DISSERTATION

HORMONAL, METABOLIC, AND SKELETAL MUSCLE ADAPTATIONS FOLLOWING WEIGHT LOSS: EFFECT OF DIETARY FAT TYPE

Submitted by

Dean Joseph Calsbeek

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DEAN J. CALSBEEK ENTITLED HORMONAL, METABOLIC, AND SKELETAL MUSCLE ADAPTATIONS FOLLOWING WEIGHT LOSS: EFFECT OF DIETARY FAT TYPE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Chris Melby

Colin M. Clay

Alan Tucker

Co-Advisor

Advisor

Barbara M. Sanborn

Department Head

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ABSTRACT OF DISSERTATION

HORMONAL, METABOLIC, AND SKELETAL MUSCLE ADAPTATIONS FOLLOWING WEIGHT LOSS: EFFECT OF DIETARY FAT TYPE

These studies investigated the effect of dietary weight-loss and a specific dietary fat type, ALA(18:3n-3), on the hormones leptin and insulin and the peroxisome proliferator activated receptor (PPAR) family of nuclear receptors in the skeletal muscle of obese humans. It was hypothesized that a 10% weight-loss induced by a hypocaloric diet would result in decreased protein concentrations of PPARα and PPARβ but increased protein concentrations of PPARγ, but dietary supplementation with ALA(18:3n-3) would diminish this effect. Furthermore, it was hypothesized that plasma concentrations of leptin and insulin would decrease with weight loss, but that ALA(18:3n-3 supplementation would also reduce this effect.

All subjects were prescribed hypocaloric diets at 80% of resting metabolic rate until 10% weight-loss was achieved. In addition, a subset of subjects exchanged some of their dietary fat with a flaxseed oil capsule supplement (~60% ALA(18:3n-3)) thereby increasing the dietary intake of ALA(18:3n-3) from a typical 0.5% of total caloric intake (control group) to 5% of total caloric intake (ALA group). The dietary composition of the group diets were significantly different for ALA(18:3n-3). This difference was reflected in erythrocyte membrane fatty acid composition, as flaxseed oil supplemented subjects experienced a significant increase of ALA(18:3n-3) composition in the erythrocyte membranes. Muscle biopsies were taken before and after weight-loss in both groups. Western blots for PPAR isoforms showed that PPARβ significantly increased with dietary weight-loss. The effect of weight-loss was reversed, however, in subjects supplemented with ALA(18:3n-3). A similar, but

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non-significant trend was seen for PPARα, and the opposite effect (also non-significant) was observed for PPARγ.

These observations indicate that skeletal muscle concentrations of the PPAR isoform proteins fluctuate to reflect the metabolic state of the tissue rather than to protect the tissue from energy deprivation, as hypothesized. These changes, however, may be negated by supplementing the diet with ALA(18:3n-3). Plasma concentrations of leptin and insulin decreased significantly for both groups, but there was no effect of ALA(18:3n-3) supplementation for either hormone. These findings may be important in future treatment of obesity. The significance and relationship of these observations is discussed, in addition to ideas for future research.

Dean Joseph Calsbeek
Department of Physiology
Colorado State University
Fort Collins, CO 80523
Spring 2003

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CHAPTER 1

LITERATURE REVIEW

Obesity is a chronic health condition most simply defined as an excessive accumulation of adipose tissue. It is a condition that raises the risk for morbidity from hypertension, dyslipidemia, type 2 diabetes, coronary heart disease, stroke, gallbladder disease, osteoarthritis, sleep apnea and respiratory problems, and endometrial, breast, prostate, and colon cancers (267). Because of the significant and independent association of obesity and adult weight gain with morbidity and mortality (332), the National Heart, Lung, and Blood Institute (NHLBI) and the World Health Organization (WHO) have published criteria and a classification of overweight and obesity according to a body mass index (BMI) (258, 267). BMI is the division of a person's mass by height squared (kg/m^2). Persons whose BMI is less than 18.5 are considered underweight and those with a BMI greater than 24.9 fall into four categories: 25-29.9, overweight; 30-34.9, Class I obesity; 35-39.9, Class II obesity; and $\geq$ 40, Class III obesity (258, 267).

The Prevalence of Obesity

Approximately 20% of people in the United States are obese, and the prevalence is even higher in certain subgroups such as non-whites (101). There is evidence of an increase of obesity worldwide, especially in countries experiencing

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rapid economic growth (321). Logical causes of the rise in obesity are increased quantities of energy dense food available per capita and lower physical activity. Mechanization of many types of work and changes in transportation has allowed people to be sedentary more of the time. Furthermore, socio-economic factors, age, and gender contribute to the development of weight gain and obesity (321). The obesity epidemic is a complex but preventable condition that needs to be combated with lifestyle modification on a global level.

The Economic Cost of Obesity

The epidemic of obesity, as it is now commonly called, has a profound economic impact. Although estimates vary, the direct and indirect costs of obesity have increased over the last 20 years (57, 58, 277, 389, 390). Colditz estimated that in 1986, obesity had an economic cost of $39.3 billion in the U.S., accounting for approximately 5.5% of U.S. health care costs (57). Wolf and Colditz estimated that the cost of obesity in the U.S. had risen to $68.8 billion by the year 1990 (389). In a separate publication, Wolf and Colditz approximated that $45.8 billion (6.8% of health care expenditures) could have been saved in 1990 alone if obesity had been prevented (391). Estimates of the cost of obesity in the U.S. in 1995 range from $70 to $99.2 billion (58, 390). The cost of obesity is not a problem only in the U.S. Similar costs have been reported in other western, industrialized countries (26, 201, 215). Oster *et al.* described the economic benefits of weight loss for obese persons (269). In addition to increasing life expectancy, a mere 10% weight-loss can reduce

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expected medical costs up to $5,300 (269). The magnitude of an individuals' obesity-related health costs are influenced by the degree of adiposity (350). Associated disease risks and concomitant costs increase with increasing BMI (350). In summary, the lifetime health and economic consequence of obesity are extensive and imply that efforts to prevent or reduce this problem are eminent.

Co-morbidities of Obesity

Coronary heart disease (CHD) has been closely linked to obesity. The Framingham Heart Study (113, 161), the Nurses' Health Study (229), and the U.S. Male Health Professionals Study (296) have each demonstrated that obesity is an independent risk factor for CHD. In the Nurses' Health Study (229) and the U.S. Male Health Professionals Study (296), overweight individuals with an elevated BMI (25-29.9 kg/m^2) doubled their risk for CHD. In fact, the risk for CHD progressively increased across all BMI categories. CHD is not the only disease to be significantly associated with excess adiposity. Excess body weight is also the strongest single predictor of hypertension (241). According to articles by Eckel (87) and Heyka (146), 40-70% of U.S. hypertension cases are associated with obesity. As reviewed by Melanson (241), overweight and obesity are also commonly associated with other risk factors such as dyslipidemia, Type 2 diabetes mellitus, and hemostatic risk factors that exacerbate the incidence of cardiovascular disease.

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Lipid biochemistry and digestion

The word lipid refers to a heterogeneous group of substances, having in common the property of insolubility in water, but solubility in non-polar solvents such as chloroform, hydrocarbons and alcohol. Lipids are hydrocarbons representing highly reduced forms of carbon, and when metabolized, yield large amounts of energy. Although lipids in general include a large array of molecules, the focus of this discussion is on fatty acids rather than sphingolipids, waxes (long-chain alcohols), and terpenes.

Fatty acid nomenclature is based primarily on the longest chain of carbons within the hydrocarbon chain, counting from the hydroxyl carbon. Most fatty acids have an even number of carbons due to the nature of fatty acid synthesis, but odd numbered fatty acids do exist. The focus of this discussion is on saturated (straight chain) and unsaturated fatty acids; fatty acids can also be categorized as branched, cyclic-, oxy-, and conjugated fatty acids. The saturation or unsaturation of a fatty acid refers to the absence or presence of carbon to carbon (carbon-carbon) double bonds, whereby saturated fatty acids have no double bonds and the carbon atoms are 'saturated' with hydrogen atoms. Likewise, unsaturated fatty acids can have one (monounsaturated fatty acid, MUFA) or more carbon-carbon double bonds (polyunsaturated fatty acid, PUFA). PUFAs are ultimately derived from MUFAs by processes of elongation and desaturation but have unique physical and metabolic properties due to multiple double bonds. The number and location of double bonds largely determines the metabolic fate of unsaturated fatty acids. Furthermore,

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unsaturated fatty acids are differentially classified by the number of carbons between the terminal carbon (-CH_3, the *omega* (ω) carbon) and the last double bond. For example, an 18-carbon unsaturated fatty acid with a double bond at the fifteenth carbon-carbon bond is termed an omega-3 (ω-3, n-3) fatty acid, where had the last double bond of the hydrocarbon been at the twelfth carbon-carbon bond it would be called an omega-6 (ω-6, n-6) fatty acid. This is referred to as the omega nomenclature. Delta nomenclature includes the direction of each carbon-carbon double bound (i.e. cis- or trans-), the location of each double bond, the total number of carbons in the fatty acid, and then the number of double bonds. Examples include cis-9-18:1 (oleic acid), all-cis-9, 12-18:2 (linoleic acid) and cis-9, trans-11, trans-13-18:3 (alpha-eleostearic acid). The majority of the following discussion will use the omega (ω) nomenclature.

Dietary fat (i.e. triglycerides) is degraded for absorption mostly in the stomach and duodenum. Short-chain fatty acids are easily absorbed by the villi lining the intestinal mucosa and redistributed via the blood stream. Long-chain fatty acids, on the other hand, are absorbed by epithelial cells of the villi where new triacyl-glycerides are formed and subsequently packaged into chylomicrons. Chylomicrons are then transported via the lymphatic system into the bloodstream and to other tissues to be utilized or stored (24).

Fatty acids are chiefly catabolized by a cyclical process called β-oxidation where the bond between the alpha and beta carbons of the hydrocarbon chain is cleaved, releasing a two-carbon acetyl-CoA. Fatty acids are also synthesized within

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cells. The primary process of mammalian fatty acid biosynthesis produces saturated fatty acids at a maximum length of sixteen carbons. Separate processes are utilized to desaturate and further elongate these hydrocarbon chains. Mammalian cells lack the ability to add double bonds between the terminal methyl carbon (omega) and a double bond at the ninth position (between carbon 9 and 10). Plants, however, readily desaturate oleic acid (18:1n-9), for example, at the twelfth position to create linoleic acid [LA(18:2n-6)], or at the twelfth and fifteenth position to create α-linolenic acid [ALA(18:3n-3)]. Fatty acids such as these are important for normal growth and life in mammalian cells, but can only be incorporated into cells via digestion rather than biosynthesis. It is for this reason that humans must digest what are commonly called *essential fatty acids*. A few examples of essential fatty acids are LA(18:2n-6), columbinic acid (18:3n-6), γ-linolenic acid (18:3n-6), and ALA(18:3n-3). Essential fatty acids are important components of a daily diet not only because of an inability to synthesize them, but because they are precursors for other important biological molecules. Linoleic acid (18:2n-6) is an essential precursor for arachidonic acid [AA(20:4n-6)], a subsequent precursor for class 2 prostaglandins.

The Health Benefits of PUFAs

The health benefits of dietary polyunsaturated fatty acids were first proposed by Bang and Dyerberg (12, 85), who noted the relationship between a low rate of death from cardiovascular disease and a high consumption of fish among the Greenland Inuit. The typical Inuit diet, comprised of mostly fatty fish, is low in

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saturated but high in polyunsaturated fatty acids. It was then hypothesized that the long-chain polyunsaturated fatty acids eicosapentaenoic acid [EPA(20:5n-3)] and docosahexaenoic acid [DHA(22:6n-3)], fatty acids found exclusively in marine phytoplankton and marine animals, were the key components of the Inuit diet providing cardiovascular disease risk benefits. It is highly unlikely that a complex disease such as cardiovascular disease is attributable to a single dietary component; however, fish oil intervention studies have provided evidence of improving the status of risk factors associated with cardiovascular disease such as hypertension, elevated triglycerides, and increased platelet aggregability. Epidemiological studies have provided additional evidence to support this relationship.

Hypertension is a risk factor for cardiovascular disease (340) that can be improved with the consumption of fish oils. Pauletto *et al.* (273) found mean blood pressure to be lower in a fish-eating group of Tanzanian Bantu villagers than in a nearby vegetarian group. Kestin *et al.* (180) reported a significant decrease in systolic blood pressure after six weeks of EPA(20:5n-3) and DHA(22:6n-3) supplementation (3.4 grams/day). Lastly, Yosefy *et al.* (396) observed a significant reduction of systolic and diastolic pressures in hypertensive individuals after only 13 days of Alsepa fish oil consumption. However, a recent study by Kriketos *et al.* (194) produced results contrary to these reports. A weight loss paradigm of approximately 10% in moderately obese, moderately hypertensive subjects was effective in reducing blood pressure, irrespective of the dietary treatment (saturated fatty acid, n-3 PUFA, or n-6 PUFA). The decrease in blood pressure was highest in the n-3 PUFA-fed

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group during the weight loss phase of the study. However, the added benefit of weight loss with an n-3 PUFA-enriched diet on reduced blood pressure was lost during the weight maintenance phase as compared to the saturated fatty acid fed group.

Elevated triglycerides are an independent cardiovascular risk factor (9). This condition can be improved with the consumption of fish lipids (140, 180, 205, 239, 273, 326). A study of hypertriglyceridaemic patients showed a decrease in triglycerides after two months of daily fish oil supplements (3.9 grams/day) while soya oil-fed subjects had an increase in triglycerides. Interestingly, there was an inverse relationship between the amount of fish consumed and the effect of fish oil supplementation on hypertriglyceridaemia (326). McManus *et al.* (239) also reported a lowering effect of fish oil supplementation on plasma triglycerides levels. Furthermore, Gerster (118) reported that reduced platelet aggregability and increased bleeding time have consistently been observed after fish oil supplementation, without a pathological tendency to bleed.

Epidemiological studies have provided confirming evidence that moderate fish consumption has a cardioprotective effect. A 20-year follow-up study of men in Zutphen, the Netherlands, showed that death due to coronary heart disease was greater than 50 percent lower in men who consumed 30 or more grams of fish per day than those who did not eat fish (197). Daviglus *et al.* (74) categorized 1,822 cardiovascular disease-free men, aged 40 to 55 years, based upon a detailed diet history of fish consumption. A 30-year follow-up determined that mortality from

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coronary heart disease is inversely associated with the amount of fish consumed. The Health Professional Follow-up Study discovered that the risk of death from coronary heart disease was 20% lower in men that ate moderate amounts of fish than in those that ate none. Furthermore, more than one or two portions per week did not provide any additional benefit in reducing CHD risk (7). These, and many other studies, indicate that even two portions of fatty fish per week (35 g of fish daily) are associated with a diminished risk of death from coronary heart disease. The British Nutrition Foundation interpreted these findings and subsequently recommended eating fatty fish two to three times a week (117).

The recommendation by the British Nutrition Foundation to consume two to three portion of fish per week is comparable to 3 to 4 grams of standard fish oil per day, or 1.25 grams of EPA(20:5n-3) and DHA(22:6n-3). The typical Western diet consists of approximately 0.25 grams per day (117). Whether or not this void can be improved by consuming ALA(18:3n-3), the parent fatty acid of EPA(20:5n-3) and DHA(22:6n-3), has been a topic of research interest.

ALA(18:3n-3) is not an extremely prevalent dietary fatty acid, but can be obtained from several sources. Soya oil, rapeseed oil, and flaxseed oil have the highest concentrations of ALA(18:3n-3) at 7-10%, 10%, and 45-50%, respectively (117). The metabolic means by which ALA(18:3n-3) is converted to EPA(20:5n-3) and DHA(22:6n-3) is displayed in Figure 1-1. ALA(18:3n-3) competes for the elongation and desaturation enzymes with another 18-carbon fatty acid, LA(18:2n-6), which is ultimately converted to AA(20:4n-6). The Δ6-desaturase enzyme

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preferentially desaturates ALA(18:3n-3) over LA(18:2n-6), but because LA(18:2n-6) is consumed at levels far exceeding ALA(18:3n-3) consumption, ALA(18:3n-3) is at a competitive disadvantage. Emken *et al.* (89) used deuterated LA(18:2n-6) and ALA (18:3n-3) to investigate the effect of dietary LA(18:2n-6) on the conversion of LA(18:2n-6) and ALA(18:3n-3) to their respective n-6 and n-3 metabolites. ALA(18:3n-3) was estimated to have a 7-fold higher conversion rate than LA(18:2n-6). However, when LA(18:2n-6) was increased from 15 to 30 grams per day, both LA(18:2n-6) and ALA(18:3n-3) conversion decreased by 40-54%. These metabolic pathways are thought to be modified by other dietary factors such as n-6:n-3 ratios and polyunsaturated:saturated (P:S) fatty acid ratios (38, 186). There have been several studies investigating how plausible ALA(18:3n-3) is as a source of EPA(20:5n-3) and DHA(22:6n-3). Lipid profiles of vegetarians have been used to study ALA(18:3n-3) conversion to EPA(20:5n-3) and DHA(22:6n-3) because a plant food diet contains only scarce amounts of EPA(20:5n-3) and DHA(22:6n-3), but normal to high levels of ALA(18:3n-3). Sanders *et al.* (305) compared the fatty acid profiles of vegans versus omnivore controls. The AA(20:4n-6) levels were similar between the two groups, but vegan levels of EPA(20:5n-3) and DHA(22:6n-3) were less than half of omnivore levels. Unfortunately, dietary fatty acid intakes were not recorded for that study. A similar study was performed by Ågren *et al.* (1) in which erythrocyte and platelet AA(20:4n-6) concentrations were similar between vegans and omnivore controls, but EPA(20:5n-3) was only a third and DHA(22:6n-3) a half of omnivore levels. Dietary analysis revealed that omnivores had considerably lower

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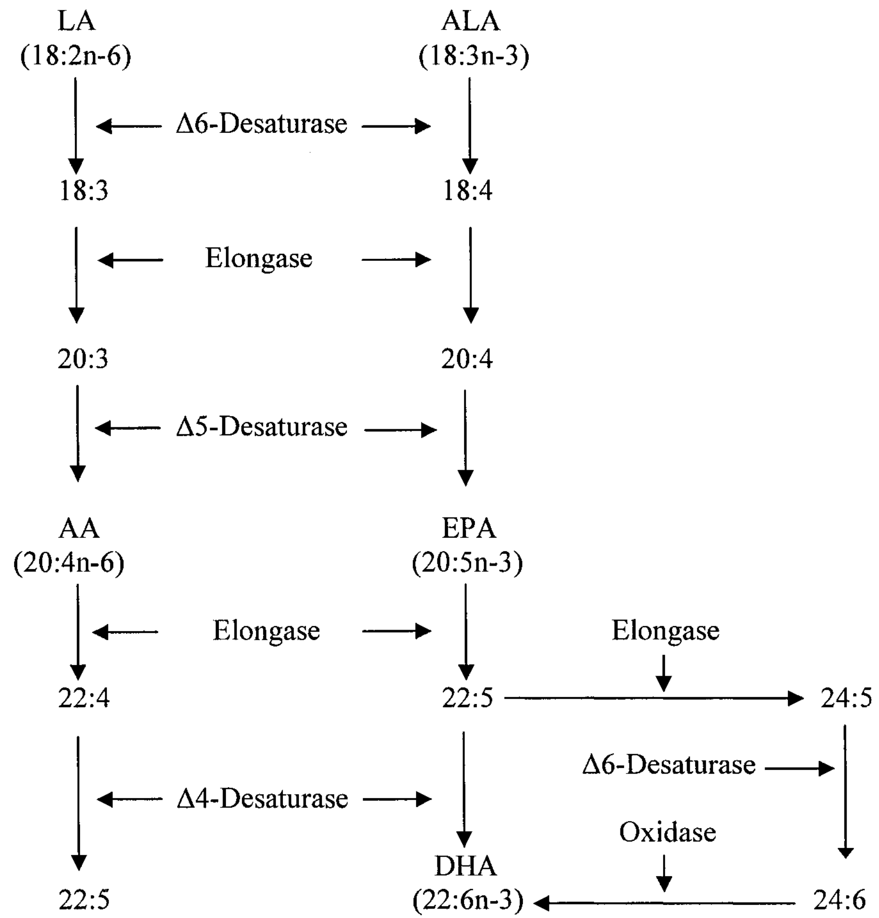

adapted from Gerster H. (1998) *Internat. J. Vit. Nutr. Res.* **68**: 159-173.

FIGURE 1-1

Metabolic pathway of LA(18:2n-6) and ALA(18:3n-3) conversion to their long-chain metabolites.

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consumption of LA(18:2n-6) and ALA(18:3n-3), but higher levels of saturated fat consumption. Dietary EPA(20:5n-3) and DHA(22:6n-3) were nonexistent in vegan diets. Altogether, this indicates that even relatively high levels of dietary ALA(18:3n-3) is inadequately converted to compensate for lacking levels of EPA(20:5n-3) and DHA(22:6n-3), which are otherwise ingested at moderate levels in a mixed diet. Two distinct Tanzanian communities were studied by Pauletto *et al.*, one eating a diet extremely high in fish and the other strictly vegetarian (273). As mentioned earlier, the fish-eating tribe had significantly lower blood pressures, but not mentioned were the significantly lower plasma concentrations of total cholesterol, triglycerides, and lipoproteins. The proportion of EPA(20:5n-3) and DHA(22:6n-3) in plasma lipids were significantly lower in the vegetarian group (2.3 versus 0.7% for EPA(20:5n-3) and 5.7 versus 1.5% for DHA(22:6n-3)) (273).

More studies investigating ALA(18:3n-3) to EPA(20:5n-3) and DHA(22:6n-3) conversion have not involved vegetarians. Platelet EPA(20:5n-3) levels increased moderately in healthy volunteers after supplementing their diets with flaxseed oil [9.4 grams ALA(18:3n-3)], but DHA(22:6n-3) levels were not affected (306). However, when the same subjects were subsequently given marine oil [6 grams EPA(20:5n-3) + DHA(22:6n-3)], both metabolites were increased significantly. Singer *et al.* (328) supplied subjects with high daily amounts (60 gm) of flaxseed oil [38 grams ALA(18:3n-3)] or sunflower seed oil [45 grams LA(18:2n-6)], or two cans of mackerel [5 grams EPA(20:5n-3) + DHA(22:6n-3)]. Similar to other studies, flaxseed oil was unable to increase the levels of EPA(20:5n-3) and DHA(22:6n-3) in

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plasma cholesterol esters. A controlled double-blind study resulted in a 7-fold and 2-fold increase in plasma triglycerides values of EPA(20:5n-3) and DHA(22:6n-3), respectively, after 6 weeks of fish oil supplementation. Six weeks of flaxseed oil supplementation resulted in a 2-fold increase in plasma EPA(20:5n-3) levels, but had no effect on DHA(22:6n-3) (180). Other studies have produced similar results, indicating rather conclusively that ALA(18:3n-3) conversion to its longer metabolites, EPA(20:5n-3) and DHA(22:6n-3), is inefficient and cannot compete with the levels of EPA(20:5n-3) and DHA(22:6n-3) obtained from a mixed diet (71, 86, 177, 230, 307, 362).

As mentioned previously, ALA(18:3n-3) conversion is affected by other dietary factors. A reduction of the LA(18:2n-6):ALA(18:3n-3) ratio from 27.4 to 2.7 doubled the EPA(20:5n-3) content in phosphatidylcholine, phosphatidylethanolamine, and alkenylacyl glycerophosphoethanolamine. DHA(22:6n-3) levels decreased, although not significantly (47). Layne *et al.* (205) studied ALA(18:3n-3) conversion to its long-chain metabolites by prescribing diets with a high (0.87) or low (0.48) ratio of dietary polyunsaturated fat to saturated fat (P/S). Flaxseed oil supplementation significantly increased plasma EPA(20:5n-3) for the high P/S diet but not for the low P/S diet, indicating that the efficiency of ALA(18:3n-3) conversion is partly dependent on the composition of diet. The study by Emken *et al.* (89) concluded that ALA(18:3n-3) conversion to its long-chain n-3 metabolite is seven times more efficient than that of LA(18:2n-6), but conversion of both parent fatty acids is reduced when dietary LA(18:2n-6) is increased from 15 to 30 grams per day. The authors

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conclude that two grams of ALA(18:3n-3), in the context of a typical Western diet, would provide 300 milligrams of n-3 long-chain PUFAs [EPA(20:5n-3) + DHA(22:6n-3)], or about 25% of the British Nutrition Foundation's dietary recommendation. Alternatively, Mantzioris *et al.* (230) did not find a preferential conversion of ALA(18:3n-3) over LA(18:2n-6). When LA(18:2n-6) consumption was low, there was no relationship between LA(18:2n-6) and AA(20:4n-6), while ALA(18:3n-3) was correlated to EPA(20:5n-3) concentrations in the phospholipids of neutrophils, mononuclear cells, erythrocytes, and platelets at the same dietary consumption level. However, at a higher level of LA(18:2n-6) consumption (20 grams), AA(20:4n-6) comprised 11% of plasma phospholipid and 13% of neutrophil phospholipids. The same amount of ALA(18:3n-3) consumption resulted in only 2.5% EPA(20:5n-3) in plasma phospholipids and 0.8% in neutrophil phospholipids. Lastly, a rat study found that the amount and composition of dietary protein influenced the activity of Δ6-desaturase, thereby modifying the amount of AA(20:4n-6) and DHA(22:6n-3) converted from their precursors LA(18:2n-6) and ALA(18:3n-3) (286).

ALA(18:3n-3) is less than equivalent to EPA(20:5n-3) and DHA(22:6n-3) regarding health benefits, most likely because of insufficient conversion of ALA(18:3n-3) to its long-chain metabolites. Layne *et al.* (205) fed subjects 35 mg/kg body weight/day of flaxseed oil had no effect on plasma triglycerides, whereas a comparable amount of fish oil resulted in a significant decrease. Silva *et al.* (326) fed 12 grams of soya oil [10% ALA(18:3n-3)] or fish oil to subjects for 4 weeks. The

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soya oil fed group experienced a 20% increase in triglycerides while the fish oil group had a beneficial 28% decrease in triglycerides. Ferretti and Flanagan (98) modified the n-6:n-3 content of diets from 28:1 to 1:1 and found urinary excretion of 11-dehydrothromboxane B2 and 2,3-dinor-6-oxo- prostaglandin F1 alpha to be reduced by 34% from baseline levels. The authors conclude that ALA(18:3n-3) modulated thromboxane and prostacyclin biosynthesis effectively and further expect that eicosanoid-mediated effects of ALA(18:3n-3) are similar to those elicited by marine lipids. Lastly, de Lorgeril *et al.* (77) studied the effect of an ALA(18:3n-3)-enriched diet on the reoccurrence of myocardial infarction. The experimental group (n=302) consumed less LA(18:2n-6) but more oleic acid (18:1n-9) and ALA(18:3n-3) than the control group (n=303). As a result, there were more cardiac events in the control group (16 deaths, 17 non-fatal) than in the experimental group (3 deaths, 5 non-fatal) during the following 27 months.

Effect of PUFAs on transcription

Polyunsaturated fatty acids have been shown to have the unique ability to modify, over a short time course, the expression of key lipogenic and glycolytic enzymes (6, 28, 172). For example, there is a marked decreased in rat hepatocytes of fatty acid synthase (FAS) and S14 mRNA expression after only three hours of PUFA ingestion (55, 172). When the PUFA was removed from the diet, the suppression of gene transcription was lost in less than 3 hours (55). Some of the factors altered in this way include glucokinase (GK), pyruvate kinase (PK), fatty acid synthase (FAS),

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malic enzyme (ME), and the S14 protein. Studies on cultured hepatocytes showed that ALA(18:3n-3), LA(18:2n-6), AA(20:4n-6), and EPA(20:5n-3) were equally effective in suppressing the transcription of PK, FAS, and S14 mRNA (172). The means by which PUFAs effect transcription in such an acute manner have remained unclear. However, a subclass of the steroid receptor super-family was recently cloned in several tissues. The novel ligand-dependent transcription factors are called peroxisome proliferator-activated receptors (PPAR) (168, 357).

Nuclear receptors have similar structures with an N-terminal domain (A/B) that varies in size and sequence homology, a highly conserved DNA binding domain (C), a weakly defined hinge region (D), a ligand-binding domain (E), and a variable C-terminal domain (F) (See Figure 1-2). Like other nuclear receptors, PPARs contain a DNA-binding domain that distinguishes response elements in the promoters of target genes. This response element is referred to as a PPAR-response element (PPRE), and is a direct repeat, with a one base pair spacing, of the sequence TGACCT(T/A)TGACCT (196). PPAR DNA-binding domains contain two zinc fingers that specifically bind the PPRE sequence in the regulatory region of target genes (204). The ligand-binding domain is comprised of 13 α-helices and a small β-sheet. Crystal structure studies have determined that the ligand-binding domain area is relatively large, compared to other nuclear receptors, and could explain how PPARs are able to network with a broad range of natural and synthetic ligands (263, 393). To that end, a ligand-dependent activation domain, termed AF-2, is involved in

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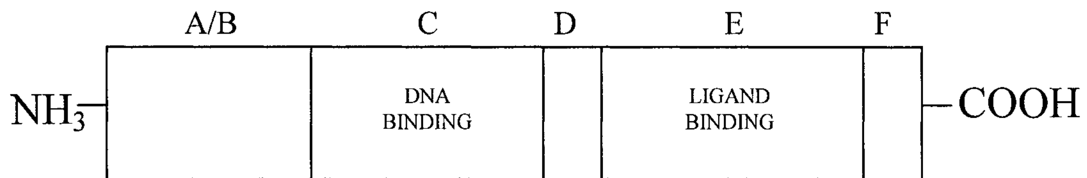

FIGURE 1-2

The general structural organization of nuclear receptors. The N- and C-terminals (A/B, F) vary in size and sequence homology. The DNA binding region (C) is highly conserved among nuclear receptors. The hinge region (D) is not well-defined while the ligand-binding domain (E) is receptor specific, but highly conserved between PPAR isoforms, as it is involved with ligand binding, dimerization, and transcriptional activation.

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the binding of co-activators within the ligand-binding domain (263). Alternatively, a ligand-independent activation function, AF-1, is located in the A/B domain at the N-terminus (382).

Three PPAR isoforms have been described, namely PPARs α (168), β(δ) (313), and γ (88, 126, 401). PPAR function is dependent on heterodimerization with the cofactor retinoid X receptor (RXR) (249). The RXR has three isoforms, α, β, and γ, which do not necessarily correspond with the PPAR isoforms (i.e. RXRα dimerizing with PPARα). Each of the RXR isoforms bind to and are activated by 9-*cis* retinoic acid (228).

The study of PPARs has uncovered a plethora of putative roles and functions for the nuclear receptors. PPARα and PPARγ have been implicated in vasculature-related events, including atherosclerotic plaque formation and stability, vascular tone, and angiogenesis (279). PPARγ has been proven to have an anti-inflammatory role in colon inflammation (345). All three isoforms have been studied as regulators of cellular differentiation (354) and the pathophysiology of carcinogenesis (84, 143, 272). PPARβ function has been more difficult to elucidate, but recent reports have shown that PPARβ has a role in the central nervous system. PPARβ may function in the process of myolinogenesis and glial cell maturation (35). PPARβ has also been researched in the area of fertility, where it has been shown that it may affect embryo implantation (218). Only very recent studies have indicated that PPARβ may play a critical role in lipid metabolism (254). Despite the numerous areas of research in

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which PPARs have been studied, the remainder of this discussion will focus primarily on the role of PPARs in lipid homeostasis and glucose metabolism.

Because each of the PPAR isoforms respond preferentially to different ligands, are expressed preferentially in different tissues, and regulate the transcription of different factors, they will be reviewed individually.

PPARα

PPARα, the first of the isoforms to be identified, is expressed most highly in liver and mediates transcription effects of drugs that induce peroxisome proliferation (168). PPARα was first discovered when Issemann *et al.* were searching for the molecular target of peroxisome proliferating agents in rodent livers (168). Since then, numerous natural and synthetic ligands have been identified.

PPARα can be activated by a wide variety of saturated and unsaturated fatty acids (103, 125, 185). Palmitic (16:0), oleic(18:1n-9), LA(18:2n-6), and AA(20:4n-6) bind PPARα with micromolar affinity, but it is unclear if these concentrations reflect cellular concentrations (185). Forman *et al.* (103) reported that short-chain FAs and medium chain FAs were poor and weak activators of PPARα, respectively. The PUFAs LA(18:2n-6), ALA(18:3n-3), γ-linolenic acid [GLA(18:3n-6)], AA(20:4n-6), EPA(20:5n-3), and DHA(22:6n-3), however, all bind to and effectively activate PPARα. Fatty alcohols and very-long-chain unsaturated fatty acids fail to bind and activate PPARα. The authors concluded that natural PPARα ligands can be generally described as long-chain monocarboxylic acids (103).

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Amphipathic carboxylic acids known as fibrates (phenoxyisobutyrate) are also PPARα ligands. Clofibrate was the first of the fibrate class used to elicit lipid-lowering effects almost 30 years prior to the discovery of PPARα (352). Other fibrates such as fenofibrate, bezafibrate, and WY-14643, a 2-arylthioacetic acid analogue of clofibrate, have shown to be effective agonists for PPARα (and weakly for PPARγ) and are given in high doses (300-1200 mg/day) to humans to elicit a lipid-lowering affect. The ureidofibrate GW9578 has been shown to be a ligand selective for PPARα at nanomolar concentrations and still be efficacious in lowering lipids in humans (37).

In normal rats, PPARα mRNA and protein is preferentially expressed in tissues known for high levels of mitochondrial and peroxisomal β-oxidation activity such as liver, kidney, heart, and intestine (356). This pattern of expression is similar in humans with high levels of PPARα expression in liver, kidney, heart, and skeletal muscle, and low levels in the brain and lungs (35, 252). An interesting observation is that hepatic PPARα mRNA and protein levels follow a diurnal pattern, similar to that of circulating glucocorticoids (211, 212), indicating that PPARα transcription may be regulated by stress. This response was not seen in the hippocampus, however, emphasizing the fact that PPARα regulation, and possibly function, is tissue specific.

Once activated, PPARα stimulates lipid metabolism by inducing peroxisomal β-oxidation and FA ω-hydroxylation (316). In rodents, PPARα activation brings about peroxisome proliferation in the liver, as well as hepatomegaly and

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hepatocarcinogenesis (168). Although PPARα is activated by the same agents in humans, these toxic effects are not evident in human tissues (41), indicating that there are differential effects of PPARα ligands between species.

An important role linked to PPARα is the regulation of cellular uptake and metabolism of fatty acids. The mRNA expression and activity of lipoprotein lipase, which hydrolyzes triacylglycerols, is upregulated by PPARα (315). PPARα has been shown to induce the expression of two fatty acid transporters, fatty acid transport protein (FATP) (234) and fatty acid translocase (FAT) (251). Intracellular fatty acid concentrations are imported across the plasma membrane by these proteins to be esterified for the prevention of subsequent cellular efflux. Acyl-CoA synthetase (acyl-CoA ligase or fatty acid thiokinase) activates (i.e. esterifies) the fatty acids as an initial step preceding the β-oxidation pathway with the formation of a thiol ester bond between the fatty acid and thiol group of coenzyme A. Several studies have shown that PPARα ligands stimulate an increase in the transcription of acyl-CoA synthetase (233, 317). Acyl-CoA oxidase, the analogous enzyme in peroxisomes, is also upregulated by PPARα (83). Before acyl-CoA complexes (i.e. activated fatty acids) are fully oxidized via the β-oxidation pathway, they are transported into the mitochondrial matrix. The transport protein carnitine palmitoyltransferase (CPT I) is the enzyme that catalyzes this transport and is strongly induced by PPARα ligands (36, 235, 248, 397). Mitochondrial β-oxidation enzymes such as acyl-CoA dehydrogenases (5, 127) and hydroxymethylglutaryl-CoA synthase (297) are also

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regulated by PPARα. In summary, the role of PPARα is to sense and respond to cellular lipid concentrations by regulating the transcription of factors involved in the catabolism of those lipids. As a result, PPARα knockout mice have a massive accumulation of lipid in their livers after a chronic feeding of a high fat diet. Alternatively, PPARα knockout mice had hypoglycemia, hypoketonemia, hypothermia, and elevated plasma free fatty acids levels when fasted for 24 hours. This indicates that the liver cells were prevented from performing fatty acid uptake and oxidation due to the inability to efficiently induce the transcription of necessary genes (179). Another PPARα knockout study showed that PPARα-null mice experience progressive dyslipidemia and maturity onset obesity. Interestingly, there was a sexual dimorphism in weight gain and lipid accumulation where the accumulation of fat in female mice was 238% of that in control mice while transgenic male mice exceeded control mice by only 150%. Male mice void of PPARα displayed considerable hepatic enlargement as compared to control mice, while no difference was noted in females (65). The reason for sexual dimorphism in weight/fat gain and liver hypermegaly/steatosis has yet to be elucidated, but the study does demonstrate that PPARα is a major player in rodent lipid homeostasis, fat storage, and obesity (65).

Studies of PPARα expression and activity in human tissues have been less prevalent. However, the studies that have been performed provide evidence to support the observations seen in other species. Minnich *et al.* (248) found

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mitochondrial β-oxidation (of palmitate, in particular) to be increased via PPARα in hamster soleus muscle, but also in cultured human liver (HepG2) and skeletal muscle (hSKMU) cells. Furthermore, there was a dose-response to UF-5, a potent PPARα agonist, in decreasing plasma triglycerides (248). Another study used the potent PPARα agonist, GW7647, to elucidate its effect on [^{14}C]oleate metabolism and gene expression in primary human skeletal muscle cells. Consistent with previous observations, PPARα protein increased during cell differentiation and corresponded in a dose-dependent manner to oleate oxidation. GW7647 decreased oleate esterification into triacylglycerides by 45%. Additionally, the mRNA expression of the key β-oxidation genes carnitine palmityltransferase 1, malonyl-CoA decarboxylase, and pyruvate dehydrogenase kinase 4 increased by 2-fold, 2-fold, and 45-fold, respectively (255).

PPARβ

The PPARβ isoform, also known as PPARδ and NUC-1, was first cloned in human tissues in 1992 (314). The expression of this gene is nearly ubiquitous, but has higher levels of expression in brain, adipose, cardiac, skeletal muscle, intestinal and skin tissues (4, 35). Specific gene targets of PPARβ have not yet been identified, but it is evident that it is stimulated by ligands similar to that of PPARα and PPARγ. Natural ligands include saturated and unsaturated fatty acids, notably the PUFAs dihomo-γ-linolenic acid [DGLA(20:3n-6)], EPA(20:5n-3), and AA(20:4n-6) have low

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micromolar affinities for PPARβ (103). The fatty acid selectivity of PPARβ is reported to be intermediate between PPARα and PPARγ (393). A number of eicosanoids and prostaglandins have been shown to activate PPARβ including PGA1, PGD2, and carbaprostacyclin, a semisynthetic prostaglandin (103, 398). Two synthetic ligands have been identified. Both are α-substituted carboxylic acids and were developed in an attempt to define the physiological role of PPARβ. The ligand L-165041 has a 30-fold selectivity for PPARβ over PPARγ, and was inactive on murine PPARα (210). Additionally, Oliver *et al.* reported that GW501516 is PPARβ selective and is beneficial in altering serum lipid parameters in obese insulin-resistant rhesus monkeys (266).

As stated previously, the specific role of PPARβ is unclear, but the protein has been implicated in a wide range of mechanisms. PPARβ is expressed at uterine implantation sites, indicating that the receptor is involved in reproduction. COX-2 null females are unable to implant a blastocyst in the uterus. COX-2 regulates the rate-limiting step in generating the natural PPARβ ligand, prostacyclin. When carboprostacyclin or the PPARβ-selective agonist L-165041 is introduced, implantation was restored in the COX-2 null mice (218). It has been established that PPARβ is the target of tumor suppressor agents in colorectal cancer cells (143). $PPAR\beta^{-/-}$ colorectal cancer cell lines have a decreased ability to form tumors in mice compared to $PPAR\beta^{+/-}$ cancer cells (272). It has also been suggested that PPARβ plays a role in lipid metabolism, neuronal signaling, and myelination in the central

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nervous system (23). Similar to the other PPAR isoforms, α and γ, PPARβ has been shown to be involved in cholesterol and triglyceride metabolism, as well as associated with fasting insulin levels. The PPARβ-specific agonist GW501516 causes a dose-dependent rise in serum HDL, decrease in LDL, fasting triglycerides, and insulin levels in insulin-resistant obese rhesus monkeys. The changes in serum HDL were shown to be a result of changes in the expression of the reverse cholesterol transporter ATP-binding cassette A1, which induces apolipoprotein A1-specific cholesterol efflux (266). This evidence supports an earlier observation in which the PPARβ agonist L-165041 caused modest increases in HDL-cholesterol levels in db/db mice (210).

A recent study provided evidence that PPARβ plays a critical role in lipid homeostasis. PPARα-knockout mice were metabolically challenged with exercise or starvation to induced the transcription of pyruvate-dehydrogenase kinase 4 and uncoupling protein 3, regularly robust gene targets of PPARα. There was no difference in pyruvate-dehydrogenase kinase 4 and uncoupling protein 3 transcription between wild-type and knockout mouse skeletal muscle, but there was in heart tissue. Further analysis of human and rodent myocytes showed that the PPARβ selective agonist GW742, induced a 2-fold increase in β-oxidation and an increased expression of several lipid regulatory genes, including pyruvate-dehydrogenase kinase 4 and uncoupling protein 3 (254). This indicates that in the absence of a potent regulator of skeletal metabolism, PPARα, the high expression of PPARβ compensates and

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regulates lipid oxidation. This is not to say that even in the presence of PPARα, PPARβ does not also play an important role in lipid homeostasis.

PPARγ

Of the three PPAR isoforms, PPARγ has been the most studied. PPARγ is expressed, as a result of alternative splicing and alternative promoter usage, in three forms, PPARγ1, PPARγ2, and PPARγ3. PPARγ2 is expressed primarily in adipose tissue; PPARγ3 is expressed exclusively in adipose and intestinal tissue, while PPARγ1 is expressed nearly ubiquitously, but primarily in heart, skeletal muscle, colon, small and large intestines, kidney, pancreas, spleen, and adipose tissues (93, 222). Despite differences in where the isoforms are expressed, there is no evidence that the PPARγ isoforms have separate roles. Furthermore, there is no indication within the literature that the isoforms differ in ligand preferences or affinity.

PPARγ preferentially binds PUFAs, particularly the essential fatty acids LA(18:2n-6), ALA(18:3n-3), AA(20:4n-6), and EPA(20:5n-3) (393). PPARγ is only weakly responsive to monounsaturated fatty acids (393) and to an AA(20:4n-6) metabolite, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-J_2) (105). The synthetic analogs of 15d-J_2 are called thiazolidinediones (TZD) and have, in addition to being PPARγ agonists, been effective antidiabetic and insulin-sensitizing agents. This family of agents includes, but is not limited to, rosiglitazone, pioglitazone, englitazone, and ciglitazone. Of these, rosiglitazone has the highest binding affinity (K_d of ~40 nM) (22, 386).

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PPARγ is a necessary agent for the differentiation and development of adipose (16, 357), cardiac, and placental tissues (16). This phenomenon has been observed in transgenic mice. Heterozygous PPARγ-null mice have reduced amounts of adipose tissue (16, 199, 299). Dominant-negative PPARγ mutants, when expressed in 3T3-L1 cells, inhibit the differentiation of cells into adipocytes (128). The effect of PPARγ on cellular processes, such as differentiation and adipocyte hypertrophy, may be via the regulation of a number of genes. For example, PPARγ has been shown to regulate a number of genes involved in lipid metabolism. The genes expressed as the proteins aP2, phosphoenolpyruvate carboxykinase (PEPCK), acyl-CoA synthase, fatty acid transport protein –1 (FATP-1), and lipoprotein lipase (LPL) all have a PPRE within their regulatory region and are subsequently regulated by PPARγ (234, 315, 317, 354, 355). Furthermore, genes associated with energy homeostasis are also regulated by PPARγ. Mitochondrial uncoupling proteins (UCP1, UCP2, and UCP3), potential uncouplers of energy harvested from the metabolism of fats and sugars, are also regulated by PPARγ (178). The physiological consequence of this relationship is not known because of the remaining uncertainty regarding UCP function. Leptin mRNA and secretion is down regulated with TZD treatment, most likely mediated by PPARγ, in 3T3-L1 adipocytes (173) and rodents (78), providing more evidence of PPARγ involvement in energy homeostasis. The use of TZDs to treat diabetes and insulin resistance has been rather successful. The means by which TZDs improve insulin sensitivity is through the regulation of a number of genes related to insulin signaling. Tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine, has

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been linked to insulin resistance and impaired insulin signaling. TZD treatment of obese rodents decreased TNF-α expression (150). Furthermore, mice were rescued from TNF-α – induced insulin resistance by treatment with PPARγ agonists (244). Insulin receptor substrate – 2 (IRS-2), a proven role player in insulin signal transduction, increased in cultured adipocytes and human adipose tissue when provided with PPARγ agonists (331). Another protein involved in the insulin signaling cascade is the c-CBL-associated protein (CAP). It has a PPRE within the regulatory region of its gene (19), and is thereby increased via PPARγ activation (291). Lastly, PPARγ agonist treatment increases the levels of the protein associated with decreased glucose, triglycerides, and free fatty acids, called adipocytes-related complement protein 30 (Acrp30) (110). Although the exact mechanism linking Acrp30 to type II diabetes has yet to be determined, type II diabetic patients have decreased levels of Acrp30 (156). Suppressed levels of Acrp30 are normalized with the treatment with the TZD rosiglitazone (61). The general function of PPARγ is to regulate the expression of genes that will promote the uptake and storage of fatty acids and increase the responsiveness of insulin-sensitive tissues by suppressing the levels and actions of factors such as TNF-α and increasing the levels and actions of potential sensitizers such as Acrp30.

Effects of obesity and weight loss on PPAR isoforms

PPARs clearly play a role in adipocyte differentiation and in energy homeostasis, particularly in lipid metabolism. Only a couple of studies have

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investigated the distribution of PPAR isoforms in skeletal muscle as it relates to obesity. Loviscach *et al.* (222) report that all three isoforms are expressed in human skeletal muscle. PPARα protein is expressed more than seven-fold higher in skeletal muscle than adipose tissue, PPARβ protein levels are similar between adipose tissue and muscle, and PPARγ protein is lower in human skeletal muscle, approximately two-thirds of the levels found adipose tissue (222). Kruszynska *et al.* reported that human skeletal muscle PPARγ1 mRNA expression was 10-15% of the level found in adipose tissue, but no PPARγ2 mRNA was detected in skeletal muscle (198). Together, these results indicate that PPARγ expression in skeletal muscle is low as compared to other isoforms, or may be regulated more at the translational level versus the transcriptional level.

The expression of the PPAR isoforms may be dependent on the level of adiposity of an individual. Vidal-Puig *et al.* reported that obese individuals express higher levels of PPARγ2 mRNA in adipose tissue than lean controls (368) but no differences in levels of PPARγ1. There was a strong correlation between PPARγ2/γ1 expression and BMI of the subjects. When the obese subjects were fed a low calorie diet until a 10% weight loss was achieved, PPARγ2 mRNA fell by 25%. This reduction was reversed to pretreatment levels, however, over the course of a 4-week maintenance phase (368). Similar findings were reported by Ribot *et al.* (292). A 6% weight loss was achieved in obese women, resulting in lower PPARγ2 mRNA expression compared to controls. Interestingly, this difference was observed in subcutaneous fat but not in omental fat (292). Bastard *et al.* (18) subjected obese

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women to a very low calorie diet induced weight loss (6 kg) and observed a 13% reduction in PPARγ mRNA in abdominal subcutaneous adipose. None of these studies investigated the level of expression of the other PPAR isoforms (α and β /δ) in adipose tissue or the effect on any of the PPAR isoforms in skeletal muscle.

Hormonal mediators of energy homeostasis

It has been thought for many years that metabolism and satiety are largely regulated by factors circulating throughout the body. Normal or homeostatic levels of energy substrates is critical for whole body function. For example, blood glucose levels must be maintained at 4-5 mM (90-100 mg/dL). The endocrine system, with a coordinated effort from other physiological systems such as the autonomic nervous system, manages the control of blood glucose under conditions presenting excess or shortage of the macronutrient. Important hormones such as insulin, glucagon, thyroid hormone, cortisol, and possibly leptin, mediate these effects. This review will highlight work previously reported regarding leptin and insulin.

Leptin

Caro *et al.* (40) eloquently summarized the classical physiological work of G.R. Hervey in which a ventromedial hypothalamic lesion resulted in obesity in the lesioned, but starvation in the non-lesioned rat of parabiotic rat pair (145). Hervey proposed that a circulating satiety factor was produced excessively by the lesioned animal resulting in (and as a result of) insensitivity, while the non-lesioned parabiont

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starved because of high levels of the circulating satiety factor crossing the parabiotic union. The circulating factor that Hervey hypothesized has now become known as leptin (400).

Leptin is the product of the *ob* gene (400). The word leptin comes from the Greek root leptos, meaning 'thin'. In addition to the parabiosis experiments performed by Hervey (145), other parabiosis experiments were instrumental in understanding the putative role of leptin years before it was cloned in 1994.

Mice with a mutated *ob* gene (*ob/ob*) were prevented from developing obesity when parabiotically unified with normal lean mice (141). Alternatively, the rodent obesity models of *db/db* mice and *fa/fa* rats, defective in their respective gene products, caused normal littermates to die of starvation when parabiotically unified (59, 139). It is now known that *ob/ob* mice do not produce functional leptin (400), while *db/db* mice and *fa/fa* rats produce a mutated leptin receptor (48, 51, 167, 349).

Leptin is a 16 kDa protein that is secreted almost exclusively by adipose tissue (400) and circulates in bound and unbound forms (329). The expression of leptin mRNA in white adipose tissue is in direct proportion to the size of the adipocytes (132). More recent evidence suggests that leptin is also expressed in skeletal muscle (372), stomach (10), brain (90, 385), and placenta (213). Zhang *et al.* (400) report that there is signaling sequence present at the N-terminus of the protein and a disulfide bond present at the C-terminus, which is apparently important for activity (400).

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The leptin receptor (OB-R) is the product of the *db* gene in mice and the *fa* gene in rats (48, 51, 206, 349). OB-R has considerable homology with cytokine receptors, particularly that of the cytokine interleukin-6 (IL-6) (20, 349). Due to alternative splicing, there are several forms of OB-R that can be generally classified as either short (OB-R_S) or long (OB-R_L), although they have been individually named as OB-$R_{a\text{-}f}$ (48, 119, 166, 167, 206, 278, 349). The biological effects of leptin are mediated primarily through the longest form, OB-R_b (349), which has the longest cytoplasmic domain of all of the identified receptors (119, 206). Meanwhile, OB-R_e lacks a transmembrane domain and evidence indicates that this short form is soluble and makes up 10% of circulating binding proteins for leptin (329). In response to leptin, OB-R_b is capable of signaling to insulin receptor substrate (IRS-1), mitogen-activated protein kinase (MAPK) via Janus kinase (JAK), and STAT (signal transducers and activators of transcription). Shorter isoforms, with the exception of OB-R_e, are also capable of signaling through JAK, but at substantially weaker levels (27, 361).

Several reports suggest that the hypothalamus is the primary target for leptin. As mentioned earlier, ventromedial hypothalamus lesions result in obesity (145), and leptin has a more potent anorectic effect when administered centrally rather than peripherally (39, 364). Autoradiographic and immunohistochemical studies have shown that leptin binds at hypothalamic plasma membranes (339, 349), and the leptin receptor is expressed in the hypothalamus (130, 160, 320, 349). Leptin might also act peripherally as several of the leptin receptor isoforms are expressed in peripheral

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tissues (48, 151, 206, 349, 384). To that end, cultured hepatocytes (56), adipocytes (11, 111), haemopoietic cells (111, 119), and pancreatic islet cells (323) respond to leptin *in vitro*.

There are several candidate effector molecules involved in mediating the downstream effects of activation of the hypothalamic leptin receptor. Neuropeptide Y (NPY) and agouti-related peptide (AGRP), both also produced in the hypothalamus, are potent stimulators of food intake. Expression of NPY and AGRP is inhibited by leptin administration (242, 339, 387). NPY-mutated mice remain susceptible to leptin administration indicating that NPY does not act alone. Other candidate genes, which are also centrally active, are melanocyte-stimulating hormone (97, 375), glucagon-like peptide-1 (123, 129), corticotropin-releasing hormone (64, 158), and melanin-concentrating hormone (159, 187, 324).

Shortly after cloning the *ob* gene in 1994 (400), Jeffrey Friedman's group tested the effect of recombinant leptin administered to *ob/ob* and *db/db* mice (131). Daily intraperitoneal injections of recombinant leptin caused *ob/ob* mice to lose significant weight within four days and lost 40% of their original weight in 33 days. The food intake of the treated *ob/ob* decreased significantly within two days, as compared to control mice. Pair-fed *ob/ob* mice also lost weight, but significantly less than those given recombinant leptin, indicating that leptin may also have an effect on metabolic rate. In the same experiment, recombinant leptin administration had no effect on food intake or body weight in *db/db* mice (131). Similarly, Campfield *et al.* (39) found a dose-dependent response to intraperitoneal injections of leptin in *ob/ob*

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mice and mice with diet-induced obesity (DIO). Food consumption was significantly reduced, but returned to normal levels when the leptin treatment was withdrawn. Injections of leptin were also administered to the lateral ventricle through chronically implanted intracerebroventricular (ICV) cannulae. Food intake was also blunted in these treated mice and pre-injection weight loss recidivism was attenuated. No effect was observed in *db/db* mice with either peripheral or central leptin administration (39).

These results were supported by later studies by Banks *et al.* (13) and Van Heek *et al.* (364), who showed that rodents with diet-induced obesity are hyperleptinemic and become resistant to the effects of exogenous leptin administration. Both groups found, however, that when much smaller doses of leptin were administered centrally (i.e. intracerebroventricular cannula), leptin was (and remained) effective. Together, these results indicate that a mechanism at the blood-brain barrier may play a role in the development of leptin resistance.

It has been determined that one of the short leptin receptor isoforms ($OB\text{-}R_a$) is expressed at the choroid plexus and acts to transport leptin across the blood-brain barrier into the cerebrospinal fluid (13, 206). Radioisotope-labeled leptin was transported across the blood-brain barrier at rates comparable to other blood-borne substances of similar size, such as interleukin-1α and TNF-α. Unlabeled leptin inhibited the transport of radiolabeled leptin, indicating that transport mechanism of leptin is saturable (13).

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The administration of recombinant leptin was a common theme of other early animal studies. When *ob/ob*, lean, and diet-induced obese mice were administered leptin they lost significant weight by reducing their food intake and increasing their energy expenditure (17, 274, 376). Similarly, leptin caused a 56% decrease in food intake and a subsequent 4.1% decrease in body weight of *ob/ob* mice (319). Lastly, acute leptin administration resulted in a 31% increase in plasma hypertriglyceridemia by altering the lipid uptake into rat adipose and skeletal muscle tissue.

Recombinant leptin administration trials have also been performed in humans. Heymsfield *et al.* (147) organized a study in which 54 lean and 73 obese men and women self-administered recombinant human leptin by subcutaneous injection at increasing doses of 0 (placebo), 0.01, 0.03, 0.1, or 0.3 mg/kg. Weight loss increased with increasing doses of leptin among all subjects after 4 weeks of daily administration (147). Pre-breakfast appetite decreased when pegylated leptin (PEG-OB) was given to obese men at doses of 20 mg/week for 12 weeks. Hunger and appetite remained higher for those receiving a placebo. There were no changes, however, in body composition, energy expenditure, or body mass loss between groups (383). Similarly, weekly PEG-OB doses of 20 mg (163) or 60 mg (164) to obese men had no effect on amount of weight loss or markers of inflammation (163, 164). Others have found that leptin administration has no effect on the autonomic nervous system (225). Although exogenous leptin administration has profound effects on food intake and energy metabolism in animals genetically deprived of leptin, it has negligible effect on energy metabolism and weight loss in humans. This

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conclusion has inspired a plethora of studies investigating the regulation of serum leptin levels.

In contrast to *ob/ob* mice, obese humans have elevated plasma leptin levels (62), and only a small number of obese humans with a congenital leptin deficiency comparable to that of the *ob/ob* mouse model have been identified (250, 344). Recombinant leptin was administered to one such individual, a 9-year-old with severe early-onset obesity (94). In that study, daily treatment with leptin resulted in a sustained reduction of body weight. It was determined that the body weight reduction, predominantly from a loss of body fat, was due to changes in energy intake rather than changes in total energy expenditure (94). The following paragraphs will describe other studies regarding the physiology of leptin in human obesity, starting with the characterization of serum leptin concentrations in obese versus lean humans.

The cloning of the human *ob* gene was followed by the development of RIA, Western, and ELISA techniques to measure circulating leptin concentrations in human serum (62, 154, 155, 224, 227). The initial studies found that lean individuals had lower levels of circulating leptin when compared to obese individuals. The leptin concentration was found to increase with increased body fat percentage (62, 227). Gender is an important factor as women consistently have higher levels of circulating leptin, independent of body fat percentage or fat mass (304). The strongest difference between sexes in the organization of serum leptin concentrations is in the amount of leptin released (or removed) per unit of time (217).

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The regulation of plasma leptin levels is multi-factorial and therefore complex. As mentioned previously, leptin levels increase with increasing fat mass (62, 227). Energy imbalances that effectively alter the amount of fat storage also modify serum leptin levels. Fasting results in substantially decreased leptin levels (2, 29, 135, 188, 281, 377), whereas overfeeding increases leptin levels (190, 214). Trayhurn *et al.* found that fasting induced a significant decrease in *ob* mRNA in lean mouse white adipose tissue which was rapidly reversed by refeeding (359). Fasting had no effect on *ob* levels in *ob/ob* mice. Frederich *et al.* (107) found similar results, and in addition, found serum levels of leptin protein to be reduced as a result of fasting.

Weight loss expectedly results in a reduced level of leptin (114, 300, 351, 363, 365, 388, 392), primarily because of a loss of fat mass, the tissue origin of leptin. Wing *et al.* (388) subjected 52 overweight and obese women to a weight-loss program. An average of 8.1 kg was lost over a 4-month period resulting in a decrease of leptin concentrations from 30.1 to 20.4 ng/mL. Subjects who maintained their weight loss also maintained a lower level of circulating leptin whereas leptin concentrations returned to higher levels in subjects who did not maintain their weight-loss (388). Another weight loss study found that weight reduction by a 800 kcal/day diet resulted in a significant and rapid decline in leptin within 3 weeks as compared to BMI-matched controls consuming a normal diet (114). Thong *et al.* (351) investigated the effects of diet versus exercise as a means of reducing leptin concentrations. This study found that both diet-induced and exercise-induced weight

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loss induced lowered leptin concentrations, but exercise had no independent effect. To support the observations that leptin levels reflect a change in body weight, Rosenbaum *et al.* (301) instructed subjects to either gain 10% or lose 10% or 20% of their weight. Weight gain resulted in an increase in circulating leptin concentrations, while weight-loss resulted in a dose-dependent (10% or 20% weight loss) decrease in circulating leptin levels (301). The lessening of circulating leptin via weight loss may be the cause for weight recidivism. Rosenbaum *et al.* (300) measured leptin concentrations after a 10% weight loss and found (similar to previous studies) that it was reduced. They then administered recombinant human leptin to restore pre-weight loss concentrations. Twenty-four hour energy expenditure measurements decreased by 500 kcal/day during weight loss, but increased by 335 kcal/day during recombinant leptin administration, an effect possibly mediated by thyroid hormone (300). These findings indicate that maintenance of circulating leptin levels during weight-loss, despite decreased fat mass depots, could help to eliminate the prevalence of weight recidivism after weight loss.

Hormonal factors also regulate leptin levels. Sustained, but not acute, hyperinsulinemia markedly increases plasma leptin concentrations (189, 371). Similarly, glucocorticoids also cause an acute increase in leptin synthesis and secretion (79, 203, 243, 270, 330, 371). Alternatively, isoproterenol (a sympathomimetic compound) and β_3-adrenergic receptor agonists invoke decreases in leptin expression and secretion (80, 216, 232, 294, 358). There is also evidence that

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cytokines such as TNF-α and interleukin-1α (IL-1α) increase serum leptin concentrations (169, 311, 402).

The precise molecular mechanisms that induce the transcription of leptin remain indefinable. Several reports indicate that the peroxisome proliferator activated receptor gamma (PPARγ) (18, 78, 152, 173, 193, 264, 290), sterol regulatory element binding protein-1 (SREBP-1) (290) and CCAAT/enhancer binding protein alpha (C/EBPα) (152, 165, 193, 236, 245) are involved.

The composition of the diet (macro- and micronutrients) also regulates leptin levels. For micronutrients, a few reports have found a link between the intake of zinc and increased leptin concentration (50, 231). Jenkins *et al.* (170) found that a decreased serum leptin concentration was strongly correlated with a reduction in carbohydrate intake, but not changes in protein or fat. The authors suggest that leptin may act to defend the body's carbohydrates stores. Schrauwen *et al.* (318) kept non-obese subjects in energy balance but provided a high-fat diet for one week. Serum leptin concentrations did not change in response to the short-term manipulation of dietary composition. Although not affecting daily mean concentrations of leptin, high-fat diets acutely reduced the diurnal variation of leptin (45). In contrast to these reports, Cooling *et al.* (63) found a higher plasma leptin level among individuals on a high fat diet compared with those on a low fat diet, and observed a correlation between plasma leptin levels and the dietary intake of fat. Reseland *et al.* (289) found that a year of decreased fat consumption resulted in decreased levels of circulating

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leptin beyond that accounted for by losses in fat mass. Yet other studies have investigated the effect of dietary fat *type* on leptin levels.

Rojo-Martinez *et al.* (298) found that serum leptin was significantly correlated with habitual dietary intake of stearic acid (18:0) and EPA (20:5 n-3) in men and LA(18:2n-6) and AA(20:4n-6) in women. There was also a strong correlation between the AA(20:4n-6):LA(18:2n-6) ratio and serum leptin in women (298). The reason for the differences between sexes has yet to be explained. Raclot *et al.* (283) reported that dietary n-3 PUFAs, especially DHA (22:3n-3), modify the concentrations of leptin mRNA in the retroperitoneal, but not the subcutaneous, tissue of rats. Cha and Jones (46) fed rats at 100, 85, or 75 percent of *ad libitum* caloric intakes with either (20% wt/wt) fish oil, safflower oil, or beef tallow for 10 weeks. The changes in serum leptin differed depending on the type of fat consumed. At 100% of *ad libitum* energy intake, beef tallow-fed rats had a 60% lower level of plasma leptin. Furthermore, fish oil- and safflower oil-fed rats experienced a significant decrease in leptin levels with energy restriction whereas no changes occurred in leptin levels for the beef tallow-fed group. The authors concluded that the response of leptin concentration to energy restriction is dependent on dietary fat type, particularly polyunsaturated fatty acids (46).

Insulin

Much like the discovery of leptin, the discovery of insulin was preceded by many years with a general knowledge of its function. J. von Mering and O.

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Minkowski reported in 1889 that complete removal of the pancreas resulted in diabetes (369). Even prior to this event, Paul Langerhans described pancreatic islets in his doctoral thesis in 1869 (284). Although others had postulated it, the internal secretion of the endocrine pancreas was proven to be insulin by Banting and co-workers in 1922 (14, 15).

The structure of insulin was not completed until 1955 by F. Sanger (308), but by 1947 it was already known that insulin lowers blood glucose; stimulates glucose uptake and glycogen synthesis in skeletal and cardiac muscles *in vitro*; inhibits gluconeogenesis in liver; raises the respiratory quotient by inhibiting fat oxidation; is required for fat synthesis from carbohydrate; inhibits protein catabolism; and is required for protein deposition (68, 115, 284). Krahl reported in 1952 that insulin also stimulates the uptake of glucose into adipocytes (191), and in 1956, that insulin stimulates protein synthesis (192).

The insulin gene was one of the first to be isolated (360). It is located on the short arm of human chromosome 11 (137). The expression of the insulin gene, of which most species have a single copy, occurs only in the pancreatic β-cell because the 5'-flanking region contains cell-type specific response elements (109, 338).

Sanger's painstaking effort to determine the structure of insulin revealed a relatively simple protein, consisting of two short chains of amino acids (308). The shorter chain, A (21 amino acids), is held to the longer chain, B (30 amino acids), by two disulfide bridges. There is an additional disulfide bond within the A chain (309). Several theories existed to explain how these two chains were assembled (175, 200),

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but it was eventually established that a longer single-chain protein containing both the A and B chains was originally synthesized and subsequently modified by cleaving an intermediate peptide called C-peptide (53, 335-337). Figure 1-3 depicts the primary structure of human insulin.

Insulin is synthesized and secreted from the β-cells of the pancreas. The primary stimulants of insulin secretion include the substrates that insulin control: glucose, amino acids, and some fatty acids (282, 374). Glucose, however, is the prerequisite; without it, few stimuli can elicit insulin release (240). The rates of insulin transcription and degradation are also regulated by glucose (116, 120, 261, 380), but the most potent effects of glucose are on translation of preproinsulin mRNA (275, 381). Lengthy exposure to glucose leads to highly significant increases in insulin production (268), as well as eventual increases in β-cell mitosis and hyperplasia (348).

Circulating nutrients such as glucose, fatty acids, and amino acids indirectly regulate the release of insulin from pancreatic β-cells into circulation (260). Of these, glucose is unique because it can stimulate insulin secretion independent of the presence of other fuels. Glucose does not utilize cell-surface receptors to elicit a secretion of insulin; rather, it enters β-cells via a Na^+-independent glucose transporter (GLUT), particularly the GLUT 2 isoform (21). The glucose molecule is subsequently metabolized to eventually alter the ATP:ADP x P_i ratio, serving as a signal to indicate that glucose is abundant. The increased concentration of ATP

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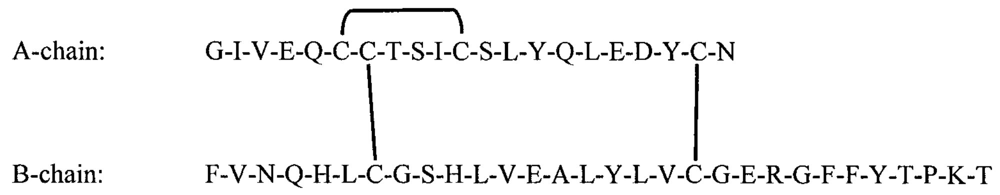

FIGURE 1-3.

The primary structure of human insulin. The dark lines represent disulfide bonds.

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inhibits ATP-sensitive potassium channels (K^+_{ATP}) (8), resulting in membrane depolarization and Ca^{++} influx (247). Increased cellular Ca^{++} triggers the exocytosis of insulin vesicles (247); this mechanism is not well understood. The release of insulin, therefore, is limited by the availability of glucose and the rate it can be metabolized by the pancreatic β-cell.

Glucose-stimulated insulin release is also altered by fatty acids. Incubation of islet cells with long-chain fatty acids results in increased insulin release during glucose stimulation (374). Increased plasma NEFA concentrations by heparin treatment also caused increased insulin release in healthy volunteers (30). Furthermore, the type of fatty acid is important in this effect. In *in vitro* studies, glucose-stimulated insulin release is more pronounced with increased fatty acid chain length and saturation (3, 374). Glucose-stimulated insulin release in perfused rat pancreas was effected particularly by stearic (18:0) and palmitic acids (16:0) (334). Results from human studies are somewhat conflicting. Beysen *et al.* recently reported that PUFAs and MUFAs have the greatest effect on insulin secretion rate, while SFAs elicit no effect (25). Fasching *et al.* found that insulin secretion was not affected by one week of a defined fat exposure (95). Adding to the contradiction, some studies have reported greater stimulation of insulin secretion after diets high in saturated fatty acids in T2DM subjects (285), while others have reported a more pronounced effect with PUFAs (202). The effect of dietary fat type on insulin secretion will need to be the topic of future research.

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Insulin acts upon its target tissues by binding a cell-surface receptor (69), initiating a sequential cascade of phosphorylations and dephosphorylations to activate or inactivate particular molecular structures, enzymes or kinases that mediate insulin's effect. What is currently known about the players of this rather elaborate cascade has been well characterized in recent reviews (174, 181, 276, 353).

Insulin resistance refers to the inability of insulin to elicit its metabolic effects, despite an abundance of it. Most well established is the resistance to insulin-mediated glucose disposal (91, 121). Insulin resistance precedes the development of type 2 diabetes mellitus (T2DM) and is often associated with obesity (75).

The incidence of insulin resistance and compensatory hyperinsulinemia is characterized by impaired tolerance to glucose. This is observed by measuring plasma glucose concentrations after consumption of a glucose load. This oral glucose tolerance test (OGTT) provides a glucose curve, indicating the rate at which the glucose load was disposed and reflects peripheral insulin sensitivity (76). A more-advanced method for measuring insulin resistance has been developed; it is called a euglycemic insulin clamp. The plasma insulin concentration is acutely raised and maintained at approximately 100 μU/mL by a prime-continuous infusion of insulin. The plasma glucose concentration is held constant with a variable glucose infusion. Therefore, the glucose infusion rate equals glucose uptake by insulin-dependent tissues and is a measure of tissue sensitivity to insulin (76). Recently, less-invasive techniques have been utilized for estimating insulin sensitivity based on fasting plasma levels of glucose and insulin (176, 237).

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It has been shown that a non-linear relationship between obesity and insulin resistance exists (31, 265). There is also ample evidence indicating that weight loss, by either exercise or diet, increases insulin sensitivity in obese individuals (60, 106, 108, 124, 262, 295, 303, 346). For example, a 6 week hypocaloric diet, causing a 10.6% decrease in body weight (85% from fat mass), was associated with improved insulin sensitivity (106). Also, Ross and colleagues subjected 52 obese men to diet-induced weight-loss, exercise-induced weight loss, or exercise without weight loss. Both weight loss groups achieved improvements in glucose disposal (insulin sensitivity) far exceeding any improvements made by the exercise without weight-loss group. The authors concluded that weight loss induced by increased daily physical activity without caloric restriction substantially reduces insulin resistance in obese men (303).

Much research interest has focused on dietary factors to better understand insulin resistance. There are some indications that limited amounts of alcohol may benefit (92) while a very high salt intake might impair (81) insulin sensitivity. Epidemiological studies have indicated that fat intake is positively correlated with plasma insulin and negatively correlated with insulin sensitivity (293). Intervention studies, however, have not found increases in fat ranging from 20-40% intake to impair insulin sensitivity (32, 112, 162, 271). Only extremely high fat intakes have been able to elicit a decrease in insulin sensitivity (49). Therefore, studies have steered interests toward the effect of the *type* of dietary fat on insulin resistance.

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In vitro evidence suggests that insulin binding and glucose transport differs between cells grown with different types of fat in the incubation medium (343). Sprague-Dawley rats fed a diet containing 30% fat as fish oil had elevated (n-3) PUFAs incorporated into adipocyte membranes and greater insulin-stimulated glucose transport than rats fed control oils. The insulin sensitivity was positively correlated with the fatty acid unsaturation index in membrane phospholipids (223). The fatty acid profiles of insulin-dependent tissues have been shown to be an indicator of insulin sensitivity (341). Storlien *et al.* found a diet high in saturated, monounsaturated (n-9), or polyunsaturated (n-6) fatty acids resulted in insulin resistance in rats. When the (n-6) PUFA diet was partially substituted with fish oil fatty acids (n-3), the insulin resistance was normalized (342). Kim *et al.* testified that substituting fish oil for corn oil in the context of a high-fat diet partially protected against insulin resistance (184). While the results of rodent studies convincingly demonstrate an effect of dietary fat type, particularly PUFAs, on insulin sensitivity, human studies are more controversial.

Short-term dietary interventions have shown either a modest increase (96, 280) or no effect (33, 95, 122) on insulin sensitivity in humans. Heine *et al.* did find improved insulin action after manipulating the PUFA:SFA ratio in T2DM subjects (144). Furthermore, Stefan *et al.* injected different lipid emulsions for twenty-four hours and reported that blood serum with high levels of PUFAs reduced insulin sensitivity less than blood serum with lower levels of PUFAs (333).

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Epidemiological studies have shown a positive relationship between the percentage of MUFA intake and insulin resistance (99, 157). In the Insulin Resistance Atherosclerosis Study (238), there was a significant inverse correlation between insulin sensitivity and dietary n-3 PUFAs.

Recently Vessby *et al.* (367) reported findings from the KANWU study. This was a study performed at five locations (Kuopio, Aarhus, Naples, Wollongong, and Uppsala), where subjects were put on an isoenergetic diet containing a high proportion of SFAs or MUFAs. Insulin sensitivity significantly decreased during the SFA diet by 10%, but did not change on the MUFA diet. The addition of fish oil (ω-3) capsules did not influence insulin sensitivity in either group (367). Although a controversy remains regarding the influence of dietary fat *type* on insulin resistance/sensitivity in humans, previous studies *generally* indicate that unsaturated fatty acids, MUFAs in particular, have a positive influence on insulin sensitivity.

Leptin - Insulin Relationship

Throughout the many studies of leptin and insulin physiology, observations have led to the hypothesis that a unique relationship exists between the two metabolic hormones. In rodent models of genetic obesity, hyperinsulinemia is associated with overexpression of mRNA for the *ob* protein leptin (73). Furthermore, the diabetic phenotype of *ob/ob* mice was corrected by leptin treatment (256). However, it has been reported that insulin action is impaired by leptin in human hepatic cell lines, isolated rat adipocytes, and skeletal muscle (56, 253, 347).

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Ceddia *et al.* (44) provided evidence that insulin stimulates leptin production in adipocytes, indicating the existence of an "adipoinsular axis". Furthermore, Ceddia *et al.* reported that leptin, under normoglycemic and normoinsulinemic conditions, shifts the flux of metabolites from adipose tissue to skeletal muscle. They concluded that this might be a peripheral mechanism to control body weight and prevent obesity. Conversely, Dagogo-Jack *et al.* found that leptin secretion was not the result of insulin and that hyperleptinemia was not the result of hyperinsulinemia in obese human individuals (73).

Although the topic remains controversial, a recent review (42) provided an outline of the seemingly paradoxical observations regarding leptin and insulin sensitivity. The authors concluded that leptin does appear to play a role in insulin sensitivity. The leptin resistance observed in obese individuals may be tissue selective in that the central effects of leptin are blunted while peripheral effects dominate. The authors caution that apparently conclusive *in vitro* studies must be interpreted carefully. The effects of leptin observed *in vitro* may be masked when integrated with the rest of the body, especially in the context of obesity (42). Future studies are obviously necessary to resolve the precise relationship between leptin and insulin sensitivity.

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CHAPTER 2

EFFECT OF WEIGHT-LOSS AND ALA(18:3n-3) SUPPLEMENTATION ON ERYTHROCYTE FATTY ACID COMPOSITION

Abstract

Numerous studies have proven dietary fat to be a major culprit in the development of obesity and related co-morbidities. Unsaturated fatty acids, however, have beneficial health effects. In particular, the PUFAs found in fish oil, EPA(20:5n-3) and DHA(22:6n-3), have cardioprotective effects when consumed in higher amounts. The metabolic precursor of EPA(20:5n-3) is ALA(18:3n-3). This study investigated the effects of ALA(18:3n-3) supplementation in the context of a hypocaloric diet. It was hypothesized that erythrocyte plasma membrane lipid content of ALA(18:3n-3) and its metabolites would increase for those subjects who supplemented their diet with flaxseed oil capsules (~60% ALA(18:3n-3)). Two groups of obese, non-diabetic subjects were prescribed a hypocaloric diet at 80% of RMR assessments. One of these groups exchanged a portion of their dietary fat with flaxseed oil capsules thereby increasing the ALA(18:3n-3) dietary intake to 5% of total caloric consumption. The fatty acid composition of erythrocyte membranes was analyzed before and after a 10% weight loss. Subjects who increased their dietary intake of ALA(18:3n-3) to 5% of total caloric intake had an ~600% increase in ALA(18:3n-3) content of erythrocyte plasma membranes while control subjects

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showed no change during weight loss. The ALA(18:3n-3) metabolites, EPA(20:5n-3) and DHA(22:6n-3), did not increase for either group, indicating that ALA(18:3n-3) is not readily metabolized or that ALA(18:3n-3) metabolism is not reflected in erythrocytes. What is important is that ALA(18:3n-3) was readily taken up into the cell membrane. The physiological benefit of increased ALA(18:3n-3) will need to be assessed in future studies.

Introduction

Dietary fat has been implicated as a major culprit in the development of obesity and related co-morbidities (312). The American Heart Association and the American Diabetes Association recommend that diets contain less that 30% of its calories in the form of fat (87). Furthermore, only 10% of dietary calories should be in the form of saturated fat, bringing attention to the *type* of dietary fat consumed. The beneficial health effects of unsaturated fatty acids are of particular research interest. The typical Western diet has a high ratio of n-6 to n-3 fatty acids (327). Dietary linoleic acid [LA(18:2n-6)] and α-linolenic acid [ALA(18:3n-3)] are essential fatty acids that are desaturated and elongated into the 20-carbon fatty acids arachidonic acid [AA(20:4n-6)] and eicosapentaenoic acid [EPA(20:5n-6)], respectively. The transformed fatty acids can then be metabolized to form eicosanoids via the lipoxygenase and cyclooxygenase pathways. Eicosanoid products of AA(20:4n-6) metabolism are potent proinflammatory and proaggregatory agonists by comparison with the eicosanoids derived from EPA(20:5n-6) (208). Increased

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dietary consumption of EPA(20:5n-6), which is indicated in the ratio of n-6 to n-3 cell membrane fatty acids, has been shown to have beneficial effects in preventing coronary heart disease, hypertension, and inflammatory disorders (136, 153). Whether or not ALA(18:3n-3) consumption elicits similar beneficial effects is an issue of intense research interest.

Previous experimental studies have investigated methods of increasing cell membrane EPA(20:5n-6) content. Fish oil has been proven to be an effective treatment in enhancing cellular EPA(20:5n-6) concentrations. Flaxseed oil, which has a high content of ALA(18:3n-3), the precursor of EPA(20:5n-6), has also effectively increased cellular EPA(20:5n-6) concentrations, although to a lesser degree. Mantzioris et al. (230) reported the effect of increasing the dietary content of ALA(18:3n-3) and decreasing the dietary content of LA(18:2n-6) by >50% on cellular and non-cellular fatty acid concentrations in normal healthy males over a 4-week period. The results showed a significant increase in ALA(18:3n-3) content in plasma phospholipid, cholesterol esters, and triglycerides fractions, and neutrophil phospholipids after only four weeks of dietary ALA(18:3n-3) supplementation. The present study proposed to study the effects of dietary ALA(18:3n-3) supplementation on the concentration of ALA(18:3n-3) and other regulatory fatty acids in erythrocyte membranes in the context of a hypocaloric diet in obese individuals.

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Subjects and methods

Subjects

After approval from the Human Research Committee of Colorado State University (Appendix A), 11 obese subjects (6 males, 5 females) were recruited from volunteers responding to a weight-loss study advertisement. The subjects were aged 20 to 53 years (35.45 $\pm$ 9.21 years) with a body mass index (BMI; kg/m^2) range of 30.9 to 40.1 (34.82 $\pm$ 3.49). None of the subjects presented a history of hyperlipidemia or inflammatory disorders as indicated by a medical health history questionnaire (Appendix B). Written informed consent was received from each subject prior to any experimental procedures (Appendix C).

Diets and experimental design

Subjects were randomly assigned to one of two treatment groups: control group (CON) and ALA group (ALA). A registered dietician taught the subjects how to record their daily food consumption, and the subjects continued to do so for the duration of their participation in the study. The subjects reported to the Human Performance and Clinical / Research Laboratory at Colorado State University on a weekly basis to be weighed and to turn in their diet records for the previous week. After four weeks of weight stability, the pre-weight-loss assessments of resting metabolic rate, height, mass, body composition, and a blood draw were performed. These measurements were taken during the mid-follicular phase (days 7-10) of

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female subjects' menstrual cycle. The procedures by which these assessments were performed are explained below.

After pre-weight-loss assessments were made, a registered dietician prescribed the AHA Step 1 diet to each subject to achieve a 10% weight loss. The total caloric content of each individual's diet was determined by calculating 80% of the subject's resting metabolic rate, or at a minimum, 1200 kcal/day. A typical diet is shown in Table 2-1. The experimental group (n=6) increased its ALA (18:3n-3) content to 5% of their total caloric intake by consuming a prescribed number of flaxseed oil capsules. Subjects in both groups were advised not to consume other foods high in ALA (18:3n-3) and EPA (20:5n-3). ALA subjects were given a fixed number of flaxseed oil capsules every other week, allowing for compliance to be estimated by the number of remaining capsules. After 10% of the starting body weight had been lost, the diet was manipulated (increased caloric consumption) so that each subject could maintain his/her new weight for 4 consecutive weeks. At that time, post-weight-loss assessments were administered. The diet diaries were analyzed by using the Food Intake Analysis System (FIAS 3.98, University of Texas School of Public Health, 1998). Figure 2-1 provides a diagram of the study design.

Anthropometric measurements

Subjects' height and weight were determined using a physician's balance scale (Detecto, Webb City, MO). The Lunar DPX-IQ Dual-energy x-ray absorbtiometry (DEXA) scanner (Lunar Corp., Madison, WI) was used to determine

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Table 2-1 Food exchanges for caloric levels, 1200 to 2000 kcal/day

Calorie Levels	1200	1300	1400	1500	1600	1700	1800	1900	2000
Nonfat Dairy (90 calories)	2	2	2	2	2	2	2	2	2
Lean Protein (55 kcal/oz)	4	4	5	5	5	6	6	6	6
Vegetables (25 calories)	4	4	4	5	5	5	5	5	5
Fruit (60 calories)	2	2	2	3	3	3	3	4	4
Starch (80 calories)	6	7	8	8	9	10	11	11	12
Extra Calories*	100	100	100	100	100	100	100	150	150

*Extra calories can be from any source including fats, alcohol, condiments, sweets, or the food groups listed in the table. (1 fat exchange = 5 grams of fat and 45 calories)

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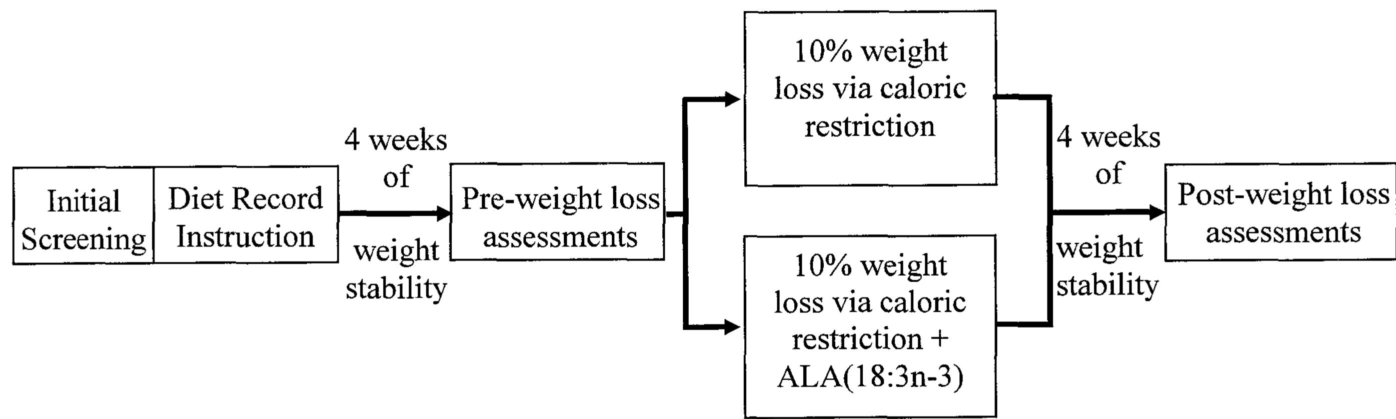

Figure 2-1. Study design.

body composition. The procedure required subjects to remove all metal articles and to lie flat on the DEXA bed. The rate of the scan was determined by the thickness of the chest.

Resting Metabolic Rate

Resting metabolic rate was determined by indirect calorimetry using the ventilated hood method of the Vmax 29 Series (SensorMedics, Yorba Linda, CA). The subjects arrived at the lab after a 12-hour fast and a normal night of sleep. The subjects were instructed to stay awake, and not make any voluntary limb movements. Expiratory gases were collected for 60 minutes while the subject was in a supine position, and the last 30 minutes were analyzed and then extrapolated to determine 24-hour energy requirements.

Blood collection and analysis

Blood samples were collected from an antecubital vein after an overnight 12-hour fast. The blood was collected in vacutainers containing 0.081 mL of 15% (K_3) EDTA solution (Becton Dickinson, Franklin Lakes, NJ). The blood serum was removed after centrifugation for 15 minutes at 2800 RPM and 4°C. The remaining blood was resuspended at an approximate 50% hematocrit in a 2% (K_3) EDTA saline solution. The cells were washed by vortexing for 30 seconds and centrifuging for 15 minutes at 2800 RPM and 4°C. This washing process was repeated for a total of three

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washes. Thereafter, the cells were resuspended at an approximate 40% hematocrit in a 2% (K_3) EDTA saline solution and stored at -80°C until analysis.

Erythrocyte lipids were extracted using a modified Folch extraction (102). Fatty acid methyl esters were prepared by hydrolysis of phospholipids using 0.5N NAOH (Heat for 10 min at 70°C, cool to room temperature) and transmethylation using 14% BF3 methanol (Supelco, Inc., SupelcoPark, PA) (Heat for 10 min at 70°C, cool to room temperture) with C23:1 as an internal standard. The fatty acid methyl esters were analyzed using a temperature-programmed HP6890 Gas Chromatography (HP-Agilent Technologies, Palo Alto, CA), with flame ionization detection (FID) on a Supelco 30mm x 0.25mm ID SP-2380 column. Initial temperature was 165°C and held for 20 minutes, then ramped at 5°C per minute to 185°C and held for 34 minutes. Individual fatty acid methyl esters were identified by comparison of retention time with known fatty acid methyl ester standards (Nu-Chek, Elysian, MD).

Statistics

The Statistical Package for the Social Sciences 10.0 (SPSS 10.0; SPSS, Chicago, Ill) was used for all statistical procedures. Comparisons between groups (CON vs. ALA) were performed via independent samples t-tests, with significance set at $p \leq 0.05$. Experimental effect was determined by repeated measures analysis of variance. Correlations between dietary and erythrocyte fatty acids were analyzed by using Pearson correlation coefficients.

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Results

Subjects

Eleven subjects successfully completed this trial (CON 3 Female, 2 Male; ALA 2 Female, 4 Male). There was no statistically significant difference in BMI ($t=2.078$, $P=0.067$), body fat percentage ($t=-0.306$, $P=0.766$), fat mass ($t=0.705$, $P=0.498$), or age ($t=0.331$, $P=0.748$) between the two groups before the weight-loss intervention. There was a significant decrease in BMI ($F=107.97$, $P<0.001$), fat mass ($F=41.43$, $P<0.001$) and body fat percentage ($F=19.12$, $P=0.002$) for both groups, as expected, over the course of the trial. There was also no significant statistical difference between groups with regard to the change in percent body fat ($t=-0.643$, $P=0.54$) over the course of the weight-loss period (Table 2-2).

Dietary Intake (Table 2-3)

Dietary analysis was performed on one of the pre-weight loss dietary records, two of the intervention weight loss records, and one of the post-weight loss dietary records for each of the subjects. Statistical analysis showed that prior to weight-loss, there was no statistical difference between groups for any of the 62 factors analyzed. Total caloric intake decreased significantly for both groups at the beginning of the weight-loss phase of participation. During the weight-loss phase, there were only two significant differences between groups: (1) the ALA group had a higher consumption polyunsaturated fat expressed as total grams ($t = 3.241$, $P = 0.01$) or as a percentage of total caloric consumption ($t = 3.322$, $P = 0.009$), and (2) the ALA group consumed

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more ALA (18:3n-3) than the control group when expressed as total grams ($t = 7.26$, $P < 0.001$) or as a percentage of total calories ($t = 7.119$, $P < 0.001$). This indicates that the difference between groups for PUFA consumption is primarily due to the increased ALA(18:3n-3) consumption and no other PUFA.

The same differences were present for the comparisons between groups for the post-weight loss weight maintenance phase. PUFA consumption was higher in the ALA group when expressed as grams ($t = 2.372$, $P = 0.042$) or as a percentage of total calories ($t = 3.027$, $P = 0.014$). ALA(18:3n-3) consumption was higher in the ALA group when expressed as grams ($t = 3.865$, $P = 0.004$) or as a percentage of total calories ($t = 4.378$, $P = 0.002$). Vitamin E consumption was also higher in the ALA group during the weight-maintenance phase when expressed as mg ($t = 2.570$, $P = 0.03$) and as mg per 1000 kcal ($t = 3.591$, $P = 0.006$). Lastly, the control group consumed more butyric acid (4:0) ($t = 2.627$, $P = 0.027$), caproic acid (6:0) ($t = 2.524$, $P = 0.033$), capryllic acid (8:0) ($t = 2.446$, $P = 0.037$), capric acid (10:0) ($t = 2.480$, $P = 0.035$), and myristic acid (14:0) $t = 2.267$, $P = 0.050$) when expressed as a percentage of total calories.

Erythrocyte Membrane Fatty Acid Concentrations (Table 2-4)

Prior to the weight loss intervention, there was no significant difference between groups for any of the following fatty acids: palmitic acid (16:0; $t = 0.415$, $P = 0.688$), stearic acid (18:0; $t = -1.209$, $P = 0.258$), LA (18:2n-6; $t = 0.556$, $P = 0.592$), gadoleic acid (20:0; $t = -1.138$, $P = 0.285$), ALA (18:3n-3; $t = 0.485$, $P =$

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Table 2-2.

Characteristics of the control and ALA groups prior to and after weight loss[1]

	Control group (n=5)		ALA group (n=6)	
	Pre	Post	Pre	Post
Age (yrs)	34.40 ± 12.34		36.33 ± 6.77	
Height (cm)	67.60 ± 3.29		68.33 ± 12.34	
Mass (kg)	96.32 ± 9.21	86.64 ± 7.89*	110.30 ± 15.21	98.33 ± 13.80*
BMI (in kg/m^2)	32.74 ± 1.61	29.46 ± 1.41*	36.55 ± 3.79	32.55 ± 3.39*
% body fat	40.02 ± 7.94	36.58 ± 7.86*	38.52 ± 8.23	36.08 ± 8.33*
Fat mass (kg)	36.39 ± 6.96	29.42 ± 5.66*	39.69 ± 8.29	33.36 ± 7.28*
Lean mass (kg)	55.02 ± 13.7	51.75 ± 10.2	64.28 ± 13.7	59.94 ± 12.8

[1] $\bar{\chi} \pm$ SD

*Post value significantly lower than Pre value, P<0.05.

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TABLE 2-3
Estimated daily dietary intake of nutrients in the control (CON) and flaxseed (ALA) group during the weight-loss intervention[1]

	Control group (n=5)	ALA group (n=6)
Energy intake (kcal/day)	1489.43 ± 158.35	1571.42 ± 287.63
Carbohydrate		
(grams/day)	191.95 ± 34.72	188.53 ± 39.67
(% total kcal)	50.51 ± 5.01	47.20 ± 6.69
Fat		
(grams/day)	49.48 ± 6.54	48.29 ± 20.47
(% total kcal)	29.64 ± 4.38	32.38 ± 8.44
SFA		
(grams/day)	15.54 ± 4.10	15.68 ± 7.21
(% total kcal)	9.23 ± 2.03	8.65 ± 3.35
MUFA		
(grams/day)	18.77 ± 2.00	19.78 ± 7.99
(% total kcal)	11.24 ± 1.46	10.91 ± 3.44
PUFA		
(grams/day)	10.98 ± 1.71	17.98 ± 4.53*
(% total kcal)	6.65 ± 1.56	10.11 ± 1.83*
Protein		
(grams/day)	71.78 ± 5.72	74.50 ± 9.05
(% total kcal)	19.13 ± 0.81	18.90 ± 2.27
LA (18:2n-6)		
(grams/day)	9.76 ± 1.61	9.69 ± 2.79
(% total kcal)	5.92 ± 1.45	5.40 ± 0.90
ALA (18:3n-3)		
(grams/day)	0.93 ± 0.15	7.94 ± 2.13*
(% total kcal)	0.56 ± 0.11	4.51 ± 1.23*

[1] $\chi \pm$ SD.
*Statistical difference between CON and ALA group, $P < 0.05$.

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0.639), erucic (cetoleic) acid (20:2, n-9; t = 0.632, P = 0.543), dihomogammalinoleic acid (DGLA,20:3n-6; t = 2.111, P = 0.064), AA (20:4n-6; t = -0.551, P = 0.595), EPA (20:5n-3; t = 0.216, P = 0.834), lignoceric acid (24:0; t = -0.773, P = 0.459), nervonic acid (24:1n-9; t = -0.951, P = 0.366), and DHA (22:6n-3; t = 2.255, P = 0.051). There was a significant difference between the two groups for oleic acid (18:1n-9; t = -2.569, P = 0.030) and eicosanoic acid (20:1n-9; t = -2.775, P = 0.022). This difference between groups was later evident in a group effect for oleic acid (18:1n-9; F = 6.994, P = 0.027), and eicosanoic acid (20:1n-9; F = 8.236, P=0.018) when performing repeated measures comparison of means. The hypocaloric intervention had an effect on DGLA (20:3n-6; (F=10.282, P=0.011) and AA (20:4n-6; F=7.064, P=0.026). A paired sample t-test revealed that DGLA (20:3n-6) decreased for the ALA group (t = 3.221, P = 0.024) and CON group (t = 3.927, P = 0.017), resulting in no significant interaction (F=1.607, P=0.237). The percentage of AA (20:4n-6) increased in the CON group (t= -2.609, P = 0.059), although not significant. This increase was attenuated in the ALA group (t = -0.431, P = 0.684), nearly resulting in a significant interaction between the intervention and the groups for AA (20:4n-6; F=5.037, P=0.051). Similarly, palmitic acid (16:0) decreased in both groups, but was only significantly lower in the CON group (t = 3.683, P = 0.021). There was a group (F = 26.94, P = 0.001) and time (F = 9.532, P = 0.013) effect for ALA (18:3n-3), resulting in an interaction between the intervention and the groups (F=11.682, P=0.008). These effects were due to an increase in ALA(18:3n-3) content for the ALA group (t = -3.616, P = 0.015), but not for the CON group (t = 1.420, P = 0.229).

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Diet versus erythrocyte FA composition (Table 2-5)

Select dietary and erythrocyte FA variables of interest were analyzed by performing a Pearson's correlation, significance set at 0.05. Pre-weight loss dietary records and pre-weight loss erythrocyte fatty acid compositions were analyzed to investigate the viability of the specific erythrocyte fatty acids LA(18:2n-3), oleic acid (18:1n-9), ALA (18:3n-3), and palmitic acid (16:0) as biomarkers of dietary intake, as had been indicated in previous literature (310).

The LA (18:2n-6) percentage of erythrocyte membranes was not significantly correlated to dietary PUFAs (r = 0.20, P = 0.57) or dietary LA(18:2n-6) (r = 0.38, P = 0.29). Dietary MUFAs did not correlate significantly with oleic acid (18:1n-9) (r = 0.25, P = 0.48) or ALA (r = 0.20, P = 0.58). Lastly, the palmitic acid (16:0) percentage of erythrocyte membranes did not correlate with dietary SFA (r = -0.38, P = 0.28) or dietary palmitic acid (16:0) (r = -0.38, P = 0.27).

A similar analysis was performed between post-weight loss erythrocyte fatty acid compositions and dietary records from the treatment phase of the study. Erythrocyte LA (18:2n-6) composition was not significantly associated with dietary PUFA content (r = 0.056, P = 0.869) or dietary LA (18:2n-6) content (r = 0.25, P = 0.46). Erythrocyte oleic acid (18:1n-9) and ALA (18:3n-3) composition was not related to dietary MUFA content (r = 0.25, P = 0.46 and r = -0.09, P = 0.80, respectively). Lastly, erythrocyte palmitic acid (16:0) concentration was not related to dietary SFA content (r = -0.03, P = 0.92) or dietary palmitic acid (16:0) content (r = -0.60, P = 0.054).

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TABLE 2-4

Fatty Acid area percent of red blood cell membranes for both dietary groups.[1]

	Control group (n=5)		ALA group (n=6)	
	Pre	Post	Pre	Post
SFA[2]				
16:0	17.42 ± 1.28	16.66 ± 1.25*	17.80 ± 1.68	17.15 ± 0.58
18:0	18.68 ± 2.00	17.72 ± 0.69	17.11 ± 2.26	17.03 ± 2.50
20:0	0.11 ± 0.03	0.06 ± 0.06	0.07 ± 0.07	0.07 ± 0.07
24:0	2.62 ± 2.63	3.66 ± 2.28	1.57 ± 1.89	2.48 ± 1.61
MUFA[3]				
18:1n-9	16.49 ± 0.92	16.91 ± 1.24	14.40 ± 1.60†	14.49 ± 1.70
20:1n-9	0.45 ± 0.02	0.45 ± 0.02	0.37 ± 0.06†	0.39 ± 0.05
24:1n-9	0.92 ± 0.27	0.93 ± 0.26	0.77 ± 0.26	0.68 ± 0.22
PUFA[4]				
18:2n-6	9.75 ± 1.68	9.62 ± 2.02	10.37 ± 2.00	10.29 ± 1.99
18:3n-3‡	0.03 ± 0.04	0.02 ± 0.04	0.05 ± 0.10	0.29 ± 0.09*
20:2n-9	0.26 ± 0.15	0.31 ± 0.08	0.30 ± 0.05	0.29 ± 0.05
20:3n-6	1.65 ± 0.17	1.49 ± 0.20*	1.87 ± 0.17	1.58 ± 0.31*
20:4n-6	15.60 ± 0.66	16.86 ± 1.31	14.89 ± 2.79	15.00 ± 2.43
20:5n-3	3.12 ± 0.74	2.81 ± 0.28	3.23 ± 0.88	3.21 ± 0.71
22:6n-3	2.85 ± 0.47	3.39 ± 0.93	3.62 ± 0.64	3.66 ± 0.83

[1] $\chi \pm$ SD.
[2] SFA = saturated fatty acid
[3] MUFA = mono-unsaturated fatty acid
[4] PUFA = poly-unsaturated fatty acid
* significantly different from respective "Pre" measurement, $P<0.05$
† significantly different from respective "CON" measurement, $P<0.05$
‡ significant interaction between group and time, $P<0.05$

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Table 2-5

Pearson correlation coefficients between the proportion of fatty acids in erythrocytes (%) and in dietary intake of fatty acid (% of total fatty acid intake).

Fatty Acid	PUFA	MUFA	Linoleic Acid (18:2n-6)	SFA	Palmitic acid
Linoleic Acid (18:2n-6)					
Pre-weight loss	0.20	-----	0.38	-----	-----
Post-weight loss	0.06	-----	0.25	-----	-----
Oleic Acid (18:1n-9)					
Pre-weight loss	-----	0.25	-----	-----	-----
Post-weight loss	-----	0.25	-----	-----	-----
α-linolenic acid (18:3n-3)					
Pre-weight loss	-----	0.20	-----	-----	-----
Post-weight loss	-----	-0.87	-----	-----	-----
Palmitic acid (16:0)					
Pre-weight loss	-----	-----	-----	-0.38	-0.38
Post-weight loss	-----	-----	-----	-0.03	-0.60

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Discussion

Previous studies have demonstrated that dietary n-3 PUFA consumption has a positive impact on a number of health parameters. The fish oil PUFAs EPA(20:5n-3) and DHA(22:6n-3) have received the majority of research interest. Bang and Dyerberg (12, 85) were the first to observe that a diet high in fish oil PUFAs, but low in SFAs, was strongly related to a low rate of death from cardiovascular disease. Others have found that high fish oil consumption is associated with lowered blood pressure (180, 273, 396), lowered triglycerides (140) (180, 205, 239) (273, 326), and reduced platelet aggregability (118), all factors intimately related to cardiovascular disease. Fish oil consumption results in increased incorporation of EPA(20:5n-3), and to a lesser extent DHA(22:6n-3), into erythrocyte membrane phospholipids (117). The metabolic precursor of EPA(20:5n-3) and DHA(22:6n-3) is ALA(18:3n-3) (117). The current study results demonstrate that an increased consumption of ALA(18:3n-3) during a hypocaloric diet will cause an increased incorporation of ALA(18:3 n-3) into the plasma membrane of erythrocytes without altering the content of EPA(20:5n-3) or DHA(22:6n-3).

The potential health benefits of ALA(18:3n-3) remain controversial. Layne *et al.* fed subjects 35 mg/kg body weight/day of flaxseed oil [50-60% ALA(18:3n-3)] and found that this intervention had no impact on plasma triglyceride levels, but a similar diet of fish oil resulted in a significant decrease (205). These results were supported by other studies that showed that ALA(18:3n-3) is less than equivalent to EPA(20:5n-3) and DHA(22:6n-3) regarding cardiovascular health benefits (98, 326).

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Alternatively, de Lorgeril *et al.* (77) showed that an ALA(18:3n-3)-enriched diet resulted in a lower rate of myocardial infarction reoccurrence. The impact of ALA(18:3n-3) on health will need to be resolved in future studies.

The fatty acid composition of erythrocytes reflects relative patterns of fatty acid intake. Sarkkinen *et al.* (310) reported Spearman correlation coefficients between the proportions of fatty acids in erythrocytes at the end of a 4.5-month dietary intervention and dietary intake of fatty acids expressed as a percentage of total fatty acid intake. The authors found that erythrocyte LA(18:2n-6) composition correlated with dietary LA(18:2n-6) and dietary polyenes, that erythrocyte oleic acid (18:1n-9) and ALA(18:3n-3) correlated with dietary monoenes, and that erythrocyte palmitic acid (16:0) correlated with dietary saturated fat and dietary palmitic acid(16:0). None of these relationships were significant in the present study, most likely because of a very small study sample ($n = 11$), whereas the sample reported by Sarkkinen *et al.* had an $n = 159$. The erythrocyte FA composition was very comparable between the present study and that of Sarkkinen *et al.* (310).

In conclusion, this study demonstrates that increased ALA(18:3n-3) incorporation in erythrocyte membranes is a result of higher ALA(18:3n-3) consumption. The impact of this incorporation on other physiological parameters will need to be the topic of future research.

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CHAPTER 3

EFFECT OF WEIGHT-LOSS AND ALA(18:3n-3) SUPPLEMENTATION ON PPAR ISOFORM CONCETNRATIONS IN HUMAN SKELETAL MUSCLE

Abstract

The metabolic plasticity of human skeletal muscle has been a topic of research interest, particularly in the context of obesity and weight loss. A family of nuclear receptors called peroxisome proliferator-activated receptors (PPAR) have been identified and implicated to play a role in lipid metabolism. It was hypothesized that upon a hypocaloric diet, the skeletal muscle protein concentrations of the three PPAr isoforms would change to reflect a thrifty phenotype. It was also hypothesized that an increased caloric ocnsumption of ALA(18:3n-3), a natural ligand of the PPARs, would attenuate any fluctions of PPAR concentrations. Contrary to these hypotheses, the PPAr isoforms changed ina manner that more reflected fat oxidation versus fat storage. These effect were attenuated or reversed with dietary ALA(18:3n-3) supplementation. Future experiments are needed to further elucidate the importance of the PPAR isoforms to lipid metabolism of skeletal muscle.

Introduction

The condition of human obesity is increasingly prevalent (101, 321). The excessive accumulation of adipose tissue is associated with several comorbidities

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(267) and has a profound negative economic impact (58, 277). For these reasons it is imperative that the understanding of the molecular aspects of obesity be increased.

The cause of obesity has been attributed to interactions between the environment and genetics (148). Since the rate of increased obesity far exceeds any changes in the human genome, it is therefore more likely that obesity is due to changes in the environment rather changes in the genome. The current environment, particularly of industrialized countries, is characterized by an essentially unlimited supply of convenient, inexpensive, highly palatable, energy-dense foods. This is coupled with a technologically advanced lifestyle requiring limited physical activity for survival. This type of environment results in an energy imbalance; that is, a high-energy intake and low energy expenditure.

The role of dietary fat in obesity development has been the topic of intense research interest and debate (72, 82). Organizations such as the American Heart Association, American Diabetes Association, and American Cancer Society have advocated diets with a maximum of 30% of calories from fat, and no more than 10% of calories from saturated fat (87). Further interest in dietary fat *type* has been amplified by epidemiological observations regarding the prevalence of dietary long-chain omega-3 (n-3) fatty acids derived from fish oil and a decreased incidence of cardiovascular disease (153).

The essential polyunsaturated fatty acids (PUFAs) (e.g. n-3 and n-6) are important as an energy source and in membrane structure and signal transduction. Other studies have indicated that PUFAs are also involved in the regulation of gene

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expression (54). Of particular interest is the regulation of genes involved in lipid and carbohydrate metabolism (322). To that end, a fatty-acid regulated family of nuclear receptors called peroxisome proliferator-activated receptors (PPARs) was discovered in the early 1990's (125, 168). Three PPAR isoforms, i.e. PPARα (168), PPARβ (313), and PPARγ (88, 126, 401), have been identified. Interestingly, the natural ligands for these nuclear receptors include fatty acids (103, 104, 125).

PPARα reportedly regulates genes involved in the cellular uptake and metabolism of fatty acids, e.g. lipoprotein lipase (315), fatty acid transport protein (234), acyl-CoA synthetase (233), carnitine palmitoyltransferase (36), and others. In contrast, some evidence indicates that PPARγ is involved in the regulation of genes involved in cellular uptake and storage of fatty acids. A working example is that heterozygous PPARγ-null mice have reduced amounts of adipose tissue (16, 199), and 3T3-L1 adipocytes are inhibited from differentiation and hypertrophy by a dominant-negative PPARγ mutant (299). The precise role that PPARβ plays in cellular metabolism remains unresolved, but recent evidence indicates that this isoform is also closely related to lipid metabolism (254).

The effect of weight loss on PPAR isoform expression in skeletal muscle has not been studied. Some studies have shown, however, that subcutaneous adipose tissue responds to hypocalorically-induced weight loss with a reduction of PPARγ mRNA (18, 292, 368). Neither of the other PPAR isoforms (α or β) was studied.

The following study was designed to better understand the regulation of PPAR isoform proteins levels in skeletal muscle. More specifically, we tested the

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impact of an increased consumption of ALA(18:3n-3) in the context of weight loss on protein concentrations of the three PPAR isoforms in skeletal muscle. We hypothesized that a 10% weight-loss induced by a hypocaloric diet will result in decreased protein concentrations of PPARα and PPARβ, but increased protein concentrations of PPARγ; the increased consumption of ALA(18:3n-3), however, would inhibit the reduction of PPARα and PPARβ and increase the protein concentrations of PPARγ.

Subjects and methods

Subjects

The Human Research Committee of Colorado State University approved the recruitment of human subjects for this study (Appendix A). Eleven obese subjects (6 males, 5 females) were recruited from a pool of volunteers responding to a weight-loss study advertisement and provided informed consent before participating (Appendix B). All other volunteers were screened but did not fulfill one or more requirements (i.e. oral birth control, smoking, medications, etc.). The subjects ranged from 20 to 53 years of age (35.45 $\pm$ 9.21 years). The body mass index of the subjects, per recruitment qualifications, ranged from 30.9 to 40.1 kg/m^2 (34.82 $\pm$ 3.49 kg/m^2) thus classifying all subjects as obese (258, 267). A medical health history questionnaire indicated that none of the subjects presented a history of hyperlipidemia or inflammatory disorders (Appendix C).

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Diets and experimental design

After establishing that a potential subject qualified to participate and provided written consented to do so, they were randomly assigned to one of two diet groups. Regardless of the diet group assignment, a registered dietician taught the subjects how to accurately record their daily food consumption (e.g. portion sizes, method of food preparation, product names) and the subjects were instructed to record their diets for the duration of their participation in the study. The subjects reported to the Human Performance and Clinical / Research Laboratory at Colorado State University on a weekly basis to be weighed and to turn in their diet records for the previous week. The diet diaries were analyzed by using Food Intake Analysis System (FIAS 3.98, University of Texas School of Public Health, 1998).

While recording daily food consumption, the subjects maintained a stable weight for 4 consecutive weeks after which pre-weight-loss assessments were administered. Resting metabolic rate, height, mass, body composition, and muscle biopsies were performed as explained below.

Immediately after all pre-weight-loss assessments were made, a registered dietician prescribed the AHA Step 1 diet to each subject in order to initiate a 10% weight loss. The total caloric content of each subject's diet was determined by calculating 80% of the subjects resting metabolic rate. The minimal caloric restriction was set at 1200 kcal/day. Table 2-1 illustrates the composition of the prescribed diets based upon daily caloric consumption. The experimental group (ALA) increased their dietary ALA(18:3n-3) content to 5% of their total calories by

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consuming a prescribed number of flaxseed oil capsules. Both groups were advised not to consume other foods high in ALA(18:3n-3) and EPA(20:5n-3). The subjects in the ALA group were given a fixed number of capsules every other week, allowing for compliance to be estimated by the number of remaining capsules. When 10% of the starting body weight had been lost, the diets were manipulated (increased caloric consumption) so that subjects would no longer lose weight, but could maintain their new weight for 4 consecutive weeks. At the end of 4 weeks of post-weight-loss weight maintenance, all assessments were re-administered. Figure 2-1 provides a diagrammatic description of the study design.

Anthropometric measurements

A physician's balance scale (Detecto, Webb City, MO) was used to determine the subjects' height and weight to the nearest centimeter and 0.1 kg, respectively. Body mass index (BMI) was calculated from body weight and height (kg/m^2). Body composition was determined with a Lunar DPX-IQ dual-energy x-ray absorbtiometry (DEXA) scanner (Lunar Corp., Madison, WI). The anthropometric variables of percent body fat, fat-free mass (kg), and lean body mass (kg) were recorded from each DEXA scan.

Resting Metabolic Rate

Resting metabolic rate was determined via indirect calorimetry using the Vmax 29 Series (SensorMedics, Yorba Linda, CA) ventilated hood system. The

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subjects arrived at the Human Performance Clinical / Research Laboratory after a 12-hour fast and a normal night of sleep. The assessment took place in a darkened, thermo neutral room. The subjects were instructed to stay awake, and not make any voluntary limb movements. Expiratory gases were collected for 60 minutes while the subject lie in a supine position. The last 30 minutes were analyzed using the Weir equation (379) and then extrapolated to determine 24-hour energy requirements.

Muscle Biopsy

A muscle biopsy was taken from the vastus lateralis, under local anesthesia, to determine PPAR isoform content in skeletal muscle. Approximately fifty to one hundred milligrams of tissue were harvested with a 5mm Bergström cannula (Bignell Surgical, Inc., Arundel, England). The muscle sample was immediately frozen in liquid nitrogen and stored at -80°C until analyzed for the protein abundance of PPARα, PPARβ, and PPARγ.

Immunoblotting

The ice-thawed muscle aliquots were weighed and subsequently homogenized in 7X volume ice-cold homogenization buffer (50 mM HEPES, pH 7.2, 10 mM NaF, 30 mM $Na_4P_2O_7$, 2 mM EDTA, 150 NaCl, 1% Triton X-100, 10% Glycerol, 1:1000 protease inhibitor cocktail (Sigma, St. Louis, MO)). Tissue lysates were solubilized with gentle mixing on a lab rotator at 4°C for 45 minutes and then centrifuged for 15 minutes at 18,000 x g for 15 minutes. The supernatant was saved and analyzed for

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total protein content with the bicinchoninic acid assay (Pierce, Rockford, IL). The supernatant was then diluted with Laemmli Sample Buffer to make a final protein concentration of 7.0 mg/mL.

The solubilized proteins (35 μg) were resolved by 10% SDS-PAGE and transferred to a polyvinyl-difluoride (PVDF) membrane. Broad-range pre-stained molecular weight markers (Bio-Rad Laboratories, Hercules, CA) were also electrophoresed to ensure adequate transfer and for determining protein sizes. For blot-to-blot comparisons, an internal standard of human skeletal muscle lysates was loaded at the same concentration. A positive control of Marmot (*Marmota flaviventris*) white adipose tissue (kindly provided Greg Florant, Colorado State University) was used for PPARβ and PPARγ, and a Hep-G2 whole cell lysate was used as a positive control for PPARα. The membrane was blocked overnight at 4°C in Tris-buffered saline (TBS) with 0.5% Tween-20 and 5% nonfat dry milk. The PVDF membrane was then incubated in a PPARα, PPARβ, or PPARγ antibody dilution (1:5000) for 2 hours in TBS with 0.5% Tween-20 and 5% nonfat dry milk. The PPARα antibody (sc-9000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was a rabbit polyclonal antibody raised against a recombinant protein corresponding to the N-terminal amino acids 1-98 of human PPARα. The PPARβ antibody (sc-7197, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 2-75 of human PPARβ. The PPARγ (sc-7196, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody was also a rabbit polyclonal antibody raised against a recombinant protein

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corresponding to the amino acids 6-105 of human PPARγ1. The blots were subsequently washed 3 x 5 min in TBS with 0.5% Tween-20, and incubated with a goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:10,000) (sc-2768, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour. The membranes were washed 3 x 5 minutes in TBS with 0.5% Tween-20. The protein bands were visualized using the Enhanced Chemiluminescence Plus System (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified with AlphaImager (Alpha Innotech Corp., San Leandro, CA). Protein bands were compared to the internal standard from each blot. The data are expressed as a percentage of internal standard intensity for that particular blot.

Statistical Analysis

The Statistical Package for the Social Sciences 10.0 (SPSS 10.0; SPSS, Chicago, Ill) was used for all statistical procedures. A repeated measures analysis of variance was performed to test the interaction of weight loss and ALA(18:3n-3) supplementation. Pre-weight loss comparisons between groups were done via independent sample t-tests and pre- to post-weight loss comparisons within groups were done via paired samples t-tests. Significance was set at $\alpha = 0.05$.

Results

Subject characteristics are provided in Table 2-2. The CON and ALA groups consist of both men and women (CON 3 Female, 2 Male; ALA 2 Female, 4 Male),

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but because of small group size the genders have been pooled. The groups did not differ significantly in age ($t = 0.331$, $P = 0.75$), height ($t = 0.405$, $P = 0.695$), or in pre-weight loss mass ($t = 1.791$, $P = 0.107$). Furthermore, there were no significant differences between groups for pre-weight loss BMI ($t = 2.078$, $P = 0.067$), percent body fat ($t = 0.0306$, $P = 0.766$), fat mass ($t = 0.705$, $P = 0.498$), or lean mass ($t = 1.232$, $P = 0.249$). The treatment of 10% weight loss was successful in significantly reducing the weight ($F = 144.50$, $P < 0.001$), BMI ($F = 107.974$, $P < 0.001$), and lean mass (35.626, $P < 0.001$) of both groups.

Dietary intake of energy and macronutrients during the weight-loss treatment is shown in Table 2-3. There were only two significant differences between the groups. Polyunsaturated fat consumption was higher in the ALA group vs. the CON group when PUFAs were expressed as total grams ($t = 3.241$, $P = 0.01$) or as a percentage of total caloric consumption ($t = 3.322$, $P = 0.009$). This differences was most likely a result of increased ALA(18:3n-3) intake. The ALA group consumed more ALA(18:3n-3) than the control group when expressed as total grams ($t = 7.26$, $P < 0.001$) or as a percentage of total calories ($t = 7.119$, $P < 0.001$).

Western blots were generated to measure the concentration of the different PPAR isoforms in skeletal muscle before and after weight loss. To correct for blot to blot variability, an internal control of human skeletal muscle was processed with the study muscle samples. Protein quantity study muscle sample was calculated as a percentage of the protein quantity observed in the internal standard for the same

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immunoblot. The values presented are mean percentages (± SE). Representative western blots are displayed in Figure 3-1.

Pre-weight loss measurements of PPAR isoforms were not different between groups (PPARα $t = 0.989$, $P = 0.348$; PPARβ $t = 1.438$, $P = 0.184$; PPARγ $t = 1.456$, $P = 0.179$). There was not a significant interaction between weight-loss and dietary ALA(18:3n-3) supplementation for PPARα ($F = 1.889$, $P = 0.203$) or PPARγ ($F = 1.728$, $P = 0.221$), but there was for PPARβ ($F = 7.336$, $P = 0.024$). The mean percentages are displayed in Table 3-1 and Figures 3-2 – 3-4.

Discussion

The peroxisome proliferator-activated receptors (PPARs) are a subfamily of nuclear receptors. The three isoforms, PPARα, PPARβ, and PPARγ, play a critical physiological role as lipid sensors and regulators of lipid metabolism (23). Generally speaking, PPARα has been implicated in the regulation of genes involved in cellular uptake and subsequent oxidation of fatty acids (233, 234, 251, 315). PPARγ is also involved with the cellular uptake of fatty acids, but promotes the storage of fatty acids rather than catabolism (16, 357). PPARβ has been shown to increase circulating levels of HDL-cholesterol and decrease circulating levels of triglycerides in rhesus monkeys (266). A recent study suggests that PPARβ is intimately involved in fatty acid oxidation, as it was upregulated in the absence of PPARα (254). Furthermore, the specific gene targets of PPARβ have recently been identified to parallel that of PPARα (254).

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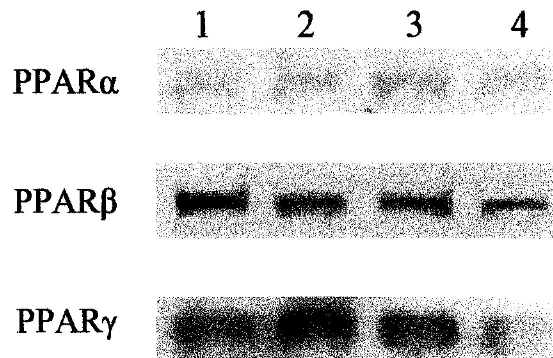

Figure 3-1. Representative western blots of PPARα, PPARβ, and PPARγ in human skeletal muscle. Lanes 1 and 3 are pre-weight loss blots, and Lanes 2 and 4 are post-weight loss blots.

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TABLE 3-1

Mean PPAR isoform protein concentrations in human skeletal muscle before and after weight-loss.[1]

	Control group (n=5)		ALA group (n=6)	
	Pre	Post	Pre	Post
PPARα	93.33 ± 24.15	104.67 ± 35.40	108.33 ± 25.72	98.33 ± 9.92
PPARβ	77.23 ± 31.98	106.25 ± 36.00	102.78 ± 27.06	83.98 ± 23.22
PPARγ	170.42 ± 84.00	103.15 ± 58.01	108.83 ± 56.03	115.32 ± 52.40

[1] χ ± SD

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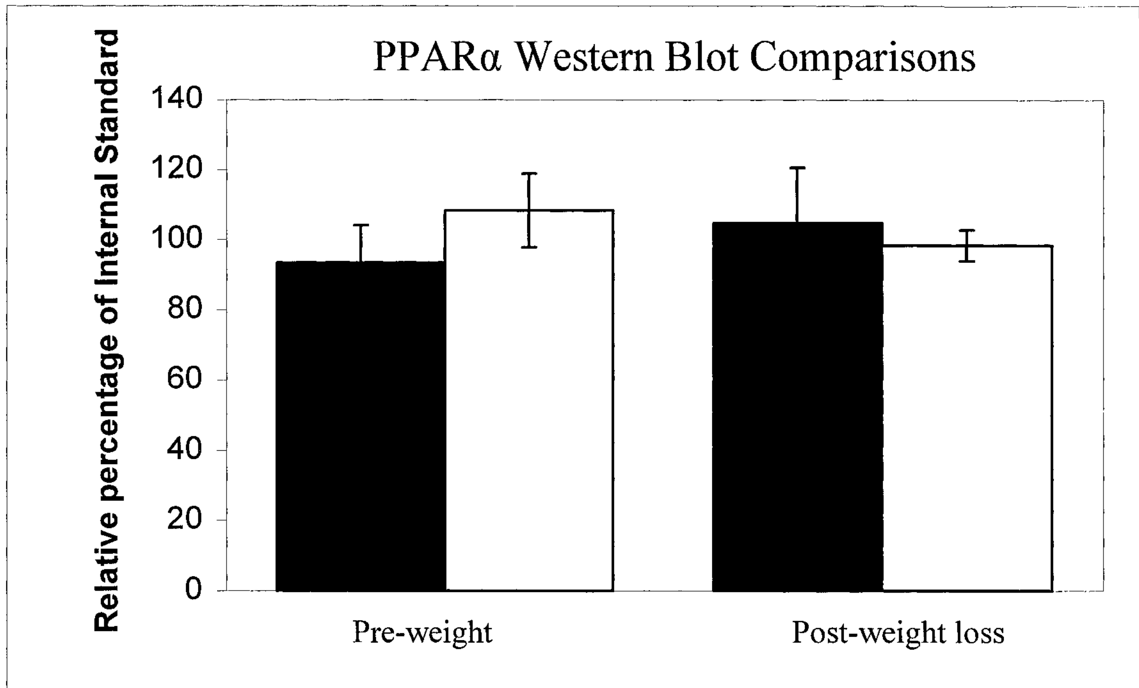

Figure 3-2. Relative percentages of PPARα protein before and after weight loss. The darkened bars represent mean percentages (± SE) of CON subjects, while the empty bars represent mean percentages (± SE) of ALA subjects.

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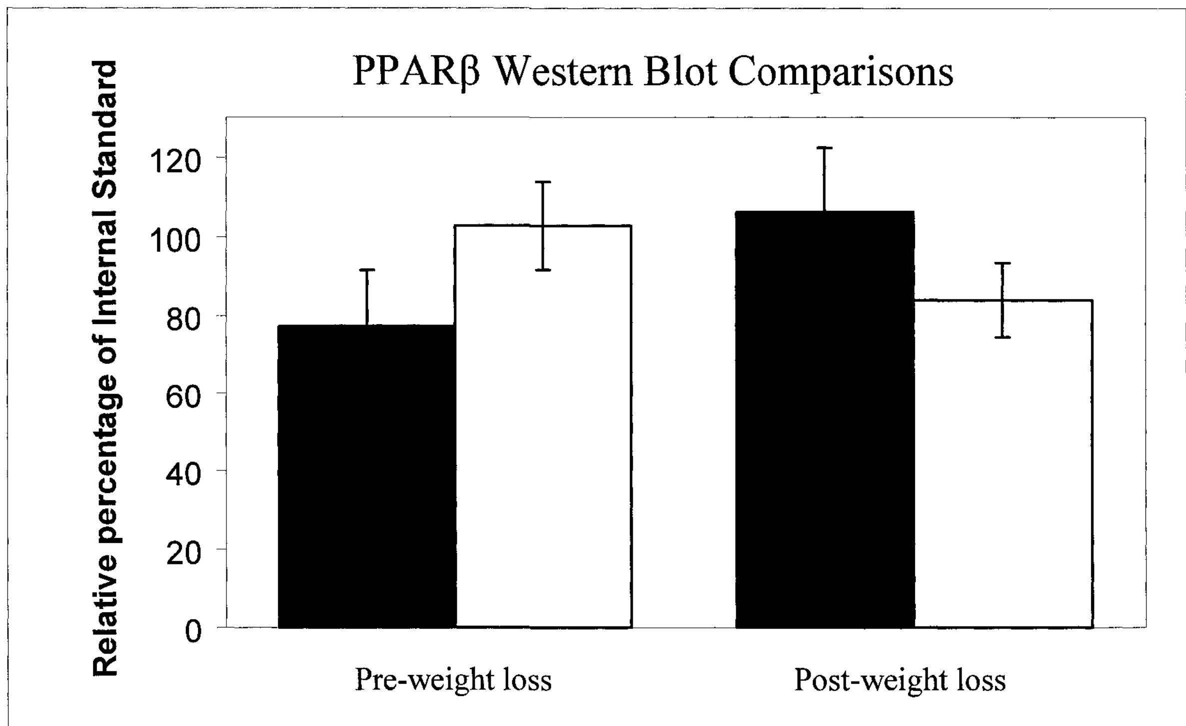

Figure 3-3. Relative percentages of PPARβ protein before and after weight loss. The darkened bars represent mean percentages (± SE) of CON subjects, while the empty bars represent mean percentages (± SE) of ALA subjects.

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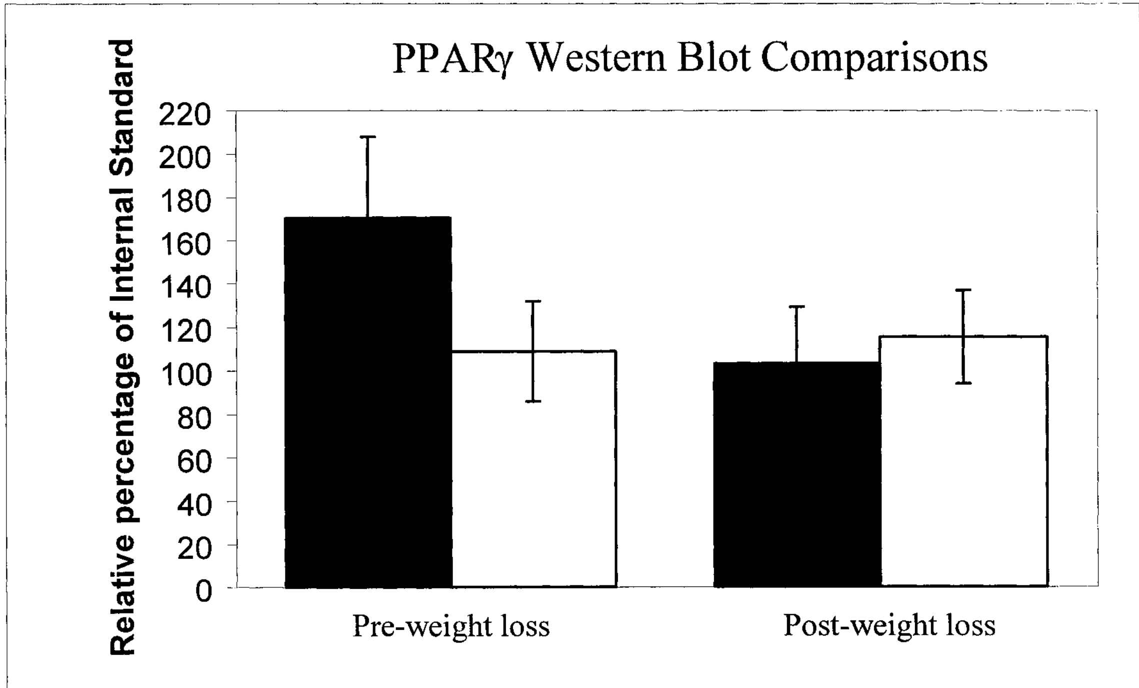

Figure 3-4. Relative percentages of PPARγ protein before and after weight loss. The darkened bars represent mean percentages (± SE) of CON subjects, while the empty bars represent mean percentages (± SE) of ALA subjects.

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The "thrifty genotype" and "thrifty phenotype" hypotheses have been proposed to explain the increase in obesity. Briefly described, the "thrifty genotype" theory hypothesizes that during a long-term famine the environment preferentially selected nmetabolically "thrifty" individuals to survive. A thrifty metabolism is ideal for an energy-deprived society, but when an affluent era arises, a thrifty metabolism results in excessive energy storage and thus, obesity (259). The "thrifty phenotype" hypothesis explains that childhood and adult-onset obesity and other metabolic diseases are the result of metabolic conditioning occurring in the womb or neonatally as a result of malnutrition (220). It has been suggested that the PPAR subfamily of transcription factors may be involved in one or both of these hypotheses.

A high fat diet is a major cause of obesity and insulin resistance in humans (100, 149) and in rodents (219). PPARγ-deficiency (heterozygous knockout) partially protected mice from high-fat diet-induced obesity and insulin resistance, indicating that PPARγ abundance is critical in the hypertrophy of adipose tissue and development of insulin resistance under a high-fat diet (199). In humans, a Proline to Alanine substitution at the twelfth amino acid residue of PPARγ has been associated with increased insulin sensitivity in obese and overweight individuals. The frequency of this polymorphism is significantly lower among diabetic individuals (134). Based upon these findings and the "thrifty genotype" hypothesis, it was hypothesized that under a hypocaloric diet, factors involved in lipid storage (e.g. PPARγ) would be upregulated to divert energy away from oxidation and toward sites of fat storage in a condition of energy-deprivation. Likewise, it was hypothesized that factors involved

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in lipid catabolism (e.g. PPARα, PPARβ) would be downregulated in skeletal muscle, allowing for the storage rather than oxidation of lipid substrates. It was further hypothesized that dietary supplementation with ALA(18:3n-3), a long-chain essential fatty acid known to bind all three PPAR isoforms (103, 393), would attenuate any changes observed with only hypercaloric restriction, thereby helping to maintain the pre-weight loss metabolic state and therefore prevent weight recidivism.

In the present study, PPARα and PPARβ protein concentrations exhibited an increasing trend with dietary weight loss. This effect was insignificant for PPARα, likely due to small samples and large variability among subjects. Another explanation, perhaps, is that PPARβ is more susceptible to dietary regulation. ALA(18:3n-3) supplementation resulted in the opposite trend, with PPARα and PPARβ protein concentrations decreasing under the combination of dietary weight loss with ALA(18:3n-3) supplementation. Also contrary to expectations, PPARγ protein concentrations decreased with dietary weight loss, albeit non-signficantly. This reduction was seemingly attenuated by ALA(18:3n-3) supplementation, which resulted in no significant change.

Why, if considered "thrifty" genes, would the PPAR isoforms change in a manner predicting subsequent fat oxidation rather than fat storage under dietary restriction? One potential reason is that the perturbation applied to these subjects did not invoke enough of a metabolic stress to warrant "thriftiness". The subjects consumed, at the least, 1,200 kcal/day; a diet relatively gluttonous when compared to victims of the Dutch Famine of 1944-1945 who had daily rations of 400-800 kcal

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(287, 288). These types of conditions cannot, for obvious ethical reasons, be reproduced in a research setting. However, a "thrifty" response may be evident after a 72-hour fast. The novel uncoupling protein isoform, UCP3, is specifically expressed in skeletal muscle (34) and significantly upregulated under fasting conditions (246, 378). Thiazolidinedione, which stimulates PPARγ, upregulated the expression of UPC3 (395), suggesting that UCP3 upregulation under fasting conditions may be an effect of PPARγ. Other studies have shown that PPARα is necessary to induce increased β-oxidation gene expression. Genes such as acyl-CoA oxidase, enoyl-CoA hydratase and 3-ketoacyl-CoA thiolase were upregulated under fasting conditions in wild-type mice but not in PPARα-/- mice (65, 179, 195, 207).

Another explanation for the observed results is that skeletal muscle is not a "thrifty" tissue. The weight lost by the subjects in the present study was primarily fat stores, a result of fatty acid mobilization from adipose tissue and oxidation at fat burning tissues, i.e. skeletal muscle. A trend toward increased expression of factors involved in lipid oxidation indicates that the negative energy balance is induced not only by a hypocaloric diet, but also a higher capacity for oxidizing fatty acids. It is important to note that PPARα and PPARβ are expressed at much higher levels in skeletal muscle that PPARγ, which is expressed primarily in adipose tissue (222). Therefore, changes in skeletal muscle expression of PPARγ may be insignificant with respect to the relatively dominant expression of the PPARs α and β. Changes in PPARα and PPARβ, on the other hand, may be more indicative of the metabolic capacity of skeletal muscle, but not of metabolic thriftiness.

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The supplementation of hypocaloric restriction with ALA(18:3n-3) caused a decrease in PPARβ protein expression. ALA(18:3n-3) binds and effectively activates PPARα, PPARβ, and PPARγ (103, 393). The affinity for ALA(18:3n-3) may differ between isoforms, potentially resulting in a feedback loop for the isoforms with higher affinity. More specifically, *if* PPARβ has a higher binding affinity for ALA(18:3n-3) it may be activated for a larger period time and have more potent effect on β-oxidation gene upregulation. Little is known about the binding affinities of the PPAR isoforms for ALA(18:3n-3). Forman *et al.* (103) reported that PPARβ has low micromolar affinities for the PUFAs DGLA(20:3n-6), EPA(20:5n-3), and AA(20:4n-6), and Xu *et al.* (393) estimated that PPARβ selectivity is intermediate between PPARα and PPARγ. Alternative to this argument, it is possible that ALA(18:3n-3) actually hinders the effects of PPARβ. If this study had been conducted in an inpatient setting, it may have been possible to link ALA(18:3n-3) consumption to the ability to lose weight.

In summary, the results from this experiment show that a hypocaloric diet causes a significant increase in skeletal muscle concentration of PPARβ, a transcription factor closely related to increased fatty acid oxidation. This observation was reversed, however, when the hypocaloric diet was supplemented with ALA(18:3n-3). Although not significant, similar trends were observed for PPARα, a family member also closely related to β-oxidation. The effect of weight-loss by a hypocaloric diet resulted in the reduction of PPARγ protein concentrations. These results do not reflect the "thrifty" genotype hypothesis, but the dietary perturbation

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may not have been metabolically stressful enough to induce a "thrifty" response. Further experiments are needed to understand the effect of fasting on skeletal muscle protein concentrations of PPAR isoforms.

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CHAPTER 4

EFFECT OF WEIGHT-LOSS AND ALA(18:3n-3) SUPPLEMENTATION ON PLASMA INSULIN AND PLASMA LEPTIN CONCENTRATIONS

Abstract

The hormonal resonse to weight loss is the topic of many research studies. The hormones leptin and insulin have been found to decrease with weight loss. Leptin is believed to decrease because of a reduction of fat mass, the origin of the circulating protein. Insulin is believed to decrease because of a reduction in insulin resistance associated with weight loss. This study hypothesized that previous observations would be replicated but that dietary supplementation with ALA(18:3n-3) would attenuate the reductions in plasma leptin and insulin concentrations otherwise seen with weight-loss. The results from this study indicate that dietary supplementation of ALA(18:3n-3) has no effect on the response of leptin and insulin concentrations to weight-loss.

Introduction

The specific role of dietary fat in obesity development has been the topic of intense research interest and debate (70, 82). Organizations such as the American Heart Association, American Diabetes Association, and American Cancer Society have advocated diets with a maximum of 30% of calories from fat, and no more than 10% of calories from saturated fat (87). Further interest in dietary fat *type* has been

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amplified by epidemiological observations regarding the prevalence of dietary long-chain omega-3 (n-3) fatty acids derived from fish oil and a decreased incidence of cardiovascular disease (77, 153).

Research evidence also supports the potential importance of dietary fat *type*. Rats fed an isocaloric diet high in polyunsaturated fatty acids (PUFA) have less body fat accumulation and lower respiratory quotients (RQ) than rats fed a diet high in saturated fatty acids (SFA) (325). In humans, isocaloric supplementation of long-chain PUFA (fish oil) results in increased fat oxidation and lower body mass (66). This may be especially important in obese humans, as they partition less SFAs toward oxidation than do lean humans (171). Furthermore, increasing PUFA content in low fat diets enhanced the oxidation of select medium and long chain fatty acids (52).

The long-chain fatty acid α-linolenic acid (ALA18:3n-3) is the fatty acid of interest in the presented research. ALA(18:3n-3), a plant-based 18 carbon n-3 fatty acid (70), can be elongated and desaturated to produce the 20 and 22 carbon n-3 fatty acids that are found in high amounts in fish oil, eicosapentanoic acid (EPA, 20:5n-3) and docosahexanoic acid (DHA, 22:6n-3) (70, 370). Interestingly, ALA(18:3n-3) competes for the same elongation and desaturation pathways as linoleic acid LA(18:2n-6), an 18-carbon omega-6 (n-6) fatty acid and precursor for arachadonic acid (AA, 20:4n-6) (70). The dietary n-6:n-3 ratio has been shown to be important with regard to human health, particularly in decreasing the risk of coronary heart disease (136).

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The composition of diets may also be important in regulating plasma insulin and leptin concentrations, both hormonal mediators of energy balance and nutrient utilization. A positive correlation exists in rodent models between increased fat consumption (50-60% of total energy) and increased leptin levels, but there seems to be no effect in humans (318). This evidence is further supported by work from Havel *et al.*, who found that weight maintenance diets containing 14, 23 or 31% of energy from fat, had no effect on leptin in human women (142). More germane to this study is the examination of the proportion and types of macronutrients, specifically fatty acids, and their effect on plasma leptin and insulin levels. Cha and Jones concluded that there was a hyperleptinemic effect in rats fed a diet rich in polyunsaturated fatty acids (PUFA), especially fish oil (46). High fat intake has been associated with insulin resistance (138), while fish oil consumption has been shown to improve insulin action (223). In addition, both insulin and leptin plasma concentrations have been shown to decrease in response to a dietary weight loss (114, 300, 301, 351). Therefore, the aim of the this study was to examine the effect of ALA(18:3n-3) supplementation on changes in leptin and insulin during dietary weight loss.

Subjects and methods

Subjects

The Colorado State University Human Research Committee approved the following protocol (Appendix A), and 11 obese ($40\ kg/m^2 > BMI > 30\ kg/m^2$) subjects were recruited from volunteers responding to a weight-loss study advertisement. Potential subjects completed a health history questionnaire (Appendix

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B) and were also not allowed to participate if they presented any of the following conditions: pregnant, irregular menstrual cycle, hypo- or hyperthyroidism, hypertension, tobacco use, diabetes mellitus, history or eating disorders, any irregular metabolic condition, or taking any metabolism-altering medications. Subjects were required to be weight stable ($\pm$ 2.5 kg) for the previous 6 months. Each volunteer provided written consent to participate in the study (Appendix C).

Diets and experimental design

Subjects were instructed how to properly record their diets (e.g. portion sizes, method of preparation, brand names). Records were reviewed by a registered dietician for completeness and satisfactory detail before analysis with the Food Intake Analysis System (FIAS 3.98 nutrient analysis program, University of Texas School of Public Health, 1998). Pre-weight-loss measurements (e.g. height, mass, body composition, resting metabolic rate, blood collection) were administered after four weeks of weight stability. Subjects were then randomly assigned to the control group (CON) or the ALA(18:3n-3) supplementation group (ALA) and prescribed the respective diet. Both diets were designed to induce a 10% weight loss by using the American Heart Step I diet. The ALA diet, however, had an exchange of fatty acids so that 5% of daily caloric consumption was in the form of ALA(18:3n-3). ALA subjects were provided with flax seed oil capsules for this purpose. The prescribed daily caloric intake was determined by calculating 80% of 24-hour resting metabolic rate, with a minimum consumption of 1200 kcal/day. Table 2-1 illustrates the composition of the prescribed diets based upon daily caloric consumption. Both diet

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groups were told not to eat food with a high content of ALA(18:3n-3) or EPA(20:5n-3). After approximately 10% of a subjects starting weight had been lost, daily caloric consumption was manipulated so that the post-weight-loss weight could be maintained for 4 weeks and then post-weight loss measurements were re-administered. Figure 2-1 provides a diagram of the study design.

Anthropometric measurements

A physician's balance scale (Detecto, Webb City, MO) was used to measure height and weight to the nearest centimeter and 0.1 kg, respectively. Body mass index (BMI, kg/m^2) was calculated from the body mass and height measurements. A Lunar DPX-IQ Dual-energy x-ray absorbtiometry (DEXA) scanner (Lunar Corp., Madison, WI) was used to determine body composition. From this assessment, the variables of percent body fat, fat-free mass (kg), and lean body mass (kg) were obtained.

Resting Metabolic Rate

The Vmas 29 Series (SensorMedics, Yorba Linda, CA) ventilated hood system was used to determine a subject's resting metabolic rate. The subjects were instructed to arrive at the Human Performance Clinical / Research Laboratory after fasting for 12 hours and a normal night of sleep. The assessment transpired in a darkened, thermo neutral room. The subjects were told to stay awake, and to not make any voluntary limb movements. Expiratory gases were collected for a total of

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60 minutes. The last 30 minutes of the assessment was analyzed using the Weir equation (379) and then extrapolated to determine 24-hour energy requirements.

Blood collection and analysis

Subjects were instructed to fast for 12-hours before arriving at the Human Performance Clinical / Research Laboratory. Blood was collected from an antecubital vein into vacutainer containing 0.081 mL of 15% (K_3) EDTA solution (Becton Dickinson, Franklin Lakes, NJ). The sample was centrifuged for 15 minutes at 2800 RPM and 4°C. The blood plasma was removed and stored at –80°C until further analysis.

Plasma leptin and insulin concentrations were determined using the ACTIVE™ Human Leptin ELISA (DSL-10-23100, Diagnostic Systems Laboratories, Inc., Webster, TX) and the ATIVE™ Human Insulin ELISA (DSL-10-1600, Diagnostic Systems laboratories, Inc., Webster, TX) assays, respectively. The instructions provided by the manufacturer were followed.

Statistics

Statistical analysis was made using the Statistical Package for the Social Sciences (SPSS) 10.0 (SPSS, Chicago, Ill.). Repeated measures analysis of variance was used to test the presence of an interaction between dietary weight loss and ALA(18:3n-3) supplementation, with $\alpha \leq 0.05$. Differences between groups prior to and after weight loss were analyzed with an independent samples t-test, $\alpha \leq 0.05$.

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Results

Subject characteristics

Eleven obese subjects completed the weight-loss experiment. Prior to starting the weight-loss diet there were no significant differences between the CON group and the ALA group for BMI ($t = 2.078$, $P = 0.067$), body fat percentage ($t = -0.306$, $P = 0.766$), fat mass ($t = 0.705$, $P = 0.498$), or age ($t = 0.331$, $P = 0.748$). Weight loss had a significant effect by reducing BMI ($F = 107.97$, $P < 0.001$), fat mass ($F = 41.43$, $P < 0.001$) and body fat percentage ($F = 19.12$, $P = 0.002$) as expected. There was no significant difference between groups with regard to the change in percent body fat ($t = -0.643$, $P = 0.54$) over the course of the weight-loss period. Table 2-2 provides the subject characteristics before and after weight loss.

Dietary analysis

Analysis of dietary records was performed to examine the differences between the diets consumed by the CON and ALA groups. Prior to weight loss, there were no statistical differences between groups. Upon entering the weight-loss phase, both groups significantly reduced their total caloric consumption. During the weight-loss phase, there were only two statistical differences between groups. First, the ALA group consumed more polyunsaturated fat when expressed as total grams ($t = 3.241$, $P = 0.01$) or as a percentage of total caloric consumption ($t = 3.322$, $P = 0.009$). Secondly, the ALA group consumed more ALA(18:3n-3) than the control group when expressed as total grams ($t = 7.26$, $P < 0.001$) or as a percentage of total calories ($t = 7.119$, $P < 0.001$). Together, this indicates that higher PUFA consumption of the

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ALA group is due to the increase ALA(18:3n-3) consumption. These data are presented in Table 2-3.

Plasma Insulin

There was no statistical difference between groups for plasma insulin concentrations prior to weight loss ($t = -0.164$, $P = 0.873$), but mean value do indicate that both groups were relatively hyperinsulinemic (see Table 4-1). The dietary weight-loss had a significant effect on plasma insulin levels as both groups declined ($F = 9.972$, $P = 0.012$). There was no interaction between weight-loss and ALA(18:3n-3) supplementation ($F = 0.859$, $P = 0.378$), as the decrease experienced by ALA group was not statistically different than the CON group. The group means for plasma insulin concentrations are graphically displayed in Figure 4-1.

Plasma Leptin

Similar to plasma insulin concentrations, plasma leptin concentrations did not differ between subjects prior to weight-loss ($t = 0.594$, $P = 0.599$). Weight-loss also had a significant effect on plasma leptin concentrations ($F = 20.770$, $P = 0.001$), but there was no interaction between weight-loss and ALA(18:3n-3) supplementation ($F = 0.258$, $p = 0.624$). The group means for plasma leptin concentrations are displayed in Table 4-1 and Figure 4-2.

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Table 4-1.
Plasma hormone concentrations of CON and ALA groups prior to and after weight loss[1]

	Control group (n=6)		ALA group (n=5)	
	Pre	Post	Pre	Post
Insulin (pmol/L)	95.05 ± 26.23	80.24 ± 26.05	92.44 ± 26.35	65.31 ± 18.86
Leptin (ng/mL)	34.68 ± 20.92	17.33 ± 13.47	41.75 ± 21.75	20.03 ± 9.74

[1] $\chi \pm$ SD

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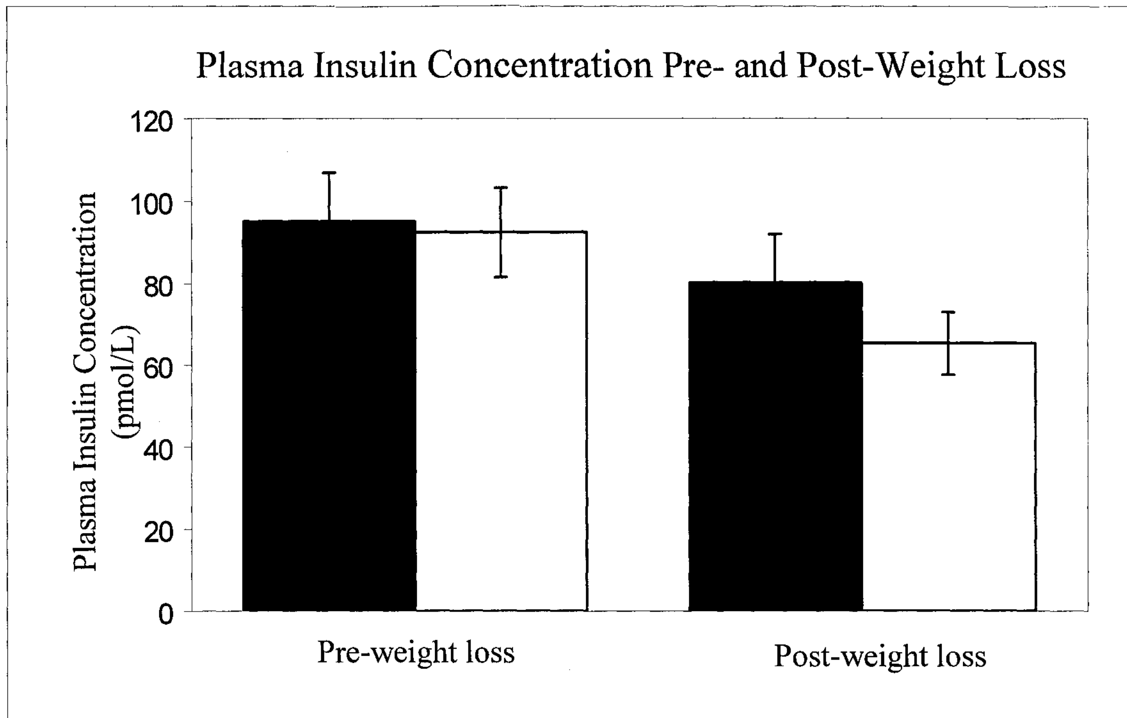

Figure 4-1. Plasma insulin concentrations (pmol/L) before and after weight loss in CON (black) and ALA (white) subjects (mean ± SE).

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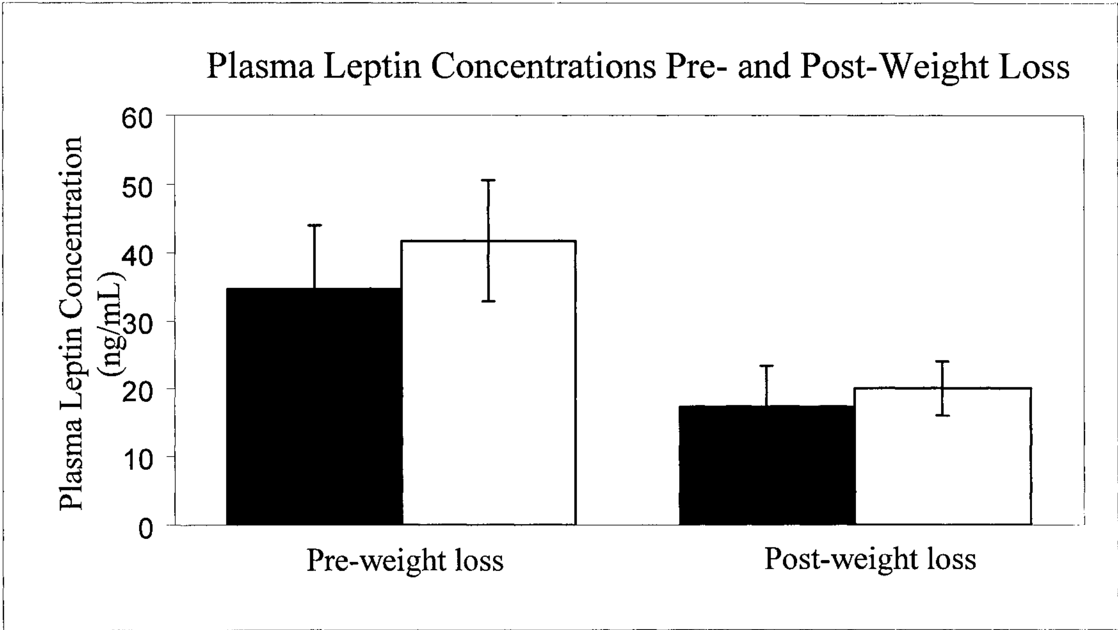

Figure 4-2. Plasma leptin concentrations (ng/mL) before and after weight loss in CON (black) and ALA (white) subjects (mean $\pm$ SE).

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Discussion

The present study was designed to investigate the effect of weight loss and dietary ALA(18:3n-3) supplementation on plasma insulin and leptin concentrations. Previous reports have consistently shown that weight loss results in decreased insulin resistance and decreased hyperinsulinemia (302, 303, 392). Zarkovic *et al.* (399) found that in addition to improved insulin sensitivity, the number of insulin pulses is reduced and regularity of insulin pulses is improved after weight loss in obese subjects. The present results are consistent with previous studies showing that dietary weight-loss is beneficial in reducing hyperinsulinemia. Insulin sensitivity estimates were not calculated for this study, therefore it can only be assumed that the decline in plasma insulin concentrations are a reflection of increased peripheral insulin sensitivity, as has been shown in numerous other studies (257, 303).

The increased consumption of ALA(18:3n-3) had no apparent effect on the decreased levels of plasma insulin with weight loss. It has been well established that glucose is the most potent regulator or insulin secretion (240). Glucose-stimulated insulin release is also altered by fatty acids. *In vitro* studies have shown that glucose-stimulated insulin release is more pronounced with increased fatty acid chain length and saturation (3, 374). Human studies, however, do not provide a clear indication as to which fatty acid type is more potent in regulating insulin release. Beysen *et al.* found that dietary PUFAs and MUFAs increase and SFAs have no impact on insulin release (25). On the contrary, a high saturated fat diet stimulated a higher insulin secretion rate in Type II diabetic subjects (285), while others reported that insulin release was more pronounced as a result of PUFA intake (202). In the case of the

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present study, the dietary weight loss may have resulted in improved insulin sensitivity, and therefore decreased plasma glucose concentrations. The potential effect of ALA(18:3n-3) supplementation on insulin secretion, therefore, may be masked by a decrease of the more potent stimulator of insulin release, i.e. plasma glucose levels.

Dietary weight loss has previously been shown to reduce plasma leptin concentrations, largely due to a decrease in adipose tissue depots, the source of leptin synthesis (114, 300, 351, 363, 365, 388, 392). The present results are consistent with previous observations as plasma concentrations declined significantly after dietary weight loss. The main objective of this study, however, was to study the effect of supplementary ALA(18:3n-3) consumption on the reduction of leptin.

A previous study reported that fish oil- and safflower oil-fed rats had significantly higher plasma leptin concentrations. Caloric restriction resulted in a significant decrease in plasma levels in these groups, but beef tallow-fed rats experienced no change. The authors concluded that the response of leptin concentration to energy restriction is dependent on dietary fat type, particularly polyunsaturated fatty acids (46). Since ALA(18:3n-3) is a metabolic precursor to EPA(20:5n-3), a predominant fatty acid of fish oils, we tested the effect of an ALA(18:3n-3)-rich diet on leptin concentrations after weight-loss. We observed similar results, in that the plasma leptin concentrations did decrease with dietary weight-loss and ALA(18:3n-3) supplementation, but the same observation was made in the CON subjects. It is possible that obese humans are more sensitive to dietary restriction than dietary fat *type*. Larger sample sizes, however, may have revealed a

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more robust decrease in the ALA group than the CON group. Future studies will be needed to further elucidate the complexity of leptin regulation. It should be noted that mixed gender groups might have had an effect on the results. Rojo-Martinez *et al.* found that dietary stearic acid (18:0) and EPA(20:5n-3) were significantly correlated with serum leptin levels in men, while in women, the correlation was significant for dietary LA(18:2n-6) and AA(20:4n-6) (298), providing another level of complexity to leptin regulation. The cause of the gender-specific relationship has yet to be elucidated and the present study did not provide ample sample sizes for this type of analysis.

Numerous leptin and insulin studies have been performed that support the hypothesis that there is a unique relationship between the two hormones - an "adipoinsular axis" (44). In support of this theory, hyperinsulinemia is associated with hyperleptinemia (73) and the diabetic phenotype of *ob/ob* mice was corrected with leptin treatment (256). Leptin is also thought to play a direct role in decreasing insulin secretion (182). This effect is likely concurrent to leptin's effect on improving insulin sensitivity at peripheral tissues (183, 221, 373), which also leads to decreased pancreatic insulin release. Contrary to this, however, are results indicating that insulin action is impaired by leptin treatment to human hepatic cell lines, isolated rat adipocytes, and skeletal muscle (56, 253, 347). Although some observations are paradoxical, Ceddia concluded in a recent review that leptin does play a role in insulin sensitivity (42). The results of this study do not provide any novel evidence that would help to clarify this issue.

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In conclusion, an ALA(18:3n-3) rich diet has no effect on the leptin- and insulin-reducing effect of dietary weight-loss. This observation might be due to obese humans being more sensitive to dietary restriction, rather than dietary fat type. Furthermore, the results of this study do not help to elucidate the existence of an adipoinsular axis.

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CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The results presented in the previous chapters demonstrate that weight loss via caloric restriction results in changes in plasma hormones and skeletal muscle factors important in metabolism. Furthermore, the supplementation of ALA(18:3n-3) in the diet has an effect on some of these changes. This chapter will highlight some of these findings, point out the limitations of interpreting the data, and provide some ideas for future studies.

CHAPTER 2 - Effect of Weight-loss and ALA(18:3n-3) Supplementation on Erythrocyte Fatty Acid Composition

The first study showed that manipulation of the diet with a particular fatty acid is reflected in similar changes erythrocyte plasma membranes. Most importantly, this showed that the subjects instructed to consume an ALA(18:3n-3) supplement were compliant. There was no difference between groups for erythrocyte membrane concentrations of the ALA(18:3n-3) metabolites EPA(20:5n-3) and DHA(22:6n-3). This result was not unexpected as others have shown that diets relatively high in ALA(18:3n-3) result in lower erythrocyte and platelet concentrations of EPA(20:5n-3) and DHA(22:6n-3) compared to a mixed diet (1).

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There is no evidence to indicate that the enzymes necessary for ALA(18:3n-3) to EPA(20:5n-3) and DHA(22:6n-3) conversion are present in erythrocytes, but this should not limit their incorporation into erythrocyte plasma membranes. There is some evidence that indicates that Δ5- and Δ6-desaturase activity and expression are regulated by their substrates. More specifically, the amount of ALA(18:3n-3) metabolism by Δ5- and Δ6-desaturase is regulated by the amount of competing substrate, LA(18:2n-6). Since LA(18:2n-6) consumption was not regulated in the present study, it is not prudent to speculate on Δ5- and Δ6-desaturase activity. It should be noted, however, that any potential health benefits of increased ALA(18:3n-3) content in erythrocyte or any tissue membrane could be optimized by concomitantly decreasing LA(18:2n-6) consumption. This is not to say that ALA(18:3n-3) does not have an impact on membrane properties, gene expression, metabolic signaling, eicosanoid production, or energy expenditure independent of its conversion to EPA(20:5n-3) and DHA(22:6n-3) (366).

CHAPTER 3 - Effect of Weight-loss and ALA(18:3n-3) Supplementation on PPAR Isoform Concentrations in Human Skeletal Muscle

The results of the second study presented demonstrated that skeletal muscle concentrations of the three PPAR isoforms change with dietary weight loss. The only significant change occurred in PPARβ, as it increased from pre- to post-weight loss measurements. The other lipolytic isoform, PPARα, also increased while the lipogenic isoform, PPARγ, decreased. These two observations were not statistically significant, likely because of the small sample size. PPARβ is expressed at levels

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much higher than PPARα and PPARγ. The higher concentration of PPARβ might have allowed for any changes to be detected, where any changes in PPARα and PPARγ were less detectable.

The PPAR family of nuclear receptors, particularly PPARγ, has been implicated as role players in metabolic thriftiness (133). It was expected that because of the dietary restriction, PPARγ concentration would increase to upregulate genes involved with lipid storage. Alternatively, it was expected that PPARα and PPARβ concentration would decrease to allow for more lipid storage and less lipid oxidation. Since the capacity of skeletal muscle to store lipids is remarkably lower than adipose tissue, it is possible that skeletal muscle does not participate in metabolic thriftiness. That is, the dietary restriction may have very well caused the expected changes in adipose tissue. This would not allow, however, for the release of stored fat from adipose tissue to be oxidized by skeletal muscle where the changes in PPAR isoforms reflect a higher metabolic state. Future studies should consider studying both tissues simultaneously.

An interesting outcome of the study was that ALA(18:3n-3) supplementation attenuated any changes in the skeletal muscle concentrations of PPAR isoforms due to dietary weight-loss. For example, while PPARβ concentration significantly increased in the subjects losing weight via dietary restriction, PPARβ concentration decreased or stayed the same in the subjects losing weight via ALA(18:3n-3) supplemented dietary restriction. This trend, although not significant, was also observed for PPARα. The opposite was true for PPARγ. Skeletal muscle concentrations of PPARγ decreased with dietary weight loss, but with the

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supplementation of ALA(18:3n-3), PPARγ stayed the same. These observations are perplexing because the same ligand, ALA(18:3n-3), is attenuating the *decrease* of PPARγ and attenuating the *increase* of PPARα and PPARβ. Due to limited muscle sample sizes, this study was restricted from measuring PPAR mRNA in addition to protein. It is possible that a regulatory factor recognizes a higher concentration of energy density (unsaturated vs. saturated fatty acids), and therefore causes the dampening of lipid metabolism gene regulation via PPAR isoforms. Another possibility is that the PPARs have a higher affinity for ALA(18:3n-3), and therefore require less change of abundance in order to elicit an effect on the transcription of target genes.

Chapter 4 - Effect of Weight-loss and ALA(18:3n-3) Supplementation on Plasma Insulin and Plasma Leptin Concentrations

The final study does not provide any new evidence regarding the effect of weight-loss on plasma leptin and plasma insulin concentrations. Dietary weight loss caused a decrease in plasma leptin and plasma insulin, but ALA(18:3n-3) supplementation had no effect on either change. The decrease of adipose tissue mass is likely the strongest reason that plasma leptin decreased, since adipose tissue is the primary source of the hormone. The increased insulin sensitivity associated with weight loss is a likely reason for the decrease in plasma insulin.

Conclusions and Future Directions

The studies presented in the previous chapters describe some interesting observations regarding obesity, weight loss, and dietary fat. Although interesting in

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their own right, this final section is an attempt to integrate these findings and relate their significance to metabolic physiology. The data obtained from this study are not sufficient for accurately predicting the mechanisms by which they occur; however, it may be of value to the design of future studies to speculate why the observations made in the presented studies occurred. The most important relationships to be examined are that between the metabolic hormones leptin and insulin, and the skeletal muscle nuclear receptors, PPARs α, β, and γ.

The major stimulant for change in these studies was dietary weight-loss. This caused an increase in PPARβ protein in skeletal muscle as well as an apparent trend of increased PPARα protein. Together, this indicates a status of higher lipid oxidation in skeletal muscle. The negative energy imbalance caused by the weight-loss diet and higher PPARα/β expression could have conceivably resulted in elevated fat mobilization from adipose tissue. The decrease in adipose cell size probably resulted in decreased leptin expression and subsequent secretion (67).

The increased level of skeletal muscle fatty aid oxidation, as reflected by increased PPARs α and β and decreased PPARγ may have also led to increase insulin sensitivity. Previous reports have shown that heterozygous (+/-) PPARγ-deficient mice display increased insulin sensitivity as compared to wild-type control mice (209). Although the decrease of PPARγ observed in this study was not significant, an increase of fatty acid oxidation decreases skeletal muscle triglyceride (as well as liver triglyceride), possibly potentiating any decrease of PPARγ. This is, as one study concluded, a means by which thiazoladinediones increase peripheral insulin sensitivity (394). Although not tested, a decrease of PPARγ in adipose tissue would

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have likely led to an increase in leptin mRNA and secretion. The leptin gene promoter contains a PPRE and several studies have shown that leptin is down-regulated by PPARγ agonists (152). This supports the idea that PPARγ likely increased in adipose tissue, the opposite of what was observed in skeletal muscle, and that future studies need to investigate both skeletal muscle and adipose.

The potential increase in insulin sensitivity, as well as a subsequent decrease in the primary insulin secretion stimulant, may have reduced the need for insulin and thus, decreased insulin production. Additionally, a decrease of leptin could also directly cause a decrease of insulin secretions (43, 226). Direct measurements of neither insulin secretion nor clearance were measured in these studies, making these kinds of predictions difficult. These, of course, are only potential mechanisms by which the observation described in the previous chapters may be related. Future studies will need to examine these relationships more closely.

Study Shortcomings

Several criticisms could be made of the present experiments. First of all, and most obvious, is the need for larger sample sizes. The original design of these studies called for 12 subjects in each group. This was not accomplished because of a lack of response by *qualified* volunteers to the study advertisement. Secondly, the time course of study may not have been appropriate. A better study design might have taken muscle and blood samples immediately after the 10% weight-loss and again after a longer period of weight stability. The length of weight stability enforced in these studies may not have been sufficient for appropriate post-weight-loss

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observations. A longer period of weight stability may provide a better indication of the effect of weight loss on the measured parameters, and by measuring at two time-points after weight-loss would help discern between the effect of caloric restriction and the effect of prolonged weight-loss. Lastly, it would also have been beneficial to obtain both skeletal muscle and adipose tissue samples.

Recommendations

Potential follow-up research should consider expanding the sample sizes and including two other controls groups: a lean control and an obese control group, each supplemented with ALA(18:3n-3) but not undergoing dietary weight-loss. Although not ethical by most research standards, ongoing research in this area could investigate the effect of a 10% weight gain. It may also be beneficial to provide some meals for the subjects in order to have better compliance and potentially faster weight-loss. As mentioned in previous paragraphs, post-weight-loss measurements should be planned carefully, making sure to test the result of reduced adiposity and weight-loss rather than hypocaloric stress. In addition to measuring PPAR isoform protein levels, mRNA expression should be quantified, as well as the gene targets of the different PPAR isoforms. This was not performed for the presents studies because of small muscle tissues sizes. Furthermore, both lipid storage and lipid oxidation genes should be investigated in adipose tissue and skeletal muscle. To better assess insulin sensitivity, euglycemic/hyperinsulinemic clamps could be utilized. On that note, measurements could also be made to assess insulin secretion and clearance. Lastly, little is known about the effect of different fatty acids on the regulation of PPAR gene

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expression. This study provided some evidence to indicate that dietary weight-loss and ALA(18:3n-3) may play a role , but *in vitro* experiments may be necessary to fully characterize the promoters of the different isoforms.

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APPENDIX A

HUMAN RESEARCH COMMITTEE APPROVAL

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Office of Regulatory Compliance
Office of Vice President for Research
and Information Technology
Fort Collins, CO 80523-2046
(970) 491-1563
FAX: (970) 491-2293

MEMORANDUM

TO: Matthew Hickey, Health and Exercise Science, 1582

FROM: Celia S. Walker, Administrator
Human Research Committee

SUBJECT: PROJECT APPROVAL
Title: Effect of a-Linolenic Acid on 24 Hours Energy Expenditure and Nutrient Oxidation Following Weight Loss in Obese Humans.
Protocol No.: 99-266H H
Funding Agency: USDA/Colorado Agricultural Experiment Station
Funding Agency Deadline: N/A

DATE: January 19, 2000

I am pleased to inform you that the above-referenced project was approved by the Human Research Committee on Januuary 12, 2000 for the period January 12, 2000 through January, 2001 with the condition that the attached consent form is signed by the subjects and each subject is given a copy of the form. It is the investigator's responsibility to obtain this consent form from all subjects. *NO changes may be made to this document without first obtaining the approval of the Committee.* **Approval is for 24 participants.**

A status report of this project will be required within a 12-month period from the date of approval. You will be sent a reminder approximately two months before the protocol expires. The Principal Investigator will report on the numbers of subjects who have participated this year and project-to-date, about problems encountered, and provide a verifying copy of the consent form or cover letter used. The necessary form (H-101) is available from the Regulatory Compliance web page (see below). Should the protocol not be renewed before expiration, all activities must cease until the protocol has been re-reviewed.

It is the responsibility of the investigator to immediately inform the Committee of any serious complications, unexpected risks, or injuries resulting from this research. It is also the investigator's responsibility to notify the Committee of any changes in experimental design, participant population, or consent procedures or documents. This can be done with a memo which completely describes the changes and their consequences (new consent form or cover letter, or altered survey instrument, for example). Students serving as Co-Principal Investigators may not alter projects without first obtaining PI approval. The PI is ultimately responsible for the conduct of the project.

This approval is issued under Colorado State University's OPRR Multiple Projects Assurance M-1153-01 issued August 1, 1996. If approval did not accompany a proposal when it was submitted to a sponsor, it is the researcher's responsibility to provide the sponsor with the approval notice.

Please direct any questions about the Committee's action on this project to me for routing to the Committee.

Additional information is available from the Regulatory Compliance web site at www.research.colostate.edu/regulatory/

Attachment
xc: Kevin Davy w/attachment
Jennifer Iron

Animal Care & Use • Drug Review • Human Research • Institutional Biosafety • Radiation Safety
410 University Services Center

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APPENDIX B

HEALTH HISTORY QUESTIONNAIRE

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COLORADO STATE UNIVERSITY

CONFIDENTIAL HEALTH HISTORY QUESTIONNAIRE

STUDY______________________ **DATE**______ **SUBJECT ID #** __________________

Reviewed by (must be PI or MD):_____________________________

Current Age_______ **Height**______ **Weight**___________

PLEASE PRINT

GENERAL MEDICAL HISTORY

	YES	NO
Do you have any current medical conditions?	☐	☐

If Yes, please explain:

	YES	NO
Have you had any major illnesses in the past?	☐	☐

If Yes, please explain:

	YES	NO
Have you ever been hospitalized or had surgery?	☐	☐

If Yes, please explain: (include date and type of surgery, if possible)

	YES	NO
Have you ever had an EKG?	☐	☐

If Yes, please explain:

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Are you currently taking any medications, including aspirin, hormone replacement therapy, or other over-the-counter medications? YES ☐ NO ☐
If Yes, please explain:

Medication	Reason	Times taken per Day	Taken for how long?

Are you currently taking any nutritional supplements, such as Ginko, St. John's Wort, or others?

YES ☐ NO ☐ If Yes, please explain:

Supplement	Reason	Times taken per Day	Taken for how long?

Have you been diagnosed with diabetes? YES ☐ NO ☐
If Yes, please explain:

Age at diagnosis__________

Have you been diagnosed with a thyroid disorder? YES ☐ NO ☐
If Yes, please explain, including any medications taken:

FAMILY HISTORY

Please indicate the current status of your immediate family members.

	Age (if alive)	Age of Death	Cause of Death
Father	________	________	____________
Mother	________	________	____________
Brothers/Sisters	________	________	____________
	________	________	____________
	________	________	____________
	________	________	____________

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Do you have a **family** history of any of the following: (Blood relatives only, please give age at diagnosis if possible)

Diagnosis	**YES**	**NO**	**Relation**	**Age at**
a. High blood pressure	☐	☐	________________	
b. Heart Attack	☐	☐	________________	
c. Coronary bypass surgery	☐	☐	________________	
d. Angioplasty	☐	☐	________________	
e. Stroke	☐	☐	________________	
f. Diabetes	☐	☐	________________	
g. Obesity	☐	☐	________________	
g. Other (Please list)	☐	☐	________________	

TOBACCO HISTORY (check any that apply)

CURRENT TOBACCO USE

				(if applicable) **# per day**
None	☐			
Quit	☐	(when)______		
Cigarette	☐		Cigarette	___________
Cigar	☐		Cigar	___________
Pipe	☐		Pipe	___________
Chew Tobacco	☐		Chew Tobacco	___________
Snuff	☐		Snuff	___________

Total years of tobacco use_______

CARDIORESPIRATORY HISTORY

	YES	**NO**
Presently diagnosed with heart disease?	☐	☐
History of heart disease?	☐	☐
Heart murmur?	☐	☐
Occasional chest pain or pressure?	☐	☐
Chest pain or pressure on exertion?	☐	☐
Heart valve problem?	☐	☐
Abnormal heart rhythm?	☐	☐
Edema (fluid build up)?	☐	☐
High cholesterol?	☐	☐
History of rheumatic fever?	☐	☐
Episodes of fainting?	☐	☐

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Daily coughing?	☐	☐
High blood pressure?	☐	☐
Shortness of breath?		
At rest?	☐	☐
Lying down?	☐	☐
After 2 flights of stairs?	☐	☐
Asthma?	☐	☐
Emphysema?	☐	☐
Bronchitis?	☐	☐
History of bleeding disorders?	☐	☐
History of problems with blood clotting?	☐	☐

If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.

MUSCULOSKELETAL HISTORY

	YES	NO
Any current muscle injury or illness?	☐	☐
Any muscle injuries in the past?	☐	☐
Muscle pain at rest?	☐	☐
Muscle pain on exertion?	☐	☐
Any current bone or joint (including spinal) injuries?	☐	☐
Any previous bone or joint (including spinal) injuries?	☐	☐
Painful joints?	☐	☐
Swollen joints?	☐	☐
Edema (fluid build up)?	☐	☐
Pain in your legs when you walk?	☐	☐

If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.

NEUROLOGICAL HISTORY

	YES	NO
History of seizures	☐	☐

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Diagnosis of epilepsy	☐	☐
History of fainting	☐	☐

GASTROINTESTINAL HISTORY

	YES	NO
History of ulcers?	☐	☐
History of colitis?	☐	☐
History of chronic diarrhea?	☐	☐
History of chronic constipation?	☐	☐

REPRODUCTIVE HISTORY

	YES	NO
Currently pregnant?	☐	☐
Think you might be pregnant?	☐	☐
Planning on becoming pregnant in the near future?	☐	☐
Currently using Oral Contraceptives?	☐	☐
History of menstrual cycle irregularities?	☐	☐
Hysterectomy?	☐	☐

DIET HISTORY

	YES	NO
Have you ever dieted?	☐	☐

If YES, have you dieted within the past 12 months or are you currently on a diet?

YES	NO
☐	☐

If you have dieted within the past 12 months, please describe the diet:

a). Name (if applicable):_______________________________________

	YES	NO
b). Prescribed by a Physician/nutritionist?	☐	☐
c). Have you lost weight?	☐	☐

d). Duration of diet________

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What was your weight 12 months ago? __________

What is your current weight?_______

	YES	NO
Have you dieted other than in the past 12 months?	☐	☐

If YES, please answer the following:

a). How many times have you dieted?

b). How old were you?

c). Weight loss (amount)?

	YES	NO
History of eating disorders?	☐	☐

You may be asked to complete a more detailed diet survey if you are volunteering for a research study.

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APPENDIX C

INFORMED CONSENT FORM

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INFORMED CONSENT TO PARTICIPATE IN A RESEARCH PROJECT

TITLE OF PROJECT: Effect of α-Linolenic Acid on 24 hour energy expenditure and nutrient oxidation following weight loss in obese humans.

NAME OF PRINCIPAL INVESTIGATOR: Matthew S. Hickey, Ph.D.

NAME OF CO-INVESTIGATOR: Kevin P. Davy, Ph.D.

CONTACT NAME AND PHONE NUMBER FOR QUESTIONS/PROBLEMS:
Matthew Hickey, 491-5727

SPONSOR OF THE PROJECT: USDA-Colorado Agricultural Experiment Station

PURPOSE OF THE RESEARCH: The purpose of the present study is to determine the effect of a specific type of dietary fat on the metabolism during weight loss. This project is part of a larger effort by the Department of Health and Exercise Science to develop a better understanding of the biological basis of human obesity. It is anticipated that each participant will be in the study for 3-6 months.

PROCEDURES/METHODS TO BE USED: You will be asked to complete a health history questionnaire prior to participation, which will take about 15-20 minutes. If you agree to participate and the investigators determine you are eligible, you will be scheduled for several visits to the Human Performance Clinical/Research Laboratory (HPCRL). You will not be able to participate in this study if you are currently using prescription or over-the-counter medications known to alter metabolism, oral contraceptives, or tobacco. These will be determined from a health history questionnaire you will complete before starting the study. In addition, if your normal diet contains an unusually high intake of a specific type of fat, you will not be eligible for the study. Should you meet any of these exclusion criteria, we will fully inform you as to the reason for the exclusion.

The preliminary tests include:

1). Resting Electrocardiogram The electrical activity of your heart will be monitored while you lie quietly on a cot in the HPCRL. This requires the placement of small adhesive foam electrodes (10 total) on your chest. This procedure takes about 15 minutes and will be performed one time, at the beginning of the study.

2). Body composition (fat and lean tissue) - This will be performed using a machine called a dual energy X-ray absorptiometer (DEXA). This unit uses 2 low energy X-rays to determine the amount of body fat you have. You should be advised that the amount of radiation exposure in this procedure is very low, about 1/10,000 of the normal radiation exposure you receive yearly from what is called "background" radiation from the environment. Put another way, the exposure from a DEXA scan is less than the normal exposure in a flight from Denver to Chicago, and about 1/250th the exposure from a normal stomach X-ray you might receive at a hospital. This test will be performed in room 124 in the HPCRL. You will be asked to lie quietly on a bed in shorts and a T-shirt for about 15 minutes while the scan is performed. **This procedure will be performed twice: once during the preliminary phase and once at the end of the protocol.**

 Subject's Initials____________ Date_________

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3). **Fasting Blood sample:** You will be asked to report to the HPCRL following a 10-12 hour fast for a blood sample. You will lie on a cot, and a blood sample will be taken from a vein in your forearm. We will be taking a 10 cc sample, which is equivalent to about 2 teaspoons. The blood will be analyzed for the presence of the hormones which are thought to be involved in metabolism. In addition, we will measure glucose and fat levels in your blood. This procedure will take about 15 minutes. **This procedure will be performed 2 times**: once during the screening period at the beginning of the study and after the completion of the protocol.

4). **Muscle Biopsy:** You will be asked to report to the HPCRL for a muscle biopsy. The biopsy will be obtained from the vastus lateralis, which is a large muscle in your thigh. The procedure involves numbing the skin using lidocaine, an anesthetic similar to novacaine, which you may have received at the dentist If you are allergic to novacaine or have had any reaction to novacaine from your dentist, you should notify Dr. Hickey immediately and should not participate in this study. After numbing the skin, a small incision (less than the width of a pencil) is made in the skin over the muscle. The biopsy is obtained using a sterile needle. The muscle sample obtained is generally ~ ½ the size of an eraser on the end of a pencil. This procedure will take 30-45 minutes, including preparation time. It is not uncommon to experience some mild soreness in the muscle that lasts for about a day. You should NOT restrict your activity, although you should also not perform any unusual or extremely vigorous activity for a few days. You will be provided with written instructions regarding proper care of the incision, and a telephone contact should you have any questions. **This procedure will be performed 2 times**: once during the preliminary phase and at the end of the protocol.

5). **Diet Analysis:** You will be asked to complete a 7 day diet diary in which all food and drink you eat will be recorded. We will analyze your diet using a computer program and determine the percentage of fat, carbohydrate and protein as well as vitamin and mineral status. **This procedure will be performed once during the screening phase to determine your normal diet intake and determine your eligibility for the study. The diet analysis will be repeated twice per month during the weight loss and weight stabilization phases of the study. Because the duration of time needed to achieve the desired weight loss will vary from subject to subject, we cannot state exactly how many diet analyses will be performed. We anticipate 6-10.**

6). **24 hour energy expenditure** - This measure involves reporting to the HPCRL between 7:00 and 8:30 am after a 12-hour overnight fast. You will be asked to spend a 24 hour period in a whole-room calorimeter. This calorimeter is a 10x10 room which allows us to accurately measure the number of calories you expend over a 24h period. We will provide all food and drink during this period. You are allowed to bring any reading materials or work with you during your visit. In addition, should you become uncomfortable in the room at any time, you may leave immediately. A staff member will be on duty in the building at all times during your stay in the chamber. **This procedure will be performed three times: twice** during the preliminary phase and once at the end of the protocol. **Please be advised that only a subset of the study population will undergo these tests (i.e., not every subject). You will be informed in advance of whether we would like you to complete the 24 hour energy expenditure tests or the resting metabolic rate tests described below.**

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7). **Resting Metabolic Rate** - This measure involves reporting to the HPCRL between 7:00 and 9:30 am after a 12-hour overnight fast. You will be asked to lie down quietly on a hospital-type bed for about 45 minutes while breathing normally so that your expired air can be collected. You will be breathing in a large clear (see-through) canopy, similar to a very large plastic helmet. Your are not required to use a mouthpiece for this procedure. This test will provide information regarding the number of calories burned in the resting state. **This procedure will be performed twice: once during the preliminary testing and once after the weight loss. Please be advised that only a subset of the study population will undergo these tests (i.e., not every subject). You will have EITHER the 24h energy expenditure procedure described in #6 above or the resting metabolic rate measurement. We will inform you in advance of which procedure we would like you to complete.**

WEIGHT LOSS PROTOCOL:

You will be asked to participate in a weight loss protocol designed to reduce your body weight by 10%. This will be conducted on a supervised outpatient basis. You will be provided with a diet menu that includes a list of recommended items to meet the diet goals. One experimental group will be provided with a liquid diet supplement (that will provide you with a specific type of fat) that can be picked up (2x per month) at the HPCRL. No other diet groups will have this supplement. The study diets will have roughly the same amount of carbohydrate, fat, and protein as your normal diet, but we will ask you to reduce the total amount of calories as part of the weight loss program.

If you are randomized to the control group, you will be asked to consume your normal diet and NOT make any attempts to lose body weight during the course of the study. We will provide specific, individualized diet information to members of the control group following completion of the study. All members of the study will be asked to complete weekly body weight logs and will complete 7 day diet diaries twice each month of the study. You can expect this study to last somewhere between 3-6 months. The duration varies because not everyone loses weight at the same rate. The objective of this research project is to study adaptations to a 10% reduction in body weight. If you have not achieved the target 10% weight loss following 16 weeks of supervised outpatient dieting, we will be unable to complete the research protocol using your data. We will NOT ask you to complete post-testing, and will prorate your remuneration (you will be paid $150). In addition, while you will no longer be considered a research participant, we will make diet and exercise recommendations available to you so that you can continue to make progress at your own pace.

RETENTION OF BLOOD AND MUSCLE SAMPLES

You should understand that we plan to keep any extra muscle and blood samples that are not used in the analysis for this study. In other words, if we have any "extra" blood or muscle we will keep them in a freezer in our lab. It is very possible that we will use all of the blood and muscle obtained in this study and will have none left, but in the event that we do, we would like your permission to keep the samples in the event that they can be used for further research. We will use these samples in the future solely for additional research on obesity and metabolism; specifically, all future research will simply be an extension of what we hope to accomplish with the current study. We may simply analyze your blood for the presence of other hormones or metabolites, or analyze your muscle for other enzymes, etc. We have NO plans to store DNA in this study. Your stored samples will be coded in such a way that your confidentiality will be

 Subject's Initials____________ Date_________

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maintained. Only the Principal Investigator (Dr. Hickey) will have access to the coding system for your samples. There is a possibility that your samples may be shipped to other departments on the CSU campus, or to colleagues at other Universities for assistance with analysis. Under such circumstances, the same coding system will be used, so researchers in other labs will not be able to identify you. We do not anticipate ANY commercial product development from your tissue, the samples will be used solely for research purposes. You should be advised that we do NOT have plans to recontact you in the future regarding any additional analyses, but will seek full approval of the CSU Regulatory Compliance Office prior to initiating any further research on your samples.

By checking "Yes" below and signing on the accompanying line, you are agreeing to allow the investigators retain any muscle and blood samples obtained during this study. If you do not wish the investigators to retain any samples, please check the box marked "No" and also sign on the accompanying line.

The investigators may keep any muscle or blood samples obtained during the course of this study for future research on obesity and metabolism YES ☐ NO ☐

____
Signature Date

RISKS INHERENT IN THE PROCEDURES:

1). Resting Electrocardiogram: There is no risk associated with a resting electrocardiogram other than some minor skin irritation at the site where the electrodes are placed.

2). DEXA: The risks associated with the DEXA are very low. The radiation you will receive in this study is less than 1/3000th of the FDA limit for annual exposure. Put another way, you could receive 3000 DEXA scans in a single year and still not meet the FDA limit for radiation exposure. In this study, you will receive 2 scans over a 16-24 week period. The more radiation you receive over the course of your life, the more is the risk of having cancerous tumors or of inducing changes in genes. The radiation in this study is not expected to greatly increase these risks, but the exact increase in such risks is not known. Women who are pregnant or could be pregnant should receive no unnecessary radiation and should not participate in this study.

3). Blood Samples: The risks associated with blood drawing include hematoma/bruising, slight risk of infection, local soreness, and fainting. These are all very minor risks and if present, are generally resolved in less than a day.

4). Muscle Biopsy: The risks associated with the muscle biopsy include discomfort, localized soreness, bruising, infection, and minor scarring. The discomfort and localized soreness are likely, but generally last only 24-48h. Temporary scarring is also expected, the natural course of wound healing varies substantially from individual to individual, but the scar will only be 8-10 mm long, and is generally difficult to distinguish within 8-12 months after the biopsy. The risk of bruising is low, and infections are extremely rare.

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5). 24 hour energy expenditure: There are no know physical risks associated with whole-body calorimetry. Because you will be asked to stay in a 10x10 room for 24h, there is some risk of discomfort from being confined to the small space. You will be allowed to bring reading materials, work, etc. In addition, the room has a TV/VCR and computer access.

6). Resting Metabolic Rate measurement: There is no known risk associated with this procedure. You may experience some minor discomfort associated with this measurement if you have claustrophobia, but this is very unlikely. The canopy used is a large, see-through plastic bubble. There is adequate space, breathing is unrestricted, and it does not require the use of a mouthpiece.

7). Diet Supplement: There is no risk associated with the diet supplement used in this study. It is a commercially available liquid form of a specific type of dietary fat that we believe is a "healthy fat". We have used this supplement in previous studies at CSU, and it can be purchased in any health food store.

BENEFITS:
You will receive detailed diet and body composition data, and information on weight loss. In addition, you will be paid $300.00 upon completion of this study. Should you decide not to complete the study, you will be paid $50 for completion of the preliminary tests (individuals completing the 24h chamber visits will be compensated an additional $50) , $100 for completion of weight loss, and $150 for completion of the post-training tests.

CONFIDENTIALITY:
Your data will be coded and kept in a locked file cabinet on the CSU campus. A copy of the coded data must be sent to the sponsor of this project. However, you will note be identified in relation to your data at any point.

LIABILITY:
Because Colorado State University is a publicly-funded, state institution, it may have only limited legal responsibility for injuries as a result of participation in this study under a Colorado law known as the Colorado Government Immunity Act (Colorado Revised Statutes, section 24-10-101, et seq.). In addition, under Colorado law, you must file any claims against the University within 180 days after the date of the injury. In light of these laws, you are encouraged to evaluate your own health and disability insurance to determine whether you are covered for any injuries you might sustain by participating in this research, since it may be necessary for you to rely on individual coverage for any such injuries. If you sustain injuries which you believe were caused by Colorado State University or its employees, we advise you to consult an attorney. Questions concerning treatment of subject's rights may be directed to Celia S. Walker at (970) 491-1563.

PARTICIPATION:
Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled. Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 6__ pages.

Page 5 of 6 Subject's Initials____________ Date_________

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______________________________ ______________________________
Participant Name (print) Date | Participant Signature

______________________________ ________________________
Investigator or co-investigator Signature | Date

Page 6 of 6 Subject's Initials____________ Date_________

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