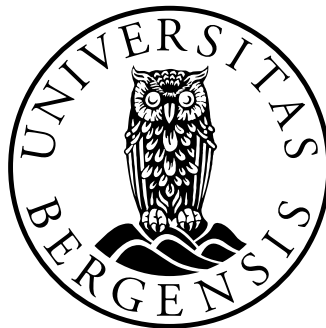


Dietary linoleic acid induces obesity through excessive endocannabinoid activity

Anita Røyneberg Alvheim



Dissertation for the degree of Philosophiae Doctor (PhD)

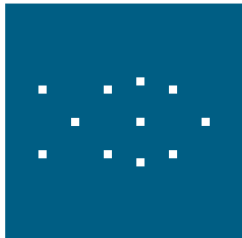
University of Bergen, Norway
2012

Scientific environment

The present PhD was completed at the National Institute of Nutrition and Seafood Research (NIFES), Bergen, Norway, under the supervision of Dr. Marian Kjellekvold Malde.

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Don't cry because it's over. Smile because it happened - Dr. Seuss

Bergen, January 2012

Anita

Abstract

Background: Dietary intakes of the n-6 fatty acid linoleic acid (LA, 18:2n-6) have increased dramatically during the 20th century. Replacing fish oil (FO) with vegetable oil (VO) in feed for farmed fish introduces LA and alters the fatty acid composition in Atlantic salmon (*Salmo salar* L.). LA is the precursor of arachidonic acid (AA, 20:4n-6) the backbone of the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA). A sustained hyperactivity of the endocannabinoid system is believed to play a causal role in the development of obesity and associated metabolic disorders. Here we posit that excessive dietary intake of LA, the precursor of AA, would induce endocannabinoid hyperactivity and promote obesity.

Design: LA was isolated as an independent variable to reflect the dietary increase in LA from 1 percent of energy (en%) to 8 en% occurring in the US during the 20th century. Male C57BL/6j mice were exposed to 1 en% LA and 8 en% LA in diets of 35 en% and 60 en% fat from last week of gestation and 14 weeks from weaning (Paper I), and in diets of 12.5 en% and 35 en% fat for 16 weeks from 6 weeks of age (Paper II). To reduce tissue n-6 highly unsaturated fatty acids (HUFA), 1 en% eicosapentaenoic acid (EPA, 20:5n-3)/docosahexaenoic acid (DHA, 22:6n-3) were supplemented to the 8 en% LA diets in Paper I. Atlantic salmon, 340 g, was fed fish oil and soybean oil (SO) for 6 months. Male C57BL76j mice, 6 weeks of age, were fed diets of 35 en% fat based on FO salmon fillet (1 en% LA) and SO salmon fillet (8 en% LA) for 16 weeks (Paper III).

Results: Increasing dietary LA from 1 en% to 8 en% elevated AA in phospholipids (AA -PL) with a subsequent elevation in liver 2-AG and anandamide associated with higher food intake, feed efficiency, weight gain and adiposity and increased hypertrophy and inflammation of adipose tissue. Selectively reducing LA to 1 en% reversed the obesogenic properties of a high fat diet. Reducing AA -PL by EPA/DHA supplementation resulted in metabolic patterns resembling 1 en% LA diets. Replacing fish oil with soybean oil in feed for Atlantic salmon elevated tissue LA and AA, and increased endocannabinoid activity and lipid accumulation in salmon liver. Mice fed SO salmon gained more weight, had larger

adipocytes and more adipose tissue inflammation than mice fed FO salmon.

Conclusion: Dietary LA of 8 en% LA induces hyperactivity of the endocannabinoid system and increase the risk of developing obesity and associated metabolic disorders in mice. In a balanced diet, the adipogenic effect of LA can be prevented by consuming sufficient EPA and DHA to reduce the AA -PL pool and normalize endocannabinoid tone. A dietary approach addressing an underlying cause of endocannabinoid hyperactivity may prove to be a safe and viable alternative for preventing and decreasing obesity.

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List of publications

Paper I Anita R. Alvheim, Marian K. Malde, Douglas Osei-Hyiaman, Yuhong Lin, Robert Pawlosky, Lise Madsen, Karsten Kristiansen, Livar Frøyland, Joseph R. Hibbeln. (2012). Dietary linoleic acid elevates endogenous 2-AG and anandamide and induces obesity. *Obesity (Epub ahead of print doi:10.1038/oby.2012.38)*

Paper II Anita R. Alvheim, Bente E. Torstensen, Yu Hong Lin, Haldis H. Lillefosse, Erik-Jan Lock, Lise Madsen, Livar Frøyland, Joseph R. Hibbeln, Marian K. Malde. (2012). Dietary linoleic acid elevates the endocannabinoids 2-AG and AEA, and induces weight gain and inflammation in mice fed a low fat diet. *PLoS One (submitted)*

Paper III Anita R. Alvheim, Bente E. Torstensen, Yu Hong Lin, Haldis H. Lillefosse, Erik-Jan Lock, Lise Madsen, Joseph R. Hibbeln and Marian K. Malde. (2012). Dietary linoleic acid elevates endogenous 2-AG and anandamide in Atlantic salmon (*Salmo salar* L.) and mice, and induces weight gain and inflammation in mice. *Journal of Nutrition (submitted)*

Paper I has been granted reprint permission from journal. The papers will be referred to by their Roman numbers.

Abbreviations

AA	Arachidonic acid (20:4n-6)
ACC1	Acetyl-coenzyme-A carboxylase-1
AD	Anno domini
AEA	Anandamide
ALA	α -linolenic acid (18:3n-3)
BAT	Brown adipose tissue
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CB1^{-/-}	Cannabinoid receptor 1 knock-out
CHD	Coronary heart disease
CVD	Cardiovascular heart disease
Δ^9-THC	Delta ⁹ -tetrahydrocannabinol
DHA	Docosahexaenoic acid (22:6n-3)
DPA	Docosapentaenoic acid (22:5n-3)
EFA	Essential fatty acid
EFSA	European Food Safety Authority
en%	Energy percent
EPA	Eicosapentaenoic acid (20:5n-3)
eWAT	Epididymal white adipose tissue
FAS	Fatty acid synthase
FO	Fish oil
GC-MS	Gas chromatography-mass spectrometry
GPR120	G-protein coupled receptor 120
HDL	High density lipoprotein

H&E Hematoxylin and eosin

HF High fat diet (60 en% fat)

HU210 Synthetic agonist for cannabinoid receptor 1

HUFA Highly unsaturated fatty acids (> 20 carbons)

IHC Immunohistochemistry

ISSFAL International Society for the Study of Fatty Acids and Lipids

iWAT Inguinal white adipose tissue

LA Linoleic acid (18:2n-6)

LF Low fat diet (12.5 en% fat)

MUFA Monounsaturated fatty acids

MF Medium fat diet (35 en% fat)

NIFES National Institute of Nutrition and Seafood Research

n- Fatty acids will be notated with the n- designation of positional isomers rather than the omega-terminology.

n-3 omega-3

n-6 omega-6

OECD Organisation for Economic Co-operation and Development

PPAR α Peroxisome proliferator-activated receptor α

PEA Palmitoylethanolamide

PGC-1 α Peroxisome proliferator-activated receptor- γ coactivator 1 α

PL Phospholipid

PUFA Polyunsaturated fatty acid

RBC Red blood cells

RDA Recommended dietary allowances

RT-qPCR Real-time reverse-transcription - quantitative polymerase chain reaction

rWAT Retroperitoneal white adipose tissue

SEM Standard error of the mean

SFA Saturated fatty acids

SO Soybean oil

SR141716 Rimonabant, synthetic antagonist for the cannabinoid receptor 1

SREBP-1c Sterol regulatory element-binding protein-1c

TAG Triacylglycerol

UCP1 Uncoupling protein 1

VO Vegetable oil

VSI Visceral somatic index

WHO World Health Organization

Chapter 1

Preface

In the 1960s, Americans consumed 45 en% of their energy from fats, 13% of the population was obese (Flegal et al., 1998) and 1 % had diabetes type 2 (NDIC, 1995). In 2008, 72% of men and 64% of women were overweight or obese (Flegal et al., 2002), 8% had diabetes, most of which were type 2 (CDC, 2011) and energy intake from fat was reduced to 32 en% presumably due to dietary recommendations to reduce dietary fat to 20-35 en% (USDA, 2010). In the 1970s, Norwegians consumed 40 en% fat and 14% were overweight (HOD, 2010). In 2008, 32% of Norwegian men and 21% of women were overweight and dietary fat intakes were reduced to 35 en% (HOD, 2010). Although low in a global perspective, the percent of obese Norwegian adults has nearly doubled since 2000 (Figure 1.1).

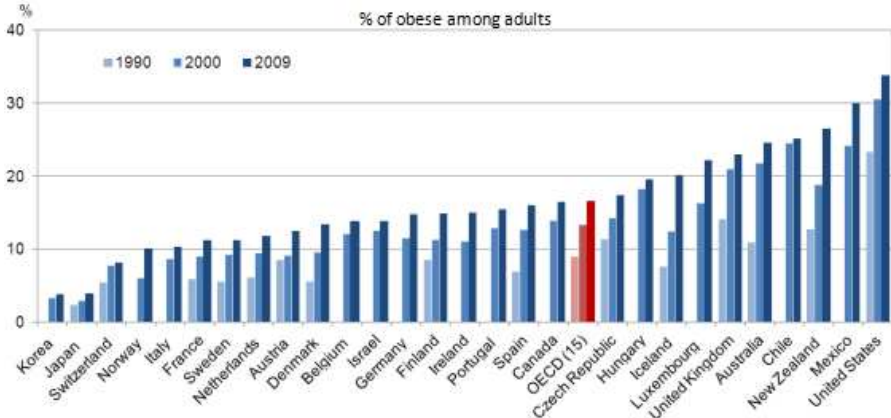


Figure 1.1: The percent of obese adults worldwide. The prevalence of obesity in Norway is low compare to countries like the United States and Mexico. Important to note, the increase in obese adults in Norway from 2000 to 2009 is considerably higher than most other countries. ©OECD.

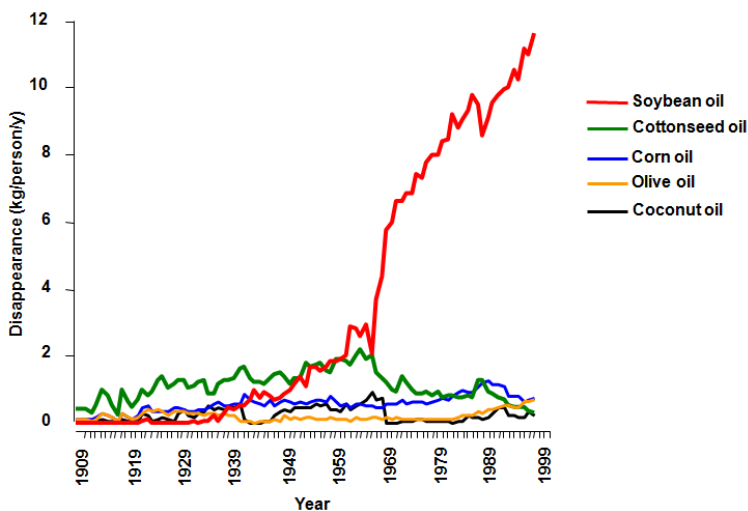


Figure 1.2: Intake of soybean oil in the US. Estimated consumption of soybean oil (in red), the major dietary source of n-6, in the US during the 20th century. Adapted with permission to reprint from authors (Blasbalg et al., 2011).

The cause of obesity is multifactorial. Physical inactivity and poor diet, and especially dietary fat have been associated with the development of obesity (Bray and Popkin, 1998). As there is no link between a low fat diet and reduced weight and disease (Beresford et al., 2006; Howard et al., 2006a,b), the type of dietary fat specifically the imbalance in n-6 to n-3 polyunsaturated fatty acids (PUFA), is emerging as a risk factor for developing obesity (Massiera et al., 2003; Ailhaud et al., 2008; Madsen et al., 2008; Matias et al., 2008a,b). Dietary advices to replace saturated fat (SFA) with PUFA and increasing use of vegetable oils in cooking have lead to a dramatic increase in human consumption of soybean oil, one of the major dietary sources of LA (Figure 1.2). In the US, the availability of soybean oil increased from 2.2 en% to 7.3 en% during the 20th century whereas the availability of α -linoleic acid (ALA, 18:3n-3), EPA and DHA decreased in the same period (Blasbalg et al., 2011) causing a disequilibrium in the intakes of n-6 to n-3 PUFA. Norwegian dietary guidelines recommend increasing use of vegetable oils for cooking (Nasjonalt Råd for Ernæring, 2011) and the consumption of vegetable oils in Norway has doubled since 1980 (HOD, 2010).

The endocannabinoid system is involved in the control of appetite and energy balance, and is important in maintaining energy homeostasis. Overactivity of the endocannabinoid system is believed to be one of the underlying causes of obesity,

hyperglycemia, dyslipidemia, insulin resistance and type 2 diabetes (Di Marzo, 2008; Kunos et al., 2008), but has not as of yet been linked to the worldwide epidemic of obesity. Suppressing a hyperactive endocannabinoid tone is believed to be a critical target for reducing obesity (Osei-Hyiaman et al., 2005). Pharmaceutical drugs interrupting endocannabinoid signaling serve as potential candidates for therapy and treatment of obesity and associated metabolic disorders (Després et al., 2005; Van Gaal et al., 2005; Christopoulou and Kiortsis, 2011). However serious psychiatric side-effects caused marketplace withdrawal of rimonabant, a selective cannabinoid receptor antagonist (Christensen et al., 2007). No pharmaceutical alternatives are currently available, which urges the need for a dietary alternative to reduce endocannabinoid hyperactivity. A dietary approach to prevent endocannabinoid hyperactivity is likely to have widespread beneficial public health implications related to obesity and metabolic syndrome.

Chapter 2

Introduction

Unless care is exercised in selecting food, a diet may result which is one-sided or badly balanced - that is, one in which either protein or fuel ingredients (carbohydrate and fat) are provided in excess [...] The evils of overeating may not be felt at once, but sooner or later they are sure to appear - perhaps in an excessive amount of fatty tissue, perhaps in general debility, perhaps in actual disease. - Wilbur Olin Atwater, 1902

2.1 Dietary changes in n-6 and n-3 intakes

Humans evolved on a diet based on lean meat, fish, green leafy vegetables, fruits, nuts and berries shaping the modern human's genetic nutritional requirements (Simopoulos, 1999). Major dietary changes in terms of dietary fat, especially during last 150 years have changed the type and amount of fat for human consumption. The evolutionary diet was based on a balanced intake of n-6 to n-3 fats. Cereal grains are one of the major contributors to the world's food supply and usually contain high levels n-6 and low levels of n-3 (Simopoulos, 1999). The introduction of cereals into the human diet has contributed to a shift in the n-6 to n-3 ratio. The use of grain feed in modern agriculture together with emphasis on production have decreased the n-3 content in animal meat and animal products, vegetables, eggs and fish (Simopoulos, 1999). In aquaculture, farmed Atlantic salmon have traditionally been fed diets based on fish oil and fish meal (Turchini et al., 2009). Today, marine resources are limited and the steady increase in aquaculture production volume of 8-10% per year (Tacon et al., 2006) has resulted in increased use of alternative protein and oil sources in aqua feeds (Figure 2.1). Vegetable oils are recognized as suitable alternatives to fish oils (Torstensen et al., 2005; Turchini et al., 2009) but these oils are devoid of EPA and DHA and contain high levels of LA and monounsaturated fat (MUFA). Replacing fish oil with vegetable oil in feed for farmed fish reduce EPA and DHA and increase LA con-

tent in fish fillet (Grisdale-Helland et al., 2002; Torstensen et al., 2005; Turchini et al., 2009). To ensure sustainability, fish farming implies a trade-off between the amount of wild fish used in feed for Atlantic salmon and the amount of n-3 PUFA in salmon fillet (NIFES, 2011a). There have been increasing concerns about the decreasing content of EPA and DHA in farmed Atlantic salmon. Norwegian surveillance data (NIFES, 2011b) report a moderate increase in LA levels in fillets of farmed Atlantic salmon from 1.1 g / 100 g in 2005 to 1.6 g / 100 g in 2010 and a decrease in EPA + DHA from 2.7 g / 100 g to 2.1 g / 100 g in the same period.

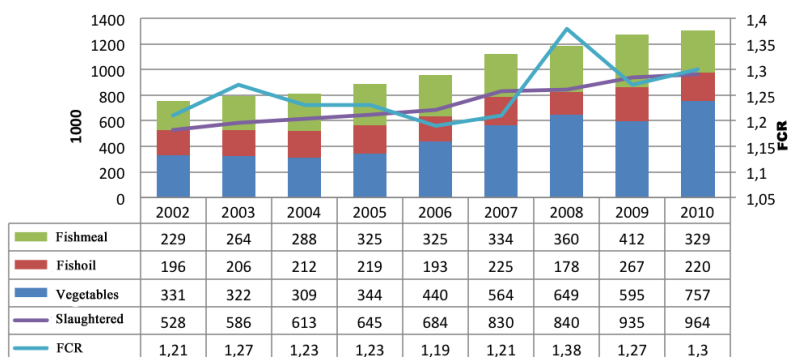


Figure 2.1: Composition of Norwegian-produced fish feed. The increase in aquaculture production, presented as slaughtered fish (purple line), has led to a replacement of fish meal (green bar) and fish oil (red bar) with alternative plant protein and oil sources (blue bar) in Norwegian-produced aqua feeds from 2002 - 2010. The fish conversion ratio (FCR, blue line) has remained fairly constant since 2002. Permission to reprint from Norwegian Seafood Federation (FHL).

Technological advancements in the beginning of the 20th century lead to the industrial production of vegetable oils for cooking. The production of vegetable oils was further augmented by the use of solvent extraction which made large scale production of vegetable oils more efficient and profitable (Simopoulos, 1999). The increased availability of vegetable oils and the current public recommendation to replace saturated fat with vegetable oil due to the cholesterol lowering and cardiovascular protective properties of PUFA (Kannel et al., 1964, 1971; Hegsted et al., 1965; Keys et al., 1965; Keys and R.W., 1966; Harris et al., 2009) have caused an imbalance in the intake of n-6 to n-3 fat. The intake of soybean oil, one of the major dietary sources of LA, has increased dramatically in the US during the 20th century, whereas the availability of ALA, EPA and DHA decreased in the same period (Blasbalg et al., 2011). Technological advancements, changes in modern agri- and aquaculture and current dietary guidelines are contributing factors in the

shift from an evolutionary ratio of n-6 to n-3 of 1:1 to the current US ratio of 10-20:1 (Simopoulos, 2001). Compared to the dietary changes, our genetic profile has remained fairly constant and we now live in a nutritionally environment that is very different from which our genetic constitution was selected (Simopoulos, 1999).

2.1.1 Dietary guidelines

The US Department of Agriculture (USDA) by Wilbur Olin Atwater published its first dietary recommendations in 1894. Prompted by President Franklin Roosevelt a National Nutrition Conference took place in 1941 which resulted in the first Recommended Dietary Allowances (RDA) for caloric intake and essential nutrients to provide “standards to serve as a goal for good nutrition” (National Research Council, 1943). In 1961 the American Heart Association published dietary recommendations regarding fat and heart disease “based on the best scientific information available at the present time” stating that “the reduction or control of fat consumption under medical supervision, with reasonable substitution of polyunsaturated for saturated fats, is recommended as a possible means of preventing atherosclerosis and decreasing the risk of heart attacks and strokes” (Page et al., 1961). The Norwegian National Board of Nutrition, established in 1946, published its first dietary advices in 1954 (Meltzer et al., 2004). A national comitée led by Dr. Nicolaysen concluded in 1963 that saturated fat was a major reason for the increase in infarct mortality and therefore recommended to reduce dietary fat to less than 30 percent of total energy intake (Hjort, 1963).

Dietary advices have changed during the last 40 years from a reason to protect against deficiency diseases to prevent chronic diseases such as diabetes type 2, coronary heart disease and cancer (Meltzer et al., 2004). The primary objectives for current dietary recommendations are to 1) assure primary needs 2) give premise for good health and decrease the risk of diet-induced diseases and 3) avoid excessive intake that may cause adverse effects. In Norway, the National Board of Nutrition, part of the Norwegian Directorate of Health, presented updated dietary advices in 2011. The aim of the new dietary guidelines is to promote public health with focus on primary prevention of chronic diet-induced diseases within the adult population in Norway (Nasjonalt Råd for Ernæring, 2011). The report recognizes that a major part of the Norwegian population needs to adjust or change their dietary habits in order to achieve better public health. The guidelines recommend increased use of vegetable oils with high level of unsaturated fat, such as rapeseed, sunflower, olive and soybean oil

PUFA requirements

An essential nutrient is needed for normal development and functions throughout the life cycle, and must be provided by the diet. The requirement can vary with species, gender, age and physiological and pathological challenges (Cunnane, 2003). Traditionally, the only fatty acids considered essential were LA and ALA. Humans and mammals are not able to make these fatty acids *de novo* and are thus dependent upon dietary intakes. Pragmatics will also include AA and DHA as essential fatty acids since these fatty acids are not made in sufficient amounts by newborn infants to guarantee normal development (Crawford, 1993; Carlson and Neuringer, 1999), or to sustain brain accumulation of DHA equivalent to breast-fed infants (Cunnane et al., 2000).

A requirement should be at the minimum amount to maintain apparently normal physiology (Lands, 1992). LA and ALA requirements were set at levels that prevented biochemical and physiological symptoms of deficiency (skin problems, hair loss and growth retardation) and optimized tissue PUFA content. In the 1950-1960s, Ralph Holman and colleagues estimated LA requirement to be 1% of dietary energy intake (Holman, 1960). In 1976, Cuthbertson (1976) noted that minimum requirements for essential fatty acids in infancy were “set far too high and are in fact less than 0.5 en%”.

The majority of studies to determine LA requirement used diets that were depleted in both LA and ALA, or based requirements on the level necessary to reach a plateau of n-6 in tissues (Cunnane, 2003). In rodents, as long as minimum quantities of dietary ALA was present (200 mg/100 g diet), dietary LA of 150 mg/100 g diet covered the minimal requirements, whereas 1200 mg/100 g diet was required to reach a plateau of n-6 PUFA in liver (Bourre et al., 1990b). n-3 deficiency exacerbate n-6 deficiency symptoms and higher amount of LA is needed to correct deficiency symptoms when dietary ALA is absent (Greenberg et al., 1950; Mohrhauer and Holman, 1963a). In the presence of 0.5 en% ALA, 0.5 en% LA appears to be sufficient for the growing rat (Guesnet et al., 2011). An n-6 “deficient” diet (10% of LA requirement for rodents) caused no adverse effects as long as minimum requirements of ALA were present, but decreased AA and increased EPA and DHA in tissues compared to an n-6 adequate diet (Igarashi et al., 2009). Therefore current LA requirements are believed to be overestimated (Cunnane, 2003; Ailhaud et al., 2008) and cannot be determined in the absence of dietary ALA (Cunnane, 2003).

There are no well controlled studies to establish the minimum required intake of n-6 PUFA in healthy human adults (ISSFAL, 2004). The human requirements for LA are based on estimates from experimental measurements. It has been suggested that 1 en% LA is sufficient to meet n-6 PUFA requirements in healthy adults (Cunnane, 2003). 2 en% LA and 0.7 en% ALA are considered adequate

for healthy human adults, although LA requirements may be higher during pregnancy, lactation and early development but should not exceed 3 en% (ISSFAL, 2004). EPA and DHA were traditionally not regarded as essential fatty acids and thus no minimum requirements have been established for these fatty acids.

PUFA recommendations

Providing the minimum requirement of a nutrient to avoid deficiency symptoms may not be sufficient in prevention of lifestyle-related diseases resulting from an excess intake of certain nutrients. Dietary recommendations offer quantitative estimates of nutrient intakes applicable to healthy individuals. Intakes outside the acceptable range increase the risk for deficiency and diseases due to excessive intakes (IOM, 2006).

Despite several consensus' and research stating the importance of lowering dietary n-6 PUFA and increasing n-3 PUFA to achieve a healthier diet and reduce the burden of diseases such as cardiovascular and mental illnesses (Lands, 1992, 2009; Simopoulos, 1999; ISSFAL, 2004; Hibbeln et al., 2006; Global Omega-3 Summit, 2011), most of the current n-6/PUFA recommendations are set at 5-10 en% (Table 2.1).

There is no conclusive scientific evidence with regards to the issue of a safe upper limit of dietary n-6 PUFA but there is a controversy regarding the health properties of a LA intake above an adequate intake of 2 en% (ISSFAL, 2004) especially related to cardiovascular disease (CVD). Several meta-analyses (Gordon, 1995; Mozaffarian et al., 2010), and reviews (Harris, 2008; Harris et al., 2009; Czernichow et al., 2010) support the protective properties of PUFA, and n-6 PUFA in particular, in cardiovascular heart disease. The American Heart Association recommends 5-10 en% LA to improve heart health (Harris et al., 2009). Important to remember, PUFA from the n-3 and n-6 series are chemically and nutritionally distinct fatty acids, and are precursors of metabolites with distinct physiological responses. The inconsistent use of PUFA makes it difficult to distinguish the effect of n-6 PUFA from that of n-3 PUFA. In contrast to Mozaffarian et al. (2010), Ramsden et al. (2010) found significant different effect on cardiovascular disease when separating studies using a mix of n-3 and n-6 from n-6 specific PUFA. Randomized controlled trials using a mix of n-3 and n-6 PUFA significantly reduced the risk of non-fatal myocardial infarction and coronary heart disease (CHD). By contrast, n-6 specific PUFA diets increased the risk of all CHD endpoints, pointing out the importance of distinguishing between n-3 and n-6 PUFA (Ramsden et al., 2010).

Although not regarded essential by all, EPA and DHA deficiency is slowly being recognized as detrimental to human health. Dietary recommendations of EPA and DHA to healthy adults varies from 250 mg/day set to prevent the de-

Table 2.1: PUFA recommendations

Organization	Year	PUFA (en%)	n-6 PUFA (en%)	n-3 PUFA	
				ALA (en%)	EPA+DHA (g)
Norway ¹	2005	5 - 10		1 en% ¹¹	
Japan ²	2005		< 10	2.2 - 2.6 g ¹²	
Nordic Nutrition Recommendations ³	2004	3 ¹³		> 1 en%	
British Nutrition Foundation ⁴	2009	6, < 10	> 1	> 0.2	
Eurodiet Core Report ⁵	2001		4 - 8	2	0.2
National Institute of Medicine ⁶	2005		5 - 8 ¹⁴	0.5	
American Heart Association ⁷	2009		5 - 10		1 - 4 ¹⁵
American Dietetic Ass and Dietetians of Canada ⁸	2007		3 - 10	1.2	0.5
EFSA ⁹	2010			0.5	0.25 ¹⁶
ISSFAL ¹⁰	2004		2 ¹⁷	0.7 ¹⁸	> 0.5 ¹⁹

¹ (Nasjonalt Råd for Ernæring, 2011), ² (Ministry of Health, Labor and Welfare, Japan, 2004), ³ (NNR, 2004), ⁴ (BNF, 2009), ⁵ (Eurodiet Core Report, 2001), ⁶ (IOM, 2005), ⁷ (Harris et al., 2009), ⁸ (Kris-Etherton et al., 2007), ⁹ (EFSA, 2010), ¹⁰ (ISSFAL, 2010). ¹¹ at least 1 en% from n-3 PUFA, ¹² 2.2 g for adult women and 2.6 for adult men, ¹³ provide some margin above minimum requirement, ¹⁴ 5 en% for women and 8 en% for men, ¹⁵ 1 g for patients with cardiovascular disease and 2-4 g for patients with high triglycerides, ¹⁶ for healthy adults, ¹⁷ adequate intake, ¹⁸ healthy intake, ¹⁹ expected to reduce risk for cardiovascular deaths in healthy adults. Abbreviations: EFSA European Food Safety Authority, en% energy percent, ISSFAL International Society for the Study of Fatty Acids and Lipids, < no more than, > at least.

velopment of cardiovascular disease (EFSA, 2010) to 2.6 g/day to maintain and promote health and to prevent lifestyle-related diseases (Ministry of Health, Labor and Welfare, Japan, 2004). Norway has no specific dietary recommendations for EPA and DHA, but recommend that at least 1 of the 5-10 en% PUFA should be n-3 PUFA (Table 2.1). In line with several other countries and organizations (Table 5.2 in (VKM, 2011)) Norwegians are recommended to eat 300-450 g fish per week (of which 200 g should be fatty fish (Nasjonalt Råd for Ernæring, 2011), whereas American guidelines recommend 8 ounces (226 g) of seafood, equivalent to 2 servings of seafood a week (USDA, 2010). Seafood is a good source of EPA and DHA and Norwegians and Americans are recommended to eat more seafood (USDA, 2010; VKM, 2011).

2.1.2 Dietary n-6

The n-6 fatty acid LA is our main dietary PUFA and the precursor of arachidonic acid (AA). LA is a significant component of western type diets and occurs naturally in seed oils and high levels can be found in vegetable oils such as safflower oil (75%), sunflower oil (69%), soybean oil (55%) and maize oil (45%).

The pro-adipogenic effect of linoleic acid

The adipogenic effect of dietary LA was already reported in 1966 when dietary vegetable oil replaced saturated fat in veterans (Dayton et al., 1966). Men receiving vegetable oil had elevated serum levels of LA, increased adipose tissue mass and they gained more weight than men on the conventional diet of saturated fat (Dayton et al., 1966). In 1985, Cunnane et al. (1985) reported higher body weight in mice fed evening primrose oil (72% LA) compared to mice fed cod liver oil (16% EPA and DHA). Several studies support the findings of higher body weight and adiposity from vegetable oils rich in LA (Ikemoto et al., 1996; Okuno et al., 1997; Takahashi and Ide, 2000; Pellizzon et al., 2002; Massiera et al., 2003, 2010; Javadi et al., 2004; Madsen et al., 2008). An epidemiological report link increased LA intake over the last 40 years, especially in infant feeding, to the increased prevalence of obesity and postulate that AA-induced elevations in the endocannabinoid 2-AG may have altered energy balance towards obesity (Ailhaud et al., 2008). Savva et al. (2004) reported a positive association between adipose tissue AA and BMI in overweight children.

On the other hand, lower adipose tissue accumulation has been reported in mice fed soybean and safflower oil compared to saturated fat (Shimomura et al., 1990; Takeuchi et al., 1995; Matsuo et al., 2002). The opposing effect of LA on adipose tissue development have been explained by diet-induced differences in cyclic AMP (cAMP) levels, which can be altered by the carbohydrate/protein ratio (Madsen et al., 2008).

2.1.3 Dietary n-3

The n-3 fatty acid ALA, found in vegetable oils, is the precursor of EPA and DHA, occurring naturally in marine sources. The majority of beneficial effects of n-3 PUFA in terms of cardiovascular protection and mental health are attributed to EPA and DHA.

Anti-adipogenic effect of EPA and DHA

Fatty acids of the n-3 and n-6 series are not equipotent in promoting adipose tissue development (Ailhaud et al., 2006) and they affect gene expression of white and brown adipose tissue differently (Takahashi and Ide, 2000). Fish oil upregulate uncoupling protein 1 (UCP1) in brown adipose tissue (Takahashi and Ide, 2000) which induce thermogenesis (Cannon and Nedergaard, 2004), downregulate the expression of the sterol regulatory element-binding protein-1c (SREBP-1c) in liver which is responsible for regulating genes involved in *de novo* lipogenesis (Kim et al., 1999, 2002; Tandy et al., 2009; Piscitelli et al., 2011) and

upregulate intestinal lipid metabolism (Mori et al., 2007). Krill oil is superior to fish oil in elevating the levels of palmitoylethanolamide (PEA) an activator of the peroxisome proliferator-activated receptor α (PPAR α) (Piscitelli et al., 2011). Activation of PPAR α and down-regulation of SREBP-1c mRNA in liver is believed to be related to the hypolipidemic effect of fish oil in a high fat diet (Kim et al., 1999).

There is a general notion that fish oil rich in EPA and DHA limits diet-induced obesity in rodents (Belzung et al., 1993; Hill et al., 1993). An effect that is associated with reduced tissue levels of AA -PL (Cunnane et al., 1985). EPA and DHA from fish oils are also associated with weight reduction in humans. Epidemiological studies report that fish consumption within a healthy eating pattern is associated with lower body weight (Shubair et al., 2005; Schulze et al., 2006), and dietary intervention studies support the notion that fish consumption helps to control body weight (Mori et al., 1999; Kunesova et al., 2006; Thorsdottir et al., 2007). Inclusion of fish or EPA and DHA supplements to calorie restricted diets resulted in higher weight loss than an isocaloric control diet (Thorsdottir et al., 2007) and greater reduction in BMI and hip circumference than placebo (no EPA and DHA) or diet alone (Kunesova et al., 2006). Changing to a Mediterranean diet lowered the ratio of n-6 to n-3 in plasma and was associated with reduced body weight, BMI and inflammatory markers, and improved insulin sensitivity and elevated adiponectin levels (Esposito et al., 2011).

Important to note, the obesogenic potential of diets rich in both n-6 and n-3 PUFA is increased by elevating the levels of dietary carbohydrates (Ma et al., 2011; Madsen et al., 2008), demonstrating the importance of the background diet on the effect of dietary fat in adipose tissue development.

2.2 Linoleic acid lowering to increase tissue EPA and DHA

The proportion of n-6 HUFA in tissue can be used as an indicator of disease risk (Lands, 2009). Cardiovascular deaths correlate with the percent of n-6 HUFA in tissues for populations worldwide (Lands, 2003). A tissue composition of 50% n-3 HUFA is estimated to protect 95% of population against mental illness and cardiovascular disorders (Hibbeln et al., 2006; Global Omega-3 Summit, 2011). As the fatty acid composition in tissues is reflected by dietary fat intake, the amount of EPA and DHA necessary to achieve 50% n-3 HUFA in tissue depend on the dietary amount of LA (Lands et al., 1992). An intake of 4 - 8 en% LA will require 1 - 2 g of EPA and DHA daily to achieve 50% n-3 HUFA in tissues (Table 2.2). A meal containing 100 - 200 g salmon or 600 g - 1.2 kg cod will provide 1 - 2 g

EPA and DHA and assure 50% n-3 HUFA in a high LA diet (Lands et al., 1992; NIFES, 2011b).

Approximately 3.1 billion people live in countries with low fish and low ALA availability. This results in a large segment of the world's population that is likely to have serious consequences due to n-3 PUFA insufficiency (Petrova et al., 2011). 8.5 % of the population with low fish/ALA availability lives in Eastern Europe where sunflower oil is the main oil used for cooking. Sunflower oil contain 69% LA giving a high LA and very low ALA and DHA content in adipose tissue (Petrova et al., 2011).

To ensure sustainability and improve the n-3 HUFA tissue composition, especially in people living in areas with low fish availability, emphasis should be on lowering dietary LA to increase tissue EPA and DHA. The proportions of dietary LA and ALA alter the synthesis and conversion of ALA to EPA and DHA (Bourre et al., 1990b; Lands, 1992; Guesnet et al., 1997). In a “western” diet with high intake of LA, the conversion of ALA to EPA, and especially DHA, is not sufficient to sustain tissue DHA (Brenna et al., 2009). But substantial reduction in dietary LA increases tissue EPA and DHA in both humans and animals (Clark et al., 1992; Jensen et al., 1996; Liou et al., 2007; Igarashi et al., 2009; Novak et al., 2008; Munakata et al., 2009; Guesnet et al., 2011), and highlight the importance of lowering dietary LA to increase tissue concentrations of EPA and DHA.

Table 2.2: PUFA intakes and n-3 HUFA in tissue

Country	Concurrent dietary intake (en%) ¹			Tissue target	n-3 HUFA to meet tissue target ²	
	LA	ALA	AA	% n-3 in HUFA	en% ³	mg/d
Philippines	0.80	0.08	0.06	50	0.06	133
Denmark	2.23	0.33	0.09	50	0.26	578
Iceland	2.48	0.33	0.10	50	0.31	689
Colombia	3.21	0.24	0.04	50	0.30	667
UK	3.91	0.77	0.07	50	0.39	567
Netherlands	4.23	0.28	0.08	50	0.50	1111
Australia	4.71	0.49	0.07	50	0.51	1133
Italy	5.40	0.51	0.06	50	0.56	1244
Germany	5.57	0.62	0.06	50	0.57	1267
Bulgaria	7.02	0.06	0.05	50	0.73	1622
Israel	7.79	0.67	0.07	50	0.85	1889
USA	8.91	1.06	0.08	50	0.98	2178

¹ Percent energy per day, ² appears to protect 98% of the population from disease risks, ³ based on a 2000 kcal/d diet. Abbreviations: AA arachidonic acid, ALA α -linolenic acid, en% percent energy, HUFA highly unsaturated fatty acids (> 20 carbon), LA linoleic acid. Adapted and reprinted with permission from authors (Hibbeln et al., 2006).

2.3 Dietary linoleic acid and early development of obesity

The prevalence of overweight and obesity among youths continue to rise despite decreasing intakes of total energy as fat (Troiano et al., 2000). Since the 1970s the percentage of US children between 6 and 11 months of age above the 95th percentile of the weight-for-length growth reference curve has increased (Ogden et al., 1997). Breast milk reflects the dietary fat consumed by the mothers. The content of LA in breast milk of US women has increased from 5% to 17% between 1945 and 1995 (Jensen et al., 1989; Jensen, 1996, 1999), and are considerably higher than in breast milk of European and Australian women (Ailhaud et al., 2006). The higher prevalence of infant adiposity emphasizes the qualitative issues of dietary fat since it is not likely be explained by an increased energy and fat intake or by increased sedentarity (Ailhaud et al., 2006).

In rodents, the maternal diet during gestation and lactation can have a significant impact on nutrient preference in offspring (Walker et al., 2008). Maternal consumption of a high fat diet can program hypothalamic pathways that regulate feeding (Grove et al., 2005) and cause hyperphagia as adults (Sullivan et al., 2011). Dams consuming a high fat/lard diet increased the preference to a high fat diet in offspring whereas a high fat/fish oil maternal diet did not (Nakashima, 2008).

Adipocyte formation is an irreversible process. In humans, the proliferative capacity of adipose precursor cells from subcutaneous adipose tissue is highest during the first year of life and before puberty (Massiera et al., 2003; Ailhaud et al., 2006). Thus, an early age is a highly sensitive period during which adipose tissue expands dramatically. Several animal studies report higher body weight, increased fat accumulation, hyperplasia and hypertrophy of adipose tissue and elevated plasma leptin levels in offspring from mothers fed a high fat/high n-6 diet compared to offspring from mothers fed high fat/high n-3 rich oils (Cleary et al., 1999; Korotkova et al., 2002; Massiera et al., 2003; D'Asti et al., 2010). A recent study demonstrate how quantity and quality of maternal dietary fat during the perinatal period directly influences neonatal metabolism, fatty acid composition in phospholipids and sensitivity to endocannabinoid system manipulation (D'Asti et al., 2010). Another recent study show that mice fed a "western-like" diet of 35 en% fat containing 18 en% LA and 0.6 en% ALA for 4 generations resulted in a gradual transgenerational increase in adiposity through hyperplasia and hypertrophy with no significant change in food intake. The enhanced adiposity was apparent in pups at weaning and maintained in adulthood (Massiera et al., 2010). Continuous exposure to high levels of LA also increased inflammatory stimuli (Massiera et al., 2010). In humans, nonobese offspring from obese parents

had higher C-reactive protein levels compared to offspring from one or no obese parent, indicating that obese parents transmit a susceptibility to predispose offspring to systemic inflammation and neurohormonal activation (Lieb et al., 2009). Changes occurring during development appear to be persistent and affect disease outcome later in life.

2.4 The endocannabinoid system

The endocannabinoid system includes the cannabinoid receptors 1 and 2 (CB1 and CB2) and the endogenous ligands of which the two best characterized are 2-arachidonoyl-glycerol (2-AG) and N -arachidonylethanolamine (anandamide or AEA). The endocannabinoids are endogenous marijuana-like lipid mediators synthesized on demand in animals, including humans, from the pool of 20 carbon n-6 fatty acids present in membrane phospholipids (Wang and Ueda, 2009).

The endocannabinoid system was discovered in the 1960s when the major psychoactive component of *Cannabis sativa* and marijuana Δ^9 -tetrahydrocannabinol (Δ^9 -THC) was identified (Mechoulam and Gaoni, 1967). The CB1 receptor, one of the most abundant G-protein-coupled receptors in the central nervous system, was not identified until 1988 (Devane et al., 1988). The first endogenous agonist for the cannabinoid receptor to be discovered was anandamide named after the Sanskrit word for “bliss” (Devane and Axelrod, 1994), followed by the identification of 2-AG in 1995 (Mechoulam et al., 1995; Sugiura et al., 1995). Several cannabinoid analogs have been synthesized (Howlett et al., 2004). Synthetic Δ^9 -THC (dronabinol) is approved for use in the USA to treat nausea and vomiting in cancer chemotherapy, and to stimulate appetite in AIDS wasting syndromes (Mechoulam and Hanu, 2001). The first specific antagonist for the CB1 receptor was SR141716 (rimonabant) which proved to be effective in reducing weight gain and improve cardiovascular risk factors (Després et al., 2005; Van Gaal et al., 2005; Christensen et al., 2007; Christopoulou and Kiortsis, 2011).

The presence of the cannabinergic system in most animal systems, and the high degree of conservation of the endocannabinoid system components (Elphick and Egertova, 2001) points out the importance of the endocannabinoid system in the control of basic physiological activities such as feeding and energy homeostasis (Yamaguchi et al., 1996; Sepe et al., 1998; De Petrocellis et al., 1999; Soderstrom et al., 2004; Valenti et al., 2005). However, the presence of the endocannabinoids should not in itself be interpreted as evidence that they necessarily function as part of an endocannabinoid system. Anandamide is found to be present in chocolate (di Tomaso et al., 1996) but it is not believed that cannabinoid signaling pathways exist in the cocoa bean (Elphick and Egertova, 2001).

In terms of evolution, nature has selected the endocannabinoid system whose

main physiological function appears to reorientate energy balance towards energy storage (Piazza et al., 2007). The importance of the endocannabinoid system was more apparent 4 million years ago when humans lived as hunter/gatherers with unstable food supply. People ate when hungry to counteract the decrease in endogenous nutrient levels. The introduction of agriculture provided more stable conditions and has along with recent technological advances in food preservation shifted the nutritional environment to an excess of food supply and people now eat when food is available. Thus an excessive food supply and an overactivation of the CB1 receptor can lead to overeating and an orientation of metabolism towards excessive energy storage (Piazza et al., 2007).

2.4.1 Endocannabinoids and appetite

Hyperphagia following cannabis intoxication is a widely accepted phenomenon known as the “the munchies”. The appetite stimulant properties of marijuana was recorded as early as 300 AD (Abel, 1971). In 1976, Greenberg et al. (1976) reported increased food intake and weight gain in men smoking marihuana for 21 days (Figure 2.2). Food intake stabilized to pre-experimental levels after 7 days of smoking, whereas body weight continued to increase independent of food intake. The ability of cannabinoid activation to affect appetite appears to be transient whereas the metabolic effects remain active longer (Greenberg et al., 1976; Colombo et al., 1998).

Central stimulation of the CB1 receptor by 2-AG and anandamide increases hunger-induced elevation in food intake, and decrease in satiety (Williams and Kirkham, 1999; Kirkham et al., 2002). The endocannabinoid system controls food intake at two levels; 1) it reinforces the motivation to find and consume foods with high incentive value, and 2) induces appetite by regulating levels and/or action of orexigenic and anorectic mediators (Di Marzo and Matias, 2005). The hyperphagic properties of endocannabinoids were reported in 1999 when anandamide injected peripherally stimulated overeating in satiated rats, an effect that was attenuated by selectively blocking the CB1 receptor by SR141716 (Williams and Kirkham, 1999). Anandamide caused a modest hyperphagia that appeared over a longer time course compared to Δ^9 -THC (Williams and Kirkham, 1999). Injection of 2-AG into the nucleus accumbens shell, an area of the brain associated with appetite stimulation, induced eating in rats (Kirkham et al., 2002). The increase in food intake from 2-AG injections was prevented by pre-treatment with SR141716, while the CB2 antagonist SR144258 had no effect. Thus demonstrating that the hyperphagic properties of anandamide and 2-AG are specifically mediated by central CB1 receptors (Williams and Kirkham, 1999; Kirkham et al., 2002).

Endocannabinoid activity seems to represent an amplification, or potentiation, associated with the normal episodic pattern of meal-taking in rats, thereby in-

creasing the size of meals (Kirkham and Williams, 2001). Endocannabinoid levels decline once eating is initiated, indicating that endocannabinoid activity is not needed for maintenance of food intake (Kirkham et al., 2002). The functional relationship between endocannabinoids and dopaminergic activity in the brain may be important for the incentive value and hedonic evaluation of food (Williams and Kirkham, 2002). The incentive value of a food is acquired through experience (Balleine and Dickinson, 1998). Preference and hunger state are factors that contribute to the incentive value of a food (Balleine and Dickinson, 1998). Endocannabinoids are believed to increase the incentive value of food and to be implicated in the processes underlying the motivation to eat, and not primarily involved in food reward during eating (Kirkham and Williams, 2001). Leptin, released by adipocytes, may act through down-regulation of hypothalamic endocannabinoid levels (Di Marzo and Matias, 2005), which may be the mechanism of which endocannabinoids reduce the general incentive value of food and restrict feeding.

Central endocannabinoid signaling seems to be important in regulating energy homeostasis. An elevated endocannabinoid tone in brain is sufficient to induce insulin resistance in liver, and high endocannabinoid levels in brain can impair hypothalamic insulin action in liver and adipose tissue (O'Hare et al., 2011).

2.4.2 Endocannabinoids and energy homeostasis

Genetic and chronic pharmacological impairment of the CB1 receptor have demonstrated the role of the endocannabinoid system in energy homeostasis through inhibition of food intake and reduction in body weight occurring independent of energy intake (Cota et al., 2003; Ravinet Trillou et al., 2003; Poirier et al., 2005)(Figure 2.2).

An early endocannabinoid-mediated increase in *de novo* lipogenesis appears to be a critical component in diet-induced obesity (Osei-Hyiaman et al., 2005). In liver, dietary LA and pharmacological stimulation of the CB1 receptor increase *de novo* fatty acid synthesis through the induction of the lipogenic transcription factor SREBP-1c and its target enzymes fatty acid synthase (FAS) and acetyl-coenzyme-A carboxylase-1 (ACC1), which was blunted by CB1 antagonist (SR141716) and absent in CB1 knock-out mice (Osei-Hyiaman et al., 2005). Mice with a specific knock-out of liver CB1 receptors develop a similar degree of obesity as wild-type mice but are not insulin and leptin resistant and have less hyperglycemia and dyslipidemia compared to wild-type mice (Osei-Hyiaman et al., 2008). This finding delineate the contribution of the endocannabinoid system/CB1 receptor in liver to specific components of the metabolic syndrome induced in mice fed a high fat diet (Osei-Hyiaman et al., 2008).

In adipose tissue, the endocannabinoid system is up-regulated immediately

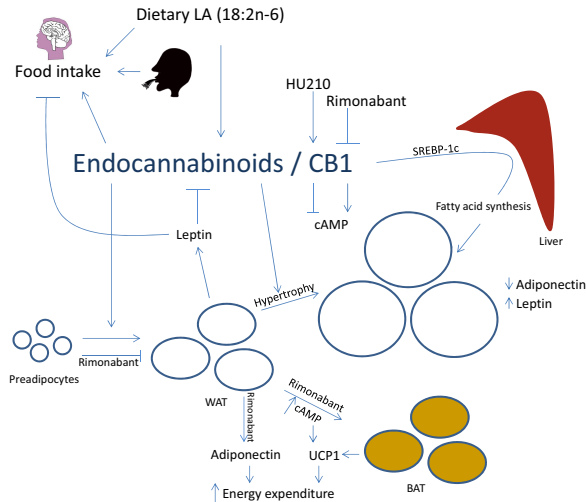


Figure 2.2: Endocannabinoids, CB1 action and energy metabolism. Activation of the CB1 receptor centrally and peripherally, favors metabolic processes that stimulate appetite and increase food intake, increase fatty acid synthesis and increase adipose tissue development. Overactivity of CB1 stimulation may therefore lead to obesity and associated metabolic disorders. Pharmacological blocking of the CB1 receptor by the selective antagonist rimonabant reduces weight gain by preventing preadipocyte cell proliferation, inducing energy expenditure and affecting adiponectin secretion. Abbreviations: BAT brown adipose tissue, cAMP cyclic AMP, CB1 cannabinoid receptor 1, HU210 cannabinoid receptor agonist, UCP1 uncoupling protein 1, WAT white adipose tissue.

before adipocyte differentiation, possibly inducing differentiation and lipogenesis via CB1 mediated inhibition of cAMP formation (Matias et al., 2006b). The mature adipocytes produce leptin which may act to turn off endocannabinoid action (Matias et al., 2006b) (Figure 2.2). Pharmacologically blocking the CB1 receptor by rimonabant modulates the expression of genes involved in maturation of adipocytes (Jbilo et al., 2005), inhibits preadipocyte cell proliferation and prevents lipid accumulation in adipocytes (Gary-Bobo et al., 2006), increases the expression of UCP1 by inducing transdifferentiation of white adipocytes to a thermogenic brown phenotype (Perwitz et al., 2010) and is implicated in the control of adiponectin secretion (Bensaid et al., 2003; Gary-Bobo et al., 2006; Matias et al., 2006b). In contrast to pharmacological blocking of the CB1 receptor, chronic overactivation by the CB1 agonist HU210 reduced the expression of adiponectin (Matias et al., 2006b). Adiponectin is a hormone secreted from the adipose tissue

that can induce fatty acid oxidation and cause weight reduction (Fruebis et al., 2001), reverse insulin resistance and decrease hyperinsulinemia (Yamauchi et al., 2001b), improve insulin sensitivity and decrease triglyceride storage in liver and muscle (Yamauchi et al., 2001b) and has anti-inflammatory and anti-atherogenic properties (Diez and Iglesias, 2003). Adiponectin levels are decreased in serum of humans with insulin resistance, obesity, type II diabetes mellitus and heart disease (Diez and Iglesias, 2003; Hotta et al., 2000).

2.4.3 Dietary fat and endocannabinoid levels

The endocannabinoids anandamide and 2-AG are endogenous lipid derivatives of the n-6 fatty acid arachidonic acid in the sn-1 and sn-2 position of phospholipids (AA -PL) respectively (Banni and Di Marzo, 2010a). Because humans cannot synthesize AA *de novo* tissue concentrations in phospholipids are dependent upon 1) dietary intakes of the AA, 2) the AA precursor LA and 3) content of competing fatty acids such as EPA and DHA (Mohrhauer and Holman, 1963b; Bourre et al., 1990a; Cunnane et al., 1985; Lands et al., 1992). It has therefore been hypothesized that an excess of dietary n-6 or intakes of n-3 fatty acids alter the availability of biosynthetic precursors and subsequently tissue concentrations of endocannabinoids (Matias et al., 2008b; Batetta et al., 2009; Banni and Di Marzo, 2010b; Piscitelli et al., 2011). Figure 2.3 indicates how the shift in available seed oils changes the estimated tissue composition of n-6 HUFA described in Blasbalg et al. (2011), and influences the precursor level for endocannabinoid synthesis.

Dietary fatty acids can modulate and cause profound tissue-specific changes on endocannabinoid levels after short-term (Artmann et al., 2008; Wood et al., 2010) and long-term exposure (Osei-Hyiaman et al., 2005; Matias et al., 2008b; Starowicz et al., 2008; Piscitelli et al., 2011). *In vivo* and *in vitro* studies have demonstrated that n-3 deficiency and dietary AA increase (Berger et al., 2001; Artmann et al., 2008; Matias et al., 2008a), whereas dietary EPA and DHA decrease endocannabinoid levels (Watanabe et al., 2003; Artmann et al., 2008; Batetta et al., 2009; Di Marzo et al., 2010; Wood et al., 2010). There are indications that physiological functions and pathological conditions involving endocannabinoids could be correspondingly modified by the manipulation of dietary fatty acids, such as a beneficial effect of n-3 PUFA and an exacerbating effect of n-6 PUFA in obesity, dyslipidemia and insulin resistance (Banni and Di Marzo, 2010b).

2.4.4 Hyperactivity of the endocannabinoid system

The body's ability to keep a relatively stable body weight indicates that energy balance is controlled in a way to maintain constancy of total body energy stores. Multiple signals and systems are involved in maintaining energy homeostasis. A

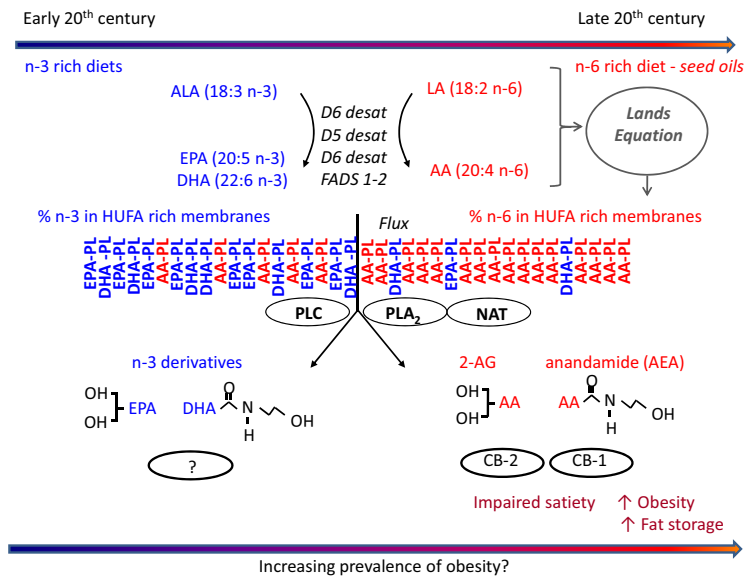


Figure 2.3: The endocannabinoids 2-AG and anandamide are synthesized on demand from AA in membrane phospholipids (AA-PL). AA in the phospholipid precursor pool can be elevated by dietary LA or diminished by consumption of n-3 fatty acids notably EPA and DHA. Activation of the CB receptors by 2-AG and anandamide centrally and peripherally affects satiety and fat storage. The figure indicates the hypothesis that these shift in diet, and resulting endocannabinoid levels, may contribute to the increasing prevalence of obesity in the US during the 20th century.

sustained hyperactivity of the endocannabinoid system is believed to have a causal role in obesity and to be one of the underlying factors in the development of hyperglycemia, dyslipidemia, ectopic fat accumulation, insulin resistance and type 2 diabetes (Di Marzo, 2008; Kunos et al., 2008).

The hypothalamus, known as the brains “hunger center”, is an important region the control of satiety and hunger, and in maintaining energy homeostasis. The hypothalamus receives and synthesizes hormonal stimuli to coordinate the central nervous system to the endocrine system. Leptin is a hormone that signals nutritional status and modulates food intake and energy balance by suppressing appetite and stimulating lipid oxidation. In normal animals leptin is involved in the up-regulation of anorectic signals (neuropeptides) and down-regulate orexigenic signals expressed in the hypothalamus (Friedman and Halaas, 1998). In

obese subjects the actions of leptin are absent or inefficient resulting in leptin resistance (Friedman and Halaas, 1998) which may elevate endocannabinoid levels in hypothalamus (Di Marzo et al., 2001). Higher hypothalamic endocannabinoid levels in leptin deficient subjects may lead to a chronic state of what has been described as perceived starvation (Friedman and Halaas, 1998). Thus the hyperphagia exhibited by genetically obese rodents is believed to arise from a motivational state that mimics the natural hunger generated by food deprivation (Kirkham et al., 2002).

Mice with diet-induced obesity display elevated tissue endocannabinoid levels compared to lean controls and CB1 knock-out mice (Osei-Hyiaman et al., 2005; Matias et al., 2006a; Starowicz et al., 2008). Overactivity of the endocannabinoid system in pancreas is believed to underlie the hyperinsulinemia that characterizes obesity by causing β -cell hypertrophy and damage that eventually might lead to the development of type 2 diabetes (Matias et al., 2006b). Insulin is an anabolic hormone that stimulates adipocyte differentiation and adipose tissue expansion (Madsen et al., 2010). In continued presence of insulin adipocytes becomes hypertrophic and have high levels of CB1 receptors and 2-AG (Matias et al., 2006b). In a state of insulin resistance, more insulin is required to promote glucose uptake by peripheral tissues, and insulin deficiency disrupts the regulation of glucose production in the liver, glucose uptake in the muscle and release of fatty acid from adipose tissue (Esposito et al., 2008; Di Marzo et al., 2011). Insulin resistant adipocytes lose their ability to regulate endocannabinoid metabolism (D'Eon et al., 2008) and are thus unable to decrease intracellular endocannabinoid pool in response to insulin stimulation (D'Eon et al., 2008; Di Marzo et al., 2009b).

Endocannabinoid signaling seems to be upregulated in obese humans and in subjects with high visceral adiposity and hyperglycemia (Engeli et al., 2005; Bluher et al., 2006; Matias et al., 2006b; Pagano et al., 2007; Di Marzo et al., 2009b). Plasma endocannabinoid levels in obese men correlate with changes in visceral adipose tissue and metabolic risk factors (Di Marzo et al., 2009a), and high circulating levels of 2-AG are in direct relationship to intra-abdominal adiposity (Bluher et al., 2006; Cote et al., 2007). Endocannabinoids are local mediators therefore the higher circulating levels of endocannabinoids observed in obese subjects are most likely a "spill-over" effect from excessive levels in peripheral tissues (Di Marzo et al., 2011). It has been speculated that an overactivity of the endocannabinoid system in some adipose tissue depots contributes to lower levels of adiponectin observed in obesity and hence to insulin resistance and atherosclerosis (Di Marzo et al., 2011). By contrast, a lower endocannabinoid tone in subcutaneous versus visceral fat, as seen in obese patients with type 2 diabetes (Annuzzi et al., 2010), may eventually contribute to excessive accumulation of visceral fat at the expense of the more "beneficial" subcutaneous depots (Di Marzo et al., 2011). The potential causes and metabolic responses of endocannabinoid overactivity is

described in Figure 2.4.

Growing evidence of upregulated endocannabinoid signaling in obesity lead pharmaceutical companies to develop CB1 receptor antagonists. In 2006, the European Medicines Agency approved rimonabant, a CB1 receptor inverse agonist, following impressive clinical trial results in both obese and diabetic patients. Specifically, placebo-controlled trials demonstrated considerable weight-loss and significant improvements in waist circumference, high density lipoprotein (HDL)-cholesterol, triglycerides, inflammatory markers, blood pressure, fasting glucose and fasting insulin (Després et al., 2005; Van Gaal et al., 2005; Christopoulou and Kiortsis, 2011). In 2008, however, rimonabant was pulled from the market due to serious psychiatric side effects, including suicide risk (Christensen et al., 2007; Christopoulou and Kiortsis, 2011). Because pharmacological blockade of the CB1 receptor is effective in treating obesity and related metabolic derangements, a dietary approach to diminish endocannabinoid hyperactivity may represent a safer alternative to pharmaceuticals. Addressing an underlying cause of endocannabinoid overactivation may have widespread beneficial public health implications related to obesity and metabolic syndrome.

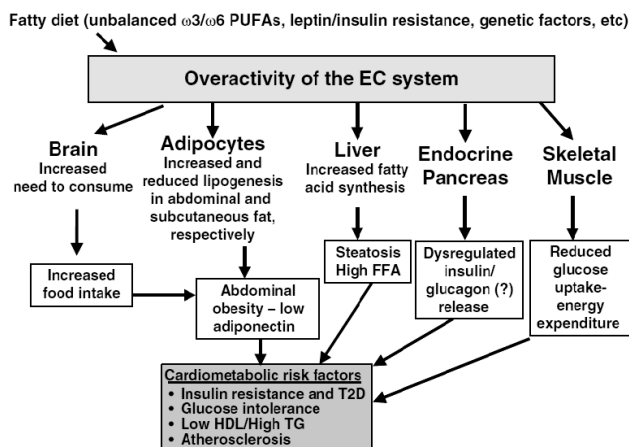


Figure 2.4: Overactivity of the endocannabinoid system. Potential causes of endocannabinoid hyperactivity in peripheral tissues and the consequences on metabolic risk factors. Abbreviations: EC endocannabinoid FFA free fatty acids, HDL high density lipoprotein, T2D type 2 diabetes, TG triglycerides. Permission to reprint from authors (Di Marzo et al., 2011).

2.5 Aims

Efforts and courage are not enough without purpose and direction. - John F. Kennedy

We aimed to investigate if elevating a single molecular species in the diet, LA, an essential n-6 fatty acid and the precursor of arachidonic acid, the backbone of endocannabinoids could cause endocannabinoid hyperactivity and induce obesity. We postulate that by addressing an underlying cause of endocannabinoid hyperactivity using a dietary approach, we may find a safe and viable alternative for decreasing the development of obesity.

The overall aims of the PhD were to:

- Compare the effect of excessive endocannabinoid activity from high dietary intake of LA in different levels of dietary fat (low - medium and high fat diets).
- Investigate the metabolic effect of different time points of exposure to dietary LA - prenatally versus adulthood.
- Study the endocannabinoid response to dietary LA in Atlantic salmon and mice - and investigate the metabolic effect in mice fed Atlantic salmon containing different levels of LA.

The specific objectives of the PhD were to determine if elevating dietary LA from 1 en% to 8 en%:

- Increases arachidonic acid and lowers EPA and DHA in tissue phospholipids.
- Elevates endocannabinoid system activity.
- Affects food intake and feed efficiency.
- Induces obesity.
- Affects adipose tissue morphology.
- Increase adipose tissue inflammation.

Chapter 3

Summary of Results

Dis-moi ce que tu manges, je te dirai ce que tu es¹.
- Brillat-Savarin, 1825

Paper I

Dietary linoleic acid elevates endogenous 2-AG and anandamide inducing obesity.

1. Design

C57Bl/6 mice were exposed to diets of 35 en% fat and 60 en% fat containing 1 en% LA, 8 en% LA and 8 en% LA + 1 en% EPA/DHA from the last week of gestation, through lactation and for 14 weeks after weaning.

2. Major findings

- There is a strong ecological relationship between human consumption of the major dietary sources of LA (soybean oil, poultry and shortening) and the increasing prevalence of obesity in the US during the 20th century.
- 8 en% LA in a high fat diet increased food intake.
- 8 en% LA increased adiposity (Figure 3.1).
- 8 en% LA increased tissue AA -PL and induced hyperactivity of the endocannabinoid system by elevating 2-AG and anandamide in mice liver (Figure 3.2).

¹Tell me what you eat, and I will tell you what you are.

- Reducing dietary LA to 1 en% elevated tissue EPA and DHA (Figure 3.2), and prevented the development of obesity normally induced by a high fat diet (Figure 3.1(b)).
- Supplementing the 8 en% LA diets with 1 en% EPA + DHA prevented the elevation of tissue AA -PL and endocannabinoid levels (Figure 3.2), and reversed the adipogenic effect of the 8 en% LA diets (Figure 3.1).

3. Conclusion

Dietary LA of 8 en% increased tissue AA -PL, subsequently elevating 2-AG and anandamide associated with the development of diet-induced obesity. The adipogenic effect of LA can be prevented by consuming sufficient EPA and DHA to reduce the AA -PL pool and normalize endocannabinoid tone.

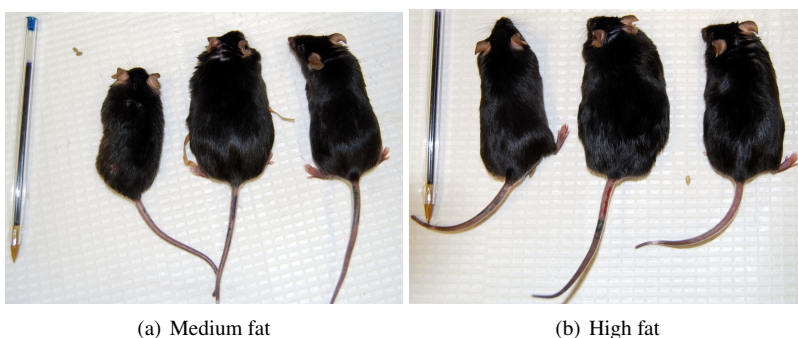


Figure 3.1: Dietary LA induces obesity. Dietary LA increases adiposity in mice fed (a) medium fat diets (35 en% fat) and (b) high fat diets (60 en% fat). In each photo, mice to the left were fed 1 en% LA, mice in the middle were fed 8 en% LA and mice to the right were fed 8 en% LA + 1 en% EPA/DHA.

Paper II

Dietary linoleic acid elevates the endocannabinoids 2-AG and anandamide and induces weight gain and inflammation in mice fed a low fat diet.

1. Design

C57Bl/6 mice were exposed to diets of 12.5 en% and 35 en% fat containing 1 en% LA and 8 en% LA for 16 weeks from 6 weeks of age.

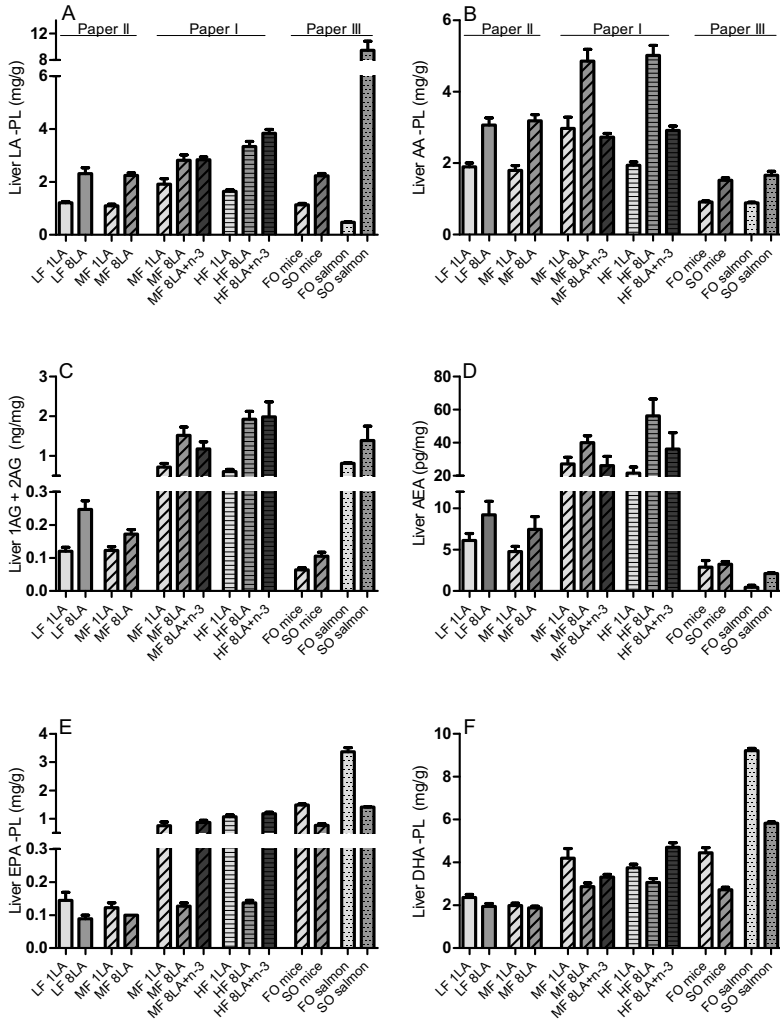


Figure 3.2: Dietary LA increases AA and endocannabinoid activity and decreases EPA and DHA in liver of mice and salmon. Selective elevation of dietary LA elevates LA-PL (A), AA-PL (B) and 2-AG (C) and anandamide (D) in liver. Compared to 8 en% LA, diets of 1 en% LA allow endogenous accretion of EPA (E) and DHA (F). The fatty acids in salmon are presented as amount of total fat. Open bars represent low fat diets (12.5 en%), coarse bars medium fat diets (35 en%), vertical lines high fat diets (60 en%) and dotted bars Atlantic salmon. Bar colors represent light gray 1 en% diets, gray 8 en% LA and dark gray 8 en% LA + 1 en% EPA/DHA. Abbreviations: FO mice fed fish oil (FO) salmon, HF high fat, LA linoleic acid, LF low fat diet, MF medium fat, PL phospholipid, SO mice fed soybean oil (SO) salmon. Statistical differences are described in individual papers.

2. Major findings

- Dietary LA of 8 en% increased tissue AA -PL and elevated endocannabinoid activity by elevating 2-AG and anandamide in liver (Figure 3.2).
- 8 en% induced weight gain in a low fat diet (Figure 3.3).
- 8 en% LA elevated adipocyte size and adipose tissue inflammation.

3. Conclusion

Increasing dietary LA from 1 en% to 8 en% with a subsequent elevation in liver endocannabinoid levels may increase the risk of developing obesity and associated metabolic complications also in low a fat diet.

Paper III

Dietary linoleic acid elevates endogenous 2-AG and anandamide in Atlantic salmon (*Salmo salar* L.) and mice, and induces weight gain and inflammation in mice.

1. Design

Atlantic salmon (340 g) were fed diets with fish oil or soybean oil for 6 months. C57Bl/6 mice, 6 weeks of age, were fed diets of 35 en% fat based on fillets from Atlantic salmon raised on fish oil or soybean oil for 16 weeks.

2. Major findings

- Replacing fish oil with soybean oil in feed for Atlantic salmon increased liver AA and endocannabinoid levels (Figure 3.2) and elevated liver lipids in Atlantic salmon.
- 8 en% LA in a SO salmon diet elevated AA -PL and endocannabinoids in mice liver (Figure 3.2).
- The SO salmon diet caused higher weight gain, increased adipocyte cell size and caused higher adipose tissue inflammation in mice.

3. Conclusion

Dietary LA of 8 en% elevated endocannabinoid levels similarly in liver of Atlantic salmon and mice. In mice, high dietary intakes of salmon fed soybean oil increased weight gain and counteracted the anti-inflammatory properties of EPA and DHA.

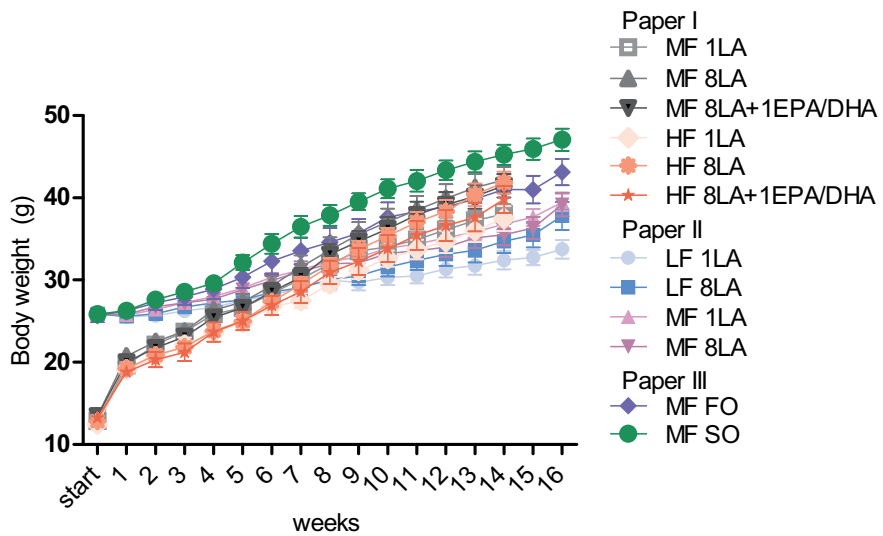


Figure 3.3: Weekly weight gain. Mice in Paper I were exposed to diets for 14 weeks after weaning (start) and for 16 weeks from 6 weeks of age (start) in Paper II and III. Abbreviations: FO mice fed fish oil (FO) salmon, HF high fat (60 en%), LA linoleic acid, LF low fat diet (12.5 en%), MF medium fat (35 en%), SO mice fed soybean oil (SO) salmon, 1LA 1en% LA, 8LA 8 en% LA, 1EPA/DHA 1 en% EPA+DHA. Statistical differences are described in individual papers.

Table 3.1: Diet composition and fatty acid profile of the diets

Parameter	Paper II				Paper III		Paper I					
	Low fat		Medium fat		Salmon		Medium fat		High fat			
	1LA	8LA	1LA	8LA	FO	SO	1LA	8LA	8LA+ IEPA/DHA	1LA	8LA	8LA+ IEPA/DHA
Cocosa	4	—	129	85	84	38	—	—	—	—	—	—
HCOC	—	—	—	—	—	—	108	70	61	280	228	219
HLA Safflower	1	44	—	42	—	7	—	43	45	—	—	—
HO Safflower oil	—	—	—	—	—	—	—	—	—	—	42	18
HO Sunflower oil	—	—	—	—	—	—	50	42	42	60	7	25
Olive oil	44	—	33	7	13	18	—	—	—	—	—	—
Soybean oil	—	—	—	—	—	—	—	3	—	—	—	74
Flaxseed oil	2	7	3	7	2	2	—	9	8	9	11	—
Fat from salmon fillet	—	—	—	—	—	—	—	—	—	—	—	—
Total oil added (g/kg)	50	50	165	165	99	65	167	167	167	350	350	350
E nergy (% of energy) derived from ¹ :												
Fat	11	11	32	34	35	32	35	35	35	60	60	60
Carbohydrate	71	71	45	44	44	47	45	45	45	20	20	20
Protein	18	18	23	22	21	21	20	20	20	20	20	20
			% energy of diet									
Sum SFA	2.5	1.5	27	19	17	14	25	18	16	51	44	42
Sum MUFA	9	2	10	8	8	8	8	8	9	7	7	7
18:2n-6	1	8	1	8	1	8	1	8	8	1	8	8
18:3n-3	0.3	0.9	0.4	1	0.4	1	1	1	1	1	1	1
20:5n-3	—	—	—	—	1	0.5	—	—	0.6	—	—	0.6
22:6n-3	—	—	—	—	1.7	1.3	—	—	0.36	—	—	0.34
n-6/n-3	3	9	3	8	0.3	3	1	7	4	1	8	4
Estimated n-6 HUFA (%)	56	72	53	72	8	33	42	72	41	42	72	41
Actual n-6 HUFA (%) in RBC	62	73	64	73	19	39	47	74	45	43	72	38
Estimated n-3 index	11	5	11.5	5.5	26	18	15	5.5	16	15	4.5	16
Actual n-3 index	9	5	8	5	23	16	12	4.5	14	14	5	16

¹ Fat and protein were measured in Paper II and III and were based on estimated values in Paper I. n-6 HUFA calculated from the Lands equation (Lands et al., 1992) ($[\text{sum n-6 HUFA}/\text{sum n-6 HUFA} + \text{sum n-3 HUFA}] * 100$), n-3 index ($[\text{n-3 HUFA} * 0.32] - 3.5$) based on (Lands, 2009; Harris and von Schacky, 2004). Abbreviations: HCOC hydrogenated coconut oil, HLA high linoleic acid, HO high oleic acid, HUFA highly unsaturated fatty acids (>20carbons), MUFA monounsaturated fatty acids, SFA saturated fatty acids. Statistical differences are described in individual papers.

Table 3.2: Physiological, biochemical and metabolic parameters in mice

Parameter	Paper II				Paper I				Paper III			
	Low fat		Medium fat		Medium fat		High fat		8LA + IEPA/DHA		Salmon diets	
	ILA	8LA	ILA	8LA	ILA	8LA	ILA	8LA	8LA + IEPA/DHA	FO	SO	
BW start (g)	25.8±0.8	25.9±0.5	25.9±0.6	25.8±0.5	12.7±0.4	13.0±0.3	12.4±0.3	13.3±0.3	13.1±0.2	25.7±0.8	25.8±0.5	
BW final (g)	33.7±1.1	37.8±1.7	39.4±1.1	39.2±1.5	37.7±1.0	41.7±1.0	36.1±1.3	43.5±1.0	40.4±1.4	43.1±1.6	47.1±1.3	
BW gain (g)	7.9±0.8	11.9±1.4	13.5±0.9	13.4±1.2	24.9±0.7	28.7±0.7	24.7±1.4	30.3±1.0	26.9±1.4	17.4±0.9	21.2±1.1	
BW increase (%)	23±2	31±2	34±2	34±2	66±1	68±1	66±1	69±1	67±1	40±1	45±1	
Food intake (g)	357±8	361±10	322±7	323±5	288±8	290±3	247±7	292±10	298±14	330±10	347±13	
Calorie (kcal)	1398±33	1443±39	1387±28	1361±19	1221±32	1230±15	1278±40	1532±52	1563±72	1465±43	1504±57	
Feed eff(g/meal)	5.6±0.5	8.1±0.8	9.7±0.5	9.8±0.8	20.5±0.7	23.4±0.7	22.0±0.9	19.1±1.0	19.9±0.7	12.0±0.7	14.1±0.6	
Liver (g)	1.5±0.0	1.5±0.1	1.9±0.1	1.7±0.1	1.8±0.1	1.9±0.1	1.9±0.1	1.4±0.1	1.3±0.1	1.7±0.2	2.3±0.2	
iWAT (g)	0.4±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.8±0.1	1.1±0.1	0.9±0.1	0.7±0.1	1.0±0.1	0.7±0.1	0.9±0.1	
eWAT (g)	1.0±0.2	1.4±0.2	1.4±0.2	1.6±0.1	1.7±0.1	2.2±0.1	1.9±0.1	2.9±0.1	2.3±0.1	1.9±0.2	1.8±0.1	
rWAT (g)	0.4±0.1	0.6±0.1	0.5±0.1	0.7±0.1	0.7±0.0	1.0±0.1	0.9±0.1	1.2±0.0	0.9±0.1	0.8±0.1	0.9±0.1	
Muscle (mg) ¹	142±0.0	140±0.0	148±0.0	153±0.0	-	-	-	-	-	157±0.0	165±0.0	
Adiposity Index (%)	6±0.9	8±0.7	7±0.9	9±0.7	11±0.7	13±0.6	11±0.8	16±0.4	13±0.7	10±0.6	10±0.6	
Leptin (ng/ml)	4.4±1.3	11.4±2.5	6.1±0.8	13.4±1.3	16.8±1.6	25.5±3.5	21.4±3.0	11.0±2.6	16.5±3.3	17.0±2.0	19.0±2.1	
Adiponectin (ug/ml)	2.4±0.2	2.9±0.1	1.6±0.1	2.8±0.5	12.0±0.9	8.1±1.2	11.8±1.4	10.5±1.6	9.0±1.0	10.5±2.0	3.1±0.2	
Adiponectin (ug/ml/g fat)	1.6±0.3	1.4±0.2	0.6±0.1	1.2±0.2	4.9±0.1	4.6±0.7	5.0±0.2	3.5±0.8	4.0±0.5	3.7±0.6	8.8±0.7	
Insulin (ug/L)	2.4±0.5	3.2±0.5	3.7±0.5	2.9±0.6	4.9±0.1	4.6±0.7	5.0±0.2	3.5±0.8	4.0±0.5	8.8±0.7	9.0±0.2	

¹ Muscle weight was not recorded in Paper I. Adiposity index (subcutaneous + retroperitoneal + inguinal fat pads)/eviscerated body weight * 100), feed efficiency ((body weight gain/calorie intake)*100). Abbreviations: BW body weight, eWAT epididymal white adipose tissue, feed eff feed efficiency, FO fish oil, iWAT inguinal white adipose tissue, rWAT retroperitoneal white adipose tissue and SO soybean oil. Statistical differences are described in individual papers.

Chapter 4

Discussion

Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning. - Sir Winston Churchill, November, 1942

4.1 Linoleic acid and the prevalence of obesity

We found a positive correlation between soybean oil, shortening and poultry, the major dietary sources of LA, and the rise in obesity in the US during the 20th century (Paper I). Data on the apparent soybean oil consumption is robustly associated with adipose concentrations of PUFA across 11 countries (Petrova et al., 2011) and consistent with an epidemiological report linking increased intake of LA to the increased prevalence of obesity (Ailhaud et al., 2008) suggesting that AA-induced elevations in the endocannabinoid 2-AG may have altered energy balance towards obesity (Ailhaud et al., 2008). Also, adipose tissue AA and BMI were associated with overweight status in children. (Savva et al., 2004).

Sugar was weakly correlated with the prevalence of obesity. In contrast, changes in total energy consumption and total fat consumption were not significantly correlated with the increasing rates of obesity (Paper I). A shift from physical labor occupations including farmers, farm laborers and laborers was negatively correlated with the prevalence of obesity. We note that the shift away from physical labor occupations is likely to be a significant factor, however the increasing prevalence of obesity is also found separately within physical and non-physical labor occupations (Caban et al., 2005). This ecological examination was not intended to test for causality, but permitted the design of an animal model which isolated LA as an independent variable and modeled differences in LA intake comparing consumption at the beginning and the end of the 20th century.

4.2 Dietary fat, endocannabinoids and energy metabolism

4.2.1 Dietary fat and endocannabinoid levels

The aim this thesis was to provide a dietary approach in which decreasing substrate availability for endocannabinoid synthesis could reduce hyperactivity of the endocannabinoid system and improve metabolic functions associated with obesity. Dietary influence of the endocannabinoid system is a new field of research. The current understanding of the endocannabinoid system in energy homeostasis is mainly generated from pharmacological stimulation of the CB1 receptor and the use of knock-out mice lacking the CB1 receptor (CB1^{-/-}). Several studies have demonstrated that dietary n-6 and n-3 PUFA influence endocannabinoid levels (Berger et al., 2001; Watanabe et al., 2003; Matias et al., 2006b; Artmann et al., 2008; Di Marzo et al., 2010; Wood et al., 2010; Piscitelli et al., 2011) and affect energy metabolism (Matias et al., 2006a, 2008a; Starowicz et al., 2008).

Dietary n-6 increases endocannabinoid levels

The work presented here is the first to demonstrate the critical importance of dietary LA to tissue AA -PL concentrations to endocannabinoid hyperactivity, previously proposed by others (Matias et al., 2008b; Batetta et al., 2009; Piscitelli et al., 2011; Banni and Di Marzo, 2010a). We show that increasing dietary LA from 1 to 8 en% significantly increased AA in the phospholipid pool subsequently elevating liver 2-AG (50% Paper I, II) and anandamide (30-40% Paper I and II). The highest increase in liver 2-AG and anandamide was found in mice fed a high fat diet containing 8 en% LA (68% and 57% respectively, Paper I, Figure 3.2). Artmann et al. (2008) did not find elevated endocannabinoid levels from a diet with high level of LA, however the short term exposure of one week did not allow adequate time for the alteration of the AA -PL precursor pool.

Dietary LA of 8 en% elevated endocannabinoid levels centrally and peripherally despite higher circulating leptin levels (Paper I, II and III). The expression of leptin mRNA in adipose tissue (Paper I, Supplementary) was not affected by dietary LA suggesting that mice fed 8 en% LA were leptin resistant (Paper I) similar to what is observed in obese subjects (Friedman and Halaas, 1998). Under normal circumstances leptin exerts a negative control of endocannabinoid levels in hypothalamus (Di Marzo and Matias, 2005) and adipocytes (Matias et al., 2006b; O'Hare et al., 2011). In an obese state, defective leptin signaling is associated with elevated endocannabinoid levels (Di Marzo and Matias, 2005). Leptin is secreted from adipose tissue, thus higher circulating levels of leptin may reflect an increased adipose tissue mass (Paper I). Although not obese, mice fed 8 en% LA in low fat and medium fat diets displayed elevated leptin levels (61% and 54%

respectively) compared to 1 en% LA (Paper II, Table 3.2) indicating that leptin resistance may occur independent of obesity.

Dietary EPA and DHA decrease endocannabinoid levels

In our studies, tissue levels of AA -PL seems to be an important determinant of endocannabinoid levels (Figure 3.2). Supplementing 8 en% LA diets with 1 en% EPA/DHA prevented the elevation in tissue AA -PL and liver anandamide observed in mice fed 8 en% LA diets (Paper I). Mice fed dietary salmon (2-3 en% EPA and DHA) had the lowest liver levels of 2-AG and anandamide (Paper III, Figure 3.2). Our results support previous studies reporting decreased endocannabinoid levels from dietary long-chain n-3 fatty acids (Watanabe et al., 2003; Artmann et al., 2008; Di Marzo et al., 2010; Wood et al., 2010; Piscitelli et al., 2011).

The differential effect of EPA/DHA on 2-AG and anandamide levels in liver and brain may result from the different origin of 2-AG and anandamide in the phospholipid. Anandamide is derived from AA esterified in the sn-1 position, whereas 2-AG is derived from AA in the sn-2 position (Banni and Di Marzo, 2010a). *In vitro*, the amount of AA esterified in the sn-2 position of adipocyte phospholipids determined the amount of 2-AG (Matias et al., 2008a). ALA and DHA affected 2-AG levels the same way as they decreased AA in the sn-2 position (Matias et al., 2008a). Dietary fatty acids can alter endocannabinoids without affecting AA -PL (Berger et al., 2001; Batetta et al., 2009; Di Marzo et al., 2010; Wood et al., 2010). The exact mechanism of how dietary fatty acids alter brain endocannabinoid levels is not clear. The dietary n-3 PUFA status seems to be an important factor to influence brain endocannabinoid levels (Watanabe et al., 2003). Deficiency of n-3 PUFA elevated 2-AG without affecting brain AA -PL, whereas n-3 PUFA supplementation reduced brain AA -PL and 2-AG, suggesting that DHA exhibit inhibitory properties on 2-AG synthesis in brain (Watanabe et al., 2003). Krill oil appears to be superior to fish oil in lowering 2-AG levels (Di Marzo et al., 2010; Banni et al., 2011; Piscitelli et al., 2011), indicating that the structural form of n-3 PUFA also influences endocannabinoid levels (Di Marzo et al., 2010; Banni et al., 2011).

4.2.2 Endocannabinoids and energy homeostasis

The role of cannabinoid receptor activation in energy homeostasis is summarized in Figure 4.1.

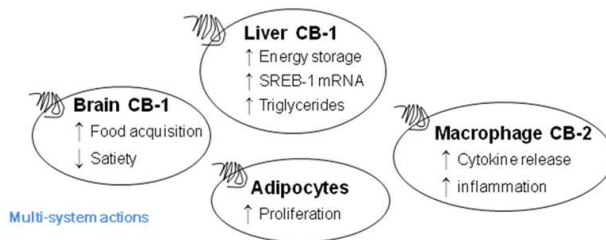


Figure 4.1: Cannabinoid receptor activation and energy metabolism. Activation of the cannabinoid receptors (CB1 and CB2) both centrally and peripherally, favors metabolic processes that stimulate appetite, increase food intake, activates fat storage pathways, promote adipocyte inflammation, and down regulates catabolism resulting in adipose accretion.

4.2.3 Central effects of endocannabinoid activation

Here we show that long-term exposure to 8 en% LA resulted in a persistent elevation in brain endocannabinoid levels (Figure 4.2). Long-term exposure of 8 en% LA elevated n-6 HUFA in hypothalamus thereby increasing the precursor availability for endocannabinoid production consequently elevating 2-AG and anandamide in the thalamus/hippocampal region¹.

Increasing dietary LA from 1 en% to 8 en% resulted in a similar increase 2-AG and anandamide concentrations in the thalamus/hippocampal region of prenatally exposed mice (Paper I) and cerebral cortex² of mice exposed from adulthood (Paper II and III, Figure 4.2). Mice fed 8 en% LA in a high fat diet had the highest increase in brain levels of 2-AG (22%) and anandamide (23%, Paper I), together with mice fed SO salmon (24% increase in anandamide compared to FO salmon diet, Paper III). In accordance with previous studies (Watanabe et al., 2003; Di Marzo et al., 2010; Wood et al., 2010), supplementation of EPA and DHA decreased brain levels of 2-AG ($P < 0.013 - 0.05$, Paper I) with no effect on brain anandamide (Figure 4.2). The low amount of fat in the low fat diets was not sufficient to influence brain levels of 2-AG and anandamide (Paper II).

Our results show that duration of exposure and amount of dietary fatty acids appears to be important factors in regards to brain 2-AG and anandamide concentrations. The adult brain is less susceptible to dietary manipulation than the young brain (Rapoport et al., 2007; DeMar et al., 2008), and retain a relatively stable

¹There was not enough hypothalamus tissue left after fatty acid analysis to accurately measure endocannabinoid levels.

²The brain was cut in half at the time of sacrifice which made it difficult to dissect out hypothalamus or the thalamus/hippocampus region.

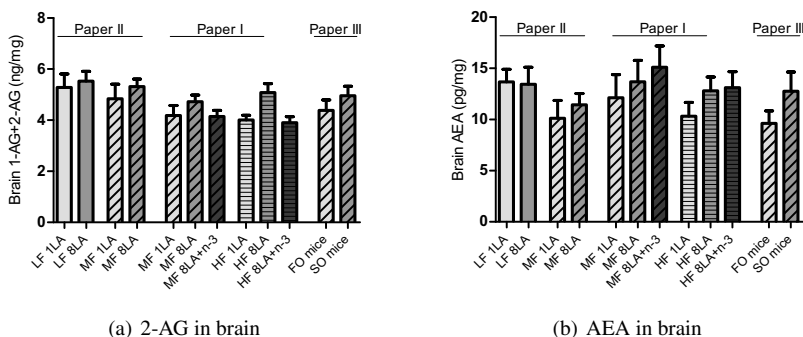


Figure 4.2: Dietary LA increases endocannabinoid levels in brain. Increasing LA from 1 en% to 8 en% elevated (a) 2-AG and (b) AEA in hypothalamus/hippocampus region (Paper I) and cerebral cortex (Paper II and III). (c) The increase (%) in brain endocannabinoid levels was independent of brain structure and time-point of exposure. Open bars represent low fat diets (12.5 en%), coarse bars medium fat diets (35 en%), vertical lines high fat diets (60 en%) and dotted bars represent Atlantic salmon (35 en% fat). Bar colors indicate light gray 1 en% diets, gray 8 en% LA and dark gray 8 en% LA + 1 en% EPA/DHA. Statistical differences are presented in individual papers. Abbreviations: AEA anandamide, FO mice fed fish oil (FO) salmon, LF low fat diet, HF high fat, MF medium fat, SO mice fed soybean oil (SO) salmon.

fatty acid composition as long as minimum requirements are met (Lauritzen et al., 2001). Although the increase in brain 2-AG and anandamide caused by the 8 en% LA diets were not statistically significantly from diets of 1 en% LA, the biological significance remains to be elucidated.

Brain levels of 2-AG and anandamide were less affected by dietary LA than the liver. The increase in brain n-6 HUFA (9 %) was considerably lower than in peripheral tissue such as liver (42 %) and RBC (37 %, Paper I), and eWAT (29% Paper II and 72% Paper III). The lower sensitivity of dietary LA on endocannabinoid levels in brain compared to peripheral tissues suggests that brain lipids and endocannabinoid levels are physiologically more stable and less influenced by changes in dietary fatty acids (Wood et al., 2010; Di Marzo et al., 2010).

Endocannabinoids and appetite

The higher food intake in mice fed 8 en% LA compared to 1 en% LA in the high fat diets (Paper I) is likely to be induced by a sustained hyperactivity of the endocannabinoid system both peripherally and centrally to increase appetite and reduced satiety (Williams and Kirkham, 1999; Kirkham et al., 2002). The elevation of brain 2-AG and anandamide levels in mice fed 8 en% in medium fat diets (Paper I, II and III) may not have been sufficient to stimulate the hyperphagic behavior seen in mice fed 8 en% LA in a high fat diet (Paper I). Endogenous endocannabinoid stimulation cause a modest overeating compared to exogenous cannabinoid stimulation (Williams et al., 1998; Williams and Kirkham, 1999).

The food intake fluctuated during the 14-16 weeks of feeding, peaking every 2 (Paper II and III, Figure 4.3 (a)) and 3 weeks (Paper I, Figure 4.3 (b)). The high fat diets of 8 en% LA followed the fluctuations but at a higher intake than the other diets (Paper I, Figure 4.3(b)) indicating that the overeating is an amplification of endocannabinoid activity associated with the normal, episodic pattern of meal-taking (Kirkham and Williams, 2001).

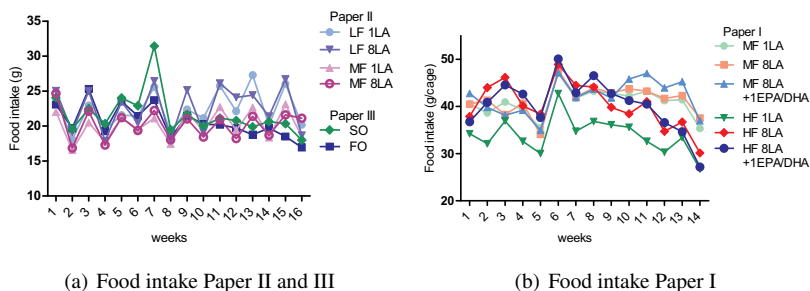


Figure 4.3: Weekly food intake. (a) Increasing dietary LA from 1 en% to 8 en% increased food intake in high fat diets (60 en%). (b) Food intake and eating pattern were similar in mice fed low fat (12.5 en%) and medium fat diets (35 en%, Paper II and III). Mice in Paper I were housed 2 per cage whereas mice in Paper II and III were housed individually. Statistical differences are presented in individual papers. Abbreviations: FO diet of salmon fed fish oil, HF high fat diet, LF low fat diet, MF medium fat diet, SO diet of salmon fed soybean oil.

4.2.4 Peripheral effect of endocannabinoid activation

Increasing dietary LA from 1 en% to 8 en% significantly increased liver AA -PL with a subsequent elevation in liver 2-AG and anandamide (Paper I, II and III, Figure 3.2). The elevated endocannabinoid activity in mice fed 8 en% LA compared to 1 en% LA was associated with higher feed efficiency; resulting in higher weight

gain per calorie consumed, which caused adipose tissue accumulation regardless of amount of dietary fat (Paper I, II and III) and increased adiposity (Paper I, Table 3.2). Dietary LA of 8 en% resulted in adipocyte hypertrophy, adipose tissue inflammation and elevated circulating leptin, factors that may increase the risk of metabolic disorders associated with obesity (Paper II, III).

Energy balance

The most striking findings in the present work are 1) that a low fat diet can be made obesogenic by selective inclusion of 8 en% LA (Paper II) and 2) the obesogenic properties of a high fat diet, commonly used to induce obesity in animal models, can be reversed by selectively reducing dietary LA to 1 en% (Paper I). Our findings imply that low fat human diets could be made more effective in reducing obesity if LA is lowered to near 1 en%. Indeed, total dietary fat intake may not need to be lowered if LA is selectively lowered.

Diets of 1 en% containing higher level of monounsaturated and saturated fat (Table 3.1) did not increase weight gain or adiposity compared to 8 en% LA diets demonstrating that dietary LA is more obesogenic than saturated and monounsaturated fat. Our data support the notion that weight gain and adipose tissue accumulation depends on the quality of the fat rather than quantity (Massiera et al., 2003; Ailhaud et al., 2008; Madsen et al., 2008; Matias et al., 2008a,b).

Several studies report higher feed intake, weight gain and greater adiposity from oils rich in LA such as soybean and safflower oil (Dayton et al., 1966; Cunnane et al., 1985; Ikemoto et al., 1996; Okuno et al., 1997; Takahashi and Ide, 2000; Massiera et al., 2003; Pellizzon et al., 2002; Javadi et al., 2004; Madsen et al., 2008). But the adipogenic effect of n-6 PUFA is controversial. Lower adipose tissue accumulation has been reported in mice fed soybean and safflower oil compared to saturated fat (Shimomura et al., 1990; Takeuchi et al., 1995; Matsuo et al., 2002). Differences in experimental setup, type and amount of carbohydrates, length of feeding and the use of different rodent strains may influence the result leading to different effects of dietary LA (Madsen et al., 2010). The opposing effect of LA on adipose tissue development was found to involve diet-induced differences in cAMP levels which can affect adipose tissue differentiation and the expression of UCP1 in adipose tissue (Madsen et al., 2008). Higher protein/carbohydrate ratio (Madsen et al., 2008) and pharmacological blockade of CB1 (Esposito et al., 2008; Sugamura et al., 2009) increase cAMP levels. Elevated cAMP levels induce thermogenesis by increasing expression of PGC-1 α and UCP1 in brown adipose tissue (Cannon and Nedergaard, 2004). Pharmacologically blocking the CB1 receptor induced UCP1 expression in white adipocytes, accompanied by an increased mitochondrial biogenesis and insulin sensitivity (Perwitz et al., 2010). We aimed at measuring UCP1 in white adipose tissue

to investigate if the leaner phenotype in mice fed 1 en% LA and 8 en% LA + 1 en% EPA/DHA was a result of higher energy expenditure. Lower CB1 activation in mice fed 1 en% LA and supplemented with EPA/DHA may have induced UCP1 expression and transdifferentiation of white adipocytes to a brown phenotype as observed in cell culture (Perwitz et al., 2010). However, long-term storage (18 months in -80°) and eWAT thawing in transit during shipment degenerated mRNA quality, making it unsuitable for real time-qPCR.³

Lipid metabolism

The higher adiposity in mice fed 8 en% LA could not be explained by differences in liver mRNA expression of the lipogenic transcription factor SREBP-1c and its target genes ACC1 and FAS. An increased basal rate of fatty acid synthesis in liver by pharmacological and dietary activation of the CB1 receptor suggest that an early endocannabinoid-mediated increase in *de novo* lipogenesis is a critical component of diet-induced obesity in mice (Osei-Hyiaman et al., 2005). As previously reported (Kim et al., 1999, 2002; Tandy et al., 2009; Piscitelli et al., 2011) we found that the expression of SREBP-1c, ACC1 and FAS were more closely related to the total PUFA content in the high fat diets than dietary LA (Paper I, Supplementary). Sugar is a strong inducer of SREBP-1c (Madsen et al., 2010) and may explain why mRNA levels of SREBP-1c, FAS and ACC1 did not differ in mice fed medium fat diets (Paper I).

Supplementing the 8 en% LA diets with 1 en% EPA/DHA reduced AA -PL and 2-AG in liver (Figure 3.2) and reversed the adipogenic effect of the 8 en% LA diets (Paper I, Table 3.2). We only observed lower liver mRNA expression of SREBP-1c, ACC1 and FAS in mice supplemented with EPA and DHA in the high fat diet, but not statistically different from mice fed 8 en% LA (Paper I). Our results suggest that altered expression of SREBP-1c and its target enzymes is not the main pathway involved in preventing weight gain in diets of 1 en% LA after long-term feeding (Paper I).

The leaner phenotype of mice fed 1 en% LA and 8 en% LA + EPA/DHA may be due to higher adiponectin levels caused by lower CB1 activation in peripheral tissue (Bensaid et al., 2003; Gary-Bobo et al., 2006; Matias et al., 2006b). Adiponectin can increase fatty acid oxidation and energy consumption (Yamauchi et al., 2001a). Mice fed 8 en% LA had lower adiponectin and higher adiposity than mice fed 1 en% LA (Paper I) which pose a greater risk for developing insulin resistance (Yamauchi et al., 2001b). Insulin levels did not differ between the dietary treatments (Paper I and II) suggesting that the mice were still insulin sensitive.

³Most tissue samples had RNA Integrity Number (RIN, analyzed by Bioanalyser) of less than 6 which we considered to low for RT-qPCR.

Insulin resistant adipocytes lose their ability to regulate endocannabinoid action (D'Eon et al., 2008; Di Marzo et al., 2009b) which may further aggravate the metabolic state of mice ingesting 8 en% LA.

Adipose tissue

Mice fed 8 en% LA had higher concentration of AA in eWAT than mice fed 1 en% LA (Paper II). Higher substrate availability for endocannabinoid synthesis may have influenced CB1 activity in adipose tissue. An overactivity of CB1 activation in mice fed 8 en% LA can have contributed to the higher adiposity (Paper I) and adipose tissue hypertrophy (Paper II and III) by a more rapid differentiation of pre-adipocytes to mature adipocytes (Matias et al., 2006b), increased adipocyte proliferation (Gary-Bobo et al., 2006), induction of accumulation of lipid droplets (Matias et al., 2008b) and facilitated fatty acid uptake by the adipocytes (Cota et al., 2003). Histology was only intended to visualize differences between the dietary treatments, therefore adipose tissue was only collected from only two animals per diet, representing the average body weight of their respective treatments. When histology was performed (18 months years after sacrifice) the techniques, equipment and software available were improved, but we did not have sufficient power ($n = 2$) to perform statistics (Paper II and III). However, our finding that dietary LA affect adipose tissue morphology is consistent with previous reports (Ezaki et al., 1992; Cleary et al., 1999).

Immunohistochemical analysis of adipose tissue by staining for the macrophage marker F4/80⁴ demonstrated more macrophage infiltration in eWAT of mice fed 8 en% LA than in mice fed 1 en% LA in both low fat and medium fat diets (Paper II and III). The higher inflammation in adipose tissue of mice fed 8 en% LA compared to 1 en% LA further supports the hypothesis of a more obesity-prone phenotype from high dietary levels of LA. It was recently shown that the expression of inflammatory markers and macrophage marker genes were higher in iWAT than eWAT (Du et al., 2011). We were not able to measure the expression of inflammatory markers in adipose tissue due low quality of mRNA and loss of tissue during shipment as previously mentioned.

The lower macrophage infiltration in adipose tissue of mice fed 1 en% LA (Paper II) and FO salmon (Paper III) may be due to higher tissue levels of EPA and DHA (Figure 3.2). Diets enriched with n-3 PUFA reduce adipose tissue inflammation in diet-induced obesity (Todoric et al., 2006; Huber et al., 2007; Perez-Echarri et al., 2008). The mechanism by which EPA and DHA reduce macrophage-induced adipose tissue inflammation is mediated by stimulation of the fatty acid receptor GPR120 (Oh et al., 2010). EPA and DHA can be converted

⁴F4/80 is a glycoprotein that increases as macrophages mature (Lin et al., 2005)

to metabolic products such as resolvins and protectins with anti-inflammatory actions independent of the state of obesity (Bannenberg et al., 2005). In our work dietary LA of 8 en% appears to decrease the anti-inflammatory properties of EPA and DHA (Paper III). Sucrose counteracts the anti-inflammatory effect of fish oil in adipose tissue (Ma et al., 2011) and together these data demonstrate that the background diet influence the anti-adipogenic and anti-inflammatory properties of EPA and DHA. The type of dietary fat seems to be an important determinant in adipose tissue inflammation (Paper II and III).

In contrast to mice prenatally exposed to 1 en% (Paper I), long-term feeding of 1 en% LA from adulthood did not prevent weight gain and adiposity compared to 8 en% LA (Paper II). Prior to arriving at our facility the mice were fed a diet based on soybean oil (NIH #31M, Paper II and III). We estimate, using the Lands equation (Lands et al., 1992) that the NIH #31M diet yields 72% n-6 HUFA in tissue phospholipids, identical to our 8 en% LA diets (Table 3.1). Metabolic changes such as induced lipogenesis and deposition of lipid droplets in response to increased endocannabinoid activity have been shown to occur before onset of obesity (Xu et al., 2003; Osei-Hyiaman et al., 2005; Matias et al., 2008b). Therefore, an already elevated endocannabinoid tone combined with abundant carbohydrates and fat may have promoted adipose tissue expansion possibly overriding the anti-adipogenic effect of 1 en% LA (Paper II). Mice fed 8 en% in the low fat diet gained considerably more weight than mice fed a low fat diet of 1 en% LA after 8 weeks of feeding (Figure 3.3). We cannot exclude that the same is the case for medium fat diets after 16 weeks of feeding. Thus when reducing dietary LA in adults, especially in diets with high fat content, longer feeding periods appear to be necessary to affect body weight and weight gain (Paper II).

4.2.5 Endocannabinoid levels in Atlantic salmon

In this work we demonstrate the presence of endogenous 2-AG and anandamide in liver of Atlantic salmon (Paper III). Endogenous cannabinoids has previously been reported in fish brains (Cottone et al., 2005; Valenti et al., 2005). Replacing fish oil with soybean oil in feed for Atlantic salmon introduce high dietary levels of LA (Table 3.1) altering the fatty acid profile of salmon fillet and liver. Salmon fed soybean oil have higher levels of LA and lower levels of EPA and DHA in liver and fillet compared to salmon fed fish oil 6 months feeding (Paper III). Similar to what we observed in mice (Paper I and II), increasing dietary LA elevated liver AA (89%), subsequently elevating 2-AG (42%) and anandamide (36%) in salmon liver with considerably higher levels 2-AG than anandamide (Paper III, Figure 3.2).

We observed higher liver lipid accumulation in Atlantic salmon when fish oil was replaced by soybean oil, consistent with previous studies replacing vegetable

oil with fish oil in feed for Atlantic salmon (Jordal et al., 2007; Torstensen et al., 2011). Osei-Hyiaman et al. (2005) found that stimulation of the CB1 receptor in mice liver induce the expression of SREBP-1c, FAS and ACC1 activity and stimulate *de novo* fatty acid synthesis . In Atlantic salmon, replacing fish oil with a blend of vegetable oils (rapeseed, palm and Camelina oils) up-regulated the expression of SREBP-1c and FAS (Morais et al., 2011a), and affected cholesterol and lipoprotein metabolism (Morais et al., 2011b). It is therefore likely that an elevated endocannabinoid tone in Atlantic salmon fed soybean oil stimulated hepatic *de novo* fatty acid synthesis (Paper III).

We did not find any effect on body weight or visceral somatic index (VSI) in salmon when soybean oil replaced fish oil for 6 months. Higher body weight has previously been reported in Atlantic salmon fed vegetable oils for 12 months (Torstensen et al., 2005, 2011), suggesting that longer feeding period (> 6 months) may be required in to detect differences in body weight and visceral fat stores in Atlantic salmon.

In the present study Atlantic salmon was fed soybean oil, one of the major dietary source of LA, resulting in 2.5 g LA / 100 g fillet and 0.7 g EPA + DHA / 100 g fillet. Thus replacing fish oil with soybean oil represents an extreme model in which vegetable oils are used as alternative lipid source in feed for Atlantic salmon (Table 4.1). Our findings of elevated liver endocannabinoid and lipid accumulation in Atlantic salmon fed soybean oil suggest that future fish oil replacement in farmed Atlantic salmon should pay attention to the choice of vegetable oils with regards to LA content.

Table 4.1: LA and EPA/DHA in a 200 g serving of farmed Atlantic salmon

Source of salmon	LA (en%) ¹	EPA/DHA (en%)
Norwegian farmed ²	1.4	1.9
Paper III (FO)	0.4	1.6
Paper III (SO)	2.3	0.6

¹ Based on a 2000 kcal diet, ² (NIFES, 2011b). No data available for wild salmon (NIFES, 2011b). Abbreviations:

FO salmon fed fish oil, SO salmon fed soybean oil.

4.3 Fish versus supplements - source of EPA and DHA

Supplementing the 8 en% LA diets with 1 en% EPA/DHA from fish oil prevented the increase in tissue AA -PL, liver anandamide and brain 2-AG levels, and reduced the adipogenic effect of the 8 en% LA diets (Paper I). Consistent with a

recent report (Ibrahim et al., 2011), we found that mice fed Atlantic salmon accumulated more adipose tissue and had higher leptin and insulin levels (Paper III) than mice fed a casein diet (Paper II), despite the lowest endocannabinoid levels in liver. Higher levels of EPA and DHA in salmon diets (2-3 en%) did not prevent the development of obesity as we observed by supplementation of 1 en% EPA/DHA from fish oil (Paper I). The salmon based diets promoted obesity (Paper III). The finding of higher adiposity in mice fed salmon compared to casein suggests an interacting effect between dietary fat and salmon protein in terms of weight gain, which is supported by the finding of higher body weight in mice fed salmon protein (defatted, no EPA and DHA) compared to whey -and casein protein (Haldis H. Lillefosse personal communication). Chronic long-term consumption of Atlantic salmon, soybean oil fed in particular (Paper III), resulted in higher liver (20-40% higher than Paper I and II) and muscle weights (Table 3.2). Higher organ weights of mice fed Atlantic salmon may result from higher ectopic fat deposition. Mice fed Atlantic salmon also had higher plasma levels of insulin (Paper III) compared to mice fed casein diets (Paper II) which may indicate insulin resistance and a diabetic phenotype (Ibrahim et al., 2011). Insulin resistance is associated with ectopic fat accumulation and increase the risk of developing type 2 diabetes (Gastaldelli, 2011). Insulin resistant adipocytes lose their ability to regulate endocannabinoid action (D'Eon et al., 2008; Di Marzo et al., 2009b), which may explain the exacerbated effects of the SO salmon diet compared to the FO salmon diet in terms of adipose tissue hypertrophy and inflammation (Paper III).

Enhanced fat absorption in mice fed Atlantic salmon (Ibrahim et al., 2011) may have lead to higher weight gain in mice fed Atlantic salmon (Paper III) compared to casein (Paper II). The macronutrient digestibility and bioavailability may be differentially affected by salmon and milk protein (Gilbert et al., 2011). Atlantic salmon provide a better amino acid balance than milk protein (casein), which is low in amino acids such as glycine and lysine and depleted of cysteine⁵. A different amino acid composition may influence the availability of macronutrients. In a diet with excess calories (Paper III) and limited physical activity higher bioavailability and macronutrient utilization from salmon protein may not be beneficial in terms of body weight. In humans, inclusion of fish (cod or salmon 3 x 150 g/week) contributed to additional weight loss compared to an isocaloric control diet in an energy restricted regime (Thorsdottir et al., 2007), and decreased inflammatory markers (Ramel et al., 2010). Daily fish intake in an energy restricted diet had beneficial effects on glucose, insulin and lipid metabolism (Mori et al., 1999). Cod protein protected against obesity-linked insulin resistance and glucose intolerance in rats fed a high fat/high sucrose diet (Lavigne et al., 2001),

⁵cystein was added to casein diets

and improved insulin sensitivity in insulin-resistant subjects (Ouellet et al., 2007, 2008) suggesting differential effect of different fish proteins. Epidemiological studies have shown that a Mediterranean type diet rich in fruits, vegetables and fish is health promoting and may facilitate weight maintenance (Shubair et al., 2005; Schulze et al., 2006) emphasizing the importance of a balanced diet. A long-term intake of a diet based solely on proteins from Atlantic salmon does not appear to be beneficial, at least in terms of weight management and hormonal levels.

4.4 Linoleic acid lowering to increase tissue EPA and DHA

We used the Lands equation (Lands et al., 1992) to predict AA -PL composition as the percent n-6 HUFA from dietary intakes of LA, ALA, AA and EPA/DHA, with good concurrence between calculated and experimental values in all papers (Table 3.1). As predicted, increasing dietary LA from 1 to 8 en% increased the percent of n-6 HUFA in RBC from 47% to 74% (Paper I) and 62% to 73% (Paper II), simultaneously decreasing the n-3 index from 12 to 4.5 (Paper I) and 9 to 5 (Paper II). The n-6 HUFA can be used as an indicator of disease risk as it models the relative amount of AA -PL available as precursors for eicosanoid derivatives of AA, and the proportion of n-6 in tissue HUFA is associated with CVD rates (Lands, 2003). An n-3 index of 8 or higher is associated with low risk of cardiovascular disease whereas 4 or lower increase CVD risk (Harris and von Schacky, 2004). It was recently stated that an n-3 index of 8-11, and/or tissue n-3 HUFA of more than 50% would protect 98% of the population from mental illness (Hibbeln et al., 2006; Global Omega-3 Summit, 2011).

Consistent with prior studies (Bourre et al., 1990a; Liou et al., 2007; Novak et al., 2008; Igarashi et al., 2009; Munakata et al., 2009; Guesnet et al., 2011) our results demonstrate that the elongation and desaturation of ALA to EPA and DHA, and/or the acylation of EPA and DHA into the sn-2 position of tissue phospholipids are considerably more effective when dietary LA is 1en%. Increasing dietary LA from 1 en% to 8 en% significantly reduced EPA in RBC, liver (Paper I, II and III) and eWAT (Paper II and III). A high dietary exposure of 8 en% LA from gestation caused a more pronounced reduction in DHA (liver; MF 32% and HF 18%, RBC; MF 30% and HF 27%, Paper I) compared to adulthood (liver; LF 18% and MF 5%, RBC; LF 8% and MF 12%, Paper II). Our results demonstrate that dietary fat cause more persistent changes when given in high doses early in life. In humans, the conversion of ALA to EPA and DHA is believed to be low, and ALA is not considered to be a significant source of EPA and DHA in the human diet

(Brenna et al., 2009). High dietary intake of LA is preventing the conversion of ALA to EPA and DHA as several human trials have demonstrated that sufficient lowering of dietary LA increase tissue levels of EPA and DHA (Clark et al., 1992; Liou et al., 2007; Munakata et al., 2009).

The aquaculture industry currently requires more than 1 kg wild fish raw material to produce 1 kg farmed salmon (FKD, 2009). Dietary guidelines recommend intakes of 4-10 en% n-6 PUFA (Table 2.1) which will require 1 - 2 g EPA/DHA per day to achieve 50% n-3 in tissue HUFA (Table 2.2), estimated to reduce the burden of diseases such as cardiovascular and mental illnesses (Hibbeln et al., 2006). Our data show that a dietary intake of 1 en% LA and 1 en% ALA (Paper I) results in 50% n-3 HUFA in tissue of mice without EPA and DHA supplementation. When dietary ALA was reduced from 1 en% (Paper I) to 0.5 en% (Paper II), the amount of n-3 HUFA in RBC decreased by 40%. However, the n-3 index was still ≥ 8 (Table 3.1, sufficient to give good cardiovascular protection (Harris and von Schacky, 2004). At least in rodents, reducing LA to increase EPA and DHA appears to be more efficient than supplementing DHA to high LA diet (Novak et al., 2008). Therefore, in order to elevate tissue EPA and DHA, emphasis should be on reducing dietary LA in addition to EPA/DHA supplementation.

4.5 Human relevance

There is dramatic progress in obesity research, but no safe and potent weight-reducing drugs are currently available. The discovery of leptin in 1994 was overshadowed by clinical studies finding that the fat-reducing effects of leptin were limited to a few patients with congenital leptin deficiency (Farooqi et al., 1999). The endocannabinoid system represents a new and promising target to reduce obesity. Pharmacological blockade of the CB1 receptor is effective in treating obesity and related metabolic derangements (Bensaid et al., 2003; Jbilo et al., 2005; Van Gaal et al., 2005; Després et al., 2005; Matias et al., 2006b; Christopoulou and Kiortsis, 2011). However serious psychiatric side effects, including suicidal risk (Christensen et al., 2007), caused marketplace withdrawal of rimonabant, a selective CB1 antagonist, and have impaired further pharmaceutical development. In lieu of a better anti-obesity drug, we here present a dietary approach to resolve an underlying cause of endocannabinoid hyperactivity. LA lowering may prove to be a safe and viable way to reduce obesity and associated metabolic disorders by reducing substrate availability for endocannabinoid synthesis.

4.5.1 Doses of dietary fatty acids

Dietary LA of 8 en% is based on current US intakes (Blasbalg et al., 2011), considered adequate by the Institute of Medicine of the US National Academy of Sciences (IOM, 2005) and within most dietary recommendations of 5-10 en% n-6 PUFA (see Table 2.1). The increasing use of soybean oil in the US has also increased the availability of ALA to 1 en% (Blasbalg et al., 2011). The doses of LA, ALA and EPA/DHA used in the individual papers underlying the present thesis are listed in Table 4.2 with the rationale for the dose used.

Table 4.2: Doses of fatty acids in Paper I, II and III

Paper	Fatty acid	Dose (en%)	Rationale
I, II	LA	1	evolutionary levels
		8	current US intake
III		1	present in FO salmon
		8	present in SO salmon
I	ALA	1	evolutionary levels
II		0.4	to match ALA in FO salmon
		0.9	to match ALA in SO salmon
I	EPA/DHA	1	to reduce tissue n-6 HUFA to levels from 1en% LA diet
III		2.7	level in FO salmon
		1.8	level in SO salmon

Abbreviations: en% percent energy, FO fish oil, HUFA highly unsaturated fatty acid (> 20 carbons), SO soybean oil.

In Paper I we calculated the amount of EPA and DHA needed in a 8 en% LA diet to obtain the same amount of n-6 HUFA (AA -PL) in RBC as a diet of 1 en% LA by using the Lands equation (Lands et al., 1992). The different levels of fatty acids were added to diets of 35 en% fat, the upper limit of recommended fat intake (Nasjonalt Råd for Ernæring, 2011; USDA, 2010). Dietary fat of 60 en% fat corresponds to a very high fat diet commonly used to induce obesity animal models.

In Paper II, the dietary fatty acid profile of LA and ALA was set up to match the fatty acid profile of Atlantic salmon raised on fish oil or soybean oil (Paper III) in a low fat (12.5 en%) and a medium fat (35 en%) diet.

In Paper III, replacing fish oil with soybean oil in feed for Atlantic salmon increased dietary LA from 1 en% to 10 en%, and dietary ALA from 0.4 to 1 en%, if salmon fillet was the sole source of dietary fat. A blend of oils were added to the salmon diets to increase dietary fat content to 35 en% and preserve the fatty acid profile of LA and ALA in fish fillets (Table 3.1).

4.5.2 Time-point of exposure to linoleic acid

Dietary LA appears to be more adipogenic, resulting in higher adiposity, when high amounts (8 en%) are introduced prenatally (Paper I), compared to adulthood.

Early exposure to high dietary levels of fat (35 and 60 en% fat) lead to persistent elevated endocannabinoid levels in peripheral tissue (Figure 3.2). Increased endocannabinoid levels were associated with higher adipose tissue accumulation and adiposity, conditions that were further aggravated when the diet contained 8 en% LA (Table 3.2). Our results support a recent study where exposure to a high fat diet in the developmental period exacerbated the effect of a high fat diet later in life (Bruce et al., 2009; Massiera et al., 2010). Changes occurring during development appears to be persistent and affect disease outcome later in life (Bruce et al., 2009). A defective leptin signaling caused by a diet of 8 en% LA may lead to a chronic state of perceived starvation (Friedman and Halaas, 1998). In addition an altered nutrient preference and increased motivation to eat arising from a chronically elevated endocannabinoid system following a high fat/high LA maternal diet may predispose children to obesity and associated metabolic disorders and/or accelerate the development of obesity and increase vulnerability to develop metabolic complications as adults.

Presently in the United States nearly 2/3 of the adult population is at least overweight (Flegal et al., 2002), many of which are diet-induced. This results in a situation where the majority of pregnancies are under conditions of less than optimal health (i.e. overweight and eating a high fat/high LA diet). There is a risk that these conditions imprint a metabolic condition onto the offspring thereby pushing the set point for body weight and adiposity higher. This could cause the next generation to be more metabolically efficient, making it harder to maintain a healthy phenotype (Grove et al., 2005). Overall, the results presented here point out the importance of a balanced diet, especially in maternal and childhood diets, with emphasis on n-3 and n-6 intakes.

Conclusions

He who knows best knows how little he knows. - Thomas Jefferson

The work presented in this PhD shows that:

- Compared to 1 en% LA, dietary LA of 8 en% increased tissue levels of AA in phospholipids and elevated 2-AG and anandamide in liver associated with higher feed efficiency, increased weight gain and adipose tissue accumulation and inflammation independent of amount of dietary fat.
- Mice prenatally exposed to 8 en% LA accumulated more adipose tissue resulting in higher adiposity index and higher leptin levels than mice exposed to 8 en% LA from young adulthood.
- High dietary levels of LA increased endocannabinoid levels in liver of Atlantic salmon. Mice fed Atlantic salmon raised on soybean oil had higher 2-AG and lower EPA and DHA concentrations in liver, gained more weight and had more adipose tissue inflammation than mice fed Atlantic salmon raised on fish oil.

We believe that elevating dietary LA from 1 en% to 8 en%, and current dietary advices regarding n-6 PUFA, contributes to the obesity epidemic by:

- Increasing AA and lowering EPA and DHA in the phospholipid pool.
- Elevating endocannabinoid activity.
- Higher caloric intake and feed efficiency.
- Inducing weight gain and adiposity.
- Adipose tissue hypertrophy.
- Increasing adipose tissue inflammation.
- Of note, a 60 en% fat diet did not induce obesity when dietary LA was 1 en%.

Future directions

The more that you read the more you will know. The more that you learn the more places you'll go! - Dr. Seuss

Human intervention trial

Dietary guidelines recommend replacing saturated fat with vegetable oils and having an n-6 PUFA/LA intake of 5-10 en% (Table 2.1). A diet of 12.5 and 35 en% fat (within the general dietary guidelines of total dietary fat (20-35 en% fat) with 9 en% PUFA (LA:ALA ratio of 8:1, Paper I, and 8:0.5, Paper II and III) elevated endocannabinoid activity, increased weight gain and adiposity, and caused adipose tissue hypertrophy and inflammation. Based on our results from modeling the ecological changes in dietary LA in mice, we hypothesize that 8 en% LA, with a low intake of n-3 PUFA, will induce endocannabinoid hyperactivity and may substantially increase the risk of metabolic disorders and obesity in humans. We acknowledge that a positive correlation between increasing intake of soybean oil, the major dietary source of LA, and the rise in the prevalence of obesity in the US during the 20th century and our animal data are not sufficient to explain causality. It is therefore of great importance to conduct a long-term human dietary intervention trial with intakes of 1 en% LA and 8 en% LA to translate the animal data and confirm the causal role of high dietary LA intakes to endocannabinoid hyperactivity and obesity in humans. Reducing dietary LA from 8 en% to 1 en% in overweight subject may prove the safe and favorable effect of lowering dietary LA to diminish endocannabinoid hyperactivity to reduce obesity and associated metabolic disorders. Such a dietary approach will have widespread beneficial health implications related to obesity and metabolic syndrome. In addition reducing dietary LA to less than 2 en% is likely to increase the conversion of ALA to EPA and DHA in human tissue to reduce the global burden of chronic diseases (Hibbeln et al., 2006).

Dietary fat, carbohydrates and endocannabinoid hyperactivity

Dietary recommendations emphasizing the replacement of saturated fat with PUFA and increase the intake of complex carbohydrate turn many people to fat-free products which often replace fats with sugar and refined carbohydrates.

The background diet affects the adipogenic effect of both n-3 and n-6 fatty acids (Madsen et al., 2008; Ma et al., 2011). A high carbohydrate diet elevate insulin levels which can induce *de novo* fatty acid synthesis and expression of SREBP-1c (Madsen and Kristiansen, 2010). The type of carbohydrates has different effect on insulin secretion. Carbohydrates with a high glycemic index, such as sucrose, increase the adipogenic effect of fat more than carbohydrates with low glycemic index, such as starch (personal communication master thesis Ragnhild Jarlsby). Hence dietary changes in insulin and cAMP signaling might translate into different effects on adipose tissue growth (Madsen and Kristiansen, 2010).

Cyclic AMP is an important second messenger in regulation of adipose tissue metabolism where it can increase lipolysis and induce the expression of UCP1 (Madsen and Kristiansen, 2010). UCP1 is exclusively expressed by brown adipocytes (Nicholls and Rial, 1999) where it regulates energy expenditure by increasing thermogenesis (Cannon and Nedergaard, 2004). Pharmacological blockage of the CB1 receptor by rimonabant increase cAMP, induce the transdifferentiation of white adipocytes to a brown phenotype and increase the expression of UCP1 which may increase and/or prevent weight loss. The work from this thesis has generated a platform from which we can study the molecular and mechanistic effects of dietary LA and endocannabinoid hyperactivity and energy expenditure. We hypothesize that a low LA diet of 1 en% will lower CB1 activity and induce the transdifferentiation of white adipocytes to a brown phenotype thus increasing energy expenditure by elevating UCP1 levels. Lower CB1 activity is likely to improve metabolic functions associated with obesity, but it remains to be elucidated if the effect of LA on CB1 activity is dependent on source and amount of dietary carbohydrates (Figure 4.4).

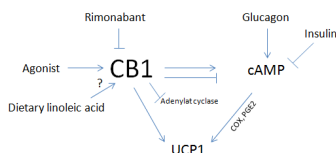


Figure 4.4: Pharmacological stimulation of the cannabinoid receptor CB1 influence cAMP and UCP1. Stimulation of CB1 inhibits adenylate cyclase and decrease cAMP levels. Antagonizing CB1 by rimonabant increases the expression of UCP1 thereby increasing energy expenditure. cAMP levels are influenced by the dietary protein/carbohydrate ratio. High dietary protein increase cAMP levels and induce the expression of UCP1 in a COX and PGE₂ dependent fashion.

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