EFFECTS OF DIETARY FATTY ACID COMPOSITION AND ENERGY RESTRICTION ON ADIPOSE TISSUE *OBESE* mRNA, FATTY ACID COMPOSITION AND SERUM LEPTIN LEVELS

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ABSTRACT

Dietary fatty acid (FA) composition and energy restriction (ER) independently affect serum leptin levels; however it is not known whether this correlates with changes in *obese* (*ob*) gene expression. Herein, we assessed whether dietary FA composition and ER influence white adipose tissue (WAT) *ob* mRNA by Northern analysis. Animals consumed diets containing tallow (BT), safflower oil (SO) or fish oil (FO) *ad libitum* or at 60% *ad libitum* intakes. Serum leptin values were not different between levels of energy intake. ER decreased weight gain and WAT weights, which positively correlated with serum leptin values. WAT *ob* mRNA levels were in the rank order: FO>SO>BT in depots of all groups with ER showing a lower level of *ob* mRNA. Data show similarity in *ob* mRNA levels between depots with discordance in circulating leptin levels. These data suggest that energy restriction exerts greater control over leptin production than dietary fat source.

RÉSUMÉ

La composition alimentaire en acides gras (AG) et la restriction énergétique (RE) influencent de façon indépendante les taux de leptine sérique. Parcontre, on ne sait pas si ces changements sont associés à une modulation dans l'expression du gène *obèse* (*ob*). Nous avons déterminé l'influence de la composition en AG et de la RE sur les taux d'ARN-m *ob* dans les tissus adipeux blancs (TAB) par analyse Northern. Les animaux ont été nourris avec une diète composée de gras de bœuf (GB), d'huile de carthame (HC) ou d'huile de poisson (HP), *ad libitum* ou à 60% de la prise alimentaire *ad libitum*. Les taux sériques de leptine n'ont pas été influencés par le niveau de prise alimentaire. La RE a diminué la prise de poids corporel et la masse des TAB, lesquels étaient positivement associés aux valeurs de leptine sérique. Dans les différents sites de TAB, les taux d'ARN-m *ob* étaient dans l'ordre: HP>HC>GB, et la RE démontrait un niveau inférieur d'ARN-m *ob*. Une similarité dans les taux d'ARN-m *ob* a été observée entre les différents dépôts de TAB, et une disconcordance a été notée dans les taux de leptine circulante. Ces données suggèrent que la restriction énergétique exerce un plus grand contrôle, en comparaison à la source de gras alimentaire, sur la production de leptine.

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LIST OF ABBREVIATIONS

ASP – Acylation-stimulating protein

BAT – Brown adipose tissue

BMI – Body mass index

BT – Beef tallow

EE – Energy expenditure

Ep – Epididymal

ER – Energy restriction

FO – Fish oil (Menhaden)

MCH – Melanocortin hormone

MCT – Medium chain triglycerides

mRNA – messenger Ribonucleic acid

MUFA – Monounsaturated acids

NPY – Neuropeptide Y

ob – obese gene

Peg-Ob - Pegylated recombinant human leptin

PPAR – Proximal proliferator activated receptor

PUFA – Polyunsaturated fatty acids

Pr – Perirenal

Rp – Retroperitoneal

SFA – Saturated fatty acids

SO – Safflower oil

TNF – Tumor necrosis factor

UCP – Uncoupling protein

WAT – White adipose tissue

CONTRIBUTION OF AUTHORS

This thesis consists of two manuscripts, both of which I am the primary author. The first manuscript is cited from an invited review that is coauthored with my supervisor, Dr. Peter Jones. The second manuscript is based on research gathered for my Master's thesis and is *in preparation* for submission to the *Journal of Lipid Research*. It is co-authored with Dr. Peter Jones, my co-supervisor, Dr. Kris Chadee and fellow graduate student, Jode Heshka.

As primary author, I was responsible for the thought, text and revision of Manuscript I in its entirety with Dr. Jones providing correction of the manuscript before submission to *Current Opinion in Lipidology*. Dr. Jones developed the idea for the research, while providing advice and suggestions during my research. The idea and experimental components of Manuscript II are a collection of input from Dr. Chadee, Dr. Jones and myself. I was responsible for the execution, data analysis and text, with the exception of the feeding trial and circulating leptin analysis, which were shared with Jode Heshka. Dr. Jones provided assistance in the preparation of the manuscripts prior to submission for publication.

CHAPTER 1. INTRODUCTION

Obesity has silently become a serious health concern in adult and adolescent populations in North America and is currently the most common nutritional disorder in Western societies (Rosenbaum *et al.* 1997). It is estimated that approximately one-third of this population is considered obese and this number is reaching epidemic proportions (WHO 1998). Obese humans are at increased risk for developing heart disease, diabetes, stroke and hypertension (Spiegelman and Flier 1996). In many situations obesity is the result of increased energy intake that exceeds normal requirements and coincidental decline in energy expenditure as reflected by decreased physical activity.

There is a great deal of controversy regarding the major determining factor in the development of obesity as genetic, environmental or both. It is generally agreed that dietary intake is a key contributor to the development of obesity. Of the dietary macronutrients, fat is the most energy dense; thus it serves as the major fuel and storage nutrient for humans. Consumption of a high-fat energy dense diet is positively associated with increased weight gain and is attributed to the observed growth of fat mass cited in human (Romieu *et al.* 1988, Tucker *et al.* 1992) and animal studies (Faust *et al.* 1978, Klyde and Hirsch 1979, Boozer *et al.* 1995).

It is now accepted that the adipose tissue fatty acid composition is reflective of the dietary fat consumed (London *et al.* 1991, Garland *et al.* 1998, Cha and Jones, 1996 and 1998). The cell membrane fatty acid composition determines its structure and fluidity, thereby playing a critical role in the control of metabolic processes within the cell. More

clearly, changes in membrane fatty acid composition has been shown to alter insulin action, glucose transport and membrane function (Clandinin *et al.* 1985, Field *et al.* 1990, Pan and Storlien 1993). Cha and Jones (1996) examined influences of dietary fat and energy restriction on adipose tissue fatty acid composition and observed increased concentrations of linoleic acid (18:2 n-6). In situations of reduced energy availability there is a physiological adjustment to retain essential fatty acids. The significance of this process requires further exploration. In summary it can be extrapolated from the literature that varied sources of fat are metabolized and affect cellular processes differently.

Apart from the influence of dietary fat consumption and energy intake on determining the fate of lipids in nutrient metabolism, it is now understood that specifically the dietary fatty acid profile is a key factor in this process and as such, may influence weight gain. Generally, polyunsaturated fatty acid (PUFA) consumption is associated with decreases in visceral adipose stores, versus high-saturated fatty acid (SFA) diets. Leyton *et al.* (1987), demonstrated an increased oxidation of PUFA as compared to SFA, which may explain the reduced adiposity observed with some PUFA-rich diets (Shimomura *et al.* 1990 and Okuno *et al.* 1997). Shimomura et al. (1990) studied the effect of diet fat type on adiposity in rats fed diets differing in SFA, monounsaturated fatty acid (MUFA) and PUFA composition. These researchers observed less body fat accumulation when rats were fed safflower oil (SO) as opposed to rats fed high SFA containing beef tallow (BT). Hence, the weight-reducing effects observed with feeding differing fat types may depend on the concentration or the type of PUFA, which can have varied differences in the metabolism of fat through shifts in lipid biosynthesis or oxidation through regulation of

adipocyte growth. Recently, this theory was examined in rats fed high fat diets containing perilla oil, BT and SO (Okuno *et al.* 1997). The findings of researchers supported a fatty acid structure dependant process as demonstrated by the disparity in accretion between perilla oil rich in 18:3 (n-3) fatty acids, which reduced accretion of visceral adipose tissue versus a SO diet high in 18:2 (n-6) fatty acids. Molecular analysis further revealed a specific down-regulation of PPAR receptor-α, adipsin and adipocyte P2 in adipose tissues with perilla oil feeding, resulting in a reduced synthesis of adipocytes. Thus, dietary fatty acid composition influences weight gain in animals through direct fatty acid oxidative stimulation or via modification of adipocyte proteins but the specific mechanism requires further research as it may be depended on fat quantity or energy intake.

In recent years, there has been a considerable amount of research exploring the function of leptin as a central regulator of body weight through hypothalamic centers controlling satiety and energy expenditure (Zhang *et al.* 1994, Stephens *et al.* 1995, Campfield *et al.* 1995, Halaas *et al.* 1995). Leptin, a 16-kDa protein product of the *obese* gene, is produced primarily from white adipocytes. After cloning of the *obese* gene it was determined that mutations in either (i) *obese* gene or (ii) leptin receptor were responsible for the observed morbid obesity, hyperphagia, hyperglycemia and hyperinsulinemia in mice and rodents (Zhang *et al.* 1994). Methods of increasing circulating leptin levels, either by recombinant leptin injection or through stimulating increased endogenous production as observed with adenoviral gene therapy, eradicates these abnormalities (Campfield *et al.* 1995, Koyama *et al.* 1997). Circulating levels of leptin correlate

strongly with body weight, but more specifically body fat mass (Maffei *et al.* 1995, Cha and Jones 1998). Additionally, there has been a report of large variability in circulating leptin levels in both animals and humans (Maffei *et al.* 1995). Whether this variability is associated with differences in dietary factors influencing *ob* mRNA production is unknown. One such factor that has been investigated is dietary fat source. Current research suggests the existence of a unique inter-relationship between dietary fat, leptin expression and leptin action within the peripheral system. Recent studies suggest that the reduction in adipose hypertrophy observed with n-3 PUFA-containing fish oil feeding might involve a leptin-specific process (Raclot *et al.* 1997). A large amount of evidence supports direct functioning of leptin in peripheral lipid metabolism *in vivo* and *in vitro*. It is possible that consumption of n-3 PUFA will maintain an efficient level of circulating leptin, thus preventing leptin insensitivity and weight gain.

Induction of supraphysiological leptin levels (i.e. recombinant leptin injection) in animals reduces body weight and food intake (Stephens *et al.* 1995, Campfield *et al.* 1995, Halaas *et al.* 1995); however, it is unknown whether leptin is as effective at physiological levels. Studies in humans and rodents have demonstrated a positive correlation between leptin levels and adipose stores, suggesting leptin is a marker of stored triglyceride and not a weight reducing protein. It has also been shown that restricting energy intake reduces circulating leptin levels, regardless of changes in body weight. Furthermore, dietary fatty acid composition is an important determinant of circulating leptin levels in dietary induced obese and normal animals. The mechanism by which dietary fatty acid composition and energy restriction (individually or interactively) influences leptin

production and action is unknown but undoubtedly involves fatty acids in the regulation of gene expression. Modifications in dietary fat composition influences adipose tissue fatty acid composition and while energy restriction further modifies tissue compositions due to selective oxidation of particular fatty acids, namely PUFA. Whether these factors influence leptin production by altering adipose tissue fatty acid composition, body weight, or a combination of the above, also remains to be elucidated.

CHAPTER 2. LITERATURE REVIEW

2.1. Dietary Fat, Energy Balance and Adipose Tissue Accumulation

2.1.1. Dietary fat and adiposity

Dietary fat is a major component of the North American diet. As such, it serves as an important fuel source; yet current evidence suggests that a reduction in the daily intake of fat by 10 % with a total intake of no greater than 30% calories could reduce the high incidence of obesity (Bray and Popkin 1998). The National Health and Nutrition Examination Survey concluded that 33% of adults in the United States between 20-74 years of age are considered overweight (Kuczmarski *et al.* 1994). This estimate is likely to be comparable with the Canadian population.

Obese and overweight individuals have an increased risk of disease namely: type II diabetes, cardiovascular disease, hypertension, and even certain forms of cancer (Bray 1996). Maintenance of a stable and healthy body weight can be achieved by sustaining a balance in energy intake and energy expenditure. Since protein and carbohydrate stores vary little under normal conditions, regulation of body weight would involve primarily a regulation of fat tissue mass (Jéquier and Tappy 1999). Unlike protein and carbohydrate, increased fat intake does not stimulate fat oxidation thus; fluctuations in energy balance will involve direct variations in adipose tissue mass. Consequently, a sustained state of positive energy balance will increase fat mass proportionately in individuals at risk for obesity who have low physical activity.

Epidemiological studies show strong, positive associations between fat intake and body weight (Danforth 1985, Lissner and Heitmann 1995). High fat diets in humans (Romieu *et al.* 1988, Tucker et al., 1992 and Doucet *et al.* 1998) and animals (Faust *et al.* 1978, Klyde and Hirsch 1979, Hill *et al.* 1992) promote greater weight gain and excess body fat deposition as compared to low fat dietary consumption. This trend was shown recently in rats fed diets containing 24, 36 or 48% fat as energy isocalorically (Boozer *et al.* 1995). Despite similar body weights between rats after the diet feeding period, total body fat and adipose depot weights were significantly increased in proportion to the percent fat in the diets.

2.1.2. Dietary fat source and adipose tissue accumulation

Apart from the quantity of fat in the diet, qualitative fat consumption has also been shown to influence body weight and triglyceride stores as exemplified in several animal studies executed in the last decade (Su and Jones 1993, Shimomura *et al.* 1990 and Okuno *et al.* 1997). Rats fed diets containing 42% of energy as olive oil, safflower oil or beef fat for 12 weeks had greater fat mass and total energy gains as estimated from accreted lean body mass and fat mass, than rats fed an equal quantity of menhaden fish oil (Su and Jones 1993). The authors speculated that the observed differences between dietary fat sources could be attributed to the efficiency of fatty acid accumulation in adipose tissue. Although, these authors observed greater fecal losses of fat with fish oil feeding, suggesting the fatty acid composition of fish oil may interfere with absorption.

Differences in fat oxidation rates have been documented in animals fed varied sources of fat (Leyton *et al.* 1987). Feeding rats diets rich in polyunsaturated fatty acids (PUFA) resulted in a much greater fatty acid oxidation rate versus saturated fat (SFA) rich diets. As well, Jones and Schoeller (1988) used indirect calorimetry to show that humans consuming diets high in PUFA had an increased contribution of fat to postprandial energy expenditure compared with a diet high in saturated fat. Therefore, there is evidence to suggest that consuming diets with a high PUFA:SFA ratio could be beneficial for increasing oxidation of longer chain fatty acids, resulting in less adipose accumulation. It has recently been shown that diets rich in saturated fat decrease diet-induced thermogenesis by reducing sympathetic activity in brown adipose tissue (BAT) and may explain the larger gains in fat mass observed in animals consuming beef tallow diets (Takeuchi *et al.* 1995). This mechanism is an unlikely scenario in humans, due to the very limited amount of BAT in the human system. It has been shown that hepatic fatty acid synthase mRNA was greater in rats fed saturated fat compared to a polyunsaturated fat diet, which could be partly responsible for the higher lipogenesis observed with consumption of a saturated fat diet (Shillabeer *et al.* 1990). Further research is required to elucidate the mechanisms involved in reducing adipose tissue mass with consumption of certain PUFA, especially n-3 and n-6 fatty acids, which may involve a structure-specific processes.

2.1.3. Diet fat composition influences adipose tissue composition

It has long been known that the pattern of dietary fatty acid composition reflects fatty acid accumulation in adipose tissue (Jacobsen *et al.* 1983; London *et al.* 1991, Cha and Jones, 1996 and Garland *et al.* 1998). Bailey *et al.* (1993) examined the specific fatty acid composition of epididymal (Ep), perirenal (Pr) and inguinal (Ing) adipose depots from adult male rats fed chow *ad libitum*, daily or every second day. They observed decreases in adipose depot myristic, palmitic and stearic acids over a period of two years with a steady growth in each fat pad examined in animals restricted to food on alternate days. Thus, as body weight, fat pad weight and PUFA concentrations increased, there was a coordinated decrease in SFA in response to reduced energy intake. The percent of these saturated fatty acids as compared to the total fatty acid content was significantly different between the three fat depots with Pr containing the most saturated fatty acids with 27.8% followed by inguinal and Ep with 24.7% and 21.6% respectively. Human studies have yielded similar findings. Bhattacharyya et al. (1990) examined human subjects with large body weights and analyzed adipose tissue fatty acid composition. These researchers found that adipose tissues from heavier subjects contained proportionately more unsaturated fatty acids than saturated, even though no information was provided on dietary fat intake.

The study of Bailey *et al.* (1993) in particular provides several points of interest. Firstly, these authors observed depot-specific differences in adipose depot fatty acid composition, which were not unique to location as the depot with greatest and lowest concentrations of saturated fatty acids came from the visceral adipose tissue. And secondly, we must

consider the potential for age-specific differences in fatty acid metabolism in a feeding study over two years in length; thus, the response may be different in a growing animal.

Hill *et al.* in 1993, examined the fatty acid composition of Ep, retroperitoneal (Rp), mesenteric and inguinal adipose depots in rats fed diets containing beef fat, corn oil, fish oil or medium-chain triglycerides (MCT). These researchers reported that the fatty acid composition of stored triglyceride was most similar to that of the diet for rats fed beef tallow and corn oil. Neither the long-chain, PUFA of the fish-oil or the medium chain fatty acids (MCFA) of the MCT-oil were apparent at a significant level, suggesting that these fatty acids were preferentially oxidized or that they underwent metabolic transformation prior to storage. In fact, it has been reported that WAT is resistant to n-3 PUFA deposition over the short-term (Sinclair and Gale 1987). The decreased deposition of n-3 fatty acids from fish oil-feeding is consistent with the decreased fat deposition observed in each adipose depot, although this is likely due to incorporation within the phospholipid bilayer. Nevertheless, these findings suggest that the partitioning of dietary fatty acids for oxidation versus retention is regulated by the dietary fatty acid composition. The higher oxidation of n-3 fatty acids would certainly explain the limited body fat deposition observed in both humans and animals consuming fish oil diets.

Similar findings have also been reported in human studies. Male and female subjects who consumed diets rich in hydrogenated oils had elevated endogenous *trans* fatty acid levels in their adipose tissue fatty acids (London *et al.* 1991). Similar results were observed in free-living female subjects with adipose tissue levels of polyunsaturated and *trans* fatty

acids reflecting quantities in the diet (Garland *et al.* 1998). Yet, there are few published reports with regard to the impact modification in the WAT environment has on the production of adipocyte proteins and whether these factors are involved in obesity.

2.1.4. Energy intake and adipose tissue accumulation

Energy restriction is the most common strategy for weight reduction but due to difficulties related largely to compliance, success is rarely achieved. In the short term, weight loss may be realized but maintenance of the new weight for the long term is limited. This evidence has led to a theory of body weight deviation as an individual "set point" (Jéquier and Tappy 1999). Generally, during the course of a lifetime, body weight fluctuates minimally (~1%) in most humans; thereby explaining the limited success of weight loss. Leibel *et al.* (1995) examined energy expenditure in human subjects challenged with situations of positive and negative energy balance. Energy expenditure increased with weight gain and decreased with weight loss, in an attempt to return to the individuals "normal weight". Weight loss during energy restriction can be influenced by the quantity of fat in the diet. Boozer *et al.* (1993) studied the effect of diet fat in animals fed energy restricted (75%) isocaloric diets differing in the percent fat for 6 weeks. These researchers observed similar body weights in rats fed energy restricted 45% fat diets versus *ad libitum* fed control animals; therefore, even with modest energy restriction, body weight remained similar between dietary regimens. Of note were significant increases in Ep and Rp WAT weights in the restricted high-fat fed animals. Therefore, weight loss can be overshadowed by dietary composition, especially when consuming a high-fat diet due to expansion of visceral adipose tissue.

2.1.5. Energy restriction and tissue fatty acid deposition

There is a substantial body of evidence to suggest that the adipose tissue fatty acid composition is not static. Energy restriction has also been shown to influence adipose tissue fatty acid accretion. Bailey *et al.* (1993), examined the main and interactive effects of exercise and food restriction in young rats. Exercise was achieved by placement of animals in a shallow pool for inducing physical activity. Alternate day feeding of standard rodent chow alone and in combination was used to study WAT fatty acid compositional changes. It was revealed that non-exercised *ad libitum* fed rats had lower concentrations of saturated fatty acids within Ep, Pr, and subcutaneous adipose depots. In food-restricted non-exercised animals, adipose depots showed higher levels of saturated fatty acids with Pr WAT containing the highest concentration of saturated fatty acids. Based on this study, there is evidence to suggest tissue-specific responses to energy restriction exist, as demonstrated with differences in SFA concentrations. Specifically, Pr WAT contained large concentrations of SFA; whereas, Ep and subcutaneous depots were composed of greater amounts of PUFA. Further research is required to examine consequences of feeding dietary sources of fat differing in concentrations of SFA, MUFA and PUFA, which may reveal structure-specific responses with regards to fatty acid composition.

In accordance with the previous study, short-term food restriction in rats increased concentrations of 18:0, 18:2 (n-6), 20:4 (n-6), and 22:6 (n-3); whereas, these authors observed reductions in 16:0, 16:1 (n-7) and 18:1 (n-9) in serum triglycerides and hepatic tissues of these animals (Chen and Cunnane 1992). These authors observed the largest

increases in the concentration of 18:2 (n-6), within these triglycerides. It was concluded that during fasting, long chain fatty acids released from adipose tissue were differentially utilized and hepatic triglyceride species were remodeled, permitting the optimal preservation of 18:2 (n-6). Thus, energy restriction plays a unique role in inducing supplementary changes in tissue fatty acid composition; ultimately promoting retention of polyunsaturated fatty acids, notably 18:2 (n-6). The significance of this response to negative energy balance requires further study.

2.1.6. Dietary fat source and energy restriction: an interactive effect on adipose tissue fatty acid accumulation

Under normal circumstances dietary fat is stored within adipose depots for use as a source of energy in times of need. Hence, after prolonged ingestion of diet fat there is an anticipated increase in the concentration of these fatty acids within the adipose tissue environment (London *et al.* 1991, Garland *et al.* 1998, Cha and Jones 1996 and 1998). Cha and Jones (1996) conducted a long-term study looking at the interactive effect of both dietary fat composition and energy intake at 85% or 68% of *ad libitum* intake on nape adipose tissue fatty acid concentrations rats. In *ad libitum* fed animals, fatty acid depositions in adipose tissue resembled the dietary fatty acid composition with the exception of olive oil feeding. Dietary fatty acid composition and energy restriction interactively influenced fatty acid composition, for concentrations of 16:0, 18:0, 18:1(n-9) and 18:2 (n-6). Although, nape fat was examined as a representative adipose tissue, it is likely that there are tissue-specific differences as demonstrated elsewhere (Bailey *et al.* 1993). Differences in the fatty acid profile of visceral adipose depots may be of interest

as there is a strong association between increased visceral adipose tissue and risk of chronic disease (Despres 2001). There is substantial evidence to confirm the role of dietary fatty acid composition and energy intake in altering WAT fatty acid composition but it is yet unknown what impact these changes have on visceral adipose tissue growth.

2.2. Nutritional Factors Affecting Leptin Production

2.2.1. Leptin: an Overview

Understanding the development of obesity has been a challenge for researchers, but the recently discovered *obese* gene product leptin is providing important clues. Leptin is synthesized and secreted primarily from WAT (Zhang *et al.* 1994) but it has been recently discovered that minor quantities of leptin are supplied from placental (Masuzaki *et al.* 1997) and stomach tissue (Bado *et al.* 1998). The first evidence of the existence and role of leptin in the regulation of body weight through actions within the hypothalamus was demonstrated using parabiotic mice (Coleman and Hummel 1969 and Coleman 1973). Parabiosis allows one to study the effects of cross-circulation of blood between animals, in this case between genetically obese and lean mice. It was observed that the genetically obese mice would lose weight, which lead to the hypothesis that these same mice lack some unknown signaling factor, which is being produced in lean mice to control body weight. Thirty-five years later, characterization of the human obese gene and the encoded protein leptin, resulted in the discovery of a 166 amino acid polypeptide, 84% and 83% identical to mouse and rat leptin respectively (Zhang *et al.* 1994). Such an extensive homology between species would suggest that leptin functioning would also be highly conserved (Zhang *et al.* 1994). Human ob gene expression in adipose tissue varies

from region to region and may depend on the size and metabolic characteristics of each region (Lonnqvist *et al.* 1997). In addition, leptin is cleared from circulation by the kidney in both human and animals, which may also play a role in the observed variability of circulating leptin (Cumin *et al.* 1996).

One year after the cloning of the *obese* gene, Tartaglia and colleagues (1995) cloned the leptin receptor gene and in the following year discovered all of the leptin receptor splice variant genes and their encoded proteins (Tartaglia 1997). Major forms of the leptin receptor include the long form (Ob-Rb) found predominantly in the hypothalamus and a short form (Ob-Re). The later lacks the transmembrane domain region and is therefore a potential soluble receptor or binding protein (Tartaglia 1997). Other minor isoforms of the leptin receptor Ob-R a, c and d, respectively, can be found in several peripheral tissues and are noted for their shortened intracellular domain. Two of the major animal models of genetic obesity, namely fatty (*fa/fa*) and diabetic (*db/db*), have mutations within their *OB-Rb* genes, resulting in the synthesis of receptors, lacking both transmembrane and intracellular domains (Phillips *et al.* 1996 and Lee *et al.* 1996). The non-functional hypothalamic receptor interaction with leptin cannot initiate the signal transduction pathway without these essential domains. Regulation of body weight is impaired, resulting in morbid obesity because the central effects of leptin are not exerted.

2.2.2. Leptin Action

Leptin administration in both leptin-deficient ob/ob and lean mice induces weight loss, almost entirely through loss of white adipose tissue (WAT), which is attributable to a decline in food intake and a boost in thermogenesis (Campfield *et al.* 1995, Pelleymounter *et al.* 1995, Halaas *et al.* 1995). Research up to this point in animals has revealed that the primary target of leptin is the hypothalamus, wherein leptin inhibits the synthesis of neuropeptide Y, a potent orexigenic agent (Stephens *et al.* 1995), and increases centrally mediated thermogenesis via stimulation of uncoupling protein (UCP) expression (Commins *et al.* 1999). Leptin administered intra-cerebroventricularly (i.c.v) to rats altered the expression of both neuropeptide Y (NPY) and corticotrophin releasing hormone (CRH), two neuropeptides that play important roles in controlling appetite (Schwartz *et al.* 1996). NPY is a well known stimulator of food intake and repressor of brown adipose tissue thermogenesis (Dryden and Williams 1996); whereas, CRH exerts a counter-regulatory role to NPY (Arase *et al.* 1988). Leptin administration decreased hypothalamic NPY mRNA levels by 24% and increased CRH mRNA by 38%. These results are consistent with earlier findings by Stephens *et al.* (1995) who demonstrated that NPY mRNA levels are elevated in the arcuate nucleus of the hypothalamus in ob/ob mice. When leptin is administered; the levels of NPY mRNA decrease significantly, suggesting that when leptin is present, it modulates NPY synthesis, subsequently regulating body weight.

Humans and rodents have higher circulating leptin levels in the obese state. These levels are strongly correlated with adipose tissue size (Havel *et al.* 1996 and Maffei *et al.* 1997).

With the exception of rare cases involving mutations in human obese genes, which bear incredible similarities to the obese mouse (Farooqi *et al.* 1999), the similarities between human and animal leptin functioning are few. Therefore, in normal situations, overweight and obese individuals have elevated leptin levels contrary to what one might expect. Elevated leptin levels in the obese state suggest that in the human condition, leptin deficiency may not be at fault but rather a likely situation of impaired leptin sensitivity or resistance.

Considine *et al.* (1996) examined serum leptin concentrations in normal-weight and obese humans. They observed a strong positive correlation between percentage body fat and serum leptin concentrations. These findings are supported by Martin *et al.* (1997), who studied serum leptin levels in normal-weight women (mean age = 48.6 years). These authors concluded that serum leptin levels were positively associated with percentage body fat, fat mass and BMI. It appears circulating leptin levels in humans are higher with obesity, and correlate strongly with adipose tissue stores (Maffei *et al.* 1997, Liuzzi *et al.* 1999), particularly subcutaneous fat (Tai *et al.* 2000). Interestingly, energy restriction lowers leptin levels; while overfeeding is associated with increased circulating leptin levels, supporting a nutritional role in the regulation of leptin production (Kolaczynski *et al.* 1996, Wisse *et al.* 1999). Of particular interest, Wisse *et al.* show a 66% reduction in plasma leptin levels after 1 week of consuming an energy restricted diet, which shows a prompt rise after refeeding. As well, changes in plasma leptin correlate with those of glucose and not with fat mass. Therefore, it can be deduced that leptin levels are also

under acute control by specific nutritional factors. Over the long term the effects of these factors is not yet known.

With the recent discovery of several tissues expressing leptin receptors, it appears that leptin may play a significant role in peripheral lipid metabolism (Tartaglia *et al.* 1995, Fruhbeck *et al.* 1999, Fruhbeck *et al.* 2000, Sobhani *et al.* 2000). Emerging molecular research supports a novel direct role for leptin in partitioning fuel sources within adipose, muscle and hepatic tissue. In addition, the recognized association of leptin with a large number of newly discovered proteins, such as peroxisome proliferator-activated receptor-γ (PPAR-γ) and tumour necrosis factor-α (TNF-α and acylation-stimulating protein (ASP), suggests a more complex metabolic cascade in the control of energy balance.

2.2.3. Leptin Production

The *obese* gene is regulated by various factors of which neuropeptide Y (NPY) would appear to be the most consistent determinant of ob mRNA levels in white adipose tissue. This has been shown in studies using *ob/ob* mice, which have elevated levels of NPY mRNA (Stephens *et al.* 1995). Schwartz and colleagues (1996) demonstrated that leptin downregulates NPY synthesis and release from the same region within the hypothalamus. Cold temperatures will suppress leptin expression as observed in mice exposed overnight to a temperature of 4°C. Ob mRNA within epididymal WAT was undetectable compared to control animals. This decrease in ob gene expression in WAT is caused by sympathetic nervous system sensitivity to cold temperature, a β_3-adrenergic agonist, (Trayhurn *et al.* 1995).

Nutritional factors, such as energy status, play an important role in the production of leptin in both humans and animals. Feeding rats twice their normal intake elevated leptin mRNA levels without a simultaneous shift in body weight as revealed by Northern blot analysis of WAT (Harris *et al.* 1996). Similarly, rats restricted to 70% of their normal intake, show a 62% decrease in plasma leptin levels which was not correlated with changes in fat mass. Furthermore, a study involving twenty-one obese women who were fed an energy-restricted (50%) diet for 1 week showed a 66% decrease in plasma leptin levels, the degree of which did not mimic corresponding changes in fat mass (Wisse *et al.* 1999). It has been shown that levels of insulin have been positively correlated with the levels of leptin in animals (Cusin *et al.* 1995) and humans (Utriainen *et al.* 1996). Most notably, Utriainen *et al.* (1996) examined changes in leptin levels at supraphysiological levels of leptin through hyperinsulinemic clamp studies. Under normal circumstances, the effect of insulin on leptin is unclear and requires further examination.

2.2.4. Regulation of leptin expression by dietary fat

It is generally accepted that leptin levels correlate strongly with body adiposity, percentage of body fat and body mass index (Jensen *et al.* 1999). However, the large variations that are seen in circulating leptin levels in humans with similar levels of adiposity, suggest that adiposity is not the primary determinant of circulating leptin levels. Reductions in 24-h circulating leptin levels were observed after high-fat meal consumption and were unrelated to weight gain (Havel *et al.* 1999). Conversely, long-term reductions in fat intake reduced circulating leptin levels beyond that attributable to weight loss (Reseland *et al.* 2001). In the latter study, participants were advised to

increase their consumption of fish and fish products; thus, it may have contributed to reductions in body weight leptin expression and production. Scientific evidence suggests nutrition, more specifically dietary fat quantity or quality, may play a role in the regulation of leptin production.

Numerous studies in rodents have shown that diets high in fat stimulate leptin production. Masuzaki *et al.* (1995) fed Sprague Dawley rats a high fat diet compared to a standard rodent diet and examined leptin mRNA levels by employing Northern blot analysis. Epidydimal, mesenteric, subcutaneous and retroperitoneal WAT had 2.2, 3.2, 2.0 and 2.1 fold higher leptin mRNA levels respectively, than WAT from rats fed standard chow. This finding was further supported by Frederich *et al.* (1995a) who fed mice a high-fat diet (21% energy) for 12 weeks and observed elevated plasma leptin and adipose tissue obese mRNA levels. They also noted that leptin levels were indicative of the state of body lipid stores; thus, they could not discount an increase in expression linked with an increase in fat mass in these animals. Human studies have not shown this trend between fat intake and leptin levels. Schrauwen *et al.* (1997) fed 12 human volunteers a high-fat diet (60% energy) and measured plasma leptin levels after 7 days of feeding. There were no observed changes in leptin levels at the end of the study but it is possible that chronic consumption of a high-fat diet may be necessary to influence leptin levels.

Animals fed n-3 fatty acid rich fish oil show reduced abdominal adipose tissue hypertrophy and fat cell lipid content (Belzung *et al.* 1993, Raclot *et al.* 1997). It is uncertain, however, whether leptin is involved in the weight-reducing effects observed

with fish oil feeding. Raclot *et al.* (1997) were the first to provide evidence for a direct effect of dietary fat composition in regulating WAT leptin expression. In that study, mice consumed diets that differed in polyunsaturated fatty acid (PUFA) composition, while changes in leptin expression were measured by Northern analysis. A significant reduction in leptin expression was observed, which was independent of weight gain and feed intake in mice fed docosahexanoic acid alone or in combination with eicosapentaenoic acid. Consequently, the observed reduction in leptin expression with fish oil feeding is contrary to the anticipated upregulation concomitant with a decrease in adipose tissue fat deposition. Later, Cha and Jones (1998) examined the role of qualitative fat type in combination with energy restriction in order to explore changes in plasma leptin levels in rats. That study indicated that feeding rats a diet that was high in n-3 fatty acid induced hyperleptinemia and significantly reduced fat deposition and fat cell weight. In contrast to the findings of Raclot *et al.* (1997), leptin gene expression was not measured. Subsequently, WAT depots that were examined for determination of depot weight and fat cell weight were derived from different anatomical regions. Discrepancies in circulating leptin levels and expression of leptin mRNA across studies suggests tissue-specific differences in leptin mRNA levels. Further more, discordance between leptin expression and plasma levels has been observed in individuals with broad ranges of adiposity (Ranganathan *et al.* 1998).

More recently, Hun *et al.* (1999) examined the effects of fish oil in combination with lard, perilla and soybean oil feeding in obese, diabetic KK-A^y mice. Those investigators examined plasma leptin levels and WAT weights after 12 weeks of feeding. As

anticipated, WAT weights and circulating leptin levels decreased in response to fish oil feeding. An elevated expression of leptin mRNA was observed previously in mouse adipocytes in eicosapentaenoic acid-containing media, illustrating the necessity of the hypothalamus for complete leptin action (Murata *et al.* 2000). Takahashi *et al.* (2000) studied the effect of high fat safflower, perilla or fish oil feeding on the expression of leptin mRNA in rats. WAT leptin expression was significantly higher with high-fat feeding of safflower oil versus perilla or fish oil in epididymal adipose tissue. Conversely, perirenal leptin mRNA expression was elevated in animals fed safflower oil and perilla oil. Differences in leptin mRNA was unrelated to weight gain, because this variable was not significantly different between the three feeding regimens. Once again, these results suggest tissue-specific differences and that safflower oil feeding induces the greatest increase in leptin mRNA expression. More recently, Nisoli *et al.* (2000) infused intralipid 20% in humans and collected gluteal subcutaneous fat for leptin expression analysis. After infusion of intralipid, an emulsion of soybean oil, leptin mRNA increased significantly; an effect that was independent of weight gain.

Following this, it can be said that fish oil feeding reduces leptin mRNA and circulating levels in animals; whereas, feeding safflower oil or other fats that are high in n-6 PUFA, have opposing effects. The decline in weight gain observed with n-3 fatty acid feeding may involve leptin but with the great disparity between studies, it is difficult to interpret the data. It is necessary to examine leptin expression and production in a feeding trial that compares the effects of diets differing in the source of fat. This will allow examination of

the effects of dietary saturated, monounsaturated and polyunsaturated fatty acids on leptin expression.

2.2.5 Regulation of leptin production by energy balance

Energy restriction in both animals and humans has been shown to reduce plasma leptin concentrations. As demonstrated by Wisse *et al.* (1999) we can expect significantly large reductions in plasma leptin outside of changes in body weight. Maffei and colleagues (1995) found that weight loss due to food restriction (500-800 kcal/day) in obese humans and fasting for obese mice was associated with a significant reduction in plasma leptin levels beyond that attributed to weight loss. These findings are supported by a recent study examining energy restriction and serum leptin levels (Weigle *et al.* 1996). In obese male volunteers, a 21% weight loss was associated with a 76% mean decrease in plasma leptin levels (Weigle *et al.* 1996). There is strong evidence to suggest that leptin may act not only as an indicator of body energy stores to the brain, but also as a sensor of energy balance. Although, studies have shown factors other than intracellular triglyceride stores, such as fat, appear to exert control over leptin expression (Houseknecht *et al.* 1998).

Animal studies have examined the interactive effects of dietary composition and energy restriction on circulating leptin. Cha and Jones, (1998) observed similar findings when feeding diets consisting of either fish oil, safflower oil, or beef tallow with graded levels of energy restriction (100, 85, and 70 % of *ad libitum* food intake). Energy restriction inhibited weight gain and decreased leptin levels more so in the fish and safflower oil fed animals than in the animals fed beef tallow. This suggests that PUFA rich diets may exert

beneficial effects on the leptin system contributing to reductions in body weight. Recently, Agus *et al.* (2000) examined the effects of dietary composition on the physiologic adaptations to energy restriction in humans. Subjects consuming isocaloric low-glycemic (30% fat) diets had lower serum leptin than the high-glycemic (18% fat) diet. Although, these changes in leptin were not significantly different, the disparate response may be attributed to the fat content. Therefore, there is strong evidence to suggest that dietary composition with simultaneous energy restriction is beneficial to development of dietary obesity in animals. In the human system this is less clear.

2.3. MANUSCRIPT I.

Leptin and its role in lipid metabolism

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2.3.1. Abstract

Since the discovery of leptin in 1994, a considerable amount of research has focused on leptin as a central regulator of body weight. In the animal model, research has demonstrated leptin action through hypothalamic centers altering both satiety and energy expenditure. In contrast to animal studies, it is unlikely that leptin functioning in the human system exerts such a profound role in body weight regulation. Human studies suggest that leptin levels are strongly correlated with both percentage fat mass and body mass index, in accordance with the proposed 'lipostatic theory'. Current research suggests the existence of a unique inter-relationship between dietary fat, leptin expression and leptin action within the peripheral system. More specifically, it has been demonstrated that polyunsaturated fatty acid (PUFA) intake influences adipose tissue expression of leptin, and of several lipogenic enzymes and transcription factors. In addition, leptin stimulates triglyceride depletion in white adipose tissue without increasing free fatty acid release, thus favoring fatty acids versus glucose as a fuel source. Recent studies suggest that the reduction in adipose hypertrophy observed with n-3 PUFA-containing fish oil feeding might involve a leptin-specific process. A large amount of evidence supports direct functioning of leptin in peripheral lipid metabolism *in vivo* and *in vitro*. It is possible that PUFAs will maintain an efficient level of circulating leptin, thus preventing leptin insensitivity and weight gain. There has been much recent progress in clinical leptin research, from energy expenditure to leptin analogue efficacy; the purpose of the present review is to summarize our current understanding of leptin functioning.

Abbreviations

ASP acylation-stimulating protein
EE energy expenditure
Peg-Ob pegylated recombinant human leptin
PUFA polyunsaturated fatty acid
UCP uncoupling protein
WAT white adipose tissue

2.3.2. Introduction

Understanding the development of obesity has been a challenge for researchers, but the recently discovered obese gene product leptin is providing important clues. Leptin, a circulating 16-kDa adipocyte-derived protein, regulates food intake and energy expenditure in animal models (Zhang et al., 1994). Specifically, leptin administration in both leptin-deficient ob/ob and lean mice induces weight loss, almost entirely through loss of white adipose tissue (WAT), which is attributable to a decline in food intake and a boost in thermogenesis (Campfield et al., 1995, Pelleymounter et al., 1995, Halaas et al., 1995). Research up to this point in animals has revealed that the primary target of leptin is the hypothalamus, wherein leptin inhibits the synthesis of neuropeptide Y, a potent orexigenic agent (Stephens et al., 1995), and increases centrally mediated thermogenesis via stimulation of uncoupling protein (UCP) expression (Commins et al., 1999).

The human adipose tissue obese gene is 84% homologous to that of mice. Such an extensive homology between species would suggest that leptin functioning would also be highly conserved (Zhang et al., 1994). Circulating leptin levels in humans are higher with obesity, and correlate strongly with adipose tissue stores (Maffei et al., 1997, Liuzzi et al., 1999), particularly subcutaneous fat (Tai et al., 2000). In addition, energy

restriction lowers leptin levels, whereas overfeeding is associated with increased circulating leptin levels (Kolaczynski et al., 1996, Wisse et al., 1999). Thus, circulating leptin in humans appears to function as barometer that reflects adipose stores, and is less likely to be a central regulator of body weight. With the recent discovery of several tissues that express leptin receptors, it appears that leptin may play a significant role in peripheral lipid metabolism (Tartaglia et al., 1995, Fruhbeck et al., 1999, Sobhani et al., 2000, Fruhbeck et al., 1999). Emerging molecular research supports a novel direct role for leptin in partitioning fuel sources within adipose, muscle and hepatic tissue. In addition, the recognized association of leptin with a large number of newly discovered proteins, such as peroxisome proliferator-activated receptor-γ, tumour necrosis factor-α and acylation-stimulating protein (ASP), suggests a more complex metabolic cascade in the control of energy balance. Therefore, it is important to review recent animal model and human system developments in order to further our understanding of leptin production and its role in lipid metabolism, and to discuss further areas for future exploration.

2.3.3. Regulation of leptin expression by dietary fat composition

It is generally accepted that leptin levels correlate strongly with body adiposity, percentage body fat and body mass index (Jensen et al., 1999). However, the large variations that are seen in circulating leptin levels in humans with similar levels of adiposity suggest that adiposity is not the primary determinant of circulating leptin levels. Reductions in 24-h circulating leptin levels were observed after high-fat meal consumption, and were unrelated to weight gain (Havel et al., 1999). Conversely, long-

term reductions in fat intake reduced circulating leptin levels beyond that attributable to weight loss (Reseland et al., 2001). In the latter study, participants were advised to increase their consumption of fish and fish products, thus possibly contributing to reductions in body weight leptin expression and production. Scientific evidence suggests nutrition, more specifically dietary fat quantity or quality, may play a role in the regulation of leptin production.

Feeding rats a diet high in n-3 fatty acid fish oil limits abdominal adipose tissue hypertrophy and fat cell lipid content (Belzung et al., 1993, Raclot et al., 1997). It is uncertain, however, whether leptin is involved in the weight-reducing effects observed with fish-oil feeding. Raclot et al. (1997) were the first to provide evidence for a direct effect of dietary fat composition in regulating WAT leptin expression. In that study, mice consumed diets that differed in polyunsaturated fatty acid (PUFA) composition, while changes in leptin expression were measured with Northern blot hybridizations. A significant reduction in leptin expression was observed, which was independent of weight gain and feed intake, in mice fed docosahexanoic acid alone or in combination with eicosapentaenoic acid. Thus, the observed reduction in leptin expression with fish oil feeding is contrary to the anticipated upregulation concomitant with a decrease in adipose tissue fat deposition. Later, Cha and Jones, (1998) examined the role of qualitative fat type in combination with energy restriction in order to explore changes in plasma leptin levels in rats. That study indicated that feeding rats a diet that was high in n-3 fatty acid induced hyperleptinaemia, and significantly reduced fat deposition and fat cell weight. In contrast to the findings of Raclot et al. (1997), leptin gene expression was not measured,

and consequently WAT depots that were examined for determination of depot weight and fat cell weight were derived from different sites. Furthermore, discrepancies in circulating leptin levels and expression of leptin messenger RNA across studies suggest site-specific differences in leptin messenger RNA expression. Also, discordance between leptin expression and plasma levels has been observed recently in individuals with broad ranges of adiposity (Ranganathan et al., 1998).

Recently, Hun et al. (1999) examined the effects of fish oil in combination with lard, perilla and soybean oil feeding in obese, diabetic KK-A^y mice. Those investigators examined plasma leptin levels and WAT weights after 12 weeks of feeding. As anticipated, WAT weights and circulating leptin levels decreased in response to fish oil feeding. An elevated expression of leptin messenger RNA was observed previously in mouse adipocytes in eicosapentaenoic acid-containing media, illustrating the necessity of the hypothalamus for complete leptin action (Murata et al., 2000). Takahashi et al. (2000) studied the effect of high-fat safflower, perilla or fish oil feeding on the expression of leptin messenger RNA in rats. WAT leptin expression was significantly higher with high-fat feeding of safflower oil versus perilla or fish oil in epididymal adipose tissue. On the other hand, perirenal leptin messenger RNA expression was elevated in animals fed safflower oil and perilla oil. The observed differences in leptin messenger RNA were unrelated to weight gain, because this variable was not significantly different between the three feeding regimens. Once again, these results suggest site-specific differences, and that safflower oil feeding induces the greatest increase in leptin messenger RNA expression. More recently, Nisoli et al. (2000) infused

intralipid 20% in humans and collected gluteal subcutaneous fat for leptin expression analysis. Intralipid, an emulsion of soybean oil, increased leptin messenger RNA increased significantly after infusion, an effect that was independent of weight gain.

In general, fish oil feeding reduces leptin messenger RNA and circulating levels in animals, whereas feeding safflower oil or other fats that are high in n-6 PUFAs have opposing effects. The decline in weight gain observed with n-3 fatty acid feeding may involve leptin, but not through a direct action of PUFA-dependant stimulation of leptin function and action. More likely, the reduction in leptin expression and production observed with fish oil feeding is a means to prevent hyperleptinaemia, and thus leptin insensitivity and weight gain. It would be of interest to examine whether fish oil feeding in humans displays similar effects on the leptin system as to those observed in animals.

2.3.4. Leptin and peripheral lipid metabolism

The discovery of expression of several functional leptin receptor isoforms in peripheral tissues has led to speculation regarding a role for leptin in lipid metabolism, without the involvement of the hypothalamus (Tartaglia et al., 1995, Fruhbeck et al., 1999, Fruhbeck et al., 2000, Sobhani et al., 2000). Adipose tissue mass is determined by the rate of rival pathways for triglyceride synthesis, versus fatty acid oxidation. Leptin administration in animals favours lipolysis, while subsequently inhibiting lipogenesis. The observed stimulation in fatty acid oxidation results in the explicit loss of adipose tissue. In addition to the effects of leptin on lipolysis and lipogenesis, it has also been shown (Lopez-Soriano et al., 1998) that acute leptin injections in rats induced hypertriglyceridaemia,

and significantly decreased the uptake of triglycerides in both adipose and muscle tissue; these effects were unrelated to any changes in lipogenesis.

2.3.4.1. Leptin stimulates lipolysis

Elevated circulating leptin levels have also been shown (Wang ZW et al., 1999a) to decrease the intracellular lipid content in several tissues via increases in lipid oxidation. Gene therapy (i.e. adenovirus-induced hyperleptinaemia) has been used in animals an alternative to recombinant leptin injections, which produce exceedingly high circulating leptin levels for short periods, whereas gene therapy moderately increases leptin levels and maintains them for a longer duration. Adenovirus-induced hyperleptinaemia reduced body fat by 36% in high-fat fed rats without the participation of the hypothalamus, as revealed through changes in the expression of cocaine- and amphetamine-regulated transcript (Wang ZW et al., 1999b). The gene that encodes this protein is known to be upregulated by leptin, and there were no observed changes in expression. This provides evidence for direct, hypothalamic-independent lipolytic action of leptin in reducing adipose depot weight. In a follow-up study (Wang ZW et al., 1999b), those investigators verified the lipolytic actions of leptin in transplanted denervated epididymal adipose tissue. Thus, acute doses of leptin from injections or adenovirus-mediated expression of leptin are functional without requirement for participation of the hypothalamus, and reduce triglyceride uptake and stimulate lipolysis to reduce adipose weight.

There is a large body of evidence that suggests that leptin functions in an autocrine or paracrine manner to regulate fat stores. Not surprisingly, lipolysis is inhibited in *fa/fa* cells that lack leptin receptor, demonstrating the necessity for a functional leptin receptor

(Wang MY et al., 1999). Specifically, Wang et al. (1999) observed a simultaneous leptin-dependant effect on lipid metabolism in adipocytes from obese Zucker diabetic rats with functional leptin receptors. Leptin upregulated acetyl coenzyme A oxidase and carnitine palmitoyl transferase I, and reduced fatty acid synthase. Thus, leptin reduces lipid synthesis, stimulates the enzymes that are necessary for β-oxidation, and in addition the reduction in carnitine palmitoyl transferase I downregulates fatty acid uptake. Bornstein et al. (1999) recently provided support for this concept, using human adipose tissue and ultrastructural immunogold labelling. Those investigators observed the presence of leptin on cell membranes of adipocytes, supporting an autocrine role of leptin in upregulating lipolytic enzymes.

2.3.4.2. Leptin reduces lipogenesis

Adipose tissue de-novo lipogenesis is a major contributor to overall fatty acid synthesis, more so than hepatic lipogenesis. Swierczynski et al., (2000) analyzed human and rodent adipose tissue samples, and found that lipogenic enzymes are in fact a very significant contributor to overall fatty acid synthesis when taking into account whole-body adipose stores. Therefore, we can assume that, if lipogenesis from adipose tissue is a large contributor to fat synthesis and storage, then reductions in WAT lipogenesis may prevent fat deposition. Recently, Bryson et al. (1999) showed that a single dose of leptin could inhibit lipogenesis in adipose and hepatic tissue, as measured after an intraperitoneal injection of tritiated water and measurement of ^{3}H-labelled fatty acids in both lean and gold thioglucose mice. In addition, Fukuda et al. (1999) showed that leptin specifically inhibits the fatty acid synthase gene by converging on the insulin/glucocorticoid response

element, thus inhibiting synthesis. More recently, stable isotopes were used by Ceddia et al. (2000) to examine the fate of ^{14}C-acetate or oleate in isolated rat white adipocytes in culture. Leptin levels of 10—100 ng/ml stimulated the production of $^{14}CO_2$ with both substrates. In addition, leptin decreased the production of ^{14}C-lipids and stimulated citrate synthase, thus indicating a leptin-mediated stimulation of the Krebs cycle and overall promotion of fatty acids as fuel.

The preference of adipocytes for fatty acids as fuel is supported by in-vitro studies (Sarmiento et al., 1997), in which inhibition by leptin of acetyl coenzyme A carboxylase, the key enzyme in fatty-acid biosynthesis, was observed. Leptin exerts a twofold effect on the fate of cellular fatty acids. Firstly, through the inhibition of acetyl coenzyme A carboxylase. Secondly, the inhibition of acetyl coenzyme A carboxylase reduces concentrations of malonyl coenzyme A, an inhibitor of carnitine acyltransferase and mitochondrial β-oxidation. Soukas et al. (2000) also showed, using microarray analysis, that leptin injections selectively inhibit genes that regulate fatty acid biosynthesis in WAT. Similar results were observed in the liver, a suggested target for leptin in regulating peripheral lipid metabolism. Cohen et al. (1998) used nondestructive ^{13}C-nuclear magnetic resonance to follow leptin kinetics in mice that received the recombinant human protein. The activities of both acetyl coenzyme A carboxylase and fatty acid synthase were decreased, resulting in a significant reduction in hepatic de-novo lipogenesis. Therefore, it appears that leptin acts by inhibiting lipogenic enzymes, which in turn stimulate lipolytic enzymes, promoting the oxidation of fat as fuel.

Interestingly, in addition to inhibition of lipogenesis by leptin, there is recently reported evidence that supports a role for leptin in the inhibition of adipose cell proliferation (Thomas et al., 1999). Bone marrow stromal cells, the precursor cells for both osteoblasts and adipocytes, were used to assess cell differentiation. Recombinant leptin suppressed adipose cell proliferation and favoured differentiation into osteoblasts. Overall, leptin appears to block fatty acid synthesis and triglyceride uptake, as well as favouring mitochondrial fatty acid uptake and ultimate oxidation of the fat as fuel. Therefore, the evidence supports a role for leptin in reducing triglyceride content of adipocytes, muscle and liver by increasing oxidation of fat as fuel. These results are certainly noteworthy, especially when considering that de-novo lipogenesis within adipose tissue contributes significantly to overall lipogenesis and storage in both animals and humans.

2.3.5. Leptin and energy expenditure

A reduced capacity to oxidize fatty acids and low energy expenditure (EE) are obvious risk factors for weight gain. Leptin treatment in animals stimulates EE, reducing weight beyond that attributable to increases in satiety. In addition to central effects of leptin on energy expenditure via hypothalamic neuropeptides, it has also been shown (Zhou et al., 1999, Scarpace et al., 2000, Wang JL et al., 1999) that leptin directly induces expression of UCPs in WAT and brown adipose tissue. By uncoupling mitochondrial β-oxidation from ATP synthesis, these proteins result in increased thermogenesis and energy dissipation. The discovery of UCP2 in WAT and UCP3 in muscle has renewed interest in examining the influences of leptin on the expression of these proteins.

In humans, EE does not appear to be affected by changes in circulating leptin levels, as demonstrated in an obese girl who was unable to produce leptin, but with normal basal EE (Farooki et al., 1999). During leptin therapy her EE remained normal, although she did lose a considerable amount of weight. Short-term changes in fat and carbohydrate intake have been shown (Havel et al., 1999, Reseland et al., 2001) to affect leptin levels, and recently changes in 24-h EE were examined after either carbohydrate or fat overfeeding (Dirlewanger et al., 2000). Neither dietary regimen was associated with any change in EE; it thus appears unlikely that leptin exerts weight-reducing effects via changes in EE.

More recently, it was shown (Doucet et al., 2000) that changes in resting EE and resting fat oxidation in obese men after weight loss were positively correlated with changes in hyperleptinaemia. In support of these findings, it has been shown in postobese individuals matched with never-obese individuals (Filozof et al., 2000); in general, previously obese individuals had impaired fat oxidation and relatively low circulating leptin concentrations. Interestingly, older men had a significant inverse correlation between fasting respiratory quotient and plasma leptin levels, whereas older women tended to have an opposite association and increased risk for weight gain (Soares et al., 2000). Such data indicate an association between leptin and reduced EE or fatty acid oxidation in individuals who are susceptible to weight gain, but further work is required to support this evidence.

2.3.6. Recent clinical evidence for leptin therapy

To date only three clinical trials have involved use of recombinant leptin or a derivative thereof as therapy for obesity and related genetic deficiency of human leptin (Farooki et al., 1999, Heymsfield et al., 1999, Hukshorn et al., 2000).

Heymsfield et al. (1999) conducted a dose-escalation trial that involved recombinant methionyl human leptin in both lean and obese persons. Daily self-administered subcutaneous injections, in concert with energy restriction (500 kcal/day), resulted in mean weight reductions of 0.7±5.4 kg for the 0.01 mg/kg dose and 7.1±8.5 kg for the 0.30 mg/kg dose. The observed fat loss was predominantly associated with fat mass loss. Unfortunately, large variations in weight loss and the large number of adverse events (62%) observed with recombinant leptin injection tends to limit the potential use of leptin as a treatment for obesity. Recombinant leptin is presently being used as a treatment for children with genetic leptin deficiency, and has shown substantial weight loss at low doses (Farooki et al., 1999).

More recently, Hukshorn et al. examined the potential use of pegylated recombinant human leptin (Peg-Ob) as an alternative to the less practical recombinant methionyl human leptin (Hukshorn et al., 2000). Peg-Ob treatment has been reported to reduce appetite in humans (Westerterp-Plantenga et al., 1999). Peg-Ob is a polymer of ethylene glycol that is covalently attached to recombinant leptin, with an increased half-life and reduced immunogenicity. Unfortunately, funding for that study was terminated prematurely, limiting the observed effect of Peg-Ob treatment. Peg-Ob was successful in maintaining moderate circulating leptin levels with single injections per week. There

were no significant reductions in weight loss, but there was a reported decrease in appetite by those individuals who received Peg-Ob. It remains to be seen whether Peg-Ob injections over the long term will be successful in reducing weight.

Obesity in humans appears to be associated with leptin resistance, due to saturation of leptin transport across the blood--brain barrier, thus limiting the amount of leptin that reaches the hypothalamus (Banks et al., 2000). Interestingly, Fujioka et al. (1999) observed increases in cerebrospinal fluid leptin in a small number of adults who received leptin injections, suggesting the existence of a nonsaturable transport system. Therefore, human recombinant leptin treatment for obesity has realistic potential, supporting the need for long-term trial with sufficient power. Leptin treatment is already successful, because peripheral leptin injections can be very useful in the treatment of genetic deficiencies in the obese gene.

2.3.7. Future work

Over the past year there has been a considerable amount of exciting work demonstrating the potential effectiveness of leptin, and insight into the very perplexing function of leptin has been gained. Future efforts should explore the interactions between leptin and ASP. Murray et al. (2000) recently demonstrated an association between ASP and circulating leptin. Specifically, ASP-deficient mice have reduced adiposity and leptin levels, in spite of increased energy intake; it is thus possible that ASP indirectly modifies EE through leptin functioning. In addition, our understanding of the development of leptin resistance is growing from exploration in the animal model over long-term feeding (Lin et al.,

2000). It now appears that there are three stages, beginning with normal feed intake, with weight gain. The second stage is characterized by reduction in food intake, but peripheral leptin resistance is responsible for increased weight gain. Finally, after 4 months of feeding, central leptin insensitivity is unable to reduce the desire for increased energy intake.

Our understanding of leptin function has led to the development of drugs to stimulate weight loss. C-75, a fatty acid synthase inhibitor, was shown (Loftus et al., 2000) to reduce food intake and body weight similar to leptin treatment, but plasma leptin measurements indicated an effect that was independent of leptin, providing more questions than answers.

Transport of leptin across the blood--brain barrier is a limiting step in the eventual action of leptin. Landt et al. (2000) examined cerebrospinal fluid leptin levels in children, and determined that only free leptin is present, suggesting that this is the only transportable, or even active form. This creates the possibility that, when leptin is bound to the nonfunctional soluble leptin receptor, it is unable to cross the blood--brain barrier and exerts its effects within the hypothalamus. Researchers are beginning to explore the leptin--receptor interaction with the hope of discovering a therapeutic treatment for insensitivity to the leptin--receptor interaction.

2.3.8. Conclusion

Since the discovery of leptin in 1994, research in this area has expanded dramatically. Data from human clinical trials are emerging, and results are cautiously optimistic. The use of leptin analogues for treatment of obesity has shown that recombinant leptin is biologically active; in some obese humans low doses reduce appetite and food intake, and at higher doses reductions in body weight and particularly in fat mass may occur.

Over the past year, there has been a significant amount of research exploring nutrient gene interactions between leptin and PUFAs, forging a unique collaboration between nutrition researchers and molecular biologists. It has been shown that the expression of leptin messenger RNA is induced by individual fatty acids in an adipose-depot-specific manner. Current evidence supports direct leptin action in peripheral lipid metabolism, demonstrated by tissue analysis and gold thioglucose-treated mice as a model for central leptin inactivity. In general, leptin functions directly in peripheral cells by inhibiting lipogenesis with concurrent stimulation of lipolysis, resulting in adipose tissue depletion. In addition, significant advances have been made in our knowledge of the association between circulating leptin, energy expenditure and fat oxidation in humans, potentially explaining why some individuals are at risk for weight gain.

Obesity is a polygenic disorder with a complex development process that involves many factors. The discovery of a cure will be very difficult, but we are gathering insight into preventive measures by increasing our understanding of the action of leptin both centrally and peripherally.

RATIONALE

The prevalence of obesity has increased dramatically in many industrialized countries. Accompanying obesity are increased risks of hyperlipidemia, diabetes and cardiovascular disease. Researchers have recently identified specific proteins, including leptin and NPY, which play an important role in regulating food intake and energy expenditure. Recombinant leptin injection produces supraphysiological levels, which reduces body weight and food intake in animals; however, it is unknown whether leptin is as effective at physiological levels. Studies in humans and rodents have demonstrated a positive correlation between circulating leptin levels and adipose stores; suggesting leptin is a marker of stored triglyceride and not a weight reducing protein. Leptin receptor interaction suppresses transcription and release of NPY, a leptin antagonist and potent orexigenic agent. It has also been shown that restricting energy intake reduces circulating leptin levels, regardless of changes in body weight. Furthermore, dietary fatty acid composition is an important determinant of circulating leptin levels in diet-induced obese and normal animals. The mechanism by which dietary fatty acid composition and energy restriction influences leptin production and action is not completely understood but undoubtedly involves fatty acids in the regulation of gene expression. Dietary fatty acid composition influences adipose tissue fatty acid composition when coupled with energy restriction, further modify tissue profiles due to a selective retention of long chain PUFA. Whether altering adipose tissue fatty acid composition influences leptin production remains to be revealed. This study will attempt to characterize the above mechanisms through the measurement of circulating leptin, *obese* mRNA levels and body weight, in order to expand the current knowledge of a role for leptin in dietary-induced obesity.

HYPOTHESIS AND OBJECTIVES

HYPOTHESES

(1) *Ho: Dietary fatty acid composition and energy restriction have no effect on circulating leptin levels, white adipose tissue (WAT) obese mRNA levels, and/or fatty acid composition of WAT depots.*

(2) *Ho: There is no association between circulating leptin levels and WAT obese mRNA levels, fatty acid composition of WAT, food consumption and weight gain in rats.*

(3) *Ho: There are no WAT depot-specific differences in leptin mRNA levels or fatty acid composition.*

OBJECTIVES

(1) To characterize the effects of manipulation of dietary fatty acid composition and energy restriction on circulating leptin levels, white adipose tissue *obese* mRNA levels and fatty acid composition of intra-abdominal adipose depots.

(2) To characterize the associations between circulating leptin levels, white adipose tissue *obese* mRNA levels, fatty acid composition of adipose depots, food consumption and body weight gain in rats, as they are influenced by dietary fatty acid composition and energy restriction in a dietary obese animal model.

(3) To determine if changes in the WAT environment induced by dietary fatty acid composition and energy restriction affect *obese* mRNA production in white adipose tissue and to examine whether this affect is depot-dependant.

CHAPTER 3. MANUSCRIPT II

EFFECTS OF DIETARY FATTY ACID COMPOSITION AND ENERGY RESTRICTION ON ADIPOSE TISSUE FATTY ACID COMPOSITION, *OBESE* mRNA AND SERUM LEPTIN LEVELS IN RATS

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3.1. Abstract

Dietary fatty acid (FA) composition and energy restriction independently affect serum leptin levels; however, it is not known whether this correlates with changes in *obese* (*ob*) gene expression. We hypothesize that in tandem dietary FA composition and energy restriction influence white adipose tissue (WAT) *obese* gene expression in a growing animal model. To examine this association we assessed *ob* mRNA levels in selected visceral WAT by Northern blot analysis and serum leptin levels by radioimmunoassay. Animals consumed diets containing beef tallow (BT), safflower oil (SO) or fish oil (FO) (20% wt/wt) either *ad libitum* or at 60% *ad libitum* intakes. Serum leptin concentrations were higher ($p<0.0001$) after 10 weeks *ad libitum* feeding. Energy restriction decreased ($p<0.0001$) weight gain, perirenal, epididymal and retroperitoneal WAT weights, all of which were positively correlated with serum leptin values ($p<0.003$). WAT *ob* mRNA levels were in the rank order: FO>SO>BT in depots of all groups with *ad libitum* groups showing a higher level of *ob* mRNA, these differences were not observed in serum leptin levels. WAT *ob* mRNA levels were higher in *ad libitum* animals in contrast to restricted fed animals consistent with circulating levels. We observed discordance between *ob* mRNA and circulating leptin levels with respect to dietary fat composition. Diets high in polyunsaturated fat (PUFA) increased *ob* mRNA levels in WAT, whereas diets rich in saturated (SFA) and monounsaturated fat (MUFA) reduced levels. Energy restriction exerts greater control over changes in *ob* mRNA and serum leptin levels than dietary fatty acid composition. Additionally, adipose tissue FA composition was reflective of diet fat consumed. We observed an interactive effect of dietary fat and energy restriction on fatty acid composition in all WAT studied. Linoleic acid (18:2 n-6) concentration increased at

the expense of palmitic acid (16:0), palmitoleic (16:1 n-7) and stearic (18:0) fatty acids. In conclusion, *obese* mRNA levels were influenced by diet fat and energy restriction but these alterations did not confer changes in circulating leptin levels.

3.2. Introduction

Adipose tissue fatty acid composition is influenced by both the amount and the source of dietary fat. Specifically, consumption of polyunsaturated fatty acids (PUFA) of marine source rich in n-3 long chain fatty acids has been shown to reduce adipose tissue growth (Su and Jones 1993, and Cha and Jones 1996 and Okuno *et al.* 1997). The mechanism for the observed reduction is thought to involve reduced absorption and/or increased oxidation of these fatty acids. Okuno and colleagues (1997) demonstrated a specific down-regulation of adipogenesis induced by perilla oil feeding in rats. As compared to a diet containing beef tallow fed, there were reduced mRNA levels of PPARα, adipocyte P2 and adipsin with perilla oil feeding after 4 months.

With the unique collaboration between molecular biology and nutrition, we are beginning to understand the effect of diet consumption at a molecular level. Dietary fatty acid profile is the primary predictor of adipose tissue fatty acid composition under normal circumstances in humans and animals (London *et al.* 1991, Garland *et al.* 1998 and Cha and Jones 1996) and changes in the membrane fatty acid composition have been shown to influence insulin action, glucose transport and other cellular processes (Clandinin *et al.* 1985, Field *et al.* 1990, Pan and Storlien 1993). Apart from dietary fat quality, negative energy balance creates a unique scenario in which certain fatty acids are preferentially retained while others are oxidized as shown in many animal studies (Chen and Cunnane 1992, Cha and Jones 1996). Consistently, it appears that adipose tissue concentrations of the essential fatty acid linoleic acid (18:2 n-6) increases under situations of energy restriction; whereas, shorter more common fatty acids, such as palmitic (16:0) and oleic

(18:1 n-9) acid, are favored for oxidation as demonstrated in growing animals (Cha and Jones 1996). As well, increased concentrations of linoleic acid have been observed in triglycerides from liver and serum in fasted rats (Chen and Cunnane 1992). The significance of this observation during energy restriction is not yet known, but likely is related to the essentiality of polyenoic acids for growth and development in these animals. Furthermore, the advent of molecular techniques has allowed researchers to examine the implications of changes in the WAT environment beyond the tissue and cellular level. Okuno *et al.* (1997) have demonstrated a direct effect of diet fat feeding on molecular processes involving specific adipocyte proteins and their role in lipid metabolism.

One such protein involved in lipid metabolism is leptin, the 16kDa protein product of the *obese* (*ob*) gene, which is primarily produced and secreted by white adipocytes (Zhang *et al.* 1994). Once secreted into the circulation, the fate of leptin is composed of two distinct paths. Firstly, leptin can cross the blood brain barrier (BBB), enabling interaction with class I cytokine receptors in the hypothalamus, thereby exerting anorexic and thermogenic effects (Stephens *et al.* 1995). Secondly, studies with animals have shown reductions in weight through the aforementioned processes upon administration of leptin (Campfield *et al.* 1995). More recently, considerable attention has been centered on the extrahypothalamic system, and more specifically, on a peripheral role in nutrient metabolism within adipocytes and non-adipocyctes (Kawakami *et al.* 1999, Koyama *et al.* 1997, Wang ZW *et al.* 1999a). It is becoming evident that the peripheral role for leptin may be of greater importance. Researchers have examined effects of alterations in

circulating levels, but saturation of leptin transport across the BBB may limit the size of the leptin signal reaching the hypothalamic centers and potentially diminishing the true effect (Banks *et al.* 2000).

Circulating leptin concentrations are a strong predictor of adipose stores in humans (Considine *et al.* 1996, Liuzzi *et al.* 1999) and animals (Maffei *et al.* 1995, Frederich *et al.* 1995). Yet, there is still very little known regarding dietary factors with the exception of fasting and refeeding which have been shown to influence leptin levels in an opposing fashion. Fasting in animals (MacDougald *et al.* 1995) and humans (Boden *et al.* 1996, Kolaczynski *et al.* 1996 a, b) has been shown to significantly reduce leptin mRNA and plasma levels independent of changes in weight or fat mass. Plasma leptin levels correlate strongly with the amount of triglyceride stored in this tissue (Caro *et al.* 1996).

Many factors have been shown to regulate leptin expression some of which include glucocorticoids, cold exposure and insulin (Cusin *et al.* 1995, Trayhurn *et al.* 1995, Russell *et al.* 1998). Raclot *et al.* (1997) observed reduced retroperitoneal (Rp) *ob* mRNA levels with docosahexanoic acid feeding, which were positively correlated with fat cell size. This suggests a role for n-3 PUFA in down-regulating not only fat cell size, but leptin expression in Rp WAT. As well, this affect was site-specific as demonstrated by a lack of response in subcutaneous WAT.

These observations suggest that the selective deposition of body fatty acids depend on both the energy status of the animal and the habitual dietary fatty acid composition.

Obese gene expression is under regulatory control of these factors as well. Whether diet fatty acid composition and energy restriction influence adiposity through changes in leptin production in a growing animal is not known. Hence, the aim of this study was to examine leptin expression in a growing animal that was fed high-fat diets differing in their source of fat and level of energy intake. White adipose depots are characterized according to anatomic location and metabolic differences (Raclot *et al.* 1997, Bailey *et al.* 1993). Tissue-specific differences in leptin expression have been demonstrated (Masuzaki *et al.* 1995 Ranganathan *et al.* 1998), which have consistently shown positive associations between *ob* mRNA levels in visceral WAT and circulating leptin concentrations; whereas, this was not observed in subcutaneous WAT. Additionally, visceral WAT is considered the primary contributor to overall circulating leptin concentrations. Therefore, the second aim of the present study was to examine whether the regulation of *obese* mRNA levels by diet fat and energy intake is tissue-dependant. Thus we investigated *obese* mRNA expression in the intra-abdominal WAT depots and compared these levels between dietary treatment groups.

This study was designed to address the interactive effects of dietary fat type and energy restriction on: (i) weight gain (ii) tissue-specific differences in abdominal WAT growth and fatty acid composition (iii) tissue-specific leptin expression and its relationship with circulating levels. The results from this study will address the importance of dietary fat source in *ad libitum* feeding as compared to negative energy balance. Ultimately, our objective is to determine whether leptin, an adipose tissue hormone, is important in physiological changes associated with diet-induced obesity.

3.3. Methods and Materials

Animals and diets

Seventy-two male Sprague-Dawley rats (275 ± 10 g) purchased from Charles River, Inc. (Quebec, Canada) were housed individually in stainless steel hanging cages at 22 ± 1° C with a 12-h light-dark cycle. After habituation to commercial rat chow for 7 days, animals were stratified into one of three dietary fat groups and fed high fat diets containing either: menhaden fish oil, safflower oil, or beef tallow as the sole fat source. Animals within each fat group were further subdivided to receive for 10 weeks, *ad libitum* diet or at 60% of *ad libitum* daily intakes (n=12). Diets were prepared fresh and were stored at -20° C when not in use, up to a max of 1 week. The fatty acid composition of each dietary fat was determined prior to feeding by gas chromatography (Table 3.1). Beef tallow diets were supplemented with 1% safflower oil to maintain adequate intakes of the linoleic acid (18:2 n-6). Food intake was calculated daily in the *ad libitum* fed animals to determine mean intakes in each fat group and subsequent reduced intakes for food-restricted animals. With the exception of cornstarch, diets consumed by food-restricted animals were modified to supply equal quantities of all nutrients including fat each day compared with the *ad libitum* fed diets (Table 3.2). Body weights were monitored weekly throughout the feeding trial. After 10-weeks animals were food deprived overnight and killed by CO_2 asphyxia. Serum was isolated from trunk blood, and immediately stored at -80°C until analysis. Epididymal (Ep), perirenal (Pr) and retroperitoneal (Rp) fat pads were rapidly collected, weighed, and frozen in liquid N_2, followed by storage at -80°C. Ethical approval for the study was obtained from the McGill University Animal Ethics Committee.

Serum analyses

Serum concentrations were performed in duplicate, using commercially available radioimmunoassay kits for rat leptin (Linco Research Diagnostics, MO) and insulin (ICN Pharmaceuticals, INC, Costa Mesa, CA) utilizing HPLC purified ^{125}I-rat leptin (Ma *et al.* 1996) or insulin as tracer. Radioactivity was determined by gamma counting (LKB Wallac, 1282 compugamma CS, Fisher Scientific Montreal Canada) and collected as counts per min (CPM). Serum values were quantified using a standard curve and automated data reduction procedures. Leptin and insulin values were expressed as ng/mL and μU/mL respectively.

Lipid extraction and fatty acid analysis

An aliquot from each WAT site was used for total lipid extraction using a modified method according to Folch *et al.* (1957) for analysis of stored triglycerides. Briefly, 50 mg of tissue was homogenized for 15 sec at 12000 rpm in 2 mL of chloroform-methanol 2:1 (v/v), with heptadecanoic acid (C-17:0) as an internal standard. The homogenate was filtered with #1 Whatman paper to remove any connective tissue remnants. Following the addition of 0.2 volumes of 0.9% saline, samples were vortexed and centrifuged for 10 min at 2500 rpm. The supernatants were discarded and 2 mL 0.5M sodium hydroxide-methanol was added before 15 min incubation at 80-90° C. After cooling to room temperature samples were acidified with 1M sulphuric acid to pH 3.0, extracted twice with hexane and dried under nitrogen. Fatty acid methyl esters were prepared with the addition of 1 mL boron trifluoride-methanol (14% solution, Sigma)-hexane-methanol 7:6:7 (v/v/v), after incubation for 55min at 90° C (Al Makedessi *et al.* 1985). After

cooling, hexane was added followed by 1 mL distilled water. After centrifugation, supernatants containing hexane and fatty acid methyl esters were collected and resolubilized in 1 mL hexane for gas chromatograph analysis. Butylated hydroxytolulene at the final concentration of 0.1 % was added to all solvent mixtures as an antioxidant.

Fatty acid methyl esters were analyzed in duplicate using a Hewlett-Packard 5890 gas liquid chromatograph (GC), (Palo Alto, CA), equipped with a 30 m x 0.2 mm SP 2330 column (Supelco, Bellafonte, PA), flame ionization detectors, and an automated injection system. The injector and detector temperatures were set at 210° C and 250° C, respectively. The GC condition were as follows; oven temperature was held at 135° C hold for 2 min and increase at 2° C/min up to 150° C and increase at 0.7° C/min up to 160° C/min then increase at 3° C/min up to 200° C /min. Helium was used as the carrier gas. Fatty acid methyl esters were identified based on the retention times of known fatty acid standards (Supelco, Bellefonte, PA).

RNA isolation and Northern blotting

Whenever possible, 1.0 g of WAT was homogenized in TRIzol Reagent (Invitrogen- Life Technologies, Burlington ON) using a Polytron power homogenizer at top speed for 30sec as per the manufactures protocol. Subsequent isolation, precipitation and washing yielded total RNA from each WAT sample and stored in Tris-EDTA buffer (pH 8.0). The optical density (O.D.) was calculated using a spectrophotometer at 260–280 nm to ensure the integrity and concentration of isolated RNA. Agarose gel (1%) electrophoresis was performed using 15 μg total RNA along with LS174T cell total RNA as negative control

and Ep control WAT total RNA as a positive control. A representation of the U.V. transilluminated ethidium bromide gel (EtBr) is shown in Figure 3.1 to allow assessment of quantity and quality of the mRNA loaded. After electrophoresis, the RNA was then transferred onto a nitrocellulose membrane (Schleicher and Schuell Inc, NH) using 20X SSC (Sodium Chloride-17.5%, Sodium Citrate-8.8%) solution overnight and cross-linked by U.V. exposure (Stratalinker, BioRad, Life Sciences Mississauga, Ontario).

Prehybridization and hybridization of the blots were performed using a solution of 5X SSPE, 5X Denhardts, 50% Formamide, 0.5% SDS, and 250 μg/mL ssDNA. Specific cDNA probes labeled with [^{32}P]-dCTP by random priming using a Nick translation kit (Invitrogen- Life Technologies, Burlington ON). The cDNA fragment (320 bp) for rat adipose tissue leptin was synthesized using 3' primer TCATTGGCTATCTGCAGCAC and 5' primer sequence AAGAAGATCCCAGGGAGGAA and ligated into a pCRT-MII vector followed by transformation into DH5α cells, and, it was in this form which was generously donated by Dr. Ruth B.S. Harris (Pennington Biomedical Research Center, LA) as per (Harris *et al.* 1996). Rodent β-actin cDNA was kindly provided by Dr. Kris Chadee and was used for normalization of initial RNA input. Blots were washed twice with 2X SSC at room temperature followed by three washes with 0.1X SSC and 0.1% SDS at 64°C. Autoradiography was performed by exposure of radioactive membranes to Hyperfilm at -80° C with intensifying screens. The hybridization signal of *ob* mRNA from each pooled WAT sample (total RNA) was determined from autoradiograms using a phosphoimaging system, which measures the product of the density and area of the hybridization signal. For each sample, the background hybridization signal was

subtracted from the value measured for *ob* mRNA. The resultant mRNA hybridization value reported for each group was the mean of 3-4 individual samples of pooled total RNA. Values of mRNA for each group of animals were expressed as arbitrary units as compared to β-actin, a constitutively expressed gene and commonly used standard.

Immunoprecipitation and Western blotting

Protein A-Sepharose beads (0.072g) were suspended in 1mL of 10mM Tris-HCl buffer and rotated for 15 min at 4°C. After centrifugation and washing, beads were incubated overnight with agitation in 1% BSA for blocking. The following day the beads were incubated overnight in 10mM Tris-HCl buffer containing 10μg rat specific leptin antibody (Research Diagnostics, Inc. Flanders NJ) producing coupled antibody Protein A-Sepharose beads. Coupled antibodies were then ready for incubation with sample, allowing precipitation and SDS-PAGE (Sodium dodecylsulfate-polyacrylamide gel electrophoresis). Whole cell lysate and serum samples were isolated as described below and were used for both immunoprecipation studies and analysis of leptin protein quantification.

Tissues aliquots were lysed in RIPA buffer (1XPBS, 1% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS) at a volume of 3mL per gram of tissue with using a Polytron power homogenizer at 4°C. Following homogenization the sample was transferred to microcentrifuge tubes and centrifuged at 10000 x g for 10 min at 4°C. Protein concentration was determined by the Bradford method and expressed as mg/g tissue (BioRad, Life Sciences Mississauga, Ontario). Whole cell lysates (50-100 μg) were

resolved by 15 % SDS-PAGE containing 15 % acrylamide and 10 % bisacrylamide, and transferred to nitrocellulose membranes. Thereafter, membranes were blocked with 5% nonfat dried milk in 0.1% Tween-TBS (T-TBS) buffer overnight at 4°C. The following day membranes were washed three times for 5 min with T-TBS, and then incubated with the primary antibody solution (0.5-1.0 μg/mL) in 10mL 0.05% T-TBS with 1% nonfat dried skim milk overnight at 4°C with shaking. Following three washes with T-TBS membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG in 10mL 0.05% T-TBS with 1% nonfat skim milk for 1hr at room temperature and washed two times with 3T-TBS, followed by two times with T-TBS at 5 min each. Proteins were detected by enhanced chemiluminescence using immunodetection reagents (Amersham Biosciences, Baie d'Urfe, Quebec Canada) and evaluated using densitometric scanning.

Statistical analyses

Data were analyzed by two-way analysis of variance, with initial body weight included in the model as a covariate, using a general linear models procedure (SAS Institute Inc, Cary NC). Normal distribution of all variables was assured using the Shapiro-Wilk test for normality. Comparisons between individual diets were made using Scheffé post-hoc test after treatment effects were determined by analysis of variance. Differences were considered to be significant at $p<0.05$. Using a multivariate model in SAS, partial correlation coefficients were calculated for all variables to examine associations between outcome measures. Data are presented as means ± SEM (n=12), except where otherwise noted.

3.4. Results

Animals and measurements on white adipose tissue

After 10 weeks, marked alterations in nutrient metabolism were observed in *ad libitum* fed rats compared with energy restricted animals. Food intake, final body weight, weight gain, visceral (epididymal, retroperitoneal and perirenal) white adipose tissue weight for rats fed diets differing in fat source and level of energy are shown in Table 3.1. Food intakes were similar between levels of food intake in beef tallow, safflower oil and fish oil fed animals; 23.0 ± 0.7, 22.7 ± 0.5 and 21.6 ± 0.6 g, respectively. Food intake for the energy restricted animals; 13.8 ± 0.1, 13.7 ± 0.1 and 13.0 ± 0.1 g, respectively, was lower ($p<0.0001$) for the main effect of energy. A main effect of energy intake ($p<0.0001$) was seen in lowering body weight in animals pair-fed the 60% *ad libitum* diets. At the end of the 10 weeks feeding trial all animals had gained weight, with body weights greater ($p<0.0001$) in animals fed *ad libitum* (>500g), which is more than 100g heavier than the energy restricted animals. Accordingly, weight gain was different ($p<0.0001$) between the level of food intake as a main effect of energy. We did not observe any main effects for fat or the interaction of these components with respect to body weight, weight gain, and food intake.

Energy restriction decreased ($p<0.0001$) weights of all adipose tissues studied (Table 3.3). When comparing experimental groups, specific WAT weights were heavier in the rank order Rp>Ep>Pr. Of particular note, Ep and Rp WAT weights were similar, and of larger mass ($p<0.025$) than Pr WAT weights. Between dietary regimens, total abdominal WAT were larger in animals fed BT>SO>FO *ad libitum* (30.5 ± 3.3, 29.2 ± 2.1, and 25.7

± 2.4 g, respectively); although, they were not different (p=0.2). Restricted feeding showed a dissimilar pattern with SO>BT>FO (8.7 ± 0.8, 7.1 ± 0.8, and 5.9 ± 0.9 g, respectively) feeding stimulating larger adipose growth. There was no main effect for the dietary source of fat or interactive effects with respect to changes in abdominal adipose tissue weight in either *ad libitum* or 60% *ad libitum* feeding groups.

Serum leptin and insulin analyses

Fasting circulating concentrations of leptin were lower (p<0.0001) in the 60% energy restricted animals as compared to *ad libitum* fed animals (Figure 2). Leptin levels were at least 70% lower in restricted (2.7 ± 0.2, 1.9 ± 0.3 and 2.6 ± 0.3 ng/mL) compare to *ad libitum* fed rats (10.1 ± 1.6, 8.9±1.3 and 11.6±2.4 ng/mL) with beef tallow, fish oil and safflower oil fed animals, respectively. However, there were no interactive effects (p=0.73) or main effect of fat composition (p=0.45) on changes in serum leptin levels. Fasting serum insulin levels were not different between any of the experimental groups in this study and were not influenced by an interaction of fat composition and energy intake (p=0.18) (Table 3.3).

Fatty acid composition of white adipose tissue

The fatty acid compositions of Pr, Ep and Rp WAT, are shown in Tables 3.4, 3.5 and 3.6, respectively. The WAT fatty acid compositions for *ad libitum* fed animals were generally representative of the dietary fat consumed. Adipose tissue concentrations of 18:0 and 18:1 (n-9) were highest in beef tallow group (p<0.03) and 18:2 (n-6) was greatest in safflower oil group (p<0.05), thus resembling the fatty acid composition in the diet

(Table 3.1). Similarly, greater concentrations of 22:6 (n-3) were found in the fish oil group ($p<0.0001$). Conversely, animals restricted in energy showed modified FA compositions of WAT but were generally reflective of the fat consumed with some notable changes; specifically, an observed increase in 18:2 (n-6) concentrations.

An interactive effect of fat composition and energy restriction was observed for Pr adipose tissue concentrations of 14:0 ($p<0.0004$, data not shown), 16:0 ($p<0.051$), 18:0 ($p<0.0001$), 18:2 n-6 ($p<0.014$) and 22:6 n-3 ($p<0.0001$) as shown in Table 3.4. In contrast, only 22:6 (n-3) concentrations were influenced by the interaction of these components of the diet ($p<0.0001$) in Ep adipose tissue (Table 3.5). Independent effects of dietary fat composition and energy intake were found for 14:0 (data not shown), 16:0, 16:1 (n-7), 18:0, and 18:2 n-6 ($p<0.05$) deposition in epididymal tissues (Table 3.5). Rp tissue concentrations of 14:0 (data not shown), 16:0, 18:0, 18:2 (n-6), and 22:6 (n-3) were influenced ($p<0.008$) by the interaction of dietary fat composition and energy restriction (Table 3.6).

Interestingly, when animals were food restricted, adipose depot concentrations of 16:0 and 16:1 (n-7) were reduced ($p<0.0001$) independent of dietary fat source consumed. Accordingly, SO and FO feeding reduced ($p<0.0001$) the sum of SFA in all adipose depots examined. Additionally, concentrations of 18:2 (n-6) increased in response to energy restriction in all animals with the exception of Pr tissue from beef tallow fed animals; although, this observation was only effected in Rp tissue from all sources of fat ($p<0.05$) and in Pr tissues from fish oil fed animals. These changes in 18:2 concentrations

are reflected in the sum of PUFA which were greater in Rp WAT from all sources of fat and in Pr WAT from rats fed SO and FO. While we observed a trend for increased SFA and MUFA accumulation in the tissues of animals fed BT, the reverse tendency was observed in SO and FO fed rats. Generally, Rp WAT was influenced to a greater extent than either Ep or Pr, with Rp depot fatty acid compositions much more reflective of the dietary fat (Table 3.6). What is more, SFA concentrations were larger in the rank order BT>SO>FO and were consistent with the source of fat.

Partial correlations between serum leptin levels and outcome measures

To further examine the changes in outcome measures, partial correlation coefficients were calculated according to serum leptin levels (Table 3.7). Circulating leptin levels correlated with established indicators of adiposity. There was a positive association between serum leptin and body weight ($r=0.39$, $p<0.0001$) and WAT ($r=0.40$, $p<0.002$). Of note was the observed positive correlation between WAT and leptin levels. Epididymal WAT was the strongest predictor of serum leptin levels. There was no association between serum leptin and insulin in this study. There was a positive association ($r=0.48$, $p<0.0001$) between leptin and Pr WAT fatty acid concentrations of 22:6 (n-3), but not with any other fatty acids or sums of fatty acids. Examination of Ep WAT revealed positive correlations between serum leptin and concentrations of 16:0 and 16:1 n-7 ($r=0.26$ $p<0.035$ and $r=0.37$ $p<0.0019$). Rp WAT sum SFA and MUFA were positively correlated ($r=0.40$ $p<0.0009$ and $r=0.27$ $p<0.0259$) with circulating leptin levels. In contrast, Rp tissue concentrations of PUFA were negatively correlated ($r= -0.40$ $p<0.0008$) with leptin levels, which is consistent with the observed accumulation of 18:2

(n-6) and 22:6 (n-3) fatty acids in this depot.

Effects of dietary fat type and energy restriction on *obese* mRNA levels

Total RNA was isolated from 6-7 animals per treatment group and pooled to provide necessary quantity for triplicate analysis. Northern blot analysis of pooled total RNA samples (20μg total RNA) from each treatment group and WAT depot is shown on Figure 3.1. The intensities of the specific signal for leptin mRNA were lower in animals fed the energy restricted versus *ad libitum* diets (Figure 3.3 and 3.4) for all depots studied, which is consistent with previous reports. In *ad libitum* animals WAT *ob* mRNA levels were generally greater in Rp WAT, with the exception of FO feeding, which resulted in greater *ob* mRNA levels in Ep WAT. Dietary fat type also exerted different regulatory control over expression in these tissues. For example, animals fed BT had a 50% increase in *ob* mRNA levels in Rp WAT whereas SO fed rats had a 3-fold increase in *ob* mRNA concentrations in Rp WAT versus Ep WAT. FO feeding increased *ob* mRNA levels by 66% in Ep versus Rp WAT. Energy restricted animals had lower levels of *ob* mRNA in all tissues, with the lowest levels observed in BT and SO fed animals. This effect was not tissue-specific, indicated by the near identical levels in both WAT types. In contrast, FO feeding stimulated Ep WAT *ob* mRNA by 50 % compared to Rp WAT, which was also similar to BT and SO levels. Thus, energy restriction with FO feeding was the only dietary regimen to influence *ob* mRNA and was tissue-specific to Ep WAT. Taken together, these results suggest that PUFA rich fat sources influence *ob* mRNA differently, by relating specifically to the source (n-3 or n-6) and the site (Ep or Rp).

Effects of dietary fat type and energy restriction on white adipose tissue leptin levels

To examine the effect of dietary fat type and energy restriction on leptin concentrations in WAT tissues, we measured protein levels using an anti-rat polyclonal antibody, which was previously shown (Maffei *et al.* 1995) to recognize plasma leptin. After several attempts it was determined that tissue leptin concentrations were either too low to detect or the method was not sensitive enough for quantification. By using an immunoprecipitation protocol we attempted to concentrate levels of leptin in WAT and serum for measurement using protein A-Sepharose beads coupled to leptin polyclonal antibodies. Unfortunately, it appeared that levels could not be quantified using general antibody methods. At present, the quantification of leptin may require highly sensitive radioisotopic methods such as those used in radioimmunoassay analysis (Ma et al., 1996), but modified for tissue quantification. The present results suggest leptin protein concentrations were beyond the level of detection in our rats following an overnight fast.

Table 3.1. Major fatty acid composition of the experimental fats

Fatty Acid (%)	Beef Tallow (BT)	Safflower Oil (SO)	Menhaden Oil (FO)
14:0	4.1	ND	8.4
16:0	27.7	6.0	15.2
16:1 (n-7)	6.0	ND	11.6
18:0	14.0	4.0	2.7
18:1 (n-9)	45.5	19.0	9.5
18:2 (n-6)	1.9	69.7	1.8
18:3 (n-3)	0.2	0.4	1.8
20:0	0.6	ND	0.2
20:1 (n-9)	ND	ND	1.3
20:4 (n-6)	ND	ND	2.3
20:5 (n-3)	ND	ND	16.0
22:6 (n-3)	ND	ND	10.8
Other	ND	0.9	18.4

ND – Non detectable

Table 3.2. Dietary composition

g / 100 g diet	Energy Intake (% of *ad libitum*) 100%	60%
Fat[1]	20.0	33.3
Casein (vitamin free)	15.0	25.0
Corn Starch (dextrinized)	45.0	8.5
Sucrose	10.0	16.7
Cellulose	5.0	8.3
AIN-93G Vitamin Mix[2]	1.0	1.7
AIN-93M Mineral Mix[3]	3.5	5.8
L-Cystine	0.18	0.3
Choline Bitartrate (98% choline)	0.25	0.42
Tert-butylhydroquinone	0.0004	0.007

[1] Dietary sources of fat included beef tallow, safflower oil or menhaden fish oil.
[2] Vitamin mix (g/kg), Nicotinic acid 3.0, D-Calcium Pantothenate 1.6, Pyridoxine HCL 0.70, Thiamin HCL 0.60, Riboflavin 0.60, Folic acid 0.20, D-Biotin 0.02, Vitamin B_{12} (0.1% titrated in mannitol) 2.50, Alpha tocopherol powder (250 U/g) 30.0, Vitamin A Palmitate (250,000 U/g) 1.60, Vitamin D_3 (400,000 U/g) 0.25, Phylloquinone 0.075, Powder sucrose 959.655.
[3] Mineral mix, Calcium Carbonate 35.7%, Monopotassium Phosphate 25.0%, Sodium Chloride 7.4%, Potassium Sulfate 4.66%, Potassium Citrate Monohydrate 2.8%, Magnessium Oxide 2.4%, Ferric Citrate 0.606%, Zinc Carbonate 0.165%, Manganese Carbonate 0.063%, Copper Carbonate 0.03%, Potassium Iodate 0.001%, Sodium Selenate, Anhydrous 0.001025%, Ammonium Molybdate $.4H_2O$ 0.000795%, Sodium Metasilicate $.9H_2O$ 0.145%.

Table 3.3. Final body mass, weight gain, adipose depot weights and food intake from animals fed beef tallow, safflower oil and fish oil at graded levels of energy intake for 10 weeks[1].

	Energy Intake	Dietary Fat Source		
		Beef Tallow	Safflower Oil	Menhaden Oil
Body weight (g)	100%	526.1 ± 14.7	537.7 ± 11.6	507.4 ± 17.2
	60%	362.6 ± 3.8*	383.3 ± 6.5*	367.9 ± 4.6*
Weight gain (g)	100%	238.7 ± 13.8	249.1 ± 9.0	217.8 ± 17.1
	60%	71.9 ± 3.7*	91.7 ± 7.1*	77.4 ± 5.6*
Ep[2] WAT (g)	100%	12.4 ± 1.6	11.4 ± 1.2	9.7 ± 0.9
	60%	2.9 ± 0.3*	3.6 ± 0.3*	2.5 ± 0.3*
Rp[2] WAT (g)	100%	14.2 ± 1.4	14.0 ± 0.9	12.4 ± 1.3
	60%	3.2 ± 0.4*	3.8 ± 0.4*	2.6 ± 0.5*
Pr[2] WAT (g)	100%	3.69 ± 0.5	3.7 ± 0.4	3.46 ± 0.4
	60%	0.92 ± 0.1*	1.1 ± 0.1*	0.71 ± 0.1*
Food intake (g)	100%	23.0 ± 0.7	22.7 ± 0.5	21.6 ± 0.6
	60%	13.8 ± 0.1*	13.7 ± 0.1*	13.0 ± 0.1*
Insulin (μU/mL)	100%	68.3 ± 0.7	69.1 ± 0.7	70.8 ± 0.5
	60%	68.5 ± 0.7	68.5 ± 0.8	68.4 ± 0.5

[1] **Data are presented as Mean ± S.E.M, (n=12).**

[2] **Abbreviations Ep – epididymal, Rp – retroperitoneal, Pr – perirenal, WAT – white adipose tissue**

* **Denotes statistically significantly different for main effect energy *ad libitum* fed animals (P<0.05)**

Table 3.4. Major fatty acids accumulated in perirenal adipose depots of rats fed beef tallow, safflower oil and fish oil at graded levels of energy intake for 10 weeks[1].

Fatty Acid (%)		Beef Tallow	Safflower Oil	Menhaden Oil
16:0	**100%**	26.3 ± 0.9^{a}	22.1 ± 1.3^{a}	21.5 ± 0.7^{b}
	60%	23.9 ± 0.5^{a}	$16.4 \pm 1.1^{*a}$	$14.5 \pm 0.9^{*a}$
16:1 (n-7)	**100%**	6.6 ± 0.4	4.8 ± 0.5	4.5 ± 0.3
	60%	$4.3 \pm 0.5^{*a}$	$2.1 \pm 0.4^{*b}$	$1.5 \pm 0.3^{*b}$
18:0	**100%**	4.5 ± 0.1^{a}	3.5 ± 0.1^{b}	3.1 ± 0.1^{b}
	60%	$7.4 \pm 0.3^{*a}$	3.6 ± 0.1^{b}	3.7 ± 0.1^{b}
18:1	**100%**	46.5 ± 2.2^{a}	31.5 ± 1.8^{b}	28.7 ± 1.9^{b}
	60%	50.3 ± 0.4^{a}	30.4 ± 1.9^{b}	25.2 ± 0.6^{b}
18:2 (n-6)	**100%**	11.5 ± 3.5^{a}	34.1 ± 3.5^{b}	38.8 ± 0.9^{b}
	60%	9.3 ± 0.5^{a}	43.4 ± 3.4^{b}	$51.1 \pm 1.6^{*b}$
22:6 (n-3)	**100%**	ND	ND	6.0 ± 0.01
	60%	ND	ND	$4.6 \pm 0.03^{*}$
Sum SFA	**100%**	32.7 ± 1.1^{a}	27.0 ± 1.4^{b}	25.8 ± 0.9^{b}
	60%	33.5 ± 0.3^{a}	$21.1 \pm 1.2^{*b}$	$19.1 \pm 0.9^{*b}$
Sum MUFA	**100%**	53.2 ± 2.6^{a}	36.3 ± 2.3^{b}	33.2 ± 2.2^{b}
	60%	54.6 ± 0.5^{a}	32.6 ± 2.6^{b}	26.7 ± 1.0^{b}
Sum PUFA	**100%**	11.9 ± 3.5^{a}	35.1 ± 3.5^{b}	40.4 ± 1.0^{b}
	60%	9.7 ± 0.5^{a}	44.3 ± 3.4^{b}	52.1 ± 1.7^{b}

ND – Non detectable.

[1] **Data are presented as Mean ± S.E.M, (n=12).**

[a] **Groups with different letters are statistically significantly different within the same level of food intake, $p<0.05$.**

[*] **Denotes statistically significantly different from *ad libitum* fed animals, $p<0.05$.**

Table 3.5. Major fatty acids accumulated in epididymal adipose depots of rats fed beef tallow, safflower oil and fish oil at graded levels of energy intake for 10 weeks [1].

Fatty Acid (%)		Beef Tallow	Safflower Oil	Menhaden Oil
16:0	100%	26.0 ± 0.8^{a}	22.6 ± 0.5^{a}	20.5 ± 0.6^{b}
	60%	$21.7 \pm 0.7^{*a}$	$16.1 \pm 0.7^{*b}$	$14.8 \pm 0.8^{*b}$
16:1 (n-7)	100%	7.8 ± 0.5^{a}	5.7 ± 0.3^{b}	4.6 ± 0.2^{b}
	60%	$4.4 \pm 0.3^{*a}$	$2.8 \pm 0.2^{*a}$	$2.2 \pm 0.3^{*b}$
18:0	100%	4.5 ± 0.2^{a}	3.1 ± 0.1^{b}	2.8 ± 0.1^{b}
	60%	5.4 ± 0.4a	3.1 ± 0.32	3.2 ± 0.06^{b}
18:1	100%	47.0 ± 1.9^{a}	31.9 ± 1.5^{b}	28.2 ± 1.8^{b}
	60%	43.1 ± 2.5^{a}	29.9 ± 1.4^{b}	27.1 ± 0.4^{b}
18:2 (n-6)	100%	9.9 ± 3.1^{a}	31.7 ± 2.2^{b}	39.6 ± 3.0^{b}
	60%	18.0 ± 4.3^{a}	43.7 ± 2.6^{b}	48.0 ± 1.5^{b}
22:6 (n-3)	100%	ND	ND	6.1 ± 0.01
	60%	ND	ND	$3.3 \pm 0.09^{*}$
Sum SFA	100%	32.5 ± 1.0^{a}	27.2 ± 0.7^{b}	24.5 ± 0.8^{b}
	60%	28.8 ± 1.2^{a}	$21.4 \pm 1.0^{*b}$	$18.8 \pm 0.8^{*b}$
Sum MUFA	100%	54.9 ± 2.2^{a}	37.7 ± 1.7^{b}	32.8 ± 2.1^{b}
	60%	47.6 ± 2.7^{a}	34.3 ± 1.5^{b}	29.4 ± 0.7^{b}
Sum PUFA	100%	10.5 ± 3.1^{a}	32.9 ± 2.3^{b}	40.1 ± 3.0^{b}
	60%	18.3 ± 4.3^{a}	42.1 ± 2.6^{b}	48.3 ± 1.5^{b}

ND – Non detectable

[1] **Data are presented as Mean ± S.E.M, (n=12).**

[a] **Groups with different letters are statistically significantly different within the same level of food intake, $p<0.05$.**

[*] **Denotes statistically significantly different from *ad libitum* fed animals, $p<0.05$.**

Table 3.6. Major fatty acids accumulated in retroperitoneal adipose depots of rats fed beef tallow, safflower oil and fish oil at graded levels of energy intake for 10 weeks [1].

Fatty Acid (%) Oil		Beef Tallow	Safflower Oil	Menhaden
16:0	**100%**	27.6 ± 0.3^{a}	22.6 ± 0.4^{b}	20.0 ± 0.3^{c}
	60%	$23.4 \pm 0.5^{*a}$	$15.3 \pm 0.6^{*b}$	$13.5 \pm 0.6^{*b}$
16:1 (n-7)	**100%**	6.7 ± 0.3^{a}	4.3 ± 0.1^{b}	3.7 ± 0.2^{b}
	60%	$3.2 \pm 0.2^{*a}$	$1.6 \pm 0.1^{*b}$	$1.0 \pm 0.1^{*b}$
18:0	**100%**	4.9 ± 0.1^{a}	3.4 ± 0.1^{b}	2.7 ± 0.08^{b}
	60%	$7.9 \pm 0.2^{*a}$	3.6 ± 0.1^{b}	$3.8 \pm 0.1^{*b}$
18:1	**100%**	51.0 ± 0.4^{a}	31.1 ± 0.4^{b}	26.7 ± 0.3^{c}
	60%	50.6 ± 0.3^{a}	29.2 ± 0.5^{b}	25.1 ± 0.5^{c}
18:2 (n-6)	**100%**	5.8 ± 0.2^{a}	35.0 ± 1.0^{b}	44.0 ± 0.7^{b}
	60%	$10.5 \pm 0.4^{*a}$	$47.0 \pm 0.9^{*b}$	$53.1 \pm 1.2^{*b}$
22:6 (n-3)	**100%**	ND	ND	5.1 ± 0.01
	60%	ND	ND	3.7 ± 0.02*
Sum SFA	**100%**	34.6 ± 0.4^{a}	27.4 ± 0.6^{b}	23.9 ± 0.4^{c}
	60%	33.5 ± 0.4^{a}	$19.8 \pm 0.6^{*b}$	$18.0 \pm 0.6^{*b}$
Sum MUFA	**100%**	57.8 ± 0.4^{a}	35.4 ± 0.5^{b}	30.4 ± 0.5^{c}
	60%	$53.8 \pm 0.4^{*a}$	$30.8 \pm 0.2^{*b}$	$26.2 \pm 0.5^{*c}$
Sum PUFA	**100%**	6.1 ± 0.3^{a}	35.8 ± 1.0^{b}	44.7 ± 0.7^{c}
	60%	$10.9 \pm 0.4^{*a}$	$48.0 \pm 0.9^{*b}$	$54.5 \pm 1.0^{*c}$

ND – Non detectable

[1] **Data are presented as Mean ± S.E.M, (n=12).**

[a] **Groups with different letters are statistically significantly different within the same level of food intake, $p<0.05$.**

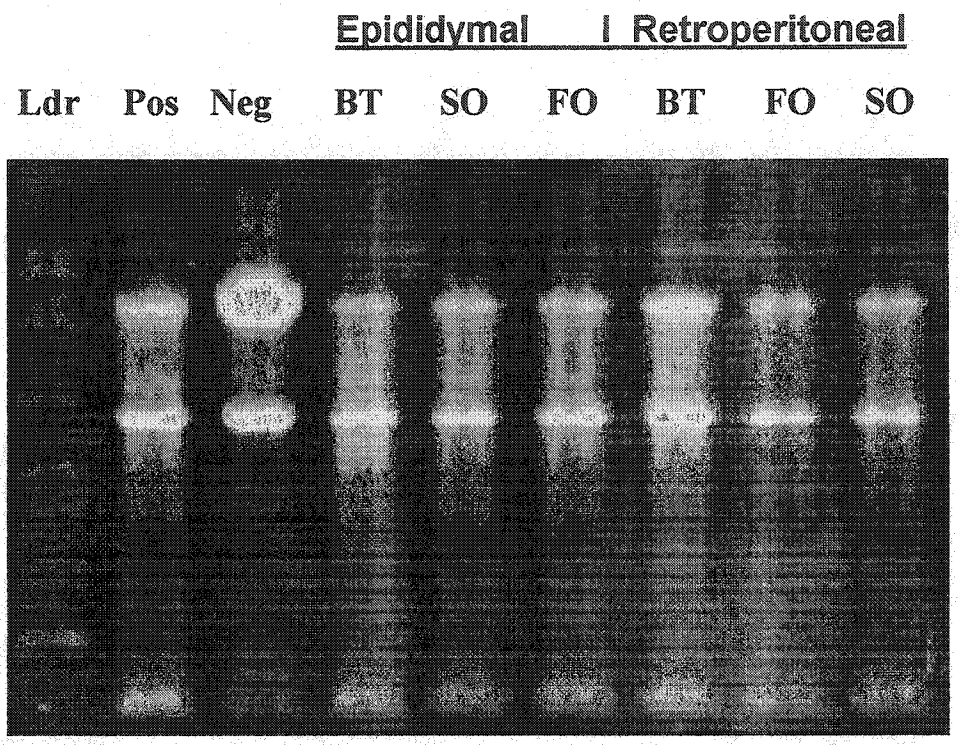
[*] **Denotes statistically significantly different from *ad libitum* fed animals, $p<0.05$.**

Table 3.7. Correlation coefficients of serum leptin with body weight, weight gain, visceral adipose tissue, epididymal, retroperitoneal, perirenal, insulin and the sum of retroperitoneal SFA, MUFA and PUFA.

	Serum Leptin		
Body weight		**Rp SFA**	
r	0.39	r	0.40
P	0.001	P	0.0009
Weight gain		**Rp MUFA**	
r	0.40	r	0.27
P	0.0009	P	0.025
Total WAT		**Rp PUFA**	
r	0.58	r	-0.40
P	<0.0001	P	0.0008
Ep WAT		**Insulin**	
r	0.58	r	0.083
P	<0.0001	P	0.5033
Rp WAT			
r	0.48		
P	<0.0001		
Pr WAT			
r	0.36		
P	0.003		

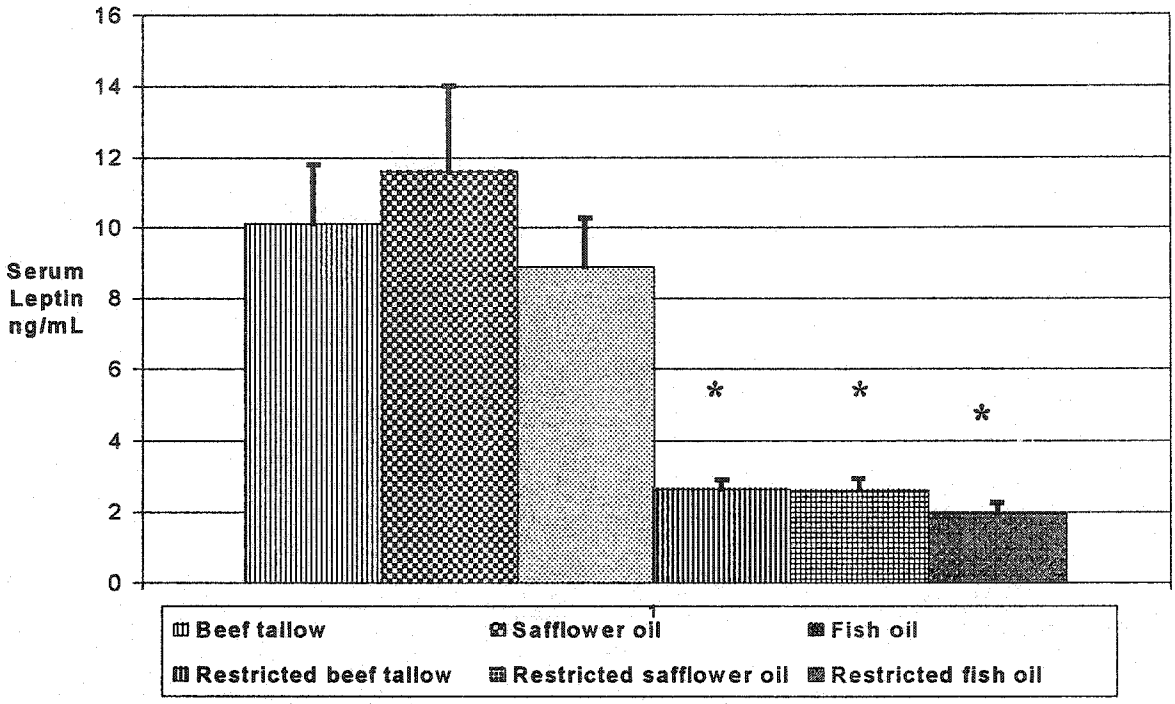
Abbreviations:
WAT – White adipose tissue
Ep – Epididymal
Rp – Retroperitoneal
Pr - Perirenal
SFA- Saturated fatty acid
MUFA – Monounsaturated fatty acid
PUFA – Polyunsaturated fatty acid

Figure 3.1. Ethidium bromide staining of total RNA electrophoresed gel



Abbreviations: BT – Beef tallow
SO – Safflower oil
FO – Fish oil
Ldr – RNA ladder
Pos - Positive control
Neg- Negative control

Figure 3.2. Serum leptin concentrations from rats fed beef tallow, safflower oil and fish oil at graded levels of energy intake[1*].

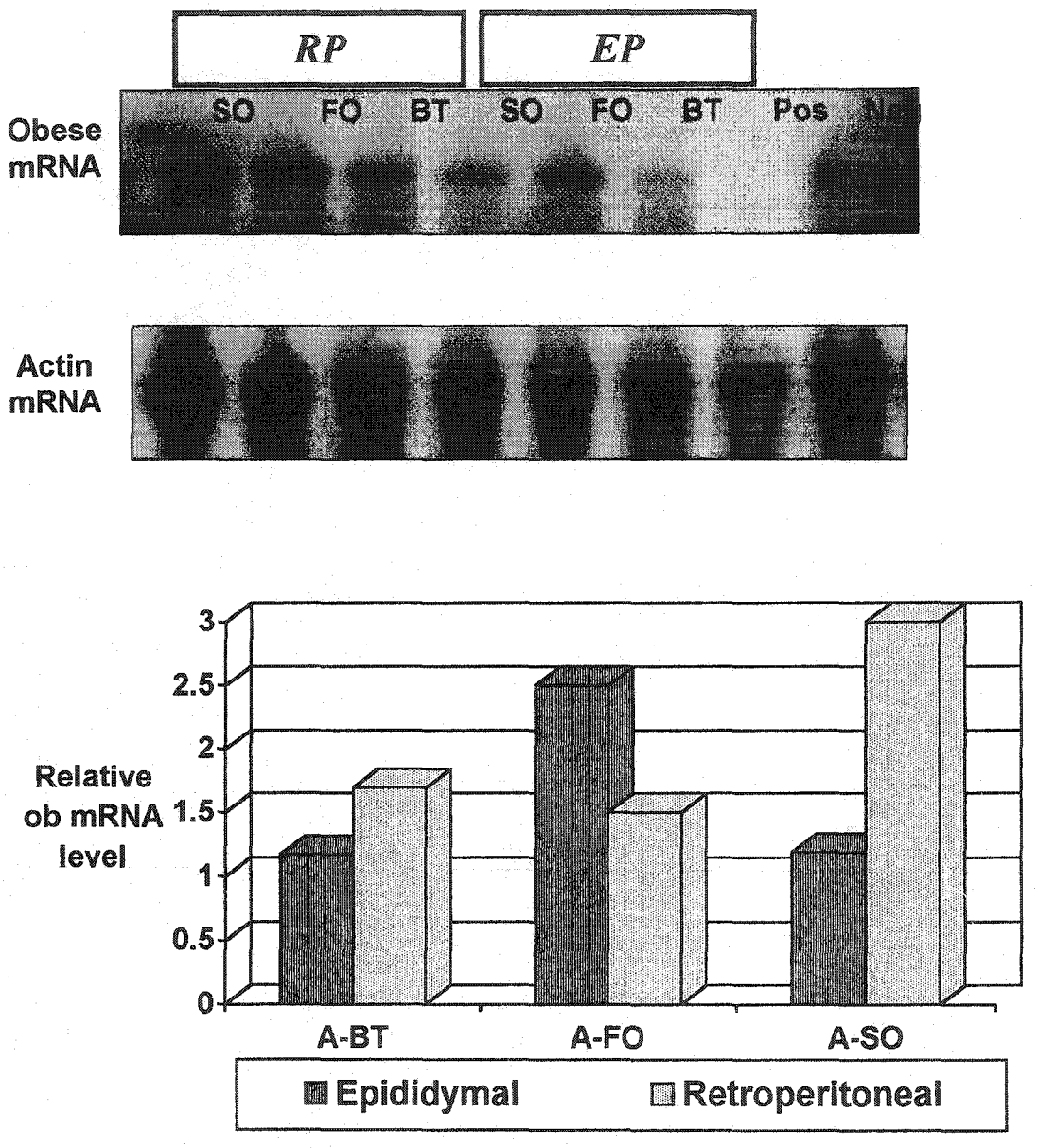


Data are presented as Mean ± S.E.M, (n=12).

[1] Energy intake significantly influenced leptin levels ($p < 0.0001$).

[*] Denotes statistically significantly different from *ad libitum* fed animals, $p<0.05$.

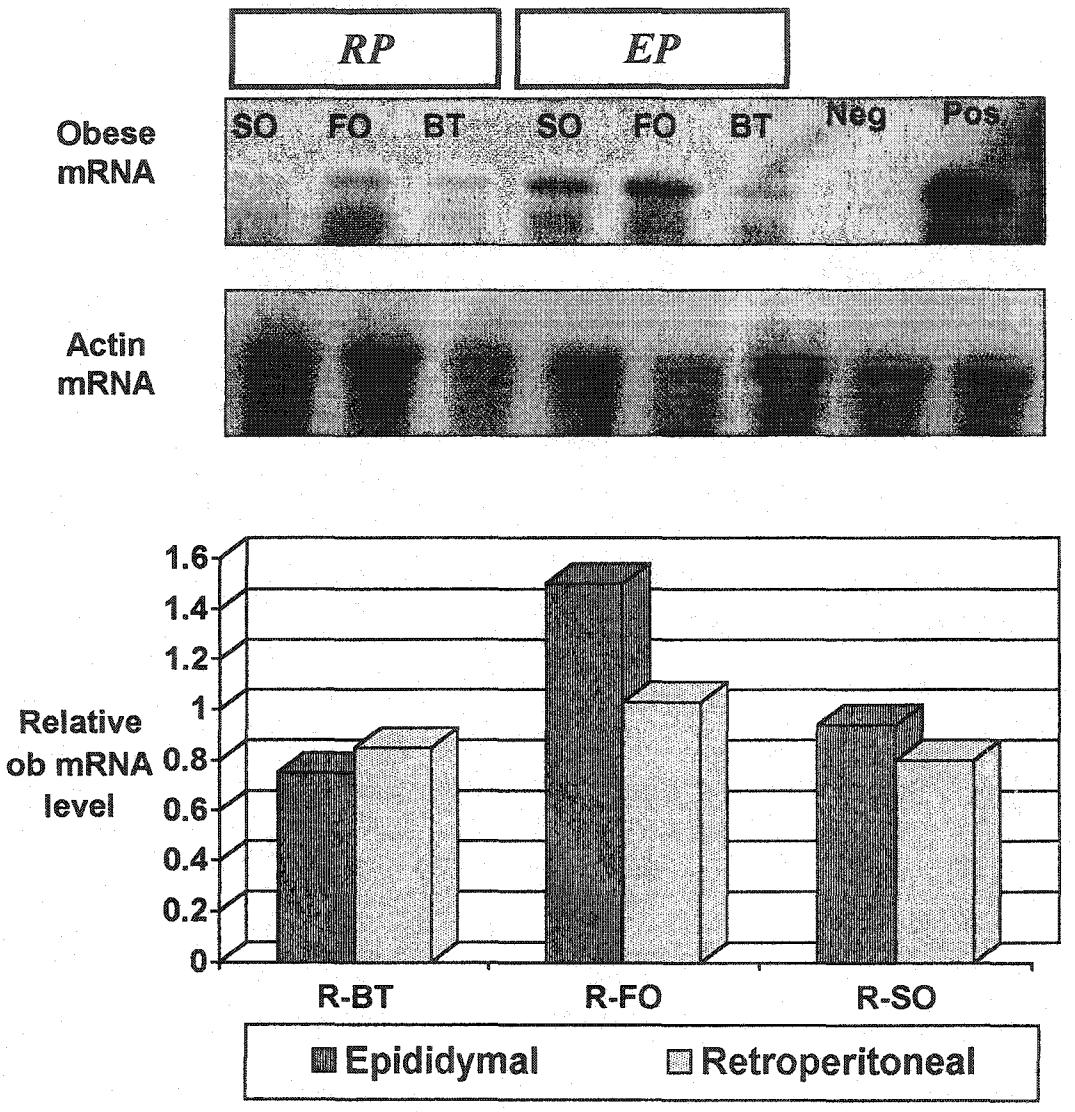
Figure 3.3. Northern blot analysis of ob mRNA levels in epididymal and retroperitoneal adipose tissue from rats fed beef tallow, safflower oil and fish oil at *ad libitum* energy intake for 10 weeks.



Abbreviations:

Ep – Epididymal
Rp – Retroperitoneal
BT – Beef tallow
SO – Safflower oil
FO – Fish oil
Pos - Positive control
Neg - Negative control

Figure 3.4. Northern blot analysis of ob mRNA levels in epididymal and retroperitoneal adipose tissue from rats fed beef tallow, safflower oil and fish oil at 60% *ad libitum* energy intake for 10 weeks.



Abbreviations:

Ep – Epididymal
Rp – Retroperitoneal
BT – Beef tallow
SO – Safflower oil
FO – Fish oil
Pos - Positive control
Neg - Negative control

3.5. Discussion

We have demonstrated an interactive effect of dietary fatty acid composition and energy restriction on white adipose tissue fatty acid composition. To our knowledge, this is the first study to observe the effects of diet fat and energy restriction on *ob* mRNA levels in rats. Our present findings demonstrate discordance between *ob* mRNA and circulating leptin levels as determined by the lack of response to dietary regimen at an equivalent level of energy intake. More specifically, there were no differences in serum leptin levels; whereas, *ob* mRNA levels were influenced by dietary fat in our animals. These parameters were profoundly influenced in response to energy intake level; in particular, *ad libitum* animals had greater levels of serum leptin and *ob* mRNA as compared to 60% *ad libitum* fed animals. The feeding design employed herein allowed assessment of lipid metabolism with adjustments in dietary fatty acid composition at varying degrees of energy intake (Cha and Jones, 1996 and 1998). Reduced food intake in our animals resulted in increased concentrations of 18:2 (n-6) in visceral adipose depots.

After 10 weeks, animals consuming an energy restricted diet had significantly lower final body weights than their counterparts who were given *ad libitum* diet. These differences can be partially explained by the growth of visceral adipose tissue. Total visceral white adipose tissue, which is the contribution from epididymal, retroperitoneal and perirenal depots, were almost 4-fold heavier in *ad libitum* animals. In our animals, we observed visceral depot weight in rank order starting with the largest mass as retroperitoneal> epididymal>perirenal tissue, respectively. Perirenal white adipose tissue weight was lower than epididymal and retroperitoneal tissue weight at both levels of energy intake. Dietary fat source did not significantly affect weight gain, feed intake or visceral white

adipose tissue at either level of energy intake. Numerous authors have shown reduced levels of visceral fat in animals fed diets high in polyunsaturated fat (Hill *et al.* 1993, Parrish *et al.* 1990, Okuno *et al.* 1997). Hill *et al.* (1993) fed rats high-fat (45% calories) diets containing beef tallow, corn oil, and fish oil and observed similar body weights after 7 months, which is consistent with our observations after 10 weeks of feeding. Thus, fatty acid composition of visceral adipose tissues is not fixed and after long-term feeding will be representative of diet fat source. In the study by Hill *et al.* (1993), the masses of epididymal and retroperitoneal depots were lower in animals fed the high-fat fish oil diet. This effect was only observed after 3 months of feeding. Therefore, a failure to demonstrate an effect of dietary fat type on white adipose tissue weight in the present study may be attributed to the length of feeding or the breed of animal. The aforementioned study did not cite the fatty acid composition of the fish oil employed and this may be responsible for discrepancies between findings.

Direct comparison between this study and others may be difficult due to different percentages of fat in the diet, which supplied 40% of the calories to the diet in the present study. Furthermore, an inherent limitation in many dietary feeding studies that examine effects of fish oil on body composition is an observed reduction in food intake. In fact, Hill *et al.* (1993) showed reduced food consumption in animals fed fish oil diets as compared to other dietary groups. It is possible that a lower energy intake may contribute to these observed reductions in adipose depot weight in animals fed fish oil.

Consistent with our previous findings (Cha and Jones, 1996 and 1998) and those of others (Bailey *et al.* 1993, Hill *et al.* 1990), WAT fatty acid composition reflected the variations in dietary fat consumed in this study. Conversely, energy restriction reduced WAT concentrations of 16:0, 16:1 (n-7) and 18:1 (n-9) in all depots studied; with the exception of perirenal adipose tissue from animals fed beef tallow. Consequently, 18:2 (n-6) concentrations increased proportionally in these same tissues. These tissue-specific changes were demonstrated by a much greater response to fatty acid accumulation from dietary fat source within retroperitoneal, as opposed to epididymal and perirenal tissues. When examining the correlations between the sum of fatty acids, serum leptin was positively associated with saturated and monounsaturated fatty acids; whereas, a significant negative correlation was observed with the sum of polyunsaturated fatty acids in retroperitoneal tissues. A recent study involving human subjects demonstrated a similar trend between serum leptin and increased intake of these fatty acids in the diet (Reseland *et al.* 2001). Examination of perirenal tissues from beef tallow fed animals showed reduced concentrations of 16:0, 16:1 (n-7) and 18:2 (n-6) with increased levels of 18:0 and 18:1 (n-9). Together, these data show a propensity for increased polyunsaturated fat concentrations in white adipose tissue that occurs in response to energy restriction, which was enforced in exchange for shorter, more common saturated fatty acids. These results are consistent with other reports demonstrating a preferable retention of 18:2 (n-6) in serum triglycerides and liver, which was accounted for through large exchanges in 16:0 and 16:1 (n-7) during energy restriction (Chen and Cunnane 1991 and 1992, Cha and Jones 1996, Takahashi and Ide 1999). Most notably, Takahashi and Ide (1999) fed rats high fat diets (20% wt/wt) containing safflower oil or olive oil *ad libitum* for 3

weeks. These authors found similar weight gain, food intake, as well as epididymal and perirenal WAT fatty acid compositions between both diet fat treatment groups. Generally, we observed similar fatty acid compositions between perirenal, retroperitoneal and epididymal adipose tissues but large differences were found between the sources of dietary fat, which is consistent with previous reports by Cha and Jones (1996). Beef tallow fed rats had more saturated and monounsaturated fatty acids in their visceral adipose tissues than fish oil and safflower oil fed rats. These differences were related to the high polyunsaturated fat content of those fats. Tissue-specific differences in FA composition has been demonstrated by Bailey et al. (1993) in rats fed standard chow *ad libitum* or food restricted by alternate day feeding for 28 months. The major FA profile of the rodent chow was as follows: 16:0, 18:0, 18:1 (n-9) and 18:2 (n-6), with concentrations 20.5%, 9.3%, 30.5% and 27.3%, respectively. Every 4 months, animals were selected randomly for fatty acid analysis of perirenal, epididymal and inguinal adipose tissue. As a result, these researchers found an increased concentration of saturated fatty acids in perirenal versus epididymal adipose tissues after 12 months. Our results do not support this finding but our shorter length of feeding may explain these differences. In contrast, food restriction increased the concentration of 18:2 (n-6) in adipose tissues of our animals; whereas, Bailey *et al.* (1993) found levels of 18:1 (n-9) to be elevated. Interestingly, in perirenal tissues of our animals fed beef tallow, we observed similar findings to those of Bailey *et al.* (1993). This is probably related to the similarities in fatty acid composition of our beef tallow and the standard chow used in the previous study.

It is not known what effect alternate day feeding may cause on the metabolic system of the animal. It seemed likely that alternate day feeding would cause greater stress on the animal than a reduced diet fed daily. Serious strain on an animal may also influence stress hormones, which have been shown to influence leptin expression in a previous study (Cusin *et al.* 1995). Since our food restricted animals received 60% of *ad libitum* animal intake, it is possible that we may have observed a stress effect; although, these animals showed consistent growth throughout the course of the feeding trial.

It remains unknown how the body regulates leptin expression acutely and chronically in response to changes in physiological state, such as adiposity. To further explore this issue we examined *obese* mRNA levels in epididymal and retroperitoneal adipose tissue from rats fed beef tallow, safflower oil and fish oil at ad libitum and 60% *ad libitum* intake. Due to the limited perirenal tissue collected, expression analysis could not be performed on this depot. As discussed earlier, the fatty acid composition of epididymal and retroperitoneal adipose tissues were similar with the exception of the higher levels of n-3 polyunsaturated fat in adipose depots from fish oil fed animals. Adipose depot *ob* mRNA levels were elevated in the *ad libitum* and reduced in the energy restricted animals in both sites. Adipose tissue *ob* mRNA levels were generally higher after polyunsaturated fat feeding, with greater levels observed in both epididymal tissues from fish oil fed and retroperitoneal tissues from safflower oil fed animals. These findings are consistent with previous reports (Reseland *et al.* 2001, Takahashi and Ide, 2000) and contrast the observations by Raclot *et al.* (1997). Raclot *et al.* observed higher *ob* mRNA levels with feeding of a lard/olive oil mix or pure 20:5 versus 22:6 or fish oil as the sole source of fat.

Interestingly, an effect of fish oil feeding may depend on the fatty acid composition; more specifically, the ratio of 20:5 to 22:6. To our knowledge, this is the first study to examine the interactive effects of energy restriction and diet fat type on *obese* gene expression. We observed similarity between the effect of beef tallow and safflower oil feeding on epididymal and retroperitoneal adipose tissue weight. However, we found elevated levels of *ob* mRNA after fish oil feeding versus other fat sources, with greater levels of *ob* mRNA coming from epididymal tissue. Our data show that energy restriction reduced the concentrations of n-3 fatty acids in depots, which may explain the observed differences in *ob* mRNA levels between food intake levels. This would appear to be the case, especially when considering that other factors were similar between adipose depots. We observed differences in expression within the same energy levels that did not contribute to changes in circulating leptin. This suggests that leptin production may be influenced by tissue concentrations of leptin protein. It is plausible that intra-abdominal adipose tissue *obese* gene expression is similar between depots. Russell and coworkers (1998) have demonstrated differences in leptin secretion from adipose tissues, which may contribute significantly to circulating levels. These researchers found a higher level of leptin secretion in subcutaneous as compare to visceral adipose tissue. We did not explore this issue in the present study because visceral adipose tissue *ob* mRNA levels in rodents are at least 3.6-fold higher than subcutaneous tissue levels; thus, supporting the belief that visceral WAT is the primary source of leptin in rodents (Zheng *et al.* 1996). When considering differences in weight gain, *ad libitum* animals were approximately 100g heavier than food restricted animals. The difference in total visceral adipose tissue weight, between *ad libitum* and 60% *ad libitum* intake groups, accounts for only 25% of

the mass. The contribution of subcutaneous tissue to whole body fat mass may be substantial but as demonstrated previously, greater *ob* mRNA levels can be observed in visceral tissues, which have been shown to be better predictors of circulating levels (Moses *et al.* 2001).

Conflicting reports regarding the depot-specific differences in leptin expression (Raclot *et al.* 1997) and discordance between circulating levels (Ranganathan *et al.* 1998) have made interpretation and extrapolation of leptin expression difficult. Serum leptin analyses revealed significantly higher circulating levels in free access versus energy restricted animals, which is consistent with several reports (Maffei *et al.* 1995, Kolaczynski *et al.* 1996, MacDougald *et al.* 1995, Boden *et al.* 1996). As well, circulating levels were correlated with weight gain, final body weight and white adipose tissue; thereby, confirming the strong association between leptin and adipose stores in animals. More specifically, epididymal tissue was a better predictor of serum levels than either retroperitoneal or perirenal depots. There were no interactive effects of energy intake and dietary fat on serum leptin in any of the animal groups studied. This is likely attributed to the large variations in ad libitum serum leptin values, which ranged from 1.9-29.9 ng/mL in the current study without any observed differences in body weight between treatment groups. Large variability in circulating leptin is supported by other authors (Maffei *et al.* 1995 and Considine *et al.* 1996) who found greater plasma leptin levels in some non-obese versus obese subjects. In this study, variability could not be attributed to dietary regimen or food intake, which was similar for rat groups engaged in equivalent levels of energy intake.

Supraphysiologic insulin levels, as induced during hyperinsulinemic clamp have been shown to influence leptin mRNA expression (Kolaczynski *et al.* 1996a,b). An objective of the current study was to determine whether insulin's influence over leptin production is significant at physiological levels and could explain the observed variability in circulating leptin. Between treatment groups insulin levels were similar and were not associated with circulating leptin, which is consistent with a previous report in rats fed a high fat diet for 14 weeks (Ainslie *et al.* 2000). Conversely, Doucet *et al.* (2000) previously found that after correction for adiposity, fasted circulating insulin and leptin were positively correlated only in subjects with high insulin levels. In the current study, at both energy intake levels rats had similar insulin values which is also contradictory to the findings of Doucet *et al.* (2000) whom observed a significant correlation between leptin and insulin after weight loss intervention. Studies demonstrating an association between leptin and insulin show that the effect is dose-dependant, as hyperinsulinemia is required for an observed shift in circulating leptin (Utriainen *et al.* 1996). Therefore, based on the present study, animals fed diets differing in fat source show no association between serum insulin and leptin, likely due to the lack of insulinemia in these animals. Alternatively, as we did not measure serum hormones during the course of the feeding trial, it is possible we may have neglected an effect at an earlier stage.

The observed differences in *ob* mRNA levels in visceral adipose and the disparity between serum leptin values suggest the synthesis of leptin protein is not regulated transcriptionally more likely is modified through post-transcriptional processes. It is probable that circulating leptin levels are controlled by tissue accumulation and thus,

leptin secretion. We attempted to examine adipose leptin concentrations to further explore this possibility. However, we were unable to detect leptin protein using a polyclonal antibody specific for an amino acid sequence in the carboxy-terminal domain. Bornquist *et al.* (2000) required a highly sensitive analytical method that employs immunohistochemistry and gold labeling to detect minute changes in leptin concentration; therefore, the sensitivity of our Western analysis may explain our inability to detect leptin. In human subcutaneous and visceral WAT from morbidly obese patients undergoing abdominal surgery or mammary reduction, no free pools of leptin were observed within the white adipocytes (Bornquist *et al.* 2000). Interestingly, minute quantities of leptin were detected bound to receptors in the membranes of adipocytes, endothelial and macrophage cells. Our inability to detect leptin protein in tissues may be explained by our choice of an overnight fast, which might not have been appropriate in this study. It has been reported elsewhere that serum leptin was reduced beyond the level of detection using similar methodology (Frederich *et al.* 1995b), in contrast, we examined tissue concentrations of leptin and chose to measure serum levels using a radioimmunoassay. It may be possible to measure leptin tissue concentrations using a modified radioimmunoassay protocol, which has increased sensitivity. This spawns two points of interest for future study. Firstly, our findings suggest that leptin production is regulated post-transcriptionally and may involve factors controlling leptin release from the adipocyte. Secondly, we have shown that changes in fatty acid composition of adipose tissue can be achieved after 10 weeks; yet, its effects on leptin binding to adipocytes and non-adipocytes are unknown. Whether this process influences leptin signaling as demonstrated in insulin binding studies is unknown as well.

CHAPTER 4. SUMMARY AND CONCLUSION

4.1. General Conclusion

In conclusion, these data are the first to show that dietary fat type affects leptin expression in a tissue-specific manner at varying levels of energy intake but it does not confer changes in circulating leptin levels. It is especially intriguing that there are differences in expression patterns within the intra-abdominal milieu; therefore, it may not be appropriate to distinguish adipose depots as either visceral or subcutaneous, since the study proves that differences exist in metabolic patterns of visceral WAT. Unlike rodents, for whom visceral fat is the largest contributor of leptin mRNA (Zheng *et al.* 1996), human subcutaneous adipose tissue is the primary site of adipose stores (accounting for ~80%) and is considered responsible for the majority of leptin production (Moses *et al.* 2001). This fact makes interpretation between animal and human systems difficult. Based on our data, energy balance is the most prominent factor affecting leptin expression and production. While we observed differences in fatty acid composition of visceral adipose tissue, it is unclear whether these factors contribute to the differences in body weight and visceral fat mass but is probable since insulin was not influenced by dietary regimen.

4.2. Significance of Findings

To our knowledge, this is the first evidence of an interactive effect of dietary fat composition and energy restriction on visceral adipose tissue *obese* gene expression. While, there was a trend for increased *ob* mRNA levels in adipose tissue from animals fed polyunsaturated fatty acid rich diets, it appears unlikely that stimulation of leptin production is specific to feeding animals diets rich in n-3 versus n-6 fatty acids. We have provided considerable evidence to support energy intake as a key factor in the regulation of the *obese* gene while the role of fat is less clear. Serum leptin levels were associated with adipose stores (with epididymal adipose tissue being the greatest predictor) thus verifying leptin's role as an indicator of adipose stores.

4.3. Future Research

We have demonstrated an interactive effect of diet fat type and energy restriction on WAT fatty acid accumulation as well as *obese* gene expression. Modification of the fatty acid composition or *obese* gene expression had an insignificant effect on visceral adiposity or leptin levels. We have provided evidence that supports the involvement of post-transcriptional processes in the regulation of leptin production. Given these results, future research should focus on the role of adipose tissue fatty acid composition in leptin binding and signalling in autocrine and paracrine roles. It would be interesting to examine the effect that changes in membrane fatty acid composition have on leptin functioning in non-adipocytes. Since peripheral leptin receptors can be found in many tissues, it is probable that leptin is involved in the regulation of lipid metabolism in these tissues, particularly those related to triglyceride storage. Current research supports leptin as a regulator of triglyceride accumulation in tissues sensitive to increasing concentrations of lipids. Undoubtedly, future research will involve studying links between leptin functioning in the development of chronic disease; the basis of which may involve excess accumulation of lipid intracellularly (i.e. diabetes, resulting from apoptosis in islet cells).

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