The Effect of Dietary Alpha-Linolenic Acid-Rich Flaxseed Oil Intervention on Immune and Adipose Tissue Function in Obese Zucker Rats

by

Maria Baranowski

A Thesis submitted to the Faculty of Graduate Studies of

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Of

Master of Science

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DEDICATION

To my Mom and Dad

ABSTRACT

Background. Obesity is associated with low-grade inflammation, altered immune response and adipose tissue dysfunction. Adipose tissue actively participates in inflammation and immunity, producing and releasing a variety of proinflammatory and anti-inflammatory factors. Dietary fatty acids have been shown to modulate immune responses and reduce inflammation.

Objective. To determine the effect of dietary omega-3 fatty acid supplementation with alpha-linolenic (ALA)-rich flaxseed oil, on mitogen stimulated *ex vivo* cytokine production, adipokines in serum and adipose tissue and adipocyte size in obese *fa/fa* Zucker rats.

Methods. Seventeen-week old male Zucker rats were randomly assigned to one of 4 groups: *fa/fa* (fa) baseline (faBASE), fa control (faCTL), lean (ln) control (lnCTL), or an ALA-rich flaxseed oil containing diet (faALA). The study period was 8 weeks. Commercial ELISA kits were used to measure fasting serum leptin and adiponectin concentrations. Pro- and anti-inflammatory cytokine production [tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-2, IL-6, IL-4, and IL-10] from splenocytes was measured using a Lincoplex kit. Adipokine levels [leptin, adiponectin, IL-6, IL-10, IL-18, TNF-α and high mobility group box 1 (HMGB1)] in peri-renal adipose tissue and adipocyte size in epididymal adipose tissue were measured by Western blot analysis and with the open-source image analysis program ImageJ, respectively.

Results. Adipocyte size in obese rats fed an ALA-rich flaxseed oil containing diet was reduced by 17% compared to the faCTL group. The ALA diet altered the distribution of visceral fat (decreased the ratio of peri-renal to epididymal adipose tissue) in *fa/fa* rats, but there were no differences in adipose tissue weight, body weight or feed intake. Pro- and anti-inflammatory splenocyte cytokine secretion and peri-renal adipose tissue adipokine protein levels were not altered in *fa/fa* Zucker rats fed ALA-rich flaxseed oil.

Conclusion. Dietary intervention with ALA-rich flaxseed oil decreases adipocyte size and alters visceral tissue distribution but does not alter cytokine or adipokine production in adult obese Zucker rats.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ACK	ammonium-chloride-potassium
AI	adequate intake
ALA	α-linolenic acid
ALT	alanine transaminase
AMDR	acceptable macronutrient distribution range
ANOVA	one-way analysis of variance
ATF	activating transcription factor
BASE	baseline group
BCA	bicinchoninic acid
BMI	body mass index
BSA	bovine serum albumin
CCAC	Canadian Council on Animal Care
CCM	cell culture medium
CD	cluster of differentiation
CLA	conjugated linoleic acid
ConA	concanavalin A
CRP	C-reactive protein
CTL	control
DHA	docosahexaneoic acid
DM	diabetes mellitus
EAE	experimental autoimmune encephalomyelitis

ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetate
ELISA/EIA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
Fa	*fa/fa*
FO	fish oil
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HMGB1	high mobility group box 1
HMS	homologous mouse serum
HRP	horseradish peroxidase
IFN-γ	interferon-gamma
IgG	immunoglobulins
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
KRH	Krebs ringer bicarbonate buffer
LA	linoleic acid
LCPUFA	long-chain polyunsaturated fatty acid
LT	leukotrienes
Ln	lean
LPS	lipopolysaccharide
MHC	major histocompatibility complex

mRNA	messenger RNA
MUFA	monounsaturated fatty acid
NF-κB	nuclear factor-kappaB
NOD	nonobese diabetic
OCT	optimal cutting temperature
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PEG-OB	long-acting pegylated human leptin
PG	prostaglandin
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene difluoride
RMR	resting metabolic rate
RP	retroperitoneal
RQ	respiratory quotient
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBST	tris buffer saline Tween
TMB	tetramethylbenzidine
TNF-α	tumour necrosis factor-alpha
TX	thromboxane
VLCD	very low-calorie diet
ZDF	Zucker diabetic fatty

LITERATURE REVIEW

Introduction

Obesity is characterized by low-grade inflammation and adipose tissue dysfunction. Omega-3 fatty acid is able to modulate immune responses and has anti-inflammatory effects. We hypothesize that diet intervention with omega-3 fatty acid will alter immune and adipose tissue function and reduce inflammation in an animal model of obesity. The current state of knowledge of obesity, adipose tissue, and dietary omega-3 fatty acid, the associations between these components, and their connections to immune function and inflammation will be presented.

Obesity

The World Health Organization defines overweight and obesity as abnormal or excessive fat accumulation that may impair health (1). Obesity is generally defined as a body mass index (BMI) equal to or more than 30. BMI is calculated as the weight in kilograms divided by the square of the height in meters (kg/m^2). In 2005, the estimated prevalence of obesity in Canadians aged 15-100 years was 23.2% and 23.7% for females and males, respectively (1). By 2015, the estimated prevalence of obesity is expected to rise to 28.2% and 27.4% for females and males, respectively.

Obesity and Infection

Obesity is associated with low-grade inflammation resulting from chronic activation of the innate immune system (2). Adipose tissue also actively participates in inflammation and immunity, producing and releasing a variety of proinflammatory and anti-inflammatory factors (3). As reviewed by Falagas & Kompoti (3), human obesity is associated with an increased frequency of infections of several organs and systems. An increased incidence of nosocomial, surgical-site, odontogenic, respiratory, and urogenital infections has been reported in obese patients compared to normal weight patients. In addition, accumulated fat may influence gastrointestinal, liver, and biliary functions and increase risk of infections.

The Immune System

Lymphocytes are a type of white blood cell, originating from red bone marrow and transported via blood to lymphatic organs and other tissues where they are involved in the immune response (4). Immunity is the ability to resist damage from unwanted invasion of foreign substances. The immune response is categorized as innate or adaptive immunity, with the difference between these systems involving specificity and memory.

Innate immunity is the ability of the immune system to recognize and destroy foreign substances in general, predominantly mediated by macrophages and neutrophils, whereas adaptive immunity can distinguish among different antigens (4). Innate immunity does not have a memory of previous encounters with foreign substances, and so the ability to destroy these substances does not improve with repeated exposure; the response is always the same. In contrast, adaptive immunity is the ability of the immune

system to recognize and remember previous exposure to foreign substances, and so its action is improved with a faster and stronger response to repeated exposure. In this way, adaptive immunity enables an individual to become immune to a particular stimulus (antigen) and thus, bacteria are destroyed before any symptoms develop.

Adaptive Immunity

Adaptive immunity can be divided into antibody-mediated (involving B cells) or cell-mediated (involving T cells) immunity (4). T cells are divided into several subpopulations, each of which is responsible for a particular aspect of cell-mediated immunity. Once they are activated, T cells undergo a series of divisions to produce effector T cells and regulatory (memory) T cells.

Effector T cells include cytotoxic T cells and delayed hypersensitivity T cells, and as their name implies, they are responsible for producing the effects of cell-mediated immunity (4). Cytotoxic T cells destroy cells by lysis or by producing cytokines, whereas delayed hypersensitivity T cells also produce cytokines that promote inflammation. Cytokines are secreted proteins or peptides that activate additional components of the immune system, such as macrophages, that are responsible for phagocytosis and inflammation. Interferon gamma (IFN-γ), interleukin (IL)-1, IL-2, IL-6, IL-18, and tumor necrosis factor-alpha (TNF-α) are examples of proinflammatory cytokines that stimulate adaptive immunity, whereas IL-10 is an example of an anti-inflammatory cytokine that inhibits the secretion of IFN-γ and interleukins. Proinflammatory and anti-inflammatory cytokines are also referred to as Th1- and Th2-type cytokines, respectively. Although these cytokines are typically classified this way and in this thesis, inflammatory

action really depends on the context. Some 'anti-inflammatory' cytokines may actually be elevated, and 'proinflammatory' cytokines reduced, in inflammatory conditions which reflects the importance of balance in maintaining and regulating the immune response.

Regulatory (memory) T cells include helper T cells and suppressor T cells, and are responsible for controlling the activities of both antibody-mediated and cell-mediated immunity (4). Memory T cells provide a secondary response and long-lasting immunity. These cells are responsible for adaptive immunity as they quickly and effectively respond to an antigen the immune system has previously encountered.

Naïve T cells are distinguished from memory T cells, as having not yet encountered an antigen. Once naïve T cells are activated by recognition of an antigen, they can further differentiate into memory T cells. Helper and suppressor T cells exert opposing action on effector T cells. The former is responsible for activating effector T cells, and the latter is responsible for inhibition.

Activation of Adaptive Immunity

Adaptive immunity begins with a macrophage presenting an antigen to a helper T cell (4). After recognition of an antigen, T lymphocytes are stimulated to divide and increase in number, particularly helper T cells.

Most lymphocyte activation involves glycoproteins on the surfaces of cells called major histocompatibility complex (MHC) molecules (4). MHC class I molecules are found on nucleated cells and function to display endogenous antigens produced inside the cells on their surfaces. MHC class II molecules are found on antigen-presenting cells, such as macrophages and monocytes. These antigen-presenting cells are specialized to

take in exogenous antigens, to process the antigens, and to use MHC class II molecules to display the foreign antigens to other immune system cells. The MHC class II/antigen complex can bind with a T cell receptor.

Helper T cells have a glycoprotein called CD4 that helps connect helper T cells to the macrophages by binding to MHC class II molecules (4). CD stands for 'cluster of differentiation' and is a system used to classify surface molecules. Cytotoxic T cells have a glycoprotein called CD8, which helps to connect cytotoxic T cells to body cells displaying MHC class I molecules. Helper T cells and cytotoxic T cells are also referred to as CD4 and CD8 cells, respectively.

Inflammation

Inflammation is part of the biological response of an organism to a stimulus or injury. Chronic inflammation leads to a progressive shift in the type of cells that are present at the site of inflammation. Cells in the affected area release or activate chemical substances (histamine, kinins, prostaglandins, leukotrienes) to trigger the recruitment of immune cells (white blood cells) to the site of injury. Cytokines are also cell-derived mediators that induce many inflammatory reactions and are responsible for the systemic effects of inflammation. Chemical mediators originating from plasma components or immune cells (macrophages, lymphocytes) are involved in chronic inflammation. High mobility group box 1 (HMGB1) is an example of a protein secreted from monocytes and macrophages that possesses pro-inflammatory and immunostimulatory properties (5). Furthermore, the production of several inflammatory factors originates from adipose tissue and their production is positively correlated with increasing amounts of obesity.

The Inflammatory State of Obesity

Markers of inflammation such as proinflammatory cytokines TNF-α, IFN-γ, IL-6, and acute-phase C-reactive protein (CRP) are overproduced in obesity, promoting a chronic sub-clinical inflammatory state (6-12). Changes in cytokine production in obesity indicate altered immune function and this may contribute to obesity-related complications (7).

Diet-induced obese mice have inflammation as indicated by increased TNF-α production by visceral adipocytes and changes in cytokine production by splenic lymphocytes (7). In isolated splenocytes cultured in the presence of a stimulant, splenocyte proliferation and production of IFN-γ and IL-4 was increased in obese mice compared to controls, however, splenocyte production of pro-inflammatory IL-2 was lower than controls. The addition of leptin to supernatants of stimulated splenocytes had different effects on cytokine production by isolated splenocytes from obese and control mice. In obese mice, IL-4 production by splenocytes was down-regulated whereas in control mice, IFN-γ production was increased and IL-2 production was suppressed. Production of IFN-γ and IL-2 was not altered by the addition of leptin in obese mice, which may reflect changes of sensitivity to leptin in obesity due to functional defects of leptin receptors or leptin resistance.

Elevated serum TNF-α has been identified in obese adults (6, 9). Serum TNF-α concentrations were significantly higher in obese women compared to normal weight subjects and decreased by 25% after a significant decrease in body weight and BMI (6). Changes in TNF-α serum concentrations and the degree of weight loss were related to basal TNF-α and basal body weight, but not to each other.

Indicators of inflammation are also present in childhood obesity, as indicated by an increase in IFN-γ-secreting cells (8). The percentage of CD4+ T cells secreting IFN-γ from peripheral blood mononuclear cells (PBMCs) in obese children was found to be significantly higher than normal weight controls but did not correlate with leptin, even though circulating leptin levels were high (8). Interestingly, the percentage of IFN-γ positive cells in obese children was also associated with nonalcoholic steatohepatitis, the hepatic component of metabolic syndrome, but not with BMI or total body fat mass. The shift to a Th1-cytokine profile, as seen by the production of proinflammatory IFN-γ in these young obese children, reflects the initial stages of inflammation.

In a population study, overweight and obese men and women were identified to be more likely to have elevated serum CRP concentrations than their normal-weight counterparts (12). CRP is an acute phase protein synthesized and released from the liver in states of inflammation and elevated levels can result from cytokine stimulation. As a marker of systemic inflammation, elevated levels of serum CRP in this study confirm that a state of low-grade inflammation exists in overweight and obese conditions in humans. In addition, elevated serum CRP is considered a strong independent predictor of cardiovascular events (13).

Inflammation has been linked to the pathogenesis of several chronic diseases, including cardiovascular disease, diabetes mellitus, metabolic syndrome, and cancer (14, 15). While obesity is also an independent risk factor for diabetes and cardiovascular disease (15), the mechanisms between these associations and the role of inflammation needs to be clarified.

Whether lifestyle modification such as diet intervention, independent of weight loss, is able to modulate the inflammatory status and metabolic profile in an obese state has yet to be investigated. In addition, the role of adipose tissue and its contribution to inflammation in obesity needs to be considered.

The Role of Adipose Tissue in the Inflammatory State of Obesity

Besides its role in fat and energy storage, adipose tissue also acts as an endocrine organ capable of producing and secreting biologically active proteins (16). Adipocytes secrete hormones such as leptin and adiponectin; as well, pro- and anti-inflammatory cytokines are released from adipose tissue (17). These molecules are referred to as adipokines or adipocytokines when they are produced by adipose tissue, however, other cells and tissues can also produce some of these molecules. Only leptin and adiponectin are considered true adipokines, as they are primarily produced by adipose tissue (13). Abnormal production of adipokines from expanded visceral fat observed in obesity contributes to an inflammatory state (13).

Adiponectin

Adiponectin is an adipose tissue-specific adipokine with anti-inflammatory properties in various cells and tissues (18). As reviewed by Ouchi and Walsh (19), adiponectin mediates the regulation of inflammatory responses in cardiac cells, endothelial cells, and macrophages. Pretreatment of adiponectin in cultured macrophages inhibits their phagocytic activity and their stimulated production of TNF-α (20). In macrophages, adiponectin also suppresses nuclear factor-κB (NF-κB) activation (21),

proinflammatory IL-6 and TNF-α production (22), and increases expression of anti-inflammatory IL-10 (22).

An inverse relationship between circulating adiponectin and leptin exists in vivo (23) and plasma adiponectin levels negatively correlate with obesity in humans (11), even though adiponectin is secreted mostly from adipose tissue (24). It follows then that weight loss increases plasma adiponectin and adipose tissue mRNA levels in obese subjects (25). An inverse relationship has been detected between plasma adiponectin and IL-6 in obese subjects, which may be explained by the fact IL-6 (plus IL-6 soluble receptor) incubation can reduce adiponectin mRNA in whole adipose tissue in vitro (25). In contrast, a positive correlation between adiponectin and IL-10 concentrations was observed in non-obese men and women with metabolic syndrome; both adipokine levels were significantly lower compared to controls without metabolic syndrome (26). In addition, CRP and IL-6 were associated with hypoadiponectinemia and subjects with metabolic syndrome had higher levels of these inflammatory markers than those without metabolic syndrome. Gender differences reveal that levels of plasma adiponectin in men are lower compared to women in both non-obese and obese groups (24).

Interleukin-6

Adipose tissue has been reported to release large amounts of proinflammatory IL-6 in vivo, with approximately 30% of the total circulating IL-6 originating from adipose tissue (27). The presence of IL-6 receptors, identified in the plasma membrane of human abdominal subcutaneous adipocytes, suggests IL-6 could operate via an autocrine mechanism on adipose tissue (28). IL-6 can reversibly down-regulate adiponectin

mRNA expression and secretion (29) and stimulate hepatic production of CRP mRNA and protein (30). Human studies confirm that circulating concentrations of proinflammatory IL-6 are increased in obese women, with or without diabetes, compared to their lean counterparts (9-11).

The Effect of Lifestyle Change on Inflammatory Markers in Obesity

The effect of lifestyle change on downregulating the inflammatory state in obesity has been investigated (9, 10, 16). Weight reduction (6.9 ± 0.1 kg) in obese individuals following a low calorie diet and exercise program for 6 months was associated with significant decreases in serum leptin and IL-6, a tendency towards lower circulating TNF-α, and in subjects with diabetes, decreased adiponectin levels were normalized (16). CRP values were not changed by the intervention.

Similar findings were reported in obese women following a very low-calorie diet (VLCD) for three weeks (10). The diet-induced weight loss (mean reduction of 3 kg in adipose tissue mass) decreased serum and subcutaneous adipose tissue IL-6 and leptin protein concentrations. Changes to serum CRP levels did not reach significance, and neither serum nor adipose TNF-α was changed after VLCD. It is possible that the lack of effect on TNF-α levels may be due to the short duration of the intervention period in this study.

A multidisciplinary approach to weight loss, including specific diet modification, has also been studied in women (9, 11). A Mediterranean-type diet (saturated fat 9 g, monounsaturated fat 17 g, and polyunsaturated fat 8 g per day) consumed for 12 months was associated with a 10% loss of initial body weight and declines in serum TNF-α and

IL-6 concentrations (9). Another study investigating lifestyle changes to achieve weight loss in obese women also included a low-energy Mediterranean-type diet (< 10% saturated fat, 10-15% monounsaturated fat, and 5-8% polyunsaturated fat per 1000 kcal) (11). Researchers found that BMI and serum concentrations of IL-6, IL-18, and CRP decreased significantly while adiponectin levels increased in the intervention group compared to controls. Of particular interest, women in the intervention group not only consumed less energy than controls, but also had a lower ratio of omega-6 to omega-3 fatty acid intake (6:1 vs. 13:1 after 2 years). Protein and carbohydrate intake changed slightly throughout the study period and was different between groups at the end of the study period (14% and 58%, respectively, of total energy at baseline and 17% and 55%, respectively, at 2 years in the intervention group compared to 14% and 59%, respectively, at baseline and 14% and 58%, respectively, at 2 years in the control group).

Together these studies suggest that weight loss achieved by a combination of lifestyle changes is a safe and effective method for downregulating inflammatory status and risk factors of cardiovascular disease in obese subjects (11). However, the contribution of diet to the improved inflammatory status in obese subjects has not been determined and the mechanism behind these improvements remains unclear. In addition, whether changes to the inflammatory profile can be observed when modification of diet quality is favoured as the primary intervention, without necessarily achieving weight loss, is unknown.

The Contribution of Adipocytes vs. Macrophages to Inflammation in Adipose Tissue

Adipose tissue may possess proinflammatory potential similar to that of macrophages in regards to production and secretion of inflammatory cytokines. However, the source of inflammatory mediators released from adipose tissue in obesity needs to be clarified. Although leptin and adiponectin are released from adipose tissue into the blood as hormones, it is unclear whether the source of other adipokines is the adipocytes or the nonadipocyte cells, such as macrophages, present in adipose tissue.

The source of adipokines released by adipose tissue of obese humans has been investigated (17, 31, 32). Earlier studies reported that adipocytes express TNF-α mRNA in vivo and are the major source of the elevated amounts of mRNA expression in adipose tissue (32). However, current research has found that the macrophages present in adipose tissue are responsible for almost all adipose tissue-derived TNF-α production and significant amounts of IL-6 production in mice (31).

In human abdominal subcutaneous adipose tissue, the number of infiltrated macrophages was positively correlated with BMI and adipocyte size, and was significantly higher in the obese compared to the lean subjects (31, 33). In agreement, Fain et al. (17) report that 90% of adipokines, excluding adiponectin and leptin, released by adipose tissue originate from cells other than adipocytes in obese individuals (17). They also found that in massively obese individuals (BMI of 45), the release of TNF-α, IL-8, and IL-10 was enhanced compared to individuals who weighed less (BMI of 32), and was due to the nonadipose cells present in adipose tissue.

The nonadipose cell fraction includes infiltrated macrophages and is considered to be a source of inflammation-related molecules within the adipose tissue (34). Macrophages are mononuclear phagocytes and reside within almost all tissues (31). Aside from tissue-specific functions, they are also involved in immune (innate and adaptive) and scavenger (eg. recognition and phagocytosis of foreign organisms) functions. In lean mice, macrophages in adipose tissue are described as uniformly small, isolated and widely dispersed among adipocytes, whereas macrophages in adipose tissue from obese animals are found in aggregates, and in extremely obese mice, some of these aggregates completely surround the adipocytes (31). In lean women, macrophages were rarely observed and they were dispersed among mature adipocytes and located near blood vessels (33). In contrast, macrophages completely surrounded single adipocytes in the adipose tissue of obese subjects.

Three months after surgery-induced weight loss in morbidly obese women, Cancello et al. (33) found a significant decrease in macrophage number in subcutaneous white adipose tissue, suggesting that a reduced number of macrophages may be responsible for the decreased expression of inflammatory markers in the stromal vascular fraction that was observed after weight loss (33). These researchers also tested the hypothesis of phenotypic change from proinflammatory to anti-inflammatory macrophages after weight loss, and found that the remaining macrophages infiltrating adipose tissue stained positive for anti-inflammatory IL-10.

Thus, several investigations have indicated that while adipose tissue may release factors that contribute to the inflammatory state of obesity, the source of these adipokines may not be the adipocyte itself, but rather reflect macrophage infiltration of adipose

tissue (17, 31, 33). These data suggest that immune cell activation may be more important than adipose tissue activation in promoting the proinflammatory state associated with obesity. The definite contribution of adipose tissue and macrophages to the inflammatory state associated with obesity are still unclear though, as a reduction in both factors after weight loss has been associated with improved inflammatory status. Further investigation to understand the mechanism responsible for macrophage recruitment and activation in adipose tissue in obesity, and the cause of associated comorbidities is required (33).

The effect of an anti-inflammatory dietary agent (eg. omega-3 fatty acid) has not been studied in isolation to determine its potential impact to the inflammatory profile seen in obesity, in the presence or absence of weight loss. It is unknown whether dietary intervention with omega-3 fatty acid supplementation is able to modulate adipokine or cytokine production, or macrophage infiltration in adipose tissue.

Leptin

In 1994, leptin was discovered to be produced by white adipose tissue as a product of the *ob* gene (35). Leptin is a peptide hormone responsible for coordinating responses to changing energy status and regulating body weight by acting on appetite and metabolism (36). This hormone is secreted in proportion to body fat levels and through action on the hypothalamus, leptin regulates food intake and increases energy expenditure.

Leptin and Obesity

Nutritional status influences the plasma concentration of leptin, as it is lowered in fasting or malnourished states (37, 38) and is increased in obese states (39, 40). However, 5-10% of obese human subjects do have relatively low levels of leptin, indicative of a reduced rate of leptin production (41). The association of mutations in leptin, and its receptor, with obesity confirms its importance in regulating human body weight (42, 43). However, these syndromes are rare, as mutations in the genes encoding leptin or its receptor have not been detected in the majority of human obesity case studies.

The most common form of human obesity is characterized by hyperleptinaemia and central and peripheral leptin resistance (41). The precise role of leptin in the connection between proinflammatory states of obesity and the risk of infection has yet to be determined (3). Leptin deficiency has been associated with increased mortality due to infections and increased susceptibility of infections in humans and animals; however, more research is needed to determine the role of leptin on immune function in leptin-resistant obese states where circulating leptin levels are high.

Leptin and Immune Function

Epidemiological associations between susceptibility of infection and malnutrition in less-developed societies have been observed, while an increased incidence of autoimmune disease has been seen in more affluent countries (44). Leptin has been proposed to be a factor that explains the inverse relationship between susceptibility to infection and autoimmunity. Therapeutic implications of the potentiating role of leptin in

immune function include reducing amounts of leptin to suppress the immune response in autoimmunity and proinflammatory conditions, or to supplement this hormone in states of immunosuppression to stimulate the immune response and fight infection. This association highlights the importance of an optimal balance between an efficient host defense to fight infection and a controlled immune response to inflammatory conditions. However, the role of leptin as a modulator of the immune response has yet to be clarified. Although its most important role may be its inhibitory effect on appetite, functional leptin receptors have also been identified on immune cells such as human monocytes and peripheral T cell populations (45). Due to growing evidence that suggests leptin is a modulator of the immune and proinflammatory response, elevated leptin levels may contribute to the low-grade proinflammatory state associated with human obesity.

Effects of Leptin in Autoimmunity

The contribution of leptin to T cell immunity can significantly affect the susceptibility and pathogenesis of autoimmune disease (46-48). In leptin-deficient *ob/ob* mice, induction of experimental autoimmune encephalomyelitis (EAE) is inhibited (46, 47). Leptin administration in *ob/ob* mice converted disease resistance to EAE disease susceptibility and transfer, and was accompanied by a switch from a Th2 to a Th1 pattern of cytokine release (enhanced IFN-γ secretion and inhibited IL-4) compared to untreated obese control mice (46). In addition, control mice receiving leptin administration had increased EAE severity, as indicated by a more severe clinical score and mortality, increased inflammatory cell infiltration and IFN-γ production, and reduced IL-4 secretion. Interestingly, while an increase in leptin levels preceded and accompanied the

acute phase of the disease in EAE-induced SJL/J female mice; this correlation was not observed in EAE-resistant mice (47), which may be a reflection of the sex differences in initial leptin concentrations, as higher circulating levels are observed in females (47).

Leptin administration can also lead to rapid acceleration of autoimmune type 1 diabetes and higher severity of disease in leptin-treated female nonobese diabetic (NOD) mice, but only when leptin therapy is initiated early in life (48). Splenocytes from leptin-treated NOD mice were also rich in IFN-γ mRNA-expressing cells, although secretion of this cytokine did not differ from the controls. These findings confirm the enhancing effect of leptin on immune response, as demonstrated by increased proinflammatory cytokine expression as well as autoimmune disease initiation, progression, and severity.

Effects of Leptin on Cytokine Production

The proinflammatory effect of leptin on cytokine production in animals and humans has been reported (45, 49). An examination of responder T cells from *ob/ob* mice showed that in the absence of leptin, Th2 responses were favoured, as a moderate amount of IL-4 was observed, but only minimal secretion of IFN-γ (45). This mouse strain is unable to produce functional leptin, weigh 3 × more than normal mice even when fed the same diet, and has a fivefold increase in body fat content (41), thereby mimicking leptin deficiency in an obese state. The addition of leptin in these animals induced a switch to a Th1-type response, as evidenced by large amounts of IFN-γ production and IL-4 suppression, in a dose-dependent manner. Leptin also increased both proliferation and IL-2 production. In addition, T cells from *ob/ob* mice had greater sensitivity to exogenous leptin compared to controls, as evidenced by a 21-fold versus 5-fold increase

in IFN-γ secretion. These findings confirm that the proinflammatory effect of leptin seems to bias T cell responses towards a proinflammatory phenotype (Th1).

Similar findings were reported in human peripheral blood mononuclear cells (PBMCs) (49). When these cells were stimulated in the absence of leptin, significant amounts of IL-2 and lesser amounts of IFN-γ were produced. In contrast, when these cells were stimulated in the presence of leptin, inhibition of proliferation and suppression of IL-2 production was observed. Furthermore, IFN-γ production was markedly increased, which again highlights the effect of leptin on proinflammatory cytokine production, but also a potential inhibitory effect of leptin on T cell function.

Obesity and Leptin Supplementation

Leptin supplementation in human states of obesity may encounter a threshold effect where leptin can no longer enhance the inflammatory response. Increasing circulating leptin levels from high physiologic to low pharmacologic levels in obese men, and even from low physiologic to high physiologic levels in lean men, did not alter serum IFN-γ, IL-10, or TNF-α after 16 weeks (50). Theses data reinforce the notion of leptin resistance in obese states and also suggest that a threshold effect of leptin may occur at even high physiological levels in lean states. However, if elevated leptin levels do contribute to the low-grade inflammation seen in obesity, leptin administration during weight loss would be expected to counteract the beneficial effects of weight loss on inflammation.

Weekly subcutaneous administration of 60 mg of long-acting pegylated human leptin (PEG-OB) given to obese subjects on a mildly hypoenergetic diet for eight weeks

did not influence weight loss compared to the placebo group (51). In addition, no significant differences in serum CRP or soluble TNF-α receptor (55 and 75) levels existed between groups at the end of the study period.

In another study by these researchers, significantly higher weight loss was reported in healthy overweight young men who received PEG-OB treatment and were fed a very low calorie diet, compared to controls who did not receive leptin therapy (52). Interestingly, leptin administration was associated with a gradual decline of proinflammatory parameters throughout the study period (46 weeks). However, only the decrease in serum TNF-α after weight loss was reported to be significant; serum CRP levels remained elevated in the PEG-OB group.

The contrasting findings from these studies may reflect differences between leptin resistance (sensitivity) among overweight and obese subjects. Greater weight loss and elevated CRP levels were observed in leptin-supplemented overweight subjects (52), but not in obese subjects receiving leptin (51), which may reflect greater sensitivity (less resistance) to leptin in overweight (versus obese) states. Their findings also indicate that the source of TNF-α is mostly from adipose tissue, which would be presumably decreased during weight loss. In summary, leptin administration does not seem to strongly counteract the beneficial effects of weight loss on the proinflammatory state in overweight or obese human subjects. However, excess leptin levels associated with overweight or obesity may indicate that leptin is ineffective in stimulating an enhanced inflammatory immune response above a given threshold in these individuals, even if inflammatory conditions improve from initial, short-term weight loss. Investigating whether sensitivity to leptin would improve with continued weight loss in the long term

would be informative to assess whether leptin resistance observed in an obese state can be reversed if a leaner state is achieved.

Although leptin administration enhances the inflammatory response in subjects with low leptin levels by promoting the switch to a Th1-type proinflammatory cytokine release (43, 45-47, 49), the same effects have not been observed in subjects with sufficient or excess leptin levels (50-52). These data indicate that the role of leptin in enhancing immune function may be more important in leptin-deficient states, and not leptin-sufficient states, in humans. However, modulating immune function by reducing leptin levels to normal physiological levels to decrease inflammation may prove to be effective in obese states.

Essential Fatty Acids: Omega-3 and Omega-6

Linolenic acid (omega-3) and linoleic acid (omega-6) are essential long-chain polyunsaturated fatty acids (LCPUFA) required for proper growth and development. Omega-3 and omega-6 fatty acids are components of all cells in the body and serve as an integral component of many bodily functions including energy generation, structure of membrane phospholipids, synthesis of many biologically active compounds, and intracellular signaling (53). Eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) are fatty acids of the omega-3 type as they are produced from linolenic acid (ALA). These LCPUFAs play an important role in visual and brain development and neural functioning (54), and are more biologically potent than ALA alone (55).

ALA (C18:3 n-3) contains 18 carbon atoms and three double bonds, with the last double bond situated three carbon atoms from the terminal methyl group (omega-3). Linoleic acid (LA) (C18:2 n-6) is comprised of 18 carbon atoms and two double bonds, with the last double bond located six carbons atoms from the terminal methyl group (omega-6). The omega-3 fatty acids EPA and DHA are most abundant in fish oils and fatty fish, while ALA is present in soybean oil, canola oil, walnuts, wheat germ oil and flaxseed oil. In addition, enrichment of omega-3 in other food items, such as eggs or margarine, has increased its availability in the food supply. Dietary sources of the omega-6 fatty acids LA include nuts, seeds, and vegetable oils such as corn, soybean, cottonseed, or safflower.

Dietary Intake Recommendations for Omega-3 and Omega-6 Fatty Acids

The Acceptable Macronutrient Distribution Range (AMDR) is the range of intake for a particular energy source that is associated with reduced risk of chronic disease while providing intakes of essential nutrients (56). The AMDR for omega-3 and omega-6 fatty acid are 0.6-1.2 % of energy and 5-10 % of energy, respectively, for female and male adults.

The Adequate Intake (AI) is believed to cover the needs of all individuals in a specified age or life group, but lack of data prevents being able to specify with confidence the percentage of individuals covered by this intake. The AI for omega-3 fatty acid is 1.6 g/day and 1.1 g/day, for male and females adults, respectively. The AI for omega-6 fatty acid for males between the ages of 19-50 years is 17 g/day and for those greater than 50 years is 14 g/day. The AI for omega-6 fatty acid for females

between the ages of 19-50 years is 12 g/day and for those greater than 50 years is 11 g/day.

Metabolism of Omega-3 and Omega-6 Fatty Acids and Immune Function

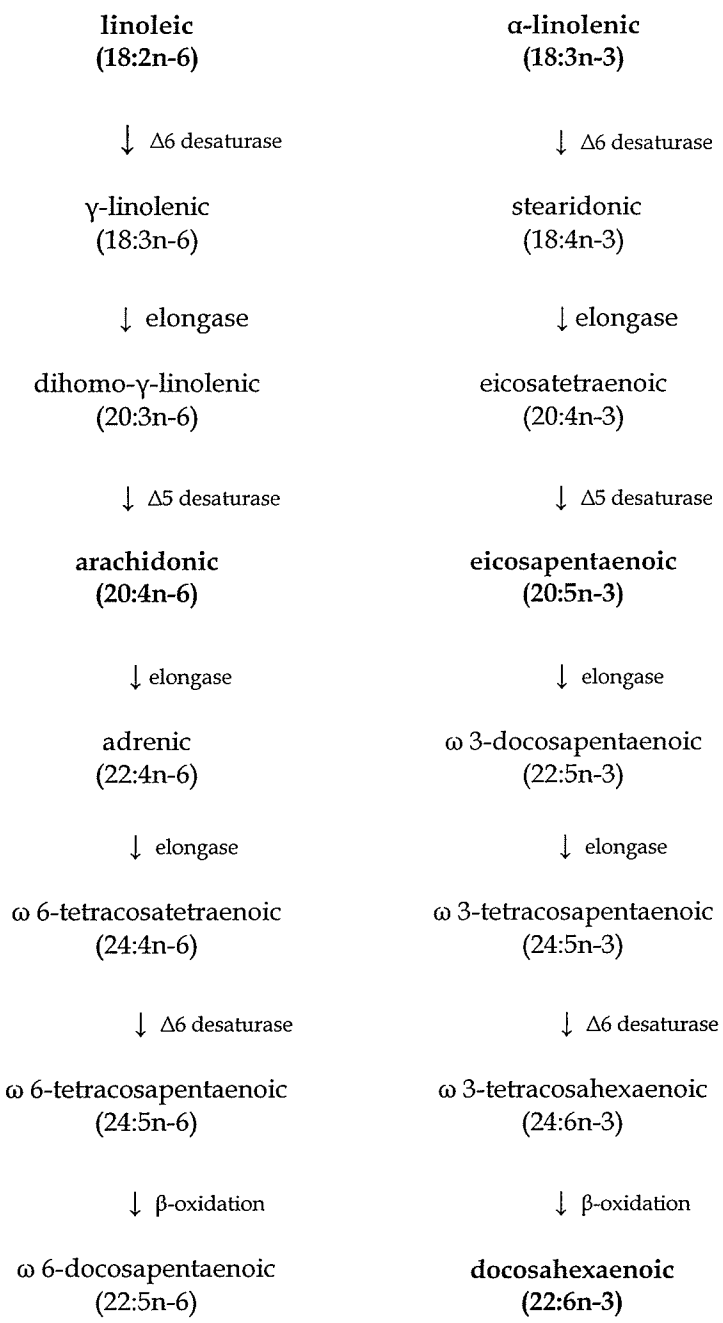
Omega-3 and omega-6 fatty acids undergo a series of desaturation and elongation reactions in the body, and just as they differ in their chemical structure, the metabolism of these LCPUFAs also leads to different end products (Figure 1). Products of omega-3 and omega-6 fatty acid elongation and desaturation reactions can be incorporated into membrane phospholipids and then be cleaved in response to injury and inflammation (54), and enter the eicosanoid pathway. ALA and LA are metabolized to EPA and arachidonic acid (AA), respectively. AA competes with EPA for the enzymes responsible for their metabolism and the synthesis of eicosanoid hormones.

Dietary fatty acids have been shown to modulate immune responses by altering membrane composition and function, modifying eicosanoid production, or changing cytokine biosynthesis (57). Eicosanoids are bio-active compounds that include prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs), and these mediators regulate the production of cytokines. Depending on the source of the LCPUFA, the resultant eicosanoids produced possess unique characteristics that modulate the immune response and modify the processes of inflammation.

Eicosanoids derived from AA are from a different series, and have different biological properties, than those produced from EPA (55). AA-derived eicosanoids include Series 2 PGs and TXs and Series 4 LTs, and those derived from EPA are of the Series 3 PG and TX and Series 5 LT type. As reviewed by Calder (58), the functional

significance of these differences is that mediators formed from EPA are usually less potent than those from AA. For example, PGE_3 is a less potent inducer than PGE_2 of IL-6 production by macrophages. EPA and DHA can also inhibit or decrease production of proinflammatory cytokines from a variety of cell types (58). Furthermore, EPA and DHA act as substrates for the generation of alternative eicosanoids, E-series and D-series resolvins, respectively, which exert anti-inflammatory action (58). The overall balance between the eicosanoid types that result from omega-3 and omega-6 fatty acid metabolism is important however, as the overall effect of the derived mediators may depend on their concentration and production, as well as the sensitivity of the target cells.

Figure 1: Biosynthesis of long-chain polyunsaturated fatty acids*.



*Adapted from Sprecher (59).

Effects of Dietary Omega-3 Fatty Acids on T Cell Function in Animals

The effects of varying dietary ratios of omega-6:omega-3 polyunsaturated fatty acids (PUFAs) (6:1, 30:1, and 60:1) on T-lymphocyte proliferation and subpopulations (helper and cytotoxic) have been investigated by altering the sunflower and fish oil composition in the diets of non-tumour-bearing and tumour-bearing male Wistar rats (60). Higher proliferation of thymus, spleen, and gut-associated lymphocytes and a decreased proportion of CD8+ lymphocytes were observed in diets richest in omega-6 fatty acid, regardless of the presence of a tumor. In contrast, thymus and spleen lymphocyte proliferation was lower when the diet contained a significant amount of omega-3 PUFA compared to omega-6 PUFA.

Further investigations have revealed that dietary omega-3 fatty acids alter the Th1/Th2 balance towards Th2 by suppressing Th1 development (61). Splenic CD4+ T cells of C57BL/6 mice fed either fish oil (FO; 4% fish oil + 1% corn oil; containing 2.0 g omega-3/100 g fatty acids) or control (5% corn oil; containing 1.1 g omega-3/100 g fatty acids) diets were cultured under neutral and Th2 polarizing conditions and then reactivated for analysis of IL-4 and IFN-γ to measure mean percentages of Th2 and Th1 cells generated. In the presence of homologous mouse serum (HMS), included to preserve the diet-induced changes in the lipid composition of cell membranes during the in vitro culture, dietary FO significantly increased the ratio of Th2:Th1 cells, indicating a clear shift towards a Th2 response. While dietary FO significantly increased the proportion of IL-4 Th2 cells under neutral conditions, no difference was observed between diets under Th2-polarizing conditions, suggesting the effect of dietary FO is not targeted to Th2 type cell differentiation. In addition, dietary FO decreased the proportion

of IFN-γ Th1 cells under neutral conditions and this suppression was maintained under Th2 polarizing conditions, indicating that the increase in the Th2:Th1 ratio by dietary omega-3 in the presence of HMS is due to suppression of Th1 development rather than enhancement of Th2 development.

Effects of Dietary Omega-3 Fatty Acids on T Cell Function in Humans

Daily consumption of capsules containing fish oil (720 mg of EPA + 280 mg of DHA), but not DHA (720 mg) or ALA (2 g) supplementation alone, was associated with a significant reduction in lymphocyte proliferation in healthy older adults (62). However, proinflammatory cytokine production (IL-2, IFN-γ) was not affected by any of the treatments, including those that significantly decreased lymphocyte proliferation.

Although favourable changes to T lymphocyte proliferation have been reported in association with omega-3 fatty acid supplementation (62), higher amounts may be necessary to alter cytokine production. On the other hand, intakes of 4.5 g of EPA and DHA/week (given as two portions of oily fish) in combination with a low ratio (2:1) of linoleic to linolenic acid, by manipulating sunflower and rapeseed oil intake, were not found to alter serum proinflammatory cytokines TNF-α and IL-6, or acute-phase protein CRP, in overweight men and women (63). Furthermore, in normal weight healthy subjects a comparison of ALA (3.5 g/day of flaxseed oil) and EPA plus DHA (0.44, 0.94, or 1.9 g/day of fish oil) consumption on markers of immune function did not show any alteration in cytokine production by stimulated mononuclear cells, except for a decrease of IL-6 production, but only with the two higher doses of fish oil (64). More research is needed to clarify the effects of the different types (ALA, EPA, DHA), forms (capsule or

foodstuff), and doses of dietary omega-3 fatty acid intake on T cell function (proliferation and cytokine production) and whether this dietary intervention would be effective in an obese state.

Effects of Dietary Omega-3 Fatty Acids on Markers of Inflammation in Humans

Observational studies of healthy men and women reveal an inverse relationship between dietary omega-3 fatty acid intake and markers of inflammation (60, 65, 66). High dietary intakes of EPA and DHA (1.12 g/day and 0.47 g/day for men and women, respectively) are inversely associated with plasma markers of TNF-α activity, such as soluble TNF receptors 1 and 2 (65). However, dietary ALA was not significantly related to inflammatory markers (IL-6, CRP). The Nurses' Health Study I cohort of 727 women also showed lower circulating concentrations of markers of inflammation, such as CRP (29%) and IL-6 (23%), among those in the highest quintile of total omega-3 PUFA intake (1.43-3.33 g/day) compared with those in the lowest quintile (0.54-0.97 g/day) (66). In addition, ALA consumption alone (quintiles ranging from 0.43-2.36 g/day) was inversely related to plasma concentrations of CRP and IL-6.

Interventional studies that have increased dietary ALA intake report changes in circulating markers of inflammation in hypercholesterolemic and dyslipidemic subjects (67-69). In hypercholesterolemic subjects, a high omega-3 fatty acid diet (6.5% of energy from ALA; total dietary intake of ALA = 19.1 g/day) decreased serum CRP more than an omega-6 fatty acid diet (12.6% of energy from LA) (67), and consumption of ALA-enriched margarine (46% LA, 15% ALA; total dietary intake of ALA = 5.9 g/day) was associated with lowered CRP levels (68). In addition, serum CRP and IL-6 levels

were also significantly decreased (38% and 10%, respectively, compared with baseline values) in dyslipidemic subjects receiving dietary supplementation of ALA (15 mL linseed oil/day containing approximately 8 g of ALA) (69). In a recent study, Zhao and colleagues (70) reported dietary ALA (6.5% of total energy; total dietary intake of ALA = 19.1 g/day) is associated with lower IL-6, IL-1, and TNF-α production by PBMCs in hypercholesterolemic overweight or obese subjects, and they identified an inverse correlation between ALA and EPA (derived from dietary ALA) in PBMC lipids and release of TNF-α. These findings indicate that dietary omega-3 fatty acid, in its ALA form, is also associated with improvements to the inflammatory status by reducing markers of inflammation, such as acute phase CRP and the proinflammatory cytokine IL-6, in human subjects.

While dietary omega-3 fatty acid intake is associated with improvements in serum markers of inflammation in normal weight and obese human subjects (65-70), further research that investigates potential improvements to adipose tissue composition and dysfunction in an obese state is needed.

Effects of Dietary Omega-3 Fatty Acids on Infection in Humans

Due to the ability of omega-3 fatty acid to modulate inflammation, recent investigations have been conducted to determine its influence on community-acquired pneumonia risk in a prospective cohort study of men aged 40-75 years (71). Pneumonia risk was found to be 32% lower in men in the highest energy-adjusted quintile of ALA intake (median of quintile = 1.53 g/day) than in men in the lowest quintile of intake (median of quintile = 0.71 g/day); for every 1 g/day increase of ALA intake, pneumonia

risk was reduced by 31%. However, because intakes of linolenic acid and linoleic acid were highly correlated, and both were associated with reduced risk, it was not possible to evaluate the interaction between them or their independent effects. In addition, a non-significant inverse relationship between pneumonia risk and frequent fish intake (5 or more times per week as compared to infrequent fish intake less than once per month) was observed. It would be interesting to further study whether high dietary intake of ALA (independent of omega-6 fatty acid intake) is associated with decreased infection risk, and whether differences are observed when only an overweight or obese population is considered.

Potential Effects of Dietary Omega-3 Fatty Acids on Inflammation in Obesity

Nutritional intervention that increases the consumption of omega-3 PUFAs provides less substrate for synthesis of eicosanoids from AA. Due to the predominant synthesis of eicosanoids from omega-3 PUFAs possessing anti-inflammatory properties and an ability to produce mediators that regulate the production of cytokines, dietary omega-3 fatty acid may have a protective effect against the damaging consequences of inflammation in states of obesity.

Dietary omega-3 fatty acids have been shown to reduce inflammation by decreasing lymphocyte proliferation in animals (60) and humans (62), through suppression of Th1 cell development (61). In addition, changes to eicosanoid production (72), decreased circulating levels of inflammatory markers such CRP (65-69) and proinflammatory cytokines (61, 65, 66, 69), and reduced risk of infection (71) have been associated with higher intakes of dietary omega-3 fatty acid. These changes are not

always associated with dietary ALA alone, or in an obese state. More research is needed to determine if improvements in immune and adipose tissue function occur with dietary ALA consumption in states of obesity. Additionally, in order to suggest potential future dietary recommendations, it is important to clarify the amount and form of dietary omega-3 fatty acid that is responsible for a favourable effect.

Leptin-Lowering Effects of Dietary Omega-3 Fatty Acids

As stated earlier, leptin is a hormone secreted in proportion to body fat and through actions on the hypothalamus, regulates food intake. Hyperleptinaemia is observed in the most common cases of human obesity, a low-grade inflammatory condition, and is likely caused by leptin resistance in this population. Since omega-3 fatty acids have anti-inflammatory potential, the link between dietary consumption of omega-3 fatty acid and leptin has been the subject of several investigations.

In human subjects receiving dietary supplementation with omega-3 fatty acid (containing 5 g of EPA and DHA), plasma leptin concentrations correlated negatively with changes to dietary intake of PUFA (73). Likewise, when Wistar rats were fed omega-3 fatty acid-enriched diets [containing 6.5% omega-3 concentrate K85, an ethylester concentrate of EPA (54.2%) and DHA (32.8%)] for 6-8 weeks, a non-significant trend for lower plasma leptin levels was observed (73). In addition, the dietary omega-3 fatty acid treatment reduced expression of leptin mRNA in epididymal tissue compared to the lard-enriched diet group. Lower plasma leptin levels have also been reported in C57B1/6J mice fed an omega-3 fatty acid-enriched high-fat diet (33.9 g of fish oil as the main source of fat; omega-3 fatty acid representing 42% of total fat) for

7 weeks whereas elevated plasma leptin levels were observed in mice fed a high saturated fat diet (74). Changing the saturated fat group to the omega-3 fatty acid diet completely reversed the hyperleptinaemia induced by the high saturated fat diet, and was more effective in this regard than changing to a low-fat diet.

Consumption of omega-3 fatty acid in its ALA form also reduces circulating leptin concentrations in humans (75). Healthy non-obese men and women were fed high-fat diets containing rapeseed oil [19.0 ± 0.4% of energy from monounsaturated fatty acid (MUFA) and 2.5 ± 0.2% of energy from omega-3 PUFA], olive oil (23.3 ± 0.6% of energy from MUFA and 0.4 ± 0.0% of energy from omega-3 PUFA), or sunflower oil (18.1 ± 0.3% of energy from omega-6 PUFA and 0.3 ± 0.00% of energy from omega-3) as the main source of fat for 4 weeks (75). Serum leptin concentrations did not change in either men or women consuming the olive or sunflower oil diets. As expected, a reduction in serum leptin levels was observed in females who consumed the rapeseed oil diet, suggesting that the high amount of omega-3 PUFA contained in this diet was responsible for the leptin-lowering effect. Interestingly, a gender bias was observed as among men fed the same diet, leptin levels significantly increased, indicating that leptin concentrations in men and women respond differently to diet. This contrast may again be a reflection of the sex differences in initial circulating leptin concentrations.

Higher intakes of the omega-3 fatty acids EPA and DHA are associated with lower circulating leptin levels in both humans and animals (73, 74). In addition, dietary intake of ALA has been associated with a reduction in serum leptin levels in healthy nonobese women (75). Other diet interventions such as conjugated linoleic acid (CLA) have been effective in attenuating hyperleptinaemia in *fa/fa* Zucker rats (76), an obese

rodent model with a mutation in the leptin receptor. Of particular interest for future research is whether dietary ALA would also be effective in lowering leptin levels when introduced in a leptin-resistant (hyperleptinaemic) obese state.

STUDY RATIONALE

The prevalence of obesity is increasing and of concern for human health and quality of life. Obesity is associated with impaired immune function and an increased risk of infections, and it is a risk factor in the pathogenesis of many chronic diseases. Human obesity is described as a subclinical low-grade inflammatory condition and in most cases is associated with elevated circulating leptin and reduced adiponectin levels. Leptin modulates immune function by enhancing T cell proliferation and production of proinflammatory cytokines. Adipose tissue is also capable of producing adipokines (leptin, adiponectin) that contribute to the inflammatory state associated with obesity. Adipose tissue dysfunction, initially associated with adipocyte hypertrophy, can further modulate the immune response. Conversely, feedback from the immune response in obesity may negatively impact adipose tissue function. Several studies indicate that dietary omega-3 fatty acid reduces inflammation and circulating leptin levels, however, more research is required to explore these effects in obese states. In addition, most research investigates the effects of marine sources of omega-3 fatty acid (EPA and DHA). More information is needed on the effectiveness of plant sources such as ALA.

HYPOTHESIS

We hypothesize that dietary intervention with omega-3 fatty acid as ALA-rich flaxseed oil will promote a decrease in the production of proinflammatory markers and an increase in anti-inflammatory markers, thereby leading to improved immune and adipose tissue function in an obese state. We expect that both cytokine (T cells) and adipokine (adipose) production of proinflammatory mediators, and adipocyte size, will be reduced and anti-inflammatory cytokine and adipokine production increased.

OBJECTIVES

We propose to study the effects of dietary omega-3 fatty acid supplementation with ALA-rich flaxseed oil on markers of inflammation associated with obesity using a rodent model. We will focus on T cell function (cytokine production) and adipose tissue function (adipokine production and adipocyte size). The objectives of our research are:

1. To determine the effect of dietary omega-3 fatty acid supplementation with ALA-rich flaxseed oil on T cell function (cytokine production) in ConA-stimulated splenocytes from obese *fa/fa* Zucker rats. Cytokines of interest include Th1 proinflammatory TNF-α, IL-2, IL-6, IFN-γ, and Th2 anti-inflammatory IL-4, IL-10.

2. To determine the effect of dietary omega-3 fatty acid supplementation with ALA-rich flaxseed oil on adipokines (leptin and adiponectin in serum; leptin, adiponectin, proinflammatory TNF-α, IL-6, IL-18, HMGB1, and anti-inflammatory IL-10 in adipose tissue) and adipocyte size in adipose tissue from obese *fa/fa* Zucker rats.

Rationale for Study Design

The *fa/fa* Zucker rat model was used in our study to investigate the effects of dietary omega-3 fatty acid on immune function and adipose tissue in an obese state. These animals have a defect in the leptin receptor (77) and exhibit obesity at 4 to 5 weeks of age. Zucker rats exhibit characteristics of the metabolic syndrome including hyperleptinaemia, insulin-resistance, muscle atrophy, hyperlipidemia, hypercholesterolemia, hypertriglyceridemia and hyperinsulinemia.

Older rats (rats were 17 weeks old when the study began and 25 weeks old when they were terminated) were fed dietary omega-3 fatty acid to investigate whether diet intervention can improve immune and adipose tissue function in an obese state during the middle adulthood period of a rodent lifespan, which is typically one year in the *fa/fa* Zucker rat model. Using animals at this age allowed us to determine whether dietary omega-3 fatty acid would be effective as a treatment to improve the inflammatory state associated with obesity, once obesity had already been established in an animal (versus investigating a preventative role of dietary omega-3 fatty acid on the inflammation associated with growth and accumulation of adipose tissue in obesity).

An 8-week study period was chosen to allow adequate time to observe diet-induced changes. Furthermore, a dietary agent (conjugated linoleic acid) administered for 8 weeks has been previously reported to be effective in reducing inflammatory markers in this rat model (78). To our knowledge, the effect of a short chain dietary plant source of omega-3 fatty acid on immune or adipose tissue function in *fa/fa* Zucker rats has not been investigated.

While the *fa/fa* genotype is characterized by hyperleptinaemia, similar to human obese states, *fa/fa* Zucker rats are leptin-receptor defective (77); mutations in the genes encoding leptin or its receptor are uncommon in human cases of obesity. In addition, because these animals are capable of converting ALA to EPA and DHA in the body, we cannot rule out the possibility that the results of the study (whether negative or positive) may be due to the effects of EPA and DHA in an obese state. However, if the anticipated beneficial effect of dietary ALA-rich flaxseed oil is observed, we can still conclude that dietary intervention with this food source is effective in improving immune and adipose tissue function in an obese state, regardless of being unable to confirm the exact form of omega-3 fatty acid in the body responsible for this effect.

Dietary omega-3 fatty acid was administered to rats in the form of ALA-rich flaxseed oil. The specific use of a food source high in omega-3 fatty acid in its ALA form may be more practical for obese individuals to incorporate into their current diets, as EPA and DHA are mostly found naturally in fish oil sources, which may be undesirable or unacceptable for some individuals to consume. ALA-containing foods may also be easier to incorporate into the diet as they are more frequently consumed and available. The ALA-rich flaxseed oil diet was formulated to contain approximately a 1:1 ratio of omega-6:omega-3 fatty acid in order to maximize the difference between diets.

One of the most important roles of the spleen is to help fight infection in the body. Although the thymus is the principal organ for T cell development, the composition of the spleen includes periarteriolar lymphoid sheaths that are rich in T lymphocytes. In addition, effective methods for isolating and culturing splenocytes had been previously established in our lab. T cell (lymphocyte) function was investigated to assess immune

function as these cells play a central role in cell-mediated immunity. Both proinflammatory (IFN-γ, TNF-α, IL-2, IL-6) and anti-inflammatory (IL-4, IL-10) cytokine production from stimulated T cells was measured to determine the effect of ALA-rich flaxseed oil on the Th1/Th2 balance. Selection of the types of proinflammatory and anti-inflammatory cytokines to measure was based on previous literature findings that showed these cytokines to be altered in an obese state. In addition, the effect of ALA-rich flaxseed oil on the mitogen-stimulated *ex vivo* production pattern of these proinflammatory and anti-inflammatory cytokines in *fa/fa* Zucker rats has not been reported.

Proinflammatory (leptin, TNF-α, IL-6, IL-18, HMGB1) and anti-inflammatory (adiponectin, IL-10) adipokine levels in adipose tissue were also measured to determine the effect of ALA-rich flaxseed oil on adipokine production. Selection of adipokines included in our analyses was determined by previous literature findings that observed altered levels of these adipokines in an obese state. Measurement of adipokine levels by Western immunoblotting determined if there were any diet-induced effects on their synthesis. Furthermore, the effect of ALA-rich flaxseed oil on adipocyte size was investigated, as an indicator of adipose tissue dysfunction. Finally, circulating leptin and adiponectin levels were quantified to assess if immediate changes to serum concentrations of adipokines are observed with dietary ALA-rich flaxseed oil intervention in an obese state.

MATERIALS AND METHODS

Adipose tissue and serum samples collected from a previous study in our lab, completed in the summer of 2006, were stored at -80°C and analyzed for this investigation. Splenocytes isolated from the same study were prepared specifically for the current study and the cell culture supernatants obtained were stored at -80°C until analysis (treatment groups at end of study but not the baseline group).

Animals and Diets

In that study, after a 5-8 day acclimatization period, 17-week old male Zucker rats (Harlan, Indianapolis, Indiana, USA) were randomly assigned to a *fa/fa* (fa) baseline group (faBASE; n=7), a fa control diet group (faCTL; n=7), a lean (ln) control diet group (lnCTL; n=7), or an omega-3 fatty acid diet intervention group containing α-linolenic fatty acid-rich flaxseed oil (faALA; n=7), for the 8-week study period. A *fa/fa* weight-matched group (n=10) fed control diet was also included in the experimental design but was excluded from analysis because diet restriction was not necessary throughout the study and no difference in body weight was observed between the faCTL and faALA groups. At the end of the study period the animals were 25 weeks old. The University of Manitoba Protocol Management and Review Committee approved the protocol for the animal care procedures and the experimental procedures were in accordance with Canadian Council on Animal Care (CCAC) guidelines (80).

The diet formulations were based on the AIN-93G diet (79) (Table 1) and were isocaloric, providing 3.87 kcal/g. Half the soy oil content in the CTL diet (42.5 g/kg diet) was replaced with flaxseed oil to create the ALA-rich diet. Based on the fatty acid

composition of the oils, it was expected that the CTL and ALA diets would contain 7.9% and 32.3% omega-3 fatty acid per total fatty acids, respectively, and that the ratio of omega-6:omega-3 in the CTL and ALA diets would be 6.8 and 1.1, respectively. The fatty acid composition was verified by analyses as described in the next section. The dry ingredients for the diets were premixed and stored at 4°C. Fresh, 6-kg batches of the diet, containing oil, were prepared weekly and stored at -20°C until used. Rats were given new feed cups with fresh feed 3 × per week. Feed intake was corrected for spillage. Rats were weighed weekly.

Table 1: Diet formulation.

Ingredients[a] (g/kg)	ALA diet	CTL diet
Cornstarch[b]	363	363
Maltodextrin	132	132
Sucrose	100	100
Egg white	212.5	212.5
Cellulose	50	50
AIN-93G-MX Mineral mix	35	35
AIN-93G-VX Vitamin mix	10	10
Choline	2.5	2.5
Biotin mix[c]	10	10
Tert-butylhydroquinone[d]	0.014	0.014
Soy oil	42.5	85
Flaxseed oil[e]	42.5	0

[a]Ingredients purchased from Harlan Teklad (Madison, Wisconsin) unless otherwise indicated
[b]Cornstarch purchased from Castco Inc. (Etobicoke, Ontario)
[c]200 mg/kg biotin mixed in cornstarch because egg white was the protein source
[d]Tert-butylhydroquinone was purchased from Sigma-Aldrich (St. Louis, Missouri)
[e]Flaxseed oil was purchased from Omega Nutrition Canada Inc. (Vancouver, British Columbia)

Extraction and Analysis of Fatty Acids in Diet

Diet samples were stored frozen at -20°C for analyses of percent fat and fatty acid profiles.

Fat Extraction

The fat extraction and fatty acid methylation protocols have been previously described (76). In a 100 mL beaker, two grams of diet were homogenized in 40 mL of 2:1 chloroform:methanol (Optima grade reagents) using a Polytron Homogenizer (speed 4) for 30 seconds. The homogenate was filtered through Whatman #1 (9 cm diameter) filter paper into a 100 mL graduated cylinder. Both the beaker and filter paper were rinsed with another 10 mL of 2:1 chloroform:methanol. The volume in the graduated cylinder was recorded and 25% of this volume was added as deionized water. The graduated cylinder was stoppered, inverted 5× to mix the solutions and left to sit overnight.

The following day, the volume of the chloroform (bottom) layer was recorded. The top layer was suctioned off as waste and a calculated volume (explained in the *Fatty Acid Methylation and Analysis* section, page 43) of the chloroform layer was set aside for fatty acid analysis. As well, a 20 mL aliquot of the chloroform layer was pipetted into a pre-weighed, 25 mL glass vial and evaporated under nitrogen at 50-70°C for approximately 1 hour or until the vial was dry. The vial was placed in a dessicator overnight and weighed.

The percent fat was calculated as follows:

$$\frac{(\text{Wt (g) of vial and oil}) - (\text{wt (g) of vial})}{02.00\text{ g sample}} \times \frac{\text{volume (mL) of chloroform layer}}{20\text{ mL aliquot}} \times 100$$

= % of total fat in sample

Fatty Acid Methylation and Analysis

To determine the fatty acid profile by gas chromatography, the fatty acids in the sample (e.g. oil) were methylated. The methyl ester form makes the fatty acid volatile when heated and easier to analyze using gas-liquid chromatography. The fractionation and eventual detection of each type of fatty acid in the sample is dependent upon the boiling point of the fatty acid and the nature of the solid adsorbant of the column used in the gas chromatograph.

i) Methylation

Solubilization and methylation of the fat extracts followed a procedure previously described in our lab (76). An aliquot of the fat extracted (part of the chloroform layer – see *Fat Extraction* section, page 42) from the diet sample was used for fatty acid analysis using gas-liquid chromatography. Determination of the aliquot volume needed for fatty acid analysis was based on the volume that would provide 10 mg of oil.

The volume required was calculated as follows:

$$2.00\ g \times 8.5\%\ \text{(by weight in diet)} = 0.17\ g \text{ or } 170\ mg \text{ oil in diet}$$

$$\frac{10\ mg \times \text{total volume (mL) of chloroform layer}}{170\ mg} = \text{volume (mL) of chloroform needed for fatty acid analysis}$$

The chloroform aliquot was placed into an 8 mL screw top tube that was flushed with nitrogen and heated in a 35°C water bath to evaporate the solvent. After evaporation, 1 mL toluene (Optima grade) was added to the tube and vortexed for 10 seconds to solubilize the fat extract. To methylate the fat sample, a 1.2 mL aliquot of 2 N methanolic hydrochloric acid was added, after which the tube was tightly capped, vortexed for 10 seconds and placed in an 80°C oven.

After 60 minutes, the tube was removed from the oven and cooled to room temperature. The cooled tubes received 1 mL deionized water and 1 mL hexane (Optima grade), were vortexed for 20 seconds and centrifuged (Model CS, International Equipment Company, Boston, Massachusetts, USA) at ¾ speed (2000 rpm) for 4 minutes. The hexane (top) layer was transferred to a clean 8 mL tube. To the hexane layer, 2 mL deionized water was added, after which the tube was capped, vortexed for 20 seconds, and centrifuged at ¾ speed for 4 minutes. The hexane (top) layer was transferred to GC vials for fatty acid analysis.

ii) Fatty Acid Analysis

The prepared samples were analyzed using a Shimadzu GC-17A Gas Chromatograph (Guelph, Ontario) with a flame ionization detector and a DB-225MS capillary column (30 m × 0.25 mm I.D. with 0.25 μm film thickness). The injector temperature was 260°C, the detector temperature was 300°C and the column was programmed from 70 to 220°C.

Tissue Collection

At the end of the study period, rats were fasted for 12 h overnight and were euthanized by carbon dioxide asphyxiation and cervical dislocation according to the Canadian Council on Animal Care Guidelines (80). Trunk blood was collected and immediately placed on ice until centrifuged to separate the serum fraction, which was stored at -80°C. Dissected peri-renal and epididymal adipose tissue was weighed; a small portion was placed in optimal cutting temperature (OTC) compound and frozen in a dry ice/ethanol bath, while the other portion was frozen in liquid nitrogen. Both portions were stored at -80°C. Dissected spleens were weighed, placed in cell culture medium (CCM; defined in 'Isolation of Splenocytes'), and splenocytes were isolated and cultured the day of termination.

T Cell Function

Isolation of Splenocytes

Spleens were weighed and placed into sterile buffer [0.5% bovine serum albumin (BSA; Sigma-Aldrich Incorportion, St. Louis, Missouri) in Krebs Ringer Bicarbonate Buffer (KRH) containing 130 mM NaCl, 10 mM HEPES, 5.2 mM KCl, 1.4 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 1.4 mM $MgSO_4$ and 10 mL antibiotic/antimycotic; pH 7.4). A 15 mL single cell suspension of splenocytes was prepared by breaking spleens apart using the blunt end of a syringe and passage of cells through nylon mesh.

Red blood cells were lysed by resuspending the splenocytes in 1-2 mL ammonium-chloride-potassium (ACK) lysing buffer [155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediaminetetraacetate (EDTA), pH 7.4]. First, ACK was added to the sample, the mixture was vortexed, left on ice for 3 minutes, and then 15 mL KRH was added. The sample was spun at 1000 rpm for 10 minutes at 4°C to pellet the cells, the supernatant was removed, and then 15 mL of CCM [RPMI 1640 (Sigma Chemicals, St. Louis, Missouri) pH 7.2 supplemented with 5% heat-inactivated fetal calf serum (Invitrogen Corporation, Burlington, Ontario), 25 mL HEPES buffer, 10 mL antibiotic/antimycotic (Invitrogen Corporation, Burlington, Ontario) and 4 μL 2-mecaptoethanol] was added. Cells (stained by addition of an equal volume of 0.4% trypan blue) were counted on a haemacytometer and adjusted to a concentration of 1.25×10^6 cells/mL. One mL of cell suspension (containing 1.25×10^6 cells) from each sample was pipeted into two 1.5 mL microcentrifuge tubes and spun at 10 000 rpm for 2 minutes

at 4°C; the supernatant was subsequently removed, and the pellets were stored at -80° for analysis not part of this thesis.

Splenocyte Stimulation

Two mLs of splenocytes (1.25×10^6 cells/mL) were stimulated with 2.5 mg/L concanavalin A (ConA; Catalogue #L2630, Sigma Chemicals, St. Louis, Missouri; CCM was used to prepare and dilute the ConA), and unstimulated cells received an equivalent amount of CCM. ConA binds to the T cell receptor and stimulates T lymphocytes to secrete cytokines. Stimulated and unstimulated splenocytes were incubated in 5 mL test tubes at 37°C in a 5% CO_2 and 95% humidified air incubator for 48 h. The 48 h incubation time had been previously determined in our lab for peak cytokine production from stimulated splenocytes; a 24 h incubation time had not been adequate. We did not test each cytokine's prime incubation time separately, so a 48 h incubation time is only an estimate to capture all cytokine production at 1 time point. Furthermore, varying concentrations of ConA would have differentially stimulated splenocytes (minimal, moderate or maximal effects) and may have revealed different patterns in cytokine production, and/or differences between obese and lean rats.

After incubation, samples were spun at 1500 rpm for 10 minutes at 4°C to pellet the cells and 2 mL aliquots of supernatant were collected for each sample. Supernatants from stimulated and unstimulated splenocytes were stored at -80°C until further analyzed for cytokine production. The remaining supernatant from each sample was spun at 10 000 rpm for 2 minutes at 4°C, the supernatant was discarded, and the pellets were also stored at -80°C for analysis not part of this thesis.

Cytokine Analysis

The supernatants were analyzed for the cytokines TNF-α, IFN-γ, IL-2, IL-6, IL-4, and IL-10 using the Rat Cytokine 6-Lincoplex Kit (Linco Research, St. Charles, Missouri). This method allows multiple cytokines to be measured simultaneously in a cell culture supernatant sample and requires only a small sample size. Antibodies conjugated to fluorescently labeled microsphere beads can be identified with laser based fluorescent analytical test instrumentation (Luminex). Luminex colour-codes tiny beads into 100 distinct sets that can be coated with a reagent specific to capture and detect the specific analytes from a sample. Within the Luminex compact analyzer, lasers excite the internal dyes that identify each microsphere particle and also any reporter dye captured during the assay.

Standards [Background (assay buffer), dilutions of 1:4096, 1:1024, 1:256, 1:64, 1:16, 1:4, and original (undiluted)], controls (Rat Cytokine Control I and II), and samples (25 μL) were pipetted into duplicate wells of a 96-well microtiter plate. Mixed beads (25 μL) conjugated with antibodies to the cytokines of interest were then added to wells and the plate was incubated with agitation on a plate shaker for 18-20 hours at 4°C.

After incubation, detection antibody cocktail (25 μL) was added to each well and the plate was incubated for 2 hours with agitation on a plate shaker at room temperature (20-25°C). After this incubation period, streptavidin-phycoerythrin (25 μL) was added to each well and the plate was incubated at room temperature (20-25°C) an additional 30 minutes with agitation on a plate shaker. The plate was analyzed on a Luminex Instrument (Model Number: Luminex SD, Lincoplex 200; Luminex Corporation, Austin,

Texas). Samples were analyzed in duplicate and a % agreement [100 - (1A-1B)/mean × 100)] greater than 85 was accepted.

Serum Adipokines

Enzyme-linked immunosorbent assay (ELISA or EIA) kits were used to measure fasting serum leptin (22-LEP-E06, Alpco, Salem, New Hampshire) and adiponectin (44-ADPR-0434, Alpco, Salem, New Hampshire) concentrations in samples from lean and *fa/fa* Zucker rats.

Leptin

The ELISA for leptin uses two specific high affinity polyclonal antibodies to quantitatively recognize mouse leptin (81). However, a high degree of cross reactivity against rat leptin allows the kit to be used for measuring rat leptin as well. Standards were prepared with recombinant mouse leptin.

The protocol enclosed with the kit was followed and is briefly described (81). One hundred μL of standards, diluted controls, and diluted serum samples (20× for lean and 250× for *fa/fa* rats) were pipetted into duplicate wells of a Costar EIA/RIA 96-well polystyrene plate (Fisher Scientific, Whitby, Ontario) and incubated at room temperature (20-25°C) for one hour on a shaker at a speed of 350 rpm. After incubation, the wells were aspirated and washed 3× with washing buffer. One hundred μL of antibody conjugate solution was added into each well and the plate was incubated for another hour on a shaker at a speed of 350 rpm.

After incubation, the wells were washed 3× and 100 µL of enzyme conjugate was added into each well, followed by a third incubation for 30 minutes on a shaker at 350 rpm. The 3× wash was again repeated before 100 µL of tetramethylbenzidine (TMB)-substrate solution was added to each well and the plate was incubated for 30 minutes in the dark. Finally, the reaction was stopped by adding 100 µL of stopping solution into each well. Colour developed in proportion to the quantity of bound leptin and intensity was measured. Absorbance was read within 15 minutes at 450 nm on a microplate reader (SpectraMax 340, Molecular Devices Corporation, Sunnyvale, California) using SOFTmax Pro software (version 1.2.0, Molecular Devices Corporation, Sunnyvale, California).

Adiponectin

For the adiponectin ELISA, a polyclonal antibody specific for rat adiponectin is pre-coated onto the 96-well microplate (82). Any present adiponectin is bound by immobilized antibody. Bound adiponectin is captured by anti-rat adiponectin monoclonal antibody and horseradish peroxidase (HRP)-conjugated mouse immunoglobulin G (IgG) is added. After washing, a substrate solution is added and colour development occurs in proportion to the quantity of bound adiponectin.

The protocol enclosed with the kit was followed and is briefly described (82). One hundred µL of standards, quality controls, and diluted serum samples (1000× for both lean and *fa/fa* rats) were pipetted into duplicate wells of an antibody coated 96-well plate (provided with kit) and incubated for one hour at 37°C. The wells were aspirated and washed 3× with wash solution after incubation. One hundred µL of secondary

antibody was added to each well and the plate was incubated for another hour at 37°C. After incubation, the washes were repeated 3× and 100 μL of detector solution was added, followed by a third hour-long incubation at 37°C. The wells were then aspirated and washed 5× with wash solution. One hundred μL of substrate solution was added to each well and the plate was incubated at room temperature and protected from light. After 20 minutes, the reaction was stopped by adding 100 μL stop solution to each well, and the intensity of the colour was read at 450 nm on a microplate reader (SpectraMax 340, Molecular Devices Corporation, Sunnyvale, California) using SOFTmax Pro software (version 1.2.0, Molecular Devices Corporation, Sunnyvale, California).

For both the leptin and adiponectin assays, standards, controls, and samples were analyzed in duplicate. Absorbance of the blank was subtracted from the mean result of each standard, control, and sample. The concentration of unknowns was determined from the standard curve and the mean result for each rat was multiplied by the dilution factor to obtain the final serum leptin or adiponectin concentration in ng/mL or μg/mL, respectively. An acceptable standard curve for both assays had a correlation coefficient of 0.9 or greater and samples had coefficients of variation less than 10%.

Serum values for leptin and adiponectin were also adjusted for blood volume [58 mL blood volume/kg rat (83)] to take into account the difference in body size between lean and *fa/fa* rats. Adjusted values are referred to as 'total' leptin or adiponectin in this thesis. Ratios of total leptin and adiponectin to body weight, peri-renal, epididymal, and visceral adipose tissue, and correlations with the stated parameters were also completed.

Adipokines in Adipose Tissue

Adipokine protein levels (leptin, adiponectin, IL-10, IL-6, TNF-α, IL-18, and HMGB1) in peri-renal adipose tissue were measured by Western blot analysis. In this procedure cellular proteins are separated on a polyacrylamide gel and transferred to a membrane filter that is subsequently incubated with protein-specific antibodies (84). The protein-bound antibody is detected with a secondary antibody. Luminescence generated by means of an enzymatic reaction is detected by using X-ray film.

Protein Extraction and Quantification

Two hundred mg of frozen peri-renal adipose tissue was ground with a mortar and pestle in liquid nitrogen and suspended in 1600 μL (8 μL/mg) of 3× sodium dodecyl sulfate (SDS) sample buffer [37.5 mL 0.5 M Tris HCl (pH 6.8), 15 mL 20% SDS, 30 mL glycerol, 17.5 mL deionized water]. The solution was clarified by centrifugation for 5 minutes at 13,000 rpm. The supernatant was removed and clarified again by centrifugation for another 5 minutes at 13 000 rpm. After the second centrifugation, the supernatant was removed and sonicated (VibraCell 50, Sonics and Materials, Danbury, Connecticut). Protein content was measured using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, Illinois).

Western Blotting

Cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 15% gel and transferred to polyvinylidene difluoride (PVDF) membrane as previously described (85). Briefly, aliquots containing equal (10 μg) amounts of extracted protein were mixed with bromophenol blue and 2-mercaptoethanol [1% (v/v) final concentration], boiled for 5 minutes to denature the proteins, and loaded onto a 15% separating (7.5% stacking) polyacrylamide gel to separate out smaller weight proteins. A molecular weight ladder was also loaded onto each gel to assist with band (protein) identification. To account for possible variation in protein loading between individual samples, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. GAPDH was used as a loading control because it is part of glycolysis and all cells need glucose. In addition, it has been previously established in our lab to be constant in adipose tissue. An internal control sample was loaded on each gel to adjust for differences in intensity among gels when multiple gels were used. After electrophoresis, the proteins were transferred to a PVDF membrane and the membranes were subsequently blocked in 3% (w/v) bovine serum albumin (BSA) in Tris buffer saline Tween-20 (TBST) solution on a shaker (DS-500 Orbital Shaker, VWR, Henry Troemner LLC, Thorofare, New Jersey) at a speed of 70 rpm for 30 minutes at room temperature (20-25°C).

Primary antibodies (Table 2) were incubated with the membrane on a shaker at a speed of 70 rpm for one hour at room temperature (20-25°C). After incubation with the primary antibody, membranes were washed 4× with 1× TBST and then incubated for an additional 45-60 minutes at room temperature (20-25°C) in blocking solution (1% BSA

in TBST solution) with secondary antibody (Table 2) diluted 1:10 000 (except for TNF-α which required a 1:15 000 dilution) with shaking at 70 rpm at room temperature (20-25°). HRP-conjugated secondary antibody was detected using the enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, United Kingdom) after the membranes had been washed 4×.

Band intensity was captured on X-ray film (CL-X Posure Film, Thermo Scientific, Rockford, Illinois) and quantified by scanning densitometry (trace analysis) with a GS800 imaging densitometer (Bio-Rad Laboratories, Hercules, California) using Quantity One software (version 4.5.0, Bio-Rad Laboratories, Hercules, California). A comparison of trace analysis and adjusted volume values was done to assess which method of measuring band density was most accurate. After several trials, trace analysis was determined to be most reflective of the band density observed. Data for each protein were expressed as an arbitrary unit by calculating the ratio of the trace analysis value of the protein of interest to the trace analysis value of GAPDH on the same blot. When multiple gels or mini-gels were used, the calculation included adjustment with the internal control sample on each gel (trace analysis of protein of interest/trace analysis of internal control/trace analysis of GAPDH).

Table 2: List of antibodies.

Name	Secondary	Dilution	Application	Size (kDa)	Catalogue #	Company
Leptin Ob (A-20)	Rabbit	1:1000	WB	16	sc-842	Santa Cruz
Adiponectin (ACRP-30)	Rabbit	1:1000	WB	30	107920	Calbiochem
IL-6	Rabbit	1:1500	WB	25	ARC00+3	Biosource International
IL-10	Rabbit	1:1500	WB	17	ARC0102	Biosource International
IL-18	Goat	1:1500	WB	24/18*	sc-6179	Santa Cruz
TNF-α	Rat	1:500	WB	25/17*	3707	Cell Signalling
HMGB1	Rat	1:1000	WB	26	ab18256	Abcam
GAPDH	Mouse	1:5000	WB	36	ab1484	Abcam

*Different molecular weights (kDa) refer to precursor/mature forms of protein

Adipocyte Size

Adipocyte size was measured using frozen sections of epididymal adipose tissue to determine size and distribution of small and large adipocytes between genotypes and diet interventions. Frozen epididymal adipose tissue (in OCT compound) was extracted and remolded into CRYO-GEL embedding medium, which facilitated sectioning thin pieces of fragile adipose tissue. Ten microns of adipose tissue (n=5 per treatment group) were sectioned using a cryotome (working temperature at -30°C, cryobar temperature at -47°C; Thermo Shandon Limited, Astmoor, Runcorn, Chesire, United Kingdom) and placed on microscope slides. Using a light microscope fitted with a camera (Olympus BH-2, Carsen Group Inc., Markham, Ontario) images were captured using 200× magnification. Computer images of each section were obtained with QCapture Pro software (version 5.1, QImaging Corporation 2003, USA). Cell area (μm^2) of 125 adipocytes per treatment group, ~25 adipocytes per rat, was measured with the open-source image analysis program ImageJ (version 1.40f, Rasband, W, ImageJ, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). The area of a circle imposed on an image of a 0.01 mm micrometer in ImageJ was used to calculate a conversion factor with which to convert arbitrary units to squared micrometers as the unit of adipocyte size.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine significant main effects and Duncan's multiple range test was used for means testing (SAS 6.04, SAS Institute, Cary, NC). Assumptions for normality and homogeneity were met and if these conditions were not met, then data was log transformed. Repeated measures ANOVA was used for weekly body weight, feed intake, energy consumption, feed intake/body weight, and feed efficiency ratio. A p-value < 0.05 was accepted as statistically significant. Correlations were calculated using Pearson's correlation coefficient. Chi-square testing was used to measure differences within cell size ranges for adipocyte size data.

RESULTS

Fatty Acid Profile of Experimental Diets

Extracted fat from experimental diets was analyzed to determine the fatty acid profile by gas chromatography. The fatty acid profile of the diets is shown in Table 3. The CTL and ALA diet contained 8.0% and 33.7% omega-3 fatty acid per total fatty acids, respectively. The ratio of omega-6:omega-3 in the CTL and ALA diets was 6.4 and 1.0, respectively. Current research suggests that the typical Western diet provides a ~15:1 ratio of omega-6:omega-3 fatty acid, and recommends an optimal ratio intake of 4:1 or less (86).

Table 3: Fatty acid profile of experimental diets

Fatty Acid % Composition*	ALA diet	CTL diet
Saturated Fat	12.2	15.8
C16:0	7.5	10.5
C18:0	3.9	4.2
Monounsaturated fat	19.1	22.5
C18:1	18.8	22.1
Polyunsaturated fat	67.3	59.9
C18:2 (n6)	33.4	51.6
C18:3 (n3)	33.7	8.0
Polyunsaturated:saturated fat	5.5	3.8
Omega-3:omega-6 fatty acid	1.0	6.4

*% Composition = g/100 g total fatty acids
Analyzed as described in section "*Fatty Acid Analysis*", page 44; only fatty acids present in amounts >1% are included.

Macronutrient Distribution of Experimental Diets

The macronutrient distribution of the experimental diets for rodents was calculated and compared to the AMDR for female and male adult humans (Table 4). The experimental diets provided carbohydrate, protein, and fat within the recommended ranges at 62.6%, 17.7%, and 19.8% of total energy, respectively. Both the CTL and ALA diets contained an omega-6 fatty acid content within the AMDR (10.2% and 6.60% of total energy, respectively), but had higher amounts of omega-3 fatty acid (1.59% and 6.65% of total energy, respectively).

Table 4: Macronutrient distribution of experimental diets

	Experimental Diets (% of total energy)	AMDR* (% of total energy)
Carbohydrate	62.6	45-65
Protein	17.7	10-35
Fat	19.8	20-35
Omega-6	10.2 (CTL), 6.6 (ALA)	5-10
Omega-3	1.6 (CTL), 6.7 (ALA)	0.6-1.2

* Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients) (56).

Feed Intake and Body Weight

Throughout the 8-week study period, weekly body weight and weekly feed intake were consistently greater in the faCTL and faALA animals compared to the lean rats (Figure 2a and 3a, respectively). The *fa/fa* rats consumed approximately 200 kilocalories (1.5-fold) more per week compared to the lnCTL group (Figure 3a) and were about 300 g (1.7-fold) heavier at the end of the study period.

Weekly gains in body weight were evident for all groups and tapered off by the last week of the study period (Figure 2b). Weekly body weight gain was less in lean rats compared to obese (significant at week 2, 3, 5, and 7). In the final week of the study there was also negative body weight change in some of the lean and obese rats (~2-3% and ~1% weight loss, respectively), reflected in Figure 2b and 4b.

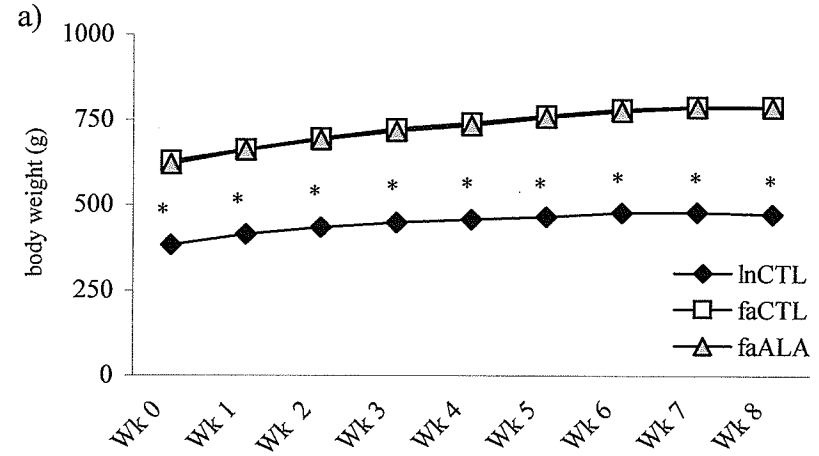
Weekly feed intake was relatively constant throughout the study period (Figure 3a). Body weight consistently increased, indicating that the diet was well tolerated by the animals, and appears to plateau by the end of the study period (Figure 2a). As an overall trend, rats consumed more feed per body weight at the beginning of the study and this decreased over time, as may be expected once growth plateaus with advanced age, and there were no significant differences among groups at week 8 (Figure 4a).

Interestingly, lean rats had a higher feed intake to body weight ratio compared to obese rats throughout the study, highlighting that despite lower body weight, lean rats had a greater appetite (Figure 4a). However, when energy intake was considered relative to body weight gain, there were no differences in feed efficiency ratio [FER; (weekly feed intake (g)/weekly weight gain (g)] between obese and lean rats (Figure 4b). An exception was noted at week 3 of the study period, where FER was higher in lean rats

compared to obese rats, which reflects less weight gain relative to feed intake in the lean rats compared to the obese.

There were no differences in weekly feed intake, body weight, or feed efficiency between the faCTL and the faALA groups.

Figure 2: Body weight. a) Weekly body weight and b) weekly body weight change are presented as means; n=7 for all groups. For a) the SEM was ± 17, 11 and 16 at week 0; ± 12, 11 and 15 at week 1; ± 12, 13 and 17 at week 2; ± 13, 12 and 18 at week 3; ± 15, 12 and 22 at week 4; ± 14, 12 and 21 at week 5; ± 15, 13 and 22 at week 6; ± 15, 13 and 23 at week 7; and ± 13, 14 and 23 at week 8 for the lnCTL, faCTL and faALA groups, respectively (not shown in figure). For b) the SEM was ± 6, 4 and 4 at week 1; ± 1, 4 and 4 at week 2; ± 1, 1 and 3 at week 3; ± 2, 3 and 4 at week 4; ± 3, 3 and 3 at week 5; ± 2, 1 and 3 at week 6; ± 3, 3 and 3 at week 7; and ±3, 3 and 0 at week 8 for the lnCTL, faCTL and faALA groups, respectively (not shown in figure). lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Significant main effects of group, time, and time × group; *indicates lnCTL statistically different from faCTL and faALA ($p \leq 0.05$).



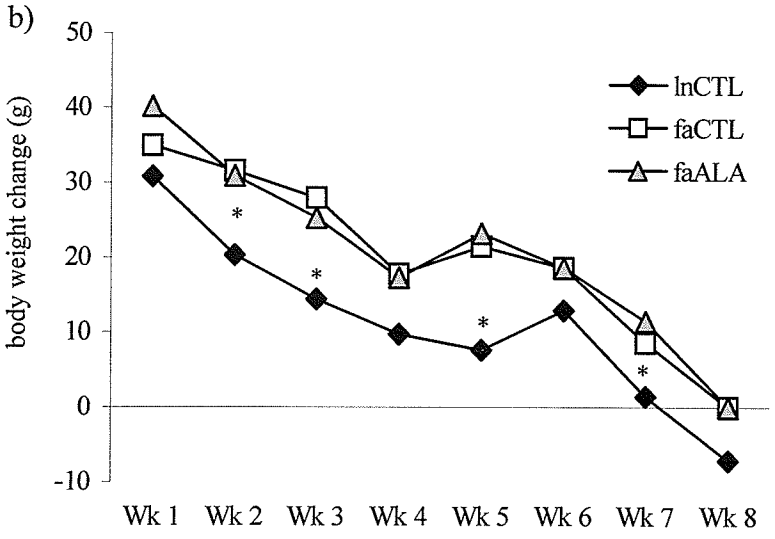


Figure 3: Feed intake. a) Weekly feed intake and b) weekly energy consumption are presented as means; n=7 for all groups. For a) SEM was ± 3, 6 and 7 at week 1; ± 4, 6 and 6 at week 2; ± 4, 5 and 8 at week 3; ± 4, 4 and 8 at week 4; ± 4, 5 and 3 at week 5; ± 5, 5 and 8 at week 6; ± 4, 6 and 4 at week 7; and ± 4, 8 and 8 at week 8 for the lnCTL, faCTL and faALA groups, respectively (not shown in figure). For b) SEM was ± 10, 23 and 28 at week 1; ± 14, 24 and 23 at week 2; ± 14, 20 and 32 at week 3; ± 16, 14 and 32 at week 4; ± 15, 20 and 10 at week 5; ± 18, 19 and 32 at week 6; ± 16, 22 and 14 at week 7; and ± 15, 30 and 32 at week 8 for the lnCTL, faCTL and faALA groups, respectively (not shown in figure). lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Significant main effects of group and time; *indicates lnCTL statistically different from faCTL and faALA ($p \leq 0.05$).

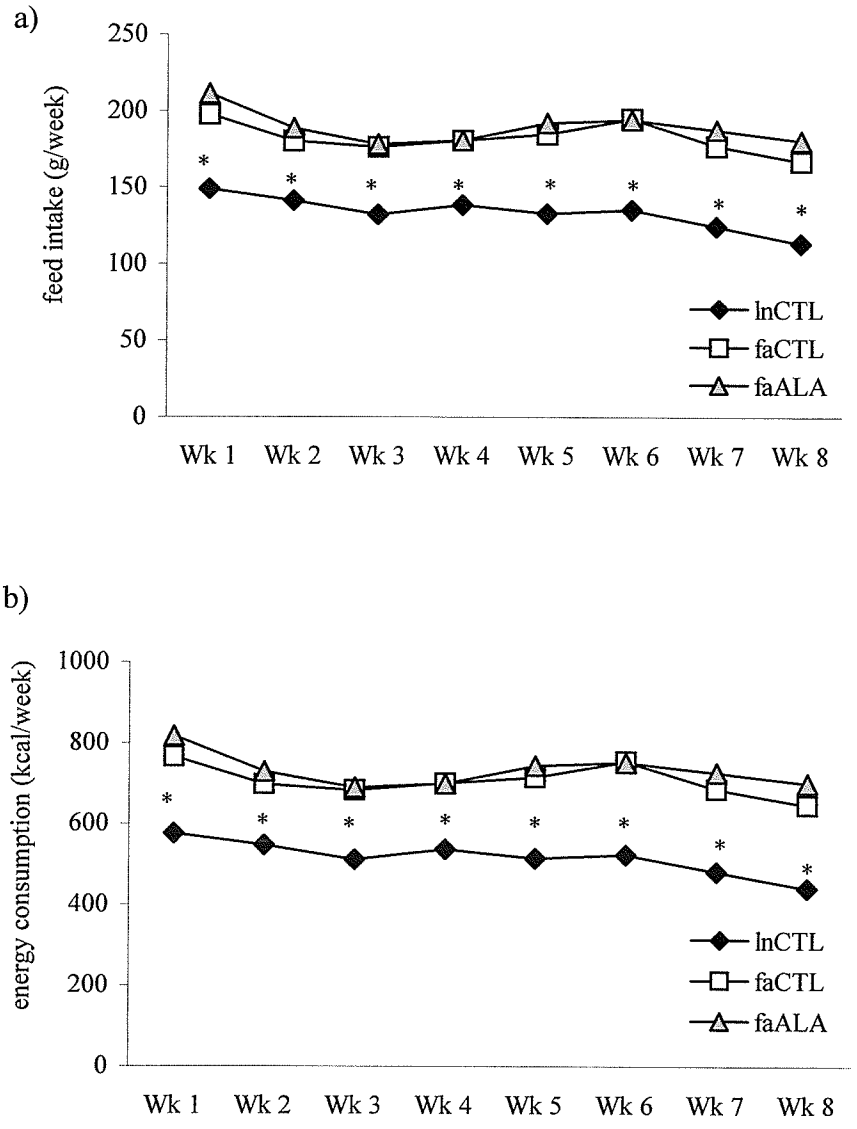
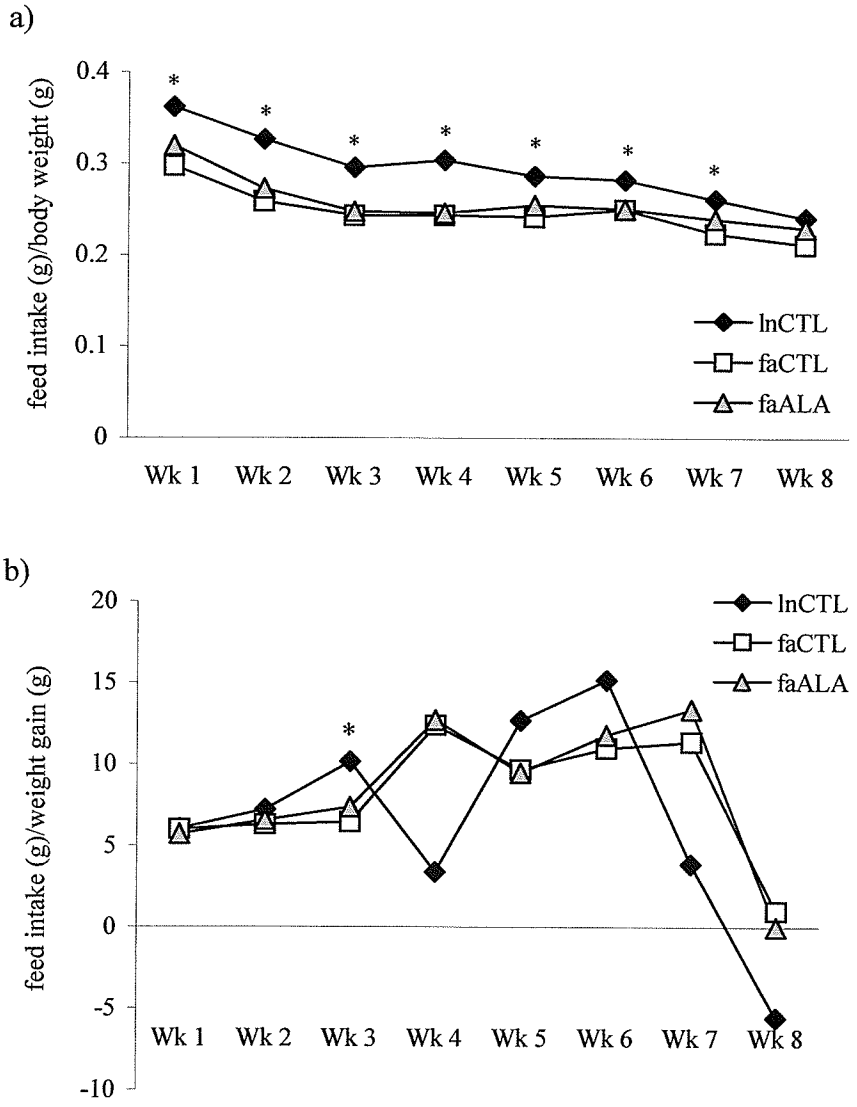


Figure 4: Relative feed intake. a) Weekly feed intake per weekly body weight and b) weekly feed efficiency ratio are presented as means; n=7 for all groups in a) and n=7 for faCTL and faALA and n=6 for lnCTL in b). For a) SEM was approximately ± 0.01 at all timepoints for all groups (not shown in figure). For b) SEM was ± 1, 1 and 1 at week 1; ± 0.5, 1 and 1 at week 2; ± 1, 1 and 1 at week 3; ± 10, 2 and 2 at week 4; ± 3, 2 and 2 at week 5; ± 5, 1 and 2 at week 6; ± 4, 3 and 3 at week 7; and ± 2, 5 and 0 at week 8 for the lnCTL, faCTL and faALA groups, respectively (not shown in figure). lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. a) Significant main effects of group, time, and time × group; *indicates lnCTL statistically different from faCTL and faALA ($p \leq 0.05$). b) Significant main effects of time; *indicates lnCTL statistically different from faCTL and faALA at week 3 ($p \leq 0.05$).



Adipose Tissue Weight

Peri-renal and epididymal adipose tissue from lean and *fa/fa* Zucker rats was dissected and weighed at termination. Visceral fat was calculated as the sum of peri-renal and epididymal white adipose tissue depots. Peri-renal, epididymal, and visceral fat pad masses relative to body weight were greater in *fa/fa* rats compared to the lean group (Figure 5). Interestingly, the amount of epididymal adipose tissue expressed relative to body weight was significantly greater in the faBASE group compared to the faCTL. However, faBASE had less peri-renal and visceral fat compared to faCTL. Dietary ALA-rich flaxseed oil did not have an effect on relative fat pad weight.

Fat distribution also differed between the genotypes, in that the proportion of peri-renal to epididymal adipose tissue was greater in the *fa/fa* rats than in lean rats (Figure 6). Obese rats fed ALA-rich flaxseed oil had a lower ratio of peri-renal to epididymal adipose tissue (2.5:1 compared to 3:1 in the faCTL group), reflecting a change in adipose tissue distribution with this dietary intervention. The shift in this ratio appears to be due to an increased amount of epididymal adipose tissue in the faALA group.

Figure 5: Adipose tissue weight. a) Peri-renal adipose tissue, b) epididymal adipose tissue and c) visceral adipose tissue are presented as mean ± SEM; n=7 for all groups. Visceral adipose tissue = peri-renal + epididymal adipose tissue. lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet, faBASE = *fa/fa* rats at baseline. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.

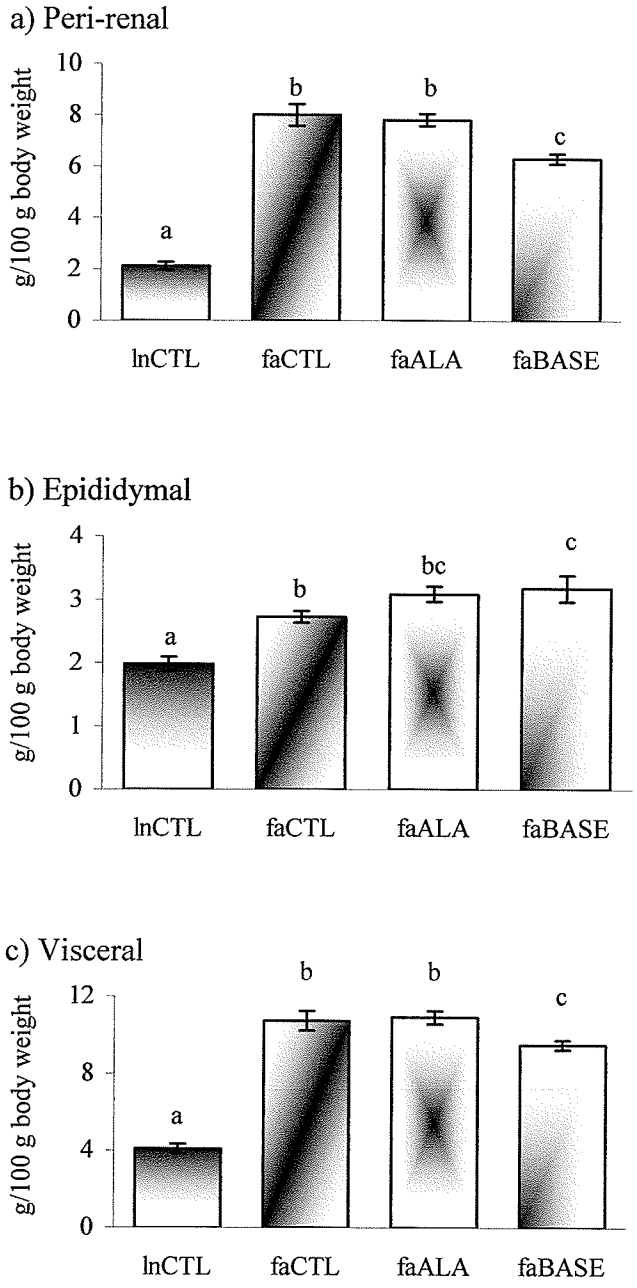
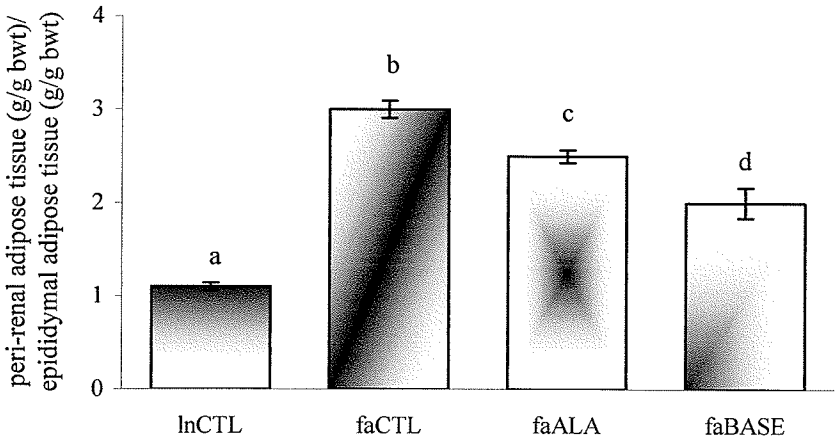


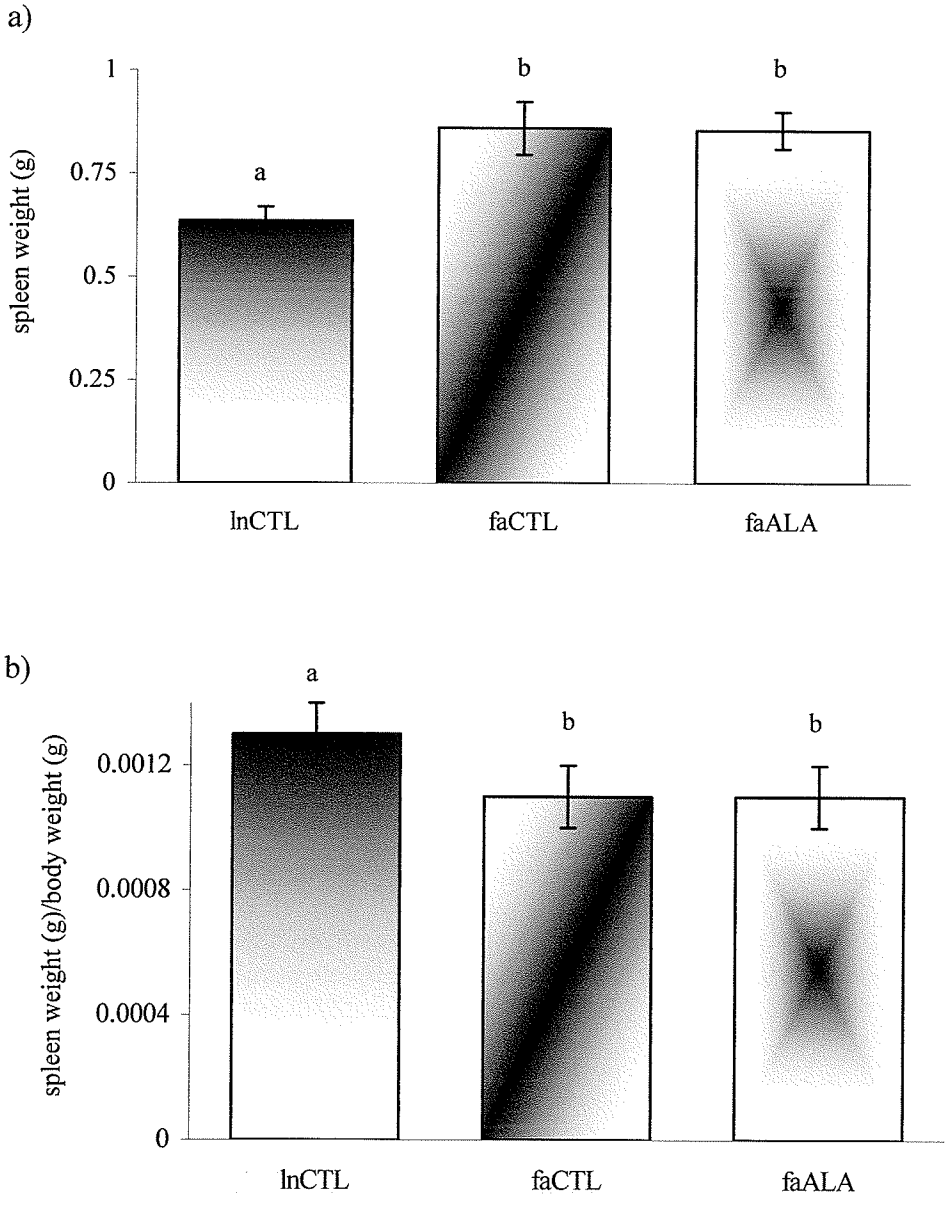
Figure 6: Visceral fat distribution. Visceral adipose tissue presented as the ratio of peri-renal to epididymal adipose tissue. Data are presented as ratios for means ± SEM; n=7 for all groups. lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet, faBASE = *fa/fa* rats at baseline. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.



Spleen Weight

The absolute and relative spleen weights from lean and *fa/fa* Zucker rats are shown in Figure 7. The absolute spleen weight from the faCTL and faALA was significantly greater than the lnCTL, and there was no difference between the faCTL and faALA groups. However, the relative spleen weight (ratio of spleen weight to body weight) reveals the opposite effect. Obese rats had a lower relative spleen weight compared to the lnCTL group.

Figure 7: Spleen weight. a) Absolute spleen weight and b) relative spleen weight are presented as mean ± SEM; n=7 for all groups. lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.



Splenocyte Cytokine Production

Splenocytes (1.25×10^6 cells/mL) from lean and *fa/fa* Zucker rats were stimulated with 2.5 mg/L concanavalin A (ConA). After a 48 h incubation, proinflammatory TNF-α, IFN-γ, IL-2, IL-6, and anti-inflammatory IL-4 and IL-10 cytokines in the culture media from both unstimulated (UNS) and ConA stimulated (STIM) splenocytes were analyzed simultaneously using a multiplex assay and Luminex Instrument. An array of proinflammatory and anti-inflammatory cytokines was selected to examine the Th1/Th2 balance.

Levels of IL-4 were undetectable (data not shown). A comparison of the cytokine production from UNS and STIM splenocytes (Figure 8) and cytokine production by STIM splenocytes alone (Figure 9) is shown. Stimulation with ConA (STIM) increased splenocyte production of both proinflammatory and anti-inflammatory cytokines in all groups. There were no significant differences among the groups in proinflammatory TNF-α, IFN-γ, IL-2, and IL-6 production by UNS or STIM splenocytes. However, anti-inflammatory IL-10 secretion from UNS and STIM splenocytes was 2.1-fold and 1.6-fold lower, respectively, in the faALA group compared to the lnCTL but not the faCTL group. This suggests that dietary ALA may influence IL-10 production in the *fa/fa* rats by reducing the production of this anti-inflammatory cytokine in the obese state. These findings indicate that dietary ALA-rich flaxseed oil intervention did not have a significant effect on proinflammatory or anti-inflammatory cytokine production from UNS or STIM splenocytes in an obese state (faALA vs. faCTL) except for IL-10.

Figure 8: Cytokine production by splenocytes. Unstimulated (UNS) and ConA-stimulated (STIM) splenocyte production of cytokines a) TNF-α, b) IFN-γ, c) IL-2, d) IL-6, and e) IL-10 were measured using a multiplex assay and Luminex Instrument. Samples were assayed in duplicate. Data are presented as the means; n=6 in a-d) and n=5 in e). UNS are presented by white bars and STIM are presented by black bars. lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) for UNS are indicated by different lowercase letters. Statistical analyses of STIM are presented in Figure 9.

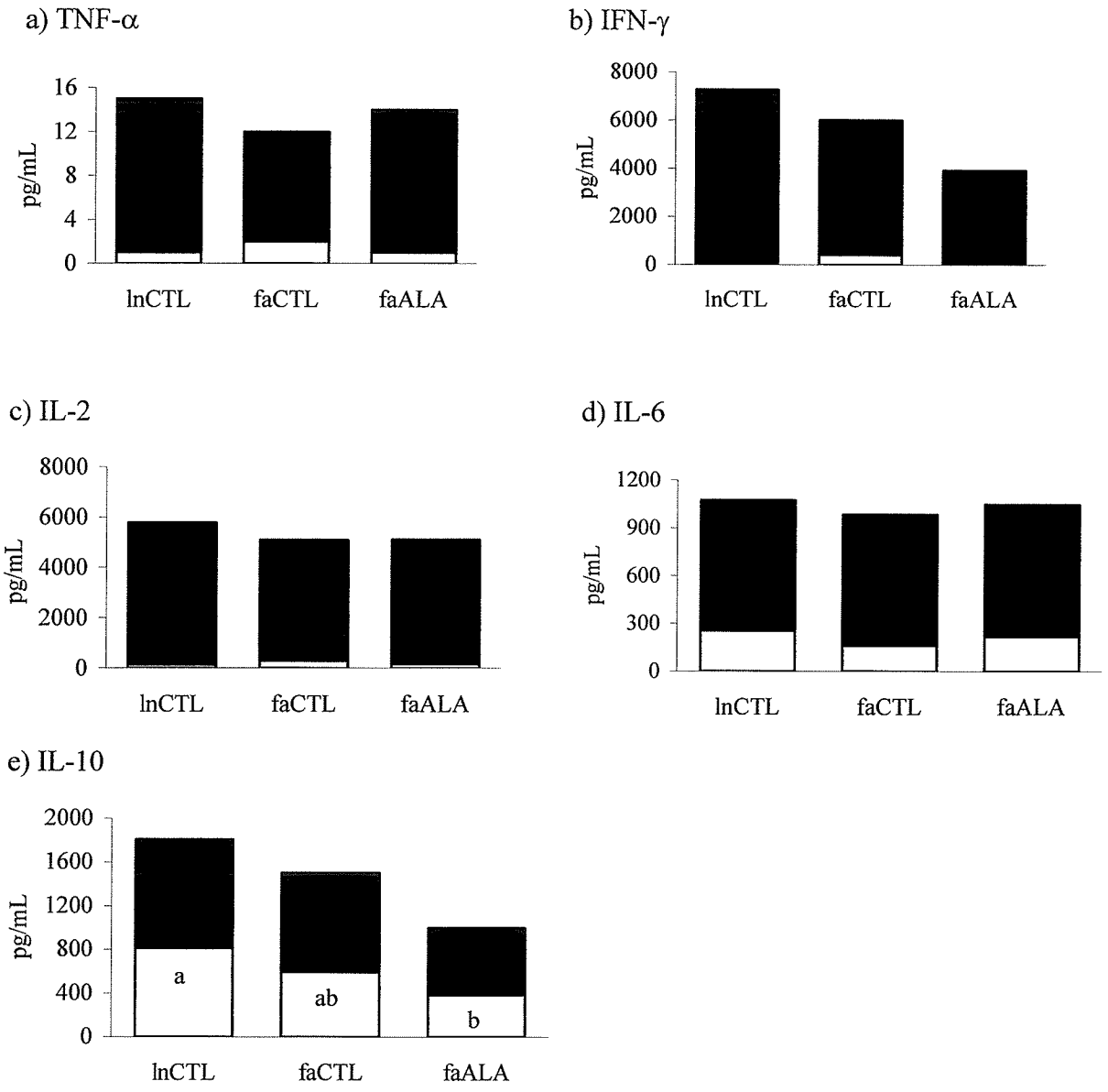
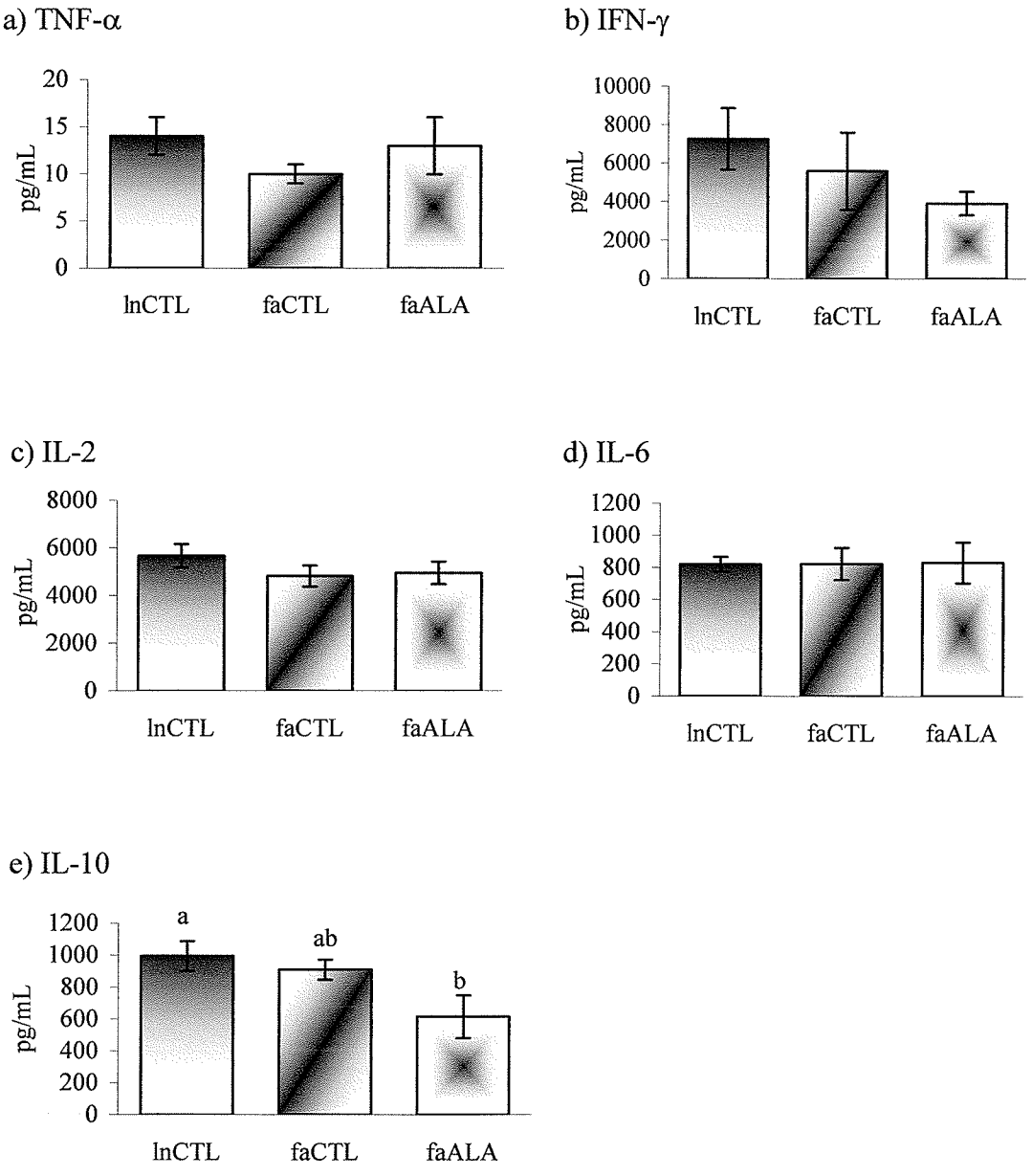


Figure 9: Cytokine production by ConA-stimulated splenocytes. ConA-stimulated splenocyte production of cytokines a) TNF-α, b) IFN-γ, c) IL-2, d) IL-6, and e) IL-10 was measured using a multiplex assay and Luminex Instrument. Samples were assayed in duplicate. Data are presented as the mean ± SEM; n=6 in a-d) and n=5 in e). lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.



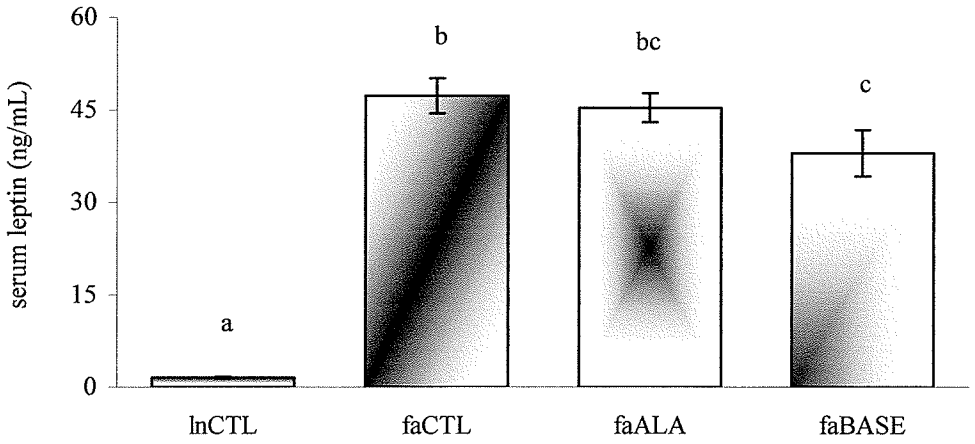
Serum Adipokines

Leptin

Fasting serum leptin concentrations from lean and *fa/fa* Zucker rats were measured using a commercial ELISA kit. As expected, serum leptin concentrations were 31-fold higher in the faCTL group compared to the lnCTL groups (Figure 10). Serum leptin concentrations were not different in the faALA group compared to the faCTL group, indicating no effect of dietary ALA-rich flaxseed oil on serum leptin.

As described in the *Materials and Methods* section (page 51), serum values for leptin were adjusted for blood volume to consider the difference in body size between lean and *fa/fa* rats ('total' leptin). Total leptin was positively correlated with body weight, peri-renal, epididymal, and visceral adipose tissue ($r = 0.97$, 0.95, 0.92, and 0.95, respectively). Total leptin expressed relative to body weight, peri-renal, epididymal, and visceral adipose tissue (Figure 21 in the *Appendix*, page 135) did not change initial findings illustrated in Figure 10.

Figure 10: Serum leptin. Fasting serum leptin concentration from lean and *fa/fa* Zucker rats was measured using a commercial ELISA kit. Samples were assayed in duplicate. Data are presented as mean ± SEM; n=6 for all groups. lnCTL = lean rats fed control diet, faCTL = fa/fa rats fed control diet, faALA = fa/fa rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.

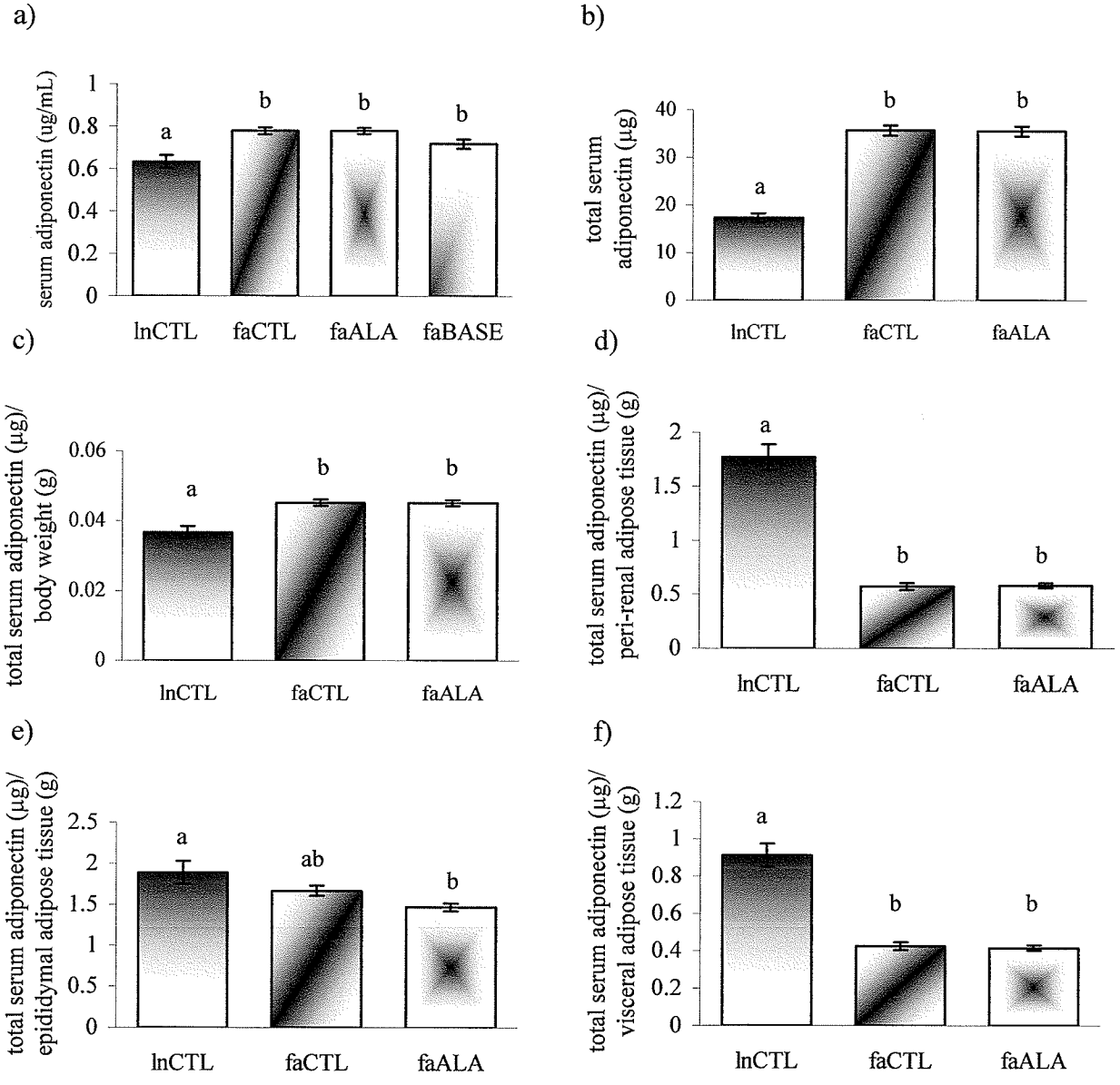


Adiponectin

Fasting serum adiponectin concentrations from lean and *fa/fa* Zucker rats were measured using a commercial ELISA kit. Interestingly, serum adiponectin was 1.2-fold higher in the faCTL group compared to their lean counterparts (Figure 11a). There was no effect of aging or dietary ALA-rich flaxseed oil on adiponectin concentrations in the obese rats.

As described in the *Materials and Methods* section (page 51), serum values for adiponectin were adjusted for blood volume to consider the difference in body size between lean and *fa/fa* rats ('total' serum adiponectin). Total serum adiponectin was positively correlated with body weight, peri-renal, epididymal, and visceral adipose tissue ($r = 0.98$, 0.96, 0.95, and 0.97, respectively) and was higher in *fa/fa* rats compared to lean (Figure 11b). However, when total adiponectin was expressed relative to peri-renal or visceral adipose tissue (Figure 11d and f, respectively), obese rats had significantly lower amounts of adiponectin compared to the lean group. A similar pattern is observed when total adiponectin is expressed relative to epididymal adipose tissue (Figure 11e) but not body weight (Figure 11c).

Figure 11: Serum adiponectin. a) Fasting serum adiponectin concentration from lean and *fa/fa* Zucker rats was measured using a commercial ELISA kit. Samples were assayed in duplicate. b) Fasting serum adiponectin values were adjusted for blood volume (58 mL blood volume/g body weight) to calculate 'total' serum adiponectin and total serum adiponectin was expressed relative to c) body weight, d) peri-renal, e) epididymal, and f) visceral adipose tissue. Data are presented as mean ± SEM; n=7 for all groups. lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.

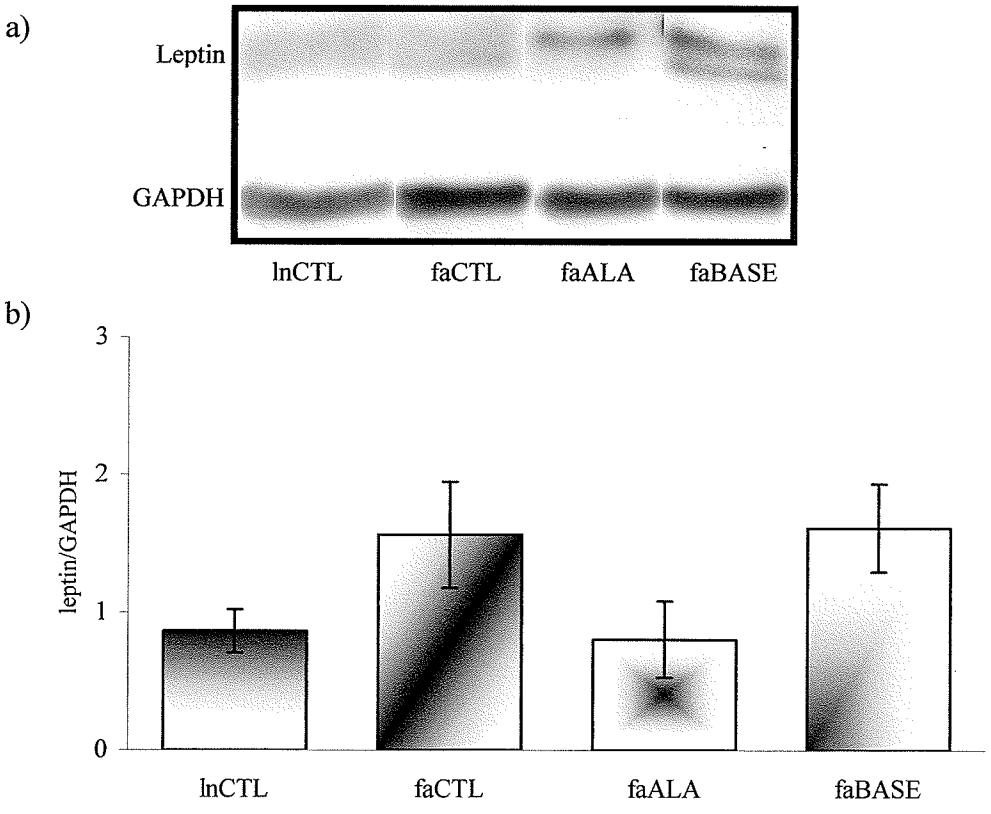


Adipose Tissue Adipokines

Leptin

Leptin protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats. Surprisingly, there were no differences between groups in leptin levels (Figure 12). Our results did not show an aging, genotype, or diet influence on adipose leptin levels.

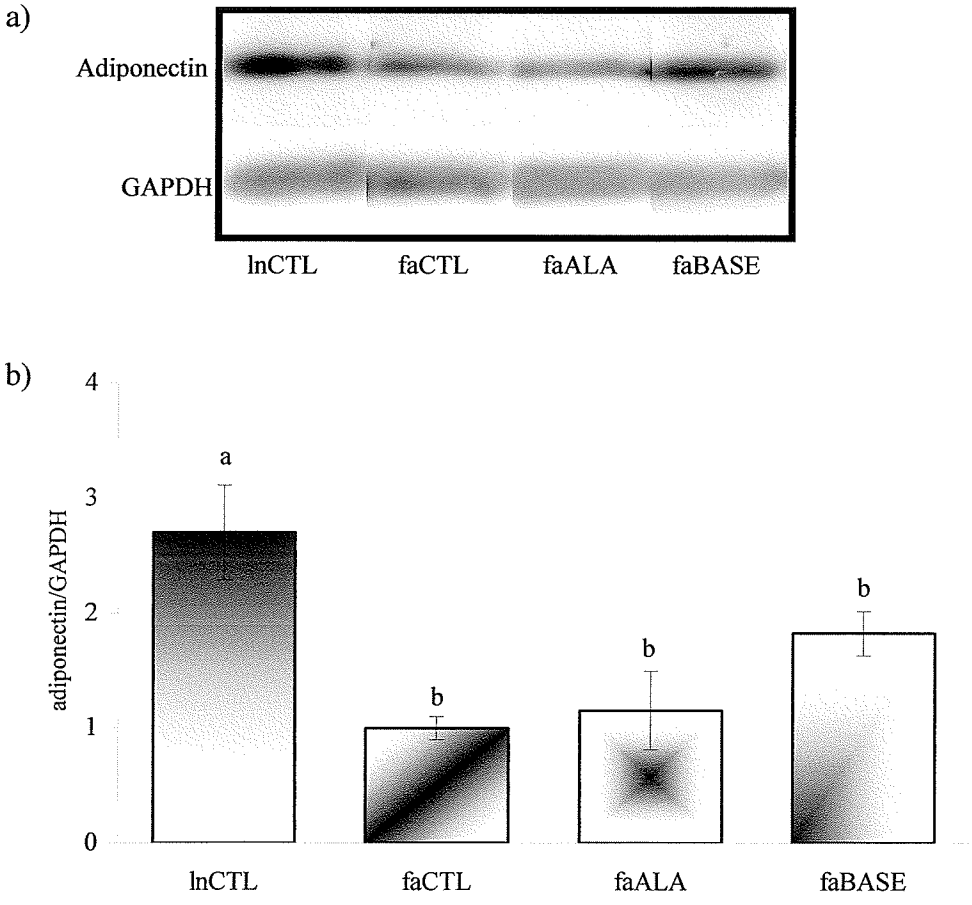
Figure 12: Leptin protein levels in peri-renal adipose tissue. Leptin protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats by Western blot analysis. a) Representative Western blot and b) corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data were derived by calculating the ratio of leptin to GAPDH and are expressed as mean ± SEM; n=7 for lnCTL, faCTL and faBASE and n=6 for faALA. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. No statistical differences were discussed.



Adiponectin

Adiponectin protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats. Adiponectin levels were 2.7-fold lower in the faCTL group compared to the lnCTL group (Figure 13). There was no effect of dietary ALA-rich flaxseed oil on adiponectin adipose tissue levels in obese rats.

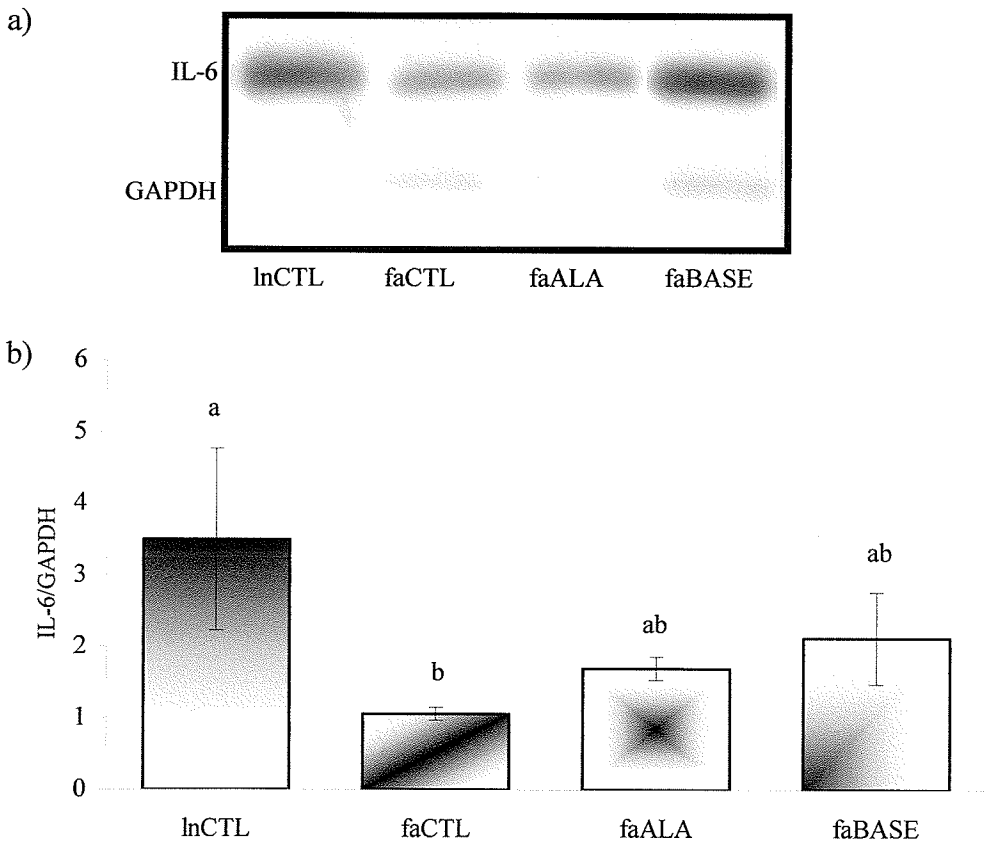
Figure 13: Adiponectin protein levels in peri-renal adipose tissue. Adiponectin protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats by Western blot analysis. a) Representative Western blot and b) corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data were derived by calculating the ratio of adiponectin to GAPDH and are expressed as mean ± SEM; n=7 for lnCTL, faCTL and faALA and n=6 for faBASE. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.



Interleukin-6

IL-6 protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats. Interestingly, levels of proinflammatory IL-6 were 3.3-fold lower in the faCTL group compared to the lean rats (Figure 14). Dietary ALA-rich flaxseed oil seemed to have a small effect in *fa/fa* rats by slightly elevating IL-6 levels, but these results were not significant.

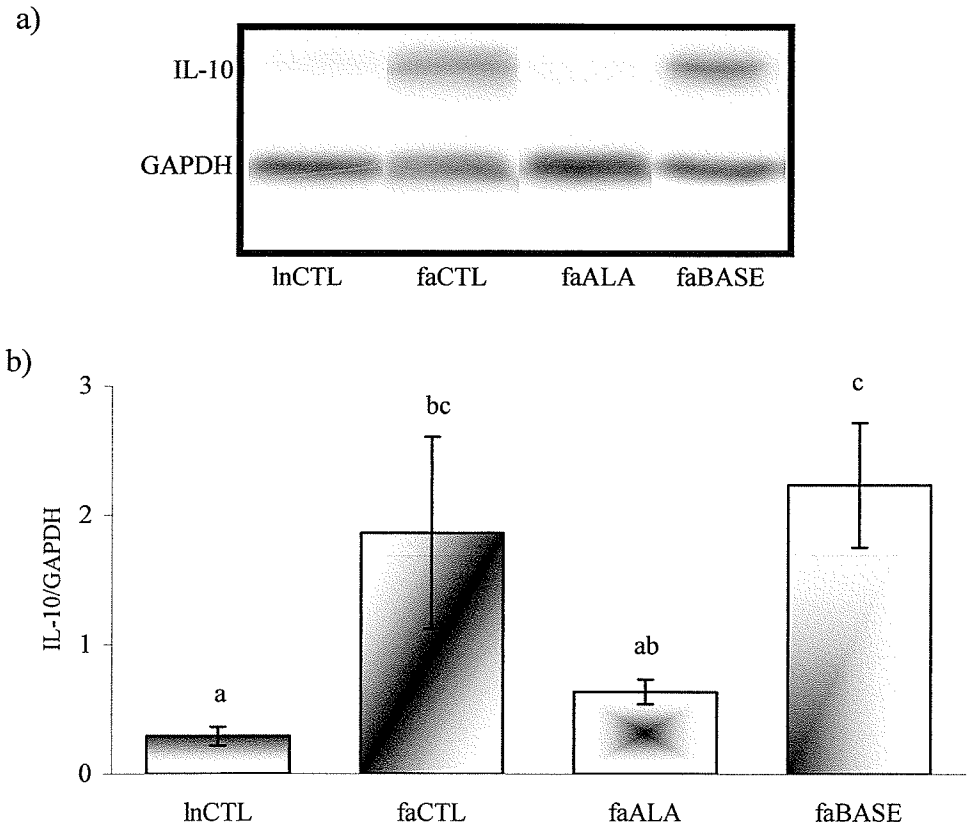
Figure 14: Interleukin-6 protein levels in peri-renal adipose tissue. Interleukin-6 (IL-6) protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats by Western blot analysis. a) Representative Western blot and b) corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data were derived by calculating the ratio of IL-6 to GAPDH and are expressed as mean ± SEM,; n=7 for lnCTL, faCTL and faALA and n=6 for faBASE. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.



Interleukin-10

IL-10 protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats. Unexpectedly, anti-inflammatory IL-10 levels were 6.4-fold higher in *fa/fa* rats compared to their lean counterparts (Figure 15). Although intervention with ALA-rich flaxseed oil reduced IL-10 by 34%, these values were not statistically different from the faCTL group. Furthermore, faALA was not different from lnCTL.

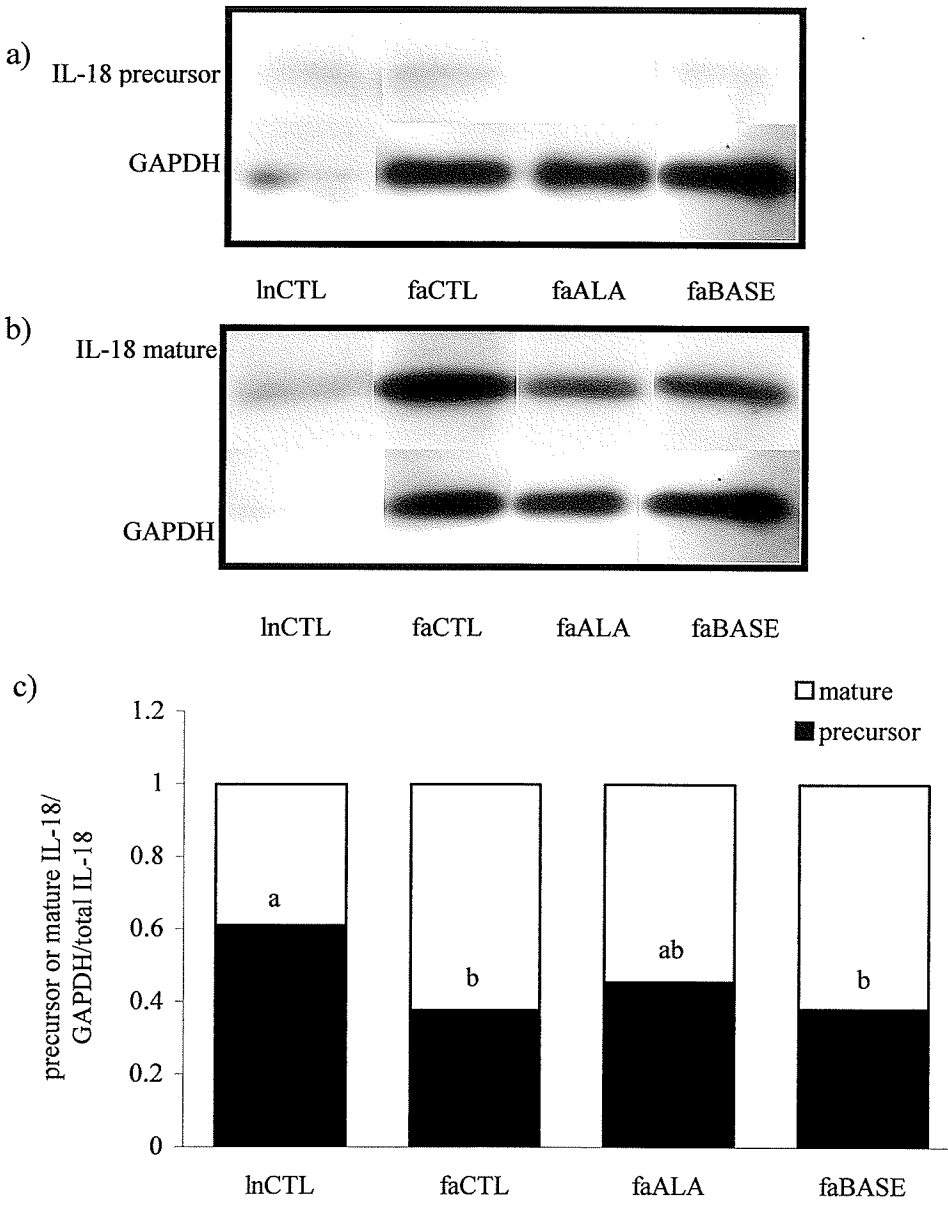
Figure 15: Interleukin-10 protein levels in peri-renal adipose tissue. Interleukin-10 (IL-10) protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats by Western blot analysis. a) Representative Western blot and b) corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data were derived by calculating the ratio of IL-10 to GAPDH and are expressed as mean ± SEM; n=7 for lnCTL and faCTL and n=6 for faALA and faBASE. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.



Interleukin-18

Precursor and mature (active) IL-18 protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats. FaCTL and faBASE had lower proportions of precursor IL-18 and higher levels of mature IL-18 compared to the lean group (Figure 16). FaALA had intermediate proportions of precursor and mature protein levels of IL-18 that were not different from faCTL or lnCTL. However, there was a trend for lower levels of mature IL-18 protein in obese rats fed ALA-rich flaxseed oil compared to the faCTL group (0.547 ± 0.122 versus 1.301 ± 0.412, respectively) (Figure 22 in the *Appendix,* page 136).

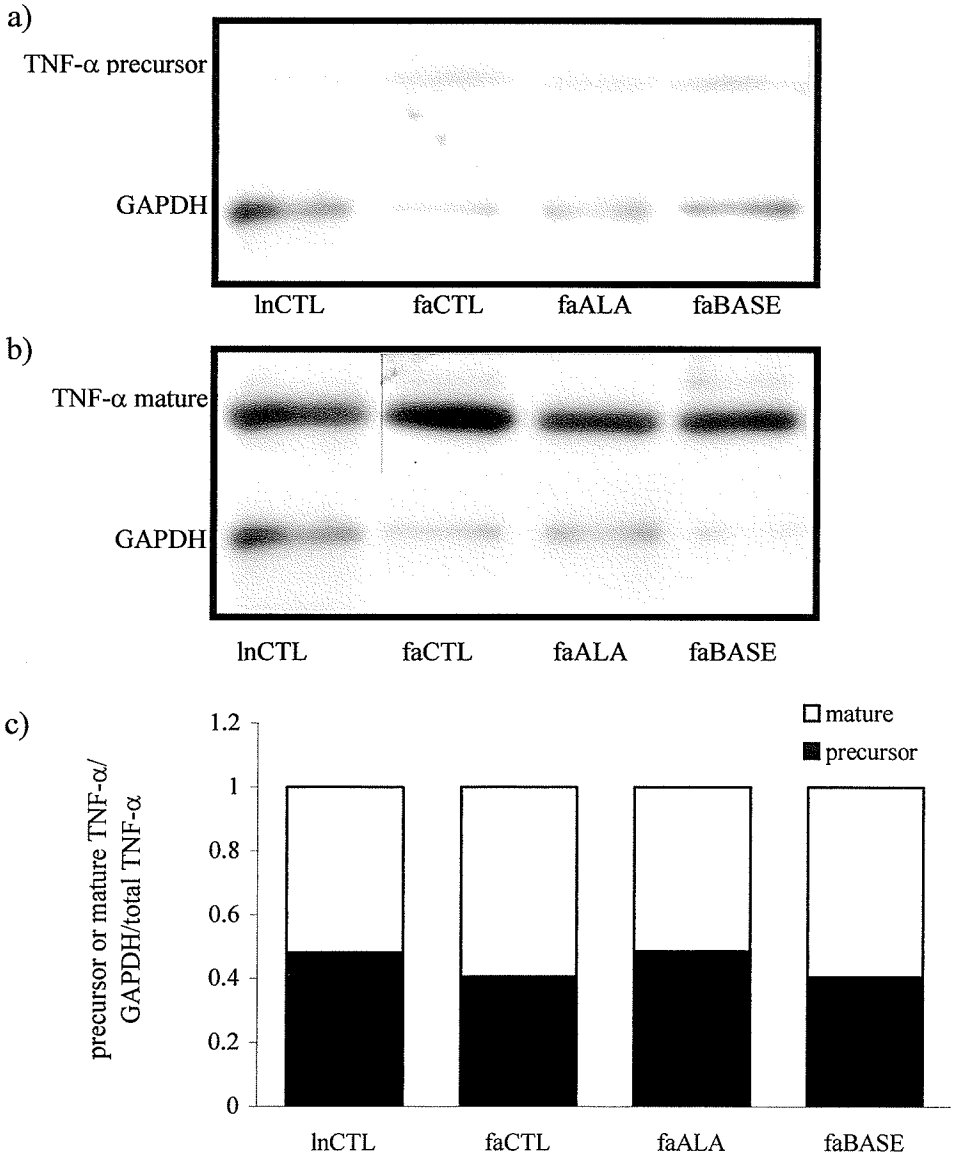
Figure 16: Interleukin-18 precursor and mature protein levels in peri-renal adipose tissue. Interleukin-18 (IL-18) protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats by Western blot analysis. a) and b) are representative Western blots of precursor and mature IL-18, respectively, and c) is the corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data were derived by calculating the ratio of precursor or mature IL-18/total IL-18 (precursor or mature IL-18 was divided by GAPDH to correct for loading differences, precursor IL-18/GAPDH and mature IL-18/GAPDH were combined to obtain total IL-18, and then precursor IL-18/GAPDH and mature IL-18/GAPDH were each divided by total IL-18 to give precursor IL-18/total IL-18 and mature IL-18/total IL-18, respectively) and are expressed as mean ± SEM; n=6 for lnCTL, faCTL and faBASE and n=7 for faALA. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet, faBASE = *fa/fa* rats at baseline. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.



Tumour Necrosis Factor-α

Precursor and mature (active) TNF-α protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats. No genotype, aging, or diet influences were detected for either precursor or mature TNF-α (Figure 17).

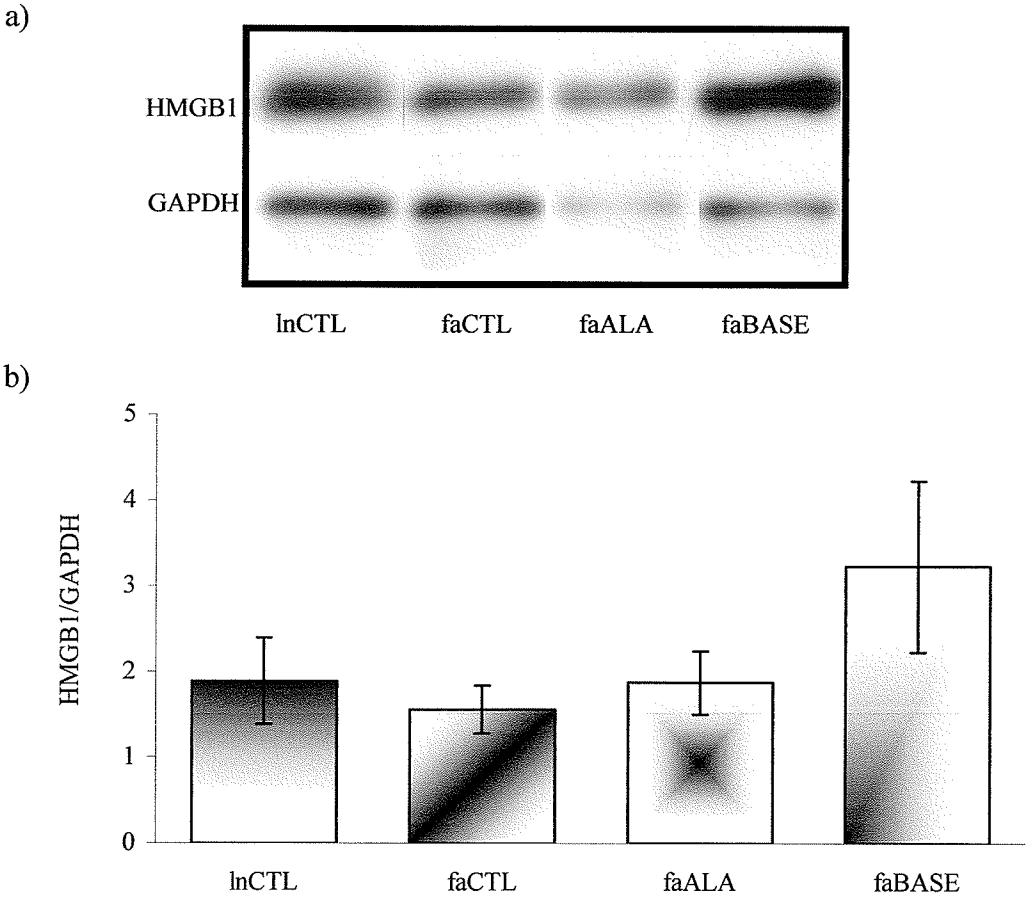
Figure 17: Tumour necrosis factor-α precursor and mature protein levels in peri-renal adipose tissue. Tumour necrosis factor (TNF)-α protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats by Western blot analysis. a) and b) are representative Western blots of precursor and mature TNF-α, respectively, and c) is the corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data were derived by calculating the ratio of precursor or mature TNF-α/total TNF-α (precursor or mature TNF-α was divided by GAPDH to correct for loading differences, precursor TNF-α/GAPDH and mature TNF-α/GAPDH were combined to obtain total TNF-α, and then precursor TNF-α/GAPDH and mature TNF-α/GAPDH were each divided by total TNF-α to give precursor TNF-α/total TNF-α and mature TNF-α/total TNF-α, respectively) and are expressed as mean ± SEM; n=6 for lnCTL, faCTL and faBASE and n=7 for faALA. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet, faBASE = *fa/fa* rats at baseline. No statistical differences were observed.



High mobility group box 1 (HMGB1)

HMGB1 protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats. There were no significant differences among groups (Figure 18).

Figure 18: High mobility group box 1 protein levels in peri-renal adipose tissue. High mobility group box 1 (HMGB1) protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats by Western blot analysis. a) Representative Western blot and b) corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data were derived by calculating the ratio of HMGB1 to GAPDH and are expressed as mean ± SEM; n=3 for lnCTL, faCTL and faALA and n=5 for faBASE. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. No statistical differences were observed.

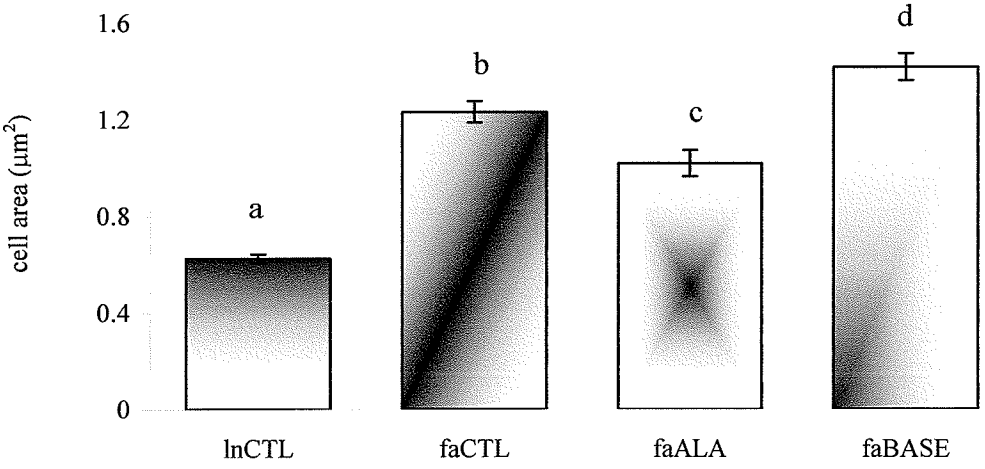


Adipocyte Size

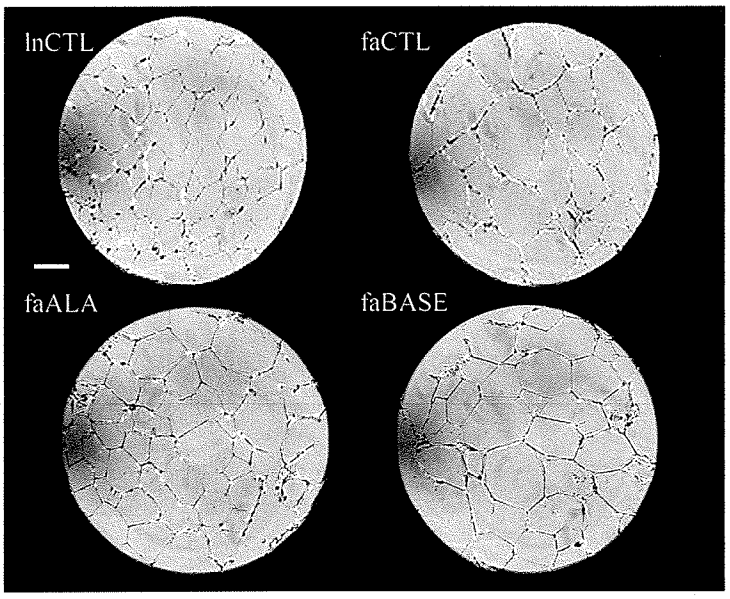
Frozen epididymal adipose tissue sections from lean and *fa/fa* Zucker rats were sectioned in order to measure adipocyte size and distribution. Adipocytes from the faCTL group were nearly double in size compared to the lnCTL (Figure 19). Interestingly, compared to the baseline *fa/fa* group, adipocyte size in obese rats decreased with age. Adipocyte size in obese rats fed ALA-rich flaxseed oil was decreased by 17% compared to the faCTL group.

Figure 19: Adipocyte size in epididymal adipose tissue. a) Adipocyte size and b) representative images of adipocytes from each group (200× magnification). Images of sectioned epididymal adipose tissue were captured using a light microscope fitted with a camera. Computer images of each section were obtained with QCapture Pro software. Cell area of 125 adipocytes per treatment group (~25 adipocytes/rat) was measured with the open-source image analysis program ImageJ. Data are presented as mean ± SEM; n=4 rats for lnCTL and faCTL and n=5 rats for faALA and faBASE. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet, faBASE = *fa/fa* rats at baseline. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters. Bar = 1 μm.

a)



b)

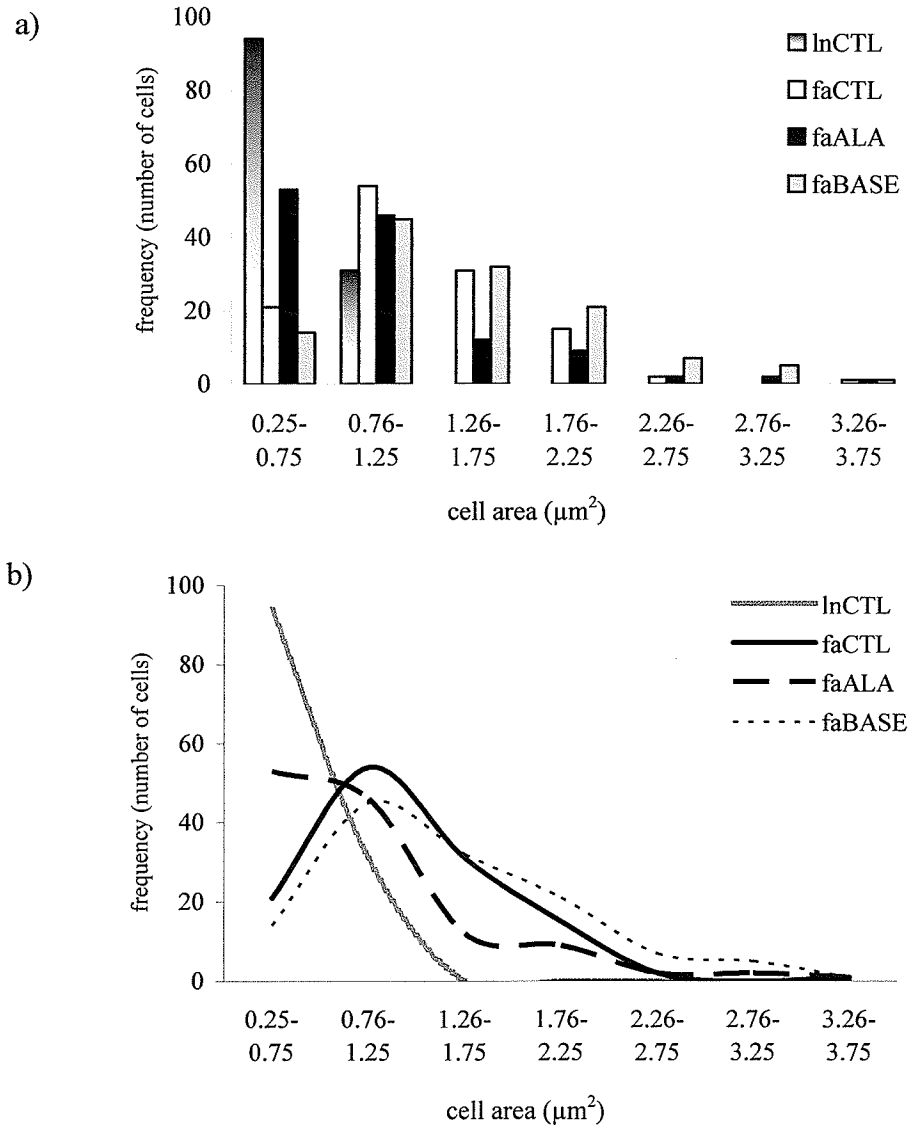


Adipocyte Distribution

The distribution of adipocyte size revealed differences between genotypes and diet interventions. The range of adipocyte size was doubled in the obese animals compared to the lean rats (Figure 20). As expected, lean rats had smaller adipocytes than obese rats. The adipocyte distribution curve for lean rats peaked at a small adipocyte area and sharply declined, reflecting a narrow range of values.

The faCTL and the faBASE distribution curves shifted to the right with a gradual ascent and tapered decline, reflecting a wider range of values. The curve representing obese rats fed the ALA-rich flaxseed oil diet followed a similar pattern to that of the lnCTL group. These rats had a greater amount of smaller adipocytes compared to the other *fa/fa* groups, so the distribution curve peaked early like the lnCTL group, and then gradually declined similar to the faCTL and faBASE groups.

Figure 20: Adipocyte size distribution in epididymal adipose tissue. a) and b) Graphs of adipocyte size frequency in each group. Images of sectioned epididymal adipose tissue were captured using a light microscope fitted with a camera at 200× magnification. Computer images of each section were obtained with QCapture Pro software. Cell area of 125 adipocytes per treatment group was measured with the open-source image analysis program ImageJ. Data are presented as frequency of cell area (μm^2); n=4 rats for lnCTL and faCTL and n=5 rats for faALA and faBASE. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet, faBASE = *fa/fa* rats at baseline. The frequency data within cell size ranges were significantly different except for the second (0.76-1.25 μm^2) and largest (3.26-3.75 μm^2) cell size ranges.



DISCUSSION

The goal of this thesis was to investigate the effects of dietary omega-3 fatty acid supplementation with ALA-rich flaxseed oil on the inflammatory state associated with obesity in a rodent model by focusing on T cell function (cytokine production) and adipose tissue function (adipokine production and adipocyte size). The major finding was that dietary ALA-rich flaxseed oil reduced adipocyte size in epididymal adipose tissue from obese Zucker rats but did not alter cytokine or adipokine levels in obese animals. As expected, energy consumption and body weight (including visceral, epididymal, and peri-renal fat pad masses) were higher in *fa/fa* Zucker rats compared to their lean counterparts. However, intervention with dietary ALA-rich flaxseed oil did not influence these parameters in the obese rats. While relative spleen weight was lower in *fa/fa* rats, there were no differences in proinflammatory cytokine (TNF-α, IFN-γ, IL-2, IL-6) secretion from UNS or STIM splenocytes among any of the intervention groups. Anti-inflammatory IL-10 secretion from UNS and STIM splenocytes was lower in the faALA group compared to the lnCTL group only. Surprisingly, both serum leptin and adiponectin concentrations were positively associated with obesity and neither was altered by dietary ALA-rich flaxseed oil. However, serum levels of adiponectin adjusted for blood volume revealed that total serum adiponectin was lower in the obese state as observed in humans. Peri-renal adipose tissue levels of pro- and anti-inflammatory adipokines were not influenced by ALA-rich flaxseed oil intervention, although there were some differences between the lean and *fa/fa* genotypes (reduced adiponectin and IL-

6 and greater IL-10 and IL-18 in obese compared to lean). These findings will be discussed in more detail in the following sections.

Effects on Physical Parameters

Increased energy consumption (~200 kcal/week) and greater body weight (~200 g) observed in obese Zucker rats compared to lean controls were logical and expected findings in our study. Other researchers using the Zucker rat model have reported similar feeding pattern and body weight differences between the lean and *fa/fa* genotypes (78, 87-89). Although our *fa/fa* rats tended to weigh more than the obese animals in the cited studies, this is most likely due to their advanced age (25 weeks vs. 12-15 weeks old) and longer duration of obesity.

Peri-renal adipose tissue mass and its contribution to visceral adipose tissue (epididymal + peri-renal adipose tissue) was notably greater in our *fa/fa* rats compared to younger animals previously analyzed (78). In our study, the ratio of peri-renal: epiddiymal adipose tissue was different between lean and *fa/fa* Zucker rats (1:1 and 3:1, respectively) and between younger and older obese rats (2:1 and 3:1, respectively). The greater proportion of peri-renal adipose tissue in obese rats explains expected adiposity differences between lean and *fa/fa* genotypes but also reflects body composition changes that occur with extended obesity (2:1 ratio of peri-renal: epididymal adipose tissue at baseline → shifted to a 3:1 ratio in obese rats after 8 weeks). Dietary intervention with ALA-rich flaxseed oil shifted the ratio of peri-renal: epididymal adipose tissue from 3:1 to 2.5:1 in obese rats, which may be a considered a favourable change to adiposity. Peri-renal adipose tissue, as its name implies, surrounds and protects the kidneys within the

inner peritoneal cavity. In an obese state, accumulation of peri-renal adipose tissue contributes to central adiposity, a component of the metabolic syndrome, which was recently identified as an important measurement to assess the risk of cardiovascular disease (90).

Higher respiratory quotient (RQ) and metabolic rate have been reported in obese Zucker rats compared to lean rats (89). A high RQ value not only suggests a lower level of fat oxidation, but also a higher level of dietary fat energy available for storage as fat (89). Furthermore, 24 hour energy expenditure in absolute values was found to be higher in obese Zucker rats compared to lean (89, 91, 92); higher energy expenditure is not explained by a greater level of physical activity but rather by greater resting metabolic rate (RMR) in obese subjects (93). In our study, even though obese rats consumed more energy and gained double the weight observed in lean rats, there were no diet or genotype differences in feed efficiency. In other studies, feed efficiency was reported to be higher in obese Zucker rats compared to leans (89, 94). Although feed efficiency was calculated as weight gain/feed intake in the cited studies (versus feed intake/weight gain in our study), interpretation of the ratio would yield the same conclusion. We may not have detected a difference in feed efficiency between genotypes in our study because the rats were at an advanced age past their growth spurt; our rats were nearly double the age of the animals in the cited studies (25 weeks vs 13 weeks). Furthermore, body weight appears to plateau by the end of the study period and in the final week there was also negative body weight change in some of the lean and obese rats (~2-3% and ~1% weight loss, respectively). Together these observations provide new information on feed and weight patterns in Zucker rats of advanced age.

Overall, older *fa/fa* rats were not that different from the baseline group in our study. We did not detect differences in any of the adipokines measured from peri-renal adipose tissue between the faBASE and the faCTL group, indicating that these parameters may not be sensitive to age or increasing obesity. It is also possible that there was no actual increase in obesity between these groups; although there were differences in body weight, the adipose to lean body mass ratio may have remained constant. Lean body mass was not measured in our study. The faCTL group had higher serum leptin concentrations and increased peri-renal and visceral adipose tissue masses compared to the faBASE group, but no differences in serum adiponectin between these groups were noted. Interestingly, epididymal adipose tissue mass declined with age, perhaps suggesting body composition changes with increasing obesity. Adipocyte size in epididymal adipose tissue also decreased over time in *fa/fa* rats and this may be related to decreased epididymal adipose tissue mass.

To our knowledge, the effect of dietary ALA-rich flaxseed oil on appetite or body weight in lean and obese Zucker rats has not been previously investigated. Other dietary agents, such as conjugated linoleic acid (CLA), have been studied in Zucker rats with varying results (78, 94). Similar to our study, dietary CLA (0.5% or 1.5%) did not affect growth rate, body weight or food intake in lean or obese Zucker rats (78, 94). Interestingly, dietary CLA intake was associated with an increase in retroperitoneal (RP) fat pad weights in obese Zucker rats; however, as in our study, epididymal pad weight was increased in obese rats but diet had no effect on this fat depot (94). Increased visceral adipose tissue in human obesity is thought to play a major role in the pathogenesis of metabolic syndrome (95). Furthermore, changes to epididymal adipose

tissue mass in male obese rats are associated with the underdevelopment of reproductive organs (96). In another study, dietary intervention with CLA (1.5%) was associated with increased epididymal, peri-renal, and visceral adipose tissue in obese Zucker rats compared to the control diet group, but no differences were detected with CLA supplementation among lean groups (78). Together these findings suggest that the obese Zucker rat model may not only be sensitive to dietary intervention, but also to the concentration of the dietary agent of interest. We can conclude that an ALA-rich diet, provided as 4.25% (w/w) flaxseed oil (where ALA contributes 33.7% of total fatty acids), changes adipose tissue distribution but does not affect body weight or feed intake among obese Zucker rats.

Effects on Immune Parameters

We are unaware of any published research that has investigated the effects of dietary ALA-rich flaxseed oil on spleen weight or ex vivo cytokine production by splenocytes from lean and *fa/fa* Zucker rats. However, the effect of longer chain omega-3 fatty acids on cytokine production has been studied in animals (61) and humans (62, 64) with mixed results. A fish oil diet was found to significantly increase the ratio of Th2:Th1 splenic T cells by suppressing IFN-γ Th1 cell development in C57BL/6 mice (61). Conversely, diets supplemented in EPA were associated with a significant reduction in stimulated T lymphocyte proliferation in healthy adults but proinflammatory cytokine production (IL-2, IFN-γ) did not change (62). In the same study, dietary ALA did not affect stimulated lymphocyte proliferation or cytokine production. Another comparison of ALA or EPA and DHA intake in healthy adults also confirmed no changes

to stimulated proinflammatory cytokine production, except for a decrease of IL-6 production, but only with the two higher doses of fish oil (64). It is possible that higher dietary intakes of omega-3 fatty acid are required to alter cytokine production. In addition, our research uniquely investigates the effect of dietary ALA-rich flaxseed oil in an obese state.

Our findings indicate that obese rats have a higher absolute spleen weight but lower relative spleen weight compared to the lean Zucker rats [similar outcomes were reported in another study focused on the effects of a CLA diet (97)] and dietary ALA had no effect on spleen weight in obese rats. One of the most important roles of the spleen is to help fight infection in the body. The spleen filters unwanted material from blood such as old or damaged blood cells and acts as a blood reservoir. The spleen also acts as a storage reservoir for lymphocytes that can be released to sites of infection via the blood. At first glance, and assuming the spleen is still functioning optimally, it appears that the greater absolute spleen weight from obese rats would provide enhanced immunity. However, it is important to consider the absolute value in proportion to body weight, because of the significant difference in body weight between obese and lean rats. From this perspective, we see that the relative spleen weight is proportionally smaller in obese rats compared to lean, suggesting that the spleen may perform suboptimally, and may explain the increased risk of infections seen in human obesity. In children and adults, spleen weight is also increased in obese states (98).

Despite this variation in relative spleen weight, there were no differences in IL-6, IL-2, IFN-γ, or TNF-α *ex vivo* cytokine secretion from UNS or ConA-STIM splenocytes between genotypes or diet groups. It is worth noting that we measured an equal number

of splenocytes from each sample, regardless of differences in spleen weight between groups. A cell volume that is calculated relative to spleen weight, even if inconsistent among samples, may have revealed differences in these measurements. Anti-inflammatory IL-10 secretion from both UNS and ConA-STIM splenocytes was 2.1-fold and 1.6-fold lower, respectively, in obese rats fed ALA compared to the lean control diet group, while the faCTL group had values not different but intermediate between the lnCTL and faALA. This finding suggests that dietary intervention with ALA-rich flaxseed oil enhances the reduction in IL-10 secretion by splenocytes from obese rats.

In contrast to our findings, Ruth et al. (97) report genotype differences in ConA-stimulated *ex vivo* cytokine production by splenocytes, including higher levels of IL-6, IFN-γ, and TNF-α, and lower levels of IL-2 in obese Zucker rats compared to lean rats and no difference in IL-10 secretion. In diet-induced obese C57BL/6 mice, LPS-stimulated splenocytes also produced greater amounts of IFN-γ and IL-4, and less IL-2 compared to control mice, although there were no differences between groups in TNF-α production (99). Interestingly, Lamas and colleagues (100) reported decreased spleen TNF-α and IL-6 mRNA levels in diet-induced obese Wistar rats compared to controls, and a negative correlation between spleen TNF-α mRNA levels and total fat pads. The pattern of cytokine mRNA levels in the study by Lamas et al. (100) and the lack of changes in *ex vivo* cytokine secretion in the present study do not coincide with the increased levels of proinflammatory cytokines typically observed in obese humans (TNF-α, IL-6, IFN-γ in serum, subcutaneous adipose tissue and PBMCs) (39, 63, 42) and diet-induced obese animals (IFN-γ) (99). The discrepancy between these results may be explained by differences in study designs, which include the animal model (rat versus

mouse) and strain, the age of the animals (25 weeks at termination in our study versus 14 and 18 weeks), and the method for measuring cytokine production (small sample size to simultaneously measuring all 6 cytokines using a Rat Cytokine 6-Lincoplex Kit read on a Luminex Instrument in our study versus individual measurements of each cytokine in separate commercial ELISA kits). Furthermore, varying results for cytokine production may be a reflection of the mitogen used for stimulation and its concentration, the incubation time, or different primary antibodies targeting separate areas of the protein of interest.

Effects on Serum Parameters

In our study, fasting serum concentrations of leptin and adiponectin were both elevated in obese rats. Hyperleptinaemia in the obese state was an expected finding and has previously been identified in *fa/fa* Zucker rats (76) and in obese individuals (40). Circulating levels of adiponectin were also surprisingly increased in obese rats compared to lean rats, which is opposite to what is typically seen in human obesity (11). However, for the *fa/fa* Zucker rat model, an increase in serum adiponectin levels in obese rats compared to lean rats seems to be the typical finding (76, 101). One recent study does report decreased plasma adiponectin levels in *fa/fa* rats compared to lean Zucker rats (102), and in another study, no difference between Zucker diabetic fatty (ZDF) rats and lean heterozygous littermates was detected when fasting levels were considered (103). Although the *fa/fa* Zucker rat model shares many common features observed in human obesity, the pathogenesis of obesity in these animals is due to a defect in the leptin receptor (77), whereas mutations in the genes encoding leptin or its receptor are rare in

human cases of obesity. We propose that this genetic difference may shift focus to the functioning adiponectin receptor and as a consequence increase the rate of production of adiponectin. Alternatively, there may be a feedback mechanism between leptin and adiponectin (an increase in one would then signal an increase in production of the other) which is required to regulate the balance of pro- and anti-inflammatory effects. In addition, as a defense mechanism to balance the immune response, anti-inflammatory adiponectin production may be enhanced to counter the high circulating leptin levels in these animals.

Further analysis of our data for circulating adiponectin levels revealed an opposite and more expectant finding. When serum adiponectin values were adjusted for blood volume ('total' serum adiponectin), to reflect the body size difference between lean and obese rats, and expressed relative to different adipose tissue depots (particularly peri-renal and visceral) but not body weight, we found that total serum adiponectin is actually less in *fa/fa* rats than in lean rats. In addition, we also show that total serum adiponectin is positively correlated with body weight, peri-renal, epididymal, and visceral adipose tissue. To explain the positive correlation between peri-renal and visceral adipose tissue with total serum adiponectin yet lower total serum adiponectin when expressed relative to peri-renal and visceral adipose tissue mass we suggest that the changes in peri-renal and visceral adipose tissue mass are not equal to the changes in adiponectin with increasing obesity in *fa/fa* rats.

We did not observe an effect of dietary ALA-rich flaxseed oil on serum leptin or adiponectin concentrations in obese Zucker rats. To our knowledge, the potential influence of dietary ALA-rich flaxseed oil on serum leptin or adiponectin levels in an

obese rodent model has not been previously investigated. In related research, a non-significant trend for lower plasma leptin levels in Wistar rats fed an omega-3 fatty acid-enriched diet was reported; however, this diet was supplemented with EPA and DHA (73). Similarly, lower serum leptin levels were observed in C57BL/6J mice fed fish oil as the main source of fat (74). Changes in dietary intake of PUFA (EPA and DHA) are also negatively correlated to changes in plasma leptin concentrations in human subjects (73). However, in an intervention study, consumption of the long chain EPA and DHA fatty acids (as two portions of oily fish per week) did not alter plasma leptin or proinflammatory cytokines (TNF-α, IL-6) in overweight adults (63).

Observational studies of healthy men and women have found low dietary ALA consumption is not significantly related to inflammatory markers (IL-6, CRP) (65), whereas others have found an inverse relationship to the same inflammatory markers when the sample included only women (66). It may be possible that markers of inflammation in women are more sensitive to dietary ALA intake compared to men. However, interventional studies report that increased dietary ALA intake is associated with decreased markers of inflammation (CRP, IL-6, TNF-α) in hypercholesterolemic and dyslipidemic men and women (67-70). In contrast, we did not observe an effect of dietary ALA on serum haptoglobin concentrations (an acute phase protein similar to CRP and an equivalent marker for inflammation in rats) in male obese Zucker rats in the present study (Rigaux et al., unpublished data). In an earlier study by Nelson & Hickey (104), decreased fasting levels of soluble IL-6 receptor concentrations were observed in normal weight and obese subjects after consuming 5% of total energy from ALA for only 4 days. However, recent work published by these authors revealed that longer term

dietary ALA intake was not effective in lowering circulating inflammatory markers such as IL-6, CRP, and TNF-α in obese men and women (105).

The lack of an effect on serum adipokine levels in response to ALA-rich flaxseed oil feeding in our study, compared to somewhat more effective EPA and DHA-enhanced diets in other studies, may be explained by the moderate biological potency of ALA compared to the longer chain omega-3 fatty acids. Perhaps more importantly, these results may indicate that the obese environment is more resistant to dietary change. For the majority of human studies, the duration and amount of ALA supplementation is similar. Contrasting results may reflect degree and duration of obesity or overweight, including clinical status and age of the sample population, how ALA was supplemented into the diet (capsule or food), or inflammatory markers measured (differences in sensitivity or measuring levels of a receptor versus the protein itself). In addition, Paschos et al. (106) report that background diet may also influence the effect of ALA supplementation on serum concentrations of inflammatory markers (including CRP and IL-6). They observed a larger anti-inflammatory effect (decrease in serum levels) in dyslipidemic men when the background diet was rich in saturated fatty acids and poor in monounsaturated fatty acids (MUFA). From this perspective, lack of an ALA diet effect in our study may be explained by similar saturated fatty acid and MUFA content between both diet groups (12.2 and 19.1 g/100 g total fatty acids of saturated and MUFA, respectively, in the ALA diet and 15.8 and 22.5 g/100 g total fatty acids of saturated and MUFA, respectively, in the control diet). Elevated baseline levels of inflammatory markers may also explain why some studies show an effect of dietary ALA intervention and others do not (105).

A recent study has reported that purified EPA fed to *ob/ob* mice and obese human subjects is associated with an increase in plasma adiponectin concentrations compared to control groups (107). However, increases in circulating levels of adiponectin have not been reported with dietary ALA intervention in human studies. Nelson et al. (108) reported a decrease in adiponectin levels in obese men and women supplemented with approximately 11 g/d of ALA (5% of total calories) for 8 weeks compared to controls. However, ALA supplementation (15 mL flaxseed oil containing 8.1 g ALA/day for 12 weeks) in overweight dyslipidemic men did not change plasma adiponectin concentrations compared to the control group (109). A diet rich in omega-3 fatty acids (3.5% of energy intake), including plant and marine sources, also did not alter total or high molecular weight (HMW) plasma concentrations of adiponectin in obese men and women (110). Paschos and colleagues (109) addressed the lack of effect of dietary ALA on adiponectin and speculated that ALA may be an inactive ligand for PPAR-γ, which is a transcription factor responsible for adiponectin synthesis (111), in adipocytes. Also, unchanged plasma TNF-α levels may also explain unaltered adiponectin levels, as TNF-α decreases the expression and secretion of adiponectin (111). Furthermore, an ELISA that employs an antibody to measure total adiponectin in the plasma, rather than HMW adiponectin, may be a less sensitive indicator of metabolic syndrome (109). The ELISA used in our study was specific for total adiponectin and did not differentiate between HMW and the globular form of adiponectin. In addition, we did not measure plasma TNF-α concentrations.

Increased adiponectin levels in diseases associated with inflammation (112) have led some researchers to investigate whether adiponectin has both anti-and

proinflammatory properties (113). As for its anti-inflammatory potential, reduced nuclear factor (NF)-κB reporter activities in response to an inflammatory stimulus (LPS) have been reported in monocytic cells preincubated with adiponectin (113). NF-κB is a transcription factor that regulates the expression of over 150 target genes; the majority of proteins encoded by these genes participate in the host immune response and inflammation (114). However, cells incubated with native adiponectin alone also had significantly increased NF-κB reporter activity compared to controls and this effect was attributed to the HMW adiponectin isoform (113). Furthermore, globular adiponectin was also found to increase NF-κB reporter activity and cytokine release (TNF-α, IL-10) (113). Despite this, no differences in NF-κB activity were found between lean and *fa/fa* Zucker rats, even though adiponectin levels were increased in the obese animals (DeClercq et al., unpublished results).

In addition to higher circulating levels of adiponectin among obese rats in our study, we also observed greater IL-10 protein levels in adipose tissue of obese rats compared to the lean controls. However, in the context of some of our other contrasting data (reduced adiponectin and IL-6 and non-significant differences in TNF-α levels in adipose tissue of obese rats compared to lean rats), it is obvious that changes to inflammatory mediators from different tissues and models of obesity are complex. For example, the unusual pattern of anti-inflammatory and proinflammatory protein levels in adipose tissue of obese Zucker rats compared to lean, may suggest that some markers of inflammation are regulated differently in adipose tissue/adipocytes in this rat model.

Effects on Adipose Tissue Parameters

Leptin, TNF-α, and HMGB1 adipokine levels in adipose tissue were unchanged among all groups in our study. Human studies have observed increased serum TNF-α (6) and leptin levels (39, 40) in obesity, and we also report increased serum leptin concentrations in obese rats compared to controls; however, our data from peri-renal adipose tissue production does not mirror the changes in serum concentrations. Production of these adipokines may be increased in obesity, but if the rate of release to serum is also increased or rate of degradation decreased, it may explain why no differences are detected in protein levels of adipose tissue when differences in serum concentrations exist.

There is limited published data on adipokine protein levels in lean and *fa/fa* Zucker rats to compare our results. In one study, adipose leptin protein levels in obese Zucker rats were reported to be even lower than lean controls, but adipose leptin mRNA had been the same between these groups, and serum levels increased in the *fa/fa* group (76). The main differences between our study design and the one cited were the age of rats (25 weeks compared to 14 weeks, respectively) and the fat depot analyzed (peri-renal versus epididymal); however, differences in technique were also identified (SDS-gel recipe and band density measurements). In addition, increased TNF-α protein has been reported in visceral adipose tissue from obese Zucker rats compared to lean (102), however, it is not clear how visceral fat was defined (measuring production in different fat depots may yield different results) and differences in technique were identified (ELISA kit versus Western blot), which may explain different findings. Furthermore,

Skopkova and colleagues (115) reported comparable TNF-α protein and mRNA levels in subcutaneous adipose tissue of obese and lean adults.

We are unaware of any published research that has investigated the protein levels of HMGB1 in adipose tissue of this rat model. HMGB1 is secreted from monocytes and macrophages, has pro-inflammatory and immunostimulatory properties, and contributes to the pathogenesis of inflammatory diseases (5). Since we did not observe differences in adipose TNF-α levels, it is not surprising we did not detect changes to HMGB1, as these proteins stimulate one another and are positively correlated [(reviewed by Voll et al. (5)].

We also observed lower adiponectin and IL-6 and higher IL-18 (mature) and IL-10 levels in peri-renal adipose tissue from obese rats compared to lean animals. Reduced adiponectin levels in *fa/fa* Zucker rats compared to lean rats was an expected finding, as adiponectin inversely correlates with obesity in humans (11). However, our results again do not match with data previously reported by Noto et al. (76) who observed no differences in adiponectin adipose tissue protein levels between genotypes of the Zucker rat model at 14 weeks of age (differences between studies have already been discussed). Alternatively, Altomonte et al. (103) also report lower mRNA levels of adiponectin in subcutaneous and visceral adipose tissue in ZDF rats compared to their lean counterparts. Interestingly, these authors also found differences in fat depot-specific expression of adiponectin between genotypes, where expression in visceral fat was higher than in subcutaneous fat from lean animals and the opposite was true for obese animals (higher adiponectin mRNA levels in subcutaneous fat compared to visceral fat).

We did not expect to see lower IL-6 levels in peri-renal adipose tissue from obese rats compared to lean, as this proinflammatory adipokine is positively correlated with

obesity in humans (9) and approximately 30% of the total circulating IL-6 originates from adipose tissue (27). We are unaware of any published work that has also investigated peri-renal adipose IL-6 levels in obese Zucker rats to directly compare our results. Elsewhere it has been reported that IL-6 mRNA levels are increased in obese Zucker rats compared to leans, but these differences were found in aorta tissue and not adipose (116). Likewise, immunostaining for IL-6 in liver tissue revealed higher IL-6 protein levels in obese Zucker rats compared to lean (117). Yet another study reports no significant differences between obese and lean Zucker rats in pancreatic, liver or lung IL-6 mRNA levels (118). Interestingly, when another inflammatory condition (acute pancreatitis) was induced, IL-6 mRNA levels in the pancreas were decreased in obese rats compared to lean. The tissue analyzed and technique employed may explain differences between these findings. Furthermore, regulatory mechanisms that deal with the inflammatory process may differ among tissues and between human and animal models of obesity. The complex balance between pro- and anti-inflammatory mediators in relation to obesity is highlighted in a study by Wallenius and colleagues (119) who reported increased subcutaneous fat pad mass and obesity in IL-6-deficient mice compared to wild-type controls. The authors suggest there may be a therapeutic interval between doses of IL-6 associated with beneficial and adverse effects.

Elevated protein levels of mature (active) IL-18, and reduced precursor IL-18, in peri-renal adipose tissue of obese rats compared to lean was an expected finding, as serum concentrations (120) and abdominal subcutaneous adipose tissue mRNA levels (121) of proinflammatory IL-18 are reportedly higher in obese individuals compared to lean controls (120). In addition, an inverse relationship between IL-18 and adiponectin in

obesity has been identified (120), which matches our data showing a decrease in adiponectin levels and an increase in IL-18 in peri-renal adipose tissue from obese rats. We are unaware of any other research that has measured IL-18 levels in adipose tissue from obese rats.

IL-10 levels in peri-renal adipose tissue were significantly greater in *fa/fa* rats compared to lean controls; this finding is in accordance with previously published work that reported higher IL-10 protein levels in subcutaneous adipose tissue of obese individuals compared to lean subjects, and indicates a possible defensive response of fat tissue to inflammation (115). Additionally, increased IL-10 mRNA levels have been observed in subcutaneous and visceral adipose tissue of obese subjects compared to lean controls, and in epididymal adipose tissue of *ob/ob* leptin-deficient mice compared to wild-type controls (122). Together these findings provide evidence to identify adipose tissue as a source of IL-10. Interestingly, serum IL-10 concentrations among obese individuals have yielded inconsistent results. Both higher (123) and lower (124) IL-10 circulating levels have been reported in obese subjects compared to lean controls. However, in the former cited study, when non-obese or obese subjects also met criteria for the metabolic syndrome, serum IL-10 levels were decreased compared to individuals without the metabolic syndrome (123), and in another study that categorized subjects based on criteria of the metabolic syndrome, similar findings were reported (26). As mentioned earlier, changes in direction for circulating and adipose tissue levels of IL-10 may be a reflection of changes in the rate of release to serum or the rate of degradation. Adipose IL-10 in lean and obese Zucker rats has not been previously reported. In one

study, no significant differences in pancreatic, liver, or lung IL-10 mRNA levels were identified between genotypes in younger Zucker rats (118).

To our knowledge, the isolated effect of dietary ALA-rich flaxseed oil on adipokine production in an obese state had not been previously investigated. We report no effect of dietary ALA-rich flaxseed oil on leptin, adiponectin, TNF-α, or HMGB1 levels in peri-renal adipose tissue from obese Zucker rats. Non-significant changes were observed with IL-6 (elevated), IL-10 (reduced), and mature IL-18 (reduced) levels in adipose tissue of obese rats fed ALA-rich flaxseed oil compared to the faCTL group. Although elevated levels of proinflammatory IL-6 and reduced levels of anti-inflammatory IL-10 may not appear to positively alter the inflammatory state of obesity, these changes, as well as the reduced IL-18, were in the direction of levels observed in the lnCTL group. Also, as previously mentioned, the overall balance between pro- and anti-inflammatory markers, rather than the levels of just one mediator, may be more important in regulating the inflammatory process in obesity.

Based on our data, dietary ALA-rich flaxseed oil does not seem to effectively alter or improve inflammatory markers in obese Zucker rats. The effect of dietary ALA-rich flaxseed oil on serum markers of inflammation in human subjects was discussed earlier. Unfortunately, differences in study designs and inconsistent results make it difficult to declare an overall positive, negative, or neutral effect of dietary ALA-rich flaxseed oil. The administration of ALA (capsule or foodstuff), the dose of ALA, duration of supplementation, and subject characteristics are just a few examples of differences. In our study as well, ALA-rich flaxseed oil may not have been potent at the dose and duration that was used, or the age of rats (and longer duration of established

obesity) may have made it more difficult to observe effects of dietary change. The genetic model of obesity used in our study also highlights the potential importance of leptin in mediating the inflammatory response, as these animals are leptin-receptor deficient. The lack of an expected difference in cytokine production and some adipokine production levels between genotypes in this study suggests that these inflammatory responses in an obese state may be leptin (and its receptor)-dependent.

Although we conclude dietary ALA-rich flaxseed oil has no effect on cytokine or adipokine production in obese Zucker rats, we do highlight some modest changes to these parameters. Of particular interest, the enhanced reduction of IL-10 secretion from stimulated splenocytes and the 34% reduction of peri-renal adipose tissue IL-10 protein levels associated with dietary ALA-rich flaxseed oil treatment in obese rats may also reflect decreased macrophage infiltration of adipose tissue.

In obesity, adipose tissue is under metabolic stress, which triggers the inflammatory response and leads to the accumulation of macrophages (125). Higher expression of macrophage markers has been reported in adipose tissue of obese subjects (115). In addition, previous reports found enhanced IL-10 release in obese individuals was due to nonfat cells present in adipose (17) and that macrophages remaining in adipose tissue after weight loss stain positive for IL-10 (33). The increased IL-10 production in obese rats compared to lean animals may be a defensive reaction to the inflammation provoked by excess adipose tissue (115), which includes the recruitment of macrophages to the site of injury. Therefore, it is reasonable to suggest that the reduced IL-10 protein levels we observed in adipose tissue of obese rats consuming an ALA-rich flaxseed oil diet may be a reflection of decreased macrophage infiltration of adipose

tissue in this group. Todoric and colleagues (126) reported that inclusion of marine omega-3 fatty acid in a high-fat diet (40% of oil volume was replaced by a concentrate of highly purified EPA and DHA) fed to genetically obese db/db mice abolished high-fat diet-induced macrophage infiltration of adipose tissue. They suggested that omega-3 long chain fatty acids could prevent adipose tissue inflammation by directly inhibiting macrophage infiltration and activation; or locally, omega-3 fatty acid may directly improve adipocyte function (ameliorate adipocyte stress, normalize adipocyte metabolism, and hinder adipocyte expression of pro-inflammatory cytokines). Importantly, their findings also indicate that dietary modulation, independent of weight loss, is able to improve adipose tissue inflammation in an obese state (126).

Although we did not measure macrophage infiltration in adipose tissue, the number of macrophages positively correlates with adipocyte size (31), and a novel finding of our study revealed that dietary ALA-rich flaxseed oil significantly decreases adipocyte size in obese rats. Adipocytes from obese rats fed ALA-rich flaxseed oil were about 17% smaller than adipocytes from obese rats fed the control diet. We can therefore hypothesize that the observed decrease in adipocyte size may also be associated with a decreased macrophage infiltration in our obese rats fed ALA-rich flaxseed oil, and that a decreased macrophage number in adipose tissue was responsible for the reduced IL-10 production. To our knowledge, this is the first study to report an effect of dietary ALA-rich flaxseed oil on adipocyte size in obese Zucker rats. In agreement with this finding, reduced adipocyte size has previously been reported with dietary supplementation of omega-3 fatty acid (EPA and DHA) in male Wistar rats (127, 128). Our lab has previously reported this animal model to be responsive to dietary intervention for this

parameter, as dietary CLA was also effective in decreasing adipocyte size in obese rats compared to controls (76). In agreement with our findings, dietary omega-3 fatty acid (25.1 mg omega-3 fatty acid/g diet; mostly EPA and DHA) supplementation of a high-fat diet (30% kilocalories from fat) was found to effectively prevent the high-fat diet-induced adipocyte enlargement in db/db mice, as mean adipocyte area was reduced to levels found in mice consuming a low-fat diet (129). These data complement our results that show dietary ALA-rich flaxseed oil is also effective in decreasing adipocyte size in a model of obesity when supplemented in a low-fat diet (~20% of kilocalories from fat); this parallelism is important, as the dietary intake of obese individuals is likely to be nutritionally imbalanced and varied. Furthermore, adipocyte diameter was previously reported to be inversely correlated with omega-6 and omega-3 fatty acid contents in the subcutaneous adipose tissue of obese subjects (130).

Metabolic differences exist between smaller and larger adipocytes (131). Early investigations showed that larger adipocytes isolated from older and fatter rats have decreased insulin-mediated glucose oxidation and decreased insulin binding to receptors compared to smaller adipocytes from younger and leaner animals. Today, fat cell enlargement is considered an independent marker of insulin resistance and DM-2 (132). Recent reviews have eloquently summarized how adipocyte hypertrophy is interrelated with other factors and contributes to adipose tissue dysfunction in obesity (132, 133). As reviewed by Goossens (132), enlarged adipocytes may be a result of impaired differentiation and failed recruitment of new adipocytes. They may be also resistant to the anti-lipolytic effect of insulin and have decreased buffering capacity for lipid storage which makes them ineffective for storing dietary fatty acids and results in lipid overflow,

an influx of lipid exposure to non-adipose tissue, and eventual fat accumulation and deposition.

Finally, adipocyte hypertrophy may contribute to adipose tissue inflammation by altering adipokine secretion, as large adipocytes differentially express inflammatory markers compared to smaller adipocytes with a shift towards pro-inflammatory adipokines. Furthermore, by modulating adipose tissue function, these large adipocytes promote recruitment of macrophages to adipose tissue (132). In addition, as described by de Ferranti & Mozaffarian (133), changes associated with adipocyte hypertrophy appear be the first steps toward adipocyte cellular dysfunction, where endoplasmic reticulum (ER) "stress" results in the dysfunction of proper folding and modification of proteins, lipid-droplet creation, and cholesterol-sensing, and oxidative stress at the level of the mitochondrion results in mitochondrial uncoupling and release of reactive oxygen species.

The mechanism of dietary ALA action on adipocyte size is unknown. We speculate that ALA may act on a variety of transcription factors and proteins responsible for regulating metabolic control and adipose tissue function that respond to cellular stress and immune system activation; changes to these proteins may signal adipocyte size modulation. Potential targets of ALA action include indicators of cell stress such as phosphorylated c-Jun N-terminal kinase (JNK), activating transcription factor (ATF)-4 and inducible nitric oxide synthase (iNOS), or indicators of adipocyte differentiation such as adipsin and PPAR-γ.

Conclusion

The favourable changes to adipocyte size and adipose tissue distribution in obese Zucker rats fed a diet supplemented with ALA-rich flaxseed oil indicates that this plant-based omega-3 fatty acid is capable of altering adipose tissue function in a short time period (8 weeks) in established obesity (25 week old *fa/fa* Zucker rats) and leads to the conclusion that this dietary change has potential to affect inflammation and modulate adipokine production in an obese state. The overall lack of an immediate positive or improved effect on levels of inflammatory markers, such as pro- or anti-inflammatory splenocyte cytokine secretion or adipose tissue adipokine secretion, serum adipokines, body weight, and feed intake suggests that an older rat model with established obesity for a longer period of its lifespan may require extended or greater dietary intervention for physiological changes to be observed. The significant decrease of adipocyte size to smaller, functional cells and the favourable shift of adipose tissue distribution to less peri-renal fat in obese rats fed ALA-rich flaxseed oil provide encouraging evidence to further study the effect of dietary ALA-rich flaxseed oil on adipose tissue and the inflammatory process in obesity. If the anatomy of the adipocyte is changing, it is reasonable to hypothesize that changes to adipocyte function would also occur.

SUMMARY OF MAIN FINDINGS

Genotype effect

Differences observed between the lean and *fa/fa* Zucker rats include:

- greater body weight and energy consumption in *fa/fa* rats
- higher feed intake to body weight ratio in lean rats
- greater peri-renal, epididymal and visceral fat pad masses in *fa/fa* rats
- greater proportion of peri-renal adipose tissue compared to epididymal adipose tissue in *fa/fa* rats
- greater absolute spleen weight in *fa/fa* rats
- greater relative spleen weight in lean rats
- higher fasting serum leptin concentrations in *fa/fa* rats
- higher fasting serum adiponectin concentrations in *fa/fa* rats
- lower total serum adiponectin (serum levels adjusted for blood volume) in *fa/fa* rats
- lower adiponectin and IL-6 levels in peri-renal adipose tissue from *fa/fa* rats
- higher IL-10 and IL-18 (mature) levels in peri-renal adipose tissue from *fa/fa* rats
- greater adipocyte size in epididymal adipose tissue from *fa/fa* rats

No effect. There were no genotype differences in unstimulated or ConA-stimulated splenocyte production of IL-2, IL-6, IL-10, IFN-γ, and TNF-α or in leptin, TNF-α, and HMGB1 levels in peri-renal adipose tissue.

Diet effect

Differences observed between the faCTL and faALA groups include:

- decreased ratio of peri-renal to epididymal adipose tissue in *fa/fa* rats fed ALA-rich flaxseed oil
- smaller adipocyte size in epididymal adipose tissue from *fa/fa* rats fed ALA-rich flaxseed oil

Aging effect

Differences observed between the faBASE and faCTL groups include:

- increased peri-renal and visceral adipose tissue mass in older *fa/fa* rats
- increased serum leptin concentrations in older *fa/fa* rats
- decreased epididymal adipose tissue mass in older *fa/fa* rats
- decreased adipocyte size in epididymal adipose tissue from older *fa/fa* rats

STRENGTHS AND LIMITATIONS

Several aspects are identified to be both strengths and limitations of our work, as described below.

- Gender. *Strength.* We studied the effects of ALA-rich flaxseed oil in male rats. Inclusion of same-sex animals ensures data are not confounded by gender-differences that may also influence results of the parameters we measured. For example, circulating adiponectin and leptin levels are lower in men compared to women (24, 47); and interventional studies have found dietary ALA supplemented as rapeseed oil is inversely related to serum leptin in females but not in males (75), suggesting there may be a difference in responsiveness to diet between genders as well. *Limitation.* While inclusion of only one gender eliminates some potential variability, it also makes it difficult to extrapolate the results to the opposite sex. Female rats may have been more or less sensitive to the same dietary treatment.

- Age. *Strength.* The rats in our study were 17 weeks old when dietary intervention began, and 25 weeks old when they were terminated at the study end. Twenty-five weeks represents an advanced (mature) life stage for the *fa/fa* Zucker rat, whose life expectancy is about 1 year, and is uncommonly studied, as other studies mostly include rats at a much younger age (13-18 weeks). This age is an important consideration of our study because our animals were past their growth spurt (from birth to 6 weeks old) and in fact, for some rats, body weight appeared

to plateau near the end of the study. These animals represented an older population that had been obese for a greater period of their lifetime and this is a unique component of our study design, which investigated the effectiveness of basic dietary intervention as treatment (rather than in a preventative role). For example, we have shown that dietary ALA-rich flaxseed oil intake for 8 weeks is capable of reducing adipocyte size in older, obese rats, indicating that both dietary intervention and ALA-rich flaxseed oil are potential positive and safe lifestyle modifications that work in an established obese state. *Limitation.* Advanced age may have also been a limiting factor in our study to study the effectiveness of dietary intervention in modulating inflammatory parameters in an obese state. Leptin- and insulin-resistance are two common features of obesity (2, 3), dem onstrating that in this state normal hormonal action is altered and ineffective. It is possible that we did not see many effects of dietary ALA-rich flaxseed oil due to a decreased responsiveness to diet that might also be present in a state of over-consumption and excess. More radical changes to cytokine or adipokine measurements may have been observed in a younger life stage; that is, investigating whether dietary ALA-rich flaxseed oil intake is more effective as a preventative tool in obesity.

- Animal model. *Strength.* The *fa/fa* Zucker rat model is an accepted rodent model of obesity. These rats exhibit obesity at 4 to 5 weeks of age and share many common features of human obesity including characteristics of the metabolic syndrome such as hyperleptinaemia, insulin-resistance, muscle atrophy,

hyperlipidemia, hypercholesterolemia, hypertriglyceridemia, hyperphagia, and hyperinsulinemia. Investigating the effects of dietary intervention in an animal model of obesity also allows us to determine potential effectiveness, safety and appropriateness for obese humans. *Limitation.* In the *fa/fa* Zucker rat model obesity is caused by a defect in the leptin receptor (77), a mutation that is rare in human cases of obesity. This genetic defect may underscore some of the differences we observed in our results between human and animal forms of obesity. For example, obese rats had increased serum adiponectin concentrations compared to lean rats, whereas circulating adiponectin levels are decreased in obese human subjects compared to normal weight controls (11). Results from any animal study are difficult to extrapolate to a human population for multiple reasons. However, for safety and ethical considerations, the rat is a convenient and accessible animal to handle and study. Findings derived from an animal study may not directly apply to humans; however, they help direct research that is aimed at providing benefit to human conditions.

- Flaxseed oil. *Strength.* Our findings indicate that the plant-based source of omega-3 fatty acid, ALA-rich flaxseed oil, improves adipocyte size in obese rats. Compared to marine sources, ALA-rich foods may be a preferred diet source of omega-3 fatty acids for individuals who do not eat fish. In addition, ALA-rich foods may be easier to incorporate into the diet, as they are more frequently consumed and readily available. Furthermore, an ALA-rich food source consumed as part of the diet, and not as a supplement, was effective to change

adipocyte size. For some individuals, consuming food instead of a capsule may be a more acceptable and desirable lifestyle modification. *Limitation.* Zucker rats are capable of converting ALA to EPA and DHA in the body (134) so we cannot rule out the possibility that our results may not be due to ALA alone. However, regardless of being able to confirm the exact form of omega-3 fatty acid responsible for decreased adipocyte size, we can still conclude that consumption of an ALA-rich food source is effective in modulating adipose tissue dysfunction in an obese state. In addition, only flaxseed oil was incorporated into the ALA diet whereas flaxseed provides additional components such as fibre and lignans that may be beneficial.

- Inflammatory markers. *Strength.* A variety of anti-inflammatory and proinflammatory markers of inflammation were measured to assess changes to immune function and the inflammatory state in obesity. In addition, attention was placed on immune-specific organs (spleen) as well as adipose tissue, which actively contribute to the inflammation observed in obesity. *Limitation.* While a host of mediators were measured in our study, the list was not exhaustive and does not represent the entirety of the complex immune response associated with inflammation in obesity.

FUTURE DIRECTIONS

- Investigate the effect of dietary ALA-rich flaxseed oil in obese female rats.
- Determine the effect of dietary ALA-rich flaxseed oil in a younger obese state.
- Increase dose of dietary ALA-rich flaxseed oil to assess a more potent or threshold effect.
- Investigate the effect of dietary ALA-rich flaxseed oil in a higher saturated fat diet.
- Increase duration of dietary ALA-rich flaxseed oil treatment to assess long-term effect.
- Compare the effect of dietary ALA-rich flaxseed oil treatment to fish oil feeding on inflammatory markers in an obese state.
- Evaluate the effect of dietary ALA-rich flaxseed oil on macrophage infiltration in adipose tissue.
- Evaluate the effect of dietary ALA-rich flaxseed oil on mRNA expression, where no changes to protein levels were observed, to determine effect at the transcription level.
- Investigate the other mechanism(s) of dietary ALA action in an obese state. For example, study the effect of dietary ALA-rich flaxseed oil on levels of transcription factors important for managing cellular stress and regulating the immune response, metabolic control, and adipose tissue function in the postprandial state such as phosphorylated JNK, ATF-4, adipsin, PPAR-γ, and iNOS.

IMPLICATIONS

Potential applications of our research project include using dietary intervention to improve the health status of obese subjects. Importantly, we have shown that 8-week consumption of plant-based omega-3 fatty acid, administered as ALA-rich flaxseed oil, reduces adipocyte hypertrophy and favourably alters adipose tissue distribution in a rodent state of obesity. Our data indicate that there are no harmful or detrimental effects associated with dietary ALA-rich flaxseed oil intake in an obese state. Further research on dietary modulation with ALA-rich flaxseed oil, independent of weight loss, is required to determine if this is a potential lifestyle modification that will help to improve adipose tissue function and inflammation in human cases of obesity.

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APPENDIX

Figure 21: Total serum leptin. a) Fasting serum leptin values were adjusted for blood volume (58 mL blood volume/g body weight) to calculate 'total' serum leptin and total serum leptin was expressed relative to b) body weight, c) peri-renal, d) epididymal, and e) visceral adipose tissue. Data are presented as mean ± SEM; n=7 for all groups. lnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters.

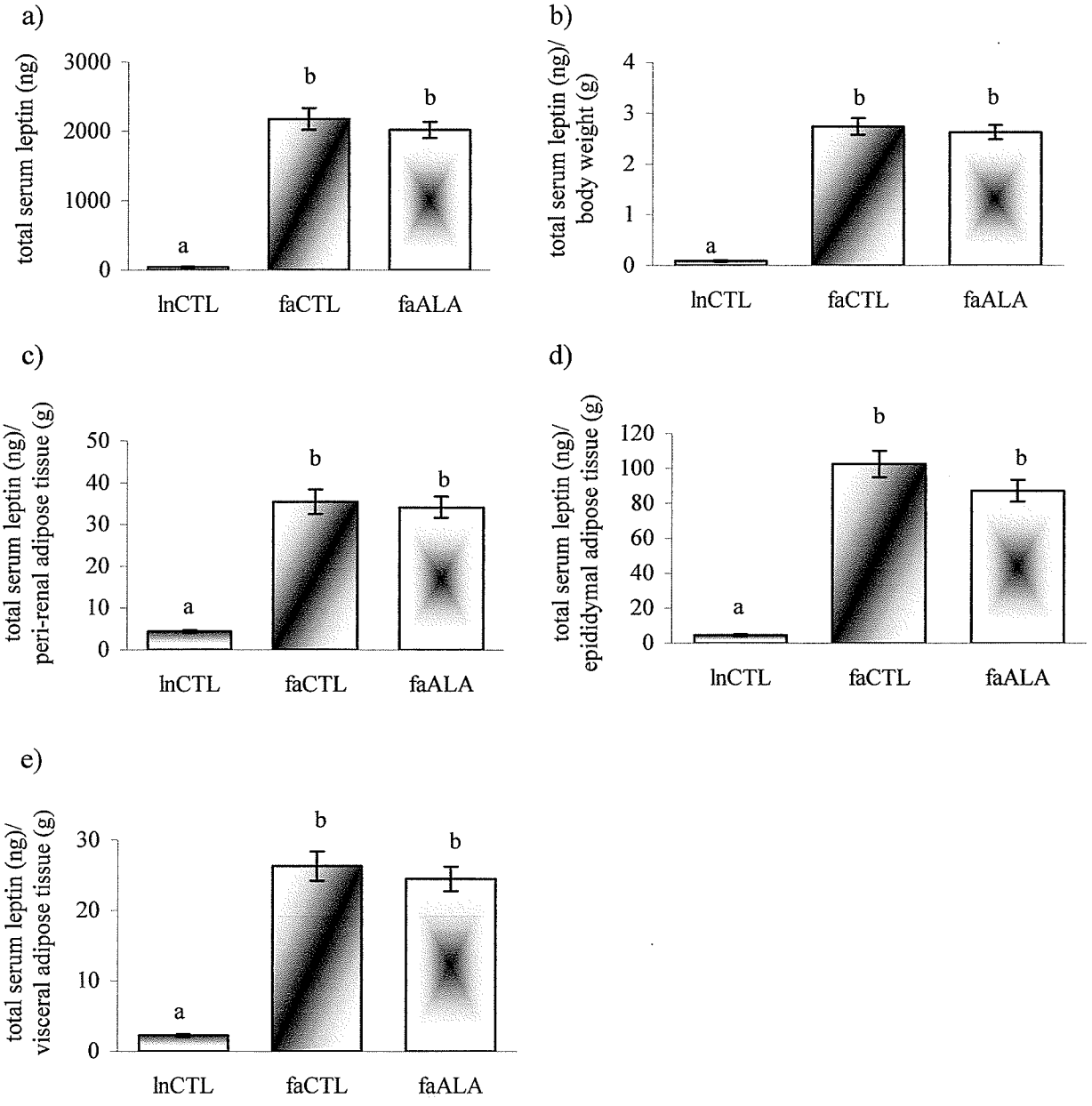


Figure 22: Mature interleukin-18 protein levels in peri-renal adipose tissue. Mature interleukin-18 (IL-18) protein levels were measured in peri-renal adipose tissue samples from lean and *fa/fa* Zucker rats by Western blot analysis. a) Representative Western blot and b) corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data were derived by calculating the ratio of mature IL-18 in absolute values to GAPDH and are expressed as mean ± SEM; n=6 for lnCTL, faCTL and faBASE and n=7 for faALA. LnCTL = lean rats fed control diet, faCTL = *fa/fa* rats fed control diet, faALA = *fa/fa* rats fed ALA diet. No statistical differences.

