTG
UCP2	Uncoupling protein 2	CTCTGGAAAGGGACTTCTCCCAATG
UCP3	Uncoupling protein 3, mitochondrial	GGTCCGTCTGCAGATCCAAGGGGAG
Bcl-2	B-cell leukemia/lymphoma 2	GATAACGGAGGCTGGGATGCCTTTG
Bax	Bcl2-associated X protein	CTGGTGCTCAAGGCCCTGTGCACTA

Gene Symbol	Gene Name	Context Sequence
Casp3	Caspase 3	TACAGCACCTGGTTACTATTCCTGG
C3	Complement component 3	TGAAGGTCGTGCCAGAAGGAATGAG
RBP-4	Retinol binding protein 4, plasma	ACACAATGGTTACTGTCAAAGCAGG

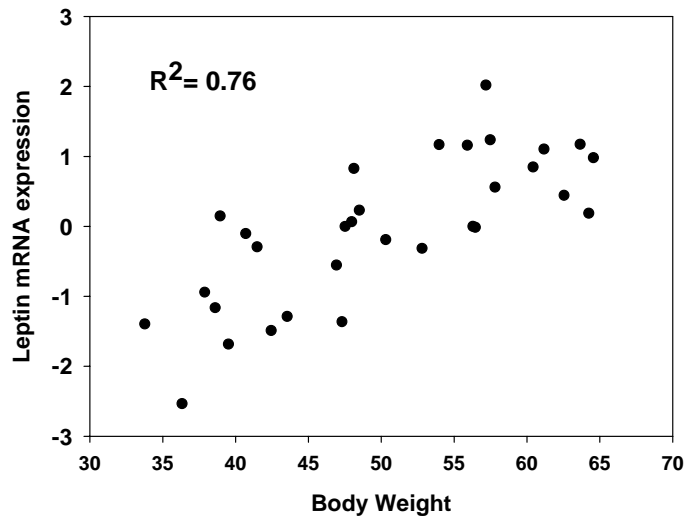
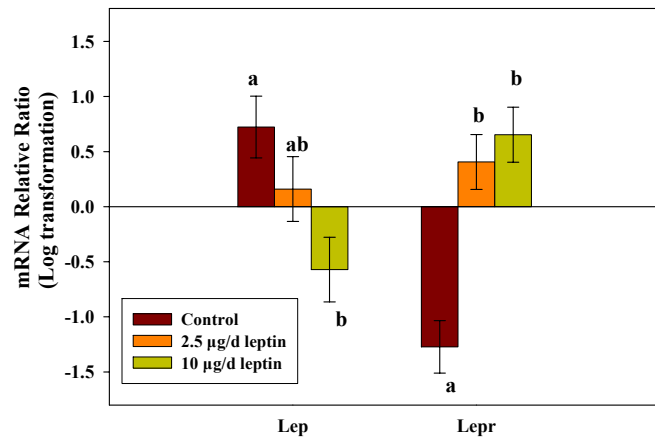


Figure 3.1. Effects of 14-day sc infusion of leptin on gene expression in adipose tissue from ob/ob mice (mean  $\pm$  S.E.M.). Total RNA extracted from inguinal adipose tissue was used for gene expression analysis of leptin and leptin receptor (Figure 3.1A). The correlation between body weight and leptin mRNA expression was examined by means of Spearman's correlation coefficients (Figure 3.1B). The Y-axis is the RQ value after log 2 based transformation. Note: Means without a common letter within each gene are different,  $p < 0.05$ . The replications for control, 2.5µg/d and 10µg/d leptin group are 11, 10 and 10, respectively.

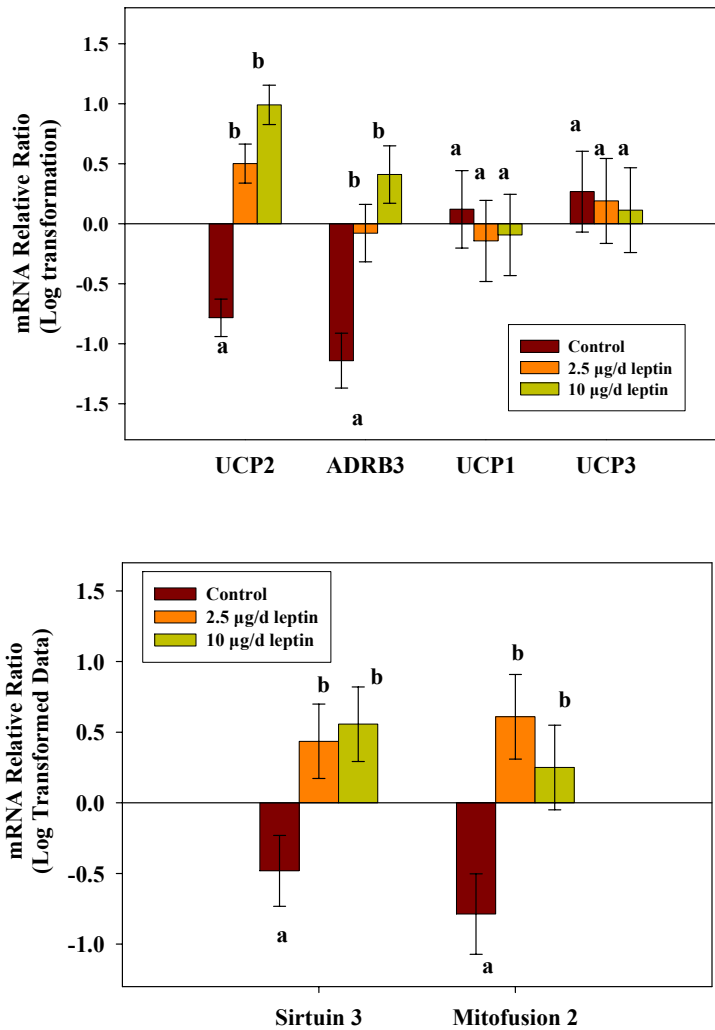


Figure 3.2. Effects of 14-day sc infusion of leptin on gene expression in adipose tissue from ob/ob mice (mean  $\pm$  S.E.M.). Total RNA extracted from inguinal adipose tissue was used for gene expression analysis of UCP1, 2, 3 and adrenergic receptor (ADRB3) (Figure 3.2A) and Sirtuin 3 and Mitofusion 2 (Figure 3.2B). The Y-axis is the RQ value after log 2 based transformation. Note: Means without a common letter within each gene are different,  $p < 0.05$ . The replications for control, 2.5µg/d and 10µg/d leptin group are 11, 10 and 10, respectively.

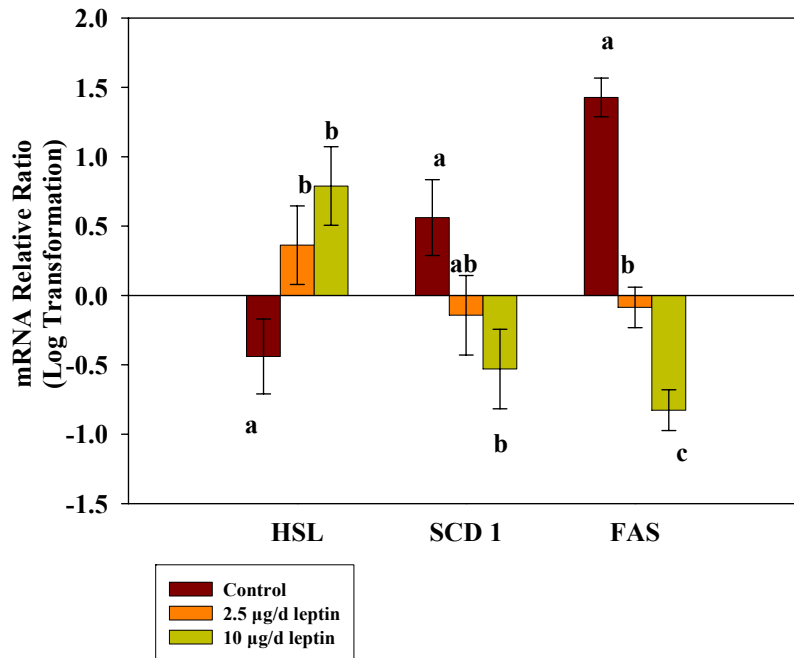


Figure 3.3. Effects of 14-day sc infusion of leptin on gene expression in adipose tissue from ob/ob mice (mean  $\pm$  S.E.M.). Total RNA extracted from inguinal adipose tissue was used for gene expression analysis of HSL, SCD1 and FAS. The Y-axis is the RQ value after log 2 based transformation. Note: Means without a common letter within each gene are different,  $p < 0.05$ . The replications for control, 2.5µg/d and 10µg/d leptin group are 11, 10 and 10, respectively.

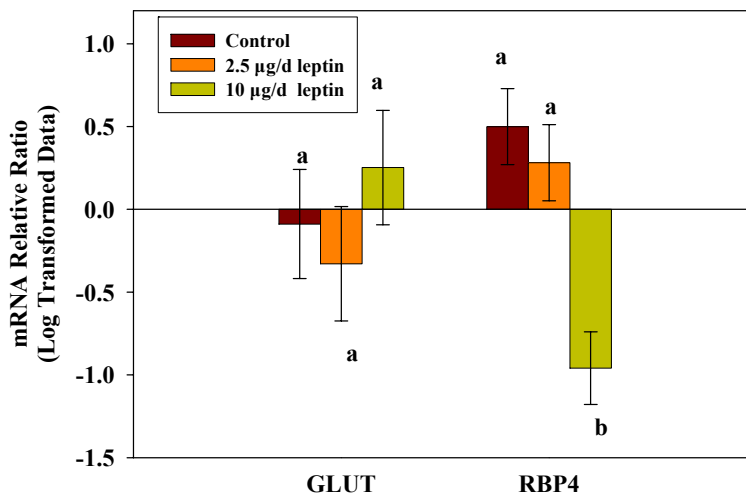


Figure 3.4. Effects of 14-day sc infusion of leptin on gene expression in adipose tissue from ob/ob mice (mean  $\pm$  S.E.M.). Total RNA extracted from inguinal adipose tissue was used for gene expression analysis of GLUT4 and RBP4. The Y-axis is the RQ value after log 2 based transformation. Note: Means without a common letter within each gene are different,  $p < 0.05$ . The replications for control, 2.5µg/d and 10µg/d leptin group are 11, 10 and 10, respectively.

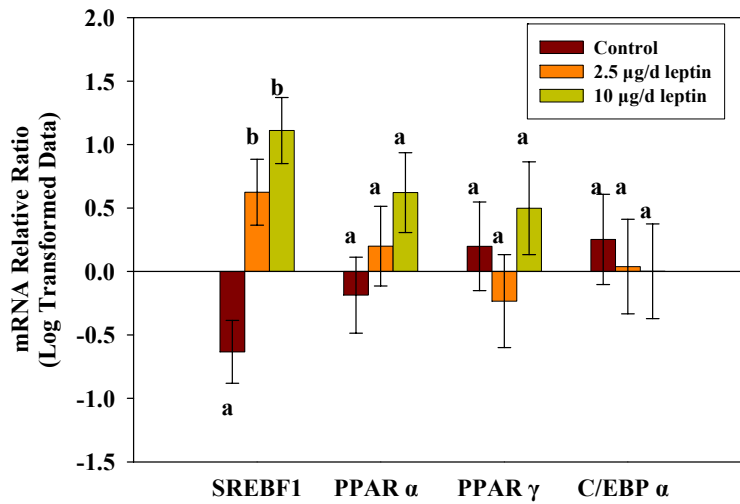


Figure 3.5. Effects of 14-day sc infusion of leptin on gene expression in adipose tissue from ob/ob mice (mean  $\pm$  S.E.M.). Total RNA extracted from inguinal adipose tissue was used for gene expression analysis of SREBF1, PPAR $\alpha$ , PPAR $\gamma$  and C/EBP $\alpha$ . The Y-axis is the RQ value after log 2 based transformation. Note: Means without a common letter within each gene are different,  $p < 0.05$ . The replications for control, 2.5 $\mu$ g/d and 10 $\mu$ g/d leptin group are 11, 10 and 10, respectively.

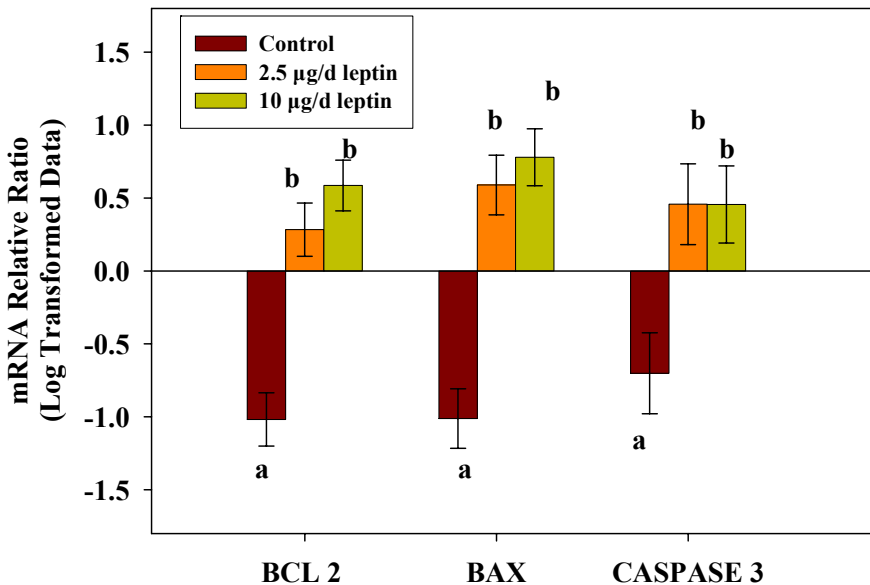


Figure 3.6. Effects of 14-day sc infusion of leptin on gene expression in adipose tissue from ob/ob mice (mean  $\pm$  S.E.M.). Total RNA extracted from inguinal adipose tissue was used for gene expression analysis of BCL2, BAX and CASPASE3. The Y-axis is the RQ value after log 2 based transformation. Note: Means without a common letter within each gene are different,  $p < 0.05$ . The replications for control, 2.5 $\mu$ g/d and 10 $\mu$ g/d leptin group are 11, 10 and 10, respectively.

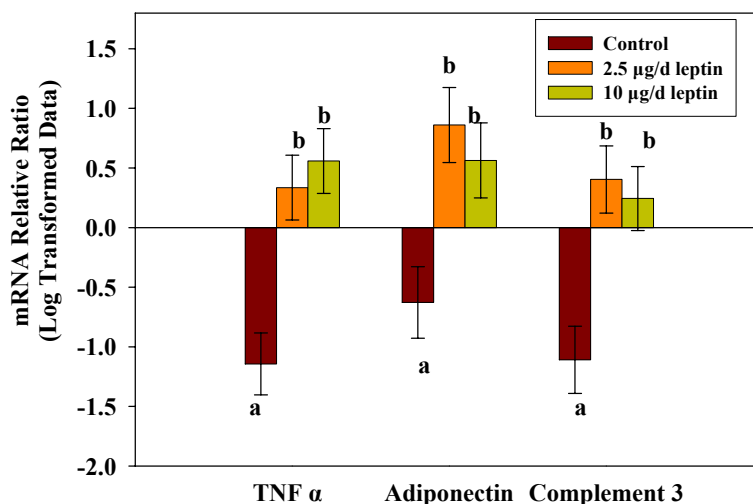


Figure 3.7. Effects of 14-day sc infusion of leptin on gene expression in adipose tissue from ob/ob mice (mean  $\pm$  S.E.M.). Total RNA extracted from inguinal adipose tissue was used for gene expression analysis of TNF $\alpha$ , Adiponectin, Complement 3. The Y-axis is the RQ value after log 2 based transformation. Note: Means without a common letter within each gene are different,  $p < 0.05$ . The replications for control, 2.5 $\mu$ g/d and 10 $\mu$ g/d leptin group are 11, 10 and 10, respectively.

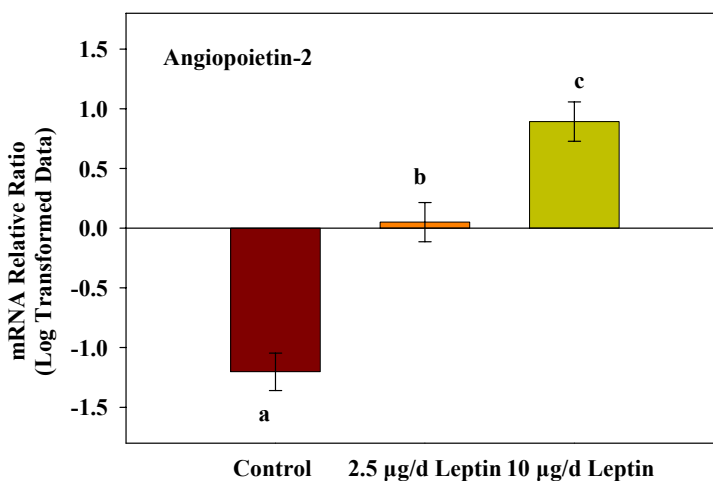


Figure 3.8. Effects of 14-day sc infusion of leptin on gene expression in adipose tissue from ob/ob mice (mean  $\pm$  SEM). Total RNA extracted from inguinal adipose tissue was used for gene expression analysis of Angiopoietin-2. The Y-axis is the RQ value after log 2 based transformation. Note: Means without a common letter within each gene are different,  $p < 0.05$ . The replications for control, 2.5 $\mu$ g/d and 10 $\mu$ g/d leptin group are 11, 10 and 10, respectively.



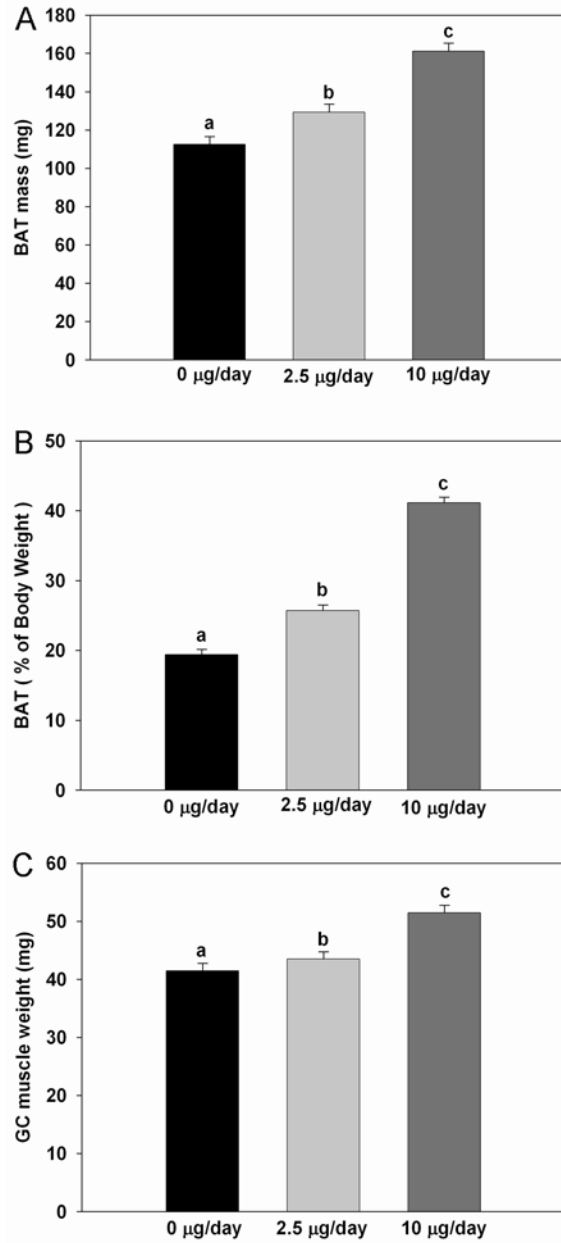


Figure 3.9. Effects of leptin on brown adipose tissue weight (Figure 3.9A), percentage of BAT vs. body weight (BW) (Figure 3.9B), and weight of gastrocnemius (GC) muscle (Figure 3.9C) from female *ob/ob* mice (mean  $\pm$  SEM). Note: Means without a common letter are different,  $p < 0.05$ . The replications for control, 2.5  $\mu\text{g}/\text{d}$  and 10  $\mu\text{g}/\text{d}$  leptin group are 11, 10 and 10, respectively.

**CHAPTER 4**

**TEST OF DIFFERENT DEGERGENT COMBINATIONS TO ENHANCE THE  
DETECTION OF PROTEOME FROM MURING ADIPOSE TISSUE**

Wei Zhang, Tracy Andacht, Pam Kirby, Yang-Ho Choi, Diane L. Hartzell, Mary Anne Della-Fera, Clifton A. Baile. Abstract to BHSI meeting (2005), Athens, GA

## **ABSTRACT**

Proteins from adipose tissue are especially difficult to be resolved for 2-D analysis due to the presence of extremely large amounts of lipid. To get the most protein spots in 2-D maps, different combinations of chaotropes (urea and thiourea), surfactants (CHAPS and ASB14) and reducing agents (DTT and 2-hydroxyethyl disulfide (HED)) were varied in the extraction buffer. Inguinal white adipose tissue from ob/ob mouse was prepared by homogenizing, ultra centrifuging and eliminating interferences by 2D clean-up kit (GE healthcare), and the extracted proteins were measured by protein assay. IPG strips (11cm, pH 3-10) were applied by in-gel sample rehydration in the first dimension IEF, and SDS-PAGE (8-16% Criterion precast gel) was used in the second dimensional electrophoresis. These gels were then stained by SYPRO Ruby<sup>®</sup> and scanned in order to be evaluated for protein patterns. We described the modification of a protocol using a combination of chaotropic agents (8.4M urea and 2.4M thiourea), zwitterionic agents (2% CHAPS and 2% ASB14), 1% IPG buffer, 15mM Tris and 50nM DTT for reducing reagents as the basis for sample preparation and IEF solutions, giving better efficiency in solubilization, separation and recovery of proteins from mouse inguinal adipose tissue.

**Key words: Proteomics, ob/ob, Leptin, Mouse, Obesity**

Proteomics could be described as the comprehensive study of the structures, functions and interactions of whole sets of proteins globally and dynamically (158). The combined use of 2-dimensional electrophoresis (DE) for protein separation and Mass Spectrometry (MS) for protein identification is the method of choice for characterization of proteins expressed at a given time under certain biological conditions in intricate samples. The ability to separate proteins with high resolution is the critical and first step for proteome analysis. Some technical improvements have been made in order to increase the resolving power in 2-DE such as the use of IPGs (159), narrow pH gradients (zoom gels) (160), long IPG strip, and IEF prefractionation (161). However, samples from adipose tissue are particularly difficult to study due to the large amounts of lipid present.

With the steadily increasing incidence of obesity all over the world, the biochemistry of white adipose tissue attracts great attention since obesity is an important risk factor in the development of type 2 diabetes and cardiovascular diseases (12). Knowledge about the role of white adipose tissue has changed from the assumption that it was only a passive organ for lipid storage to the understanding that it is an active endocrine and paracrine organ, which can secrete several adipokines such as leptin, TNF $\alpha$ , adiponectin, and resistin (162). It is these cytokines that have a role in influencing many metabolic processes, such as mobilization of lipids from adipose tissue, and stimulating glycolysis and gluconeogenesis in the liver. Therefore, one of the major interests in obesity research is to investigate these cytokines, with the objective of developing potential anti-obesity drugs.

Leptin, the product of the *ob* gene first discovered in 1994 (1), has been a major focus in the field of obesity research due to its specific role in regulation of food intake and energy expenditure (30,32). We have been interested in determining the differential expression of

proteins in adipose tissue of control ob/ob mice compared to leptin-treated ob/ob mice in order to better understand the potential mechanisms involved in weight-loss induced by leptin. However, due to its large amount of intracellular lipids, adipose tissue imposes problems in the use of 2-D analysis to separate and identify proteins. Thus, in this preliminary work, we have optimized a protocol for extraction buffer and IEF procedure with IPGs 3-10 (11cm) strips for adipose tissue from ob/ob mice, which will lay the groundwork for our further proteomic studies on adipose tissue in ob/ob mice.

The inguinal white adipose tissue from 15 week old female obese ob/ob mice (C57BL/6J background; purchased from Jackson Laboratory, Bar Harbor, ME) was used. It was frozen immediately after dissection and stored at -80° until homogenization. Before homogenization, the fat pads were weighed and thawed in the different extraction buffers (total volume 2 ml/g fat pad) as described in table 4.1. The flow chart is presented in Figure 4.1. The homogenization (Fisher Scientific PowerGen 700D, Hampton, NH) was performed on ice at 18,500 rpm for 2 minutes, followed by centrifugation at 13,000 g at 4°C for 20 minutes. Three fractions were obtained: the upper layer consisting of fat; a water soluble infranatant which was transferred to a new ultracentrifuge tube while avoiding a small amount of un-homogenized material at the bottom of the tube. The infranatant fluid was then ultra-centrifuged at 40,000 g at 4°C for 4 hours (Sorvall® Discovery™ 100 ultracentrifuge and rotor T-880, GMI Inc., Ramsey, MN, USA). Another three fractions were obtained as before and the infranatant was transferred to a new tube and further purified by 2D clean-up kit as recommended by the manufacturer (GE Healthcare), therefore the extracted proteins were purified by removing lipids, nucleic acids, salts and any other interfering contaminants under the condition of the least protein loss. Thus, proper isoelectric focusing and minimal streaking was obtained for the first dimensional electrophoresis.

The proteins were re-suspended in the corresponding extraction buffer. The protein concentration was measured by the Bradford dye-binding method (Bio-Rad, Hercules, CA) (163). The pH of the samples was adjusted to be between 8.3-8.5. The samples then were aliquoted and stored at -80°C until the 2-DE analysis was performed. A crude estimate of extraction efficiency for each extraction buffer was obtained by 1-D SDS-PAGE analysis with molecular markers as standards (data not shown). Subsequent analytical 2-D gels are able to provide more overall protein patterns and accurate extraction power between different extraction detergents. The experiments were performed in duplicate for each condition and pH range.

Criterion Tris-HCl gel (8-16%), Protean IEF cell, and Criterion Cell were from Bio-Rad. IPG Strips (11cm, pH 3 -10) were applied by in-gel sample rehydration. Each strip was rehydrated in a total volume of 185 µl rehydration solvent, which was composed of 10–50 µl of protein solution equal to 75 µg protein for each gel. The focusing was carried out in the following sequence according to the literature (164): 30V for 12h, 120V for 1h, 200V for 1h, 500V for 1h, 1000V for 1h, 2000V for 1h, 3000V for 1h, 4000V for 4h to reach a total of ~22 KVh. IPG strips were immediately equilibrated for SDS-PAGE. To achieve equilibration and reduction of disulphides, the IEF strips were incubated (5 min, gentle shaking) with 6 M urea, 2% SDS, 50 mM Tris (pH 8.8), 65 mM DTT, 30% glycerol (w/v) and 0.02% bromophenol blue after the IEF run. In a second step, alkylation of sulphhydryl groups was carried out in the same solution, except that DTT was replaced by 260 mM IAA (15min, gentle shaking). The equilibrated strips were immersed in SDS running buffer (25mM Tris, 192mM glycine and 0.1% SDS) for approximately 15s before they were sealed at the top of the second dimension gel with 0.6% agarose submerged in SDS running buffer. Molecular protein marker and BSA were

loaded as internal standards. Electrophoresis was performed in the Criterion Cell at 125 V for 2-3 h until the tracking dye, bromophenol blue, reached the anodic end of the gels.

Staining with SYPRO Ruby<sup>®</sup> was essentially performed as recommended by the manufacturer (BioRad): the gels were fixed with 30% ethanol, 7.5% acetic acid for 1 h, stained in SYPRO Ruby<sup>®</sup>, 50 ml/gel, for 12-16 h in the dark and then rinsed in 10% methanol, 6% acetic acid for 15min. For visualization of the proteins, the gels were scanned directly with a Typhoon 9400 scanner (GE Healthcare).

The “standard” IEF sample solution (8 M urea, 4% CHAPS, 50-100 mM DTT and 40 mM Tris base.) is not ideal for many membrane proteins and samples such as adipose tissue, and serum. The inclusion of thiourea in the IEF procedure greatly increases the resolution of the gel spots, especially for membrane proteins (165,166) and adipose tissue (167). Initially in the present study, IEF separations (pH 3–10) of proteins from mouse white adipose tissue were performed and a comparison between two different concentration combinations of thiourea and urea (2M thiourea + 7M urea vs. 2.4M thiourea + 8.4M urea) was made. The latter cocktail (Figure 4.1B) exerts a higher quality of protein patterns than the former one (Figure 4.1A), while no spots disappeared or showed decreased in density.

Chaotropic agents, such as urea, are strong denaturants of proteins, disrupting their hydrogen bonds, exposing their hydrophobic residues to contact with the solution, and unfolding protein structure (168). The Rabilloud group (166) first introduced the combination use of thiourea and urea to improve the solubilization of the proteins during the IEF procedure. Thiourea is a strong agent for breaking hydrophobic bonds. Therefore, the mixture of urea and thiourea offers excellent results in many cases (169,170). Although thiourea is a competent chaotrope, it requires a high concentration of urea for its solubility, and the optimal condition

includes solutions of 2-3 M thiourea plus 5-7 M urea. Based on reports in the literature (167,171), we decided to include thiourea in the extraction buffer, and two different concentrations of urea and thiourea were compared and optimized (Figure 4.1A and 4.1B). We showed that the extraction buffer and IEF solution that was the most efficient had a concentration of 2.4 M thiourea in 8.4 M urea.

Besides the use of thiourea/urea mixtures, it is usual to have at least one surfactant in the extraction buffer and IEF solution. Surfactants present help to solubilize the unfolded proteins following exposure by strong chaotropic agents. Surfactants such as the Triton X-100 and Nonidet P-40 are traditionally used. Recently, surfactant agents such as CHAPS and sulfobetaines have become first choices, especially ASB14, ASB16, as well as C8 $\phi$  (172). They have excellent compatibility with urea/thiourea mixtures compared to CHAPS due to their zwitterionic head groups and long alkyl tails (172).

Considering the commercial availability, a comparison among the zwitterionic detergents CHAPS (5%) (Figure 4.1B), ASB 14 (4%) (Figure 4.1C) and their combination (2% each) (4.1D) was made using 2.4 M thiourea and 8.4 M urea as chaotropes. It is clear that the combination of ASB14 and CHAPS of 2% each could yield better solubility power for proteins and well separated spots.

Next, based on the above chaotropes and surfactant chosen, we tested and compared two different reducing reagents of DTT and HED. Such agents are necessary to reduce disulfide bonds in order to allow complete unfolding of proteins and to stabilize them in the solvent. There are many kinds of reducing reagents in the market such as  $\beta$ -mercaptoethanol, DTT, TBP and recently reported HED (173). According to the literature for proteins extracted from adipose tissue (167,171), we decided to utilize and compare the two reducing reagents DTT and HED,



based on the above optimal detergents. As shown in Figure 4.1D and E, the protein spots seem much more round, smaller and focused when the reducing reagent DTT was applied, which is not completely consistent with the published studies (173).

In conclusion, in our study, the optimal extraction buffer was the combination of 2.4M thiourea, 8.4M urea, 2% ASB14 (w/v), 2% CHAPS (w/v), 1% IPG buffer (v/v), 15mM Tris base (pH 8.3) and 50mM DTT.

With the completion of mouse genome sequencing (174,175), the maturity of mass spectrometry technique in the analyses of protein (176), and the establishment of the protein database, 2-DE of preparative gels have become the workhorse technique to identify and characterize “unknown” proteins. Although thiourea improved the resolution of many proteins from adipose tissue, it is not sufficient to identify most of the hydrophobic/membrane proteins (164). Therefore some other methods will be considered in the future, such as stepwise fractionation (177), other chemicals or non-gel shotgun (178) or Isotope-coded affinity tagging (ICAT) techniques which avoid gel separation.(179)

#### **ACKNOWLEDGMENTS**

We wish to thank Tracy Andacht for her helpful discussion and technical advice, Pam Kirby for her assistance for 2-DE facilities. We are also grateful for Jerry Liu for assistance with the photographs.

#### **GRANTS**

This work was supported in part by the Georgia Research Alliance Eminent Scholar endowment held by CAB.

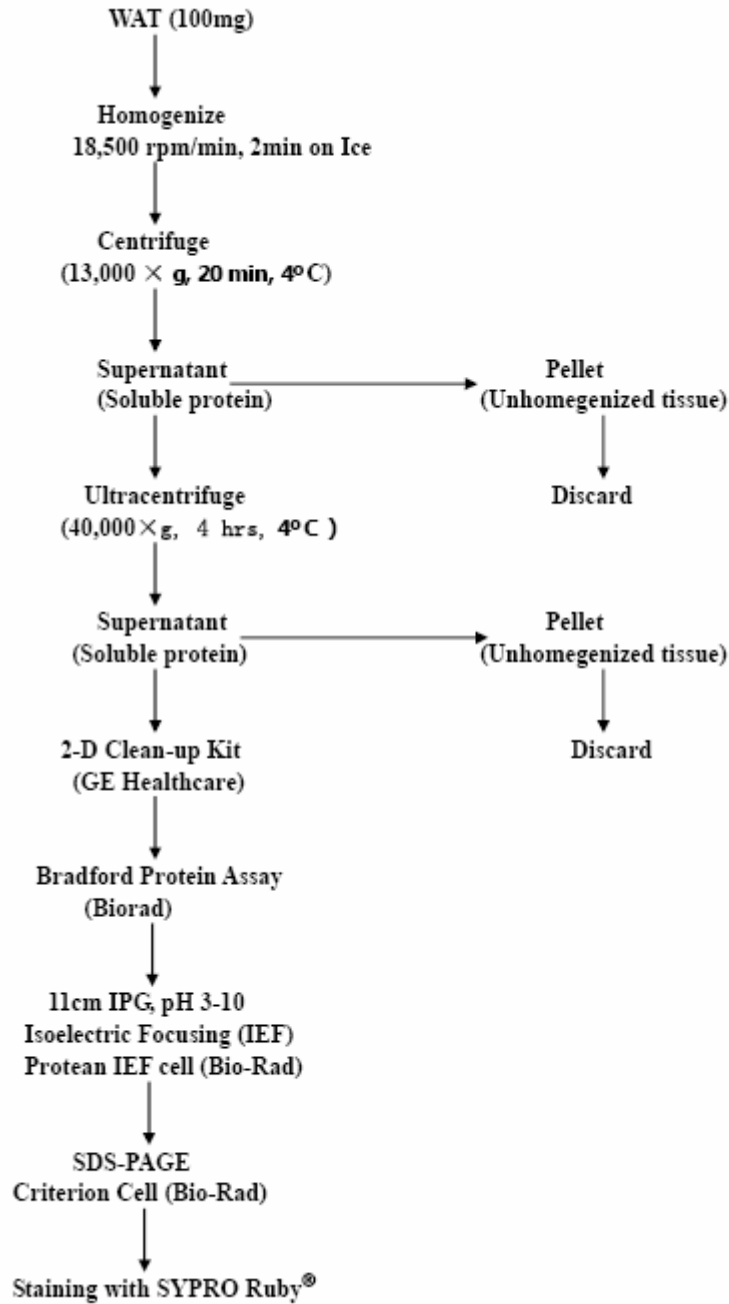


Figure 4.1. Flow chart of separation and extraction of proteins from adipose tissue of ob/ob mice

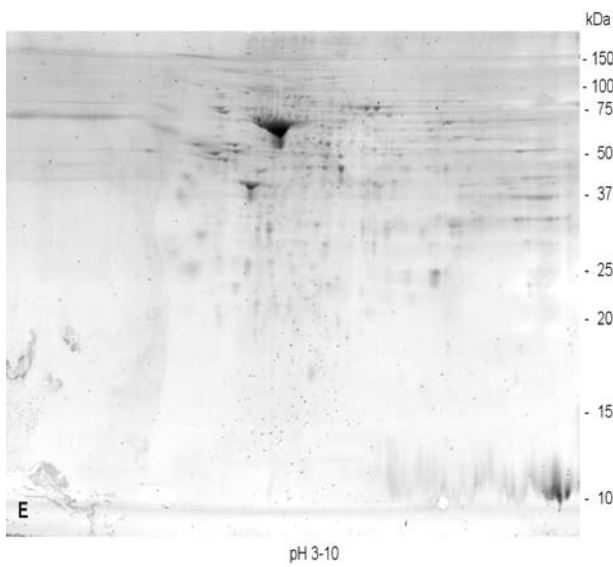
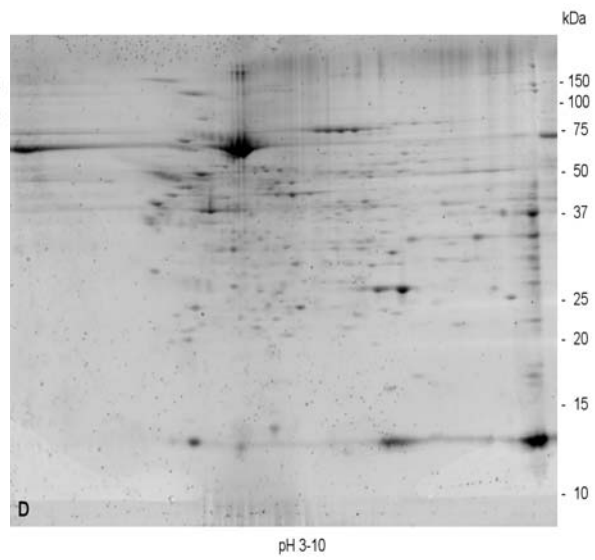
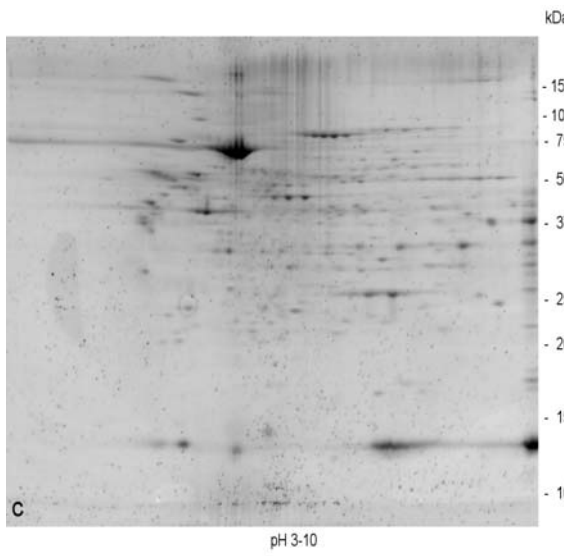
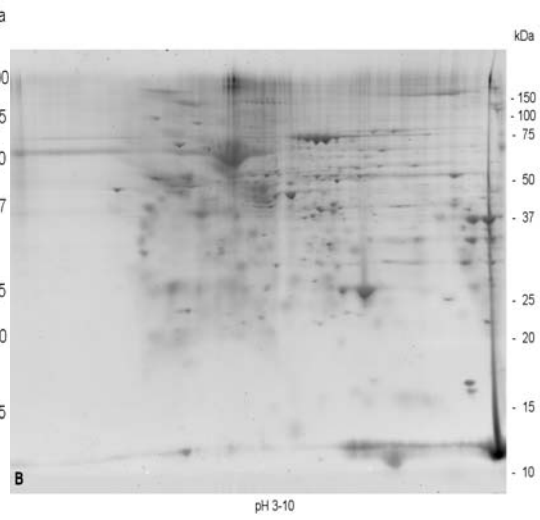
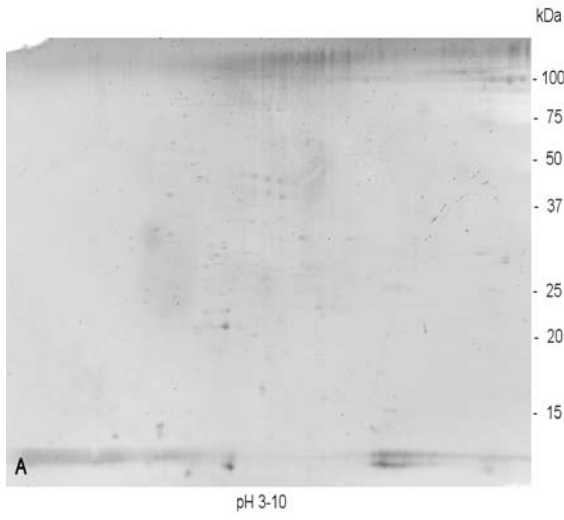


Figure 4.2. Patterns of two-dimensional gels in adipose tissue of ob/ob mice. IEF with IPG (linear 3-10 pH, 11 cm) were carried out with 75 µg of protein loaded. The second dimension SDS-PAGE gel was 8-16% and stained with SYPRO Ruby<sup>®</sup>. Detection was scanned with Typhoon 9400. The different extraction buffers were listed as below:

(A) 8.4M urea, 2.4M thiourea, 5% CHAPS (w/v), 15mM Tris base (pH8.3), 1% IPG buffer (v/v) and 50mM DTT, pH8.3;

(B) 7M urea, 2M thiourea, 4% CHAPS(w/v),15mM Tris base (pH8.3), 1% IPG buffer (v/v) and 50mM DTT, pH8.3.

(C) 8.4M urea, 2.4M thiourea, 4% ASB14 (w/v), 15mM Tris base (pH 8.3), 1% IPG buffer (v/v) and 50mM DTT, pH8.3;

(D) 8.4M urea, 2.4M thiourea, 2% ASB14 (w/v), 2% CHAPS (w/v), 15mM Tris base (pH 8.3), 1% IPG buffer (v/v) and 50mM DTT, pH8.3;

(E) 8.4M urea and 2.4M thiourea, 2% ASB14 (w/v), 2% CHAPS (w/v),15mM Tris base(pH8.3), 1% IPG buffer(v/v) and 100mM HED, pH8.3.

Table 4.1. The composition of extraction buffer in sample preparation

	Urea	Thio urea	CHAPS	ASB14	DTT	HED	Tris	IPG buffer	pH
1A	7.0M	2.0M	5%	0	50mM	0	15mM	1%	8.3
1B	8.4M	2.4M	4%	0	50mM	0			
1C	8.4M	2.4M	0	4%	50mM	0			
1D	8.4M	2.4M	2%	2%	50mM	0			
1E	8.4M	2.4M	2%	2%	0	100mM			

**CHAPTER 5**  
**A PROTEOMIC ANALYSIS OF ADIPOSE TISSUE FROM OB/OB MICE**  
**TREATED WITH LEPTIN**

Endocrine Meeting, Boston, MA (06/2006) Identification of Differentially Expressed Proteins in White Adipose Tissue of ob/ob Mice Treated with Leptin. Wei Zhang, Tracy Andacht, Diane L. Hartzell, Pamela S. Kirby, Gabriela S. Brambila, Mary Anne Della-Fera, Clifton A. Baile

## **ABSTRACT**

**Objective** The differentially expressed proteins from adipose tissue between leptin-treated and control ob/ob mouse were obtained by the method of two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Leptin may alter lipid metabolism and induce adipose tissue apoptosis through the pathways of these identified proteins.

**Research Methods and Procedures** Fifteen week old female ob/ob mice were treated with either PBS (control), 2.5  $\mu\text{g/d}$  or 10 $\mu\text{g/d}$  leptin (treatment) for 14 days via subcutaneous (sc) osmotic minipumps. Total proteins from the parametrial white adipose tissue of control and 10 $\mu\text{g/d}$  leptin-treated ob/ob mice were extracted, labeled with sensitive CyDye difference in-gel electrophoresis (DIGE) fluors, and then separated by two-dimensional electrophoresis. Some spots in the gel that showed differentially expressed were picked and digested with trypsin, and subjected to MALDI-TOF MS for protein identification. The mRNA levels of some identified proteins from the inguinal adipose tissue in three groups (control, 2.5  $\mu\text{g/d}$  or 10 $\mu\text{g/d}$  leptin) were quantitatively measured by real-time RT-PCR

**Results** For proteomics analysis, twenty-seven protein spots equivalent to twelve protein entries were found differentially expressed in adipose tissue of ob/ob mice treated with leptin compared to control ob/ob mice, including molecular chaperones and redox such as calreticulin (CRT) (+1.63- fold), protein disulfide isomerase associated 3 (PDIA3) (+2.68- fold), prohibitin (PHB) (+1.96- fold), peroxiredoxin-VI (Prx-VI) (-1.67- fold); cytoskeleton proteins such as  $\beta$  actin (+1.64- fold), desmin (+1.55- fold) and  $\alpha$  tubulin (-1.47- fold); other proteins such as pyruvate carboxylase (PC) (-2.0- fold), guanine deaminase (GDEase) (+1.45- fold), ubiquinol-cytochrome-c reductase complex core protein 1 (UQCRC) (+1.49- fold), polymerase I and

transcript release factor (PTRF) (+1.44- fold) and fibrinogen  $\beta$  chain (FGB) (+1.63- fold). The mRNA levels of CRT, PDIA3 and PHB were determined by real-time RT-PCR and found to be up-regulated ( $p < 0.05$ ), consistent with the fold change in protein expression level.

**Discussion** Our studies suggested that leptin's anti-obesity effect or more specifically the ability to enhance apoptosis can be mediated by the alterations of molecular chaperones and redox for regulating ER stress, cytoskeleton proteins for regulating mitochondrial morphology.

**Key Words** Proteomics, Mouse, Adipose tissue, Molecular Chaperone, Redox, Cytoskeleton



## 1. Introduction

Leptin, the cytokine produced from white adipose tissue, acts as an adiposity signal and functions both centrally and peripherally to specifically induce loss of adipose tissue (30,32). Since the finding of rapid loss of adipose tissue following leptin treatment and delayed recovery of fat depots (119), we hypothesized that this delayed recovery was due to apoptosis, the process of programmed cell death. The apoptotic characteristic features in adipose tissue were observed following i.c.v. injection of leptin (56), and a great number of downstream targets of leptin have been identified, such as C/EBP  $\alpha$ ,  $\beta$ ,  $\delta$  (67), TNF  $\alpha$  (180), and some mitochondria proteins such as Bcl-2 and Bax (59), but it needs further confirmation. The study of cellular mechanisms of apoptosis following leptin treatment may provide a potential effective therapeutic method for obesity, but the mechanisms involved in leptin-induced apoptosis are not clearly understood.

The significant changes in the expression of many genes and/or their protein products are usually the physiological reactions of organism (ob/ob mice) in response to external stimuli (leptin treatment). Several proteomic and genomic analyses have been done in ob/ob mice and wild type lean mice, and a number of proteins and genes have been identified as white adipose tissue fat markers during various biological treatment conditions (138,181,182). An approach that uses global analysis of protein (proteomics) is technically feasible with the complement of genome sequencing and dramatic advances in the molecular technologies such as the creation of protein databases. In the present study, we employed high-resolution 2-DE technique, sensitive DIGE technology (183), as well as MALDI-TOF MS to compare the global protein expression changes in white adipose tissue between control and leptin-treated ob/ob mice. Furthermore, quantitative PCR (TaqMan<sup>®</sup>) assays of several selected genes corresponding to the identified

proteins were performed using 384-well Low Density Array on an ABI PRISM 7900 Sequence Detection System.

In the master 2-DE gel map, 27 protein spots corresponding to 12 different protein entries have been identified, and functionally classified as molecular chaperones and redox, cytoskeleton proteins and other related proteins. These differentially expressed proteins may provide a valuable starting point for analyzing the signaling pathways through which leptin promotes adipose tissue apoptosis and regulates lipid metabolism in ob/ob mice.

## **2. Research Method**

### **2.1. Animal Experiment**

Leptin was administered subcutaneously to ob/ob mice (C57BL/6J background) (Jackson Laboratory, Bar Harbor, ME) at the dose of either 0 µg/d (control), 2.5 µg/d or 10 µg/d leptin (treatment group) for 14 days, with the replication of 11 mice in each group. At the end of the experiment, three fat depots (inguinal, parametrial, and retroperitoneal) were obtained and stored at -80° C until homogenization. A number of physiological and biochemical parameters (body weight, food intake, body temperature, physical activity), mass of three fat pads and apoptosis, body composition, and serum hormones (leptin, insulin, glucagon) of ob/ob mice were examined and detailed previously(11).

### **2.2. Reagents and Apparatus**

Urea, thiourea, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), dithiothreitol (DTT), IPG buffer (18cm, pH 3-10), 2-D clean-up kit, IPG strip (pH3-10, 18cm), Sypro Ruby<sup>®</sup> protein gel stain, CyDye DIGE Fluor Cy3,Cy5 minimal dye, iodoacetamide (IAA), N,N-dimethylformamide (DMF) were from GE Healthcare (Piscataway, NJ). Lysine, amidosulfobetainers14 (ASB 14), and  $\alpha$ -Cyano-4-hydroxycinnamic acid diethylamine salt

(HPLC) were from Sigma-Aldrich (St. Louis, MO). Tris-Glycine Gels (Gradient 8-15%) were from Jule Inc. (Milford, CT). Tris, glycine, SDS, acetonitrile, methanol, trifluoroacetic acid (TFA), ammonium bicarbonate, Bradford protein assay, and molecular protein markers (precision plus protein in all blue standards) were from BioRad (Hercules, CA). Trypsin (sequencing grade) was from Promega (Madison, WI). BSA was from Pierce (Rockford, IL). Ultra pure water was used for the preparation of all solutions.

### **2.3. Sample Preparation and CyDye labeling**

The homogenization of parametrial adipose tissue was performed in an extraction buffer (8.4M urea, 2.4M thiourea, 2% v/v ASB14, 2% v/v CHAPS, 15mM Tris base, 50mM DTT, 1% IPG buffer, pH 8.3) at 18,500 rpm for 2 minutes on ice, followed by ultra-centrifugation detailed in Chapter 4. The extracted proteins were then subjected to a 2-D clean-up kit to eliminate interferences such as lipids, and the protein concentration was assayed by the method of Bradford.

To characterize the target proteins of leptin in ob/ob mice, a total of twenty-one analytical gels in three blocks were run by performing 2D-DIGE (183). The CyDye fluor labeling was performed according to Ettan™ DIGE protocol (GE Healthcare). An internal standard was created by pooling equal aliquots of all the samples in the experiment. The use of an internal standard guaranteed that all protein spots in the samples were represented on each gel so that accurate quantification and spot statistics between gels was possible. The 50 µg internal standard labeled with Cy2 minimal dye was mixed with a 50 µg control or a 50 µg treated sample labeled both with molecular weight- and charge- matched Cy3 and Cy5 minimal dye prior to electrophoresis. The reaction was quenched with the addition of 10 nmol lysine. After labeling, 150 µg of each sample with all three different labeling were mixed and loaded on each

IPG strip via active rehydration. This experimental design (Table 5.1) had the advantage of the reduced gel-to-gel variation over the conventional 2-D PAGE.

#### **2.4. First Dimensional Isoelectric Focusing (IEF)**

All gel equipment used for separation and imaging were from GE Healthcare. IPG Strips (18cm, pH 3 -10) were applied by in-gel sample rehydration. Each strip was rehydrated for 12 h (30 V) in a total volume of 325  $\mu$ l rehydration solution (Urea 8M, CHAPS 2% (v/v), 50mM DTT, 0.5% IPG buffer 3–10 (v/v) and a trace of bromophenol blue), which contained 10-50 $\mu$ l of protein solution equal to 50  $\mu$ g protein for each gel. The strips were run under a layer of Drystrip cover fluid at 20°C in an Ettan IPGphor IEF System according to the manufacturer's instructions. The focusing was carried out in the following steps: 500 V for 1 h, 1000 V for 1 h, 2000 V for 1h, 3000 V for 1h, and 4000 V for 6.5h to reach a total of ~32 KVh. IPG strips were immediately equilibrated for SDS-PAGE.

#### **2.5. Equilibration of IPG Strips and Reduction of Disulphides**

To achieve equilibration and reduction of disulphides, the IEF strips were incubated in reduction buffer (6M urea, 2% SDS, 50 mM Tris (pH 8.8), 65 mM DTT, 30% glycerol (v/v) and a trace of bromophenol blue) for 5 minutes with gentle shaking. Then alkylation of sulphydryl groups was carried out in the same solution, except that DTT was replaced by 260 mM IAA (15min, gentle shaking).

#### **2.6. Second Dimensional SDS-PAGE**

The gels used in this study were 8-15% Tris-Glycine precast gels. The equilibrated strips were immersed in SDS running buffer for approximately 15s (25mM Tris base, 192mM glycine and 0.1% SDS) before they were sealed at the top of the second dimensional gel with 0.6% agarose submerged in SDS running buffer. Molecular protein markers and BSA were loaded as

internal standards. Electrophoresis was performed in the GE Ettan DALT Twelve Large Vertical System at 5 Watts per gel for 1~2h until the voltage reaches 2 Watts and 40-50 mA per gel, generally overnight.

## **2.7. Gel Scanning and Image Analysis**

For visualization of the proteins, the gels were scanned directly with a Typhoon 9400 scanner. Each gel was scanned at three different wavelengths, and therefore three different gels for each sample (either control or treatment) was produced and applied for the following statistical analysis. The excitation wavelengths for Cy2-, Cy3-, and Cy5-labeled images were at 488 nm, 520 nm, and 620 nm respectively. The emission wavelengths for Cy2, Cy3, and Cy5 were 520 nm, 590 nm, and 680 nm respectively. DeCyder software version 4.0 was used for gel analysis.

## **2.8. Protein Identification by MALDI-TOF MS**

The analytical gel with the best resolution was selected for spot picking and post-stained with Sypro Ruby<sup>®</sup>. The Sypro Ruby<sup>®</sup> image was matched to the analytical gels and spots of interest meeting a minimum volume cutoff were selected for spot picking. Gel plugs of 2 mm were cut using the Spot Handling Workstation automatically (GE Healthcare). In brief, the gel plugs were washed twice in 50 mM ammonium bicarbonate (ACN)/50% methanol (v/v) in water for 20 min, and once with 75% ACN (v/v) in water. After drying, the gel plugs were cleaved by sequencing-grade trypsin at 37°C overnight using a standard in-gel trypsin digestion protocol. The peptide fragments were extracted with 50% ACN (v/v), 0.1% trifluoroacetic acid (TFA) (v/v) in water twice, and dried down in a vacuum concentrator (Jouan, ThermoElectron, Milford, MA). Finally, the dissolved peptide fragments were spotted on MALDI target plates, dried and mixed with a 50% saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN (v/v),

0.1% TFA (v/v) in water. MS analyses were performed using the ABI 4700 Proteomics Analyzer (Foster City, CA) in reflector mode for positive ion detection. Mass lists were submitted to Mascot ([www.matrixscience.com](http://www.matrixscience.com)) against NCBIInr (mammals) and Swiss-Prot databases for protein identification, using the criteria of a minimum of five matching tryptic fragments with a mass tolerance of 500 ppm, and more than 25% peptide coverage. Molecular weight, isoelectric point and mass accuracy were checked for candidate proteins.

### **2.9. Real-time RT-PCR (q RT-PCR)**

Total RNA was isolated from the inguinal adipose tissue of ob mice, using Trizol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. 1.0  $\mu$ l of the 12  $\mu$ l extract RNA was used as an integrity check and for quantification by the Agilent 2100 bioanalyzer and RNA 6000 Nano Assay (Agilent Technologies, Foster City, CA). All instrument and reagents were from ABI (Foster City, CA). The experimental procedure of reverse transcription and PCR reaction and statistical method were described in chapter 3 Materials and Method section. All of the oligonucleotide primers and fluorogenic probe sets for Taqman<sup>®</sup> real time PCR were prepared by ABI (Table 5.2). Through comparing and analyzing the variability of two housekeeping genes (HKG) (18s and GADPH) from the amplification plot shown in the SDS software, the expression of a target gene was normalized by using GADPH as the reference control gene, and sample number of 2038 as a calibrator to correct the differences in the amount of total RNA added to each reaction.

### **2.10. Statistical Analysis**

The statistical analyses of differentially expressed proteins were conducted with DeCyder software version 4.0. The relative protein abundance from proteomic analysis was expressed as means  $\pm$  standard error. Statistical analysis of qPCR data was conducted with SAS (Version 9.0)

(SAS institute, Cary, NC). The whole procedure and SAS codes are in the appendix C. The Ct value can be automatically transferred to the RQ value by the software sequence detection system (SDS) (Applied Biosystems). In brief, RQ values in all samples were submitted to logarithm transformation at the base of 2 in order to create a linear range between fluorescent signal versus cycle number (95). The transformed RQ value was then winsorized for dealing with the outliers (96). Statistical significance of treatment ( $p < 0.05$ ) was assessed by a general linear model based on the transformed and winsorized data (tRQ) derived from the original RQ value. The tRQ values were expressed as means  $\pm$  standard error for all genes. Least square means was used to determine significance of differences among means ( $p < 0.05$ ). The correlation between body weight and leptin mRNA expression was examined by means of Spearman's correlation coefficients.

### **3. Results**

#### **3.1. 2-DE Gel Analysis and Identification of Proteins**

For gel analysis, gel 125 was chosen as the master gel in the following statistical analysis due to its maximal number of 1033 protein spots. All gels were then matched to the master gel (Figure 5.1). Statistical analysis was performed on these 1033 spots matched across the 21 gels by DeCyder software, and 130 proteins were selected as significant spots ( $p < 0.01$ ) with an average volume ratio between treatment and control groups greater than or equal to 1.2, and with an appearance in more than 50% of all gel images. A total of 65 spots of interest cut from analytical gels (the preparative gel can not be used since it was loaded with too much proteins of about 800 mg, and spots can not be distinguished between one to the other), trypsin digested and identified by MALDI-TOF-MS. Twenty-seven protein spots were identified, including several post-translational modifications of the same protein, therefore 12 different protein entries were

obtained, corresponding to 42% of success rate. Identified proteins and their MW, pI, peptide amount, protein score and sequence coverage were listed in table 5.3. The unresolved spots can be due to either few peptides hits from the database or poor MALDI spectrums.

### **3.2. Protein Functional Groups Modulated by Leptin**

Proteins regulated by leptin treatment were classified as three functional groups (Figure 5.2), and bar charts of fold changes of these proteins are shown (Figure 5.3). The major group identified was molecular chaperone and redox, including 1.63-fold increase of CRT, 1.96-fold increase of PHB, 2.68-fold increase of PDIA3, as well as 1.67-fold decrease of Prx VI in leptin-treated as compared with control ob/ob mice. It was of particular interest that except for peroxiredoxin VI, all of other listed proteins were up-regulated in leptin-treated ob/ob mice. Of note, the spot 545 was a co-migration protein spot including two proteins of glucose-6-phosphate 1-dehydrogenase (G6PD1) and PDIA3 (Figure 5.4.). Its total fold change was a 2.68-fold increase between leptin-treated and control ob/ob mice. The relative abundance of protein in spots 535 and 537 (both spots were identified as a single protein as G6PD1) was very close to 1, which suggested no change of G6PD1 between leptin-treated and control ob/ob mice. Therefore, we attributed the 2.68-fold change in spot 545 to the protein PDIA3.

Another major group identified were proteins associated with cellular structure and cytoskeleton organization, including a 1.64-fold increase of  $\beta$  actin, 1.55-fold increase of desmin and 1.47-fold decrease of  $\alpha$  tubulin in leptin-treated as compared with control ob/ob mice. Other proteins identified as altered in adipose tissue of the leptin-treated ob/ob mice include a 2-fold decrease of pyruvate carboxylase (PC), involved in carbohydrate metabolism; 1.45-fold increase of guanine deaminase involved in guanine degradation; 1.44-fold increase of PTRF; facilitating ribosomal RNA synthesis and possibly promoting lipolysis; 1.49-fold increase of UQCRC,



participating in energy transduction; and 1.63-fold increase of fibrinogen, which acts as a hemostatic factor.

### **3.3. Real-time RT-PCR (qPCR)**

To further validate the effects of leptin on mRNA gene expression in adipose tissue, we examined the mRNA expression of molecular chaperones (CRT, PDIA3, and PHB), fibrinogen and UQCRC. Leptin increased the mRNA expression of all three molecular chaperones ( $p < 0.05$ ) (Figure 5.5), consistent with the fold change trends of proteins identified by proteomic analysis. However, there was no change of mRNA expression for UQCRC and fibrinogen (data not shown).

## **4. Discussion**

The objective of this study was to identify and characterize the differentially expressed proteins in adipose tissue between control and leptin-treated ob/ob mice by 2D DIGE coupled to MS. These proteins and their corresponding post-translational modified forms may participate in some critical signaling pathways through which leptin regulates lipid metabolism and related systems. Notably, apoptosis was increased significantly in all three fat pads by leptin treatment in ob/ob mice (11), including the parametrial fat pads from which proteins were extracted and applied to comparative proteomics in the current study.

A major group of proteins identified in our study involved molecular chaperones and redox, including CRT and PDIA3 located in ER, as well as PHB and Prx-VI, which reside in the mitochondria. The molecular chaperones (such as CRT and PDIA3) in ER are involved in the correct folding and oligomerization of newly-synthesized proteins in order to function properly (184,185). Any factor which disturbs calcium homeostasis and restrains the formation of disulfide bonds can lead to increased amounts of unfolded or mal-folded proteins in the ER,

termed “ER stress”. In response to “ER stress”, normal cells usually increase transcription of ER-resident chaperone genes and degrade unfolded proteins. Proteins that failed to fold correctly in the ER are subjected to proteasomal degradation or the cells are deleted by apoptosis. Accumulating evidence has shown that CRT (186-188) and PDIA3 (189-191) were both up-regulated in different types of cells undergoing apoptosis, with an increased release of cytochrome c from the mitochondria (186). On the contrary, CRT deficient cells were significantly resistant to apoptosis, with a decreased release of Cytochrome c from mitochondria and low levels of caspase 3 activity (186). In addition, CRT is also a  $\text{Ca}^{2+}$ -binding chaperone and responsible for calcium concentration regulation with other proteins in ER. A great deal of interest has recently been focused on the involvement of CRT (186,187) in the modulation of apoptosis, and calcium may be the link between ER and mitochondria for handling apoptosis (192). Higher levels of CRT produced higher levels of ER luminal Ca, which increased sensitivity to apoptosis through suppressing the pro-survival kinase akt (188). In contrast, lower levels or deficiency of CRT were associated with a lower concentration of ER luminal calcium, and thus decreased sensitivity to apoptosis through reducing the activity of caspase-3 (186) or altering p53 activity (193). The molecular mechanism between calcium and apoptosis is not clearly delineated, and it was suggested that some Ca-dependent enzymes like kinases, phosphatase, and cytoplasmic Ca binding proteins may be involved in signaling pathways during the apoptotic process (188). In the present study, the up-regulated expression of CRT and PDIA3 may be the normal physiological response of adipocytes to “ER stress” mediated by leptin, and this response may help the body to delete unhealthy cells by the pathway of apoptosis.

PHB is present in the mitochondrial and acts as a molecular chaperone to stabilize newly synthesized mitochondrial respiratory chain complexes (194). It also acts as a target for a pro-

apoptotic peptide, which causes ablation of adipose tissue by endothelial cell apoptosis (195), and results in normalization of metabolism and increased energy expenditure in ob/ob mice (195). In addition, prohibitin has recently been found to inhibit glucose and fatty acid synthesis in adipocytes by specifically inhibiting the activity of pyruvate carboxylase (196). PC is an cytosolic enzyme participating in lipogenesis and gluconeogenesis (197). It catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate, providing the acetyl group and reducing potential NADPH that is required for de novo synthesis of fatty acids in the cytoplasm. The down-regulated (2-fold) expression of PC in leptin-treated mice in the present study indicates that lipogenesis was reduced in ob/ob mice, possibly one of the pathways of fat loss induced by leptin in ob/ob mice.

The peroxiredoxin family is mainly present in cytoplasm and mitochondria, and Prx I, II, V act to protect cells against oxidative stress and apoptotic stimuli, and tend to inhibit apoptosis in mice or human (198-200). Prx VI in the present study was down-regulated (1.47- fold) by leptin, which tended to increase oxidative stress or reactive oxygen species (ROS) in the cytosol, and this may be one of the pathways that leptin induced adipocyte apoptosis.

The other striking findings of the present study were that the cytoskeleton proteins ( $\alpha$  actin, desmin,  $\alpha$  tubulin) were all altered by leptin. Although the link between cytoskeleton and fat loss mediated by leptin has not been established, a growing body of evidence (201,202) suggests that interactions between mitochondria and cytoskeleton are essential for normal mitochondrial morphology, motility and distribution. Cytoskeletons include three components, microfilaments (MFs) composed of actin monomer; microtubules (MTs) composed of cylindrical polymers of  $\alpha/\beta$ -tubulin dimers; and intermediate filaments (IFs) composed of principal structural elements including desmin (203). Of these three cytoskeleton proteins, actin might be involved in the

adipocytes apoptosis mediated by leptin, while the functional studies on desmin and tubulin are very limited so far. The drug that stabilizes the actin cytoskeleton increases apoptosis in T cells and several transformed murine cell lines (204,205), while the agent for stimulating actin depolymerization (206) or actin-binding proteins repressed apoptosis (207,208). There is a large and specific decrease in biosynthetic rates for  $\beta$  and  $\gamma$  actin, vimentin,  $\alpha$  and  $\beta$  tubulin prior to morphological differentiation of 3T3 adipocytes, strongly suggesting that cytoskeleton proteins specifically take part in adipocyte differentiation and morphology (209). Therefore, leptin appears to induce adipocyte apoptosis, partly by acting as an actin stabilizer, but it needs further study.

Other identified proteins such as guanine deaminase (GDEase), UQCRC1, PTRF and fibrinogen were altered by leptin treatment. Guanine deaminase (GDEase), is involved in for purine degradation, and it recently has been shown to related to obese rats treated with tungstate (a novel anti-obesity agent) by restoring high Rho-GTPase signaling in obese to basal level (210). UQCRC1, a component of mitochondrial respiratory complex III(211), is important in redox reactions, electron transport as well as ATP production in the mitochondria (212), but it is not clear whether this change was related body weight change. PTRF had originally been identified as a nuclear factor for enhancing ribosomal RNA synthesis (213), but it was found to specifically control lipolysis by binding to HSL(214,215). Therefore, the 1.44-fold increase in PTRF expression in leptin treated ob/ob mice may be consistent with its role of fat mobilization. Several isoforms of fibrinogen were all up-regulated in leptin-treated ob/ob mice compared to control ob/ob mice. Fibrinogen which acts as one of the fibrinolytic components for blood clotting, was proposed to be a signaling link as a major risk factor between obesity and the development of cardiovascular diseases (216). The relationship has not been defined between

leptin and cardiovascular events (216-218), and further research is needed on the link between leptin and fibrinogen.

In conclusion, this study allowed, for the first time, to identify 12 different proteins (functionally classified as molecular chaperones and redox, cytoskeleton structure proteins and other related proteins) as the potential targets of leptin in the adipose tissue of ob/ob mice by the method of 2D-DIGE and MS. Some of them such as CRT, PDIA3, PHB, Prx-6 and actin have already been linked to apoptosis, some of them such as pyruvate carboxylase, GDEase, UQCRC1, PTRF or probably fibrinogen are related to obesity, others such as desmin, tubulin are not clearly delineated. Regulation of these proteins and other unknown mechanisms might be involved in the signaling pathways mediating fat loss in leptin-treated ob/ob mice. Whether these protein molecules are direct targets of leptin action, or are secondary effects to body weight loss and fat loss will be elucidated in further studies. Thus, the findings presented here gave new potential regulators of leptin on fat loss, therefore providing potential treatments for obesity.

#### **ACKNOWLEDGMENTS**

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#### **GRANTS**

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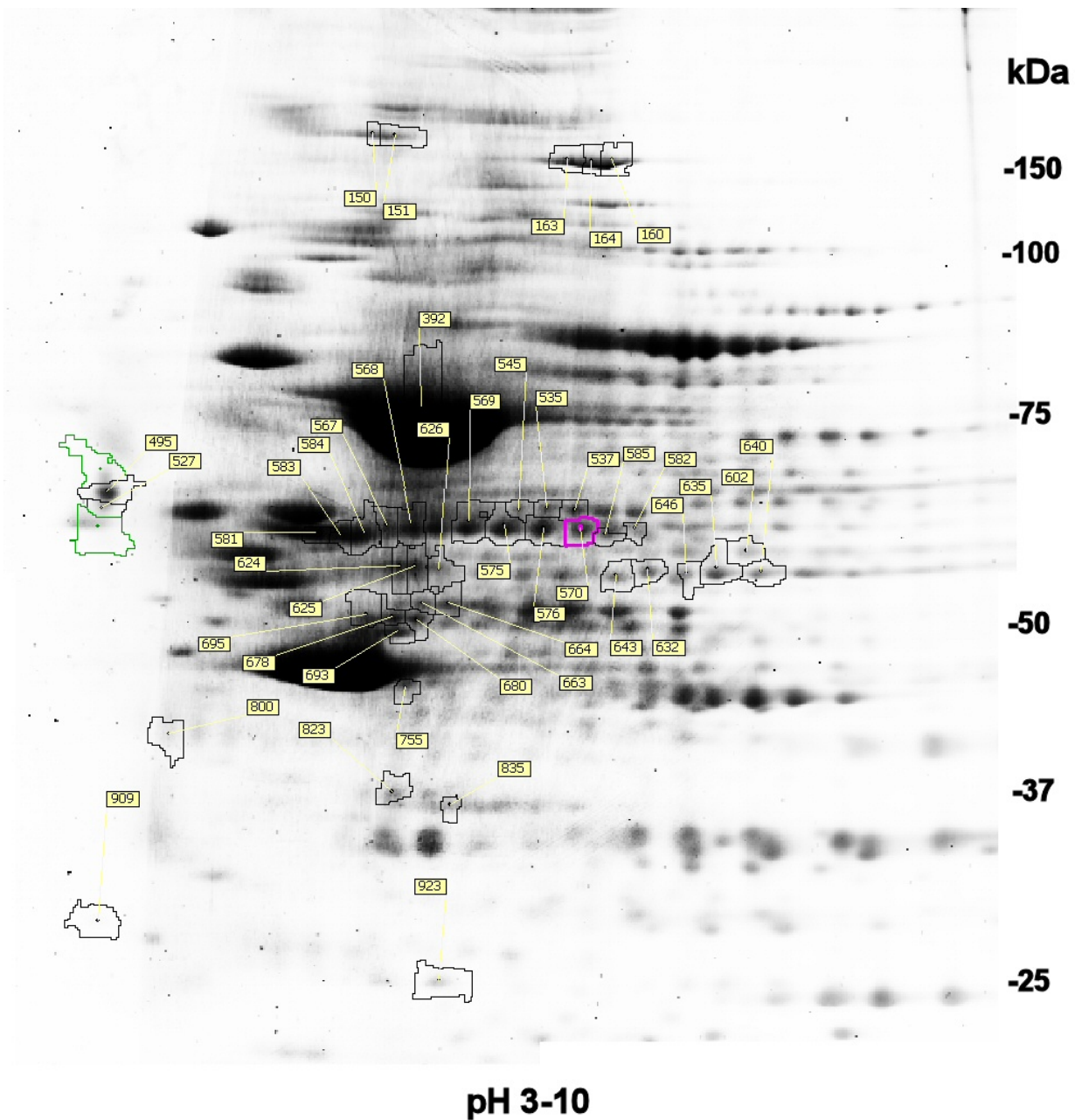


Figure 5.1. Sypro Ruby stained master 2-D gel in adipose tissue of ob/ob mice annotated with master spot numbers. The image shown is of 150  $\mu$ g sample (50  $\mu$ g each of Cy2-, Cy3 and Cy5-labeled lysate) run on a pH 3-10 linear gradient IPG strip (18cm) and 8-16% gradient polyacrylamide gel. The spot numbers of identified proteins in the 2-D gel are the following: 163, 164, 495, 527, 535, 537, 545, 570, 576, 584, 635, 693, 695, 735, 736, 923, and 959. They are further defined in Table 5.3.

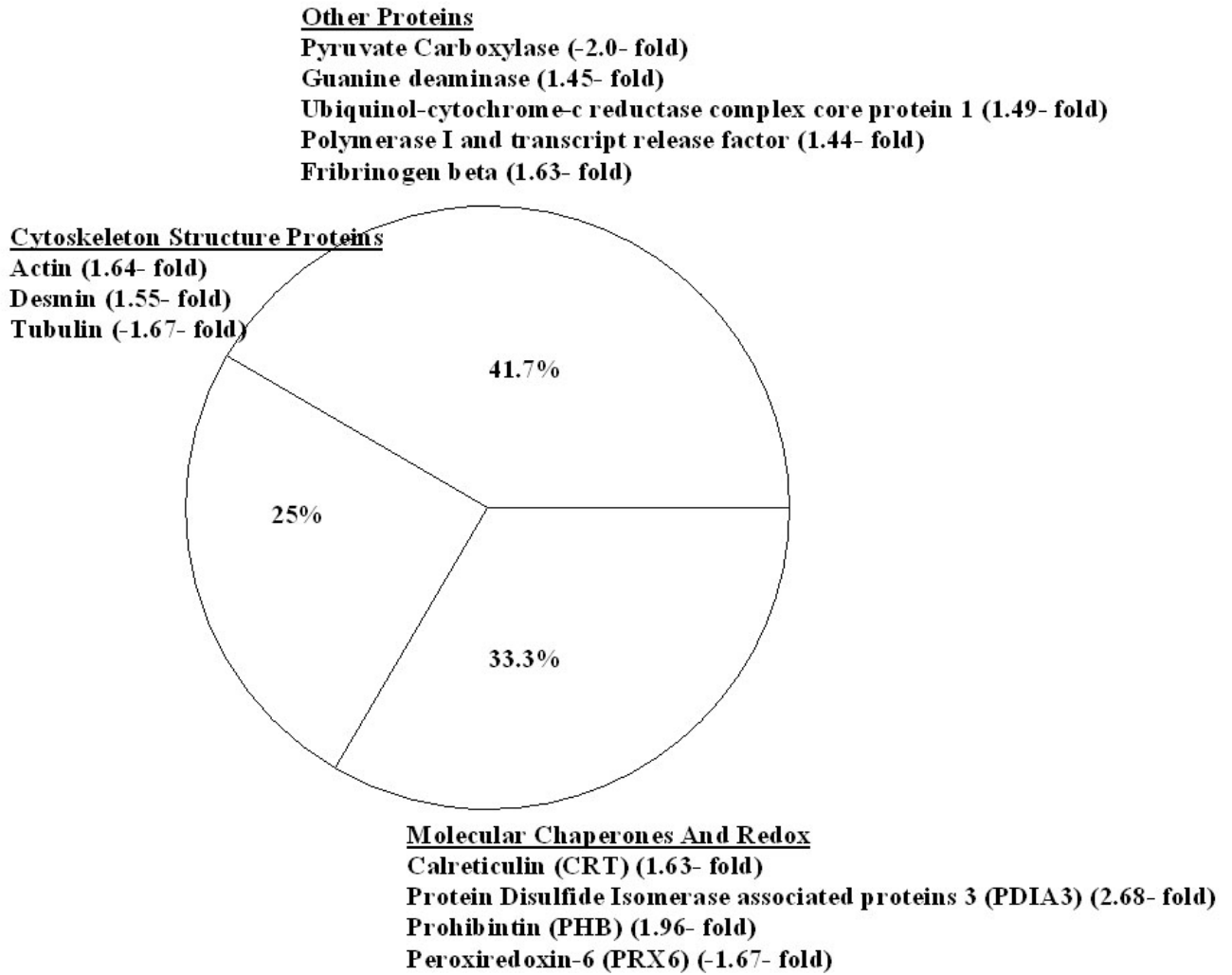
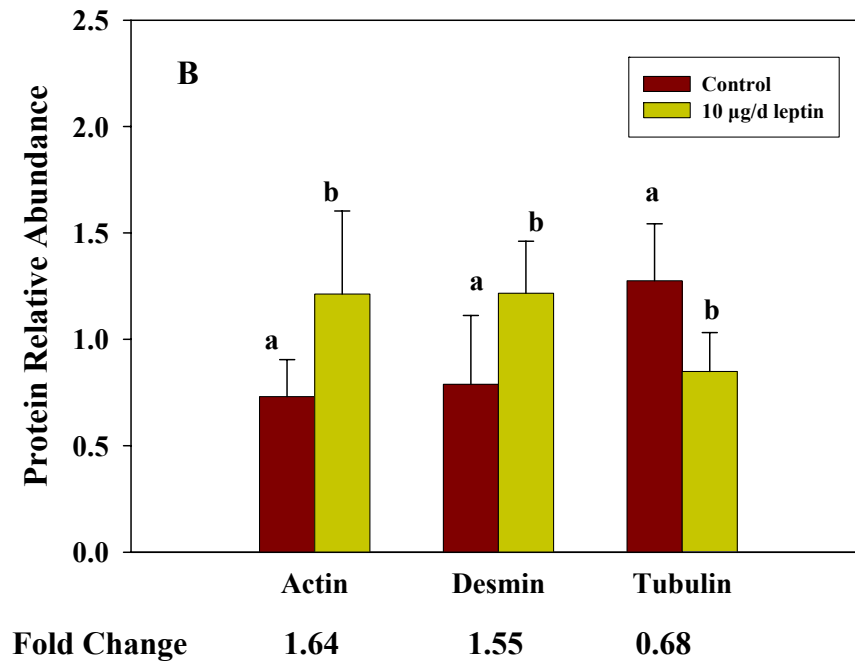
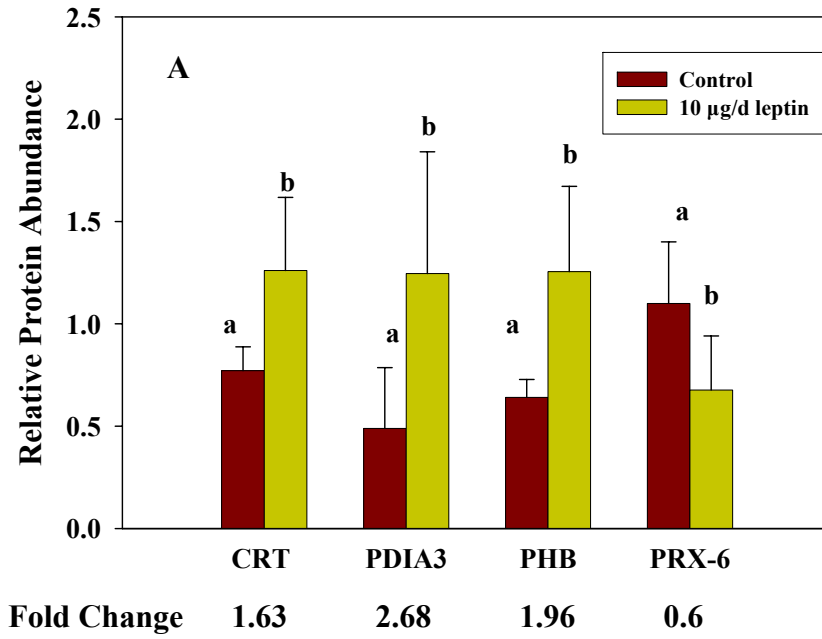


Figure 5.2. Three protein functional groups in adipose tissue of ob/ob mice treated with 10 µg/d leptin. The two major protein groups are molecular chaperons and redox group (33.3%) and cellular structure proteins (25%), other proteins represent about 41.7% of all identified proteins. Under each protein group is the differentially expressed fold change in leptin-treated ob/ob mice compared to control ob/ob mice.





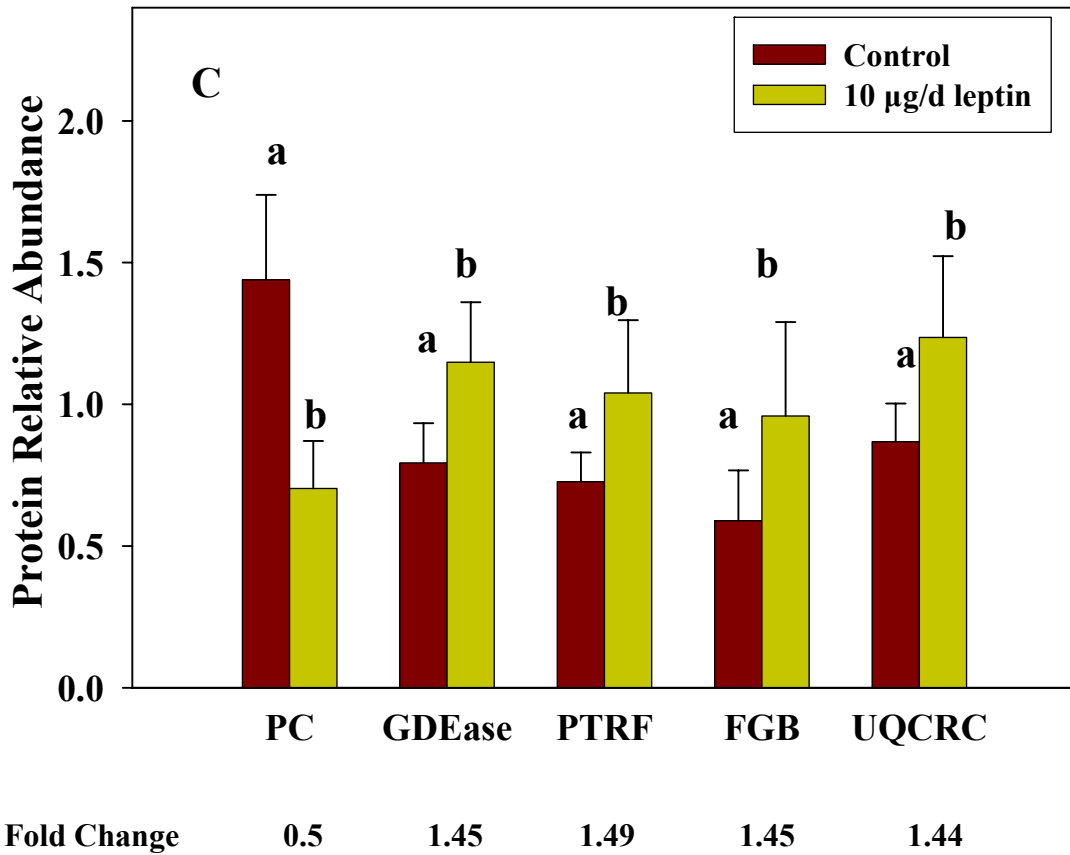


Figure 5.3. Bar charts of functional classes of proteins as molecular chaperons and redox (CRT, PDIA3, PHB and PRX) (Figure 5.3A); cytoskeleton proteins ( $\alpha$  Actin, Desmin,  $\alpha$  Tubulin) (Figure 5.3B) and other proteins (pyruvate Carboxylase (PC), guanine deaminase (GDEase), polymerase I and transcript release factor (PTRF), fibrinogen  $\beta$  chain (FGB) and ubiquinol cytochrome c reductase complex core protein1 (Uqcrc) (Figure 5.3C). The numbers below the picture are the fold change in leptin-treated ob/ob mice compared to control ob/ob mice. Values are standard abundance of proteins (mean  $\pm$  standard error). Note: Means without a common letter are different within each protein,  $p < 0.05$ . Control and treatment stand for the group of control (n=10), 10  $\mu$ g/d leptin administration (n=11).

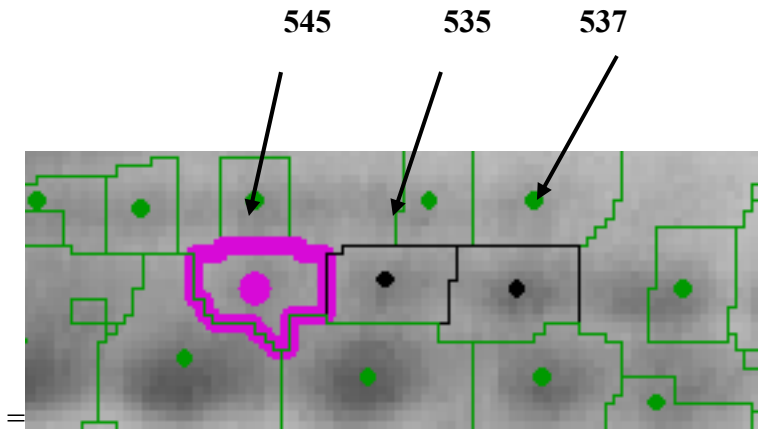


Figure 5.4. Co-migration proteins in spot 545. Both spot 535 and 537 are single protein identified as glucose-6 phosphate 1-dehydrogenase X (G6PD) with a relative ratio about 1, and spot 545 includes co-migration proteins of PDI and G6PD with fold change of 2.68-fold.

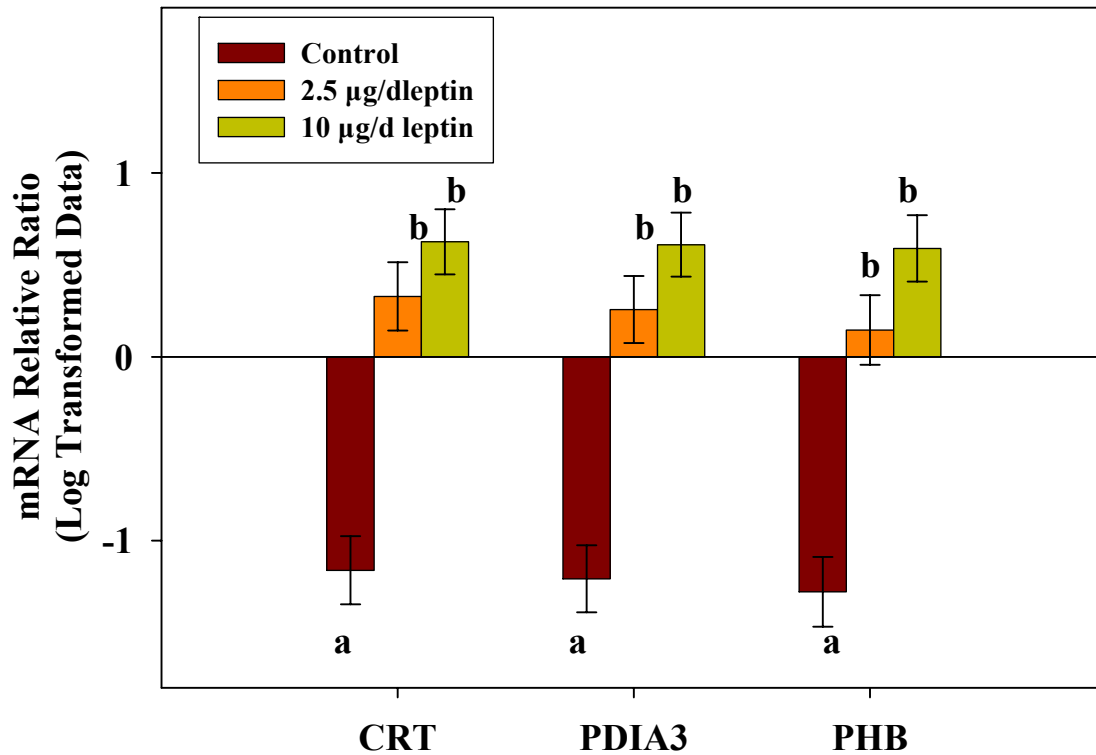


Figure 5.5. Gene expression (CRT, PDIA3 and PHB) in adipose tissue of ob/ob mice treated with leptin (mean  $\pm$  S.E.M.). Total RNA extracted from inguinal adipose tissue was used for analysis of calreticulin (CRT), protein disulfide associated protein 3 (PDIA3), and prohibitin (PHB). The Y-axis is the RQ value after log 2 based transformation as shown by mean  $\pm$  standard error. Note: Means without a common letter are different within each gene,  $p < 0.05$ . The replications for control, 2.5  $\mu\text{g}/\text{d}$  and 10  $\mu\text{g}/\text{d}$  leptin group are 11, 10 and 10, respectively.

**Table 5.1. 2D DIGE Experimental Design**

Block	Gel No.	Treatment	Cy2 (Internal Standard)	Cy3	Cy 5
1 (n=7)	121	Treatment	50 µg	50 µg	50 µg
	122	Control	50 µg	50 µg	50 µg
	123	Treatment	50 µg	50 µg	50 µg
	124	Control	50 µg	50 µg	50 µg
	125	Control	50 µg	50 µg	50 µg
	126	Treatment	50 µg	50 µg	50 µg
	128	Treatment	50 µg	50 µg	50 µg
2 (n=6)	131	Control	50 µg	50 µg	50 µg
	132	Treatment	50 µg	50 µg	50 µg
	134	Control	50 µg	50 µg	50 µg
	135	Treatment	50 µg	50 µg	50 µg
	136	Control	50 µg	50 µg	50 µg
	137	Treatment	50 µg	50 µg	50 µg
3 (n=8)	141	Treatment	50 µg	50 µg	50 µg
	142	Control	50 µg	50 µg	50 µg
	143	Treatment	50 µg	50 µg	50 µg
	144	Control	50 µg	50 µg	50 µg
	145	Control	50 µg	50 µg	50 µg
	146	Treatment	50 µg	50 µg	50 µg
	147	Control	50 µg	50 µg	50 µg
	148	Treatment	50 µg	50 µg	50 µg

**Table 5.2. Probes in Real time PCR II (www.appliedbiosystems.com)**

Gene Symbol	Gene Name	Context Sequence
Bax	Bcl2-associated X protein	CTGGTGCTCAAGGCCCTGTGCACTA
Calr	Calreticulin	TAACATCATGTTTGGTCCGGACATC
18S	Eukaryotic 18S rRNA	CCATTGGAGGGCAAGTCTGGTGCCA
Fgb	Fibrinogen, B beta polypeptide	GAAGCAAGCTCAAGTTAAAGAGAAT
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	GAACGGATTTGGCCGTATTGGGCGC
Phb;Fyb	Prohibitin; FYN binding protein	GACATCGCTGATGGTTGCATCTATG
Pdia3	Protein disulfide isomerase associated 3	ATTAGCAAAGGTGGATTGCACTGCC
Uqcrc1	Ubiquinol-cytochrome c reductase core protein 1	CATGCTACCTGCACGGTGGGAGTGT
Fabp4	Fatty acid binding protein 4	CAAGCTGGTGGTGGGAATGTGTTATG
Itlna	Intelectin a	GTGCAGTGTGGAGACTTTGCGTCAT
Lpl	Lipoprotein lipase	TGGATGGACGGTAACGGGAATGTAT
Pbef1	Pre-B-cell colony-enhancing factor	GAGGAATATGGCCATGATCTTCTCC
Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	CCCCAATGTGGTGGCCATCCCAGAC

**Table 5.3. Proteins Identified by MALDI-TOF MS in Adipose Tissue**

Spot No.	Protein Identification	Accession No.	Theory MW/pI	Peptide Count	Protein Score	Sequence coverage	Fold Change	P value
163	Pyruvate carboxylase	PYC_MOUSE	130 kDa/6.25	23	161	28%	0.5	<0.01
164	Pyruvate carboxylase	PYC_MOUSE	130 kDa/6.25	24	150	28%	0.58	<0.01
495	Calreticulin precursor	CALR_MOUSE	48 kDa/4.33	10	65	19%	1.63	<0.01
527	Polymerase I and transcript release factor	PTRF_MOUSE	44 kDa /5.43	11	65	30%	1.44	<0.01
535	Glucose-6-phosphate1-dehydrogenase X (G6PD)	G6PD1_MOUSE	59.5 kDa /6.07	13	88	31%	1.07	0.92
537	G6PD	G6PD1_MOUSE	59.5 kDa /6.07	17	133	42%	0.93	0.33
545	G6PD + PDIA 3	G6PD1_MOUSE +PDIA3_MOUSE	59.5 kDa/6.07; 57 kDa /5.98	14 ;10	97; 52	32%; 21%	2.68	<0.01
570	Fibrinogen $\beta$ chain precursor	FIBB_MOUSE	55.4 kDa /6.68	15	94	34%	1.93	<0.01
576	Fibrinogen $\beta$ chain precursor	FIBB_MOUSE	55.4 kDa /6.68	19	145	44%	1.63	<0.01
584	Desmin	DESM_MOUSE	53.5 kDa /5.21	20	150	44%	1.55	<0.01
635	Tubulin $\alpha$ -2 chain	TBA2_MOUSE	50.8 kDa /4.94	11	86	33%	0.68	<0.01
693	Guanine deaminase	GUAD_MOUSE	51.5 kDa /5.36	13	100	37%	1.45	<0.01
695	Ubiquinol-cytochrome-c reductase complex core protein 1	UQCR1_MOUSE	53.4 kDa /5.75	9	63	27%	1.49	<0.01
735	Actin, cytoplasmic 1 (Beta-actin)	ACTB_MOUSE	42.1 kDa /5.29	10	71	43%	1.63	<0.01

Continued

Spot No.	Protein Identification	Accession No.	Theory MW/pI	Peptide Count	Protein Score	Sequence coverage	Fold Change	P value
736	Actin, alpha skeletal muscle(Alpha-actin-1)	ACTS_MOUSE	42.4 kDa /5.23	12	95	45%	1.64	<0.01
923	Prohibitin	PHB_MOUSE	29.9 kDa /5.57	8	74	39%	1.96	<0.01
959	Peroxiredoxin-6	PRDX6_MOUSE	25.0 kDa /5.71	11	109	51%	0.6	<0.01

a) Accession number corresponding to Swiss-Prot databases

b) Theoretical MW and pI were calculated using the tool MW/pI from the ExPASy server.

## CHAPTER 6

### SUMMARY AND CONCLUSION

Comparative proteomics analysis and real-time RT-PCR methods were performed in adipose tissue of ob/ob mice treated with leptin in order to identify the protein and gene expression changes as the potential targets of leptin. The finding of 12 protein entries and 18 gene transcripts differentially expressed in adipose tissue with leptin treatment in ob/ob mice suggests that leptin may regulate body weight and lipid metabolism through different routes ending up with similar outcomes: negative energy balance.

This is achieved by a reduction in food intake, increase in energy expenditure, regulation of lipid metabolism, promotion of apoptosis and inhibition of angiogenesis. The increased energy expenditure was shown by increased in body temperature, physical activity in ob/ob mice treated with leptin, combined with the increased expression of UCP2, adrenergic receptor  $\beta$  3, mitofusion 2 and sirturin 3 for promoting thermogenesis, probably through the pathway of sympathetic nervous system innervated to the brown adipose tissue.

The regulation of lipid metabolism by leptin treatment in ob/ob mice was demonstrated by the reduced expression of lipogenetic enzymes such as steroyl CoA desaturase, fatty acid synthetase and increased expression of lipolytic enzyme hormone sensitive lipase, combined with significant reduction of fat mass and fat percentage in ob/ob mice treated with leptin.

The promotion of adipocyte apoptosis was confirmed by an increased expression of Caspase 3 and TNF  $\alpha$ , two factors promoting apoptotic events in mitochondrial pathways, combined with



significantly increased apoptosis by gel electrophoresis in all three fat pads in ob/ob mice treated with leptin.

The inhibition of angiogenesis was shown by the upregulated expression of angiogenesis inhibitor angiopoietin-2. Increased sensitivity of insulin mediated by leptin was demonstrated by the increased expression of adiponectin (promote insulin sensitivity) and decreased expression of retinol binding protein 4, a glucose transporter inhibitor and insulin sensitivity inhibitor, together with serum insulin in ob/ob mice treated to leptin decreasing to the level of wild-type lean mice.

For the global protein analysis, a modified protocol and extraction buffer was described in order to enhance the proteome from adipose tissue due to its difficulty of handling proteins from adipose tissue. There are three main types of detergents in the extraction buffer: 8.4M urea and 2.4M thiourea are detergents for denaturing proteins with strong efficiency; 2% CHAPS (v/v) and 2% ASB14 (v/v) are detergents for completely solubilizing the denatured proteins; and 50 mM DTT is applied as a reducing reagent for folding correctly the disulfide bonds of proteins. This optimized protocol is the basis of 'real' proteomic analysis of the proteins from adipose tissue for the study in chapter 5.

In chapter 5, 2D-DIGE combined with MALDI-TOF MS methods were used to identify differentially expressed proteins between parametrial adipose tissue of control and leptin-treated ob/ob mice. Twelve protein entries were differentially expressed, including molecular chaperones and redox proteins (CRT, PDIA3, PHB, Prx-VI), cytoskeleton proteins ( $\beta$ -actin,  $\alpha$ -tubulin and desmin) and other proteins (PC, GDEase, UQCRC, PTRF, and fibrinogen). Some of them have been shown to be related to apoptosis in other cell lines or rodents such as calreticulin, protein disulfide associated protein 3, prohibitin and peroxiredoxin-VI and actin. Others have been demonstrated to be aligned to lipid metabolism or obesity such as pyruvate

carboxylase, guanine deaminase, ubiquinol-cytochrome-c reductase complex core protein 1, polymerase I and transcript release factor, and possibly fibrinogen. Still others like desmin and tubulin are not clearly delineated, and therefore further research is needed for elucidating the role of these proteins in leptin-induced adipose apoptosis.

In total, regulation of these genes/proteins or other unknown mechanisms might be involved in the signaling pathways mediating leptin-induced adipocyte apoptosis and fat loss in leptin-treated ob/ob mice, and it provides new potential targets and regulators of leptin on adipose tissue. Thus, the findings presented here open new insights for potential treatments of obesity.

**APPENDIX A**  
**ABBREVIATIONS**

2-DE: Two-dimensional electrophoresis

ABI: Applied biosystems

ACN: Ammonium bicarbonate

ADRB3: Adrenergic receptor  $\beta$  3

Angpt2: Angiopoietin 2

ASB-14: Amidosulfobetainers 14

Bcl-2: B-cell lymphoma 2

C/EBP  $\alpha$ ,  $\beta$ ,  $\delta$ : CCAAT/ enhancer binding proteins alpha, beta and delta

CHAPS: 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate

CLA: Conjugated linoleic acid

CNTF: Ciliary neurotrophic factor

CRT: Calreticulin

DIGE: Difference in gel electrophoresis

DMF: N, N-dimethylformamide

DTT: Dithiothreitol

EGCG: Epigallocatechin gallate

FAS: Fatty acid synthase

FGB: Fibrinogen  $\beta$  chain

G6PD1: Glucose-6-phosphate 1-dehydrogenase

GDEase: Guanine deaminase

HED: 2-Hydroxyethyl disulfide

HSL: Hormone sensitive lipase

IAA: Iodoacetamide

ICAT: Isotope-coded affinity tag

IEF: Isoelectric focusing

IL: Interleukin

ING: Inguinal

MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight

MFN2: Mitofusion 2

MS: Mass spectrometry

PC: Pyruvate carboxylase

PCD: Programmed cell death

PDIA3: Protein disulfide isomerase associated 3

PHB: Prohibitin

POMC: Proopiomelanocortin

PPAR- $\alpha$ ,  $\gamma$ : Peroxisome proliferation-activated receptor- $\alpha$ ,  $\gamma$

Prx-VI: Peroxiredoxin-VI

PTRF: Polymerase I and transcript release factor

qPCR: Real-time RT-PCR

RBP4: Retinol binding protein 4

RP: Retroperitoneal

SCD1: Stearoyl-coenzyme A desaturase 1

SIRT3: Sirtuin 3

SNS: Sympathetic nervous system

SREBF 1: Sterol regulatory element binding factor 1

TFA: Trifluoroacetic acid

TNF  $\alpha$ : Tumor necrosis factor alpha

UCP2: Uncoupling protein 2

UQCRC: Ubiquinol-cytochrome-c reductase complex core protein 1

VEGF: Vascular endothelial growth factor

TAG: Triacylglycerol or Triglyceride

## APPENDIX B REAL-TIME RT-PCR AND PROTEOMIC ANALYSIS

### B.1. Introduction of Transcriptomics and Proteomics

The rapid pace of genome sequencing in the past few years and dramatic advances in the molecular technologies make it possible for biologists to develop global analysis of DNA (genomics), mRNA (transcriptomics), protein (proteomics), and metabolites (metabolomics) (Figure Appendix B.1). All these –omics methods can profile the global changes that occur in the cell, rather than the previous method of measuring one single analyte at a time. Using one technological approach alone cannot provide enough information for a comprehensive understanding of complex biological and physiological phenomena. An approach that harmoniously integrates more than one ‘omic’ study (e.g. transcriptomics, proteomics), can compare the resulting data in order to connect genes to functions (219).

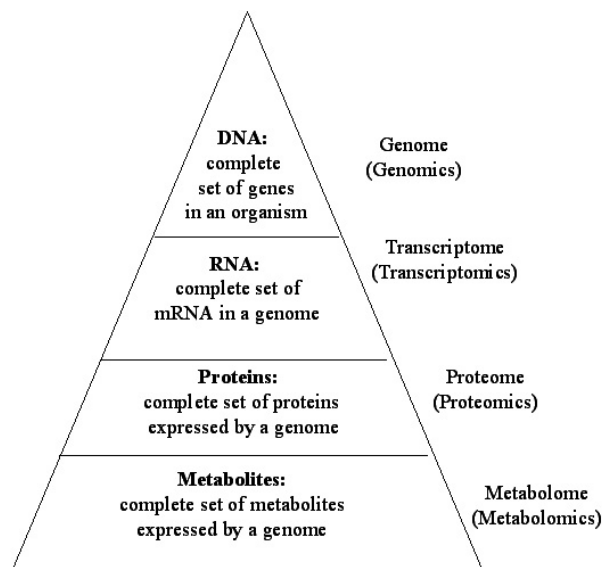


Figure Appendix B.1. Four platforms and their corresponding study of system biology: genome (genomics), transcriptome (transcriptomics), proteome (proteomics), and metabolome (metabolomics) (219)

An organism (e.g. ob/ob mice) frequently responds to various internal or external stimuli (e.g. leptin treatment) by intricately regulated systems. Cellular functions are mediated through significant changes in expression of many genes and/or their protein products. In order to elucidate the pathophysiological mechanisms involved in a disease like obesity, and to develop the proper models to clarify the dynamics of molecular and cellular function, different strategies must be integrated in a quantitative and temporal manner, particularly the study of RNA and protein expression patterns.

The transcriptome is the complete set of all mRNA molecules or transcripts in a genome. Proteome is the complete set of all proteins expressed by a genome. Transcriptomics and proteomics are the study of the transcriptome and proteome. Unlike the genome, which has a fixed number of genes expressed for a given cell population in a certain development phase, the transcriptome and proteome are dynamic, and can vary with various internal or external stimuli in a given cell type under defined treatment. Therefore, studies of the transcriptome and proteome are much more complex, and they reflect the genes/proteins that are actively expressed under defined conditions (220).

Transcriptomics and proteomics data are closely related, although different. On one hand, it is proteins, not genes that actively take part in the cellular function. On the other hand, mRNA is an intermediate step in the production of a protein, and it can be used to predict protein expression to some degree. Technologically, transcriptomic analysis using microarray technology is a high-throughput technology capable of simultaneously quantifying tens of thousands of mRNA molecules. In contrast, the traditional proteomic analysis using 2-dimensional electrophoresis is a labor-intensive and time-consuming platform for protein separation, and the target proteins are limited to proteins with high abundance, soluble, and with

a suitable pH (pH 3-10) and molecular weight (10-200 kDa). Therefore, it is imperative to integrate mRNA and protein expression into a common frame, analyzing changes in expression of genes/proteins in functional categories globally, thereby providing a broader view of systematic instead of individual alteration of genes/proteins.

## **B.2. Real-time Quantitative RT-PCR (qPCR or Real-time RT-PCR)**

The traditional method for quantification of transcription includes northern blotting, *in situ* hybridization (221), RNase protection assay (222), qPCR (223), and cDNA array (224). Of these, qPCR is widely used due to its high sensitivity, specificity and reproducibility.

### **B.2.1. Introduction of qPCR**

qPCR was first described in the 1990s (225), but it has only recently been widely accepted as a valuable technique due to the application of low-cost thermocyclers and reagents. qPCR takes advantage of special chemistries (probes) to report the presence of PCR products, dedicated instrumentation for monitoring the signal as PCR progresses (i.e. in real time) and appropriate software for quantitative analysis (226). Data from the fluorescent signal are collected throughout the exponential phases of the PCR process rather than at the end of the PCR process (end-point PCR). Therefore, it avoids the problems associated with quantitative detection in the plateau stage of traditional PCR, and there are many advantages of qPCR to quantify gene expression ([www.appliedbiosystems.com](http://www.appliedbiosystems.com)).

qPCR is highly sensitive (can even detect a single copy of a specific transcript) (227), highly sequence-specific, and reproducible (228), reliable, simple (without post-PCR processing), amenable to high sample throughput benchmark technology with wide dynamic range (7-8 log orders of magnitude) (229) for the detection and/or comparison of RNA levels quantitatively (230,231). The only disadvantages of qPCR are expensive equipment and reagents. In addition,



sound experimental design, appropriate normalization choices and reasonable models for data analysis are essential components for correct conclusions.

The general procedure of qPCR is the following: total RNA from tissue or cell lines are extracted and used as a template to reverse transcribe to cDNA, followed by exponential amplification in a PCR reaction (Figure Appendix B.2). During each cycle of amplification, the fluorescent signals attached to the probes are detected.

The instrument and reagents used in the present study were from Applied Biosystems (ABI) (Foster City, CA). The ABI instrument used for this research was a ABI 7900HT Fast Real-Time PCR System (7900HT Fast System) combined with TaqMan<sup>®</sup> Low Density Arrays (LDA). It is a medium-throughput design for 384-well format with highly sensitive laser. It is based on fluorescent chemistries to detect the gene expression quantitatively as PCR progresses. Several assay types can be performed on this system such as 96-well, 384-well and TaqMan<sup>®</sup>LDA ([www.appliedbiosystem.com](http://www.appliedbiosystem.com)). Another advantage of ABI 7900 is the “passive reference dye” (ROX<sup>™</sup>), to which the reporter dye signal can be normalized for the variation in the optics of the system (232), which leads to increased data precision.

### **B.2.2. Theory and Quantitation of qPCR**

The polymerase chain reaction (PCR) was first described in 1985 (233,234), and it is a technique for enzymatic amplifying *in vitro* DNA. In PCR, the DNA template doubles with every subsequent cycle of denaturation, annealing, and synthesis (234). RT-PCR differs from PCR only by the addition of an initial step of reverse transcribing mRNA into cDNA. Real-time RT-PCR is based on fluorescent probes to collect data throughout the PCR process allowing gene expression to be measured quantitatively.

The qPCR plot is the plot of the fluorescent signals versus PCR cycle number (Figure Appendix B.3). With each PCR cycle, the fluorescence signals become accumulated from the baseline to the threshold where they are significantly higher than background fluorescence. The threshold cycle value (Ct) is the fractional PCR cycle number at which the fluorescent signal reaches threshold, and it is the basis for the DNA/RNA relative quantification (or comparative Ct) method, which is in contrast to the absolute quantification (standard-curve) method. The comparative Ct method is specially applied when only a limited amount of RNA can be obtained or when a large number of samples need to be processed ([www.appliedbiosystems.com](http://www.appliedbiosystems.com)).

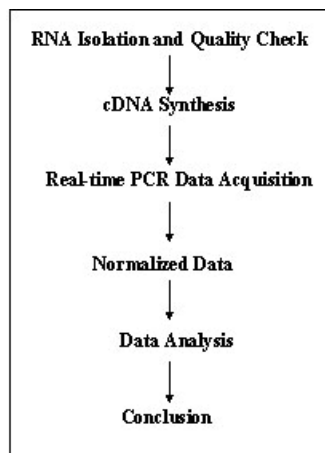


Figure Appendix B.2. Experimental steps of Real-time PCR. Total RNA from tissue or cell lines are extracted and used as a template to reverse transcribe to cDNA, followed by exponential amplification in a PCR reaction. Normalized data are obtained based on the calibrator sample after normalization to the housekeeping gene (HKG), and the correct statistical model is created and statistical analysis is performed. The conclusions are drawn based on the analytical results.

Consequently, the Ct value and the target genes in initial samples are inversely related, and the higher the initial input cDNA copy number, the faster the fluorescent signal will become greater than background fluorescence, therefore producing a smaller Ct value. Of course, the opposite is also true. Thus, the comparative Ct assays are used to analyze the changes in

expression of a nucleic acid sequence (target) in a treated sample to the same sequence relative to an untreated control sample (calibrator) after normalization to a housekeeping gene (HKG) (more detailed in the Section of Normalization) (235). Therefore, the amount of target, normalized to an endogenous control and relative to a calibrator, is calculated as the fold change (RQ), and  $RQ = 2^{-\Delta\Delta CT}$ , where  $-(\Delta\Delta CT) = -(\Delta Ct, \text{treatment} - \Delta Ct, \text{control}) = -[(Ct, \text{Target} - Ct, \text{GADPH})_{\text{treatment}} - (Ct, \text{Target} - Ct, \text{GADPH})_{\text{control}}]$  (235). The RQ method allows accurate comparison of target genes between the treated sample and untreated control samples without the application of standard curves.

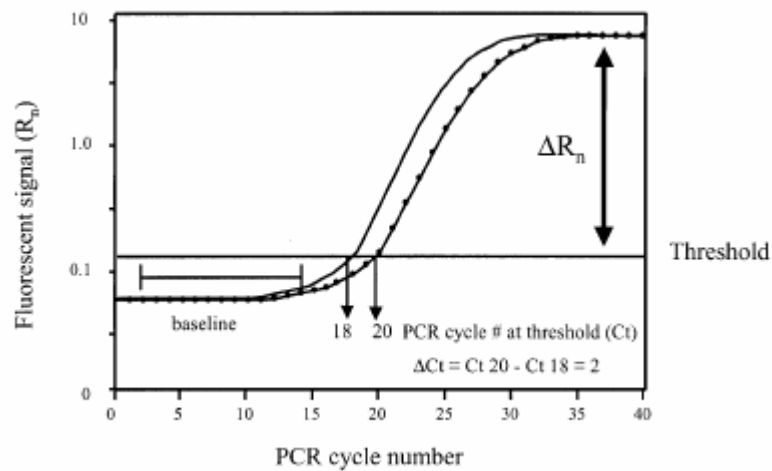


Figure Appendix B.3. Hypothetical amplification plot of qPCR (232). The amplification plot is the plot of fluorescence versus PCR number. The threshold cycle value (Ct) is the fractional PCR cycle number at which the fluorescent signal becomes accumulated enough to reach threshold for detection. Therefore, the input cDNA copy number and Ct are inversely related. The fold change (RQ) =  $2^{-(\Delta\Delta CT)}$ , where  $-(\Delta\Delta CT) = -(\Delta CT, \text{treatment} - \Delta CT, \text{control}) = -[(Ct, \text{Target} - Ct, \text{GADPH})_{\text{treatment}} - (Ct, \text{Target} - Ct, \text{GADPH})_{\text{control}}]$ .

The RQ method assumes that the ideal amplification efficiency of all target genes and the endogenous control is 1, indicating that PCR product concentration increases twofold every cycle within the exponential phase of the reaction. When the RQ method is applied by the ABI systems, there is automatic correction of amplification efficiency (i.e. if not 1) (235), as long as

the amplification efficiency of the target genes and HKGs are approximately identical, preferably over 90% or more. In addition, new primers can be designed and optimized to promise a comparable efficiency for the each of the target genes and HKGs by ABI systems, as performed in the present study. An alternative method to assess the amplification efficiency of two genes is to determine how  $\Delta\Delta CT$  varies with template dilution (235).

### **Normalization**

In theory, the purpose of the endogenous control gene, usually the housekeeping gene (HKG), is to normalize the starting material RNA for RT reaction, or the efficiency of the reverse transcription. One of the assumptions of the RQ method was that the expression level of a chosen HKG does not vary across experimental samples, and it may serve as an active reference for the qPCR reaction. The commonly applied HKGs include GAPDH,  $\beta$ -actin, and 18s rRNA, since their expression tends to be relatively stable (236). However, there are numerous reports (237) stressing that no single gene can be constitutively expressed in all cell types under all developmental phases and treatments, and every gene is regulated to some degree. Therefore, it is recommended that multiple endogenous control genes are chosen, compared and validated to identify those which are not affected by the experiment treatment. An alternative approach can be performed to validate the effect of experimental treatment on the expression of the internal control gene (235).

### **Primers and Probes**

Usually three kinds of primers are used to synthesize cDNA from mRNA: the random primers, oligo-dT, and target gene-specific primers (231). Random primers are used in the current real-time RT-PCR assays due to the advantage that RT was primed at multiple origins along every RNA template, hence producing more than one cDNA target per original mRNA

target (231). Furthermore, primers designed to span the exon-exon boundary can efficiently prevent detection of genomic DNA, therefore providing specificity (232).

### **B.2.3. Essentials of Real-time RT-PCR**

#### **Quality of RNA**

The extraction of total RNA from the sample is the initial step of qRT-PCR assay, and its quality is undoubtedly the most critical component for reproducibility and relevance of the following qRT-PCR results. RNA is tremendously susceptible to degradation once removed from its cellular environment. Therefore, its quality must be assessed for integrity before the following reverse transcription reaction. The total RNA applied in RQ experiments should not be contaminated with RT or PCR inhibitors, and be intact when visualized by gel electrophoresis. The traditional gel electrophoresis requires a significant amount of valuable RNA, and it is a time-consuming technique. The advanced technology of Agilent's Bioanalyzer 2100 and RNA Lab Chip (Palo Alto, CA) applied in the current experiment is by far the most convenient and objective way of assessing the quality and quantity of RNA.

#### **RT and PCR Reactions**

After high-quality mRNA is extracted from tissue, it is reversed transcribed to cDNA by a high capacity cDNA archive kit (PN 4322171) in order to perform the first step (RT) in the two-step RT-PCR method. The following table is optimized for thermal parameters (Table Appendix B.1) ([www.appliedbiosystems.com](http://www.appliedbiosystems.com)).

Table Appendix B.1. The thermal parameters for RT reaction

Step Type	Time (min)	Temperature
Hold	10	25°
Hold	120	37°

After RT is complete, the second step of PCR in the two-step RT-PCR is initiated in order to amplify cDNA. In this step, TaqMan<sup>®</sup> Universal PCR Master Mix is mixed with sample cDNA (Table Appendix B.2), and loaded into eight TaqMan<sup>®</sup> Array sample ports; each port feeds into 48 reaction wells for a total of 384 reactions. The TaqMan<sup>®</sup> array is then run on the 7900HT system for data collecting and quantitative analysis.

Table Appendix B.2. The components of PCR reaction

Component	Volume (µl) per fill reservoir
cDNA sample (1000 ng) + RNase-free water	50.0
TaqMan <sup>®</sup> Universal PCR Master Mix (2X)	50.0
Total Volume	100.0

For real-time PCR reactions, the following condition below is the universal default recommended thermal cycle condition is used 94.5° 15s for initial template denaturation, and 40 cycles of 97° 30s for denaturation and 59.7° 1 min for anneal/extension. This universal cycling condition eliminates the need for optimization of the thermal cycles.

#### **ABI TaqMan<sup>®</sup> Low Density Array**

The TaqMan<sup>®</sup> Low Density Array is a 384-well micro fluidic card which is preloaded with customizable probes and primer pairs of target genes from TaqMan<sup>®</sup> Gene Expression Assays, thus allowing 384 simultaneous real-time PCR reactions, and analyzes from one to eight cDNA samples according to the number of target genes using random primers on 7900HT Systems. It is a low- to medium- throughput and a standardized technique for measuring and/or screening gene expression across many samples. The array in the current experiment is a 24-format array, which contains two endogenous control genes and 22 target genes, and allows measuring 8 cDNA samples simultaneously ([www.appliedbiosystems.com](http://www.appliedbiosystems.com)).

The whole procedure is very simple to perform and without need for liquid-handling robots or multi-channel pipettors to load samples (Figure Appendix B.4). It is an efficient method to validate micro-array hits quickly and economically.

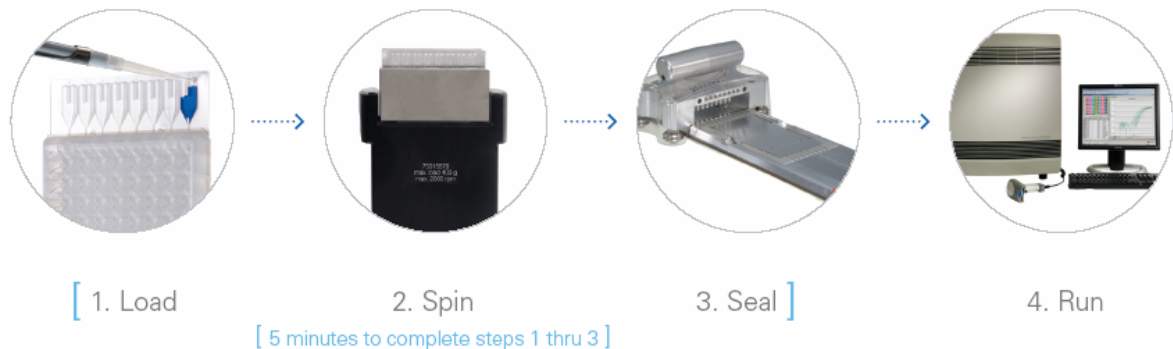


Figure Appendix B.4. The TaqMan<sup>®</sup> Low Density Array streamlines the reaction set-up process in four simple steps. Loading the array with a cDNA sample and TaqMan<sup>®</sup> Universal PCR Master Mix takes only five minutes ([www.appliedbiosystems.com](http://www.appliedbiosystems.com)).

#### **B.2.4. Conclusion of qPCR**

The combined application of TaqMan<sup>®</sup> Low Density Array (LDA) for the RQ method in the field of real-time RT-PCR undoubtedly can generate reliable, reproducible, and low- to medium throughput results for measuring mRNA expression level quantitatively. This method can be applied to measure significant changes in the expression of many genes in response to the various external/internal stimuli (such as leptin) in animal model system (such as ob/ob mice). Since the mRNA level can predict the level of protein expression to some degree, the differential expression profiles of mRNA from adipose tissue between control and leptin treated ob/ob mice can suggest some potential bio-markers responsive to leptin.

There are no previous publications that combined TaqMan<sup>®</sup> Low Density Array with qPCR method to quantitatively measure the adipose tissue mRNA expression levels in ob/ob mice

treated with leptin, and but it is an efficient method to validate micro-array hits (138) quickly and economically (238,239).

### **B.3. Proteomics**

#### **B.3.1. Introduction of Proteomics**

Proteomics, the term coined in 1994, was the linguistic equivalent to genomics. It was initially defined by Gygi et al (240) as the complete set of proteins globally and quantitatively in a cell line, tissue, or organism at a given time point. The definition of proteomics has now been broadened to embrace the study of comparative proteomics (differential display proteomics or protein expression profiling), and cell-mapping proteomics (sub-cellular distribution, protein - protein interactions) (3).

The tremendous progress in proteomics can be attributed to the complement sequencing of genomes of several species, the technological breakthrough of mass spectrometry for analyzing macromolecules, and protein databases available in the public domain. It is the product of technological advances in chemistry, physics, bioinformatics, mathematics and molecular biology. The emergence of proteomics has made it possible to automate the analysis and identification of proteins in a large-scale manner, which is greatly improved in comparison to the previous one-by-one analytical techniques of proteins. Therefore, it is a promising, high-throughput technique.

Since the proteome of a cell is only a reflection of its immediate stimuli in the environment, it is much more dynamic in nature. Basically, there's a change of the proteome of each cell at each time point under each development phase, and every picture of gel is like taking a “snapshot”. Contrary to proteomics, genomics is static, since at any time there is only one set of genes in a given organism. From this aspect, the proteome dataset is much larger and more



complex because most mammalian genes show alternative splicing of transcripts, and the proteins are functionally inactive only until they undergo different post-translational modifications in the ER.

The traditional proteomic analysis usually has two steps: protein separation by 2-dimensional electrophoresis and protein identification by MS. The first high resolution 2-DE was developed independently by O'Farrell (241), Klose (242), and Scheele in the 1970s (243), who began separating proteins from *Escherichia coli*, mouse, and guinea pig. Proteins are separated by their orthogonal characteristics, isoelectric point (pI) and molecular weight (MW). Although many technological improvements have been made for protein separation in the early stage, the science of proteomics would wait until the sensitive and rapid analytical methods for protein identification had emerged (such as the polymerase chain reaction and the automated sequencer for DNA analysis). In the 1990s, technological breakthroughs for peptide analysis by MS have marked the beginning of a new era for proteomics science.

### **B.3.2. Technology of Proteomics**

Comparative proteomics is one of the frequent applications of 2-DE. A comparison of 2-D gel images between control and treatment samples is usually performed, and the appearance or disappearance of spots, as well as the abundance of those spots offer qualitative and quantitative information about varying expression patterns of proteins. The comparison can be performed among cells treated with various treatments, or tissue of normal versus disease status. This allows identification of potential diagnosis markers, drug targets and specific biological events. This review will focus on the technology of comparative proteomics.

The standard approach of comparative proteomics is to prepare samples and solubilize proteins, and isolate proteins by 2-DE. The gels are then scanned and visualized by a special

instrument. Comparison and quantification of the protein spots are performed via highly complex software packages. Consequently, the target protein spots are selected according to the standards, and mostly cleaved and digested with amino acid-specific trypsin by in-gel digestion. The data of peptide mass fingerprints (PMFs) analyzed by MALDI-TOF. The proteins are then identified and characterized through comparing and searching protein databases (NCBI and SWISS-PROT) with the help of search engine Mascot (Figure Appendix B.5).

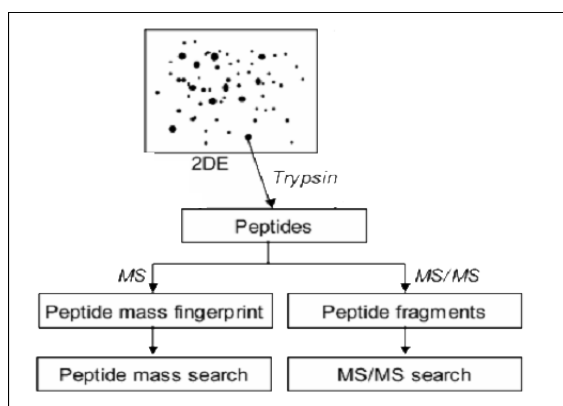


Figure Appendix B.5. Common procedure for proteomics (244)

### Sample Preparation

Sample preparation is often a critical component to perform 2-DE, and the proteins from the samples are denatured, disaggregated, and reduced in order to be solubilized reproducibly (245). It is difficult to visualize all proteins from a tissue due to the highly dynamic range of protein abundance, and the variety of proteins with respect to their various properties of MW, pI, and hydrophobicity. Although there is no single method for sample preparation that can be applied to all kinds of samples, the general rules for protein extraction are simple. With these methods protein modification can be prevented, interfering molecules such as proteolytic enzymes, lipids and nucleic acids can be removed, and the protein of interest is likely to be in a range that can be detected. Generally speaking, samples should be kept as cold as possible all the time (246).

Urea solutions should always be used fresh, and samples containing urea must not be heated to avoid the carbamylation due to the decomposition of urea (247).

Cell lysis, removal of interfering substances, and solubilization of the proteins are three major steps for sample preparation. Lysis of different types of cells can be achieved by homogenizers, liquid nitrogen mortar and pestle method, enzymatic method, sonication, bead method, or a combination of these methods. After cell lysis, it is necessary to remove or inactivate the interfering compounds such as proteolytic enzymes, salts, lipids, and all other insoluble components by high-speed centrifugation. Cold TCA/acetone precipitating technique is also a very useful method for removing salts and lipid, but protein loss may occur to some degree due to incomplete precipitation and/or resolubilization of proteins. 2-D clean-up kit (GE Healthcare) takes advantages of the TCA/acetone method for optimization, and is an alternative method (248). Spin dialysis is a commonly used method to remove salts. Proteases must be inactivated to prevent protein degradation, but high concentration of urea can effectively prevent protein degradation.

In the present study to obtain proteins from adipose tissue of ob/ob mice, homogenization was applied for cell disruption, high-speed centrifugation and ultra-centrifugation; 2-D clean-up kit and spin dialysis were all applied for removal of interfering compounds such as lipids, salts, etc.

After cell lysis and the removal of interfering substances, the individual polypeptides must be solubilized, denatured and reduced to completely break the interactions between the proteins. Sample solubilization is usually carried out in a buffer containing chaotropes (e.g. urea and/or thiourea), nonionic and/or zwitterionic detergents (e.g. CHAPS), reducing agents depending on the type of sample, and protease inhibitors. The “standard” IEF sample solution (8 M urea, 4%

CHAPS, 50-100 mM DTT and 40 mM Tris base), is not ideal for many membrane proteins and samples such as adipose tissue, and serum. The inclusion of thiourea in the IEF procedure greatly increases the resolution of the gel spots, especially for membrane proteins (165,166) and adipose tissue (167). Surfactants help to solubilize the unfolded proteins (membrane proteins) following exposure by strong chaotropic agents and is a useful detergent for the solubilization of membrane proteins in proteomics (172,177,249).

### **Separation of Proteins by 2-DE**

One of the bottleneck steps in any proteomic analysis study is protein separation. In spite of alternative technologies that have emerged (Multidimensional Protein Identification Technology, Mud PIT (250)), protein or antibody arrays (251), shotgun proteomics technique (178), isotope coded tag technique (ICAT) (252), 2-DE still remains the most effective element to resolve proteins from a complex mixture, and it is a reliable technique that can be applied to comparative expression profiles. Here the protein expression profiles of any two samples can be qualitatively and quantitatively compared, and the changed expression of target proteins can provide information about treatment/stimuli. In 2-DE, proteins are separated according to their charge by IEF in the first dimension and molecular weight by SDS-PAGE in the second dimension. Usually the standard 2-DE format can isolate and visualize 1000-3000 proteins depending on the sensitivity of staining method. The molecular weight of the proteins separated are usually about 10-300 kDa, and 1ng is the sensitivity for each spot in 2-DE. One of its greatest advantages is its ability to resolve proteins undergoing some form of posttranslational modification.

### **Isoelectric Focusing (IEF) of 2-DE**

IEF with immobilized pH gradient strip (IPG) (253) (Figure Appendix B.6A) has replaced the carrier ampholyte (CA) –based gel, and becomes the method of choice for the first dimension

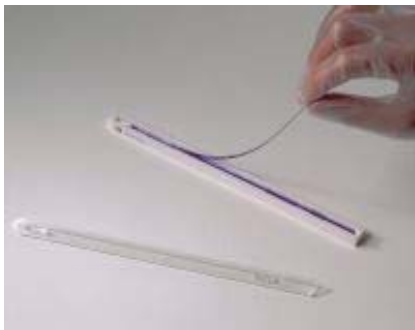
of 2-D PAGE. The advantages of application of IPGs include the increased reproducibility, the enhanced resolution ( $\Delta pI = 0.0001$ ) (159) and loading capacity (254). IPG can be linear or nonlinear (255), with different pH range (e.g. IPG 3-12, IPG 4-7, IPG 4.5-5.5), as well as in different lengths (e.g. 7-24cm) (248). Prior to IEF, the IPG dry strips must be rehydrated to their original thickness with a rehydration buffer, usually containing 8 M urea (or 2 M thiourea and 6 M urea), 2% CHAPS, 0.4% DTT as a reductant and 0.5% v/v carrier ampholyte (e.g. IPG buffer or Pharmalyte 3-10). IPG strip manipulation can be either by in-gel or cup-loading rehydration. Active rehydration (e.g. by applying low voltage under 50V) during re-swelling can be applied to IEF in order to facilitate the entry of higher molecular weight proteins (such as 150 kDa). Usually each IPG strip is set to 50mA for IEF, and temperature is an important parameter on the 2-DE gel, where 20° C is the optimal condition (256). The amount of protein loaded onto a single IPG gel strip depends on the separation distance, pH gradient and protein complexities with the aim to guarantee the maximum spot numbers and the minimum streaking/background. For analytical gels, 100 mg of protein can be typically applied on an 18cm long, wide pH range gradient (pH 3-10). For preparative gels, 5-10 times more samples can be applied.

The instrument IPGphor (GE Healthcare) (Figure Appendix B.6B) has simplified the IPG-IEF procedure of 2-DE, where rehydration and IFE steps can be completed in one-step, and the temperatures are controlled strictly and automatically (257). This instrument can accommodate up to twelve individual strip holders, therefore 12 IPG strips can be run simultaneously.

### **SDS-PAGE**

Prior to the second dimensional SDS-PAGE, the IPG strips are equilibrated in order to enhance the protein transfer from the first to the second dimension and to interact completely with SDS. SDS is an anionic detergent which denatures the proteins, unfolds them to a linear

molecular structure and imparts a negative charge. When reducing agents such as DTT is present, proteins are therefore well separated according to their mass in SDS-PAGE.



A. IPG strips



B. IPGphor

Figure Appendix B.6. IPG strips (A); IPGphor (B) ([www.amersham.com](http://www.amersham.com))

The second dimension can be performed on horizontal or vertical systems (258). Horizontal setups are suited for ready-made SDS polyacrylamide gels; whereas vertical systems allow multiple gels to run in parallel, therefore increasing the reproducibility and saving time (259). The Laemmli system has been the standard buffer used to perform SDS-PAGE (260). As with the IPGphor, the Ettan Dalt II system (GE Healthcare) allows up to 12 gels to be run simultaneously and the electrophoresis procedure often takes 4-6 hours to complete in a temperature-control manner. Protein extracts are usually separated by IPG strip with pH 3-10 in the first dimension and 9-16% linear gradient gels with format (18 X 20cm) in the second dimension in vertical setups (246). This system usually provides a general image of the proteome with molecular weight between 5–200 kDa with proper staining method (261).

### **Limitation of 2-DE**

The number and type of proteins resolved and visualized by 2-DE are limited. Only the highly abundant, soluble proteins can be identified, while the low-abundant, hydrophobic

proteins, can not be readily detected during analysis of total proteins (240). Due to their interesting roles as potential targets of drug and in signal transduction, many enrichment methods have been applied for low-copy proteins such as sub-cellular fractions and organelles (262), and enrichment of proteins from larger volumes by selective fractionation, immuno-precipitation, chromatographic methods (263-265). The introduction of thiourea and urea, as well as some novel zwitterionic detergents such as ASB(n) and CΦ8, benefit at least some membrane proteins (165).

The proteins with extreme pH and low/high molecular weight are frequently underrepresented in 2-DE (Figure Appendix B.7) (266). This picture shows the sorting proteins by charge and MW from mouse liver, and it is observed that most proteins identified have masses between 10-80 kDa and pI range 4-10 (266). Some improvements in IPGs have enabled the analysis of very acidic (pH2.5) or basic (pH12) proteins (248,267). IPGs up to 54 cm length have been applied (268).

In order to separate low or high molecular mass proteins, gels with appropriate acrylamide concentration have to be used (269,270). Tricine gels are specially applied to small proteins with a mass range of about 6-25 kDa. In addition, LC-MS integration provides isolation alternatives for the above mentioned proteins that have little chance to be separated on the 2-DE (271).

### **Staining**

Once the protein spots are separated by 2-DE, they can be stained and further visualized by the use of colorimetric and fluorescent dyes. The ideal staining dye should have a good linear relationship between quantity of protein and the staining intensity, wide dynamic range, mass spectrometry - compatible, easy-to-perform, and not too expensive. In spite of a number of different dyes available, silver and Coomassie stains are most frequently used staining methods.

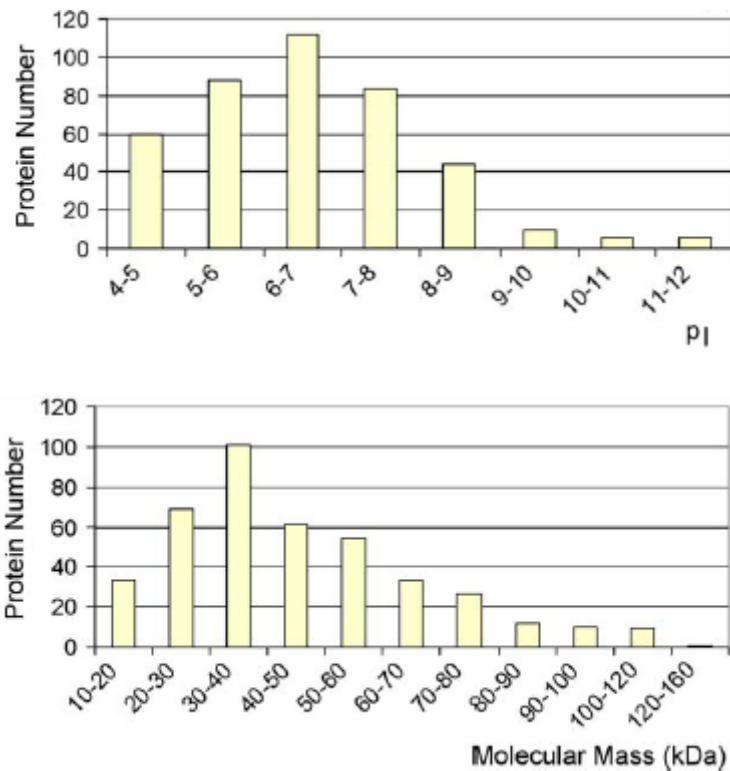


Figure Appendix B.7. The distribution of proteins in the different range of pH and molecular weight (266)

Coomassie is preferred when the gels are intended for protein identification by mass spectrometry, while silver stain is preferentially employed for gel comparison (248). Coomassie staining uses a low cost dye, and a simple procedure, and it typically only detects 8–10 ng of protein, while silver can stain trace proteins in the 1 ng range (20 fm dyes is just across 1-2 orders. Recently, the developments in fluorescence technologies have gained popularity, such as SYPRO Ruby and CyDyes, due to their high sensitivity (equal to silver stain), wide linear dynamic ranges (3 orders), MS-compatibility and highly reproducibility. Therefore, it is particularly appropriate for quantification in comparative proteomics and suitable for detecting



protein spots with low-intensity (272). The only disadvantages are that the dyes are more expensive, and a special instrument is needed to visualize the spots and to acquire the image for image analysis.

### **Differential in-gel Electrophoresis (DIGE)**

The exciting advance in 2-DE was the technology of difference in-gel electrophoresis (DIGE) (273). CyDye™ DIGE Fluor minimal dyes (Cy2, Cy3 and Cy5) are size- and charge-matched fluorescent dyes, and are specifically designed to accurately analyze differences in protein abundance between samples using Ettan™ DIGE systems. This system comprises CyDye DIGE Fluor minimal dyes, Typhoon 9400™ variable mode imager, and DeCyder differential analysis software.

Before electrophoresis, different protein samples (control, treatment and internal standard as a normalization), are individually labeled with Cy2, Cy3 and Cy5 dyes via an amide linkage of lysine residues within proteins, and are subjected to exactly the same first- and second-dimension running conditions. Therefore, the identical proteins labeled with each of the CyDye DIGE Fluor dyes will migrate to the same position on a 2-D gel. This reduces gel-to-gel variation and makes gel-to-gel matching consistent, and thus allows a more accurate and rapid quantification of changes of protein abundance between samples. After 2-DE, the same gels are scanned by Typhoon 9400 to obtain three images due to narrow and different wavelengths of each individual fluorescent dye. The three images are then overlaid and combined for further statistical analysis by DeCyder™ Differential Analysis Software (Version 4.0).

### **B.3.3. Protein Identification by MS**

Mass spectrometry (MS)-based method is an indispensable platform for the identification of gel-based peptide mass by peptide mass fingerprinting (PMF) or amino acid sequence (MS/MS)

in proteomic analysis. The location and type of protein identifications can also be determined. Protein information identified by MS can be divided into three stages: (I) sample preparation; (II) peptide mass fragment; and (III) database searching.

### **Sample Preparation**

After the sample proteins are resolved from the 2-D gel, the next challenge is to extract proteins from the gels, purify and analyze them by MS. The “in-gel” digestion with a protease is the most efficient technique for recovering proteins from 2-D gels (274), and trypsin is the favored enzyme for protein cleavage due to its low price, high specificity, and the peptides generated (usually 8-10 amino acid) are appropriate for MS analysis.

### **MALDI-TOF MS and PMF**

Mass spectrometers consist of three main components: the ionization source, the mass analyzer, and the ion detector (275). An ionization source is responsible for transferring the target molecules into gas-phase ions. A mass analyzer can separate gas-phase ions according to their different physical properties such as time-of-flight (TOF), electric or magnetic field (Quadrupole or ion trap), which subsequently is measured by the ion detector. The contribution of MS to proteomics is the technical breakthrough involving “soft” ionization that produces the protein ions, peptides-matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI).

In MALDI, analytes are first mixed with a UV-absorbing substance called matrix (e.g. 2, 5-dihydroxybenzoic acid or  $\alpha$ -cyano-4-hydroxy-cinnamic acid), and spotted on a metal plate to form a crystal. This 96-well format plate is then situated in the mass spectrometer. A pulsed laser is then fired at a target molecule, and singly charged ions are primarily produced via the transferred energy from the matrix to the embedded analytes. MALDI is frequently coupled to

TOF for mass analysis, and an  $m/z$  value is calculated as the time it takes ions to travel a certain distance to reach the ion detector.

The basis of MALDI TOF for protein identification is peptide mass fingerprinting (PMF) (276). The “unknown” proteins are digested with residue-specific protease, usually trypsin, thus generating a series of peptide mass fingerprints. For protein identification, the experimentally obtained peptide masses are compared with those of theoretical peptide masses, and the query proteins can be characterized if there is sufficient number of peptides matches in a database (244). Since PMF is not specifically for the purpose of obtaining the amino acid sequence, it is most fit for distinguishing proteins from species of which complete genome sequences have been determined. A mixture of proteins in one spot is not suitable for PMF, because it is not clear which peptide in the mixture originate from which protein. Low molecular weight proteins are the other limitation, since too few peptide fragments are produced, and not sufficient matched hits are met to characterize a specific protein. In addition, PMF is a better fit for identification of proteins separated from 2D gels, where the additional information regarding the proteins such as pI and MW is helpful in protein identification.

Database searching allows protein identification from PMF data of MALDI-TOF MS. PMF requires a number of user specified parameters such as protease used, mass type, charge and tolerance; coverage, amino acid modifications; missed cleavages; minimum number of peptide matches required as well as the database(s) to be searched (277).

The principle advantages of MALDI-TOF-MS is large-scale, high sensitivity (low fmol range), accuracy (low ppm range), and tolerance to contaminants (244,278). Because of its speed, it is frequently performed as the first line instrument for gel-based protein identification.

If proteins cannot be identified by PMF, they can be further analyzed by tandem MS/MS, where amino acid sequence can be obtained.

#### **B.3.4. Conclusion of Proteomics**

The whole procedure of proteomics is demonstrated in Figure 2.15. Proteomics and functional genomics are emerging new technologies offering promising tools to study the global analysis of proteins and mRNA transcripts in a complex biological system under certain external/internal stimuli. Although the applications of these two techniques are in their infancy and have some limitations, integrating mRNA and protein expression into a common framework has the potential to provide new insight into biology, which can not be obtained from the study of individual proteins.

## Appendix C SAS CODES FOR REAL-TIME RT-PCR DATA

```
dm 'log;clear;output;clear;';
options formdlim="*";
PROC IMPORT OUT= WORK.QPCR1
    DATAFILE= "C:\Documents and Settings\weizhang\Desktop\Applied Biosystems
files\qPCR for SAS qpcr1.xls"
    DBMS=EXCEL2000 REPLACE;
    SHEET="Sheet1$";
    GETNAMES=YES;
RUN;
/*To see the distribution of the dataset after log transformation*/
data qpcr; set work.qpcr1; lrq=log (rq); run;
/*Trim the double valuess or duplicate for each gene and each sample into the single value, get
the average */
proc sort data=qpcr; by detector mouseid; run;
proc means noprint data=qpcr; by detector mouseid; var lrq; output out=ltest mean= xlrq; id trt;
proc print data=ltest; run;
proc glm data=ltest; by detector; class trt cardno; model xlrq=trt cardno; lsmeans trt;
lsmeans trt/pdiff stderr; run;
proc gplot data=ltest; plot xlrq*detector; run;

proc univariate data=ltest; var xlrq; by detector; output out=ldtest median=mlrq p10=llrq
p90=ulrq; id trt detector; run;

proc sort data=ltest;by detector;run;proc sort data=ldtest; by detector;

data new2;merge ltest ldtest;by detector;run; proc print data=new2;run;
data new3;set new2;s=(ulrq-llrq)/2.564; z=(xlrq-mlrq)/s; if ABS(z)>2.5 then z2=2.5;
if ABS (z)<2.5 then z2=z; run; proc print data=new3; run;

/*Normal Distribution of the transformed and truncated database*/
proc gplot data=new3; plot z2*detector; run; proc sort data=new3; by detector;
run; proc print data=new3;run;

/*Normal Distribution of the database*/
options formdlim="*"; proc glm data=new3; class trt; model z2=trt;
lsmeans trt/pdiff stderr;by detector;run;
```

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