Mapping the diverse functions of dietary fatty acids via target gene regulation

Anastasia Georgiadi

Thesis committee

Thesis supervisors

Prof.dr. Michael R. Müller
Professor of Nutrition, Metabolism and Genomics
Wageningen University

Prof.dr.ir. (Sander) A.H. Kersten
Personal chair at the Division of Human Nutrition
Wageningen University

Other members

Prof. dr .ir. Jaap Keijer
Wageningen University

Prof.dr. Matthijs K.C. Hesselink
Maastricht University

Prof.dr. Esther Lutgens
University of Amsterdam

Dr. Sander M. Houten
University of Amsterdam

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

Mapping the diverse functions of dietary fatty acids via target gene regulation

Anastasia Georgiadi

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 22 May 2012
at 1:30 p.m. in the Aula.

Anastasia Georgiadi

Mapping the diverse functions of dietary fatty acids via target gene regulation.

Thesis, Wageningen University, Wageningen, The Netherlands, 2012

With references, with summary in English and Dutch

ISBN: 978-94-6173-280-4

CONTENTS

Chapter 1

General Introduction

Dietary fat is a strong predictor of chronic diseases, such as cardiovascular diseases, obesity, diabetes, dyslipidemia and metabolic syndrome [1-2]. A great number of epidemiological and observational studies clearly show that in addition to the amount of fat consumed in a diet, fat composition is an equally important factor in the development of chronic diseases. Evidence abounds indicating that adherence to a diet with high content of polyunsaturated (PUFAs) and monounsaturated (MUFAs) fatty acids such as the Mediterranean diet has substantial health benefits [3-6], while diets with high content of saturated fatty acids (SFAs) such as the Western type diet increase the risk for the development of several chronic diseases [7-9]. Nutrition science has traditionally focused on the physiological aspects of food and epidemiological studies on the relation between diet and disease risk. This approach led to the generation of a great number of dietary guidelines, which are aimed at disease prevention rather than cure. Despite specific guidelines on consumption of certain nutrients, especially dietary fats, the rates of diet related diseases remain high [10,11], leading to the search of more efficient approaches of disease prevention. In response to this need, interest has grown into understanding the molecular mechanisms underlying the diverse effects of food components, which has been the basis for the appearance of the science of molecular nutrition and nutrigenomics.

Nutrigenomics

Nutritional genomics or nutrigenomics investigates the interaction between nutrients and genes at the molecular level by using genomic tools. The advent of high throughput technology led to the emergence of a novel field generally referred to as genomics, which includes transcriptomics, proteomics and metabolomics. Currently, transcriptomics is the most developed and feasible tool used in nutrigenomics research. Transcriptomics is extensively used to measure global changes in mRNA level (the transcriptome) of cell or tissue in response to external stimuli such as nutrients, pharmacological compounds or certain diets or diseases. Taking advantage of that technology, nutrigenomics research aims to provide a clear mechanistic framework that links uptake of specific nutrients such as fatty acids to specific biological pathways and disease process. To raise our understanding on food-metabolism interactions above

purely descriptive type of information, it is important to carry out studies on mutant mice or in cell cultures, in order to identify molecular targets of nutrients [12].

As a part of nutrition research, nutrigenomics approaches have focused on understanding the early stages of disease development, as a response to consumption of a certain diet or certain food compounds. One of the main goals is to distinguish healthy individuals from those that are in a pre-diseased or diseased stage, based on information derived from gene or protein expression levels. Nutrigenomics research in humans focuses on tissues that are relatively easily accessible, including blood cells, adipose tissue and skeletal muscle. This approach is anticipated to produce novel and more sensitive markers of disease onset compared to those currently used in disease prevention. In such a way, increasing our understanding on the transition from pre-disease to the disease stage may allow early intervention that can restore health [13].

PPARα – major nutritional sensor of fatty acids

Within the field of nutrigenomics, dietary nutrients and their metabolites are seen as signaling molecules that target specific cellular response systems. An important set of signaling molecules in our diet are fatty acids. An important mechanism of action of fatty acids is via stimulation or inhibition of DNA transcription. The family of nuclear receptors represents an extensively characterized group of transcription factors that is involved in mediating the cellular responses of fatty acids [14,15]. Among them, the Peroxisome Proliferator Activated Receptors (PPARs) perhaps comprise the best recognized sensor system for fatty acids. PPARs are nuclear receptors that physically bind fatty acids and other lipophilic compounds. Ligand binding triggers a series of processes that include recruitment of specific coactivator proteins, leading to induction or inhibition of the expression of PPAR target genes. Investigating the type of target genes and their biological role has substantially improved our knowledge on the function of PPARs in different tissues. Three different subtypes of PPARs have been cloned, each characterized by an unique tissue expression pattern. PPARα shows the highest expression in tissues with high oxidative capacity, such as brown adipose tissue, cardiac muscle, skeletal muscle and liver. PPARβ/δ is found in many cell types, while PPARγ expression is more restricted in adipocytes and macrophages. **Chapter 1**

provides an extensive review on fatty acid sensing, explaining the role of Peroxisome Proliferator Activated receptors and other fatty acid sensors in fat recognition and regulation of fatty acid target genes.

Heart, a highly oxidative muscle

The mammalian heart relies highly on fat oxidation for covering its energy demands. The main site of fat oxidation is the cardiomyocyte, which covers 75% of the total number of cells in the heart. Under resting conditions fat oxidation covers up to 70% of cardiac energy demands and the remainder is covered by glucose utilization (20%-30%) [16,17]. In order to maintain the high rates of fat oxidation the heart can use a variety of metabolic fuels and its preference is determined to a large extent by the body's metabolic state. After a meal heart takes up most of the fat in the form of chylomicron triglycerides, that are formed in the small intestine [18]. Chylomicron triglycerides are lipolysed via lipoprotein lipase activity to provide the heart with free fatty acids (FFAs). A small part of FFAs is also derived from albumin-bound FFA, that are taken up by the heart without the intermediate role of LPL. Finally, in case of nutritional deprivation the heart can catabolize ketones, amino acids or lactate [19].

High rates of mitochondrial oxidation and oxygen utilization are coupled with enzymatic and non-enzymatic mechanisms, aiming to counterbalance the production of highly reactive secondary products of the respiratory chain, the reactive oxygen species (ROS) [20]. Among the best characterized enzymatic mechanisms are the catalase and glutathione peroxidase, superoxide dismutase (SODs), thioredoxin and thioredoxin reductase. Non-enzymatic mechanisms include the intracellular antioxidants, such as Vitamin E, C and beta carotene, ubiquinone, lipoic acid and urate [21]. ROS can play an important role in cardiac inflammation and their unbalanced production may lead to damage of cell membrane, organelle structures, DNA and peroxidation of lipids giving rise to lipotoxic metabolites, such as ceramide. Additionally, ROS have been described to directly regulate specific transcription factors involved in inflammation, such as NFkB and Nrf2 [22,23]. Under conditions of increased lipids uptake by the heart, such as chronic high fat diet or insulin resistance, increased rates of fatty acid oxidation, in combination with uncontrolled production of ROS and lipid intermediates may result in mitochondrial

malfunctioning and lipid accumulation [24]. Myocardial lipotoxicity refers to the accumulation of intramyocardial lipids and is associated with contractile dysfunction and even myocyte death [25].

Previous studies have suggested that PPARα plays a central role in cardiac function. At initial stages of cardiomyopathy increased PPARα activity results in upregulation of beta oxidation genes and ROS production, whereas at later stages PPARα activity decreases, leading to mitochondrial malfunction and lipid accumulation [26-28]. Since the heart takes up substantial amounts of dietary fat, we set out to investigate the direct transcriptional targets of dietary lipids in the healthy heart. In **chapter 2** we provide a detailed description of target genes and metabolic pathways that are regulated acutely after an oral gavage of triglyceride consisting of one type of fatty acid (C22:6, C18:3, C18:1 or C18:1). In addition, the role of PPARα is investigated by conducting the experiment in PPARα -/- mice. In **chapter 3,** we describe in depth a fatty acid induced mechanism that serves to inhibit the LPL dependent uptake of fatty acids and thereby protect against cardiomyocyte lipotoxicity.

LPL- Angptl4 axis and its role in regulation of lipid uptake

Plasma levels of lipoproteins are considered a risk factor for atherosclerosis and coronary heart disease. Elevated levels of low-density lipoprotein (LDL) have been shown to increase the risk for atherosclerosis developement, while high levels of high density lipoprotein (HDL) are considered to be protective. In recent years plasma triglycerides (TG) are increasingly recognized as an independent factor for cardiovascular disease (CVD). Triglycerides (TGs) circulate in the plasma in the form of VLDL remnants and chylomicrons. VLDL remnants derive from the liver, whereas chylomicrons derive from the small intestine. Thus, levels of TGs in the plasma depend on the rate of production of TG-rich lipoproteins in the small intestine and liver, and their clearance in skeletal muscle, heart and adipose tissue. Clearance of plasma triglycerides is catalyzed by lipoprotein lipase (LPL), which is anchored to the capillary endothelium via heparin sulphate proteoglycans and the protein GPIHBP1. For an extended review on the function and regulation of LPL activity the reader is referred to the review of Lichtenstein L. and Kersten S.; BBA;2010 [29]. Here we briefly describe the regulation of LPL by Angptl4, a target gene of PPARs and a sensitive fatty acid target.

Angptl4 is a secreted protein of size about 50KDa. It belongs to the family of fibrinogen/angiopoietin like proteins that includes Angptl1, Angptl2, Angptl3, and Angptl6. Similar to other angiopoietin-like proteins, Angptl4 is divided into distinct regions, which include a N-terminal signal sequence, a unique sequence, a coiled-coil domain and a large fibrinogen/angiopoietin-like domain. During protein maturation Angptl4 is cleaved to release an N-terminal and C-terminal fragment. Angptl4 is now well accepted as a potent inhibitor of LPL activity. It has been shown *in vivo* and *in vitro* that the inhibitory role of Angptl4 on LPL activity is mediated by the N-terminal domain, which favors the formation of inactive LPL monomers at the expense of active LPL dimers [30,31]. Both Angptl4 and LPL are expressed in several tissues and cell types, such as liver, skeletal muscle, adipose tissue, heart, small intestine, and macrophages. Angptl4 was originally cloned as target gene of PPARs and is highly upregulated by fatty acids. Apart from being a potent inhibitor of LPL activity, Angptl4 has been reported to have angiogenic and wound healing functions [32,33]. The C-terminal part of the protein, whose role is less understood compared to the N-terminal domain, seems to be involved in these functions.

Outline of this thesis

In **Chapter 2** we provide an overview of existing knowledge on gene regulation by fatty acids. The objective of this thesis was to provide a comprehensive analysis of gene regulation by dietary polyunsaturated fatty acids. We chose heart as the most relevant organ to investigate this question, because after a meal heart preferentially utilizes fatty acids released via LPL-mediated lipolysis of chylomicron triglycerides. **Chapter 3** is a transcriptomics study investigating the effect of different PUFAs on cardiac gene expression. We take advantage of a unique experimental design in which mice are fed a single bolus of dietary triglycerides consisting of one type of fatty acid. In addition, the role of PPARα, previously described as the master regulator of lipid homeostasis in the heart is explored.

In **Chapter 3,** we identify Angptl4 as the gene most strongly induced by PUFAs in the heart. Based on previous knowledge on the inhibitory role of Angptl4 on LPL activity, we hypothesized that increased influx of PUFA in the heart may result in upregulation of Angptl4, in order to inhibit LPL dependent

release of FFAs, thereby protecting against the toxic consequences of increased fat influx. In **Chapter 4**, we investigated in depth the transcriptional regulation of this mechanism and its contribution to regulate oxidative stress and prevent lipotoxicity in the heart.

LPL is anchored to the endothelium via heparin sulphate proteoglycans and its activity determines the plasma levels of triglycerides. Increased triglyceride levels are considered a risk factor for atherosclerosis. Therefore, in **Chapter 5** we investigate the role of Angptl4 in atherosclerosis development, using a model of whole body Angptl4 overexpression on a atherosclerosis-prone ApoE3Leiden background.

Macrophages are central players in atherosclerosis development. They function primarily as scavengers of lipids and modified lipids and excessive lipid accumulation leads to the formation of pro-inflammatory foam cells. In **Chapter 6,** we describe the identification and initial characterization of the gene Hig-2, which is sensitive to fatty acids uptake in macrophages. Finally, conclusions and suggestions deriving from this thesis are discussed in **Chapter 7**.

References

1. Kuller, L.H., Dietary fat and chronic diseases: epidemiologic overview. J Am Diet Assoc, 1997. 97(7 Suppl): p. S9-15.
2. Weisburger, J.H., Dietary fat and risk of chronic disease: mechanistic insights from experimental studies. J Am Diet Assoc, 1997. 97(7 Suppl): p. S16-23.
3. Knoops, K.T., et al., Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project. JAMA, 2004. 292(12): p. 1433-9.
4. Babio, N., M. Bullo, and J. Salas-Salvado, Mediterranean diet and metabolic syndrome: the evidence. Public Health Nutr, 2009. 12(9A): p. 1607-17.
5. Panagiotakos, D.B., et al., The current Greek diet and the omega-6/omega-3 balance: the Mediterranean diet score is inversely associated with the omega-6/omega-3 ratio. World Rev Nutr Diet, 2011. 102: p. 53-6.
6. Pauwels, E.K., The protective effect of the Mediterranean diet: focus on cancer and cardiovascular risk. Med Princ Pract, 2011. 20(2): p. 103-11.
7. van Dijk, S.J., et al., A saturated fatty acid-rich diet induces an obesity-linked proinflammatory gene expression profile in adipose tissue of subjects at risk of metabolic syndrome. Am J Clin Nutr, 2009. 90(6): p. 1656-64.
8. Hooper, L., et al., Reduced or modified dietary fat for preventing cardiovascular disease. Cochrane Database Syst Rev, 2011(7): p. CD002137.
9. Odermatt, A., The Western-style diet: a major risk factor for impaired kidney function and chronic kidney disease. Am J Physiol Renal Physiol, 2011. 301(5): p. F919-31.
10. York, D.A., et al., Prevention Conference VII: Obesity, a worldwide epidemic related to heart disease and stroke: Group I: worldwide demographics of obesity. Circulation, 2004. 110(18): p. e463-70.
11. Ahima, R.S., Digging deeper into obesity. J Clin Invest, 2011. 121(6): p. 2076-9.
12. Muller, M. and S. Kersten, Nutrigenomics: goals and strategies. Nat Rev Genet, 2003. 4(4): p. 315-22.
13. Afman, L. and M. Muller, Nutrigenomics: from molecular nutrition to prevention of disease. J Am Diet Assoc, 2006. 106(4): p. 569-76.
14. Mangelsdorf, D.J., et al., The nuclear receptor superfamily: the second decade. Cell, 1995. 83(6): p. 835-9.
15. Kersten, S., B. Desvergne, and W. Wahli, Roles of PPARs in health and disease. Nature, 2000. 405(6785): p. 421-4.
16. van der Vusse, G.J., et al., Fatty acid homeostasis in the normoxic and ischemic heart. Physiol Rev, 1992. 72(4): p. 881-940.

17. Taegtmeyer, H., R. Hems, and H.A. Krebs, Utilization of energy-providing substrates in the isolated working rat heart. Biochem J, 1980. 186(3): p. 701-11.
18. Teusink, B., et al., Contribution of fatty acids released from lipolysis of plasma triglycerides to total plasma fatty acid flux and tissue-specific fatty acid uptake. Diabetes, 2003. 52(3): p. 614-20.
19. Augustus, A.S., et al., Routes of FA delivery to cardiac muscle: modulation of lipoprotein lipolysis alters uptake of TG-derived FA. Am J Physiol Endocrinol Metab, 2003. 284(2): p. E331-9.
20. Giordano, F.J., Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Invest, 2005. 115(3): p. 500-8.
21. Nordberg, J. and E.S. Arner, Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med, 2001. 31(11): p. 1287-312.
22. Michiels, C., et al., Regulation of gene expression by oxygen: NF-kappaB and HIF-1, two extremes. Free Radic Biol Med, 2002. 33(9): p. 1231-42.
23. Sabri, A., H.H. Hughie, and P.A. Lucchesi, Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. Antioxid Redox Signal, 2003. 5(6): p. 731-40.
24. Park, T.S., et al., Lipids in the heart: a source of fuel and a source of toxins. Curr Opin Lipidol, 2007. 18(3): p. 277-82.
25. Wende, A.R. and Abel E.D., Lipotoxicity in the heart. Biochim Biophys Acta, 2010. 1801(3): p. 311-9.
26. Banke, N.H., et al., Preferential oxidation of triacylglyceride-derived fatty acids in heart is augmented by the nuclear receptor PPARα . Circ Res, 2010. 107(2): p. 233-41.
27. Finck, B.N. and D.P. Kelly, Peroxisome proliferator-activated receptor alpha (PPARα) signaling in the gene regulatory control of energy metabolism in the normal and diseased heart. J Mol Cell Cardiol, 2002. 34(10): p. 1249-57.
28. Lehman, J.J. and D.P. Kelly, Transcriptional activation of energy metabolic switches in the developing and hypertrophied heart. Clin Exp Pharmacol Physiol, 2002. 29(4): p. 339-45.
29. Lichtenstein, L. and S. Kersten, Modulation of plasma TG lipolysis by Angiopoietin-like proteins and GPIHBP1. Biochim Biophys Acta, 2010. 1801(4): p. 415-20.
30. Sukonina, V., et al., Angiopoietin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. Proc Natl Acad Sci U S A, 2006. 103(46): p. 17450-5.
31. Lichtenstein, L., et al., Angptl4 upregulates cholesterol synthesis in liver via inhibition of LPL- and HL-dependent hepatic cholesterol uptake. Arterioscler Thromb Vasc Biol, 2007. 27(11): p. 2420-7.

32. Goh, Y.Y., et al., Angiopoietin-like 4 interacts with matrix proteins to modulate wound healing. J Biol Chem, 2010. 285(43): p. 32999-3009.
33. Yang, Y.H., et al., Suppression of the Raf/MEK/ERK signaling cascade and inhibition of angiogenesis by the carboxyl terminus of angiopoietin-like protein 4. Arterioscler Thromb Vasc Biol, 2008. 28(5): p. 835-40.

Chapter 2

Mechanisms of gene regulation by fatty acids

Anastasia Georgiadi and Sander Kersten

Chapter published in Advances in Nutrition, March 2012 1;3(2): 127-134

Abstract:

Consumption of specific dietary fatty acids has been shown to influence risk and progression of several chronic diseases, such as cardiovascular disease, obesity, cancer, and arthritis. In recent years, insights into the mechanisms underlying the biological effects of fatty acids have improved considerably and have provided the foundation for the emerging concept of fatty acid sensing, which can be interpreted as the property of fatty acids to influence biological processes by serving as signalling molecules. An important mechanism of fatty acid sensing is via stimulation or inhibition of DNA transcription. Here, we will focus on fatty acid sensing via regulation of gene transcription and address the role of Peroxisome Proliferator Activated receptors, Sterol Regulatory Element Binding Protein 1, Toll-like receptor 4, G-protein coupled receptors, and other putative mediators.

Introduction

Consumption of specific dietary fatty acids has been shown to impact risk for a wide range of chronic diseases. What traditionally has been lacking is a clear mechanistic framework that links uptake of specific lipids to a biological pathway and disease process. Such a molecular framework should accommodate the often differential effects of fatty acids differing in chain length and saturation on numerous biological parameters. In recent years, insights into the mechanisms underlying the biological effects of fatty acids have progressed rapidly, partly thanks to the widespread use of in vivo and in vitro gene targeting, and have provided the foundation for the emerging concept of fatty acid sensing. Fatty acid sensing can be interpreted as the property of fatty acids to influence biological processes by serving as signalling molecules. While it is well established that fatty acid derivatives such as eicosanoids have a major signalling function, there is convincing evidence that fatty acids themselves also carry this property. Part of this regulation occurs via regulation of gene transcription, which is the topic of this review.

Trafficking and cellular sensing of dietary fat

Every day our body processes an amount of fat equivalent to almost half a cup. In the intestine, dietary triglycerides are first hydrolyzed into fatty acids and monoglycerides that together with bile acids associate into micelles in the intestinal lumen. After being taken up into enterocytes, fatty acids are reesterified into triglycerides (TG) and secreted as part of chylomicrons, initially to the intestinal lymph vessels and from there on into the blood circulation. The increase in circulating chylomicrons after a meal gives rise to the post-prandial peak in plasma triglycerides. The time-course and magnitude of the plasma triglyceride peak may differ between individuals and is elevated in obese and diabetic subjects, giving rise to post prandial lipaemia. Plasma chylomicrons undergo rapid lipolytic processing via the action of lipoprotein lipase (LPL) anchored to the capillary endothelium, leading to the release of fatty acids and their subsequent uptake into the underlying tissue [1].

One of the major sinks for meal-derived fatty acids is the adipose tissue, which acquires most of the absorbed fatty acids via elevated local LPL activity. Other tissues that substantially contribute to post-prandial clearance of

chylomicron-TG are skeletal muscle, heart, and, after conversion to chylomicron remnants, the liver [2]. In contrast to plasma TG, circulating levels of adipose tissue derived non-esterified free fatty acids decrease rapidly after a mixed meal and again increase at the end of the post prandial period. A significant portion of circulating FFAs are taken up by the liver, where together with remnant-derived fatty acids and fatty acids produced via de novo lipogenesis they form the substrate for (re-)esterification and subsequent secretion into the plasma as VLDL-TG. Depending on the tissue and feeding status, either plasma FFA or TG-derived fatty acids comprise the major share of fatty acids for tissue uptake [2]. Irrespective of the specific route of delivery, it is evident that the rate of fatty acid uptake into many tissues is very variable and influenced by numerous factors, including tissue metabolic activity, feeding status, fat intake, and the intake of other nutrients, especially carbohydrates. Furthermore, circulating concentrations and tissues fluxes of FFA and TG-derived fatty acids are often altered during obesity, type 2 diabetes or other metabolic disturbances.

A number of proteins are involved in cellular uptake of FFAs, including CD36 and various FATPs [3]. After uptake, fatty acids are bound by fatty acid binding proteins (FABPs) and can undergo a number of metabolic fates including oxidation in mitochondria and esterification and storage in lipid droplets. In addition, fatty acids can serve as signaling molecule by impacting intra- and extra-cellular receptor sensor systems either directly or after conversion to specific fatty acid derivatives. An example of these lipid sensors are the nuclear receptors, which mediate activation of gene transcription by a variety of hydrophobic compounds, including retinoic acid, steroid hormones, oxysterols and bile acids [4]. This review will provide an overview of our current knowledge on the various cellular receptor systems enabling the cell to sense the intra- or extracellular fatty acid concentration and respond by altering gene transcription.

Peroxisome Proliferator Activated Receptors

The Peroxisome Proliferator Activated Receptors (PPARs) perhaps comprise the best recognized sensor system for fatty acids (Figure 1). PPARs are transcription factors that are members of the superfamily of nuclear hormone receptors, which also include receptors for fat soluble vitamins A and D and

steroid hormones [5]. Nuclear receptors function as ligand-activated transcription factors by binding small lipophilic molecules. They share a modular structure consisting of a DNA- and ligand-binding domain and play a role in a numerous biological processes [6]. Three different PPARs subtypes have been cloned, each characterized by a unique tissue expression pattern. PPARα (Nr1c1) is found in many tissues but is predominant in oxidative tissues such as brown adipose tissue, cardiac muscle, skeletal muscle and liver. PPARβ/δ (Nr1c2) is found in many cell types, while PPARγ (Nr1c3) expression is more restricted with adipocytes and macrophages expressing the highest level [7,8]. Binding of ligand is believed to trigger the physical association of PPARs to specific DNA sequences called PPAR response elements in and around target genes. Additionally, ligand binding leads to recruitment of co-activator proteins and loss of co-repressor proteins, resulting in activation of DNA transcription [5]. Similar to many other nuclear receptors, PPARs bind to DNA as heterodimer with the nuclear receptor RXR, which binds the vitamin A derivative 9-cis retinoic acid.

PPARs serve as a receptor for structurally diverse compounds. Although substantial specificity towards one particular PPAR subtype has been achieved in the design of synthetic PPAR agonists, there seems to be comparatively little subtype specificity among endogenous PPAR agonists. In several landmark papers from the 1990's it was demonstrated that all three PPARs are able to bind fatty acids with a general preference towards long chain polyunsaturated fatty acids (PUFAs) [9-13]. Subsequent studies using a variety of biochemical techniques have firmly corroborated the direct physical association between fatty acids and PPARs and have thus established fatty acids as bona fide PPAR ligands [14-18]. In addition, numerous fatty acid-derived compounds and compounds showing structural resemblance to fatty acids, including acyl-CoAs, oxidized fatty acids (9(S)-HODE, 13(S)-HODE), eicosanoids, endocannabinoids, and phytanic acid, have been shown to activate PPARs (19-26). Whereas the eicosanoid 15-Deoxy-Delta-12,14-prostaglandin J2 behaves as a specific high affinity agonist for PPARγ, (8S)-hydroxyeicosatetraenoic acid and prostacylin PGI2 show preference for PPARα and PPARδ, respectively [9, 27-29]. Since the intracellular concentration of fatty acids (free and bound to fatty acid binding proteins) far exceeds the intracellular concentration of eicosanoids and other endogenous PPAR agonists, and since fatty acids are able to bind PPARs with high affinity, the question can be raised to what extent

eicosanoids and other fatty acid-derived compounds substantially contribute to the activation of PPARs in vivo. Rather, it can be argued that PPARs serve as general fatty acid sensors with comparatively limited ligand specificity. However, this concept is not universally embraced, and has clearly not stopped the quest to identify the potentially elusive single true endogenous PPAR ligand. Recently, Semenkovich and colleagues identified the phosphatidylcholine 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine as the lipid compound likely responsible for the activation of PPARα in mice carrying a targeted deletion of the fatty acid synthase gene [30]. Since phosphatidylcholines are abundant in any cell, it is unclear how activation of PPARα by 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine fits into the notion of PPARα being a lipid sensor that responds to changes in metabolic status and lipid fluxes.

As discussed above, dietary fatty acids mostly enter the liver as TG within chylomicron remnants, and are liberated after degradation of the remnant particles by hepatic and lysosomal lipase. It has been shown that PPARα is dominant in mediating the effects of dietary fatty acids on hepatic gene expression, including many genes involved in fatty acid catabolism, as revealed by experiments in which wildtype and PPARα -/- mice were provided with a single oral bolus of synthetic TG consisting of one type of fatty acid [17]. Lipolysis of circulating lipoproteins, whether hydrolysis of HDL by endothelial lipase or lipolysis of VLDL by lipoprotein lipase, was shown to be an important mechanism for generating ligands for PPARα in endothelial cells [31, 32], while hydrolysis of VLDL by hepatic lipase and lipoprotein lipase was shown to provide ligands for PPARβ/δ in hepatocytes and macrophages, respectively [33, 34].

In contrast and very surprisingly, circulating FFA, which primarily originate from adipose tissue lipolysis, do not seem to be able to activate PPARα, at least in the liver [35, 36]. The precise mechanism behind the differential effect of circulating FFA (“old fat”) versus dietary and endogenously synthesized fatty acids (“new fat”) on hepatic PPARα activation remain unclear but may be related to existence of distinct intracellular fatty acids pools with distinct metabolic and signaling properties [35]. In contrast, hepatic PPARβ/δ can be activated by plasma FFA (36), and likely the same is true in skeletal muscle, as revealed by the stimulatory effect of elevated FFA on expression of PPARβ/δ target Angptl4 in skeletal muscle [37, 38].

Interestingly, it was recently proposed that in the mouse heart, PPARα-mediated gene transcription requires the prior esterification of fatty acids and subsequent hydrolysis catalyzed by Adipose Triglyceride Lipase (ATGL) [39]. Conversion to TG and subsequent lipolysis seems to be necessary in order for fatty acids to become active signaling lipids, but it is unclear whether the specific routing of fatty acids leads to the formation of a specific high affinity ligand or feeds a distinct intracellular fatty acid pool. In contrast, evidence was also provided that in liver ATGL promotes PPARα activity independently of ligand-induced activation [40].

PPARα acts as a master regulator of hepatic lipid catabolism by inducing the expression of numerous genes involved in mitochondrial and peroxisomal fatty acid oxidation, as well as other lipid related pathways, inflammatory pathways, and glucose metabolism [41]. Accordingly, it can be argued that activation of PPARα by fatty acids in liver and heart is part of a feed-forward mechanism aimed at promoting oxidation of incoming fuels and thereby preventing the intracellular accumulation and consequent lipotoxicity of fatty acids by stimulating their oxidation. A similar role can be envisioned for PPARβ/δ in skeletal muscle. Besides via stimulation of fatty acid oxidation and possibly by stimulating conversion of fatty acids into triglycerides [41], activation of PPAR by fatty acids may protect against lipotoxicity by inhibiting LPL-dependent hydrolysis of circulating TG and consequent uptake of fatty acids via induction of the LPL-inhibitor Angptl4 [42].

The role of PPARs in gene regulation by fatty acids is less clear in adipose tissue. Marine oil fatty acids have major effects on adipose tissue function and metabolism, as well as on adipose tissue gene regulation [43]. Although PUFAs are direct agonists for PPARγ [12], it is unclear to what extent the observed changes in adipose gene expression upon chronic PUFA feeding reflect direct ligand-activation of PPARγ or other PPARs, or are secondary effects conferred by specific eicosanoids or other fatty acid-derived compounds. Activation of PPARγ by fatty acids may be aimed at promoting conversion of incoming fatty acids to TG and stimulating overall TG storage capacity, thereby protecting against lipotoxicity.

Sterol-regulatory element binding protein 1

Dietary PUFAs suppress hepatic expression of genes involved in fatty acid synthesis (Figure 1). The underlying mechanism involves a member of the family of basic-helix-loop-helix-leucine zipper transcription factors named sterol regulatory element binding protein-1 (SREBP-1, Srebf1). There are two SREBP isoforms, designated SREBP-1c and SREBP-2, which differ in their tissue specific expression and their target genes selectivity. Whereas SREBP-1c preferentially activates genes involved in de-novo lipogenesis, SREBP-2 has a preference towards genes involved in cholesterol synthesis and uptake, at least in liver [44]. Together, SREBPs activate the expression of more than 30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids.

Although SREBP1 and SREBP2 have both been suggested to be inhibited by PUFAs, there is a lot more evidence implicating SREBP1 in downregulation of gene expression by PUFAs. Studies over the last decade have indicated that PUFAs potently lower SREBP-1 mRNA levels and inhibit proteolytic processing of SREBP-1 [45-49]. The latter process is required for maturation of precursor membrane-bound SREBP-1 to the mature SREBP-1, which moves to the nucleus and serves as the actual transcription factor. Recently, the target of PUFAs was identified as Ubxd8, a ER membrane-bound protein that facilitates the degradation of Insig-1, which normally sequesters the SCAP-SREBP complex in the ER and prevents its activation [50]. Specifically, it was shown that PUFAs inhibit the activity of Ubxd8, thus causing the SCAP-SREBP complex to stay in the ER. In addition to the mechanism described above, evidence has been provided that DHA but not other PUFAs stimulate the removal of mature nuclear SREBP-1 via a mechanism dependent on 26S-proteosome and ERK signaling [51]. Downregulation of SREBP-1 mRNA by PUFAs has been proposed to be mediated by stimulation of SREBP-1 mRNA decay [52], or by antagonizing the activity of the nuclear receptor LXRα, a potent inducer of SREBP-1 gene transcription [53, 54]. Since a role of LXR in mediating effects of PUFAs is contentious [55], the reduction in SREBP-1 mRNA by PUFA is more likely to be secondary to inhibition of SREBP-1 maturation, which via autoregulation of SREBP-1 transcriptional activation leads to reduced SREBP-1 mRNA levels [56].

PUFAs have also been shown to reduce expression of the glycolytic gene pyruvate kinase via a mechanism independent of PPARα [57]. This effect may be mediated by inhibiting nuclear translocation of either carbohydrate responsive element binding protein (ChREBP) or MAX-like protein X (MLX) (Figure 1) [58, 59]. ChREBP and MLX form a heterodimer functioning as glucose-responsive transcription factor that induces expression of genes involved in glycolysis and lipogenesis, including pyruvate kinase, acetyl-CoA carboxylase 1, and fatty acid synthase. However, additional data need to be collected to more precisely define how PUFAs influence ChREBP or MLX nuclear translocation and what are the direct molecular target of PUFAs.

Hepatocyte nuclear factor 4α

The hepatocyte nuclear factor 4α (HNF4α, Nr2a1) is a nuclear receptor that is exclusively expressed in the gastrointestinal tract, liver and kidney [7]. Targeted disruption of HNF4α leads to early embryonic lethality related to defects in the expression of visceral endoderm proteins required for maintaining gastrulation [60]. Using liver-specific HNF4α-/- mice it was shown that liver HNF4α is important for hepatocyte differentiation and for governing the expression of genes involved in lipid homeostasis (61). In 1998 evidence was provided that saturated fatty acyl-CoA may be able to serve as agonists for HNF4α, whereas unsaturated fatty acyl-CoA were proposed to serve as antagonistic ligands [62]. These data have been contested experimentally and are not widely accepted [63]. Elucidation of the molecular structure using X-ray crystallography revealed the presence of a fatty acid that appeared to be constitutively bound [64, 65]. More recently, it was shown using affinity isolation/mass-spectrometry that HNF4α is occupied by linoleic acid in COS-7 cells as well as in liver of fed but not fasted mice, suggesting fatty acid binding is exchangeable. However, no induction of HNF4α targets by linoleic acid was observed in a human colon cancer cell line, raising questions about the purpose of binding of linoleic acid to HNF4α [66]. Overall, the binding and especially the activation of HNF4α by fatty acids or acyl-CoAs remains controversial. Indeed, there is only very limited evidence that changes in the concentration of fatty acids or acyl-CoA lead to activation of HNF4α targets.

In addition to PPARs and HNF4α, the nuclear receptors LXR, FXR and RXR have been proposed to serve as mediators of the effects of fatty acid on gene transcription. With respect to LXR, it was suggested that unsaturated fatty acids suppress Srebp1c gene expression by inhibiting LXR [53]. However, another study found that unsaturated fatty acids do not influence LXR-dependent gene regulation in primary rat hepatocytes or in liver [55].

Docosahexanoic acid was originally picked up as ligand for RXR when looking for a factor in brain tissue that activates RXR in a cell-based assay [67]. Subsequent experiments showed the direct binding of PUFAs to RXR, with strongest RXR activation observed for DHA and arachidonic acid, followed by linolenic, linoleic, and oleic acid [68]. Recent studies confirmed the direct binding of DHA to RXR, although with much lower affinity compared to 9cRA [69]. In as much as DHA also binds PPARs and PPARs form a permissive heterodimers with RXR, it is technically challenging to distinguish between DHA gene signaling via PPAR versus RXR. Interestingly, using RXR and PPARγ antagonists, it was found that DHA induces expression of Adrp (Plin2) in human choriocarcinoma cells via activation of RXR [70]. Recently, effect of DHA on despair behaviors and working memory could be attributed to activation of RXRγ [71].

NF-E2-related factor-2 (NRF2)

An oral lipid load with PUFAs causes rapid upregulation of numerous oxidative stress genes in several organs, likely representing an adaptive mechanism aimed at preventing cellular lipotoxicity [72]. Increased levels of reactive oxygen species and derivatives of fatty acid peroxidation activate the transcription factor NRF2 (NFE2L2), which governs the expression of multiple genes involved in the oxidative stress response. Compounds that activate NRF2, ranging from diphenols to hydroperoxides and heavy metals, are believed to modulate the sulfhydryl group of cysteine residues with KEAP1, which serves as NRF2-specific adaptor protein for the Cullin-3 ubiquitin ligase complex [73]. As a result, these compounds cause the dissociation of Cullin-3 and thereby inhibit NRF2 ubiquitination, leading to stabilization and nuclear translocation of NRF2 and subsequent induction of NRF2 target genes. Studies have shown that oxidation products of linoleic acid, eicosapentanoic acid and DHA can react with KEAP1, while the intact fatty acids cannot [74-76]. Thus, the effects of

(dietary) PUFA on expression of genes involved in the oxidative stress response are likely mediated by specific fatty acid oxidation products via NRF2-dependent signaling.

Toll-like receptor 4

Numerous studies have investigated the impact of fatty acids on the inflammatory response in a great variety of cell types and tissues. These studies overwhelmingly point to a pro-inflammatory effect of saturated fatty acids, whereas n-3 PUFA exhibit mostly anti-inflammatory properties [77]. Most of the modulatory effect of fatty acids on inflammation can probably be attributed to fatty acid metabolites, including prostaglandins, leukotoxins, resolvins, endocannabinoids, ceramides and diacylglycerols [77]. However, there is accumulating evidence that fatty acids may be able to directly activate or suppress inflammatory pathways.

Most of the biological activities of LPS are mediated via its lipid A moiety. It is well established that the fatty acids that are part of lipid A play an important role in ligand recognition and receptor activation of Toll-like receptor 4 (TLR4), leading to the suggestion that saturated fatty acids may promote inflammation by direct activation of TLR4 (Figure 1). Subsequent studies have provided compelling evidence that saturated fatty acids activate NF-κB and stimulate expression of NF-κB targets such as COX-2, iNOS and IL-1α in macrophages by activating TLR4 signaling in a MyD88, IRAK-1 and TRAF6 dependent manner [78-80]. In contrast, unsaturated fatty acids are ineffective or may even act as antagonists. It was reported that saturated fatty acids activate TLR4 by promoting its recruitment to lipid rafts via a mechanism involving reactive oxygen species [81]. Data showing direct physical binding of saturated fatty acids to TLR4 are still lacking, leaving open the mechanism of TLR4 activation [82]. Others have argued against TLR4 activation by saturated fatty acids [83]. Using TLR4-/- macrophages, the role of TLR4 in mediating the inflammatory effects of saturated fatty acids was convincingly demonstrated [84, 85]. Loss of TLR4 was also shown to partially protect against diet-induced obesity and insulin resistance, suggesting TLR4 may be involved in mediating the detrimental effects of chronic high saturated fat consumption [84, 86, 87].

G-protein coupled receptors

Members of the G-protein coupled receptor (GPCRs) family are involved in mediating the stimulatory effects of fatty acids on insulin secretion by pancreatic β-cells and on secretion of various gastrointestinal hormones in the gut [88, 89]. These receptors, which include GPR40 (FFAR1), GPR41 (FFAR3), GPR43 (FFAR2), GPR84, and GPR120, each exhibit preference for a specific set of fatty acids. To what extent activation of GPRs by fatty acids directly influences gene transcription remains to be determined (Figure 1). Nevertheless, due to the emerging importance of GPRs in fatty acid sensing in a variety of tissues, some discussion on GPRs is warranted.

In addition to being activated by short chain FAs such as acetate, propionate, butyrate and pentanoate, GPR41 and GPR43 have in common that they are well expressed in the colon, which is exposed to elevated concentrations of SCFAs via bacterial fermentation [88]. Furthermore, GPR41 is expressed in numerous immune cells and adipose tissue, where it was shown to be involved in regulation of leptin production [90]. The relative role of GPR41 versus GPR43 as sensor for SCFAs in the enteroendocrine system is not clear. Recently, it was proposed that GPR41 mediates the effect of gut microbiota on fat mass [91], while stimulation of GPR43 by SCFAs was shown to be necessary for the normal resolution of certain inflammatory responses [92].

In contrast to GPR41 and GPR43, GPR40 is activated by medium and long chain fatty acids , which include saturated and unsaturated fatty acids. GPR40 is expressed at high levels in pancreatic β-cells, where it mediates the stimulatory effect of fatty acids on glucose-stimulated insulin secretion [93, 94]. Apart from the pancreatic β-cells, GPR40 is known to be expressed in various other cell types such as enteroendocrine cells. In these cells, GPR40 is involved in the stimulation of production of GLP-1 and GIP by fatty acids [95].

Other relevant members of the GPCR family are GPR84, GPR119 and GPR120. GPR84 is well expressed in bone-marrow derived macrophages and has been proposed as receptor for medium chain fatty acids [96]. GPR119 has a similar expression pattern as GPR40 but the receptors shares only little homology. Endogenous ligands of GPR119 have been identified and include the fatty acid derivatives monoacyl glycerol, lysophosphatidylcholine and oleoylethanolamide [97, 98]. GPR120 is activated by saturated and unsaturated fatty acids with twelve or more carbons. GPR120 is most abundant in mouse

large intestine, lung and adipose tissue, but is also expressed in enteroendocrine cells where it mediates the effect of fatty acids on release of glucagon-like peptide-1 and cholecystokinine [99-101]. Remarkably, GPR120 was recently proposed to serve as a specific sensor for n-3 fatty acids in macrophages that may mediate the putative insulin sensitizing and anti-diabetic effects of n-3 fatty acids in vivo by repressing macrophage-induced tissue inflammation [102]. So far, evidence is lacking that activation of these receptors is directly linked to regulation of gene expression.

Conclusion

While the importance of dietary fatty acids as determinants of risk for numerous chronic diseases has been well recognized, only recently have we started to gain appreciation for the vast regulatory functions of dietary fatty acids in the human body. It is now evident that fatty acids, either directly or via its metabolites, act via a great variety of signaling pathways to influence numerous metabolic, inflammatory, and other biological processes. In the past decade, nutrigenomics has provided the ideal conceptual framework and the necessary technological tools to address the global effects of dietary fatty acids, and has importantly contributed to a major advancement in our understanding of the molecular action of dietary fatty acids. So far the focus has been on the molecular characterization of specific signaling routes, coupled to the description of the whole genome effects of dietary fatty acids. In the future, greater emphasis will have to be placed on the functional consequences of specific target gene regulation in order to fully understand the functional impact of dietary fatty acids and their potentially preventive effect in specific disease conditions. It can be foreseen that nutrigenomics will continue to make a push towards a more mechanistic and genomics-driven approach within the domain of nutritional sciences and further promote the implementation of high throughput technologies.

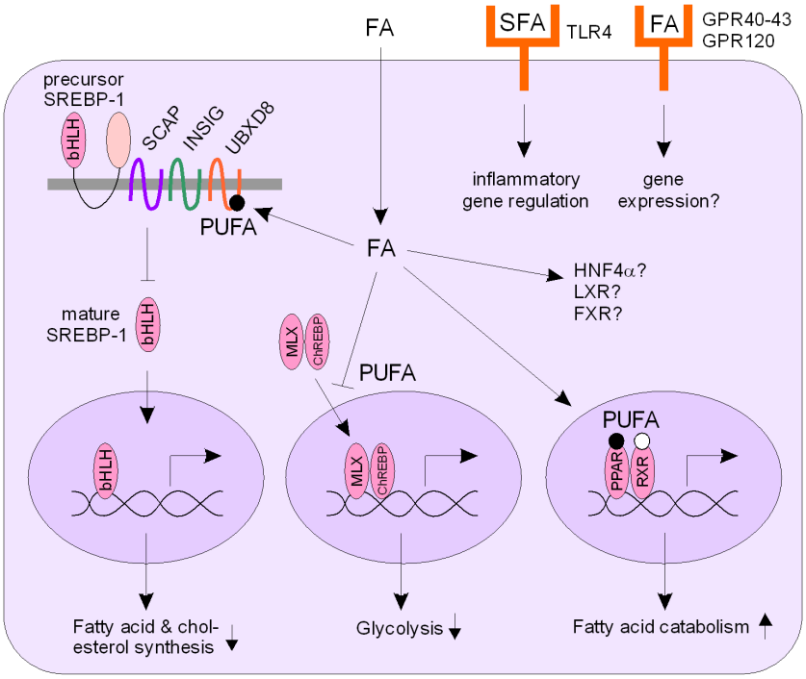


Figure 1: General mechanisms of gene regulation by fatty acids

The mechanisms shown mainly apply to hepatocytes. PUFAs reduce expression of genes involved in fatty acid and cholesterol synthesis by binding and inactivating UBXD8, thereby inhibiting proteolytic processing of SREBP-1. PUFAs reduce expression of L-type pyruvate kinase (glycolysis) in liver most likely by inhibiting nuclear translocation of MLX-ChREBP. Various fatty acids but especially PUFAs act as ligand for PPARs. Activation of PPARα by PUFAs in liver leads to stimulation of fatty acid catabolism. DHA has been reported as a ligand for RXR. GPR40-43 and GPR120 are expressed by enterocytes, enteroendocrine cells and other cell types and serve as membrane receptors for various types of fatty acids including SCFAs. It is uncertain whether they are involved in the effects of fatty acids on gene expression. TLR4 is present macrophages and other cell types and has been proposed to be activated by saturated fatty acids. bHLH, basic helix loop helix.

References:

1. Wang H, Eckel RH. Lipoprotein lipase: from gene to obesity. Am J Physiol Endocrinol Metab. 2009;297:E271-88.
2. Teusink B, Voshol PJ, Dahlmans VE, Rensen PC, Pijl H, Romijn JA, Havekes LM. Contribution of fatty acids released from lipolysis of plasma triglycerides to total plasma fatty acid flux and tissue-specific fatty acid uptake. Diabetes. 2003;52:614-20.
3. Glatz JF, Luiken JJ, Bonen A. Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiological reviews. 2010;90:367-417.
4. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. Science. 2001;294:1866-70.
5. Kersten S, Desvergne B, Wahli W. Roles of PPARs in health and disease. Nature. 2000;405:421-4.
6. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, et al. The nuclear receptor superfamily: the second decade. Cell. 1995;83:835-9.
7. Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell. 2006;126:789-99.
8. Escher P, Braissant O, Basu-Modak S, Michalik L, Wahli W, Desvergne B. Rat PPARs: quantitative analysis in adult rat tissues and regulation in fasting and refeeding. Endocrinology. 2001;142:4195-202.
9. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell. 1995;83:803-12.
10. Gottlicher M, Widmark E, Li Q, Gustafsson JA. Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. Proc Natl Acad Sci U S A. 1992;89:4653-7.
11. Keller H, Dreyer C, Medin J, Mahfoudi A, Ozato K, Wahli W. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. Proc Natl Acad Sci U S A. 1993;90:2160-4.
12. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A. 1997;94:4318-23.
13. Krey G, Braissant O, L'Horset F, Kalkhoven E, Perroud M, Parker MG, Wahli W. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of

peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. Mol Endocrinol. 1997;11:779-91.

14. Lin Q, Ruuska SE, Shaw NS, Dong D, Noy N. Ligand selectivity of the peroxisome proliferator-activated receptor alpha. Biochemistry. 1999;38:185-90.
15. Malapaka RR, Khoo SK, Zhang J, Choi JH, Zhou XE, Xu Y, Gong Y, Li J, Yong EL, et al. Identification and mechanism of a ten carbon fatty acid as a modulating ligand of peroxisome proliferator activated receptors. The Journal of biological chemistry. 2011.
16. Murakami K, Ide T, Suzuki M, Mochizuki T, Kadowaki T. Evidence for direct binding of fatty acids and eicosanoids to human peroxisome proliferators-activated receptor alpha. Biochem Biophys Res Commun. 1999;260:609-13.
17. Sanderson LM, de Groot PJ, Hooiveld GJ, Koppen A, Kalkhoven E, Muller M, Kersten S. Effect of synthetic dietary triglycerides: a novel research paradigm for nutrigenomics. PLoS ONE. 2008;3:e1681.
18. Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, Sternbach DD, Lehmann JM, Wisely GB, et al. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol Cell. 1999;3:397-403.
19. Fang X, Hu S, Xu B, Snyder GD, Harmon S, Yao J, Liu Y, Sangras B, Falck JR, et al. 14,15-Dihydroxyeicosatrienoic acid activates peroxisome proliferator-activated receptor-alpha. Am J Physiol Heart Circ Physiol. 2006;290:H55-63.
20. Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, et al. Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature. 2003;425:90-3.
21. Hostetler HA, Petrescu AD, Kier AB, Schroeder F. Peroxisome proliferator-activated receptor alpha interacts with high affinity and is conformationally responsive to endogenous ligands. J Biol Chem. 2005;280:18667-82.
22. Itoh T, Fairall L, Amin K, Inaba Y, Szanto A, Balint BL, Nagy L, Yamamoto K, Schwabe JW. Structural basis for the activation of PPARgamma by oxidized fatty acids. Nat Struct Mol Biol. 2008;15:924-31.
23. Li Y, Zhang J, Schopfer FJ, Martynowski D, Garcia-Barrio MT, Kovach A, Suino-Powell K, Baker PR, Freeman BA, et al. Molecular recognition of nitrated fatty acids by PPAR gamma. Nat Struct Mol Biol. 2008;15:865-7.
24. Moya-Camarena SY, Vanden Heuvel JP, Blanchard SG, Leesnitzer LA, Belury MA. Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARα. J Lipid Res. 1999;40:1426-33.
25. Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell. 1998;93:229-40.

26. Zomer AW, van Der Burg B, Jansen GA, Wanders RJ, Poll-The BT, van Der Saag PT. Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor alpha. J Lipid Res. 2000;41:1801-7.
27. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell. 1995;83:813-9.
28. Lim H, Gupta RA, Ma WG, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM, Dey SK. Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARβ/δ. Genes Dev. 1999;13:1561-74.
29. Muga SJ, Thuillier P, Pavone A, Rundhaug JE, Boeglin WE, Jisaka M, Brash AR, Fischer SM. 8S-lipoxygenase products activate peroxisome proliferator-activated receptor alpha and induce differentiation in murine keratinocytes. Cell Growth Differ. 2000;11:447-54.
30. Chakravarthy MV, Lodhi IJ, Yin L, Malapaka RR, Xu HE, Turk J, Semenkovich CF. Identification of a physiologically relevant endogenous ligand for PPARα in liver. Cell. 2009;138:476-88.
31. Ahmed W, Orasanu G, Nehra V, Asatryan L, Rader DJ, Ziouzenkova O, Plutzky J. High-density lipoprotein hydrolysis by endothelial lipase activates PPARα: a candidate mechanism for high-density lipoprotein-mediated repression of leukocyte adhesion. Circulation research. 2006;98:490-8.
32. Ziouzenkova O, Perrey S, Asatryan L, Hwang J, MacNaul KL, Moller DE, Rader DJ, Sevanian A, Zechner R, et al. Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an antiinflammatory role for lipoprotein lipase. Proc Natl Acad Sci U S A. 2003;100:2730-5.
33. Brown JD, Oligino E, Rader DJ, Saghatelian A, Plutzky J. VLDL hydrolysis by hepatic lipase regulates PPARβ/δ transcriptional responses. PloS one. 2011;6:e21209.
34. Chawla A, Lee CH, Barak Y, He W, Rosenfeld J, Liao D, Han J, Kang H, Evans RM. PPARβ/δ is a very low-density lipoprotein sensor in macrophages. Proc Natl Acad Sci U S A. 2003;100:1268-73.
35. Chakravarthy MV, Pan Z, Zhu Y, Tordjman K, Schneider JG, Coleman T, Turk J, Semenkovich CF. "New" hepatic fat activates PPARα to maintain glucose, lipid, and cholesterol homeostasis. Cell Metab. 2005;1:309-22.
36. Sanderson LM, Degenhardt T, Koppen A, Kalkhoven E, Desvergne B, Muller M, Kersten S. Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) but not PPARα serves as a plasma free fatty acid sensor in liver. Mol Cell Biol. 2009;29:6257-67.
37. Kersten S, Lichtenstein L, Steenbergen E, Mudde K, Hendriks HF, Hesselink MK, Schrauwen P, Muller M. Caloric restriction and exercise increase plasma ANGPTL4

levels in humans via elevated free fatty acids. Arterioscler Thromb Vasc Biol. 2009;29:969-74.
38. Staiger H, Haas C, Machann J, Werner R, Weisser M, Schick F, Machicao F, Stefan N, Fritsche A, Haring HU. Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator-activated receptor (PPAR)-delta and is of metabolic relevance in humans. Diabetes. 2009;58:579-89.
39. Haemmerle G, Moustafa T, Woelkart G, Buttner S, Schmidt A, van de Weijer T, Hesselink M, Jaeger D, Kienesberger PC, et al. ATGL-mediated fat catabolism regulates cardiac mitochondrial function via PPAR-alpha and PGC-1. Nature medicine. 2011;17:1076-85.
40. Ong KT, Mashek MT, Bu SY, Greenberg AS, Mashek DG. Adipose triglyceride lipase is a major hepatic lipase that regulates triacylglycerol turnover and fatty acid signaling and partitioning. Hepatology. 2011;53:116-26.
41. Rakhshandehroo M, Knoch B, Muller M, Kersten S. Peroxisome proliferator-activated receptor alpha target genes. PPAR Res. 2010;2010.
42. Georgiadi A, Lichtenstein L, Degenhardt T, Boekschoten MV, van Bilsen M, Desvergne B, Muller M, Kersten S. Induction of cardiac Angptl4 by dietary fatty acids is mediated by peroxisome proliferator-activated receptor beta/delta and protects against fatty acid-induced oxidative stress. Circ Res. 2010;106:1712-21.
43. Flachs P, Rossmeisl M, Bryhn M, Kopecky J. Cellular and molecular effects of n-3 polyunsaturated fatty acids on adipose tissue biology and metabolism. Clin Sci (Lond). 2009;116:1-16.
44. Horton JD. Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. Biochemical Society transactions. 2002;30:1091-5.
45. Hannah VC, Ou J, Luong A, Goldstein JL, Brown MS. Unsaturated fatty acids down-regulate srebp isoforms 1a and 1c by two mechanisms in HEK-293 cells. J Biol Chem. 2001;276:4365-72.
46. Kim HJ, Takahashi M, Ezaki O. Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. J Biol Chem. 1999;274:25892-8.
47. Mater MK, Thelen AP, Pan DA, Jump DB. Sterol response element-binding protein 1c (SREBP1c) is involved in the polyunsaturated fatty acid suppression of hepatic S14 gene transcription. J Biol Chem. 1999;274:32725-32.
48. Xu J, Nakamura MT, Cho HP, Clarke SD. Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. J Biol Chem. 1999;274:23577-83.
49. Yahagi N, Shimano H, Hasty AH, Amemiya-Kudo M, Okazaki H, Tamura Y, Iizuka Y, Shionoiri F, Ohashi K, et al. A crucial role of sterol regulatory element-

binding protein-1 in the regulation of lipogenic gene expression by polyunsaturated fatty acids. J Biol Chem. 1999;274:35840-4.

50. Lee JN, Kim H, Yao H, Chen Y, Weng K, Ye J. Identification of Ubxd8 protein as a sensor for unsaturated fatty acids and regulator of triglyceride synthesis. Proceedings of the National Academy of Sciences of the United States of America. 2010;107:21424-9.
51. Botolin D, Wang Y, Christian B, Jump DB. Docosahexaneoic acid (22:6,n-3) regulates rat hepatocyte SREBP-1 nuclear abundance by Erk- and 26S proteasome-dependent pathways. J Lipid Res. 2006;47:181-92.
52. Xu J, Teran-Garcia M, Park JH, Nakamura MT, Clarke SD. Polyunsaturated fatty acids suppress hepatic sterol regulatory element-binding protein-1 expression by accelerating transcript decay. J Biol Chem. 2001;276:9800-7.
53. Ou J, Tu H, Shan B, Luk A, DeBose-Boyd RA, Bashmakov Y, Goldstein JL, Brown MS. Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. Proc Natl Acad Sci U S A. 2001;98:6027-32.
54. Yoshikawa T, Shimano H, Yahagi N, Ide T, Amemiya-Kudo M, Matsuzaka T, Nakakuki M, Tomita S, Okazaki H, et al. Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. J Biol Chem. 2002;277:1705-11.
55. Pawar A, Botolin D, Mangelsdorf DJ, Jump DB. The role of liver X receptor-alpha in the fatty acid regulation of hepatic gene expression. The Journal of biological chemistry. 2003;278:40736-43.
56. Takeuchi Y, Yahagi N, Izumida Y, Nishi M, Kubota M, Teraoka Y, Yamamoto T, Matsuzaka T, Nakagawa Y, et al. Polyunsaturated fatty acids selectively suppress sterol regulatory element-binding protein-1 through proteolytic processing and autoloop regulatory circuit. J Biol Chem.
57. Pan DA, Mater MK, Thelen AP, Peters JM, Gonzalez FJ, Jump DB. Evidence against the peroxisome proliferator-activated receptor alpha (PPARα) as the mediator for polyunsaturated fatty acid suppression of hepatic L-pyruvate kinase gene transcription. J Lipid Res. 2000;41:742-51.
58. Dentin R, Benhamed F, Pegorier JP, Foufelle F, Viollet B, Vaulont S, Girard J, Postic C. Polyunsaturated fatty acids suppress glycolytic and lipogenic genes through the inhibition of ChREBP nuclear protein translocation. J Clin Invest. 2005;115:2843-54.
59. Xu J, Christian B, Jump DB. Regulation of rat hepatic L-pyruvate kinase promoter composition and activity by glucose, n-3 polyunsaturated fatty acids, and peroxisome proliferator-activated receptor-alpha agonist. J Biol Chem. 2006;281:18351-62.

60. Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, Prezioso VR, Bachvarova RF, Darnell JE, Jr. Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. Genes Dev. 1994;8:2466-77.
61. Yin L, Ma H, Ge X, Edwards PA, Zhang Y. Hepatic hepatocyte nuclear factor 4alpha is essential for maintaining triglyceride and cholesterol homeostasis. Arteriosclerosis, thrombosis, and vascular biology. 2011;31:328-36.
62. Hertz R, Magenheim J, Berman I, Bar-Tana J. Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4alpha. Nature. 1998;392:512-6.
63. Bogan AA, Dallas-Yang Q, Ruse MD, Jr., Maeda Y, Jiang G, Nepomuceno L, Scanlan TS, Cohen FE, Sladek FM. Analysis of protein dimerization and ligand binding of orphan receptor HNF4alpha. J Mol Biol. 2000;302:831-51.
64. Dhe-Paganon S, Duda K, Iwamoto M, Chi YI, Shoelson SE. Crystal structure of the HNF4 alpha ligand binding domain in complex with endogenous fatty acid ligand. J Biol Chem. 2002;277:37973-6.
65. Wisely GB, Miller AB, Davis RG, Thornquest AD, Jr., Johnson R, Spitzer T, Sefler A, Shearer B, Moore JT, et al. Hepatocyte nuclear factor 4 is a transcription factor that constitutively binds fatty acids. Structure. 2002;10:1225-34.
66. Yuan X, Ta TC, Lin M, Evans JR, Dong Y, Bolotin E, Sherman MA, Forman BM, Sladek FM. Identification of an endogenous ligand bound to a native orphan nuclear receptor. PLoS One. 2009;4:e5609.
67. de Urquiza AM, Liu S, Sjoberg M, Zetterstrom RH, Griffiths W, Sjovall J, Perlmann T. Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. Science. 2000;290:2140-4.
68. Lengqvist J, Mata De Urquiza A, Bergman AC, Willson TM, Sjovall J, Perlmann T, Griffiths WJ. Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor alpha ligand-binding domain. Mol Cell Proteomics. 2004;3:692-703.
69. Stafslien DK, Vedvik KL, De Rosier T, Ozers MS. Analysis of ligand-dependent recruitment of coactivator peptides to RXRbeta in a time-resolved fluorescence resonance energy transfer assay. Mol Cell Endocrinol. 2007;264:82-9.
70. Suzuki K, Takahashi K, Nishimaki-Mogami T, Kagechika H, Yamamoto M, Itabe H. Docosahexaenoic acid induces adipose differentiation-related protein through activation of retinoid x receptor in human choriocarcinoma BeWo cells. Biological & pharmaceutical bulletin. 2009;32:1177-82.
71. Wietrzych-Schindler M, Szyszka-Niagolov M, Ohta K, Endo Y, Perez E, de Lera AR, Chambon P, Krezel W. Retinoid x receptor gamma is implicated in docosahexaenoic acid modulation of despair behaviors and working memory in mice. Biol Psychiatry. 2011;69:788-94.

72. Anderson EJ, Thayne K, Harris M, Carraway K, Shaikh SR. Aldehyde stress and up-regulation of Nrf2-mediated antioxidant systems accompany functional adaptations in cardiac mitochondria from mice fed n-3 polyunsaturated fatty acids. The Biochemical journal. 2011.
73. Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol. 2004;24:7130-9.
74. Gao L, Wang J, Sekhar KR, Yin H, Yared NF, Schneider SN, Sasi S, Dalton TP, Anderson ME, et al. Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3. J Biol Chem. 2007;282:2529-37.
75. Groeger AL, Cipollina C, Cole MP, Woodcock SR, Bonacci G, Rudolph TK, Rudolph V, Freeman BA, Schopfer FJ. Cyclooxygenase-2 generates anti-inflammatory mediators from omega-3 fatty acids. Nature chemical biology. 2010;6:433-41.
76. Wang R, Kern JT, Goodfriend TL, Ball DL, Luesch H. Activation of the antioxidant response element by specific oxidized metabolites of linoleic acid. Prostaglandins Leukot Essent Fatty Acids. 2009;81:53-9.
77. Galli C, Calder PC. Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review. Ann Nutr Metab. 2009;55:123-39.
78. Lee JY, Plakidas A, Lee WH, Heikkinen A, Chanmugam P, Bray G, Hwang DH. Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. J Lipid Res. 2003;44:479-86.
79. Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem. 2001;276:16683-9.
80. Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore N, Hwang DH. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. J Biol Chem. 2003;278:37041-51.
81. Wong SW, Kwon MJ, Choi AM, Kim HP, Nakahira K, Hwang DH. Fatty acids modulate Toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. J Biol Chem. 2009;284:27384-92.
82. Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, Kopp A, Schoelmerich J, Falk W. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. Immunology. 2009;126:233-45.

83. Erridge C, Samani NJ. Saturated fatty acids do not directly stimulate Toll-like receptor signaling. Arterioscler Thromb Vasc Biol. 2009;29:1944-9.
84. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest. 2006;116:3015-25.
85. Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, Kotani H, Yamaoka S, Miyake K, et al. Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol. 2007;27:84-91.
86. Davis JE, Gabler NK, Walker-Daniels J, Spurlock ME. Tlr-4 deficiency selectively protects against obesity induced by diets high in saturated fat. Obesity (Silver Spring). 2008;16:1248-55.
87. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, Araujo EP, Vassallo J, Curi R, et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes. 2007;56:1986-98.
88. Covington DK, Briscoe CA, Brown AJ, Jayawickreme CK. The G-protein-coupled receptor 40 family (GPR40-GPR43) and its role in nutrient sensing. Biochem Soc Trans. 2006;34:770-3.
89. Kebede MA, Alquier T, Latour MG, Poitout V. Lipid receptors and islet function: therapeutic implications? Diabetes Obes Metab. 2009;11 Suppl 4:10-20.
90. Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, Yanagisawa M. Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A. 2004;101:1045-50.
91. Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, et al. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A. 2008;105:16767-72.
92. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature. 2009;461:1282-6.
93. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, et al. Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature. 2003;422:173-6.
94. Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H. The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. Cell Metab. 2005;1:245-58.
95. Edfalk S, Steneberg P, Edlund H. Gpr40 is expressed in enteroendocrine cells and

mediates free fatty acid stimulation of incretin secretion. Diabetes. 2008;57:2280-7.
96. Wang J, Wu X, Simonavicius N, Tian H, Ling L. Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. J Biol Chem. 2006;281:34457-64.
97. Hansen KB, Rosenkilde MM, Knop FK, Wellner N, Diep TA, Rehfeld JF, Andersen UB, Holst JJ, Hansen HS. 2-Oleoyl glycerol is a GPR119 agonist and signals GLP-1 release in humans. The Journal of clinical endocrinology and metabolism. 2011;96:E1409-17.
98. Overton HA, Babbs AJ, Doel SM, Fyfe MC, Gardner LS, Griffin G, Jackson HC, Procter MJ, Rasamison CM, et al. Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. Cell metabolism. 2006;3:167-75.
99. Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G. Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med. 2005;11:90-4.
100. Miyauchi S, Hirasawa A, Iga T, Liu N, Itsubo C, Sadakane K, Hara T, Tsujimoto G. Distribution and regulation of protein expression of the free fatty acid receptor GPR120. Naunyn Schmiedebergs Arch Pharmacol. 2009;379:427-34.
101. Tanaka T, Katsuma S, Adachi T, Koshimizu TA, Hirasawa A, Tsujimoto G. Free fatty acids induce cholecystokinin secretion through GPR120. Naunyn Schmiedebergs Arch Pharmacol. 2008;377:523-7.
102. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell. 2010;142:687-

Chapter 3

Detailed transcriptomics analysis of the effect of dietary fatty acids on gene expression in the heart

Anastasia Georgiadi, Mark V. Boekschoten, Michael Müller and Sander Kersten

Chapter published in Physiological Genomics 2012 Mar 19;44(6):352-61

Abstract

Fatty acids comprise the primary energy source for the heart and are mainly taken up via hydrolysis of circulating triglyceride-rich lipoproteins. While most of the fatty acids entering the cardiomyocyte are oxidized, a small portion is involved in altering gene transcription to modulate cardiometabolic functions. So far, no in vivo model has been developed enabling study of the transcriptional effects of specific fatty acids in the intact heart. In the present study, mice were given a single oral dose of synthetic triglycerides composed of one single fatty acid. Hearts were collected 6h thereafter and used for whole genome gene expression profiling. Experiments were conducted in wild-type and PPARα-/- mice to allow exploration of the specific contribution of PPARα. It was found that: 1) C18:3 had the most pronounced effect on cardiac gene expression. 2) The largest similarity in gene regulation was observed between C18:2 and C18:3. Large similarity was also observed between PPARα agonist Wy14643 and C22:6. 3) Many genes were regulated by one particular treatment only. Genes regulated by one particular treatment showed large functional divergence. 4) The majority of genes responding to fatty acid treatment were regulated in a PPARα-dependent manner, emphasizing the importance of PPARα in mediating transcriptional regulation by fatty acids in the heart. 5) Several genes were robustly regulated by all or many of the fatty acids studied, mostly representing well-described targets of PPARs (e.g. Acot1, Angptl4, Ucp3) but also including Zbtb16/PLZF, a transcription factor crucial for Natural Killer T cell function. 6) Deletion and activation of PPARα had a major effect on expression of numerous genes involved in metabolism and immunity. Our analysis demonstrates the marked impact of dietary fatty acids on gene regulation in the heart via PPARα.

Introduction

Fatty acids serve as the primary energy substrate for the contracting heart via their oxidation in the mitochondria. Oxidation of fatty acids in the heart may be altered during specific disease conditions impacting the heart, including cardiac failure, myocardial ischemia and diabetes [23]. Impaired oxidation or excess delivery of fatty acids in the heart may give rise to cardiomyocellular lipid storage. Limited variation in cardiac lipid storage can be considered normal and occurs as a direct consequence of physiological fluctuations in circulating FFA [35]. However, chronically elevated cardiac lipid storage is considered harmful and may lead to lipotoxic cardiomyopathy [24].

Fatty acids entering the cardiomyocyte can originate from two principal sources, which are circulating triglyceride-rich lipoproteins and circulating albumin-bound free fatty acids. The former pathway, shown to be the main source of fatty acids for the heart [3, 31], requires the catalytic activity of lipoprotein lipase (LPL), which is anchored into the capillary endothelium. To what extent fatty acids from different extracellular sources are channeled into different intracellular pathways in the heart remains to be investigated.

While most of the fatty acids entering the cardiomyocyte are oxidized, a small portion of the incoming fatty acids are transported towards the nucleus and alter gene transcription to modulate cardiometabolic functions. Several transcription factors are implicated in mediating effect of fatty acids on gene transcription in various tissues, including SREBP-1c, HNF4α, and PPARs [25]. PPARs are ligand-activated transcription factors that govern DNA transcription by direct binding to promoters of target genes [16]. In addition, they down-regulate gene expression by interfering with the activity of other transcription factors. The family of PPARs consists of three members encoded by distinct genes: α, δ, and γ, which are each characterized by specific tissue- and developmental patterns of expression.

PPARα serves as the molecular target for the fibrate class of drugs. In addition, PPARα is activated by fatty acids and various fatty acid derivatives such as eicosanoids and endocannabinoids. In vitro studies show that PPARα has a preference towards long-chain poly-unsaturated fatty acids (PUFAs) [12, 18, 19]. While several studies have examined the effect of fatty acids on PPAR target genes in isolated cardiomyocytes, showing induction of typical PPARα

target such as Ucp2, Cpt1a, Cd36, Fabp3, Acsl1, Acot1, and Acadl [5, 10, 32, 34], little is known about gene regulation by fatty acids in the intact heart.

Previously, we described an in vivo model that allows characterization of the transcriptional targets of PUFAs in diverse tissues in vivo [14, 26]. In this model, mice are given a single oral bolus of synthetic triglycerides composed of a single fatty acid. We showed that the Angptl4 gene is a very sensitive target of fatty acids in the heart and furthermore that its upregulation is part of a protective mechanism against cardiac lipotoxicity [14]. The present study examines the whole genome effects of individual dietary fatty acids in the heart via transcriptional profiling. By conducting these experiments in wild-type and PPARα-/- mice, the specific contribution of PPARα could be determined.

Methods

Chemicals: Wy14643 was obtained from Eagle Picher Technologies laboratories (Lenexa, KS, USA). Triolein, trilinolein, trilinolenin, tridocosahexaenoin were from Nu-Chek-Prep, Inc. (Elysian, MN, USA). Cell culture media, fetal bovine serum and penicillin/streptomycin were from Lonza (Verviers, Belgium).

Animals and oral lipid load: Pure-bred Sv129 and PPARα-/- mice (2-6 months of age) on a Sv129 background were used. In the short term experiment, animals were switched to a run-in diet consisting of a modified AIN76A diet (corn oil was replaced with olive oil to minimize baseline intake of polyunsaturated fatty acids, which are more potent activators of PPARα) two weeks before the start of the experiment (Research Diet Services, Wijk bij Duurstede, the Netherlands). Starting at 5 a.m. the animals were fasted for 4 hours followed by an intragastric gavage of 400 µL synthetic triolein, trilinolein, trilinolenin, or tridocosahexaenoin. Wy14643 was given as 400 µL of a 10 mg/mL suspension in 0.5% carboxylmethyl cellulose. The latter also served as control treatment (400uL). 6 hours after the oral gavage the mice were anaesthetized with a mixture of isofluorane (1.5%), nitrous oxide (70%) and oxygen (30%). Blood was collected by orbital puncture, followed by sacrifice of the mice by cervical dislocation. Hearts were removed, snap-frozen in liquid nitrogen and stored at -80ºC.

In the long term experiment, wild-type and PPARα-/- mice were fed a chow diet (RMH-B diet, Arie Blok, Woerden, the Netherlands) containing 0.1 % Wy14643 for 5 days. The animal experiments were approved by the animal ethics committee of Wageningen University.

Microarray analysis: RNA from total heart was extracted with TRIzol reagent and purified using RNeasy Mini kit (Qiagen, Venlo, Netherlands). RNA quality was assessed on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips using a Eukaryote Total RNA Nano assay. Expression profiling was carried out on individual mouse hearts using Affymetrix Mouse Genome 430 2.0 Arrays (short term experiment) or on pooled RNA from 4-5 mice using Affymetrix Mouse NuGO arrays (long term experiment). Hybridization, washing and scanning of the arrays were done according to standard Affymetrix protocols. Scans of the Affymetrix arrays were processed using packages from the Bioconductor project [13]. Raw signal intensities were normalized by using the GCRMA algorithm [36]. Probesets were defined according to Dai et al. using remapped CDF version 11.0.2 based on the Entrez gene database [8]. The Bioconductor R package Linear models for microarray data (LIMMA) was used to identify differentially expressed genes. All comparisons were simultaneously analysed. In order to balance between random responses and relative weak transcriptional effects by the treatments, genes that met the cut-off of mean absolute fold change > 1.2 and p-value<0.01 were considered significantly regulated. A regularized t-test was used, which has the same interpretation as an ordinary t-test except that the standard errors have been moderated across genes, i.e. shrunk to a common value, using a Bayesian model [30]. The microarray datasets have been submitted to NCBI Gene Expression Omnibus (GEO numbers pending).

Functional classification of genes: For functional classification of probe sets/genes into gene sets clusters or biological pathways, Gene set enrichment analysis (GSEA) and ingenuity software v.6.5 were used. For identifying specific pathways regulated by each treatment we used Ingenuity canonical pathways, considering only differentially expressed genes with a p-value<0.01 (fatty acids and Wy treatment) or differentially expressed genes with a p-

value<0.01 and fold-change>1.2 (baseline wildtype vs. PPARα-/-). For GSEA differentially expressed gene sets with a p-value<0.05 were considered.

Correlation Plot: Signal log ratios were calculated between intensity values for individual animals and the mean intensity value of the wildtype control group. Signal log ratios of genes significantly regulated by at least one treatment were used as input for a correlation plot in Biowisdom Omniviz 6.0.3 (Cambridge, UK).

Results

Oral feeding of synthetic triglycerides

To study the effect of individual fatty acids on in vivo gene expression in the heart, mice fasted for 4 hours were given a single oral dose (400 µL) of synthetic triglycerides (TGs) consisting of one single fatty acid, followed by collection of the heart 6 hours thereafter (26). Those dosage mirrors the amount of dietary fat provided in a post-prandial lipid test in humans. The fatty acids studied were oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and docosahexaenoic acid (C22:6). In addition, a set of mice was given a single oral dose of the synthetic PPARα agonist Wy14643. No saturated fatty acids were included because triglycerides composed of common dietary saturated fatty acids are solid at room temperature and could not be administered orally. The 6-hour time point was chosen because dietary triglycerides enter into the circulation within 1 hour after intake and a constant rate of absorption is sustained for at least 4 hours thereafter (Figure 1A). The focus of the present study is on heart since heart shows the highest relative rate of uptake of dietary fatty acids, when expressed per gram organ weight [31]. No major differences in metabolic processing of dietary fat between WT and PPARα−/− mice and between different dietary fatty acids were observed in this study [26].

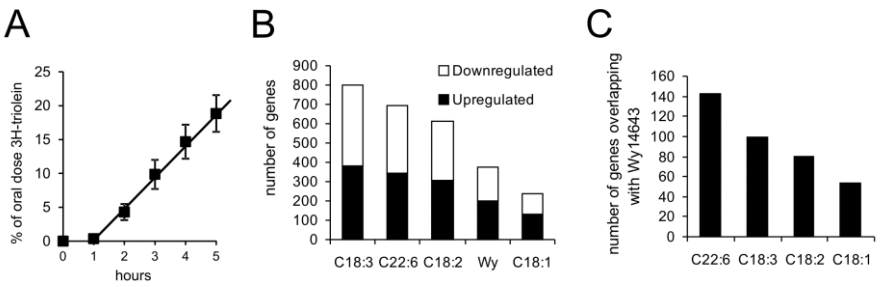


Figure 1: Whole genome effects of dietary fatty acids on gene expression in the heart. A. Mice fasted for 5h were intravenously injected with lipoprotein lipase inhibitor tyloxapol and immediately thereafter given 400 µL of olive oil containing 7µCi glycerol-tri[^{3}H] oleate (triolein). Blood was collected every hour and used to determine radioactivity, which is expressed as a percentage of total oral dose. Results illustrate the constant rate of absorption of dietary triglycerides for several hours. B. Number of genes up- or down regulated ($P<0.01$) in mouse hearts six hours after a single oral dose of Wy14643 or synthetic triglycerides containing one specific fatty acid. Mice receiving carboxymethylcellulose served as reference. C. Number of genes regulated by each of the fatty acid treatments that were also significantly regulated by Wy14643 ($P<0.01$).

Similarity in gene regulation between fatty acids

Expression profiling carried out on individual mouse hearts indicated that the largest number of genes was changed following treatment with C18:3, followed by C22:6, C18:2, Wy14643 and finally C18:1 (Figure 1B). The proportion of genes up- and downregulated was approximately equal, and was consistent throughout the various treatments. C22:6 showed the most pronounced overlap in gene regulation with Wy14643, followed by C18:3, C18:2 and C18:1 (Figure 1C). Next we studied the similarity in gene regulation between the various fatty acids via scatter plot analysis, in which the mean fold-change in expression of a gene by one treatment is expressed in one dimension, and the mean fold-change in expression of the same gene by another treatment is expressed in the other dimension. Results indicated that mean changes in

gene expression elicited by C18:3 and C18:2 were highly similar (Figure 2A), as illustrated by the limited scatter. Much less similarity in gene regulation was observed between C18:2 (or C18:3) and C22:6. Consistent with the above data, Wy14643 showed the least scatter, indicating highest similarity, when plotted against C22:6, compared to the other fatty acids. Similar results were obtained via correlation analysis, which determines the magnitude of correlation in overall gene expression between two individual mice (Figure 2B). High correlation in gene expression was observed between mice that received C18:2 and mice that received C18:3. Compared to C22:6, mice that received C18:2 or C18:3 correlated relatively poorly with mice given Wy14643.

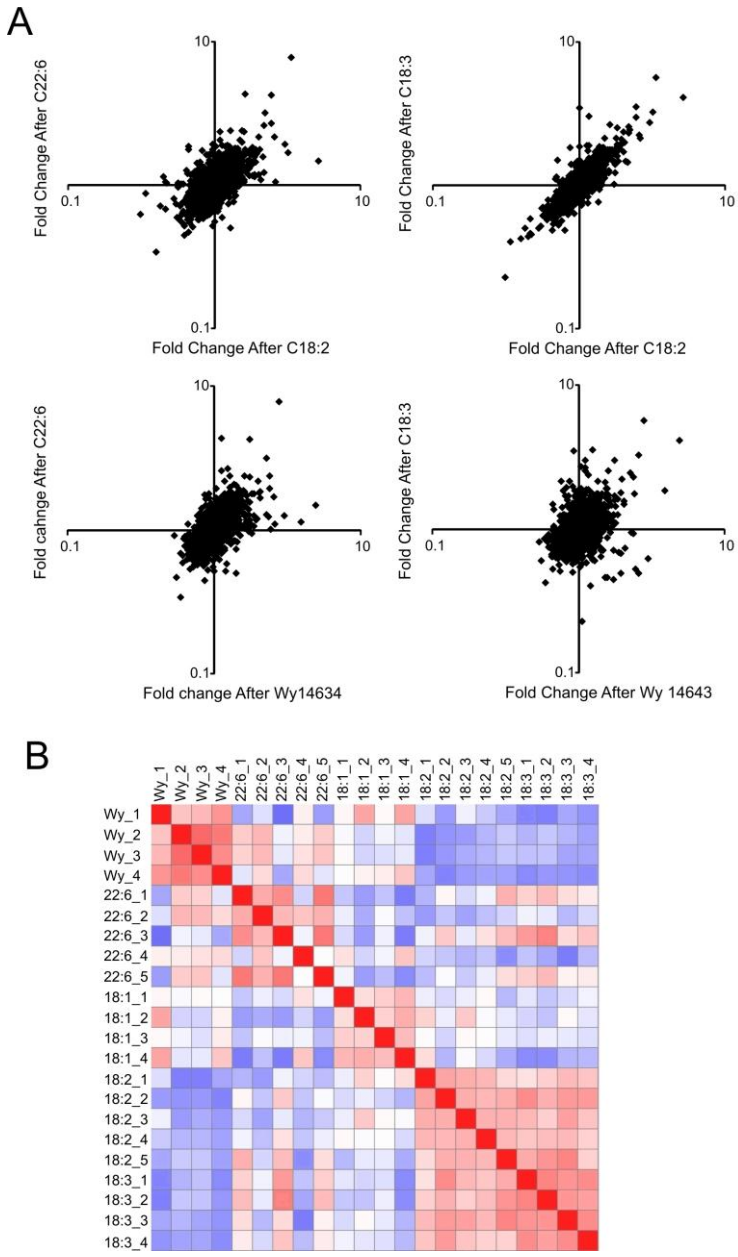


Figure 2: Similarity in gene regulation between different fatty acids. A. Scatter plots showing similarities in gene regulation between two treatments. The mean fold-change in expression of a gene by one treatment (relative to control treatment) is

expressed in one dimension (y-axis), and the mean fold-change in expression of the same gene by another treatment (relative to control treatment) is expressed in the other dimension (x-axis). The more pronounced the scatter, the lower the similarity in gene regulation. B. Correlation plot showing correlation in gene expression between individual mice. Signal log ratios were calculated between intensity values for individual animals and the mean intensity value of the control group. Signal log ratios of genes significantly regulated by at least one treatment were used as input for a correlation plot. Red indicates high correlation, blue indicates weak correlation.

To compare the effects of the various treatments, we determined the top 10 of genes most significantly up- or down-regulated by each treatment (Table 1). Some of the top upregulated genes were regulated by all five treatments (Zbtb16/PLZF) or by four treatments (Hmox1, Angptl4, Ucp3). Many of the commonly upregulated genes are involved in metabolic pathways (Hmgcs2, Acot1, Angptl4) or oxidative stress (Hmox1, Ucp3, Mt2). Genes that were consistently downregulated by Wy and fatty acids included the nuclear receptor Nurr1 (Nr4a1) and fibroblast growth factor 16 (Fgf16). To illustrate specific patterns of gene regulation, examples of genes that were regulated by all treatments, by all fatty acids, or by only one treatment are shown in Figure 3A. Only a small proportion of the genes were regulated by all five treatments or by all fatty acids (Figure 3B). Many more genes were either exclusively regulated by one particular treatment (Figure 3B) or shared between two treatments (data not shown).

Table 1: Top 10 of genes most significantly regulated by the various treatments ranked according to fold-change (FC). Genes regulated by more than one treatment are shown in bold.

	Wy			C226			C183			C182			C181		
	Entrez ID	gene name	FC	Entrez ID	gene name	FC	Entrez ID	gene name	FC	Entrez ID	gene name	FC	Entrez ID	gene name	FC
Upregulated	57875	**Angptl4**	5.0	15368	**Hmox1**	7.9	15368	**Hmox1**	7.4	57875	**Angptl4**	5.1	57875	**Angptl4**	5.7
	26897	**Acot1**	4.0	14229	**Fkbp5**	4.6	14229	**Fkbp5**	3.8	15368	**Hmox1**	4.0	15360	**Hmgcs2**	3.0
	15368	**Hmox1**	3.3	235320	**Zbtb16**	3.7	15360	**Hmgcs2**	3.8	15360	**Hmgcs2**	3.3	22229	**Ucp3**	2.9
	18787	Serpine1	3.0	320292	Rasgef1b	2.6	57875	**Angptl4**	3.7	14229	**Fkbp5**	2.8	14229	**Fkbp5**	2.5
	11492	Adam19	2.9	194231	Cnksr1	2.5	235320	**Zbtb16**	3.3	22229	**Ucp3**	2.6	235320	**Zbtb16**	2.5
	22229	**Ucp3**	2.8	17750	**Mt2**	2.5	16819	Lcn2	3.3	235320	**Zbtb16**	2.6	26897	**Acot1**	2.2
	320292	Rasgef1b	2.6	18787	Serpine1	2.4	17750	**Mt2**	2.9	17750	**Mt2**	2.2	100647	Upk3b	2.1
	15360	**Hmgcs2**	2.6	53608	Map3k6	2.4	235493	**BC031353**	2.7	12741	**Cldn5**	2.1	21847	Klf10	2.0
	235320	**Zbtb16**	2.6	226691	AI607873	2.2	22229	**Ucp3**	2.6	235493	**BC031353**	2.0	21345	Tagln	2.0
	213989	Tmem82	2.3	68487	Tmem140	2.1	241274	Pnpla7	2.4	71780	Isyna1	2.0	66180	Leprel4	2.0
Downregulated	60344	**Fign**	-2.1	80903	**Fgf16**	-3.3	15370	**Nr4a1**	-4.4	15370	**Nr4a1**	-3.3	15370	**Nr4a1**	-3.6
	22693	Zfp30	-2.1	23796	Aplnr	-2.3	80903	**Fgf16**	-3.1	18158	**Nppb**	-3.2	73333	Slc25a31	-1.8
	80903	**Fgf16**	-2.0	218763	**Lrrc3b**	-2.2	18158	**Nppb**	-2.7	80903	**Fgf16**	-2.6	81489	**Dnajb1**	-1.8
	218763	**Lrrc3b**	-1.9	170826	Ppargc1b	-2.0	27279	**Tnfrsf12a**	-2.3	228564	Frmd5	-2.3	12443	Ccnd1	-1.6
	235050	Zfp810	-1.7	27528	D0H4S114	-1.9	71699	**Slc41a3**	-2.3	71699	**Slc41a3**	-2.3	22256	Ung	-1.6
	242022	Frem2	-1.6	16716	Ky	-1.9	228564	Frmd5	-2.3	27279	**Tnfrsf12a**	-2.1	18626	Per1	-1.6
	54353	Skap2	-1.6	241303	Fam78a	-1.8	217166	Nr1d1	-2.2	109019	**Obfc2a**	-2.1	71699	**Slc41a3**	-1.6
	66952	2310030G06Rik	-1.5	67468	Mmd	-1.8	229599	Gm129	-2.1	106522	**Pkdcc**	-1.9	224860	Plcl2	-1.6
	54352	Irx5	-1.5	208177	Phldb2	-1.8	106522	**Pkdcc**	-2.1	229599	Gm129	-1.8	12767	Cxcr4	-1.6
	69150	Snx4	-1.5	60344	**Fign**	-1.8	81489	**Dnajb1**	-2.0	278279	Tmtc2	-1.8	109019	**Obfc2a**	-1.6

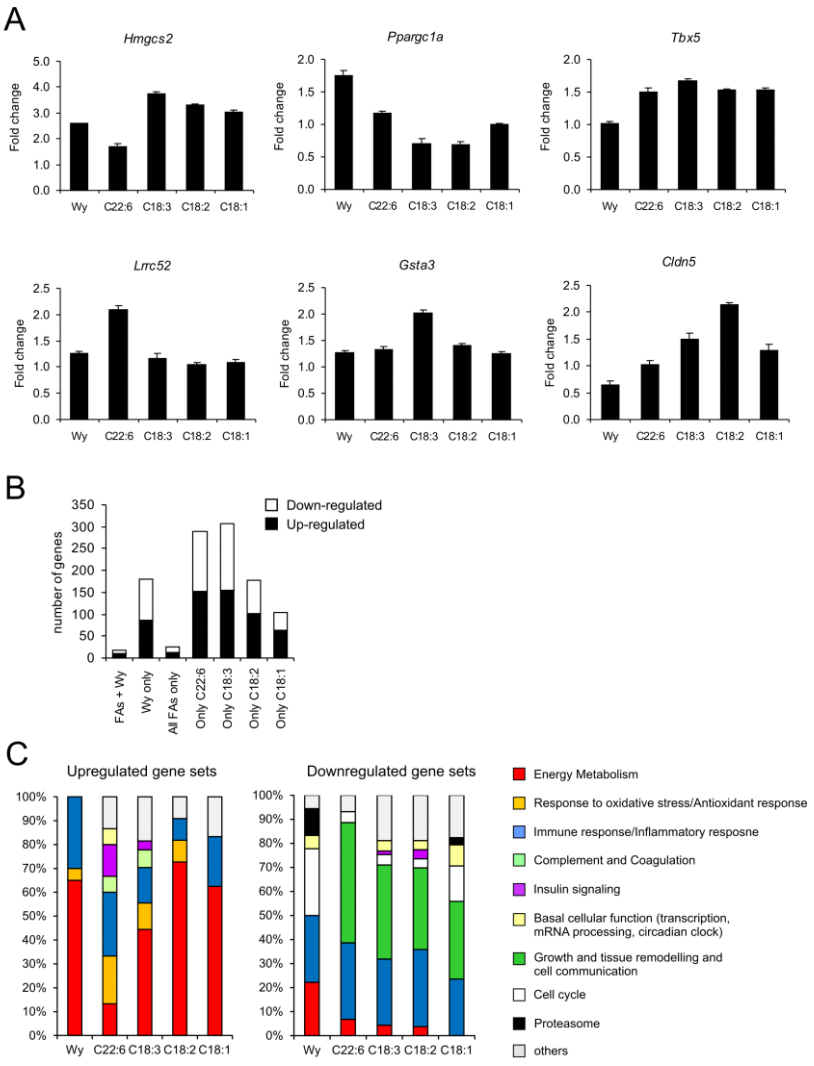


Figure 3: Fatty acid specific gene regulation in mouse heart. A. Changes in expression of selected genes in wildtype mice by the different treatments, illustrating gene regulation by all treatments (Hmgcs2), specific/selective regulation by Wy14643 (PGC1a), C22:6 (Lrrc52), C18:3 (Gsta3), C18:2 (Cldn5), and regulation by all fatty acids (Tbx5). B. Number of genes exclusively up- or down-regulated by one specific treatment or shared between one or more treatments ($P<0.01$). C. Comparative functional analysis of the whole genome effects of the various treatments. Gene sets up- and down-regulated by the various treatments as identified by GSEA ($P<0.05$) were

classified into broad functional categories. Results illustrate aberrant effect of C22:6 with respect to upregulated genes, and aberrant effect of Wy14643 with respect to downregulated genes.

We next investigated whether similarities in gene regulation between the various treatments were also observed at the level of pathways. To that end, Ingenuity pathway analysis was carried out on the changes in gene expression caused by each treatment. The results reveal that many pathways are commonly regulated by the various fatty acids, including several pathways related to metabolism of amino acids and fatty acids (Table 2). The number of pathways specifically regulated by one fatty acid was limited, with the exception of C22:6, which specifically regulated several pathways related to inflammation and cytokine/growth factor signalling. Similar data were obtained by Gene Set Enrichment Analysis (Figure 3C). Most of the gene sets enriched among genes unregulated by the various treatments were related to nutrient/energy metabolism, except for C22:6 which induced gene sets involved in a variety of biological functions. The special status of C22:6 was not evident among down-regulated gene sets. These data indicate that C22:6 induces the most diverse biological response in cardiac gene expression compared to the other fatty acids studied.

Table 2: Results of Ingenuity canonical pathway analysis considering only differentially expressed genes with a p-value<0.01.

Pathways regulated by at least three fatty acids
Butanoate Metabolism
Tryptophan Metabolism
Valine, Leucine and Isoleucine Degradation
PXR/RXR Activation
NRF2-mediated Oxidative Stress Response
RAR Activation
Fatty Acid Metabolism
LPS/IL-1 Mediated Inhibition of RXR Function
PTEN Signaling
Glutamate Metabolism
PI3K/AKT Signaling
Xenobiotic Metabolism Signaling
Aryl Hydrocarbon Receptor Signaling
TR/RXR Activation
VEGF Signaling
Pathways specific for 18:1
Eicosanoid Signaling
B Cell Development
Pyruvate Metabolism
Pathways specific for 18:3
Lysine Degradation
Nitrogen Metabolism
Tyrosine Metabolism
Glutathione Metabolism
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes
Pathways specific for 22:6
Acute Phase Response Signaling
Docosahexaenoic Acid (DHA) Signaling
Prolactin Signaling
NF-κB Signaling
p38 MAPK Signaling
IGF-1 Signaling
HMGB1 Signaling
ERK5 Signaling
Rac Signaling
Pathways specific for Wy14643
Intrinsic Prothrombin Activation Pathway

Role of PPARα in gene regulation by fatty acids

Several of the top regulated genes by fatty acids are target genes of PPARα (Table 1). In addition, significant overlap was observed between gene regulation by fatty acids and Wy14643 (Figure 1C, Figure 2A). These results suggest that PPARα plays a role in gene regulation by dietary fatty acids in heart in vivo. To better define the role of PPARα in heart, we first determined the impact of PPARα deletion on basal whole genome gene expression in the heart using PPARα-/- mice. Using a cut-off of fold-change >1.2 and P<0.01, we found that 294 genes showed elevated expression and 297 genes showed reduced expression in PPARα-/- mice (Figure 4A). Many of the top downregulated genes represented known targets of PPARα involved in lipid metabolism, including Acot1, Acot2, Ucp3, Gpam and Slc22a5 (Figure 4B). The functional roles of the top upregulated genes in PPARα-/- mice were more diverse, including genes involved in lipid metabolism (Acsl6, Angptl4) but also genes involved in immune response, cell cycle, and oxidative stress response. Pathways differentially expressed between wild-type and PPARα-/- mice according to Ingenuity pathways analysis fell into four main categories: lipid metabolism, amino acid metabolism, carbohydrate metabolism, and inflammation/immunity, reflecting the established role of PPARα in these processes (Figure 4C).

We next set out to investigate the importance of PPARα in gene regulation by dietary fatty acids in the heart. Regulation of a particular gene by dietary fatty acids or synthetic agonists was defined as PPARα-dependent when expression was statistically significantly up- or downregulated in WT but not PPARα-/- mice. As expected given the high specificity of the Wy14643 compound, gene regulation by WY14643 was almost completely dependent on PPARα, which was equally observed for up- or down-regulated genes (Figure 5A,B). The importance of PPARα in gene regulation by dietary fatty acids was less pronounced but still remarkably high. Interestingly, a very uniform picture was observed for the three PUFAs studied. Specific examples of genes showing clear PPARα-dependent or –independent gene regulation by dietary fatty acids are shownin Figure 5C. Induction of Ucp3 and Acot1 expression by Wy14643 and fatty acids was entirely dependent on PPARα, whereas induction of Zbtb16/PLZF was completely independent of PPARα. Expression of Pdk4 and Hmox1 showed a mixed picture: Whereas induction by Wy14643 was entirely

PPARα-dependent, this was not or only partially observed for the various fatty acids. Taken together, these data indicate that PPARα plays a major role in gene regulation by dietary fatty acids in heart, although other mechanisms contribute as well.

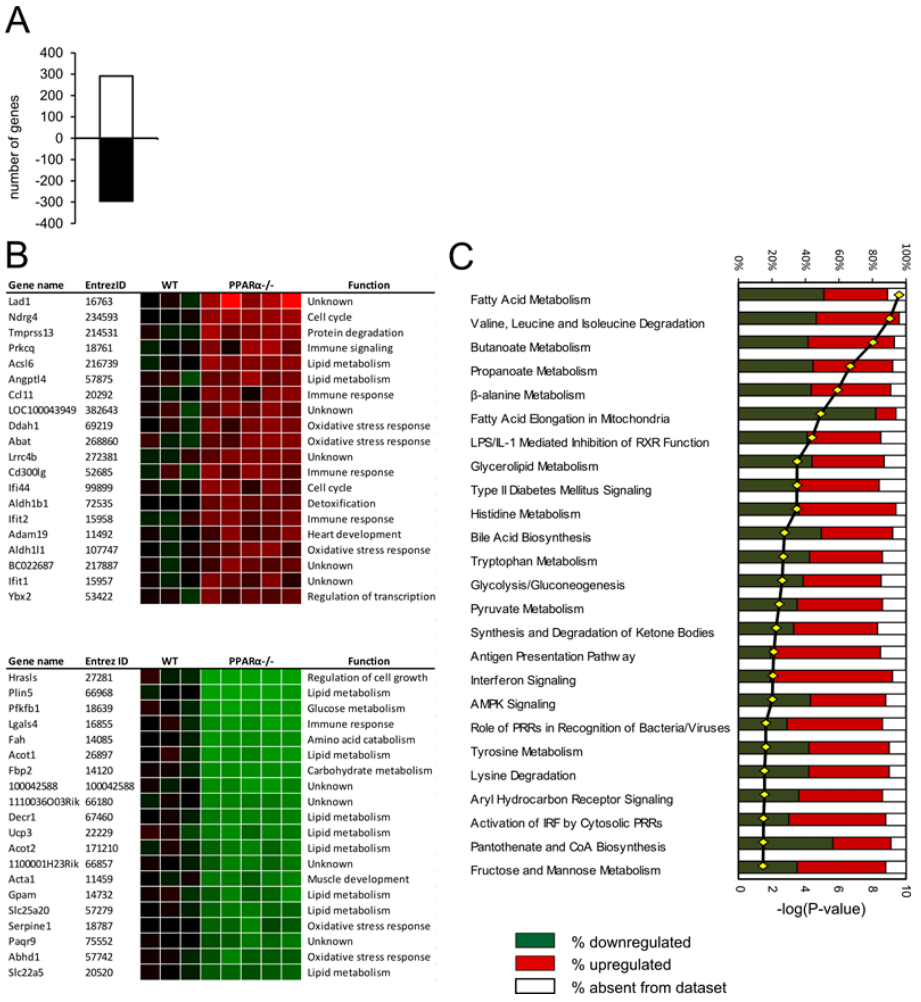


Figure 4: Effect of PPARα deletion on gene expression in mouse heart. A. Number of genes upregulated or downregulated in PPARα-/- mice compared to wildtype mice at baseline (control treatment, carboxymethylcellulose) according to P<0.01 and mean fold change>1.2. B. Heatmap showing changes in expression of the 20 genes exhibiting the highest mean fold increase (top panel) and decrease (lower panel) in PPARα-/- mice

compared to wildtype mice at baseline. Each column represents one individual mouse. Mean fold change of wildtype mice was set at 1.0. C. Ingenuity canonical pathway analysis of changes in gene expression between wildtype and PPARα-/- mice at baseline. Line reflects –log(P-value). The coloured bars reflect the percentage of genes within a particular pathway that was downregulated (green bar), upregulated (red bar) or absent from the dataset (white bars).

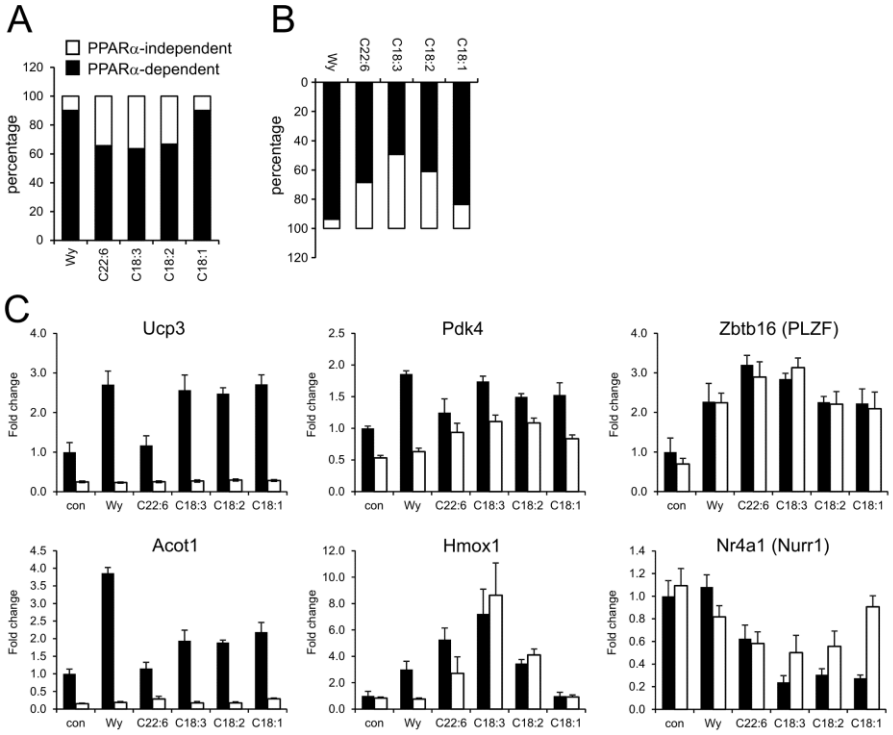


Figure 5: Role of PPARα in gene regulation by fatty acids in the heart. Bars show the percentage of genes upregulated A. or downregulated B. in the different treatment groups in a PPARα-dependent manner (black bars, changed in wildtype but not in PPARα-/- mice), or PPARα-independent manner (white bars, changed in wildtype and PPARα-/- mice). Genes were considered statistically significantly regulated if $P<0.01$. C. Changes in expression of selected genes by the various treatments in wildtype and PPARα-/- mice, illustrating complete PPARα-dependent gene regulation (Ucp3, Acot1), partial PPARα-dependent up- (Pdk4, Hmox1) and down- (Nr4a1) regulation, and PPARα-independent regulation (Zbtb16). Error bars represent SEM.

Role of PPARα in cardiac gene regulation

As mentioned above, gene regulation by Wy14643 in heart was entirely mediated by PPARα. To further understand the impact of PPARα on cardiac gene regulation, we carefully analyzed the whole genome effects of short term or long term administration of Wy14643, focusing on metabolism and immunity related genes. Genes were classified according to 1) differential expression in PPARα-/- mice in the absence of Wy14643, 2) induction after short-term Wy14643 treatment, 3) induction after long-term Wy14643 treatment. A limited number of metabolic genes were regulated in all three conditions, representing robust PPARα targets such as Ucp3, Hmgcs2, Acot1 and Ehhadh (Appendix table 1). Interestingly, many genes classified as PPARα targets based on literature were not induced by Wy14643, even though their expression was decreased in the PPARα-/- mice. Conversely, a considerable number of genes was induced by Wy14643 but was unaltered in the PPARα-/- mice at baseline. The former could be classified as baseline PPARα targets whereas the latter may be classified as inducible targets.

A large number of genes altered by PPARα deletion or by Wy14643 was related to inflammation and immunity (Appendix table 2). Although the maximal magnitude of fold-change of inflammation/immunity related genes was less compared to metabolism-related genes, the number of genes altered was at least as high, demonstrating the impact of PPARα on inflammation and immunity in heart. One gene that was robustly regulated by PPARα in all three conditions was Serpine1, also known as plasminogen activator inhibitor 1 (PAI-1). A complete list of genes can be found in Appendix table 1,2.

Discussion

In this study we set out to study the impact of individual dietary unsaturated fatty acids on whole genome gene regulation in the intact mouse heart and assess the role of PPARα. Our findings can be summarized as follows: 1) C18:3 had the most pronounced effect on cardiac gene expression. 2) The largest overall similarity in gene regulation was observed between C18:2 and C18:3, which was equally true for genes regulated in a PPARα-dependent and independent manner (Appendix Figure 1). The synthetic PPARα agonist Wy14643 and C22:6 also showed marked similarity in gene regulation. 3)

Many genes were regulated by one particular treatment only. Genes regulated by one particular treatment showed large functional divergence. 4) The majority of genes responding to fatty acid treatment were regulated in a PPARα dependent manner, emphasizing the importance of PPARα in mediating transcriptional regulation by fatty acids in the heart. 5) Several genes were robustly regulated by all or many of the fatty acids studied. A number of these genes are well-described targets of PPARs whereas others seem to be regulated via a different mechanism.

Dietary fatty acids enter the circulation packaged in triglyceride-rich chylomicron particles and enter the heart after lipolytic processing by lipoprotein lipase. The importance of hydrolysis of triglyceride-rich lipoproteins for generating endogenous ligands for cardiac PPARα has been recognized [9, 37]. Our data extend these previous findings and show that dietary fatty acids cause marked induction of several PPARα target genes. In addition, it is shown that dietary fatty acids –and therefore the lipolysis pathway- also leads to gene regulation via other signaling routes. Part of the PPARα-independent regulation may occur via PPARβ/δ (e.g. Pdk4, Angptl4), but other mediators are likely also involved, including Nrf2. Nrf2 is a transcription factor that is activated by unsaturated fatty acids after their conversion to electrophilic oxo or nitro derivatives, and stimulates anti-oxidant gene regulation [15]. An important property of PUFAs is that they not only serve as metabolic substrates, but are also sources of lipotoxic derivatives such as lipid peroxides and reactive oxygen species as secondary products of fatty acid oxidation. Thus, the effects of (dietary) PUFA on expression of genes involved in the oxidative stress response are likely mediated by specific fatty acid oxidation products via NRF2-dependent signaling. Activation of Nrf2 may explain why a large number of genes induced by dietary unsaturated fatty acids are involved in oxidative stress response. Recent data point to cross talk between regulation of lipid metabolism and the oxidative stress response. It was found that the anti-oxidant transcription factor Nrf2, besides governing oxidative stress target genes, also alters expression of numerous genes involved in lipid metabolism [17]. Conversely, PPARs, which have a primary function in lipid metabolism, directly regulate expression of a number of oxidative stress genes, exemplified by Hmox1 [1, 20, 21].

One surprising gene that was markedly and consistently induced by all fatty acids was Zbtb16/PLZF, encoding a transcription factor that controls the

development of effector functions in Natural Killer T (NKT) cells [2]. NKT cells represent an unique subset of lymphocytes that are reactive to so called lipid antigens, which include a broad range of microbial lipids that are unique structures of specific microorganisms. Based on its sensitive regulation by fatty acids, one could speculate whether Zbtb16/PLZF may link dietary lipids to NKT cell function.

Previously, we studied the impact of dietary fatty acids on gene expression in the liver under the same experimental settings [26]. When expressed per gram organ weight, the liver and heart both exhibit very high and very similar rates of fatty acid uptake from TG-rich lipoproteins [26]. Remarkably, the percentage of genes regulated by fatty acids independently of PPARα is considerably higher in heart compared to liver. Additionally, the panel and functional categories of genes regulated by fatty acids in the liver mostly reflects regulation of lipid metabolism, which is less the case in heart. Furthermore, relative inductions of PPARα target genes by dietary fatty acids are less pronounced in heart. Together, these data clearly demonstrate a less dominant role of PPARα in cardiac gene regulation by dietary fatty acids compared to the liver. Apart from differences in PPARα expression level between heart and liver, this finding may be related to differences in the route of uptake of fatty acids between the two tissues in the postprandial state. Whereas the heart takes up dietary fatty acids as non-esterified fatty acids after LPL-mediated hydrolysis, the liver internalizes dietary fatty acids as TG within chylomicron remnant particles [3, 31]. The ability of fatty acids to directly activate PPARα may depend on the form in which they are presented to the cell [7, 27]. Free fatty acids may need to be converted to TG and undergo subsequent hydrolysis before they can activate PPARα. Alternatively, it is possible that within the heart fatty acids are more efficiently shuttled towards oxidative pathways to meet the high energetic demands of the contracting heart.

It can be argued that direct comparison of the various fatty acid treatments requires comparable levels of cardiac uptake of the different fatty acids. Unfortunately, the unavailability of radioactive triglycerides except triolein makes it impossible to get precise information on the kinetic behaviour of the fatty acids used. Our previous study did not reveal major differences in metabolic processing of dietary fat between WT and PPARα-/- mice and between different dietary fatty acids [26]. Even while assuming some differences exist in kinetic behaviour between the fatty acids used, they are

unlikely to account for the major qualitative differences in gene regulation between the fatty acids studied. It is difficult to directly compare our results with in vitro data. Lockrigde and colleagues performed microarray analysis on isolated cardiomyocytes treated with various fatty acids [22]. Remarkable, the number of genes altered by saturated fatty acids was much higher compared to unsaturated fatty acids. One of the limitations of this study is that we were unable to investigate the effects of saturated fatty acids, as triglycerides composed of saturated fatty acids are solid at body temperature.

Differential gene regulation by the various fatty acids via PPARα may be hypothesized to occur via the Selective PPAR Modulator (SPARM) concept, in which different PPARα ligands may not only give rise to quantitative differences but also cause qualitative differences in gene regulation due to the differential recruitment and release of coactivators and corepressors, respectively. While we previously found support for differential coactivator recruitment between Wy14643 and DHA, no differences were found between various fatty acids [26].

Most of our understanding of the role of cardiac PPARα is based on studies of transgenic mice overexpressing PPARα in the heart. These mice are characterized by marked induction of genes involved in cardiac fat oxidation but also fatty acid uptake, resulting in cardiac lipid accumulation and subsequent cardiomyopathy [4, 6, 11]. A few genes within these pathways have been identified as direct targets of PPARα in heart. Our data underscore the importance of PPARα in lipid metabolism in the heart, and reveal a large resemblance between the PPARα transcriptome in heart and liver. Similar to the dietary fatty acids, Wy14643 was surprisingly much less potent in inducing gene expression in heart compared to liver. A possible explanation is that Wy14643 is taken up more poorly by the heart, although no actual data are available to support this notion.

Deletion of PPARα had a marked effect on cardiac gene regulation after a 10 hour fast, representing the baseline condition, which suggests that 1) PPARα has a high constitutive activity, or 2) PPARα is already mostly ligand-activated after a 10 hour fast [33], which would be consistent with the high rate of fatty acid uptake under those conditions. Although expression of numerous genes involved in lipid metabolism was markedly reduced in PPARα-/- mice, this does not result in cardiac dysfunction, at least in the absence of an additional stressor [28].

Apart from metabolism related genes, many genes involved in inflammation/immunity were altered upon PPARα deletion. Our findings are supported by recent studies describing differential expression of immunity and cellular defence related genes in hearts of PPARα-/- mice compared to wildtype mice [28, 29]. Many of those genes are regulated via NF-κB, pointing towards transrepression by PPARα. This anti-inflammatory effect of PPARα may be especially relevant in the context of cardiac hypertrophy, which is characterized by induction of inflammatory pathways.

In conclusion, our study provides the first comprehensive analysis of the acute effects of dietary fatty acids on gene expression in the heart. The data demonstrate the importance of PPARα in mediating gene regulation by dietary fatty acids in the heart.

Sources of Funding

This study was supported by the Nutrigenomics Consortium, TI Food and Nutrition, Marie Curie Research Training Network NucSys, and the Netherlands Heart Foundation (2007B046).

References

1. Ali F, Ali NS, Bauer A, Boyle JJ, Hamdulay SS, Haskard DO, Randi AM, and Mason JC. PPARβ/δ and PGC1alpha act cooperatively to induce heme oxygenase-1 and enhance vascular endothelial cell resistance to stress. Cardiovasc Res 85: 701-710, 2010.
2. Alonzo ES, and Sant'Angelo DB. Development of PLZF-expressing innate T cells. Curr Opin Immunol 23: 220-227, 2011.
3. Augustus AS, Kako Y, Yagyu H, and Goldberg IJ. Routes of FA delivery to cardiac muscle: modulation of lipoprotein lipolysis alters uptake of TG-derived FA. Am J Physiol Endocrinol Metab 284: E331-339, 2003.
4. Banke NH, Wende AR, Leone TC, O'Donnell JM, Abel ED, Kelly DP, and Lewandowski ED. Preferential oxidation of triacylglyceride-derived fatty acids in heart is augmented by the nuclear receptor PPARα. Circ Res 107: 233-241, 2010.
5. Brandt JM, Djouadi F, and Kelly DP. Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. J Biol Chem 273: 23786-23792, 1998.
6. Burkart EM, Sambandam N, Han X, Gross RW, Courtois M, Gierasch CM, Shoghi K, Welch MJ, and Kelly DP. Nuclear receptors PPARbeta/delta and PPARα direct distinct metabolic regulatory programs in the mouse heart. J Clin Invest 117: 3930-3939, 2007.
7. Chakravarthy MV, Pan Z, Zhu Y, Tordjman K, Schneider JG, Coleman T, Turk J, and Semenkovich CF. "New" hepatic fat activates PPARα to maintain glucose, lipid, and cholesterol homeostasis. Cell Metab 1: 309-322, 2005.
8. Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, Watson SJ, and Meng F. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res 33: e175, 2005.
9. Duncan JG, Bharadwaj KG, Fong JL, Mitra R, Sambandam N, Courtois MR, Lavine KJ, Goldberg IJ, and Kelly DP. Rescue of cardiomyopathy in peroxisome proliferator-activated receptor-alpha transgenic mice by deletion of lipoprotein

lipase identifies sources of cardiac lipids and peroxisome proliferator-activated receptor-alpha activators. Circulation 121: 426-435, 2010.

10. Durgan DJ, Smith JK, Hotze MA, Egbejimi O, Cuthbert KD, Zaha VG, Dyck JR, Abel ED, and Young ME. Distinct transcriptional regulation of long-chain acyl-CoA synthetase isoforms and cytosolic thioesterase 1 in the rodent heart by fatty acids and insulin. Am J Physiol Heart Circ Physiol 290: H2480-2497, 2006.
11. Finck BN, Lehman JJ, Leone TC, Welch MJ, Bennett MJ, Kovacs A, Han X, Gross RW, Kozak R, Lopaschuk GD, and Kelly DP. The cardiac phenotype induced by PPARα overexpression mimics that caused by diabetes mellitus. J Clin Invest 109: 121-130, 2002.
12. Forman BM, Chen J, and Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A 94: 4312-4317, 1997.
13. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, and Zhang J. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5: R80, 2004.
14. Georgiadi A, Lichtenstein L, Degenhardt T, Boekschoten MV, van Bilsen M, Desvergne B, Muller M, and Kersten S. Induction of cardiac Angptl4 by dietary fatty acids is mediated by peroxisome proliferator-activated receptor beta/delta and protects against fatty acid-induced oxidative stress. Circ Res 106: 1712-1721, 2010.
15. Groeger AL, Cipollina C, Cole MP, Woodcock SR, Bonacci G, Rudolph TK, Rudolph V, Freeman BA, and Schopfer FJ. Cyclooxygenase-2 generates anti-inflammatory mediators from omega-3 fatty acids. Nat Chem Biol 6: 433-441, 2010.
16. Kersten S, Desvergne B, and Wahli W. Roles of PPARs in health and disease. Nature 405: 421-424, 2000.
17. Kitteringham NR, Abdullah A, Walsh J, Randle L, Jenkins RE, Sison R, Goldring CE, Powell H, Sanderson C, Williams S, Higgins L, Yamamoto M, Hayes J, and Park BK. Proteomic analysis of Nrf2 deficient transgenic mice reveals cellular defence and lipid metabolism as primary Nrf2-dependent pathways in the liver. J Proteomics 73: 1612-1631, 2010.
18. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, and Lehmann JM. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A 94: 4318-4323, 1997.
19. Krey G, Braissant O, L'Horset F, Kalkhoven E, Perroud M, Parker MG, and Wahli W. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of

peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. Mol Endocrinol 11: 779-791, 1997.

20. Kronke G, Kadl A, Ikonomu E, Bluml S, Furnkranz A, Sarembock IJ, Bochkov VN, Exner M, Binder BR, and Leitinger N. Expression of heme oxygenase-1 in human vascular cells is regulated by peroxisome proliferator-activated receptors. Arterioscler Thromb Vasc Biol 27: 1276-1282, 2007.
21. Lin H, Yu CH, Jen CY, Cheng CF, Chou Y, Chang CC, and Juan SH. Adiponectin-mediated heme oxygenase-1 induction protects against iron-induced liver injury via a PPARα dependent mechanism. Am J Pathol 177: 1697-1709, 2010.
22. Lockridge JB, Sailors ML, Durgan DJ, Egbejimi O, Jeong WJ, Bray MS, Stanley WC, and Young ME. Bioinformatic profiling of the transcriptional response of adult rat cardiomyocytes to distinct fatty acids. J Lipid Res 49: 1395-1408, 2008.
23. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, and Stanley WC. Myocardial fatty acid metabolism in health and disease. Physiol Rev 90: 207-258, 2010.
24. Park TS, Yamashita H, Blaner WS, and Goldberg IJ. Lipids in the heart: a source of fuel and a source of toxins. Curr Opin Lipidol 18: 277-282, 2007.
25. Sampath H, and Ntambi JM. Polyunsaturated fatty acid regulation of genes of lipid metabolism. Annu Rev Nutr 25: 317-340, 2005.
26. Sanderson LM, de Groot PJ, Hooiveld GJ, Koppen A, Kalkhoven E, Muller M, and Kersten S. Effect of synthetic dietary triglycerides: a novel research paradigm for nutrigenomics. PLoS ONE 3: e1681, 2008.
27. Sanderson LM, Degenhardt T, Koppen A, Kalkhoven E, Desvergne B, Muller M, and Kersten S. Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) but not PPARα serves as a plasma free fatty acid sensor in liver. Mol Cell Biol 29: 6257-6267, 2009.
28. Smeets PJ, de Vogel-van den Bosch HM, Willemsen PH, Stassen AP, Ayoubi T, Van der Vusse GJ, and van Bilsen M. Transcriptomic analysis of PPAR{alpha}-dependent alterations during cardiac hypertrophy. Physiol Genomics 2008.
29. Smeets PJ, Teunissen BE, Planavila A, de Vogel-van den Bosch H, Willemsen PH, van der Vusse GJ, and van Bilsen M. Inflammatory Pathways Are Activated during Cardiomyocyte Hypertrophy and Attenuated by Peroxisome Proliferator-activated Receptors PPAR{alpha} and PPAR{delta}. J Biol Chem 283: 29109-29118, 2008.
30. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article3, 2004.
31. Teusink B, Voshol PJ, Dahlmans VE, Rensen PC, Pijl H, Romijn JA, and Havekes LM. Contribution of fatty acids released from lipolysis of plasma triglycerides to total plasma fatty acid flux and tissue-specific fatty acid uptake. Diabetes 52: 614-620, 2003.

32. van der Lee KA, Vork MM, De Vries JE, Willemsen PH, Glatz JF, Reneman RS, Van der Vusse GJ, and Van Bilsen M. Long-chain fatty acid-induced changes in gene expression in neonatal cardiac myocytes. J Lipid Res 41: 41-47, 2000.
33. Van der Lee KA, Willemsen PH, Samec S, Seydoux J, Dulloo AG, Pelsers MM, Glatz JF, Van der Vusse GJ, and Van Bilsen M. Fasting-induced changes in the expression of genes controlling substrate metabolism in the rat heart. J Lipid Res 42: 1752-1758, 2001.
34. Van Der Lee KA, Willemsen PH, Van Der Vusse GJ, and Van Bilsen M. Effects of fatty acids on uncoupling protein-2 expression in the rat heart. FASEB J 14: 495-502, 2000.
35. van der Meer RW, Hammer S, Smit JW, Frolich M, Bax JJ, Diamant M, Rijzewijk LJ, de Roos A, Romijn JA, and Lamb HJ. Short-term caloric restriction induces accumulation of myocardial triglycerides and decreases left ventricular diastolic function in healthy subjects. Diabetes 56: 2849-2853, 2007.
36. Wu Z, Irizarry R, Gentleman R, Martinez-Murillo F, and Spencer F. A Model-Based Background Adjustment for Oligonucleotide Expression Arrays. Journal of the American Statistical Association 99: 909–917, 2004.
37. Ziouzenkova O, Perrey S, Asatryan L, Hwang J, MacNaul KL, Moller DE, Rader DJ, Sevanian A, Zechner R, Hoefler G, and Plutzky J. Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an antiinflammatory role for lipoprotein lipase. Proc Natl Acad Sci U S A 100: 2730-2735, 2003.

APPENDIX

Appendix Table1: Table of genes altered by PPARα deletion or by Wy14643 and they are related to energy metabolism

List of genes altered in PPARα-/- mice compared to wildtype mice at baseline (column C)
List of genes altered in wildtype mice treated with Wy14643 for 6 hours compared to control treated wildtype mice (column D)
List of genes altered in wildtype mice treated with Wy14643 for 5 days compared to control treated wildtype mice (column E)
Specific criteria for inclusion are shown. List includes genes related to immunity in broadest sense based on Ingenuity canonical pathways
Genes are separated into groups based on pattern of regulation. Abs=absent from array

		Criteria			
		FC1.2 _P value<0.01	FC1.2 _P value<0.01	FC1.2_ no P value	
Gene Name	***Gene ID***	***PPARα-/- vs WT***	***WT-Wy 6h vs WT***	***WT-Wy 5d vs WT***	***Description***
Acot1	26897	-6.3	4.0	5.5	acyl-CoA thioesterase 1
Ucp3	22229	-3.9	2.8	4.7	uncoupling protein 3 (mitochondrial, proton carrier)
Acot2	171210	-3.6	1.8	3.0	acyl-CoA thioesterase 2
Pdk4	27273	-1.9	1.9	3.2	pyruvate dehydrogenase kinase, isoenzyme 4
Ehhadh	74147	-1.5	1.5	4.2	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
Hmgcs2	15360	-1.5	2.6	8.1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2
Lipe	16890	-1.5	1.6	2.3	lipase, hormone sensitive
Cpt1b	12895	-1.4	1.2	1.3	carnitine palmitoyltransferase 1b, muscle
Peci	23986	-1.4	1.1	1.4	peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase
Slc25a29	214663	1.3	-1.2	1.5	solute carrier family 25 (mitochondrial carrier, palmitoylcarnitine transporter)
Gpam	14732	-3.4	1.8	Abs	glycerol-3-phosphate acyltransferase, mitochondrial
Slc22a5	20520	-2.7	1.5	Abs	solute carrier family 22 (organic cation transporter), member 5
Mlycd	56690	-1.8	1.7	Abs	malonyl-CoA decarboxylase
Angptl4	57875	2.3	5.0	Abs	angiopoietin-like 4
Slc27a2	26458	1.2	1.5	-	solute carrier family 27 (fatty acid transporter), member 2
Plin5	66968	-10.0	-	1.3	perilipin 5
Decr1	67460	-4.1	-	1.8	2,4-dienoyl CoA reductase 1, mitochondrial
Slc25a20	57279	-3.3	-	1.6	solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase)
Cpt2	12896	-2.4	-	1.5	carnitine palmitoyltransferase 2
Ech1	51798	-2.3	-	1.5	enoyl coenzyme A hydratase 1, peroxisomal
Etfdh	66841	-2.1	-	1.2	electron transferring flavoprotein, dehydrogenase
Acadvl	11370	-1.8	-	1.3	acyl-Coenzyme A dehydrogenase, very long chain
Acads	11409	-1.8	-	1.2	acyl-Coenzyme A dehydrogenase, short chain
Gyk	14933	-1.7	-	1.4	glycerol kinase
Npc1	18145	-1.7	-	1.3	Niemann Pick type C1
Dgat2	67800	-1.6	-	2.2	diacylglycerol O-acyltransferase 2
Aldh9a1	56752	-1.6	-	1.7	aldehyde dehydrogenase 9, subfamily A1
Cidea	12683	-1.6	-	2.2	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A
Hsd17b4	15488	-1.6	-	1.3	hydroxysteroid (17-beta) dehydrogenase 4
Acadm	11364	-1.6	-	1.2	acyl-Coenzyme A dehydrogenase, medium chain
Acaa2	52538	-1.6	-	1.3	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)
Dci	13177	-1.4	-	1.7	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenyme A isomerase)
Hadha	97212	-1.4	-	1.5	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase
Acox1	11430	-1.4	-	1.5	acyl-Coenzyme A oxidase 1, palmitoyl
Txnip	56338	-1.2	-	2.6	thioredoxin interacting protein
Ucp2	22228	1.3	-	1.6	uncoupling protein 2 (mitochondrial, proton carrier)

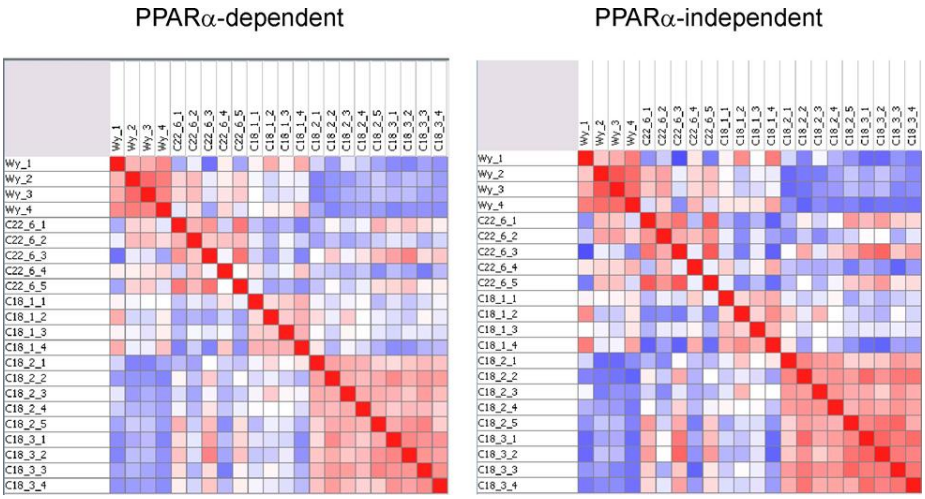
Ppara	19013	-2.6	-	-	peroxisome proliferator activated receptor alpha
Angptl3	30924	-1.9	-	-	angiopoietin-like 3
Me1	17436	-1.9	-	-	malic enzyme 1, NADP(+)-dependent, cytosolic
Scd1	20249	-1.6	-	-	stearoyl-Coenzyme A desaturase 1
Acsl1	14081	-1.6	-	Abs	acyl-CoA synthetase long-chain family member 1
Mttp	17777	-1.6	-	Abs	microsomal triglyceride transfer protein
Fdft1	14137	-1.5	-	Abs	farnesyl diphosphate farnesyl transferase 1
Scarb2	12492	-1.5	-	Abs	scavenger receptor class B, member 2
Fads1	76267	-1.4	-	Abs	fatty acid desaturase 1
Rab9	56382	-1.3	-	-	RAB9, member RAS oncogene family
Pnpla2	66853	-1.3	-	Abs	patatin-like phospholipase domain containing 2
Acat1	110446	-1.3	-	-	acetyl-Coenzyme A acetyltransferase 1
Adipor2	68465	-1.3	-	-	adiponectin receptor 2
Vldlr	22359	-1.2	-	Abs	very low density lipoprotein receptor
Cd36	12491	-1.2	-	-	CD36 antigen
Fabp4	11770	1.3	-	Abs	fatty acid binding protein 4, adipocyte
Lipa	16889	1.3	-	-	lysosomal acid lipase A
Lpin1	14245	1.3	-	Abs	lipin 1
Mgll	23945	1.3	-	Abs	monoglyceride lipase
Osbpl3	71720	1.4	-	-	oxysterol binding protein-like 3
Osbpl1a	64291	-	-1.3	Abs	oxysterol binding protein-like 1A
Cav3	12391	-	-1.2	-	caveolin 3
Aacs	78894	-	1.2	-	acetoacetyl-CoA synthetase
Agpat3	28169	-	1.3	Abs	1-acylglycerol-3-phosphate O-acyltransferase 3
Cidec	14311	-	1.4	-	cell death-inducing DFFA-like effector c
Ppargc1a	19017	-	1.7	Abs	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
G0s2	14373	-	-	-1.7	G0/G1 switch gene 2
Ces3	104158	-	-	-1.7	carboxylesterase 3
Fabp6	16204	-	-	-1.4	fatty acid binding protein 6, ileal (gastrotropin)
Soat2	223920	-	-	-1.4	sterol O-acyltransferase 2
Abcg8	67470	-	-	-1.4	ATP-binding cassette, sub-family G (WHITE), member 8
Cyp4f16	70101	-	-	-1.4	cytochrome P450, family 4, subfamily f, polypeptide 16
Acot11	329910	-	-	-1.3	acyl-CoA thioesterase 11
Ces1	12623	-	-	-1.3	carboxylesterase 1
OSBPL8	237542	-	-	-1.3	oxysterol binding protein-like 8
Acot10	64833	-	-	-1.3	acyl-CoA thioesterase 10
Osbpl6	99031	-	-	-1.3	oxysterol binding protein-like 6
Acot5	217698	-	-	-1.3	acyl-CoA thioesterase 5
Agpat4	68262	-	-	-1.2	1-acylglycerol-3-phosphate O-acyltransferase 4
OSBPL5	79196	-	-	-1.2	oxysterol binding protein-like 5
Pmvk	68603	-	-	-1.2	phosphomevalonate kinase
Gpsn2	106529	-	-	-1.2	glycoprotein, synaptic 2
Elovl4	83603	-	-	-1.2	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4
Hacl1	56794	-	-	-1.2	2-hydroxyacyl-CoA lyase 1
Apoh	11818	-	-	1.2	apolipoprotein H
Elovl3	12686	-	-	1.2	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3
Osbpl2	228983	-	-	1.2	oxysterol binding protein-like 2
Pltp	18830	-	-	1.2	phospholipid transfer protein
Fasn	14104	-	-	1.2	fatty acid synthase
Acsl5	433256	-	-	1.2	acyl-CoA synthetase long-chain family member 5
Scarb1	20778	-	-	1.3	scavenger receptor class B, member 1
Scd2	20250	-	-	1.3	stearoyl-Coenzyme A desaturase 2
Hmgcl	15356	-	-	1.3	3-hydroxy-3-methylglutaryl-Coenzyme A lyase
M6prbp1	66905	-	-	1.3	mannose-6-phosphate receptor binding protein 1
Abcg4	192663	-	-	1.3	ATP-binding cassette, sub-family G (WHITE), member 4
Acsm1	117147	-	-	1.3	acyl-CoA synthetase medium-chain family member 1
Crat	12908	-	-	1.3	carnitine acetyltransferase
Slc27a5	26459	-	-	1.3	solute carrier family 27 (fatty acid transporter), member 5
Adfp	11520	-	-	1.3	adipose differentiation related protein
S3-12	57435	-	-	1.3	plasma membrane associated protein, S3-12
Slc25a10	27376	-	-	1.3	solute carrier family 25 (mitochondrial carrier, dicarboxylate transporter)
Agpat1	55979	-	-	1.3	1-acylglycerol-3-phosphate O-acyltransferase 1
Abca1	11303	-	-	1.3	ATP-binding cassette, sub-family A (ABC1), member 1
Npc1l1	237636	-	-	1.3	NPC1-like 1
Apoc2	11813	-	-	1.4	apolipoprotein C-II
Cpt1a	12894	-	-	1.4	carnitine palmitoyltransferase 1a, liver
Dhcr7	13360	-	-	1.4	7-dehydrocholesterol reductase
Decr2	26378	-	-	1.4	2-4-dienoyl-Coenzyme A reductase 2, peroxisomal
Apob	238055	-	-	1.5	apolipoprotein B
Scd4	329065	-	-	1.5	stearoyl-coenzyme A desaturase 4
Apoc1	11812	-	-	1.5	apolipoprotein C-I
Abcg1	11307	-	-	1.6	ATP-binding cassette, sub-family G (WHITE), member 1
Gpd2	14571	-	-	1.6	glycerol phosphate dehydrogenase 2, mitochondrial
Abcd2	26874	-	-	1.7	ATP-binding cassette, sub-family D (ALD), member 2
Srebf1	20787	-	-	2.4	sterol regulatory element binding transcription factor 1
Apoa2	11807	-	-	2.6	apolipoprotein A-II
Cyp4a14	13119	-	-	8.6	cytochrome P450, family 4, subfamily a, polypeptide 14
Fabp1	14080	-	-	20.0	fatty acid binding protein 1, liver
Acaa1b	235674	-	-	21.1	acetyl-Coenzyme A acyltransferase 1B

Appendix Table2: Table of genes altered by PPARα deletion or by Wy14643 and they are related to inflammation and immunity

List of genes altered in PPARα-/- mice compared to wildtype mice at baseline (column C)
List of genes altered in wildtype mice treated with Wy14643 for 6 hours compared to control treated wildtype mice (column D)
List of genes altered in wildtype mice treated with Wy14643 for 5 days compared to control treated wildtype mice (column E)
Specific criteria for inclusion are shown. List includes genes related to immunity in broadest sense based on Ingenuity canonical pathways
Genes are separated into groups based on pattern of regulation. Abs=absent from array

		Criteria			
		FC1.2 _P value<0.01	FC1.2 _P value<0.01	FC1.2_ no P value	
Gene Name	***Gene ID***	***PPARα-/- vs WT***	***WT-Wy 6h vs WT***	***WT-Wy 5d vs WT***	***Description***
Lgals4	16855	-7.7	1.8	2.7	lectin, galactose binding, soluble 4
Serpine1	18787	-3.0	3.0	2.9	serine (or cysteine) peptidase inhibitor, clade E, member 1
Sult1B1	56362	-1.2	-1.2	1.2	sulfotransferase family 1B, member 1
Ddit3	13198	1.3	1.4	1.3	DNA-damage inducible transcript 3
Gdf15	23886	1.4	1.7	1.7	growth differentiation factor 15
Fas	14102	-1.7	-	1.4	Fas (TNF receptor superfamily member 6)
Cat	12359	-1.6	-	1.2	catalase
Cd44	12505	-1.5	-	1.3	CD44 antigen
Cited2	17684	-1.4	-	1.6	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
Gsta3	14859	-1.4	-	-1.2	glutathione S-transferase, alpha 3
Pnpla7	241274	-1.3	-	1.6	patatin-like phospholipase domain containing 7
Smox	228608	-1.3	-	1.3	spermine oxidase
Hdac9	79221	-1.3	-	-1.5	histone deacetylase 9
St6Gal1	20440	-1.2	-	-1.3	beta galactoside alpha 2,6 sialyltransferase 1
Ppp2R2A	71978	-1.2	-	-1.2	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform
Pias1	56469	-1.2	-	-2.0	protein inhibitor of activated STAT 1
Dnase1	13419	-1.2	-	1.3	deoxyribonuclease I
Ppp2R2B	72930	-1.2	-	1.2	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform
Adar	56417	1.2	-	Abs	adenosine deaminase, RNA-specific
Ifi35	70110	1.2	-	1.2	interferon-induced protein 35
Jak3	16453	1.2	-	1.4	Janus kinase 3
Tlr3	142980	1.3	-	-1.2	toll-like receptor 3
Ticam1	106759	1.3	-	-1.3	toll-like receptor adaptor molecule 1
Cnn2	12798	1.3	-	-1.2	calponin 2
Il17A	16171	1.3	-	-1.1	interleukin 17A
C1Qa	12259	1.3	-	1.7	complement component 1, q subcomponent, alpha polypeptide
B2M	12010	1.3	-	Abs	beta-2 microglobulin
Gpx1	14775	1.3	-	1.3	glutathione peroxidase 1
Cxcl9	17329	1.4	-	-1.5	chemokine (C-X-C motif) ligand 9
Mr1	15064	1.4	-	-2.2	major histocompatibility complex, class I-related
Tyrobp	22177	1.4	-	1.6	TYRO protein tyrosine kinase binding protein
Ppp1R3C	53412	1.4	-	-1.6	protein phosphatase 1, regulatory (inhibitor) subunit 3C
Hk1	15275	1.5	-	Abs	hexokinase 1
Icam1	15894	1.5	-	1.2	intercellular adhesion molecule 1
Slc2A1	20525	1.5	-	-1.2	solute carrier family 2 (facilitated glucose transporter), member 1
Ifih1	71586	1.5	-	1.2	interferon induced with helicase C domain 1
Irf7	54123	1.6	-	1.2	interferon regulatory factor 7
Aldh1L1	107747	1.9	-	Abs	aldehyde dehydrogenase 1 family, member L1
Ccl11	20292	2.2	-	1.3	chemokine (C-C motif) ligand 11
Smpd2	20598	-1.6	-	-	sphingomyelin phosphodiesterase 2, neutral
Hspb6	243912	-1.5	-	-	heat shock protein, alpha-crystallin-related, B6
Cd9	12527	-1.3	-	-	CD9 antigen
Gstk1	76263	1.2	-	-	glutathione S-transferase kappa 1
Rhot2	214952	1.2	-	-	ras homolog gene family, member T2
Cd19	12478	1.2	-	-	CD19 antigen
Psmb8	16913	1.3	-	-	proteasome (prosome, macropain) subunit
Cyba	13057	1.3	-	-	cytochrome b-245, alpha polypeptide
Smpd1	20597	1.3	-	-	sphingomyelin phosphodiesterase 1, acid lysosomal
Pde2A	207728	1.3	-	-	phosphodiesterase 2A, cGMP-stimulated
Il17A	16171	1.3	-	-	interleukin 17A
Lsp1	16985	1.4	-	-	lymphocyte specific 1
Gstt2	14872	1.5	-	-	glutathione S-transferase, theta 2
Pycard	66824	1.6	-	-	PYD and CARD domain containing
Lama5	16776	1.6	-	-	laminin, alpha 5
Ifit2	15958	2.0	-	-	interferon-induced protein with tetratricopeptide repeats 2
Acsl6	216739	2.6	-	-	acyl-CoA synthetase long-chain family member 6
Prkcq	18761	2.7	-	-	protein kinase C, theta

L3Mbtl3	237339	-	-1.3	Abs	l(3)mbt-like 3 (Drosophila)
Sash3	74131	-	-1.2	-1.2	SAM and SH3 domain containing 3
Hif1A	15251	-	1.2	Abs	hypoxia inducible factor 1, alpha subunit
Nfatc3	18021	-	1.2	1.2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3
Ache	11423	-	1.2	Abs	acetylcholinesterase
Cebpb	12608	-	1.5	2.0	CCAAT/enhancer binding protein (C/EBP), beta
Tlr4	21898	-	1.6	1.5	toll-like receptor 4
Shb	230126	-	2.0	-1.3	src homology 2 domain-containing transforming protein B
Hmox1	15368	-	3.3	2.1	heme oxygenase (decycling) 1
Rag1	19373	-	-1.3	-	recombination activating gene 1
Cdkn2C	12580	-	-1.3	-	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
Nfe2L2	18024	-	1.2	-	nuclear factor, erythroid derived 2, like 2
Hsf1	15499	-	1.3	-	heat shock factor 1
Myc	17869	-	2.0	-	myelocytomatosis oncogene
Nr4A1	15370	-	-	-2.9	nuclear receptor subfamily 4, group A, member 1
Fos	14281	-	-	-2.7	FBJ osteosarcoma oncogene
Spp1	20750	-	-	-2.1	secreted phosphoprotein 1
Itgb6	16420	-	-	-2.1	integrin beta 6
Col16A1	107581	-	-	-2.0	collagen, type XVI, alpha 1
Cd55	13136	-	-	-1.9	CD55 antigen
Cxcr4	12767	-	-	-1.9	chemokine (C-X-C motif) receptor 4
Cbl	12402	-	-	-1.9	Casitas B-lineage lymphoma
Gcnt1	14537	-	-	-1.8	glucosaminyl (N-acetyl) transferase 1, core 2
Myh11	17880	-	-	-1.8	myosin, heavy polypeptide 11, smooth muscle
Egr1	13653	-	-	-1.7	early growth response 1
Hdac11	232232	-	-	-1.7	histone deacetylase 11
Pdgfc	54635	-	-	-1.7	platelet-derived growth factor, C polypeptide
Tfrc	22042	-	-	-1.6	transferrin receptor
Atf3	11910	-	-	-1.6	activating transcription factor 3
Prok2	50501	-	-	-1.6	prokineticin 2
Il1F8	69677	-	-	-1.6	interleukin 1 family, member 8
Tfdp1	21781	-	-	-1.6	transcription factor Dp 1
Pik3R3	18710	-	-	-1.6	phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3 (p55)
Epha4	13838	-	-	-1.6	Eph receptor A4
Tgfb3	21809	-	-	-1.5	transforming growth factor, beta 3
Ppat	231327	-	-	-1.5	phosphoribosyl pyrophosphate amidotransferase
Kcnh2	16511	-	-	-1.5	potassium voltage-gated channel, subfamily H (eag-related), member 2
Mdk	17242	-	-	-1.5	midkine
Pld1	18805	-	-	-1.5	phospholipase D1
Smad3	17127	-	-	-1.5	MAD homolog 3 (Drosophila)
Gstt1	14871	-	-	-1.5	glutathione S-transferase, theta 1
Ccl2	20296	-	-	1.2	chemokine (C-C motif) ligand 2
Rap1Gap	110351	-	-	1.5	Rap1 GTPase-activating protein
Cxcl10	15945	-	-	1.5	chemokine (C-X-C motif) ligand 10
Nts	67405	-	-	1.6	neurotensin
Aoc3	11754	-	-	1.6	amine oxidase, copper containing 3
Il15	16168	-	-	1.6	interleukin 15
Sult1A1	20887	-	-	1.6	sulfotransferase family 1A, phenol-preferring, member 1
Gypa	14934	-	-	1.6	glycophorin A
Flt4	14257	-	-	1.6	FMS-like tyrosine kinase 4
Trpv2	22368	-	-	1.7	transient receptor potential cation channel, subfamily V, member 2
Ccr5	12774	-	-	1.7	chemokine (C-C motif) receptor 5
Csf1	12977	-	-	1.7	colony stimulating factor 1 (macrophage)
Agrp	11604	-	-	1.7	agouti related protein
Agrp	11604	-	-	1.7	agouti related protein
Pik3C3	225326	-	-	1.7	phosphoinositide-3-kinase, class 3
Hdac6	15185	-	-	1.7	histone deacetylase 6
Timp1	21857	-	-	1.8	tissue inhibitor of metalloproteinase 1
F2	14061	-	-	1.8	coagulation factor II
Ltc4S	17001	-	-	1.9	leukotriene C4 synthase
Slc4A1	20533	-	-	2.0	solute carrier family 4 (anion exchanger), member 1
Fcgr2B	14130	-	-	2.0	Fc receptor, IgG, low affinity IIb
Ccl9	20308	-	-	2.1	chemokine (C-C motif) ligand 9
Cd28	12487	-	-	2.3	CD28 antigen
Ccl6	20305	-	-	2.6	chemokine (C-C motif) ligand 6
Alb	11657	-	-	3.8	albumin



Appendix Figure 1: Correlation plot of PPARα dependent genes and PPARα independent genes, showing correlation in gene expression between individual mice. Signal log ratios were calculated between intensity values for individual animals and the mean intensity value of the control group. Signal log ratios of genes significantly regulated by at least one treatment were used as input for a correlation plot. Red indicates high correlation, blue indicates weak correlation.

Chapter 4

Induction of Cardiac Angptl4 by Dietary Fatty Acids Is Mediated by Peroxisome Proliferator-Activated Receptor β/δ and Protects Against Fatty Acid–Induced Oxidative Stress

Anastasia Georgiadi, Laeticia Lichtenstein, Tatjana Degenhardt, Mark V. Boekschoten, Mark van Bilsen, Beatrice Desvergne, Michael Müller and Sander Kersten

Chapter published in Circulation Research 2010 Jun 11;106(11):1712-21

Abstract

Although dietary fatty acids are a major fuel for the heart, little is known about the direct effects of dietary fatty acids on gene regulation in the intact heart. Objective: To study the effect of dietary fatty acids on cardiac gene expression and explore the functional consequences. Methods and Results: Oral administration of synthetic triglycerides composed of one single fatty acid altered cardiac expression of numerous genes, many of which are involved in the oxidative stress response. The gene most significantly and consistently upregulated by dietary fatty acids encoded Angiopoietin-like protein (Angptl)-4, a circulating inhibitor of lipoprotein lipase expressed by cardiomyocytes. Induction of Angptl4 by the fatty acid linolenic acid was specifically abolished in peroxisome proliferator-activated receptor (PPAR)β/δ$^{-/-}$ and not PPARα$^{-/-}$ mice and was blunted on siRNA-mediated PPARβ/δ knockdown in cultured cardiomyocytes. Consistent with these data, linolenic acid stimulated binding of PPARβ/δ but not PPARα to the Angptl4 gene. Upregulation of Angptl4 resulted in decreased cardiac uptake of plasma triglyceride-derived fatty acids and decreased fatty acid-induced oxidative stress and lipid peroxidation. In contrast, Angptl4 deletion led to enhanced oxidative stress in the heart, both after an acute oral fat load and after prolonged high fat feeding. Conclusions: Stimulation of cardiac Angptl4 gene expression by dietary fatty acids and via PPARβ/δ is part of a feedback mechanism aimed at protecting the heart against lipid overload and consequently fatty acid–induced oxidative stress.

Introduction

Cardiac contractility is dependent on the adequate delivery of oxygen and energy substrates to the heart followed by their efficient metabolic degradation to yield ATP. The energy requirements of the contracting heart are primarily met by fatty acid oxidation, with the remainder of energy coming from glucose and lactate [1,2]. Although fatty acids are thus of major importance to the heart, excessive uptake of fatty acids causes lipid overload or lipotoxicity and may compromise cardiac function, possibly leading to cardiomyopathy [3]. Consequently, cardiac uptake of fatty acids needs to be well adjusted to fatty acid utilization. Because most of the fatty acids taken up by the heart are derived from lipoprotein lipase (LPL)-dependent hydrolysis of circulating triglyceride-rich lipoproteins [4] the activity of LPL needs to be carefully regulated via specific activators and inhibitors, especially after a fatty meal.

Besides serving as a major fuel for the heart and a potential lipotoxic substrate, fatty acids are able to regulate gene expression [5]. In vitro experiments in rat cardiomyocytes have shown that fatty acids increase expression of uncoupling protein 2, carnitine palmitoyltransferase 1, fatty acid transporter Cd36, fatty acid binding protein 3, acyl-coenzyme (Co)A synthetase long-chain family member 1, acyl-CoA thioesterase, and long chain acyl-CoA dehydrogenase [6–9]. As these genes all represent target genes of peroxisome proliferator-activated receptor (PPAR)α [10,11] they suggest an important role of PPARα in fatty acid-dependent gene regulation in the heart [12]. However, little is known about the direct effects of dietary fatty acids on gene expression in the intact heart. In addition, it is unclear what pathways are activated by fatty acids besides their own catabolism.

Here we studied the comprehensive effects of dietary fatty acids on cardiac gene expression in vivo by giving mice a single oral bolus of synthetic triglyceride composed entirely of one single fatty acid, which were either linolenic acid (C18:3), linoleic acid (C18:2) or oleic acid (C18:1). Subsequent microarrays analysis yielded Angptl4 as the gene most highly induced in the heart after oral fat administration. The collective data suggest that induction of Angptl4 by dietary fatty acids is mediated by PPARβ/δ and is part of a feedback mechanism aimed at protecting cardiomyocytes against lipid overload and consequently fatty acid–induced oxidative stress, eg, lipotoxicity.

Methods

Materials: GW501516 was purchased from Alexis (Axxora, Raamsdonkveer, The Netherlands). Wy14643 was obtained from Eagle Picher Technologies laboratories (Lenexa, Kan). Trilinolein (9c,12c) and trilinolein (9c,12c,15c) were from Larodan Free Chemicals (Malmo, Sweden). SYBR green was from Eurogentec (Seraing, Belgium), and all other chemicals were from Sigma (Zwijndrecht, The Netherlands).

Animals: Pure-bred Sv129 PPARα−/− mice (129S4/SvJae) and corresponding wild-type mice (129S1/SvImJ) were purchased from Jackson Laboratory (Bar Harbor, Maine). The Angptl4−/− and transgenic mice were on C57Bl/6 background and have been previously described [13,14]. The PPARβ/δ−/− mice were on a mixed background (Sv129/C57Bl/6) and have been previously described [15]. Males mice were used at 2.5 to 4 months of age. Mice were anesthetized with a mixture of isoflurane (1,5%), nitrous oxide (70%), and oxygen (30%). Blood was collected via orbital puncture into EDTA tubes. After euthanasia, the hearts were excised and stored in −80°C. The animal studies were approved by Animal Ethics Committee of Wageningen University and the University of Lausanne, Switzerland.

Oral Lipid Load: Starting at 5:00 am, the animals were fasted for 4 hours, followed by an intragastric gavage of 400 μL of synthetic triglyceride (triolein, trilinolein, and trilinolein). The control group received only carboxymethylcellulose (CMC). The mice were killed 6 hours thereafter. Four to 5 mice per group were used.

High-Fat Diet: Angptl4-/-, +/+, and transgenic mice on C57Bl/6 background received a low-fat diet (LFD) or high-fat diet (HFD) for 8 weeks, providing 10 or 45 energy percent in the form of triglycerides, respectively (D12450B or D12451, Research Diets, New Brunswick). The major source of fat in the diet was palm oil, with 5 energy percent provided as soybean oil.

Cell Culture: Neonatal cardiomyocytes were isolated and cultured as described using differential plating to separate myocytes from nonmyocytes [16]. The experiments were approved by the Animal Ethics Committee of Maastricht

University. Neonatal cardiomyocytes were incubated with 1 μmol/L GW501516 or 62.5 μmol/L, 125 μmol/L, or 250 μmol/L linolenic for 6 hours as previously described [17]. In a second experiment, cardiomyocytes were incubated with 1 μmol/L GW501516, 10 μmol/L Wy14643, or 250 μmol/L linolenic acid for 24 hours.

Plasma Lipid Parameters: Plasma was obtained from blood by centrifugation for 10 minutes at 10,000 g. The plasma free fatty acids and triglyceride concentration were determined using kits from Instruchemie (Delfzijl, The Netherlands).

RNA isolation and qRT-PCR: Total RNA was isolated with TRIzol Reagent (Invitrogen, Breda, the Netherlands). 1μg of total RNA for the in vivo studies and 350ng of total RNA for the in vitro experiment was reverse transcribed using iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was amplified on BioRad MyIQ or iCycler machine using Platinum Taq polymerase (Invitrogen, Breda, the Netherlands). PCR primer sequences were taken from the PrimerBank and ordered from Eurogentec (Seraing, Belgium). Sequences of the primers used are presented on Table I. To compare expression of the three PPAR isotypes in adult mouse heart and in rat neonatal cardiomyocytes, primers were used that yielded amplicons of equal length. A standard curve was included to confirm an amplification efficiency of 100%±2 for all PPARs and for the 18S control gene. PPAR expression was calculated as 1/(2^(CtPPAR-Ct18S)), allowing for direct comparison between the PPAR isotypes (Appendix Figure 3)

Chromatin immunoprecipitation assay (ChIP): Wildtype C57Bl/6 mice were fasted for 4 hours followed by an oral gavage of Trilinolenin (n=3). Six hours thereafter the mice were killed by cervical dislocation and the hearts excised. The fresh hearts were cut into half and placed into PBS containing 1% formaldehyde. Crosslinking was stopped after 15 min by adding glycine to a final concentration of 0.125M for 5 min at room temperature. The samples were centrifuged for 5 min at 700 g at 4 °C to collect the heart pieces, the supernatant was removed and washed once again with ice-cold PBS. Fresh PBS containing protease inhibitors (Roche, Almere, Netherlands) was added and the tissue was disaggregated with a homogenizer Ultra Turrax T25 basic (Ika Werke, Staufen, Germany). The tissue was distributed into 3 tubes (2 mL each), centrifuged for 5 min at 700 g at 4°C. After the supernatant was removed, heart homogenate was resuspended in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1, protease inhibitors) and the lysates were sonicated with a Bioruptor TM (Diagenode, Liège, Belgium) to achieve a DNA length of 300- 800 bp. After removal of cellular debris by centrifugation, supernatants were diluted 1:10 in ChIP dilution buffer (150mM NaCl, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl pH 7.5, protease inhibitors). Chromatin was incubated overnight at 4oC with 2µg antibody, 25µl BSA (100 mg/ml) and 2.4µl sonicated salmon sperm (10 mg/ml). The following antibodies were used: anti-PPARα (sc-9000), and anti-PPARβ/δ (sc-7197). All were obtained by Santa Cruz Biotechnologies (Heidelberg, Germany). Immunocomplexes were collected with 25µl MagaCell® Protein A Magnetic beads (Isogen Life Science) for 1hour at room temperature, and subsequently washed with 700µL of the following buffers: ChIP was buffer 1 (150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, protease inhibitors) two times, ChIP wash buffer 2 (500mM NaCl, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, protease inhibitors), ChIP wash buffer 3 (250 mM LiCi, 1% NP40, 1% Deoxycholate, 1mM EDTA, 10 mM Tris-HCl pH 8), two times TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8). Elution of immucomplexes were carried out in 250µL elution buffer (10 mM EDTA, 0.5% SDS, 25mM Tris-HCl pH 7.5) at 64oC for 30 min. After collection of supernatant, elution was repeated with 250µl elution buffer at room temperature for 2 min. After combining the supernatants, cross-linking was reversed at 64oC overnight with 2.5µl Proteinase K (20 mg/ml) for digestion of any remaining proteins. Genomic DNA fragments were recovered by phenol-chloroform extraction with phase lock gel (Eppendorf, Wesseling-

Berzdorf, Germany), followed by salt-ethanol precipitation. Samples were diluted in sterile H2O, and analyzed with qPCR. The ChIP data are normalized against IgG to account for non-specific immunoprecipitation. A fold-enrichment value of 1 represents baseline thus no enrichment and no specific precipitation. Primers were chosen to study binding of PPARs to the transcriptional start site of the Angptl4 and Ucp3 genes, and to the previously identified PPRE within intron 3 of the Angptl4 gene. The ribosomal phosphoprotein P0 (Rplp0) was used as negative control for PPAR binding. The sequences of primers used in ChIP were as follows: Ucp3-TSS:(For:5'-GAGCCCCAGGTCACGGAAG-3',Rev:5'CTGTGCGTCTAGCCAAGGTTG-3'),

Angptl4-TSS:(For:5'CCAGCAAGTTCATCTCGTCC-3',

Rev:5'TCCCTCCCACTCCCACACC-3'),

Angptl4:PPRE:(For:5'TCTGGGTCTGCCCCCACTCCTGG-3' ,Rev:5'GTGTGTGTGTGGGATACGGCTAT-3'),Rplp0(For:5'– CGAGGACCGCCTGGTTCTC-3',Rev:5'–GTCACTGGGGAGAGAGAGG-3').

In Vivo Clearance of very low density lipoprotein (VLDL)-Like Emulsion Particles: Tissue uptake of [^{3}H]-labeled TG packaged into VLDL-like emulsion particles was measured as previously described [21]. The data shown represent the percentage of injected radioactivity taken up by the heart after 30 min

Immunohistochemistry : Deep frozen tissues (-80^{o}C) were cryosectioned (5μm) with a cryostat (Leica, CM1900 UV). Immunostaining of protein adducts of the lipid peroxidation byproduct 4-hydroxy-2-nonenal (4-HNE) was performed on freshly cut frozen sections using a rabbit polyclonal antibody (Calbiochem, San Diego, CA, USA). All steps were carried out at room temperature. The tissue was fixed in 70% ethanol for 3 min and then rinsed in PBS 1X for 3 min. To block endogenous peroxidase activity, slides were incubated with 3% H2O2 for 10 min. Incubation with the primary antibody (rabbit anti 4-HNE protein-adducts, 1:50 dilution in PBS 1X) was performed for 1h. After rinsing with PBS 1X, tissue was incubated for 45 min with the secondary antibody (Dako EnVision+® System Labelled Polymer-HRP

AntiRabbit). Visualization of the complex was carried out using AEC substrate chromogen (Dako Cytomation) for 15 minutes. Sections were mounted with Kaiser's glycerol gelatin mounting medium (Merck KGaA, Darmstadt, Germany). Normal rabbit serum was used as a negative control (Vector Laboratories). Immunostaining of Angptl4 in human heart was performed using an antibody directed against the C-terminus of Angptl4. Five-micrometer sections of paraffin-embedded human heart were mounted on Superfrost microscope slides. These sections were dewaxed in xylene and rehydrated in a series of graded alcohols. To block endogenous peroxidase activity, slides were incubated with 3% H2O2 for 20 min. Antigen retrieval was performed by placing the slides in citrate buffer (pH 6.0) and heat them in a microwave oven 5 min 700 W (without lid) and 4 times 5 min 500 W (with lid). After cooling down to room temperature, the sections were briefly washed with PBS. Prior to staining, a 20 min preincubation was performed using 20% normal goat serum (Vector Laboratories, Burlingame, CA, USA). Incubation with the primary antibody (1:50) was performed for 1h. After rinsing with PBS 1X, tissue was incubated for 45 min with the secondary antibody (Dako EnVision+® System Labelled Polymer-HRP AntiRabbit). Visualization of the complex was carried out using AEC substrate chromogen (Dako Cytomation) for 15 minutes. After counterstaining with Meyer's hematoxylin, sections were mounted with DePex mounting medium (Gurr, BDH, Poole, Dorset, UK). Negative control staining was performed using only the secondary antibody.

Tissue homigenization and quantification of oxidative stress: The extent of lipid peroxidation in heart homogenates was determined by measuring the levels of hydroxynonenal-histidine (HNE-His) protein adducts and malondialdehyde (MDA) adducts. 25mg of heart tissue were homogenized in 250µL of tissue homogenation buffer (1mM EDTA, PBS 1X pH 7.4 containing protease inhibitors). Heart tissue was homogenized over ice by needle sonication for 15sec at 40V. Heart homogenates were centrifuged at 1600xg for 10min at 4ºC. Protein content was determined in tissue supernatants by BCA Protein assay reagent. Starting from a protein concentration of 10 µg/mL 4-HNE-His protein adducts and MDA adducts content were quantified using the Oxiselect HNE-His Adduct ELISA kit (Cell Biolabs Inc., San Diego, USA) and MDA Adduct ELISA kit (Cell Biolabs Inc.), respectively. The quantity of HNE-His protein adducts was determined by using a standard curve containing known amounts

of HNE-BSA (0-10µg/ml). For the quantification of MDA adducts a standard curve of known amounts of MDA-BSA (0-120pmol/mg) was used.

Tissue triglyceride content: Triglycerides content was measured in tissue homogenates with Triglyceride LiquiColor® Test (Mono) HUMAN GmbH, kit (Instruchemie, Delfzijl, The Netherlands). 5% tissue homogenates were prepared by needle sonication over ice in tissue homogenization buffer consisting of 10mM Tris, 2mM EDTA, 0.25M sucrose pH 7.5.

Table 1: Primer Sequences used for QPCR

Primer Name	Forward sequence	Reversed sequence
mouse PPARα	TATTCGGCTGAAGCTGGTGTAC	CTGGCATTTGTTCCGGTTCT
mouse PPARβδ	TTGAGCCCAAGTTCGAGTTTG	CGGTCTCCACACAGAATGATG
mouse PPARγ	CAC AAT GCC ATC AGG TTT GG	GCT GGT CGA TAT CAC TGG AGA
rat PPARα	CACCCTTCTCTCCAGCTTCCA	GCCTTGTCCCCACATATTCG
rat PPARβδ	AACGAGATCAGCGTGCATGTG	TGAGGAAGAGGCTGCTGAAGTT
mouse Angptl4	GTTTGCAGACTCAGCTCAAGG	CCAAGAGGTCTATCTGGCTCTG
mouse Fkbp5	AAGATTCAGGCGTTATCCGTAG	CATCTCGGCAATCAAATGTCCT
mouse Gsta3	TAGAGATCGACGGGATGAAACT	CAGATCCGCCACTCCTTCT
mouse Ucp3	TGCTGAGATGGTGACCTACGA	CCAAAGGCAGAGACAAAGTGA
mouse Herpud1	CCTGGCTTCTCTGGCTACAC	GTCGGGACAAAAGTTCCTGA
mouse Lcn2	TGGAAGAACCAAGGAGCTGT	TGGAAGAACCAAGGAGCTGT
mouse Acsl1	ACCACCTTCTGGTATGCCAC	TGACATCGTCGTAGTAGTACACC
mouse Acox1	TCGAAGCCAGCGTTACGAG	ATCTCCGTCTGGGCGTAGG
mouse Cyclophilin	TGTCTTTGGAACTTTGTCTGCAA	CAGACGCCACTGTCGCTTT
mouse 36B4	ATGGGTACAAGCGCGTCCTG	GCCTTGACCTTTTCAGTAAG

Affymetrix microarray and pathway analysis: Expression profiling was carried out on individual mouse hearts. Total RNA (5 mg) was labeled using the Affymetrix One-cycle Target Labeling Assay kit (Affymetrix, Santa Clara, CA). The correspondingly labeled RNA samples were hybridized on Affymetrix Mouse Genome 430 2.0 Arrays, washed, stained and scanned on an Affymetrix GeneChip 3000 7G scanner. Packages from the Bioconductor project, integrated in an in-house developed on-line management and analysis database for multiplatform microarray experiments, were used for analyzing the scanned arrays [54]. Probesets were redefined according to Dai et al. as the genome information utilized by Affymetrix at the time of designing the arrays is not

current anymore, resulting in unreliable reconstruction of expression levels [55]. In this study probes were reorganized based on the Entrez Gene database, build 36, version 2 (remapped CDF v10). Expression estimates were obtained by GC-robust multi-array (GCRMA) analysis, employing the empirical Bayes approach for background adjustment, followed by quantile normalization and summarization. Differentially expressed probesets were identified using linear models, applying moderated t-statistics that implement empirical Bayes regularisation of standard errors [56]. A probeset was found to be significantly changed after treatment if $p<0.05$. For the pathway analysis ingenuity software version 6.5 was used.

siRNA-mediated silencing of PPARα and PPARβ/δ in H9c2 cardiomyocytes: H9c2 cardiomyoblasts at passage number 18 were seeded at a density of 40000 cells/well in 6-well plates and subsequently grown for 24h in DMEM, antibiotic-free medium containing 10% FCS. After 24h, cells were transfected for 72h with siRNA molecule (100pmol/ml) according to the DharmaFECT 1 siRNA Transfection Protocol Thermo Scientific for H9c2. The siRNA oligos used were selected from a set of 4 individual sequences (ON-TARGETplus Set of 4) designed by Dharmacon that we tested for efficient silencing of PPARα and PPARβ/δ expression in H9c2 cells. The final target sequence used for PPARα was 5'- UCACCGAGCUCACGGAAUU-3' and for PPARβ/δ 5'-CAUGAGUUCUUGCGCAGUA- 3'. Transfection medium was replaced with DMEM, antibiotic-free medium containing 10% FCS 48h after the siRNA transfection. After 72h, linolenic acid (250 μM) was added for 6h, followed by harvesting of the cells. Cell viability was assessed with trypan blue and exceeded 80 %

Results

Dietary Fatty Acids Have a Major Impact on Cardiac Gene Expression

To study the acute effects of dietary fatty acids on cardiac gene expression in vivo, SV129 mice were given a single oral gavage of synthetic TGs composed entirely of either C18:1, C18:2, or C18:3 [18], thus mimicking a postprandial lipid challenge. Animals receiving carboxymethylcellulose were used as control to study the absolute effect of dietary fatty acids. Expression profiling was carried out on individual mouse hearts collected 6 hours after the gavage using Affymetrix Mouse Genome 430 2.0 Arrays. Pathway analysis using Ingenuity revealed that the dominant pathway affected by the oral fat load was nuclear factor-like 2 (Nrf2)-related oxidative stress, indicating that the fatty acids induced oxidative stress (Appendix Figure 1). This was supported by examination of the top 20 of upregulated genes, most of which were involved in the oxidative stress response, including uncoupling protein 3 (Ucp3), heme oxygenase 1 (Hmox1), FK506 binding protein 5 (Fkbp5), lipocalin 2 (Lcn2), glutathione S-transferase A3 (Gsta3), and metallothionein 2 (Mt2) (Figure 1). A large correspondence in gene regulation between the fatty acids was observed, especially between C18:2 and C18:3. Indeed, scatter plot analysis indicated that the effects of C18:2 and C18:3 on cardiac gene expression were remarkably similar, whereas effects of C18:1 were somewhat different (Appendix Figure 2). Therefore, the remainder of the present article focuses on effects of C18:3.

Apart from genes involved in the oxidative stress response, various genes involved in lipid metabolism were also induced by the fatty acids. Interestingly, the gene most significantly and consistently upregulated by each of the dietary fatty acids was Angptl4 (Figure 1), which encodes a secreted protein involved in the regulation of plasma triglyceride levels. Previous studies have shown that Angptl4 potently inhibits LPL and accordingly plasma triglyceride clearance by converting active LPL-dimers into inactive LPL-monomers [19–21].

Although Angptl4 is known to be expressed in heart [22,23] the specific cardiac cell types that produce Angptl4 remain unclear. Accordingly, we performed immunohistological staining of Angptl4 in human heart samples. The results reveal the presence of Angptl4 protein in cardiomyocytes and vascular smooth muscle cells but not endothelial cells and fibroblasts (Figure

2A). Significant production of Angptl4 by cardiomyocytes was confirmed by the relatively low Ct values for amplification of Angptl4 cDNA from rat cardiomyocytes (Ct 22 to 23, data not shown).

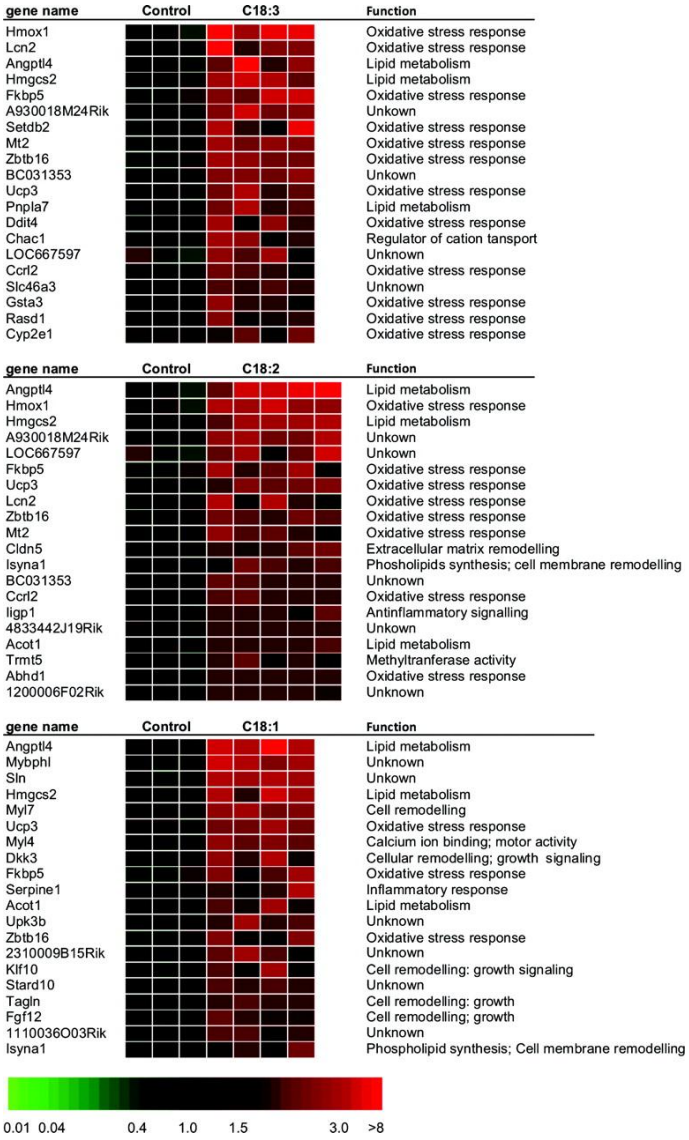


Figure 1: Cardiac Angptl4 expression is highly sensitive to dietary fatty acids. Top 20 of genes upregulated after oral gavage of synthetic triglyceride composed of either

linolenic acid (C18:3), linoleic acid (C18:2), or oleic acid (C18:1). The heat maps were generated directly from the GCRMA normalized microarray data. Genes are ranked according to mean fold change with wild-type mice receiving CMC serving as control. Only probe sets showing significant upregulation by the different fatty acids were included in the analysis ($P<0.05$).

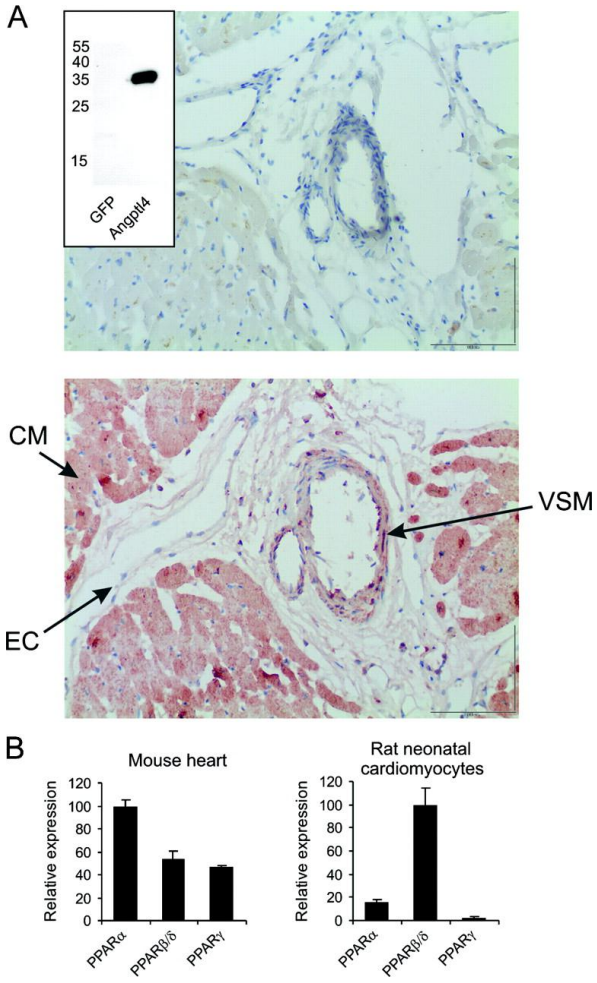


Figure 2: Angptl4 protein localizes specifically to the cardiomyocytes and not to endothelial cells. A. High-magnification image (×200) of the human heart tissue stained with an antibody against Angptl4 (bottom) or negative control (top). Arrows

point to endothelial cells (EC), vascular smooth muscle cells (VSM), and cardiomyocytes (CM). (Inset) The antibody used specifically recognizes human Angptl4. HEK293 cells were transfected with an expression vector encoding hAngptl4 and the medium was collected and used for immunoblotting. The protein recognizes the C-terminal portion of Angptl4 generated by endogenous proteolytic cleavage. B. mRNA expression of the 3 PPARs in adult mouse heart and rat neonatal cardiomyocytes as determined by quantitative PCR. Error bars represent SEM. A standard curve was included to confirm an amplification efficiency of 100±2% for all PPARs (Appendix Figure 3). PPAR expression was calculated as 1/[−2^(CtPPAR−Ct18S)], allowing for direct comparison between the PPAR isotypes.

Regulation of Angptl4 by Dietary PUFA Is Entirely Mediated by PPARβ/δ

Long-chain fatty acids are bona fide ligands for PPARs. The previous demonstration that Angptl4 is a direct PPAR target gene prompted us to investigate the role of PPARs in Angptl4 gene regulation by dietary fatty acids. We first determined the relative expression of PPARs in mouse heart. All three PPAR isotypes were well expressed in heart, with expression of PPARγ and PPARβ/δ being ≈50% of PPARα (Figure 2B). In cultured neonatal rat cardiomyocytes, expression of PPARγ was markedly lower compared to both PPARα and PPARβ/δ (Figure 2B). Because PPARγ appears to be rather insensitive to (dietary) fatty acids, [24] we focused our studies on PPARα and PPARβ/δ. PPARα−/− mice, PPARβ/δ−/− mice, and the corresponding wild-type mice were given an oral gavage of C18:3 as synthetic TG. Hearts were collected 6 hours thereafter for analysis of gene expression by quantitative PCR. Remarkably, upregulation of Angptl4 by C18:3 was entirely abolished in the PPARβ/δ−/− mice, whereas it was retained in the PPARα−/− mice (Figure 3A). In contrast, upregulation of Ucp3, another well-characterized PPAR target gene, was retained in PPARβ/δ−/− mice and completely abolished in PPARα−/− mice (Figure 3B). No compensatory increase in PPARβ/δ and PPARα expression was observed in PPARα−/− and PPARβ/δ−/− mice, respectively (Appendix Figure 4)

To examine whether regulation of Angptl4 by PPARβ/δ and not PPARα was supported by binding of PPARβ/δ to the Angptl4 gene, we performed chromatin immunoprecipitation (ChIP). Previously, we located the response element responsible for PPAR-mediated upregulation to intron 3 of the Angptl4 gene [25]. Consistent with data on Angptl4 gene regulation, ChIP on hearts of wild-type mice six hours after oral gavage of C18:3 showed enhanced binding of PPARβ/δ but not PPARα to the intronic PPRE (Figure 3C).

Nuclear receptors and other transcription factors bound to such distal sites likely contact the basal transcription machinery via DNA looping, and accordingly binding of PPAR to distant PPREs can be demonstrated by showing cross-linking of PPAR to the transcriptional start site (TSS) [26,27]. Indeed, oral gavage of C18:3 enhanced binding of PPARβ/δ but not PPARα to TSS of the Angptl4 gene (Figure 3D), whereas C18:3 enhanced binding of PPARα but not PPARβ/δ to the TSS of the Ucp3 gene (Figure 3E). No binding of either PPARα or PPARβ/δ to the negative control gene Rplp0 was observed (Figure 3F). These results demonstrate that the induction of cardiac Angptl4 gene expression by dietary C18:3 is mediated by PPARβ/δ.

The fatty acid- and PPARβ/δ-mediated induction of cardiac Angptl4 expression likely occurred in cardiomyocytes, as treatment of rat neonatal cardiomyocytes for 6 hours with C18:3 dose-dependently increased Angptl4 mRNA, which at the highest concentration was equivalent to that obtained using GW501516 (Figure 3G). To further investigate the specific role of PPARβ/δ in Angptl4 upregulation by fatty acids in cardiomyocytes, we knocked-down PPARα or PPARβ/δ in the cardiomyocyte cell line H9c2, which expresses both receptors, using siRNA and studied the effect on Angptl4 gene induction by C18:3 (Figure 3H). We observed that knock-down of PPARβ/δ almost entirely abolished the induction of Angptl4 gene expression by C18:3, whereas knock-down of PPARα had little to no effect (Figure 3I).

Our results do not imply that Angptl4 is an exclusive target gene of PPARβ/δ under any type of circumstances. Indeed, we find that in rat neonatal cardiomyocytes, Angptl4 is induced to a similar extent by synthetic PPARα and PPARβ/δ agonists, as are other cardiac PPAR targets such as Acsl1 and Acox1 (Figure 3J). Instead, our data suggest that the stimulatory effect of dietary fatty acids on cardiac Angptl4 expression is mediated specifically by PPARβ/δ.

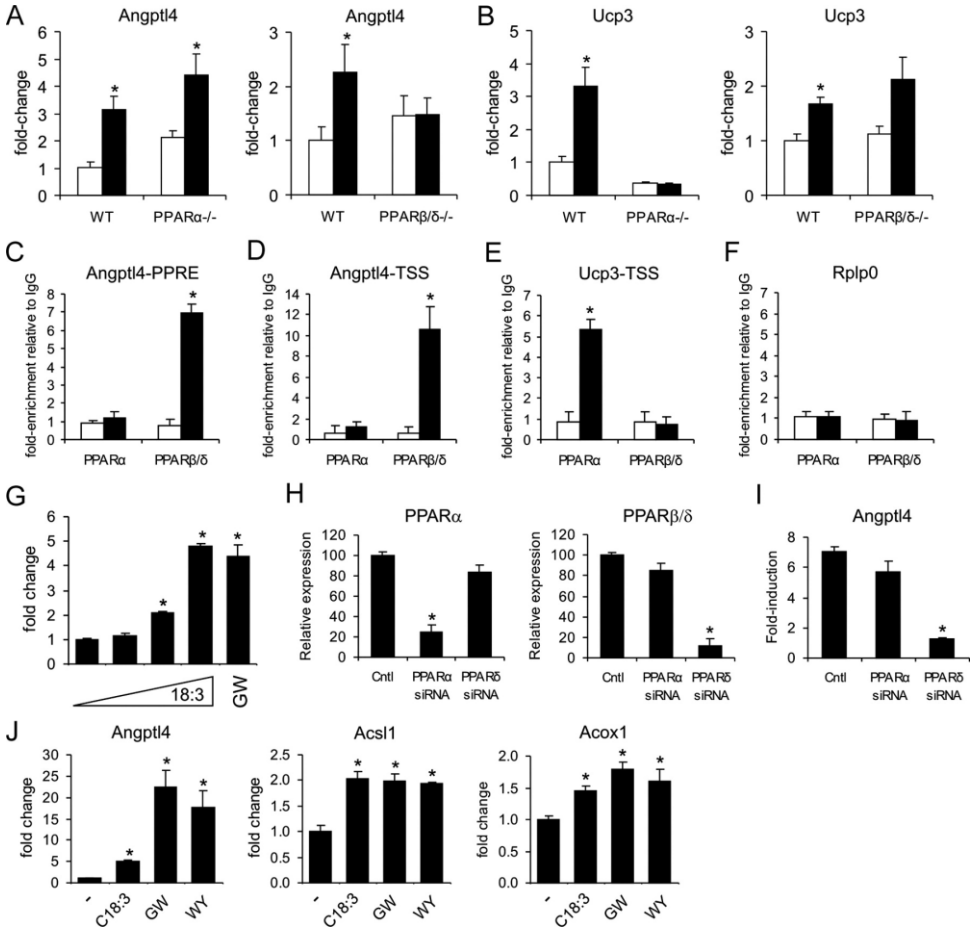


Figure 3: PPARβ/δ but not PPARα mediates the induction of Angtpl4 expression by dietary linolenic acid. Wild-type, PPARα−/−, and PPARβ/δ−/− mice were given a single oral gavage of 0.5% CMC (open bars) or synthetic triglycerides composed entirely of C18:3 (closed bars). mRNA expression levels of Angptl4 (A) and Ucp3 (B) were determined in mouse heart using real-time PCR. Results are expressed as fold change compared to the wild-type control mice. C through F, ChIP was performed on hearts of wild-type mice given an oral gavage of either CMC or C18:3. Chromatin was precipitated using antibodies against PPARα or PPARβ/δ. Rabbit IgG was used as a specificity control. Real-time quantitative PCR was performed on reverse–cross-linked chromatin templates using primers specific to the known PPRE in intron 3 of the Angptl4 gene (C), the TSS of Angptl4 (D), the TSS of Ucp3 (E), and the negative control gene RpLp0 (F). G, Angptl4 expression in rat neonatal cardiomyocytes

incubated with increasing concentrations of linolenic acid (0, 62.5, 125, and 250 μmol/L) or GW501516 (1 μmol/L) for 6 hours. H. Expression of PPARα and PPARβ/δ in H9c2 cardiomyocytes transfected with siRNA against PPARα and PPARβ/δ. I. Fold induction of Angptl4 expression by 6 hours of linolenic acid treatment (250 μmol/L) in H9c2 cardiomyocytes transfected with siRNA against PPARα and PPARβ/δ. J. Expression of Angptl4 and known PPAR targets Acsl1 and Acox1 in rat neonatal cardiomyocytes incubated for 24 hours with linolenic acid (250 μmol/L), GW501516 (1 μmol/L), or Wy14643 (10 μmol/L). Error bars represent SEM. Statistical significance was determined with a Student's t test ($P<0.05$).

Induction of Angptl4 Protects Against Fatty Acid–Induced Oxidative Stress

To study the effect of Angptl4 on the metabolic response to dietary fat, we performed the oral fat load with C18:3 triglyceride in wild-type, Angptl4−/− and Angptl4 transgenic (Angptl4-Tg) mice. In agreement with inhibition of LPL by Angptl4, the postprandial increase in plasma triglyceride was dramatically increased in Angptl4-Tg mice, whereas it was entirely blunted in Angptl4−/− mice (Figure 4A). Consistent with LPL inhibition, Angptl4 overexpression markedly reduced cardiac fatty acid uptake from [3H]triolein-labeled very-low-density lipoprotein (VLDL)-like particles (Figure 4B). These results suggest that upregulation of Angptl4 by dietary fatty acids will lead to reduced cardiac uptake of fatty acids via inhibition of LPL, thereby suppressing the stimulus that led to induction of Angptl4 expression.

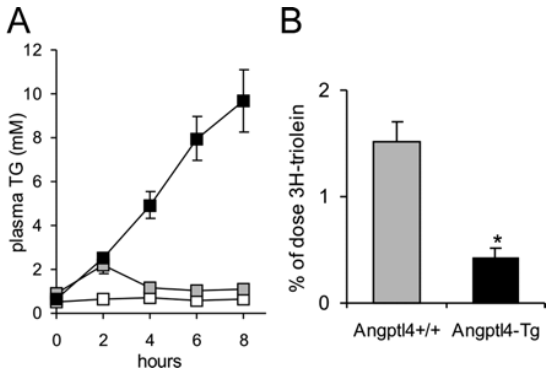


Figure 4: Angptl4 overexpression raises postprandial plasma triglyceride levels and decreases cardiac fatty acid uptake. A. Wild-type (gray squares), Angptl4−/− (open squares), and Angptl4-Tg (black squares) mice were given a single oral gavage of synthetic triglyceride (TG) composed entirely of C18:3. Plasma triglycerides were determined in blood collected via the tail vein. B. [3H]-labeled triolein was incorporated into VLDL-like emulsion particles and directly injected into the tail vein of wild-type and Angptl4-Tg mice. The heart was collected 30 minutes after the injection for determination of radioactivity. *Significantly different between wild-type and Angptl4-Tg mice according to Student's t test ($P<0.05$). Error bars represent SEM.

To examine whether the inhibitory effect of Angptl4 on cardiac fatty acid uptake is associated with reduced fatty acid-induced oxidative stress, expression of Fkbp5, Lcn2, and Gsta3 was determined 6 hours after oral gavage with either control treatment (CMC) or C18:3 triglyceride in wild-type, Angptl4−/− and Angptl4-Tg mice. All three genes represent markers of oxidative stress [28–30]. Consistent with a protective role of Angptl4 against fatty acid-induced oxidative stress, the magnitude of induction of Fkbp5, Lcn2, and Gsta3 by C18:3 was dependent on Angptl4 genotype and inversely correlated with Angptl4 expression (Figure 5A and 5B). Expression of Fkbp5, Lcn2, and Gsta3 after the oral fat load was not related to plasma free fatty acid (FFA) levels, which followed an opposite pattern (Figure 5C). Expression of the endoplasmic reticulum stress marker Herpud1 mimicked the pattern of oxidative stress markers (Figure 5A).

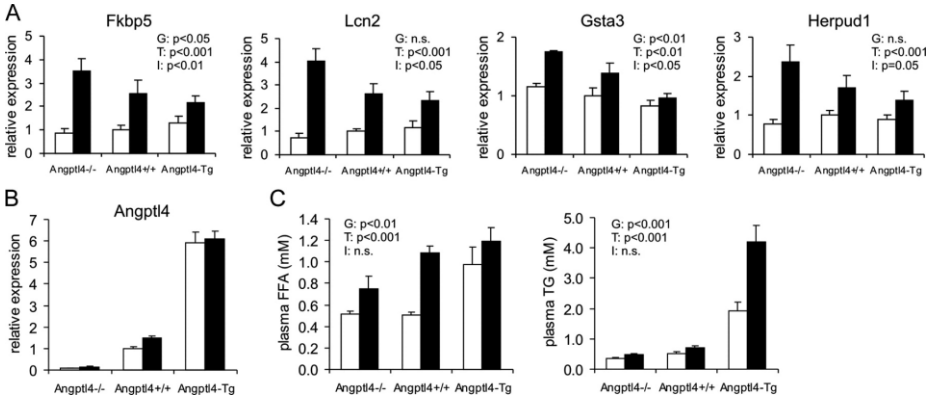


Figure 5: Markers of oxidative stress are inversely correlated with Angptl4 expression after oral fat load. Wild-type, Angptl4−/−, and Angptl4-Tg mice were given a single oral gavage of 0.5% CMC (open bars) or synthetic triglyceride composed entirely of C18:3 (closed bars). Mice were euthanized 6 hours later. A. mRNA expression levels of oxidative stress genes Fkbp5, Lcn2, and Gsta3 and endoplasmic reticulum stress marker gene Herpud1, as determined by real-time PCR. B. mRNA expression levels of Angptl4. C. Plasma levels of FFA and TG. Differences were evaluated statistically using two-way ANOVA. Significance (probability value) of effect of genotype (G), treatment (T), and interaction (I) between genotype and treatment is indicated in each graph. n.s. indicates non significant. Error bars represent SEM.

Finally, we examined whether Angptl4 may exert a similar effect in the context of a chronic fat overload. To that end, we measured expression of the oxidative stress markers and performed immunohistochemical and quantitative analysis of 4-HNE protein adducts in wild-type and Angptl4−/− mice fed a HFD for 8 weeks. 4-HNE is one of the major biologically active aldehydes formed during inflammation and oxidative stress. Formation of 4-HNE protein adducts is a marker for lipid peroxidation.

Although high fat feeding did not influence expression levels of Fkbp5, Lcn2 and Gsta3, expression was significantly higher in Angptl4−/− mice fed HFD compared to wild-type mice fed HFD (Figure 6A). A similar tendency was observed for Herpud1. Furthermore, lipid peroxidation was increased in Angptl4−/− mice fed HFD, as shown by enhanced 4-HNE staining (Figure 6B). These results were supported by quantitative analysis of 4HNE protein adducts (Figure 6C) and MDA adducts (Figure 6D), which were significantly increased in Angptl4−/− mice fed HFD compared to wild-type mice fed HFD. These data indicate that Angptl4 protects against oxidative stress in the context of a chronic fat overload. No differences in cardiac triglyceride levels were observed between wild-type and Angptl4−/− mice (Figure 6E).

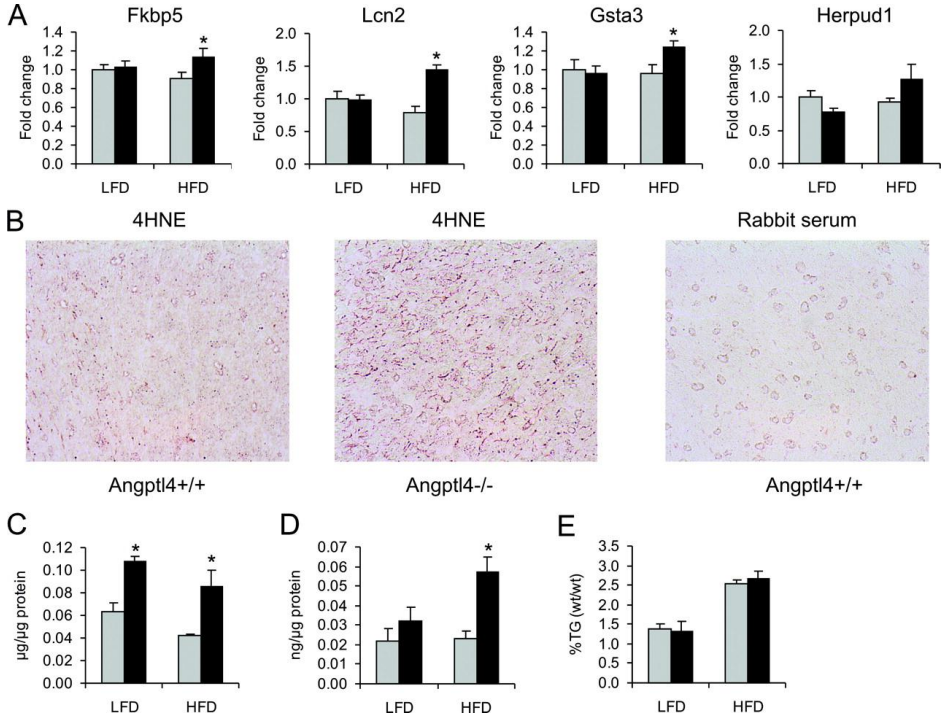


Figure 6: Angptl4 protects against oxidative stress in the context of chronic fat overload. A. Wild-type and Angptl4−/− mice were fed a LFD or HFD for 8 weeks. Expression levels of Fkbp5, Lcn2, Gsta3, and Herpud1 in the heart were measured by real-time PCR. Results are expressed as fold change compared to wild-type mice on LFD. B. Immunohistochemistry of 4-HNE protein adducts in mouse cardiac tissue from mice fed HFD for 8 weeks. Representative sections are shown. Magnification, ×400. Right, Negative control obtained using rabbit serum. Quantitative measurement of 4HNE protein adducts (C) and MDA adducts (D). E. Cardiac triglyceride content. Gray bars indicate wild-type mice; black bars, Angptl4−/− mice. *Significantly different between wild-type and Angptl4−/− mice according to Student's t test ($P<0.05$). Error bars represent SEM.

Discussion

In the present article we show that the gene most significantly and consistently upregulated by short term treatment with dietary fatty acids is Angptl4. Induction of Angptl4 by dietary fatty acids is mediated by PPARβ/δ and confers a protective effect against fatty acid-induced oxidative stress by restricting cardiac fatty acid uptake via inhibition of LPL. Overall, our data suggest that upregulation of Angptl4 by fatty acids is part of a feedback mechanism aimed at preventing myocardial fatty acid accumulation, thereby minimizing lipid-induced oxidative stress and lipotoxicity (Figure 7). Although our follow-up studies only included linolenic acid, the results are likely generalizable to other dietary fatty acids.

Lipotoxicity describes the untoward consequences of fat overload in a particular tissue and may be related to fatty acid-induced oxidative stress, accumulation of lipotoxic intermediates such as ceramides and fatty acyl-CoA, and excess storage of triglycerides [31]. Chronic lipotoxicity in the heart has been shown to promote cardiomyopathy in several animal models [12,32–34]. Most of these models are characterized by a mismatch between myocardial fatty acid uptake and utilization, as in mice with heart-specific overexpression of acyl-CoA synthetase, fatty acid transport protein 1, or lipoprotein lipase [32–34]. Although triglycerides are unlikely to be the actual culprit in cardiac lipotoxicity, they may be guilty by association as its levels may be positively correlated with lipotoxic intermediates. However, we did not see increased cardiac triglyceride levels in Angptl4−/− mice compared to wild-type mice after 8 weeks of high fat feeding, suggesting that the increase in fatty acid uptake is limited or that the incoming fatty acids are efficiently oxidized. In the present study, it was not possible to investigate the effect of Angptl4 deletion on cardiac lipid storage, oxidative stress, and parameters of cardiac dysfunction after a more prolonged period of HFD, as a cachectic phenotype progressively emerges after 12 weeks of HFD [53].

In a previous study, heart-specific Angptl4 overexpression reduced cardiac LPL activity and reversed the excessive lipid storage in hearts of lipotoxic acyl-CoA synthetase transgenic mice [22]. In agreement with these data, we find that Angptl4 overexpression reduced cardiac fatty acid uptake and protects against fatty acid-induced oxidative stress. In contrast, Angptl4 deletion aggravated oxidative stress both acutely and after chronic HFD. Because we used whole

body Angptl4 overexpression and deletion models, strictly we cannot rule out that the observed effects may be related to changes in extracardiac Angptl4 expression. However, the current literature mainly supports a paracrine function of Angptl4, and the role of Angptl4 as endocrine factor remains somewhat uncertain.

Angptl4 was discovered by screening for target genes of PPARα and PPARγ in liver and adipose tissue, respectively [35,36]. It is member of a family of angiopoietins and angiopoietin-like proteins and is produced by a variety of organs. Numerous studies using Angptl4 transgenic or knock-out mice have invariably shown a stimulatory effect of Angptl4 on plasma triglyceride levels, which is achieved by inhibiting LPL activity [13,14,19,22,37–40]. These data have established Angptl4 as an important regulator of plasma triglyceride levels. The present data suggest that Angptl4 is upregulated by dietary fatty acids to inhibit local LPL activity and consequently reduce fatty acid uptake and lipid-induced oxidative stress.

To study the role of PPARs in cardiac gene regulation by dietary fatty acids, we ideally should have used cardiomyocyte-specific PPAR−/− mice but unfortunately we did not have access to these animals. The PPAR-dependent upregulation of Angptl4 and Ucp3 by dietary linolenic acid underscores the importance of TG-rich lipoproteins as source of PPAR ligands in the heart, which are liberated via LPL [41,42] Remarkably, upregulation of Angptl4 by C18:3 in intact heart and cultured cardiomyocytes was entirely mediated by PPARβ/δ and not PPARα. These data were supported by ChIP data showing C18:3-induced binding of PPARβ/δ and not PPARα to the Angptl4 gene in the intact heart. Because we did not study PPARγ, strictly we cannot rule out a role for PPARγ in mediating the effect of (dietary) fatty acids on cardiac Angptl4 expression. However, as similar results were obtained in cultured cardiomyocytes, which express little PPARγ, the data favor a specific role of PPARβ/δ in the regulation of cardiac Angptl4 by C18:3. It is unclear what mechanism may underlie the differential role of PPARβ/δ versus PPARα in mediating induction of Angptl4 and Ucp3 by C18:3, respectively. Induction of Ucp3 by linoleic acid via PPARα rules out a PPARβ/δ-specific ligand activity that is generated by linolenic acid in heart. Gel shift and transactivation studies have failed to provide convincing evidence for the existence of response elements or promoters that are specifically or selectively bound or regulated by a particular PPAR isotype [43,44]. Indeed, in vitro experiments have revealed

that all three PPARs are intrinsically able to (trans)activate the human and mouse Angptl4 gene [25]. However, the situation may be different in vivo in the absence of PPAR overexpression or when PPARs are activated via endogenous ligands rather than via high-affinity synthetic agonists. Thus, the dominant receptor in the regulation of a particular PPAR target is likely context- and tissue-dependent and additionally depends on whether PPAR is activated via endogenous or synthetic agonists. When as in cardiomyocytes two or more PPARs are expressed in the same cell and are simultaneously activated, it is possible that specific binding of one PPAR isotype to a particular PPRE is promoted via interactions with another protein that binds adjacent to the PPRE and is expressed in a tissue-specific manner. In this context, it is interesting to mention that recent genome wide profiling of PPARα and PPARγ binding sites revealed colocalization of PPAR binding with other transcription factor binding sites and demonstrated interplay between PPARs and other transcription factors in PPAR-mediated gene regulation [45–47].

Recently, targeted PPARβ/δ overexpression in the heart was shown to have a clear differential effect on cardiac metabolism compared to PPARα overexpression [48]. In contrast to PPARα, PPARβ/δ overexpression did not impact fatty acid transport and failed to induce myocardial lipid accumulation. Based on the data presented here it can be hypothesized that PPARβ/δ is neutral toward cardiac lipid storage by inducing Angptl4 expression, which in turn feeds back on fatty acid uptake.

Multiple studies support an effect of Angptl4 on endothelial function, mostly pointing to an antiangiogenic activity of Angptl4 [49–51]. Our immunohistochemical results indicate that Angptl4 is absent from vascular endothelial cells in the heart, whereas it is abundantly present in cardiomyocytes. These data are in line with previous studies showing that Angptl4 is absent from a number of different endothelial cells, yet is dramatically induced under hypoxic conditions [50]. Hypoxia also upregulates Angptl4 in cardiomyocytes [52]. Induction of Angptl4 by hypoxia and the associated inhibition of fatty acid uptake may be an adaptive mechanism to shift fuel use toward glucose, which requires less oxygen for oxidation.

In conclusion, our data show that an acute oral load of triglycerides stimulates an oxidative stress response in the heart. The concomitant upregulation of Angptl4 by dietary fatty acids is mediated by PPARβ/δ and is part of a feedback mechanism aimed at protecting the heart against lipid

overload and consequently fatty acid–induced oxidative stress, one of the hallmarks of lipotoxic cardiomyopathy.

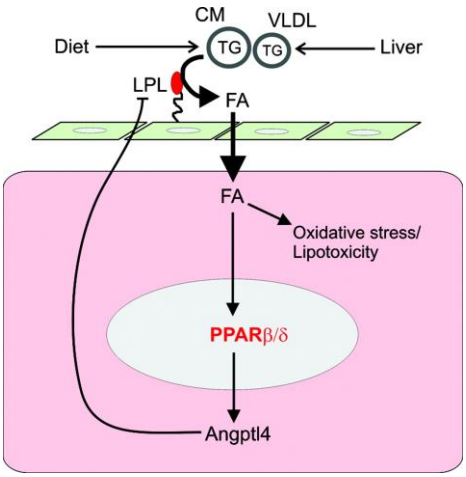


Figure 7: Model of the role of Angptl4 in the heart. Triglycerides arrive at the heart packaged into chylomicrons or VLDL particles. Plasma triglycerides are hydrolyzed by lipoprotein lipase to release fatty acids, that are taken up by the cardiomyocyte. Excess uptake of fatty acids gives rise to oxidative stress and leads to induction of Angptl4 expression via PPARβ/δ. Angptl4 will feed back on fatty acid uptake by inhibiting lipoprotein lipase.

Sources of Funding

This study was supported by the Nutrigenomics Consortium, TI Food and Nutrition, Marie Curie Research Training Network NucSys, and the Netherlands Heart Foundation (2007B046).

References:

1.Abozguia K, Clarke K, Lee L, Frenneaux M. Modification of myocardial substrate use as a therapy for heart failure. Nat Clin Pract Cardiovasc Med. 2006;3:490–498.

2. Stanley WC, Recchia FA, Lopaschuk GD. Myocardial substrate metabolism in the normal and failing heart. Physiol Rev. 2005;85:1093–1129.

3. Park TS, Yamashita H, Blaner WS, Goldberg IJ. Lipids in the heart: a source of fuel and a source of toxins. Curr Opin Lipidol. 2007;18: 277–282.

4. Teusink B, Voshol PJ, Dahlmans VE, Rensen PC, Pijl H, Romijn JA, Havekes LM. Contribution of fatty acids released from lipolysis of plasma triglycerides to total plasma fatty acid flux and tissue-specific fatty acid uptake. Diabetes. 2003;52:614–620.

5. Clarke SD. The multi-dimensional regulation of gene expression by fatty acids: polyunsaturated fats as nutrient sensors. Curr Opin Lipidol. 2004; 15:13–18.

6. Brandt JM, Djouadi F, Kelly DP. Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. J Biol Chem. 1998;273:23786–23792.

7. Durgan DJ, Smith JK, Hotze MA, Egbejimi O, Cuthbert KD, Zaha VG, Dyck JR, Abel ED, Young ME. Distinct transcriptional regulation of long-chain acyl-CoA synthetase isoforms and cytosolic thioesterase 1 in the rodent heart by fatty acids and insulin. Am J Physiol Heart Circ Physiol. 2006;290:H2480–H2497.

8. van der Lee KA, Vork MM, De Vries JE, Willemsen PH, Glatz JF, Reneman RS, Van der Vusse GJ, Van Bilsen M. Long-chain fatty acidinduced changes in gene expression in neonatal cardiac myocytes. J Lipid Res. 2000;41:41– 47.

9. Van Der Lee KA, Willemsen PH, Van Der Vusse GJ, Van Bilsen M. Effects of fatty acids on uncoupling protein-2 expression in the rat heart. FASEB J. 2000;14:495–502.

10. Mandard S, Muller M, Kersten S. Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci. 2004;61:393– 416.

11. Rakhshandehroo M, Sanderson LM, Matilainen M, Stienstra R, Carlberg C, de Groot PJ, Muller M, Kersten S. Comprehensive Analysis of PPARα-Dependent Regulation of Hepatic Lipid Metabolism by Expression Profiling. PPAR Res.

2007;26839.

12. Finck BN, Lehman JJ, Leone TC, Welch MJ, Bennett MJ, Kovacs A, Han X, Gross RW, Kozak R, Lopaschuk GD, Kelly DP. The cardiac phenotype induced by PPARα overexpression mimics that caused by diabetes mellitus. J Clin Invest. 2002;109:121–130.
13. Koster A, Chao YB, Mosior M, Ford A, Gonzalez-DeWhitt PA, Hale JE, Li D, Qiu Y, Fraser CC, Yang DD, Heuer JG, Jaskunas SR, Eacho P. Transgenic angiopoietin-like (angptl)4 overexpression and targeted disruption of angptl4 and angptl3: regulation of triglyceride metabolism. Endocrinology. 2005;146:4943– 4950.
14. Mandard S, Zandbergen F, van Straten E, Wahli W, Kuipers F, Muller M, Kersten S. The Fasting-induced Adipose Factor/Angiopoietin-like Protein 4 Is Physically Associated with Lipoproteins and Governs Plasma Lipid Levels and Adiposity. J Biol Chem. 2006;281:934 –944.
15. Nadra K, Anghel SI, Joye E, Tan NS, Basu-Modak S, Trono D, Wahli W, Desvergne B. Differentiation of trophoblast giant cells and their metabolic functions are dependent on peroxisome proliferator-activated receptor beta/delta. Mol Cell Biol. 2006;26:3266 –3281.
16. de Vries JE, Vork MM, Roemen TH, de Jong YF, Cleutjens JP, van der Vusse GJ, van Bilsen M. Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes. J Lipid Res. 1997;38:1384 –1394.
17. de Vogel-van den Bosch HM, de Wit NJ, Hooiveld GJ, Vermeulen H, van der Veen JN, Houten SM, Kuipers F, Muller M, van der Meer R. A cholesterol-free, high-fat diet suppresses gene expression of cholesterol transporters in murine small intestine. Am J Physiol Gastrointest Liver Physiol. 2008;294:G1171–G1180.
18. Sanderson LM, de Groot PJ, Hooiveld GJ, Koppen A, Kalkhoven E, Muller M, Kersten S. Effect of synthetic dietary triglycerides: a novel research paradigm for nutrigenomics. PLoS ONE. 2008;3:e1681.
19. Yoshida K, Shimizugawa T, Ono M, Furukawa H. Angiopoietin-likeprotein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. J Lipid Res. 2002;43:1770 –1772.
20. Sukonina V, Lookene A, Olivecrona T, Olivecrona G. Angiopoietin-likeprotein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. Proc Natl Acad Sci U S A. 2006;103: 17450–17455.
21. Lichtenstein L, Berbee JF, van Dijk SJ, van Dijk KW, Bensadoun A,Kema IP, Voshol PJ, Muller M, Rensen PC, Kersten S. Angptl4 upregulates cholesterol synthesis in liver via inhibition of LPL- and HL-dependent hepatic cholesterol uptake. Arterioscler Thromb Vasc Biol. 2007;27:2420 –2427.
22. Yu X, Burgess SC, Ge H, Wong KK, Nassem RH, Garry DJ, Sherry AD,Malloy CR, Berger JP, Li C. Inhibition of cardiac lipoprotein utilization by transgenic overexpression of Angptl4 in the heart. Proc Natl Acad SciU S A. 2005;102:1767–

1772.

23. Zandbergen F, van Dijk S, Muller M, Kersten S. Fasting-induced adipose factor/Angiopoietin-like protein 4: a potential target for dyslipidemia. Future Lipidol. 2006;1:227–236.
24. Tontonoz P, Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem. 2008;77:289 –312.
25. Mandard S, Zandbergen F, Tan NS, Escher P, Patsouris D, Koenig W, Kleemann R, Bakker A, Veenman F, Wahli W, Muller M, Kersten S. The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (FIAF/PGAR/ANGPTL4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment. J Biol Chem. 2004;279:34411–34420.
26. Palstra RJ, de Laat W, Grosveld F. Beta-globin regulation and long-range interactions. Adv Genet. 2008;61:107–142.
27. Saramaki A, Diermeier S, Kellner R, Laitinen H, Vaisanen S, Carlberg C. Cyclical chromatin looping and transcription factor association on the regulatory regions of the p21 (CDKN1A) gene in response to 1alpha,25- dihydroxyvitamin D3. J Biol Chem. 2009;284:8073– 8082.
28. Roudkenar MH, Kuwahara Y, Baba T, Roushandeh AM, Ebishima S, Abe S, Ohkubo Y, Fukumoto M. Oxidative stress induced lipocalin 2 gene expression: addressing its expression under the harmful conditions.J Radiat Res (Tokyo). 2007;48:39–44. 29. Edwards MG, Sarkar D, Klopp R, Morrow JD, Weindruch R, Prolla TA. Age-related impairment of the transcriptional responses to oxidative stress in the mouse heart. Physiol Genomics. 2003;13:119 –127.
30. Black AT, Gray JP, Shakarjian MP, Laskin DL, Heck DE, Laskin JD. Increased oxidative stress and antioxidant expression in mouse keratinocytes following exposure to paraquat. Toxicol Appl Pharmacol. 2008; 231:384 –392.
31. Schaffer JE. Lipotoxicity: when tissues overeat. Curr Opin Lipidol. 2003; 14:281–287. 32. Chiu HC, Kovacs A, Ford DA, Hsu FF, Garcia R, Herrero P, Saffitz JE, Schaffer JE. A novel mouse model of lipotoxic cardiomyopathy. J Clin Invest. 2001;107:813– 822.
33. Chiu HC, Kovacs A, Blanton RM, Han X, Courtois M, Weinheimer CJ, Yamada KA, Brunet S, Xu H, Nerbonne JM, Welch MJ, Fettig NM, Sharp TL, Sambandam N, Olson KM, Ory DS, Schaffer JE. Transgenic expression of fatty acid transportprotein 1 in the heart causes lipotoxic cardiomyopathy. Circ Res. 2005;96:225–233.
34. Yagyu H, Chen G, Yokoyama M, Hirata K, Augustus A, Kako Y, Seo T,Hu Y, Lutz EP, Merkel M, Bensadoun A, Homma S, Goldberg IJ. Lipoprotein lipase (LpL) on the surface of cardiomyocytes increases lipid uptake and produces a cardiomyopathy. J Clin Invest. 2003;111: 419–426.
35. Yoon JC, Chickering TW, Rosen ED, Dussault B, Qin Y, Soukas A, Friedman JM,

Holmes WE, Spiegelman BM. Peroxisome proliferator-activated receptor gamma target gene encoding a novel angiopoietin-related protein associated with adipose differentiation. Mol Cell Biol. 2000;20: 5343–5349.

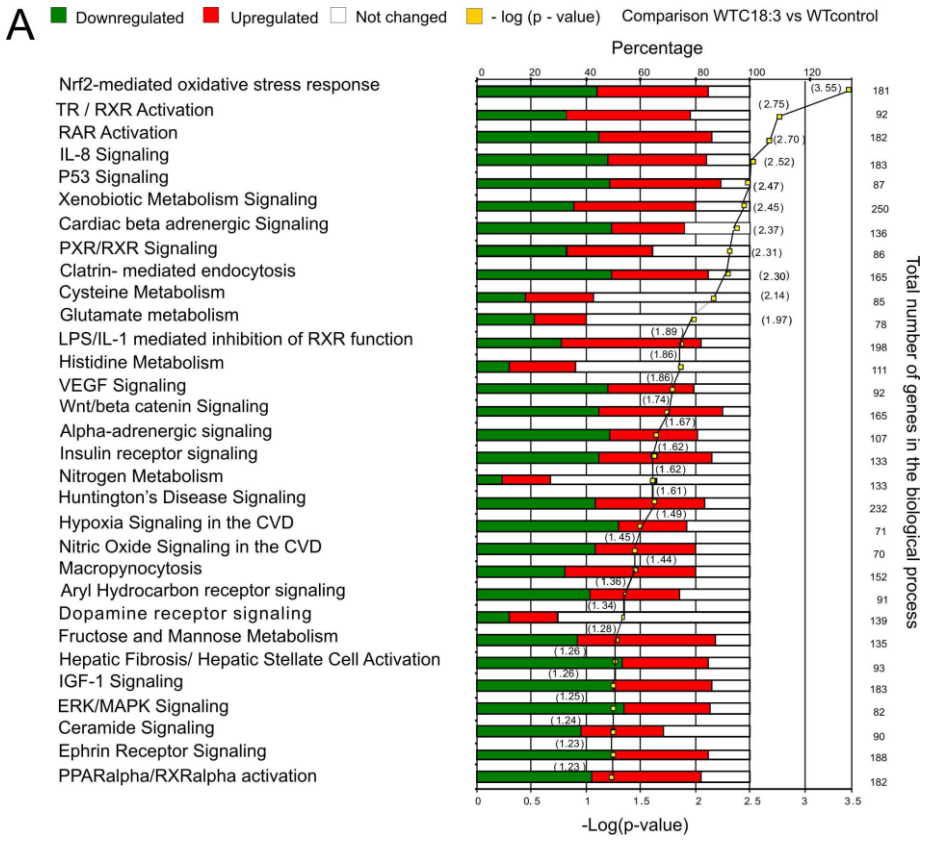
36. Kersten S, Mandard S, Tan NS, Escher P, Metzger D, Chambon P, Gonzalez FJ, Desvergne B, Wahli W. Characterization of the fastinginduced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene. J Biol Chem. 2000;275:28488 –28493.
37. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004;101:15718 –15723.
38. Ge H, Yang G, Yu X, Pourbahrami T, Li C. Oligomerization statedependent hyperlipidemic effect of angiopoietin-like protein 4. J Lipid Res. 2004;45:2071–2079.
39. Xu A, Lam MC, Chan KW, Wang Y, Zhang J, Hoo RL, Xu JY, Chen B, Chow WS, Tso AW, Lam KS. Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. Proc Natl Acad Sci U S A. 2005;102: 6086–6091.
40. Desai U, Lee EC, Chung K, Gao C, Gay J, Key B, Hansen G, Machajewski D, Platt KA, Sands AT, Schneider M, Van Sligtenhorst I, Suwanichkul A, Vogel P, Wilganowski N, Wingert J, Zambrowicz BP, Landes G, Powell DR. Lipid-lowering effects of anti-angiopoietin-like 4 antibody recapitulate the lipid phenotype found in angiopoietin-like 4 knockout mice. Proc Natl Acad Sci U S A. 2007;104:11766 –11771.
41. Ziouzenkova O, Perrey S, Asatryan L, Hwang J, MacNaul KL, Moller DE, Rader DJ, Sevanian A, Zechner R, Hoefler G, Plutzky J. Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an antiinflammatory role for lipoprotein lipase. Proc Natl Acad Sci U S A.2003;100:2730–2735.
42. Augustus A, Yagyu H, Haemmerle G, Bensadoun A, VikramadithyanRK, Park SY, Kim JK, Zechner R, Goldberg IJ. Cardiac-specificknock-out of lipoprotein lipase alters plasma lipoprotein triglyceridemetabolism and cardiac gene expression. J Biol Chem. 2004;279:25050–25057.
43. Juge-Aubry C, Pernin A, Favez T, Burger AG, Wahli W, Meier CA,Desvergne B. DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. Importance of the 5'-flanking region. J Biol Chem. 1997;272:25252–25259.
44. Heinaniemi M, Uski JO, Degenhardt T, Carlberg C. Meta-analysis ofmprimary target genes of peroxisome proliferator-activated receptors.mGenome Biol. 2007;8:R147.
45. Lefterova MI, Zhang Y, Steger DJ, Schupp M, Schug J, Cristancho A,mFeng D, Zhuo D, Stoeckert CJ Jr, Liu XS, Lazar MA. PPARgamma andmmC/EBP factors

orchestrate adipocyte biology via adjacent binding on amgenome-wide scale. Genes Dev. 2008;22:2941–2952.

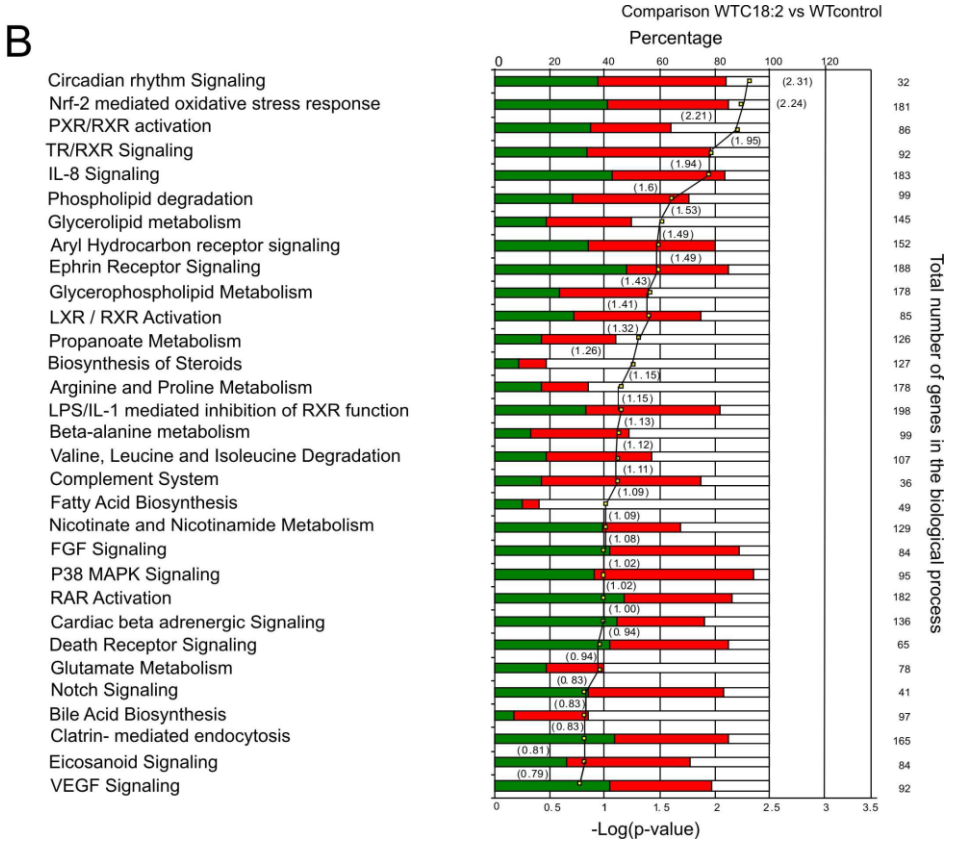
46. Nielsen R, Pedersen TA, Hagenbeek D, Moulos P, Siersbaek R, MegensmE, Denissov S, Borgesen M, Francoijs KJ, Mandrup S, Stunnenberg HG.mGenome-wide profiling of PPARgamma:RXR and RNA polymerase IIoccupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev. 2008;22:2953–2967.
47. van der Meer DL, Degenhardt T, Vaisanen S, de Groot PJ, Heinaniemi M, de Vries SC, Muller M, Carlberg C, Kersten S. Profiling of promoter occupancy by PPAR{alpha} in human hepatoma cells via ChIP-chip analysis. Nucleic Acids Res. In press.
48. Burkart EM, Sambandam N, Han X, Gross RW, Courtois M, Gierasch CM, Shoghi K, Welch MJ, Kelly DP. Nuclear receptors PPARbeta/delta and PPARα direct distinct metabolic regulatory programs in the mouse heart. J Clin Invest. 2007;117:3930 –3939.
49. Le Jan S, Amy C, Cazes A, Monnot C, Lamande N, Favier J, Philippe J, Sibony M, Gasc JM, Corvol P, Germain S. Angiopoietin-like 4 is a proangiogenic factor produced during ischemia and in conventional renal cell carcinoma. Am J Pathol. 2003;162:1521–1528.
50. Ito Y, Oike Y, Yasunaga K, Hamada K, Miyata K, Matsumoto S, Sugano S, Tanihara H, Masuho Y, Suda T. Inhibition of angiogenesis and vascular leakiness by angiopoietin-related protein 4. Cancer Res. 2003; 63:6651– 6657.
51. Cazes A, Galaup A, Chomel C, Bignon M, Brechot N, Le Jan S, Weber H, Corvol P, Muller L, Germain S, Monnot C. Extracellular matrix-bound angiopoietin-like 4 inhibits endothelial cell adhesion, migration, and sprouting and alters actin cytoskeleton. Circ Res. 2006;99:1207–1215.
52. Belanger AJ, Lu H, Date T, Liu LX, Vincent KA, Akita GY, Cheng SH, Gregory RJ, Jiang C. Hypoxia up-regulates expression of peroxisome proliferator-activated receptor gamma angiopoietin-related gene (PGAR) in cardiomyocytes: role of hypoxia inducible factor 1alpha. J Mol Cell Cardiol. 2002;34:765–774.
53. Lichtenstein L, Mattijssen F, de Wit NJ, Georgiadi A, Hooiveld GJ, van der Meer R, He Y, Qi L, Köster A, Tamsma JT, Tan NS, Müller M, Kersten S. Angptl4 protects against severe proinflammatory effects of saturated fat by inhibiting fatty acid uptake into mesenteric lymph node macrophages. Cell Metabolism. 2010 Dec 1;12(6):580-92.
54. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004;5:R80.

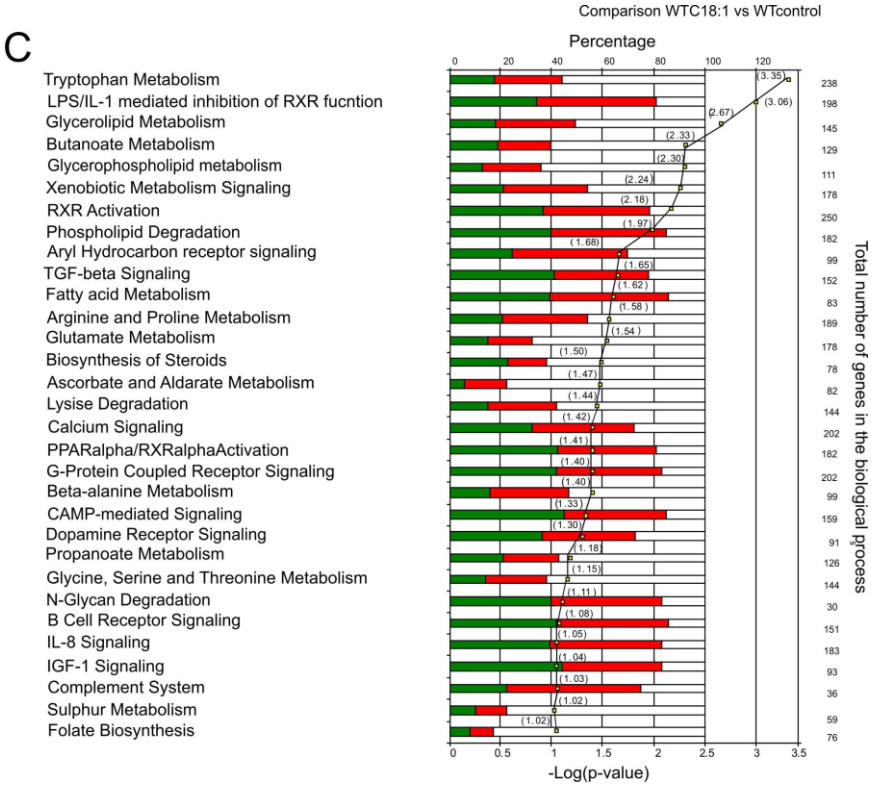
55. Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, Watson SJ, Meng F. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res.2005;33:e175.
56. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3:Article3.

APPENDIX

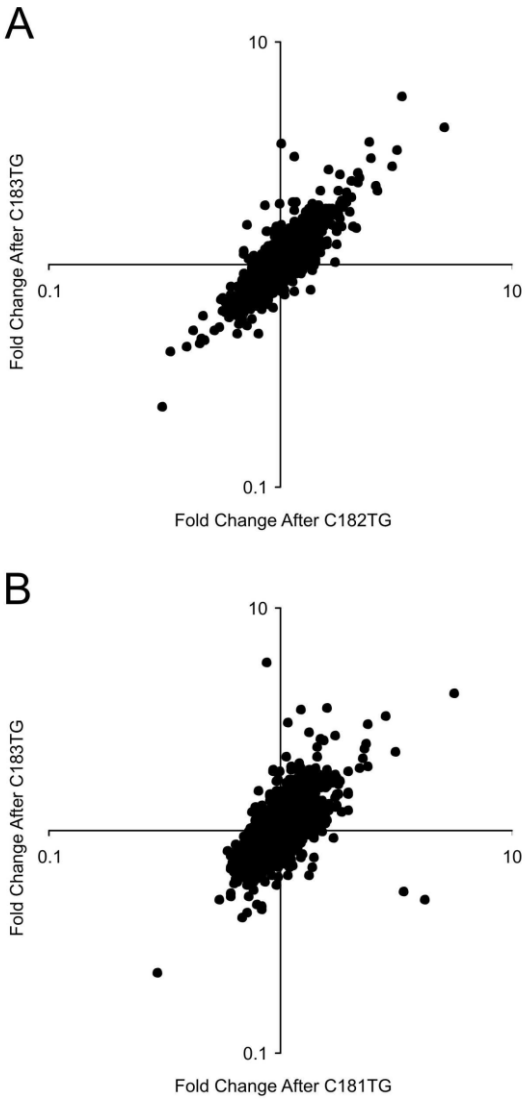

A
Downregulated
Upregulated
Not changed
- log (p - value)
Comparison WTC18:3 vs WTcontrol
Percentage
Nrf2-mediated oxidative stress response
TR / RXR Activation
RAR Activation
IL-8 Signaling
P53 Signaling
Xenobiotic Metabolism Signaling
Cardiac beta adrenergic Signaling
PXR/RXR Signaling
Clatrin- mediated endocytosis
Cysteine Metabolism
Glutamate metabolism
LPS/IL-1 mediated inhibition of RXR function
Histidine Metabolism
VEGF Signaling
Wnt/beta catenin Signaling
Alpha-adrenergic signaling
Insulin receptor signaling
Nitrogen Metabolism
Huntington's Disease Signaling
Hypoxia Signaling in the CVD
Nitric Oxide Signaling in the CVD
Macropynocytosis
Aryl Hydrocarbon receptor signaling
Dopamine receptor signaling
Fructose and Mannose Metabolism
Hepatic Fibrosis/ Hepatic Stellate Cell Activation
IGF-1 Signaling
ERK/MAPK Signaling
Ceramide Signaling
Ephrin Receptor Signaling
PPARalpha/RXRalpha activation
0 20 40 60 80 100 120
(3.55) (2.75) (2.70) (2.52) (2.47) (2.45) (2.37) (2.31) (2.30) (2.14) (1.97) (1.89) (1.86) (1.86) (1.74) (1.67) (1.62) (1.62) (1.61) (1.49) (1.45) (1.44) (1.36) (1.34) (1.28) (1.26) (1.26) (1.25) (1.24) (1.23) (1.23)
181 92 182 183 87 250 136 86 165 85 78 198 111 92 165 107 133 133 232 71 70 152 91 139 135 93 183 82 90 188 182
Total number of genes in the biological process
0 0.5 1 1.5 2 2.5 3 3.5
-Log(p-value)

B

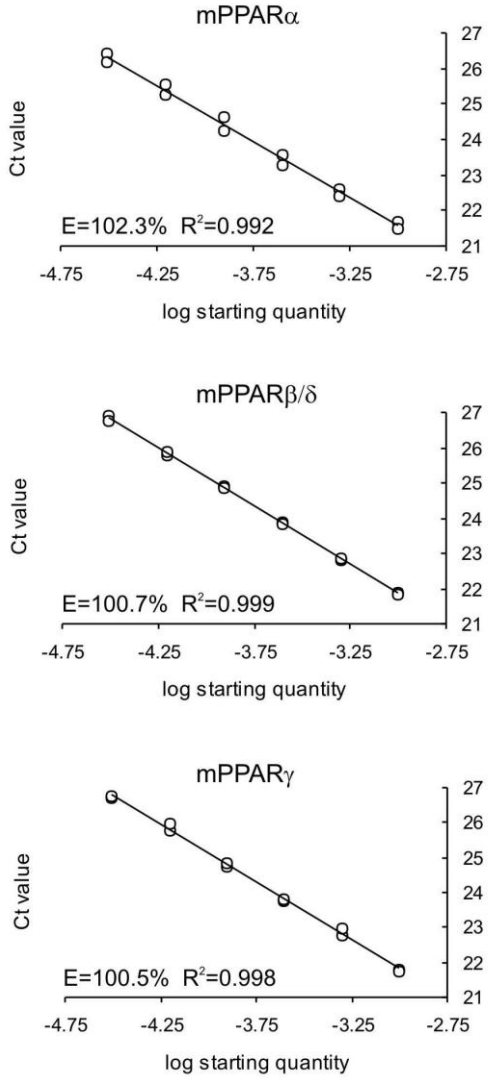

Comparison WTC18:2 vs WTcontrol
Percentage
0
20
40
60
80
100
120
Circadian rhythm Signaling
Nrf-2 mediated oxidative stress response
PXR/RXR activation
TR/RXR Signaling
IL-8 Signaling
Phospholipid degradation
Glycerolipid metabolism
Aryl Hydrocarbon receptor signaling
Ephrin Receptor Signaling
Glycerophospholipid Metabolism
LXR / RXR Activation
Propanoate Metabolism
Biosynthesis of Steroids
Arginine and Proline Metabolism
LPS/IL-1 mediated inhibition of RXR function
Beta-alanine metabolism
Valine, Leucine and Isoleucine Degradation
Complement System
Fatty Acid Biosynthesis
Nicotinate and Nicotinamide Metabolism
FGF Signaling
P38 MAPK Signaling
RAR Activation
Cardiac beta adrenergic Signaling
Death Receptor Signaling
Glutamate Metabolism
Notch Signaling
Bile Acid Biosynthesis
Clatrin- mediated endocytosis
Eicosanoid Signaling
VEGF Signaling
(2.31)
(2.24)
(2.21)
(1.95)
(1.94)
(1.6)
(1.53)
(1.49)
(1.49)
(1.43)
(1.41)
(1.32)
(1.26)
(1.15)
(1.15)
(1.13)
(1.12)
(1.11)
(1.09)
(1.09)
(1.08)
(1.02)
(1.02)
(1.00)
(0.94)
(0.94)
(0.83)
(0.83)
(0.83)
(0.81)
(0.79)
32
181
86
92
183
99
145
152
188
178
85
126
127
178
198
99
107
36
49
129
84
95
182
136
65
78
41
97
165
84
92
Total number of genes in the biological process
0
0.5
1
1.5
2
2.5
3
3.5
-Log(p-value)



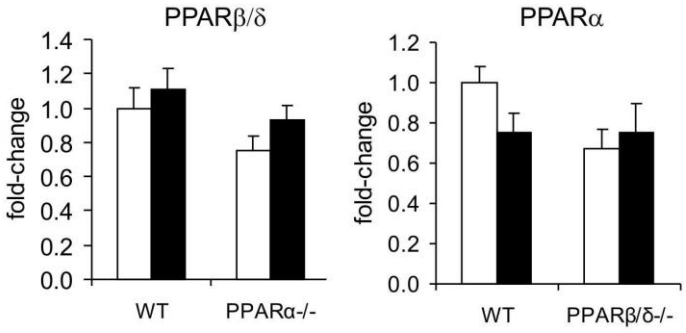
Appendix Figure 1: Induction of oxidative stress response 6 hours after the oral ingestion of linolenic acid (A), linoleic acid (B) or oleic acid (C). Bars show the percentage of upregulated genes (red) and downregulated genes (green) out of the total number of eligible genes for each pathway, based on the Ingenuity knowledge database. The white part represents the percentage of genes that do not overlap with the experimental dataset. The pathways are displayed from the direction of the most significantly regulated to least significantly regulated based on Fisher's Exact Test p-value (cut off $p<0.05$). The –log(p-value) is displayed on the top of each pathway (yellow square).



Appendix Figure 2: Similarity in cardiac gene regulation between fatty acids. Graphs show fold-change in gene expression after treatment with C18:3TG (y-axis) plotted against fold-change in gene expression after treatment with C18:2TG (A) or C18:1TG (B) (x axis). Analysis shows more significant similarity in gene regulation between C18:3 andC18:2, compared to between C18:3 and C18:1.



Appendix Figure 3: Equal efficiency of amplification of mPPARα, mPPARβ/δ, and mPPARγ in mouse heart. A standard curve was generated from cDNA prepared from mouse heart. cDNA wasamplified by qPCR using primers specific for the three PPAR isotypes. Similar efficiencies of amplification were obtained using cDNA from rat neonatal cardiomyocytes.



Appendix Figure 4: No compensatory increase in PPARβ/δ and PPARα expression in PPARα-/- and PPARβ/δ -/- mice, respectively. Wild type, PPARα-/-, and PPARβ/δ-/- were given a single oral gavage of 0.5% CMC (open bars) or synthetic TG composed entirely of C18:3 (closed bars). mRNA expression levels of PPARβ/δ (left panel) and PPARα (right panel) were determined in mouse heart using real time PCR. Results are expressed as fold-change compared to the WT control mice. Error bars represent SEM.

Chapter 5

Angptl4 overexpression protects against development of atherosclerosis in mice

Anastasia Georgiadi, Yixin Wang, Rinke Stienstra, Anton Stalenhoef, Nguan Soon Tan, Michael Müller, Patrick C.N. Rensen and Sander Kersten.

Chapter based on manuscript in Preparation

Abstract

Atherosclerosis is a multi-factorial chronic disease characterized by lipid retention and inflammation in the arterial intima. Inflammatory activation of macrophages combined with increased lipid uptake leads to formation of foam cells, which form the core of atherosclerotic plaques. Inhibition of lipoprotein lipase-mediated lipolysis by the protein Angptl4 was shown to prevent macrophage foam cell formation in vitro, as well as in mesenteric lymph nodes upon high fat feeding. Accordingly, we hypothesized that Angptl4 may affect atherosclerosis development by reducing foam cell formation. To investigate this hypothesis, we studied Angptl4 expression in atherosclerotic lesions and macrophages and determined the effect of Angptl4 transgenic overexpression in atherosclerosis prone ApoE3Leiden (E3L) mice fed a Western diet containing 0.4% cholesterol. Angptl4 was localised to macrophages in human atherosclerotic lesions and was regulated by inflammatory stimuli in human THP-1 macrophages. During the 24 weeks of diet intervention, plasma cholesterol and triglyceride level were not different between the two groups. Remarkably, Angptl4Tg.E3L mice showed a 34% decrease in lesion area compared to E3L mice. In addition, content of macrophages and numbers of adhering monocytes were decreased in Angptl4Tg.E3L mice compared to E3L mice. Furthermore, Angptl4 decreased uptake of oxLDL in mouse and human macrophages. Our results suggest that Angptl4 is involved in an anti-inflammatory signaling that suppresses development of atherosclerosis by reducing foam cell formation.

Introduction

Despite a major decrease in mortality from atherosclerosis in many Western countries in recent decades, atherosclerosis-related morbidity and mortality remain a major global health concern. Atherosclerosis is a multi-factorial chronic disease characterized by lipid retention and inflammation in the arterial intima, forming the so called atheromas. While traditionally atherosclerosis was mostly viewed as a lipid retentive disease caused by elevated plasma lipids, in the past two decades the interplay between inflammatory mechanisms and dyslipidemia in development of atherosclerosis has been increasingly recognized. It is well accepted that circulating monocytes responding to chemotactic stimuli adhere to endothelial cells and subsequently migrate into the sub-endothelial space where they become activated. Activated macrophages may subsequently accumulate lipid and become foam cells by scavenging oxidized lipoprotein remnants - mainly oxidised LDL (oxLDL) - trapped in the sub-endothelial space [1].

Uptake of oxLDL and formation of foam cells via macrophage scavenger receptors are thus considered key events in the development and progression of arterial intimal inflammation. According to this view, scavenger receptors are proatherogenic proteins, although recent data present a more complicated picture in which these multifunctional receptors may both protect and exacerbate atherosclerosis development [2-5]. Furthermore, innate immune mechanisms also appear to contribute to macrophage activation and subsequent fat accumulation in macrophages [6]. Specifically, the finding that oxidized lipoproteins or their component oxidized lipids may act as ligands for Toll-like receptors (TLRs) coupled with the recent demonstration that mice deficient in TLR2 or TLR4 exhibit reduced atherosclerosis points to a key role of Toll-like receptor signaling in atherosclerosis development [7, 8].

While macrophage foam cell formation has been studied primarily in the context of atherosclerosis, they also participate in other pathologies including multiple sclerosis [9], non-alcoholic fatty liver disease, and kidney disease. Recently, we described the accumulation of macrophage foam cells in mesenteric lymph nodes of mice fed a high saturated fat diet. Foam cell formation was specific for mice lacking Angptl4, an endogenous inhibitor of the triglyceride hydrolyzing enzyme lipoprotein lipase (LPL), which catalyzes uptake of circulating lipids into tissues [10, 11]. Specifically, the N-terminal

portion of Angptl4 irreversibly inhibits LPL activity by converting active LPL dimers into inactive monomers [10]. As a consequence, overexpression of Angptl4 leads to hypertriglyceridemia and reduced fatty acid uptake into tissues, whereas Angptl4 deletion results in lowering of circulating triglyceride levels [12-16]. Angptl4 is a protein secreted by a variety of tissues including adipose tissue, liver, skeletal muscle, and intestine. In addition, Angptl4 as well as LPL are well expressed in macrophages [17]. Angptl4 expression in macrophages is governed by PPARβ/δ and PPARγ.

Considering the importance of foam cells in atherosclerosis and the stimulatory effect of LPL on atherosclerosis [18], we were interested to examine the potential impact of Angptl4 on atherosclerosis development. For these studies we took advantage of the atherosclerosis prone ApoE3Leiden (E3L) mouse model. E3L mice carry a partially disabled ApoE gene that is associated with familial hypercholesterolemia in humans [19, 20]. E3L mice represent an unique human-like model for studies on atherosclerosis characterized by: 1) plasma cholesterol levels that are proportional to the cholesterol content in the diet, 2) development of diet-induced atherosclerosis in the presence of the LDL receptor and apoE. In the present study, we show that whole body overexpression of Angptl4 protects against atherosclerosis as manifested by a decreased size of atherosclerotic plaques.

Materials and Methods

Materials: Human Recombinant Angptl4 (full length 4487-AN) was purchased by R&D systems. Mouse recombinant MCP-1 was purchased by ITK Diagnostics, The Netherlands. Intralipid was purchased from Fresenius Kabi. TLR agonists were purchashed from Sigma, Fluka, Brunwich amd InvivoGen.

Animals: Animal studies were done using pure-bred WT and Angptl4Tg mice on a C57BI/6 background [13]. The ApoE3Leiden were also on C57BI/6 background (n=19-18). Mice were housed under standard conditions with a 12-hour light/dark cycle and had free access to food and water. Female mice were fed a Western type diet with 15% w/w cacao butter (diet T, Hope Farms, Woerden, the Netherlands) supplemented with 0.4% (wt/wt) cholesterol, (Sigma-Aldrich, Zwijndrecht, the Netherlands). Detailed description of diet T is shown in Apprendix Table 1.

Plasma Lipids: Blood was collected into EDTA containing tubes to prevent coagulation. Blood samples were placed on ice and centrifuged at 4°C for 10 minutes at 10,000 g. The plasma concentration of triglycerides was determined using the Triglycerides liquicolor mono kit by Instruchemie (Delfzijl, Netherlands). Plasma cholesterol was determined using the Elitech cholesterol PAP SL from Sopachem (Wageningen, Netherlands).

Lipoproteins profiling: Plasma lipoproteins of Angptl4Tg.E3L and E3L mice were separated using fast liquid chromatography (FPLC). For fast protein liquid chromatography (FPLC) fractionation of lipoproteins, 18µl were pooled from each mouse up to 0.4ml per group. 0.35ml of pooled plasma was injected into a Superose 6B 10/300 column (GE Healthcare Bio-Sciences AB, Roosendaal, Netherlands) and eluted at a constant flow of 0.5 mL/minute with phosphate buffered saline (pH 7.4). The effluent was collected in 0.5 mL fractions and TG and cholesterol levels were determined as described above.

Atherosclerosis quantification and plaque composition (immunohistology): After 24 weeks on diet T + 0.4% cholesterol, final blood collection was withdrawn from the eye, after the mice have been anaesthetized with injection of VDF (Vetranquil, Dormicum, Fentanyl). Mice were killed by CO_2 inhalation and organs were perfused followed by heart perfusion with PBS. Hearts were isolated and later fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin, and cross-sectioned (5 µm), throughout the aortic root area. Four sections per mouse with 50-µm intervals were used for atherosclerosis measurements. For further histological analysis, sections were stained with hematoxylin-phloxin-saffron (HPS staining). Lesions were categorized for severity according to the guidelines of the American Heart Association, adapted for mice [37, 38]. Briefly, we describe the criteria: severity type 0 -no lesion, type 1- early fatty streaks containing up to 10 foam cell macrophages in the intima, type 2- early fatty streaks with more than 10 foam cell macrophages in the intima, type 3- early fatty streak, containing foam cells in the intima, covered by a fibrous cap, type 4- advanced lesions with foam cell macrophages in the intima, signs of fibrosis, lipid core, but no disruption of the media, type 5 - advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization, and/or necrosis. Lesion area was determined with Leica Qwin image analysis software (Image J). AIA 31240 antiserum (1:3000, Accurate Chemical and Scientific, Westbury, NY) was used

to quantify the macrophage area and the number of monocytes adhering to the endothelium. Sirius Red was used to quantify the collagen area and M0851(1:800, Dako) against smooth muscle cell actin to quantify the smooth muscle cell area.

Paraffin preserved sections from *human carotid artery* embedded in paraffin cross sectioned (5 μm) and used for histological analysis. A general HE (hematoxylin eosin) staining, to visualize the structure of the tissue was performed. Human Antibody for Cd68 staining was purchased from Abdserotec and human Angptl4 antibody, which recognizes the C-terminus was a gift from Andrew Tan. The first day, sections were left overnight at 37^{o}C. Second day, we performed deparafinization of the sections, followed by inhibition of endogenous peroxidases for 30min. Antibody for Cd68 and for Angplt4 was used in dilution 1:100. Staining with primary antibody was performed overnight. Negative control was not incubated with primary antibody. Secondary antibody was used in dilution 1:200 and it was applied on the sections for 45min. Visualization of the complex was done with DAB staining. Hematoxyline staining was perfomed, as well. Areas of positive staining obtained a brown colour.

Migration assay: QCM™ Chemotaxis 96 well (5 iM) Cell Migration Assay Fluorimetric assay from Millipore was used. Boyden chambers with filters of 5μm pore size were used. Mouse bone marrow cells were differentiated into macrophages for 9 days in DMEM medium, containing 10% heat inactivated FCS and 20% L929 conditioned medium. Medium was changed every day after day 9 and cells were used at day 12. Before the migration assay cells were starved from FCS for 24hrs (medium containing only 1,5% of FBS deriving from L929 medium). At the upper part of the filter we added 2000 cells in 100uL serum free DMEM medium with additional 1% BSA. At the bottom part of the filter, we added serum free DMEM medium with additional 1% BSA, with or without mouse recombinant MCP-1 (5μg/ml). Migration was stopped after 3hours. Cells attached at the bottom of the filter were detached, via incubation with a detachment buffer, provided with the kit and pooled with those that have migrated towards the MCP-1 containing medium at the bottom chamber. A mix of lysis buffer/DNA binding fluorescent dye (CyQuant GR Dye) was added on the migrated cells for 15 min at RT. Fluorescence was measured at 480/520nm.

RNA isolation and qRT-PCR: Total RNA was isolated with TRIzol Reagent (Invitrogen, Breda, the Netherlands). 1µg of total RNA was reverse transcribed using Fermentas (Bio-Rad, Veenendaal, the Netherlands). cDNA was amplified on BioRad iCycler machine using Sensimix (Invitrogen, Breda, the Netherlands). PCR primer sequences were ordered from Eurogentec (Seraing, Belgium). List of primers used are shown in Table 1.

Table 1: Sequences of primers used for qRT-PCR.

Primer Name	Forward sequence	Reverse sequence
hAngptl4	CACAGCCTGCAGACACAACTC	GGAGGCCAAACTGGCTTTGC
mAngptl4	GTTTGCAGACTCAGCTCAAGG	CCAAGAGGTCTATCTGGCTCTG
mPtgs2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
mCxcl2	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
mDdit3	CTGGAAGCCTGGTATGAGGAT	CAGGGTCAAGAGTAGTGAAGGT
mGdf15	CTGGCAATGCCTGAACAACG	GGTCGGGACTTGGTTCTGAG

Oil red O staining on fixed cells: Oil red O stock solution was prepared by dissolving 0.5 g oil red O (Sigma, #O0625) in 500 ml isopropanol. Oil red O working solution was prepared by mixing 30 ml oil red O stock with 20 ml dH2O, followed by filtration. Attached cells were washed twice with PBS and fixed for 10 min in formal calcium (4% formaldehyde, 1% CaCl2). After that cells were washed twice with PBS and covered with Oil Red O working solution for 20min, followed by two rinses with dH2O.

Lipoprotein isolation and tritium labelling: Isolation of human lipoproteins was achieved by sequential ultracentrifugation at the respective density at 40,000 rpm in a Ti-50 fixed-angle rotor (Beckman Instruments, Geneva, Switzerland) for 18h at 5°C, followed by dialysis at 4°C overnight against PBS, pH 7.4. VLDL and LDL fraction was isolated and labelled with tritium, using glycerol tri^{3}[H]oleate (^{3}[H]TO) and ^{3}[H]cholesteryl oleoyl ether (^{3}[H]COEth), respectively. Labeling protocol has been previously described [39]. Protein concentrations in the lipoporteins fractions were determined with BCA Pierce assay using a BSA standard curve.

Quantification of cellular uptake of tritium labelled lipoproteins: After incubation of macrophages with tritium labelled lipoproteins, cells were washed twice with 500μL PBS and cell lysates were precipitated in 500μL of 0,1M NaOH, with gentle shaking for 15min. 250μL of cell lysates were used for quantification of radioactivity. dmp values were normalised to the total amount of protein (mg) present in 250μL of cell lysates. Protein from cell lysates was quantified with BCA Pierce assay.

LDL oxidation with CuSO4 : LDL preserved in Kbr was dialysed overnight in PBS. Oxidation of LDL was done with CuSO4 at a final concentration of 20uM for 3hours in 37^{o}C. Oxidation was terminated by adding 200uM EDTA and palce the tube on ice. EDTA and CuSO4 were removed with overnight dialysis in PBS.

Cells: *Bone marrow cells* were grown in DMEM +10% heat inactivated (HI) FCS +1% P:S with additional 20% L929 conditioned medium DMEM (10% HI FCS, 1% P:S), in order to stimulate macrophage differentiation. Differentiation was allowed up to 7-9 days. During that period fresh medium was added to the cells without removing the old medium. After that period medium was renewed every day. Bone marrow derived macrophages were used for experiments at day 10 or 11.

THP-1 monocytes were differentiated into macrophages after 2 days incubation in RPMI medium, 10% HI FCS, 1% PS containing 100ng.ml phorbol-12-myristate-13-acetate (PMA). After 2days, PMA was washed away and THP-1 macrophages were kept in complete medium without PMA, for 2 days further and then used for experiments.

RAW 264.7 cells were grown in DMEM supplemented with 10% FCS and 1% P:S.

Intralipid was purchased from Sigma and used in final concentration of 2mM.

Recombinant Angptl4 was used in concentration 1.5ug/ml

Activation of macrophages with TLR agonists: The different TLR agonists used and their concentrations are shown in Table 2

Table 2: Targeting receptors and experimental concentrations of TLR agonists

TLR ligands	Receptor	Concentration
TLR4	LPS	1ug/ml
TLR1+2	Pam3Cys	1ug/ml
TLR5	Flageline	10ng/ml
TLR2+6	FSL-1	1ug/ml
TLR3	Poly(I:C)	2ug/ml
Dectrin 1+TLR2+6	b-glucan	4ug/ml
NOD1	MTP	18nM
NOD2	MDP	10ug/ml
NOD1	Tri-dap	5ug/ml

Results

Angptl4 is induced by Toll- like receptors 3 and 4 in macrophages

Previous studies have shown that Angptl4 expression is highly upregulated by chylomicrons and fatty acids in peritoneal macrophages [17]. In line with these data we observed that incubation of RAW 264.7 macrophages with a triglyceride emulsion, which causes foam cell formation and macrophage activation (Figure 1A), increases Angptl4 mRNA expression, as well as expression of several inflammatory markers and ER stress marker, Ddit3 (Figure 1B). Similar results were obtained in bone marrow derived macrophages (BMDMs) (Figure 1C,D). To investigate whether activation of macrophages independent of foam cell formation may induce Angptl4 expression, macrophages were treated with various Pattern Recognition Receptor (PRR) agonists. Human THP-1 macrophages were used to enable measurement of Angptl4 protein secretion using ELISA [21]. TLR3 and TLR4 agonist potently induced Angptl4 mRNA and protein secretion (Figure 1E,F), whereas the other agonists were weak or not effective. These data show that macrophage activation induced by PRR agonists or lipid overload leads to upregulation of Angptl4 gene expression.

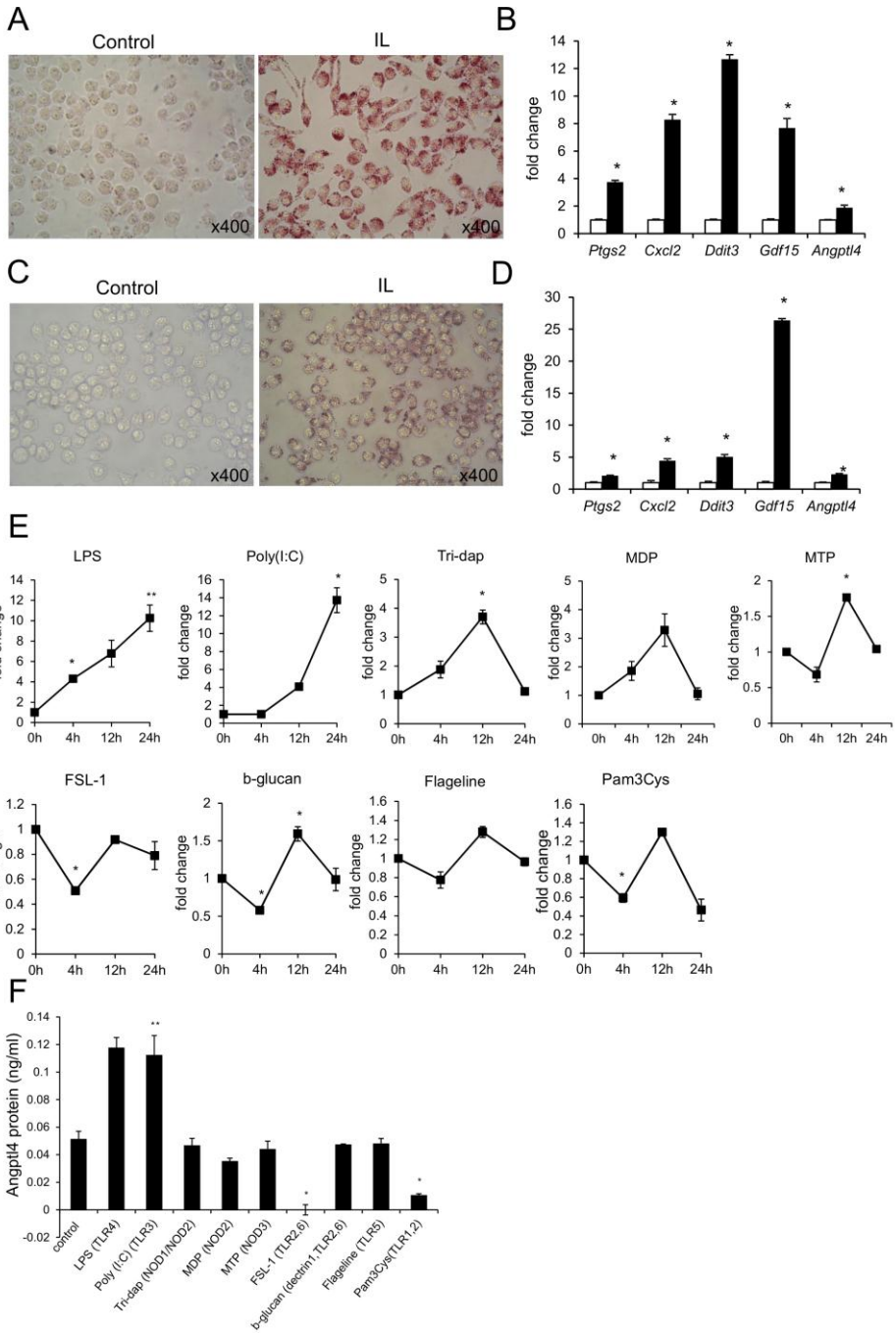

A
Control
IL
x400
x400
B
fold change
Ptgs2
Cxcl2
Ddit3
Gdf15
Angptl4
C
Control
IL
x400
x400
D
fold change
Ptgs2
Cxcl2
Ddit3
Gdf15
Angptl4
E
LPS
Poly(I:C)
Tri-dap
MDP
MTP
FSL-1
b-glucan
Flageline
Pam3Cys
fold change
0h
4h
12h
24h
F
Angptl4 protein (ng/ml)
control
LPS (TLR4)
Poly (I:C) (TLR3)
Tri-dap (NOD1/NOD2)
MDP (NOD2)
MTP (NOD3)
FSL-1 (TLR2,6)
b-glucan (dectrin1,TLR2,6)
Flageline (TLR5)
Pam3Cys(TLR1,2)

Figure 1: Angptl4 expression is induced in macrophages, after lipid overload or activation with TLR4 and TLR3 agonists A. Oil red O staining of RAW 264.7 mouse macrophages treated 6hrs with intralipid (IL) B. Mean fold change of mRNA levels of highly responding genes to 6 hrs intralipid (IL) treatment of RAW 264.7 cells black bars-IL, white bars-control C. Oil red O staining of bone marrow derived mouse macrophages (BMDMs) treated 6hrs with intralipid (IL) D. Mean fold change of mRNA levels of highly responding genes to 6 hrs intralipid (IL) treatment of bone marrow derived macrophages, black bars-IL, white bars-control (Mean fold change over the control,which is 1) E. THP-1 macrophages treated for 4h, 12h and 24h with a panel of TLR agonists. Human Angptl4 mRNA levels were quantified with qRT-PCR and expressed as mean fold change over its basal levels for each time point. F. Protein levels of Angptl4 were quantified with ELISA, in medium collected from THP-1 macrophages treated 24 hrs with different TLR agonists. Error bars represent SEM. *Significantly different between treatment and control, according to Student's t-test (*P<0.05, ** P<0.01).

A pathological condition characterized by macrophage activation and lipid overload is atherosclerosis. To study the potential role of Angptl4 in atherosclerosis, we first ascertained the presence of Angptl4 in human atherosclerotic plaques. Staining of serial sections from human carotid tissue with antibodies against Cd68 and Angptl4 revealed co-localization of Angptl4 with Cd68, suggesting Angptl4 is present in macrophages (Figure 2).

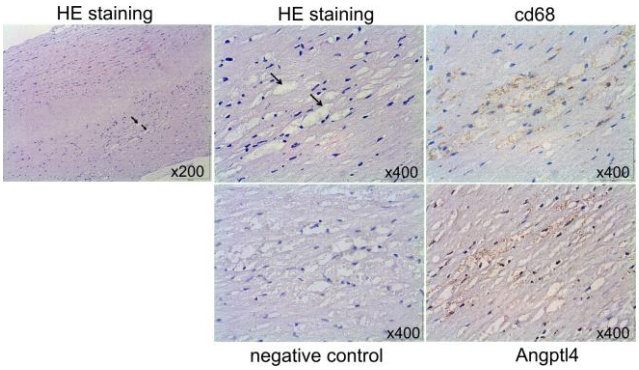


Figure 2: Angptl4 protein localizes to the macrophages at the arterial wall A. Staining of serial sections of human carotid artery. Top left, top middle: High magnification of x200 and x400 of section of human carotid artery stained with HE staining in order to inspect the structural morphology of the arterial wall. Arrows point to lipid-loaded macrophages (foam cells). Top right: Staining with human Cd68

recognizing antibody, diluted 1:100, magnification x400. Bottom right: Staining with human Angptl4 recognizing antibody, diluted 1:100, magnification x400. The antibody recognizes specifically the C-terminal portion of Angptl4. Bottom left: negative control.

Overexpression of Angptl4 on an E3L background does not affect plasma cholesterol and triglyceride levels

To further explore the role of Angptl4 in atherosclerosis development, we crossed mice with whole body overexpression of Angptl4 with ApoE3 Leiden (E3L) mice to generate the Angptl4Tg.E3L mice. E3L mice represent an unique human-like model for studies on atherosclerosis characterized by: 1) plasma cholesterol levels that are proportional to the cholesterol content in the diet, 2) development of diet-induced atherosclerosis in the presence of the LDL receptor and apoE. Both Angptl4Tg.E3L mice and control E3L mice were fed a Western type diet containing 0.4% cholesterol for 24 weeks. Weight gain was equal between the two groups (Figure 3A). In contrast, Angptl4Tg.E3L ate slightly less than the E3Lgroup (Figure 3B). After 4 weeks all animals were hypercholesterolemic and plasma cholesterol levels remained high until the end of the study. Importantly, plasma cholesterol levels were not significantly different between the two groups (Figure 3C). Whereas plasma triglycerides were increased in Angptl4Tg.E3L mice up to week 4, triglycerides subsequently dropped to levels that were not significantly different from the E3L group (Figure 3D). Elevated plasma cholesterol and triglyceride levels in Angptl4Tg.E3L and E3L mice could be attributed to elevated VLDL/LDL levels, as determined by fast liquid protein chromatography (FPLC) (Figure 3E).

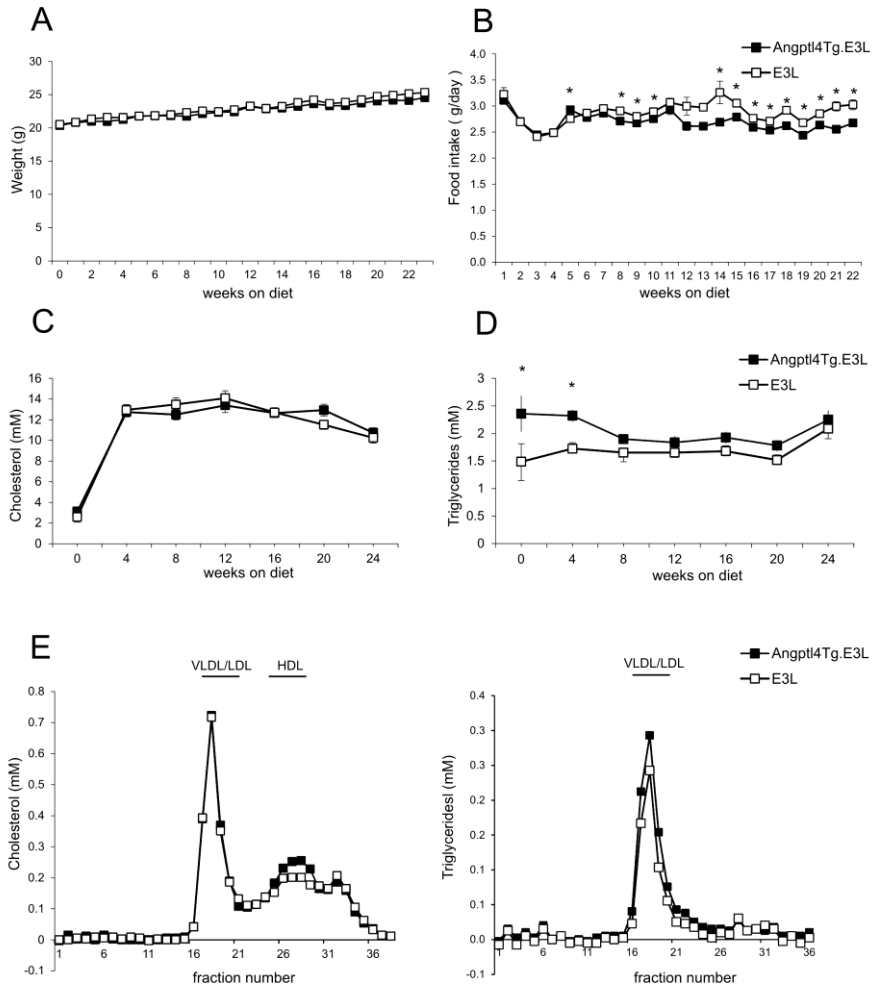


Figure 3: No difference in plasma cholesterol and triglyceride levels between Angptl4Tg.E3L and E3L after 24 weeks on Western diet + 0.4% cholesterol. A. Body weight, in grams, of Angptl4Tg.E3L and E3L mice after 24 weeks on a Western diet + 0.4% cholesterol B. Food intake expressed in grams/day C,D. Plasma cholesterol and triglycerides levels (mM). E. Quantification of plasma cholesterol and triglycerideslevels in FPLC fractions. Black squares refer to Angptl4Tg.E3L group and white squares to ApoE3Leiden group. Y-axis values represent mean, error bars represent SEM.* Significantly different between Angptl4Tg.E3L and E3L, for the specific time point, according to Student's t-test ($P<0.05$). Numbers of animals per group were 16 to 18.

Angptl4Tg.E3Leiden mice developed smaller lesions

We further investigated the severity of atherosclerosis by measuring the lesion area and by assigning lesions to different levels of severity based on cellular composition of the plaques (for criteria see Methods/Materials). Whereas Angptl4Tg.E3L mice developed on average an equal number of plaques compared to the E3L mice (Figure 4A), average lesion size was reduced by 34% in Angptl4Tg.E3L mice (Figure 4B). We next classified lesions according to severity and determined the distribution of lesions according to severity in the two groups. Angptl4Tg.E3L mice showed a tendency towards the development of less severe lesions compared to E3L mice (Figure 4C). We next evaluated the effect of Angptl4 overexpression on monocyte recruitment and lesion composition including macrophage content, collagen content and smooth vascular muscle cells content. Angptl4 overexpression significantly decreased the number of adhering monocytes to the vessel wall compared to E3L mice (Figure 5A). The decrease in adhering monocytes was accompanied by a 2-fold ($P<0.05$) decrease in macrophage content in the intima of Angptl4Tg.E3L mice (Figure 5B). No statistically significant differences in collagen and smooth vascular muscle cells content were observed between the two groups (Figure 5C,D). Thus, overexpression of Angptl4 reduces lesion size compared to the E3L group and leads to a less inflammatory lesion phenotype characterized by decreased monocyte/macrophage accumulation.

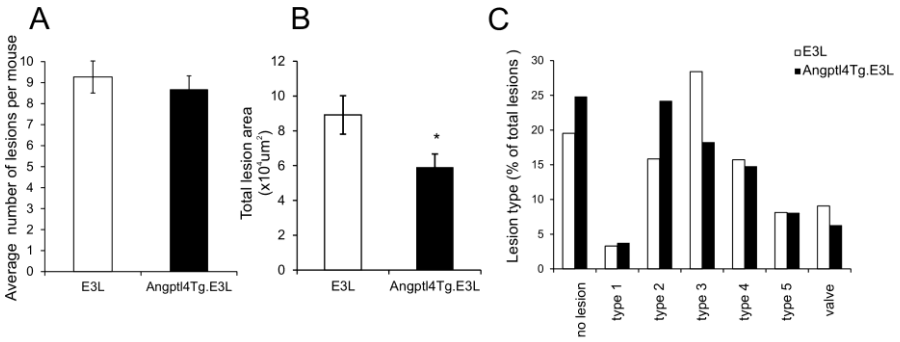


Figure 4: Angptl4Tg.E3Leiden mice developed smaller lesions A. Average number of lesions in Angptl4Tg.E3L and E3L mice (E3L n=15, Angptl4Tg.E3L n=16) Number of lesions were counted in four sections per mouse, each of those containing three segments B. The total lesion area (μm^2) in Angptl4Tg.E3L and E3L mice. Lesion area was counted in four sections (50-µm intervals) per mouse, three segments per section. C. Percentage of lesions per type of severity over the total number of lesions belonging in each type of severity are shown. Numbers of animals per group were 15-16. *Significantly different between Angptl4Tg.E3L and E3L group, according to Student's t-test ($P<0.05$). Error bars represent SEM.

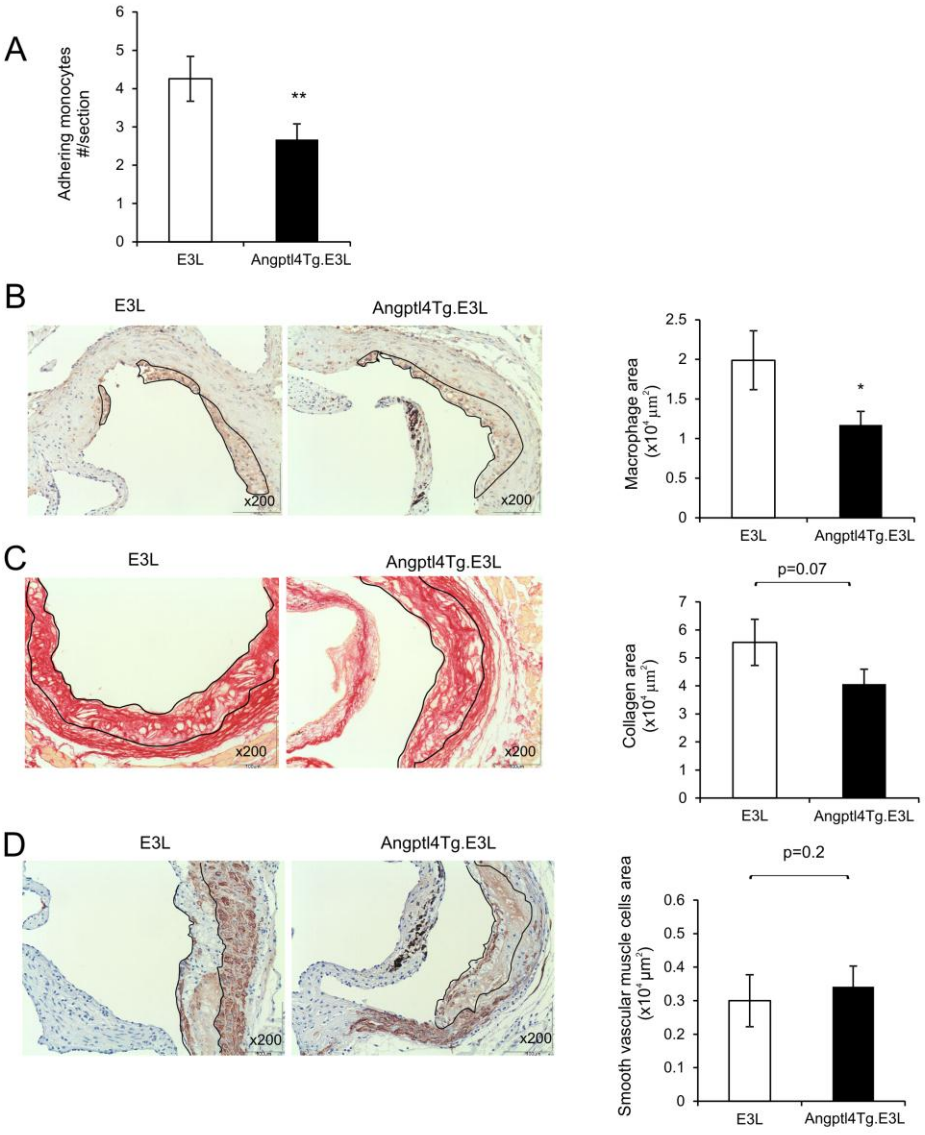


Figure 5: Decreased monocyte and macrophage content in atherosclerotic plaques of Angptl4Tg.E3L mice A. Counted numbers of monocytes adhered to the endothelial layer. B, C, D. (Right) Absolute area (μm^2) of staining is shown. (B, right) Mean area of AIA 31240 antiserum staining for macropahage area (brown staining), (C,right) Sirius Red staining collagen area (red staining) and (D,right) a-actin staining for smooth vascular muscle cell area (brown staining) is shown. (Left) Representative images of

stained atherosclerotic lesions of camplarable size are presented. A black line is drawn to define the lesion area. Y-axis represent mean in Angptl4Tg.E3L and E3L group and error bars SEM. Numbers of animals per group were 15-16. *Significantly different between Angptl4Tg.E3L and E3L group, according to Student's t-test (*P<0.05, **P<0.01).

In the early stages of atherogenesis, monocytes/macrophages are recruited to the vessel wall in response to chemokines such as MCP-1 produced by the inflamed endothelium [22]. To further investigate the effect of Angptl4 on the chemotactic recruitment of macrophages, we performed an *in vitro* macrophage migration assay. BMDMs from Angptl4-Tg mice, characterized by 4-fold elevated Angptl4 mRNA levels (Figure 6A), migrated significantly less towards chemotactic signal (MCP-1) compared to WT macrophages (Figure 6B). These results suggest a suppressive effect of Angptl4 overexpression on macrophage migration and chemotaxis.

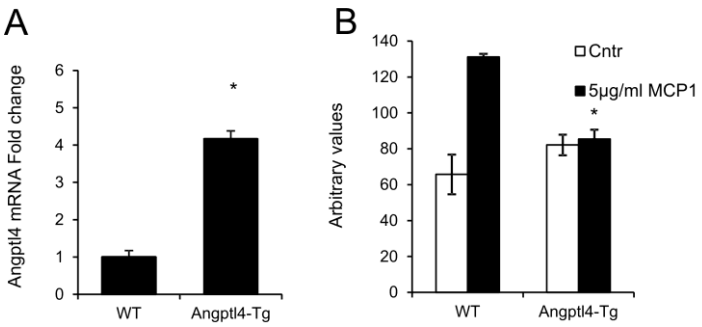


Figure 6: Decreased migration of Angptl4-Tg BMDMs towards MCP-1 A. Mean fold change of mouse Angptl4 mRNA expression levels in BMDMs derived from Angptl4-Tg mice, compared to BMDMs derived from WT mouse. Quantification was done with qRT-PCR (Ct values: 25-27) B. BMDMs from WT or Angptl4-Tg mice were added on the top chamber of QCM™ Chemotaxis 96 well (5 ìM) Cell Migration Assay plate from Millipore. At the bottom chamber 5µg/ml MCP-1 mouse recombinant protein was added. Number of cells migrated to the bottom chamber were quantified via measurements of fluorescence DNA binding substance introduced to the cells during cell lysis. Y-axis represent mean and error bars SEM. *Significantly different between Angptl4-Tg and WT group, according to Student's t-test (P<0.05)

Angptl4 decreased the uptake of oxLDL in macrophages

Oxidized LDL (oxLDL) is known to promote atherogenesis by causing foam cell formation and inducing secretion of proinflammatory cytokines like MCP-1 [23]. Since Angptl4 overexpression decreased the recruitment of monocytes/macrophages, we were interested to assess its role in oxidized LDL uptake and subsequent formation of foam cells. For this purpose, we used BMDMs from WT and Angptl4-Tg mice. We first assessed whether BMDMs may transform into foam cells after uptake of oxLDL. OxLDL efficiently promoted foam cell formation in WT and Angptl4-Tg BMDMs. In contrast, native LDL did not have any effect on foam cell formation (Figure 7A). In order to quantitatively assess macrophage uptake of oxLDL, we incubated BMDMs from WT and Angptl4-Tg mice with [^{3}H] labelled oxLDL and LDL for 48hrs. Uptake of oxLDL was lower in Angptl4-Tg compared to WT macrophages, while uptake of LDL was unaltered (Figure 7B).

TG-rich VLDL particles have been also shown to stimulate foam cell formation and provoke the release of proinflammatory cytokines [24, 25], which may further stimulate uptake of oxLDL and thereby accelerate the formation of foam cells [26]. To mimic that sequence of events, we first treated THP-1 with VLDL for 2h in order to induce lipid uptake and a pro-inflammatory phenotype. Thereafter, after 2hours, VLDL was washed away, and cells were treated with oxLDL in the presence of human recombinant Angptl4 for 6 hours. Interestingly, recombinant Angptl4 reduced the uptake of oxLDL by 2-fold, but had no effect on uptake of native LDL (Figure 7C).

Previously, we showed that by inhibiting LPL activity, Angptl4 reduces macrophage uptake of TG-derived fatty acids and impairs macrophage activation [17], which may be suspected to indirectly lead to decreased uptake of oxLDL. To explore this possibility, THP-1 macrophages were incubated with VLDL in the presence of Angptl4 for 24 hours followed by a wash and treatment of the cells with oxLDL for 6 hours. Human recombinant Angptl4 decreased the uptake of triglycerides from VLDL (Figure 7D), which led to decreased uptake of oxLDL but not LDL (Figure 7E). Overall, the data indicate that Angptl4 suppresses oxLDL uptake.

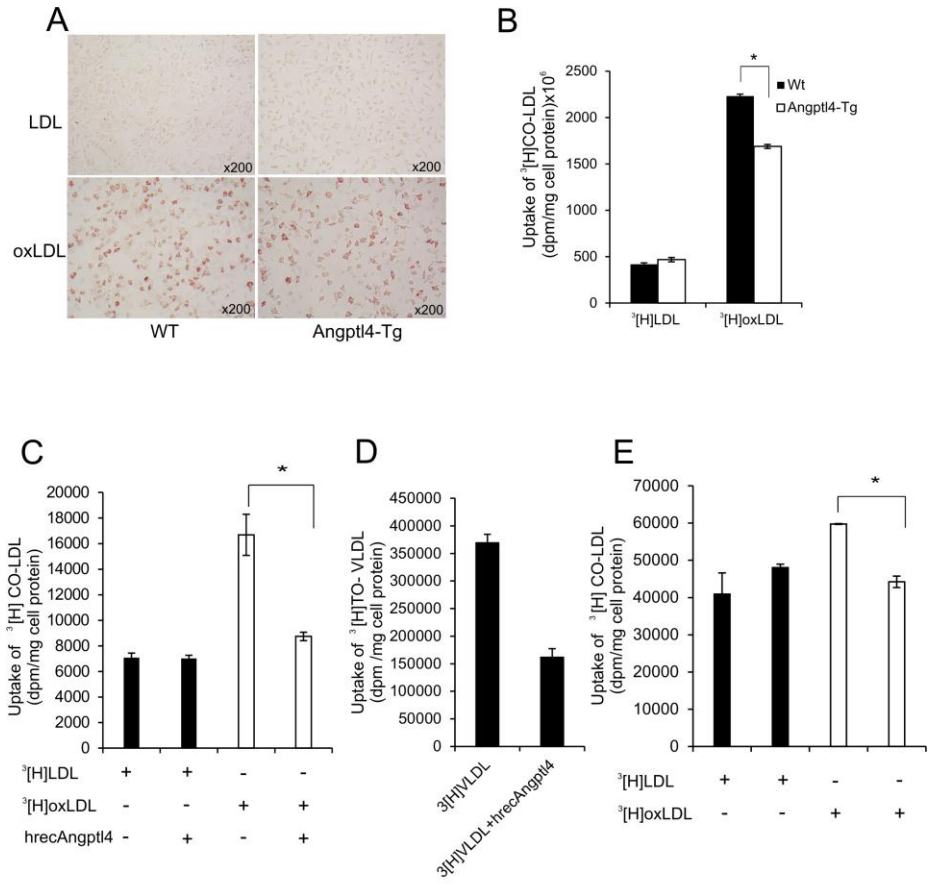


Figure 7: Angptl4 reduced the uptake of oxLDL in macrophages A. Oil Red O staining on BMDMs derived from WT and Angptl4-Tg mice, treated with 10ug/ml oxLDL or LDL for 24hrs B. Quantification of the uptake of tritium labelled ^{3}[H]oxLDL and ^{3}[H]LDL from BMDMs of WT and Angptl4-Tg mice, after 48hrs of incubation with each of the lipoproteins C. Quantification of the uptake ^{3}[H]LDL and ^{3}[H]oxLDL. THP-1 macrophages were preincubated for 2hrs with VLDL (150ug protein /ml), subsequently washed and treated for 6hrs with ^{3}[H]LDL or ^{3}[H]oxLDL, in the absence of presence of human recombinant protein Angptl4 (full length,1.5ug/ml) D. Quantification of the uptake of ^{3}[H] VLDL from THP-1 macrophages after 24hrs incubation with ^{3}[H]VLDL (30ug protein /ml), in the presence of absence of human recombinant protein Angptl4 (full length,1.5ug/ml) E. Quantification of the uptake of ^{3}[H]LDL and ^{3}[H]oxLDL. THP-1 macrophages were preincubated for 24hrs with VLDL (30ug protein /ml) in the presence or absence of human recombinant protein Angptl4 (full length,1.5ug/ml), subsequently washed and incubated with ^{3}[H]LDL and

^{3}[H]oxLDL, for 6hrs. Values are expressed as mean of dpm 3[H] /mg of protein (see methods). Errors bars represent SEM. * Significantly difference between the compared groups is indicated in the graph, according to Student's t-test (*P<0.05,**P<0.01).

Recently, it was suggested that atherosclerosis and NASH (non-alcoholic steatohepatitis) are two aspects of a shared disease characterized by infiltration of activated macrophages in the arterial wall and liver, respectively [27]. To study the potential impact of Angptl4 overexpression on macrophage abundance in liver, we measured Cd68 and F4/80 mRNA expression. Although, F4/80 did not show any difference between Angptl4Tg.E3L and E3L mice, Cd68 mRNA was reduced by approximately 20% in Angptl4Tg.E3L mice (Appendix Figure 1). Despite the small reduction in Cd68 mRNA levels, these data provide a hint of less macrophage infiltration in livers of Angptl4Tg.E3L mice.

Discussion

In this study we have investigated the role of Angptl4 in atherosclerosis development. For that purpose we crossed Angptl4-Tg mice characterized by whole body overexpression of Angptl4 with atherosclerosis prone E3L mice. Here, we show that after 24 weeks on an atherogenic diet, Angptl4Tg.E3L mice showed less atherosclerosis. This effect was independent of the plasma cholesterol and triglyceride levels, since levels were similar between the two groups. Importantly, Angptl4Tg.E3L exhibited a less pro-inflammatory phenotype with decreased accumulation of monocytes/macrophages in the atherosclerotic plaque, suggesting an anti-inflammatory role of Angptl4 in atherosclerosis development.

The impact of Angptl4 on atherosclerosis has been previously investigated by Adachi H. et al. [28]. In that study Angptl4-/- mice on a ApoE-/- background developed less atherosclerotic lesions on a chow diet compared to control mice. ApoE-/- mice are characterized by a severe deficiency in clearance of VLDL remnants and develop severe atherosclerosis from birth, whereas E3L mice only develop hyperlipoproteinemia when fed a diet rich in fat and cholesterol, which we believe better mimics the lifestyle-dependent development of atherosclerosis in humans. Angptl4-/- mice on ApoE-/- background exhibited a significant decrease in circulating LDL-C and triglyceride levels, which very likely

accounted for the improvement in atherosclerosis in that model. In contrast, plasma VLDL/LDL-cholesterol and total cholesterol as well as plasma triglycerides were unaffected in Angptl4Tg.E3L mice compared to E3L mice after several weeks on the atherogenic diet. Previously, we found that whole body Angptl4 overexpression was associated with elevated plasma total and VLDL-cholesterol and triglycerides in FVB and C57Bl/6 mice. This effect was prominent in the fasted state and minimal in the fed state, and could be attributed to inhibition of LPL-dependent lipolytic processing of triglyceride-rich lipoproteins[10, 13]. The exact reason why Angptl4 overexpression did not alter plasma triglycerides and cholesterol in E3L mice is not clear but maybe the inhibitory effect of Angptl4 on LPL is dependent on a fully functional ApoE molecule.

The absence of any changes in plasma lipoproteins suggests that Angptl4 overexpression reduces atherosclerosis via a mechanism independent of inhibition of LPL-mediated systemic lipid clearance and consequent lipid lowering. Previously, we have shown that Angptl4 dramatically reduced foam cell formation and inflammatory gene expression in peritoneal macrophages incubated with chylomicrons in a LPL-dependent manner [17]. The present study indicates that inhibition of LPL-dependent lipid loading and activation of macrophages by Angptl4 also leads to downregulation of subsequent oxLDL uptake. In addition, Angptl4 seems to directly inhibit oxLDL uptake by macrophages. Consistent with Angptl4 exerting its effect via LPL, externally added and endogenously-produced LPL was previously found to enhance binding and uptake of oxLDL in macrophages [29-32]. Supporting a stimulatory effect of macrophage LPL on atherosclerosis in vivo, macrophage-specific overexpression of LPL was found to stimulate the formation of atherosclerotic lesions and accumulation of macrophage-derived foam cells, which occurred in the absence of any changes in circulating lipoproteins [18]. It was shown using transgenic mice expressing catalytically active or inactive LPL that the noncatalytic bridging function of LPL is sufficient for its proatherogenic effect [33]. Whereas Angptl4 is known to potently inhibit LPL catalytic activity, it is unclear whether Angptl4 inhibits the bridging function of LPL. It should be realized that Angptl4 may lower oxLDL uptake via a mechanism entirely independent of LPL and that may be mediated by its C-terminal Angiopoietin-like domain. Future studies will have to address this issue in more detail.

Interestingly, Angptl4 inhibited oxLDL uptake when macrophages were preloaded and activated with VLDL. Accumulation of intracellular triglycerides may trigger inflammatory pathways and ER stress that may cause upregulation of oxLDL uptake [6], [17], [26]. In this context it would be of interest to determine the effect of preloading of macrophages with lipid on oxLDL receptors and study the potential impact of Angptl4.

Besides lowering oxLDL uptake, Angptl4 seems to reduce atherosclerosis by reducing chemotaxis. Specifically, we found that Angptl4-overexpression led to decreased accumulation of monocytes/macrophages in the atherosclerotic plaque. Additionally, Angptl4-Tg macrophages exhibited a decreased chemotactic response in an in vitro migration assay. Recently we showed that the C-terminal portion of Angptl4 binds to integrin β1 and β5 and stimulates integrin-linked signaling pathways [34, 35]. Since integrin β1 and β5 are highly expressed by macrophages (http://biogps.org; data not shown), these data raise the prospect that Angptl4 may influence macrophage chemotaxis via integrin β1 and/or β5. Alternatively, it is possible that Angptl4 impacts chemotaxis via its N-terminal domain. To our knowledge, there are no data linking macrophage LPL to chemotaxis.

Expression of Angptl4 in macrophages was markedly induced by TLR4 and TLR3 agonists, as well as by lipid loading. More modest induction was observed upon treatment with NOD2 agonist muramyl dipeptide. A stimulatory effect of LPS on Angptl4 mRNA has also been observed in vivo in heart, skeletal muscle and adipose tissue [36]. In the same study, several pro-inflammatory stimuli including LPS, IL-1β and TNFα upregulated Angptl4 expression in 3T3-L1 adipocytes. The stimulatory effect of lipids (as fatty acids or lipoproteins) on Angptl4 expression has been extensively studied and confirmed in a variety of cell types, suggesting Angptl4 may mediate effects of lipids. In intimal macrophages, induction of Angptl4 by lipids and other inflammatory stimuli may be a protective mechanism to reduce foam cell formation and mitigate anti-inflammatory responses, resembling its role in mesenteric lymph node macrophages [17].

There is increasing interest in the link between obesity and associated adipose tissue inflammation, and atherosclerosis. Despite the chronic feeding of a high fat/high sucrose diet, animals showed only minor weight gain and were not obese at the end of the experiment. The inhibitory effect of Angptl4 overexpression on macrophage recruitment in atherosclerotic plaques may also

be manifested in the adipose tissue, possibly leading to lower inflammatory status of adipose tissue. Detailed analysis of the inflammatory status of adipose including determination of the abundance of numerous immune cells will be the subject of future investigations.

In conclusion, the present study reveals a protective role of Angplt4 in atherosclerosis development that is independent of changes in levels of plasma lipoproteins. Furthermore, the study suggests an inhibitory effect of Angptl4 on macrophage oxLDL uptake and chemotaxis.

Sources of Funding

This study was supported by the Nutrigenomics Consortium, TI Food and Nutrition and the Netherlands Heart Foundation (2007B046).

References:

1. Glass, C.K. and J.L. Witztum, Atherosclerosis. the road ahead. Cell, 2001. 104(4): p. 503-16.
2. Moore, K.J. and M.W. Freeman, Scavenger receptors in atherosclerosis: beyond lipid uptake. Arterioscler Thromb Vasc Biol, 2006. 26(8): p. 1702-11.
3. Kuchibhotla, S., et al., Absence of CD36 protects against atherosclerosis in ApoE knock-out mice with no additional protection provided by absence of scavenger receptor A I/II. Cardiovascular research, 2008. 78(1): p. 185-96.
4. Febbraio, M., et al., Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. The Journal of clinical investigation, 2000. 105(8): p. 1049-56.
5. Moore, K.J., et al., Loss of receptor-mediated lipid uptake via scavenger receptor A or CD36 pathways does not ameliorate atherosclerosis in hyperlipidemic mice. The Journal of clinical investigation, 2005. 115(8): p. 2192-201.
6. Nicolaou, G. and C. Erridge, Toll-like receptor-dependent lipid body formation in macrophage foam cell formation. Current opinion in lipidology, 2010. 21(5): p. 427-33.
7. Seneviratne, A.N., B. Sivagurunathan, and C. Monaco, Toll-like receptors and macrophage activation in atherosclerosis. Clinica chimica acta; international journal of clinical chemistry, 2012. 413(1-2): p. 3-14.
8. Cole, J.E., et al., Unexpected protective role for Toll-like receptor 3 in the arterial wall. Proceedings of the National Academy of Sciences of the United States of America, 2011. 108(6): p. 2372-7.

9. Boven, L.A., et al., Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain, 2006. 129(Pt 2): p. 517-26.
10. Lichtenstein, L., et al., Angptl4 upregulates cholesterol synthesis in liver via inhibition of LPL- and HL-dependent hepatic cholesterol uptake. Arterioscler Thromb Vasc Biol, 2007. 27(11): p. 2420-7.
11. Sukonina, V., et al., Angiopoietin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. Proceedings of the National Academy of Sciences of the United States of America, 2006. 103(46): p. 17450-5.
12. Ge, H., et al., Oligomerization state-dependent hyperlipidemic effect of angiopoietin-like protein 4. J Lipid Res, 2004. 45(11): p. 2071-9.
13. Koster, A., et al., Transgenic angiopoietin-like (angptl)4 overexpression and targeted disruption of angptl4 and angptl3: regulation of triglyceride metabolism. Endocrinology, 2005. 146(11): p. 4943-50.
14. Mandard, S., et al., The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. J Biol Chem, 2006. 281(2): p. 934-44.
15. Xu, A., et al., Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. Proc Natl Acad Sci U S A, 2005. 102(17): p. 6086-91.
16. Yu, X., et al., Inhibition of cardiac lipoprotein utilization by transgenic overexpression of Angptl4 in the heart. Proc Natl Acad Sci U S A, 2005. 102(5): p. 1767-72.
17. Lichtenstein, L., et al., Angptl4 protects against severe proinflammatory effects of saturated fat by inhibiting fatty acid uptake into mesenteric lymph node macrophages. Cell metabolism, 2010. 12(6): p. 580-92.
18. Ichikawa, T., et al., Macrophage-derived lipoprotein lipase increases aortic atherosclerosis in cholesterol-fed Tg rabbits. Atherosclerosis, 2005. 179(1): p. 87-95.
19. van den Maagdenberg, A.M., et al., Apolipoprotein E*3-Leiden allele results from a partial gene duplication in exon 4. Biochemical and biophysical research communications, 1989. 165(2): p. 851-7.
20. de Knijff, P., et al., Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. The Journal of clinical investigation, 1991. 88(2): p. 643-55.
21. Kersten, S., et al., Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids. Arterioscler Thromb Vasc Biol, 2009. 29(6): p. 969-74.
22. Charo, I.F. and M.B. Taubman, Chemokines in the pathogenesis of vascular disease. Circulation research, 2004. 95(9): p. 858-66.

23. Cushing, S.D., et al., Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proceedings of the National Academy of Sciences of the United States of America, 1990. 87(13): p. 5134-8.
24. Gianturco, S.H., et al., Hypertriglyceridemic very low density lipoproteins induce triglyceride synthesis and accumulation in mouse peritoneal macrophages. The Journal of clinical investigation, 1982. 70(1): p. 168-78.
25. Saraswathi, V. and A.H. Hasty, The role of lipolysis in mediating the proinflammatory effects of very low density lipoproteins in mouse peritoneal macrophages. Journal of lipid research, 2006. 47(7): p. 1406-1415.
26. Ishiyama, J., et al., Unsaturated FAs prevent palmitate-induced LOX-1 induction via inhibition of ER stress in macrophages. Journal of lipid research, 2011. 52(2): p. 299-307.
27. Bieghs, V., et al., NASH and atherosclerosis are two aspects of a shared disease: Central role for macrophages. Atherosclerosis, 2011.
28. Adachi, H., et al., Angptl 4 deficiency improves lipid metabolism, suppresses foam cell formation and protects against atherosclerosis. Biochemical and biophysical research communications, 2009. 379(4): p. 806-11.
29. Hendriks, W.L., et al., Lipoprotein lipase stimulates the binding and uptake of moderately oxidized low-density lipoprotein by J774 macrophages. Biochem J, 1996. 314 (Pt 2): p. 563-8.
30. Makoveichuk, E., A. Lookene, and G. Olivecrona, Mild oxidation of lipoproteins increases their affinity for surfaces covered by heparan sulfate and lipoprotein lipase. Biochemical and biophysical research communications, 1998. 252(3): p. 703-10.
31. Wang, X., J. Greilberger, and G. Jurgens, Calcium and lipoprotein lipase synergistically enhance the binding and uptake of native and oxidized LDL in mouse peritoneal macrophages. Atherosclerosis, 2000. 150(2): p. 357-63.
32. Wang, X., et al., Endogenously produced lipoprotein lipase enhances the binding and cell association of native, mildly oxidized and moderately oxidized low-density lipoprotein in mouse peritoneal macrophages. Biochem J, 1999. 343 Pt 2: p. 347-53.
33. Gustafsson, M., et al., Retention of low-density lipoprotein in atherosclerotic lesions of the mouse: evidence for a role of lipoprotein lipase. Circ Res, 2007. 101(8): p. 777-83.
34. Goh, Y.Y., et al., Angiopoietin-like 4 interacts with integrins beta1 and beta5 to modulate keratinocyte migration. Am J Pathol, 2010. 177(6): p. 2791-803.
35. Goh, Y.Y., et al., Angiopoietin-like 4 interacts with matrix proteins to modulate wound healing. J Biol Chem, 2010. 285(43): p. 32999-3009.
36. Lu, B., et al., The acute phase response stimulates the expression of angiopoietin like protein 4. Biochemical and biophysical research communications, 2010. 391(4): p. 1737-41.

37. Gijbels, M.J., et al., Progression and regression of atherosclerosis in APOE3-Leiden transgenic mice: an immunohistochemical study. Atherosclerosis, 1999. 143(1): p. 15-25.
38. Volger, O.L., et al., Dietary vegetable oil and wood derived plant stanol esters reduce atherosclerotic lesion size and severity in apoE*3-Leiden transgenic mice. Atherosclerosis, 2001. 157(2): p. 375-81.
39. van der Hoogt, C.C., et al., Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. J Lipid Res, 2007. 48(8): p. 1763-71.

APPENDIX

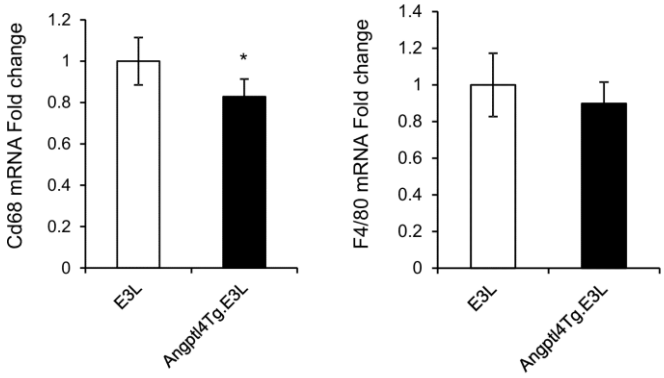
Appendix Table 1: Composition of diet T(4021 04)

Purified diet T

Composition	Weight (g)	Percentage
8500 STAND.VIT.PREMIX	37,50	0,25
8503 ST.SPOR.PREMIX	37,50	0,25
7559 CaHPO4.2H2O (LAAG F)	195,00	1,30
7560 CaCO3 REINST/Me.2069	150,00	1,00
7547 KH2PO4	105,00	0,70
7546 KCl.	105,00	0,70
7083 ZOUT (GEZAKT).	45,00	0,30
7611 MgSO4.7H2O	60,00	0,40
7552 MgO SCHWER REINST	30,00	0,20
7082 METHIONINE SYNTH. DL	30,00	0,20
7514 CHOLINE CL 50%	300,00	2,00
7599 ZURE CASEINE.	3.000,00	20,00
7584 DICACEL2+4/cellulose	930,00	6,20
7579 MAISZETMEEL GEL INST	1.500,00	10,00
7639 MAISOLIE/CORN OIL.	150,00	1,00
7529 CACAO-BOTER.	2.250,00	15,00
7154 SUIKER/sucrose	6.075,00	40,50
	15.000,00	**100,00**

Analysis	Unit	Portion
1 Cr.Prot	g/kg	176,16
2 Cr. Fat	g/kg	160,45
3 Cr.Fiber	g/kg	62,69
4 Minerals	g/kg	23,62
5 Moisture	g/kg	29,33
6Sug.+St.	g/kg	495,16
7 Nfree ex	g/kg	508,47
8 Dry Mat.	g/kg	967,82
9 Lysine	g/kg	12,42
10 Methion.	g/kg	6,78
12 Cystine	g/kg	0,52
13 Threonin	g/kg	7,12
14 Tryptoph	g/kg	2,32
15 Isoleuc.	g/kg	10,08
16 Arginine	g/kg	6,12
17 Phenylal	g/kg	7,28
18 Histidin	g/kg	4,64
19 Leucine	g/kg	19,04
20 Tyrosine	g/kg	9,44
21 Valine	g/kg	11,76
22 Alanine	g/kg	3,96
23 Asp.acid	g/kg	9,26
24 Glut.ac.	g/kg	35,10
25 Glycine	g/kg	5,12
26 Proline	g/kg	16,06
30 Calcium	g/kg	7,12
31 Phos.tot	g/kg	4,17
34 Potass.	g/kg	5,68
35 Magnes.	g/kg	1,57
36 Sodium	g/kg	1,15
37 Chlorine	g/kg	7,59
38 Sulfur	g/kg	0,54

49	C8-C12:0	g/kg	-
50	C14:0	g/kg	-
51	C16:0	g/kg	40,10
52	C16:1	g/kg	-
53	C18:0	g/kg	52,70
54	C18:1	g/kg	55,05
55	C18:2	g/kg	10,50
56	C18:3	g/kg	0,10
57	C20-C22	g/kg	1,51
61	Vit. A	IU/g	18,00
64	Vit. D3	IU/g	2,00
65	Vit. E	mg.	62,67
67	Vit. K3	mg.	10,00
68	Vit. B1	mg.	20,00
69	Vit. B2	mg.	11,56
70	Vit. B6	mg.	15,33
71	Niacin	mg.	39,20
72	Pant.ac.	mg.	15,90
73	Vit.B12	mcg.	50,00
74	Folic.ac	mg.	7,84
75	Choline	mg.	7.457,80
76	Biotin	mcg.	306,65
77	Inositol	mg.	499,98
82	Starch	g/kg	78,50
83	Sugars	g/kg	405,00
86	Lactose	g/kg	0,40
91	Cellulos	g/kg	55,80
93	Glucose	g/kg	3,70
96	Iron	mg.	129,98
97	Mangan.	mg.	63,50
98	Zinc	mg.	52,40
99	Copper	mg.	17,97
100	Cobalt	mg.	0,14
101	Iodine	mg.	0,47
102	Selenium	mg.	0,19
104	Chromium	mg.	0,49
105	Nickel	mg.	0,07
107	Fluorine	mg.	2,12
109	Arsenic	mg.	0,07
111	Lead	mg.	0,80
113	Alumin.	mg.	3,31



Appendix Figure 1: Mean fold change of mRNA levels of macrophage markers Cd68 and F4/80, in liver of Angptl4Tg.E3L mice compared to E3L control, fed for 24 weeks dietT with 0.4% additional cholsterol. Quantification was done with qRT-PCR. *Significantly different between E3L and Angptl4Tg.E3L, according to Student's t-test (*P<0.05).

Chapter 6

The Hypoxia inducible gene (Hig-2) is a novel target gene of fatty acids in macrophages

Frits Mattijssen, Anastasia Georgiadi* and Sander Kersten.

Chapter based on manuscript in Preparation
(Present version written by *author)

Abstract

Most of the modulatory effects of fatty acids on inflammation can probably be attributed to fatty acid metabolites, including prostaglandins, leukotoxins, resolvins, endocannabinoids, ceramides and diacylglycerols. However, there is accumulating evidence that fatty acids are able to directly activate or suppress inflammatory pathways. The aim of the present study was to elucidate transcriptional targets of fatty acids in macrophages, as part of a general goal to elucidate mechanisms through which fatty acids exhibit a direct role in modulating inflammatory processes in macrophages. To that aim we performed microarray analysis in elicited mouse peritoneal macrophages treated with fatty acids, oleic (C18:1) or palmitic acid (C16:0), intralipid or chyle for 6 hours. We identified Hig-2 to be strongly upregulated by all treatments, which prompted us to further characterize its function. We found expression of Hig-2 to be the highest in peritoneal macrophages and white adipose tissue. Hig-2 was increased in lipid loaded mouse macrophages together with another lipid droplet coated protein Plin2. Hig-2 was highly induced by hypoxia in mouse macrophages, while intralipid and hypoxia showed a synergistic effect. Transcriptional regulation of Hig-2 was independent of PPARs. Further characterization of the transcriptional regulation of Hig-2 in macrophages is currently in progress. Chronic high fat feeding increased Hig-2 expression levels in adipose tissue but not in liver. Immunohistochemistry indicated colocalization of Hig-2 with Cd68 in infiltrating macrophages as part of crown-like structures. We propose that Hig-2 has a specific role in macrophages and may function as an interesting target in the study of obese adipose tissue.

Introduction

Numerous studies have examined the impact of fatty acids on inflammatory pathways in tissues and cells. These studies point to a proinflammatory effect of saturated fatty acids, while n-3 PUFA have mostly anti-inflammatory properties [1]. Most of the modulatory effects of fatty acids on inflammation can probably be attributed to fatty acid metabolites, including prostaglandins, leukotoxins, resolvins, endocannabinoids, ceramides and diacylglycerols [1]. However, there is accumulating evidence that fatty acids may be able to directly activate or suppress inflammatory pathways.

Immune cells and particularly macrophages are equipped with a number of receptors that are able to recognize antigens, called pattern recognition receptors (PRR). Among those, the TLR family has been the most extensively characterized. Saturated fatty acids have been demonstrated to elicit TLR4-dependent and TLR2-dependent responses in several cell types, including macrophages [2-5]. Subsequent studies have provided compelling evidence that saturated fatty acids activate NF-κB and stimulate expression of NF-κB targets such as COX-2, iNOS and IL-1α in macrophages by activating TLR4 signaling in a MyD88, IRAK-1 and TRAF6 dependent manner. In contrast, unsaturated fatty acids are ineffective or may even act as antagonists [2], [6]. Apart from TLR receptors, GPR120, a member of the G-protein coupled receptor (GPCRs) family was recently proposed to serve as a specific sensor for n-3 fatty acids in macrophages that mediates the putative insulin sensitizing and anti-diabetic effects of n-3 fatty acids in vivo by repressing macrophage-induced tissue inflammation [7].

The aim of the present study is to explore transcriptional targets of free fatty acids in macrophages, as part of the general goal to elucidate mechanisms through which fatty acids exhibit a direct role in modulating inflammatory processes in macrophages.

Methods and Materials

Materials: GW501516, Rosiglitazone was purchased from Alexis (Axxora, Raamsdonkveer, The Netherlands). Wy14643 was obtained from Eagle Picher Technologies laboratories (Lenexa, Kan). SYBR green was from Eurogentec (Seraing, Belgium), and all other chemicals were from Sigma (Zwijndrecht, The Netherlands). Fatty acids were from Larodan Free Chemicals (Malmo, Sweden). Chyle was isolated from rats fed a palm-oil based diet overnight, as previously described [8].

Cell culture: *Peritoneal macrophages* were obtained from C57Bl/6 mice by intraperitoneal injection with 1 mL 4% thioglycolic acid. After three days, mice were anesthetized with 1.5% isoflurane, bled by orbital puncture and killed with CO_2. The macrophages were collected from the peritoneal cavity by washing it with 10 mL DMEM supplemented with 1% penicillin:streptomycin (P:S). Erythrocytes were removed by incubating the cell pellets with red blood cell lysis buffer (RBC) for 5 min on ice. After removal of the RBC, the cells were suspended in warm DMEM with 10% heat inactivated fetal calf serum (HI FCS) and seeded in a density of $3*10^5$ cells/cm^2 and incubated at 37°C in a humidified 5% CO_2 incubator. Three hours post plating, the cells were washed twice with warm PBS to remove non-adherent cells. Attached macrophages were kept always in fresh medium for two more days, before the experiments.

RAW 264.7 cells were grown in DMEM supplemented with 10% HI FCS and 1% P:S.

Human THP-1 and U937 monocytes were grown in RPMI 1640 medium supplemented with 10% HI FCS and 1% P:S. They were differentiated into macrophages for 2 days with 100ng/mL phorbol-12-myristate-13-acetate (PMA) and after PMA was washed away, cells were kept for 2 days in fresh complete medium.

Bone marrow derived cells macrophages were obtained from bone marrow cells via the following process: Bone marrow cells were obtained from the long bones of the hind legs of C57Bl/6 mice. The bones were removed, rubbed clean with 70% ethanol and washed with PBS. Bones were cut at the edges and the

bone marrow was flushed in 5ml of DMEM, with the use of a syringe. Acquired pellet was washed twice with PBS and resuspended in differentiation-medium consisting of 80% DMEM with 10% HI FCS and 1% P:S and 20% L929 conditioned DMEM (10% HI FCS,1% P:S). The cells were seeded in a density of $1*10^5$ cells/cm^2 and incubated for two days. After two days, 50% of the original volume of differentiation-medium was added and incubated again for three days. The medium was refreshed again and the cells were incubated 24h again before initiation of the treatment.

Binding of free fatty acids to BSA: Before applied to the cells, free fatty acids were bound to BSA (5ug/mL per 1L) by incubation at 37^{o}C for 30 min, as previously described [9]. Fatty acids were used in concentration of 250μM.

Intralipid was used in concentration of 2mM

PPAR agonists: The PPARα agonist Wy14643 was used in a concentration of 1μM, the PPARβ/δ agonist GW501516 and PPARγ agonist rosiglitazone were used at a concentration of 0.5μM.

Induction of hypoxia: Hypoxia was induced by the addition of 100μM iron chelator 2,2'-dipyridine (DP).

Microarray Analysis: RNA from peritoneal macrophages was extracted with TRIzol reagent and purified using RNeasy Mini kit (Qiagen, Venlo, Netherlands). RNA quality was assessed on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips using a Eukaryote Total RNA Nano assay. RNA from three different wells per condition was pooled and gene expression was measured with microarray analysis, using Affymetrix Mouse Genome 430 2.0 Arrays. Hybridization, washing and scanning of the arrays were done according to standard Affymetrix protocols. Scans of the Affymetrix arrays were processed using packages from the Bioconductor project. Raw signal intensities were normalized by using the GC-RMA algorithm [10]. Probesets were defined according to Dai et al. using remapped CDF version 12.0.0 based on the Entrez gene database [11]. In order to balance between random responses and relative weak transcriptional effects by the treatments, genes that met the cut-off of mean absolute fold change >1.2

were considered significantly regulated. Since we used pools of RNA per conditions, no statistics were feasible.

Immunobloting: Rabbit-produced antibody against mouse Hig-2 was purchased from Santa Cruz. The antibody was used at 1:1000 dilution. Secondary antibody was a peroxidase-conjugated goat anti-rabbit IgG, purchased from Jackson Immunoresearch, West Grove US, and used at 1:5000 dilution. Immunoprecipitation was performed in standard RIPA buffer. 18 ug of protein from cell lysates, collected in RIPA buffer was loaded.

High-Fat Diet: Mice were purchased at The Jackson Laboratory (Bar Harbor, ME) and bred in our local facility. C57Bl/6 received a low-fat diet (LFD) or high-fat diet (HFD) for 18 weeks providing 10 or 45 energy percent in the form of triglycerides (D12450B or D12451, Research Diets, New Brunswick, NJ). The lard component in these diets was replaced by palm oil. At the end of the feeding experiment, liver and epididymal white adipose tissue were dissected, weighed, and immediately frozen in liquid nitrogen. The animal experiments were approved by the animal experimentation committee of Wageningen University.

Immunohistology: Parafin preserved sections of liver and adipose tissue were used. Antibody for Cd68 was purchashed by Abdserotec and for Hig-2 from Bioconnect. The first day sections were left overnight at 37^{o}C. Second day, we performed deparafinization of the sections, followed by inhibition of endogenous peroxidases for 30min. Hig-2 and Cd68 antibodies were used in dilution 1:500, overnight. Negative control was not incubated with primary antibody. The third day incubation with senondary antibody was performed. Secondary antibody was used in dilution 1:200 and it was applied on the sections for 45min. Visualization of the complex was done with DAB staining. Hematocylin staining was perfomed as well. Areas that were stained obtained a brown colour. Furthermore, a general HE (hematoxylin eosin) staining was performed to visualize the structure of the tissue.

RNA isolation and qRT-PCR: Total RNA was isolated with TRIzol Reagent (Invitrogen, Breda, the Netherlands). 1µg of total RNA for the in vivo studies and 350ng of total RNA for the in vitro experiment was reverse transcribed using iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was amplified on BioRad MyIQ or iCycler machine using Sensimix polymerase (Invitrogen, Breda, the Netherlands). PCR primer sequences are shown in table 2.

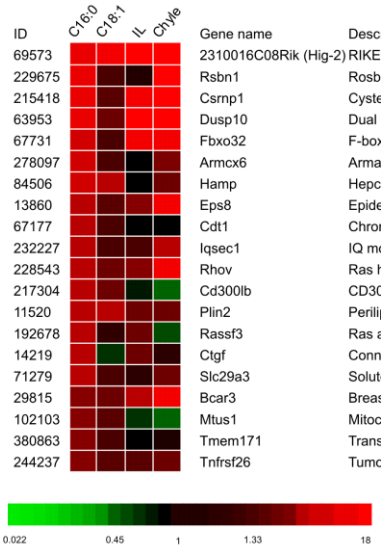
Table 2: Primer Sequences used for QPCR

Primer Name	Forward sequence	Reverse sequence
mHig-2	TGCTGGCGATCATGTTGACC	TGACCCCTCGTGATCCAGG
hHig-2	AAGCATGTGTTGAACCTCTACC	TGTGTTGGCTAGTTGGCTTCT
mPlin2	GCCTCTCAACTGGCTGGTAG	ACAGCAAAAGGGGTCATCTG
hPlin2	ATGGCATCCGTTGCAGTTGAT	GATGGTCTTCACACCGTTCTC
mCd68	CCAATTCAGGGTGGAAGAAA	CTCGGGCTCTGATGTAGGTC
mCd36	GAGCAACTGGTGGATGGTTT	TCACTTCCTGTGGATTTTGC

Results

Hig-2 is a novel target of fatty acids in mouse macrophages

To screen for novel targets genes of fatty acids in macrophages, we treated thioglycolate elicited mouse peritoneal macrophages with palmitic acid (C16:0) or oleic acid (C18:1) for 6 hours and performed Affymetrix microarrays. Alternatively, peritoneal macrophages were treated with lipid emulsions in the form of rat chyle or Intralipid. Genes were ranked according to fold-induction by C16:0 and displayed in a heat map. Fold-induction by C18:1, intralipid, and chyle are shown in parallel. Interestingly, the RIKEN gene 2310016C08 was found to be the most highly upregulated gene by fatty acids (Figure 1). RIKEN 2310016C08 was first described as a novel gene, that is inducible by hypoxia and glucose deprivation, giving rise to the name hypoxia inducible gene 2 (Hig-2) [12]. Later it was described as a lipid droplet associated protein and a specific target of HIF-1, but not HIF-2 in human cancer cell lines. Our data show that Hig-2 is the gene most highly upregulated in response to fatty acids in mouse peritoneal macrophages. The official gene symbol of RIKEN 2310016C08 and Hig-2 is Hilpda.



ID	Gene name	Description	Function
69573	2310016C08Rik (Hig-2)	RIKEN cDNA 2310016C08 gene	Response to hypoxia
229675	Rsbn1	Rosbin, round spermatid basic protein 1	MAPKinase signaling
215418	Csrnp1	Cysteine-serine-rich nuclear protein 1	Apoptosis
63953	Dusp10	Dual specificity phosphatase 10	MAPKinase signaling
67731	Fbxo32	F-box protein 32	Postivie regulation of TLR signaling
278097	Armcx6	Armadillo repeat containing, X-linked 6	Unknown
84506	Hamp	Hepcidin antimicrobial peptide	Response to hypoxia
13860	Eps8	Epidermal growth factor receptor pathway substrate 8	Cell migration
67177	Cdt1	Chromatin licensing and DNA replication factor 1	Cell cycle
232227	Iqsec1	IQ motif and Sec7 domain 1	Phagocytosis
228543	Rhov	Ras homolog gene family, member V	Unknown
217304	Cd300lb	CD300 antigen like family member B	Immune receptor
11520	Plin2	Perilipin 2	Lipid storage
192678	Rassf3	Ras association (RalGDS/AF-6)	Unknown
14219	Ctgf	Connective tissue growth factor	Cell migration/Cell proliferation/Apoptosis
71279	Slc29a3	Solute carrier family 29 (nucleoside transporters)	Unknown
29815	Bcar3	Breast cancer anti-estrogen resistance 3	Cell migration and cell death
102103	Mtus1	Mitochondrial tumor suppressor 1	Cell cycle
380863	Tmem171	Transmembrane protein 171	Unknown
244237	Tnfrsf26	Tumor necrosis factor receptor superfamily	Unknown

Figure 1: HIG-2 is the highest regulated gene by free fatty acids. Microarray analysis on macrophages treated 6 hours with palmitic acid (C16:0), oleic acid (C18:1), intralipid (IL) and Chyle. Differentially expressed genes ranked from the highest to the lowest according to C16:0 group. Fold change of the top 20 differentially expressed genes and their functions are shown. Colours correspond to fold change values compared to the control. Red represents upregulated gene and green represents downregulated genes.

We next determined the expression profile of mouse Hig-2 gene in several mouse tissues. Remarkably, Hig-2 was most highly expressed in thioglycolate-elicited mouse peritoneal macrophages, followed by white adipose tissue>colon>brown adipose tissue>skeletal muscle (Figure 2). Based on its elevated expression, we could speculate that Hig-2 may have a specific role in macrophages.

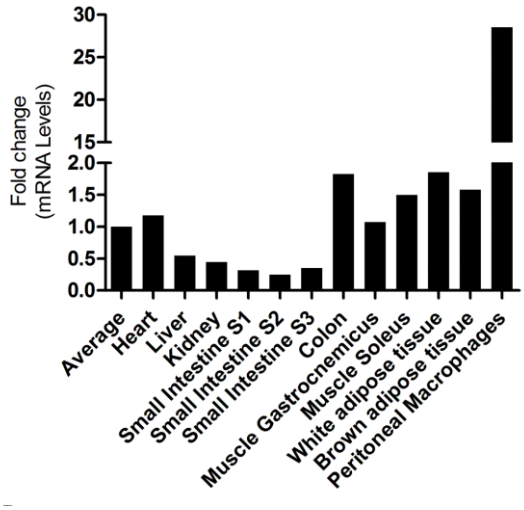


Figure 2: Mouse Hig-2 shows the highest expression levels in mouse peritoneal macrophages and white adipose tissue. qRT-PCR quantification of Hig-2 mRNA levels, on mouse tissues. Expression levels of Hig-2 was normalized to the average values of 4 different housekeeping genes (36B4, U18S, actin and cyclophilin). mRNA levels of Hig-2 per tissue were divided by the Average mRNA expression level of Hig-2 in all tissues measured, which was set at 1.

In order to test the consistency of induction of Hig-2 mRNA levels by fatty acids in macrophages, we treated several macrophage cell lines and primary cells with oleic acid (C18:1). Despite a dramatic increase in macrophage lipid accumulation (Figure 3A), Hig-2 expression was only modestly or not increased by C18:1, in M1 and M2 human monocyte derived macrophages, respectively (Figure 3B). A similar lack of increase in Hig-2 by C18:1 despite enhance lipid accumulation was observed in human U937 macrophages and human THP-1 macrophages, even though expression of the well characterized lipid droplet coating protein Plin2 was induced by C18:1 (Figure 3C,D). In contrast, expression of Hig-2 and Plin2 mRNA were increased in parallel by C18:1 in mouse RAW 264.7 and mouse peritoneal macrophages (Figure 3D).

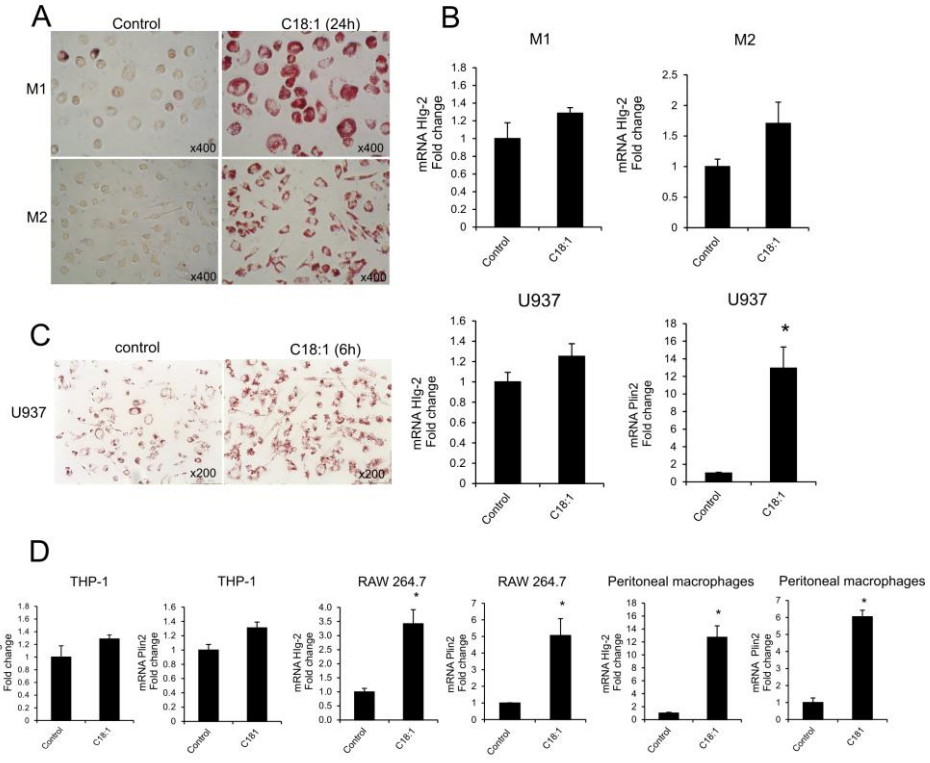


Figure 3: Hig-2 regulation by fatty acids in different macrophage cells lines A. Oil red O staining on human M1 and M2 differentiated human monocyte derived macrophages, treated with C18:1 (250uM) for 24 hours B. qRT-PCR quantification of mRNA levels of Hig-2 in M1 and M2 macrophages treated with C18:1 (250uM) for 24hrs C. Oil red O staining on human U937 macrophages treated with C18:1 for 6h (left). qRT-PCR quantification of mRNA levels of Hig-2 and Plin2 in U937 macrophages treated with C18:1, for 6hrs (right) D. qRT-PCR quantification of mRNA of Hig-2 and Plin2 in mouse and human cell line and primary cells after 6hours incubation with oleic acid (C18:1). Y-axis represents mean fold change. Errors bars represent SEM.*Significantly different between treatement (C18:1) and control, according to Student's t-test (*P<0.05).

Similar inductions of Hig-2 mRNA in RAW264.7 and peritoneal macrophages were observed in response to intralipid (Figure 4A). Upregulation of Hig-2 mRNA by intralipid was associated with marked induction of Hig-2 protein in both types of macrophages (Figure 4B).

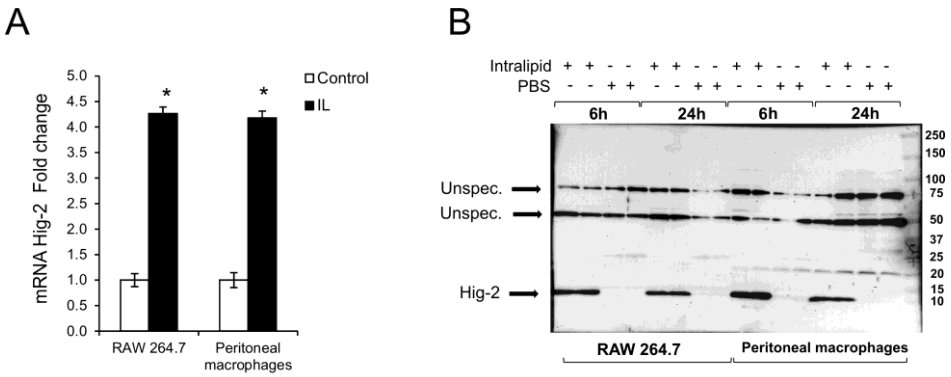


Figure 4: Upregulation of Hig-2 mRNA and protein in mouse RAW264.7 and mouse peritoneal macrophages after incubation with intralipid A. Mean fold change of mRNA levels of Hig-2 gene, quantified with qRT-PCR. Mouse RAW246.7 cells and mouse peritoneal macrophages were incubated for 6 hours with intralipid.* Statistically different between the treated (IL) and control group, according to Student's t-test (*P<0.05). B. Immunoblot showing that Hig-2 protein levels are increased in mouse RAW 264.7 and mouse peritoneal macrophages cell lysates, after 6 hours and 24 hours incubation with intralipid.

Hig-2 expression is upregulated by chemical hypoxia

In order to investigate whether Hig-2 is upregulated by hypoxia in macrophages, we exposed mouse peritoneal macrophages, mouse RAW 264.7 and BMDMs to chemical hypoxia by incubating them with the iron chelator 2,2'-dipyridine (DP), an inhibitor of the respiratory chain reaction, and compared the effect with intralipid. Both DP and intralipid were almost equally potent in inducing Hig-2 in all tested macrophages, while together they produced a synergistic increase in Hig-2 expression. In contrast, Plin2 expression did not respond or was even reduced by DP, while intralipid alone or in combination with DP effectively increased Plin2 mRNA levels (Figure 5A).

Hig-2 induction in lipid loaded macrophages is PPAR independent

Considering that fatty acids act as ligands for PPARs and that Plin2 is a PPAR target gene [13,14], we explored the possible regulation of Hig-2 by PPARs. To that end we treated mouse peritoneal macrophages, mouse RAW 264.7, human U937 and human THP-1 macrophages with pharmacological agonists for PPARα (WY14643), PPARβ/δ (GW501516) and PPARγ (Rosiglitazone). Whereas Plin2 was consistently upregulated by agonist for PPARβ/δ and PPARγ, we did not observe any effect of PPAR agonists on Hig-2 mRNA levels, except of a 1.8-fold increase by GW501516 in RAW 264.7 macrophages. There results suggest that in contrast to Plin2, Hig2 is probably not a PPAR target gene, at least in macrophages.

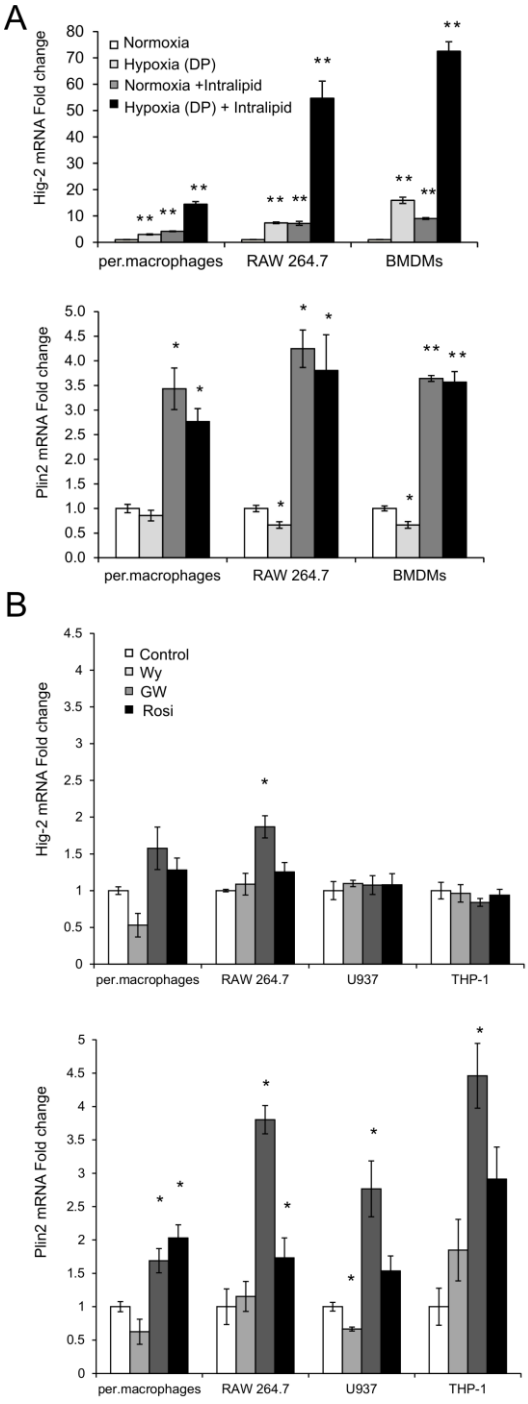

A
Hig-2 mRNA Fold change
Normoxia
Hypoxia (DP)
Normoxia +Intralipid
Hypoxia (DP) + Intralipid
per.macrophages
RAW 264.7
BMDMs
Plin2 mRNA Fold change
per.macrophages
RAW 264.7
BMDMs
B
Hig-2 mRNA Fold change
Control
Wy
GW
Rosi
per.macrophages
RAW 264.7
U937
THP-1
Plin2 mRNA Fold change
per.macrophages
RAW 264.7
U937
THP-1

Figure 5: Hig-2 is induced by hypoxia mimetics and not by PPAR agonists in macrophages A. Mean fold change of mRNA levels of Hig-2 and Plin2 measured in mouse peritoneal macrophages, mouse RAW 264.7 macrophages and BMDMs, under normoxia, hypoxia (induced by hypoxia mimetic DP, 100uM), intralipid (2mM) and combined DP and intralipid. Duration of incubation was 6 hours. B. Mean fold change of Hig-2 and Plin2 mRNA levels in mouse peritoneal macrophages, mouse RAW 264.7 macrophages, human U937 and human THP-1 macrophages treated for 6 hours with PPARα (WY 14643), PPARβ/δ (GW 501516) and PPARγ (Rosiglitazone). Expression levels were quantified by qRT-PCR. Error bars represent SEM.*Significantly different between treatment and control group, according to Student's t-test, (* $P<0.05$, ** $P<0.01$).

Hig-2 is induced in adipose tissue upon high fat diet

Obesity is associated with macrophage infiltration in adipose tissue. We found Hig-2 to be expressed at high levels in white adipose tissue and in peritoneal macrophages. Thus, we set out to investigate the expression of Hig-2 in liver and adipose tissue in lean mice and mice rendered obese by chronic high fat feeding. Whereas 18 weeks potently induced Cd36 in the liver in concert with development of fatty liver (Figure 6A,C), expression of Hig-2 as well as macrophage marker Cd68 were not increased. In contrast, chronic high fat feeding significantly increased Hig-2 and Cd68 mRNA in adipose tissue (Figure 6B). Immunohistochemistry indicated colocalization of Hig-2 with Cd68 in infiltrating macrophages as part of crown-like structures (Figure 6D)

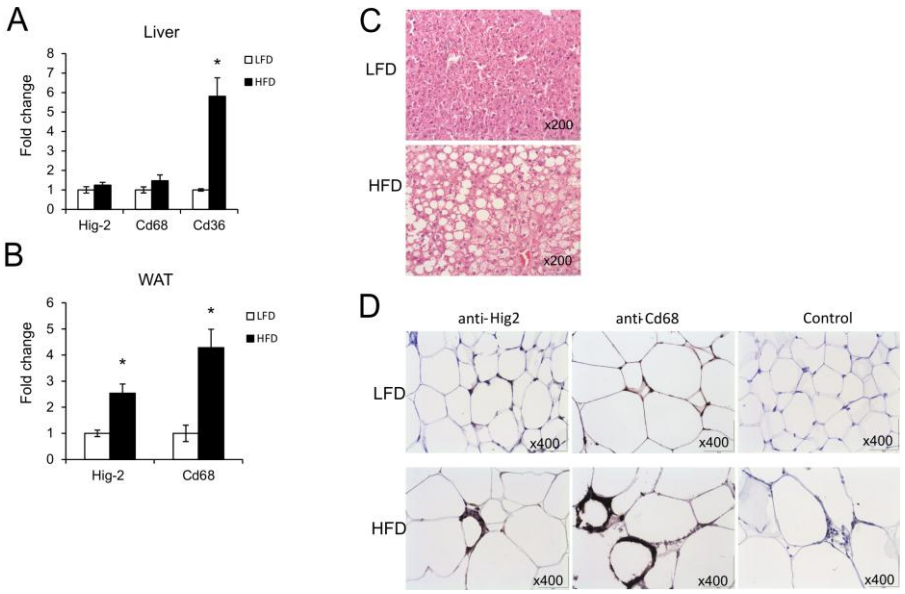


Figure 6: Hig-2 is upregulated in adipose tissue upon high fat diet and it colocalizes with the macrophages. Gene expression of Hig-2 and Cd68 and Cd36 in liver (A) and Hig-2 and Cd68 in white adipose tissue (WAT) (B) after 18 weeks high fat diet C. Hematoxylin eosin (HE) staining showing liver steatosis after 18 weeks on HFD. Low fat diet (LFD) is used as a control D. Immunhistological staining of white adipose tissue (WAT) sections from low fat diet (LFD) and high fat diet (HFD) group. Hig-2 staining is observed together with Cd68 macrophage staining (brown colour), around crown like structures.

Discussion

Fatty acids have been shown to regulate a wide range of genes in several cell types. However, the impact of fatty acids on gene expression in macrophages has not been extensively investigated. Using microarray we identified Hig-2 as the most sensitive target gene of fatty acids in macrophages. Hig-2 has been previously described to be induced by hypoxia, chemical hypoxia and lipid accumulation in human renal carcinomas cells, human HeLa cells and human HuF7 cancer cell lines [12], [15]. In line with these data, we also found Hig-2 mRNA and protein levels to be upregulated under conditions of lipid droplet accumulation as it is induced by intralipid in mouse RAW 264.7 and mouse peritoneal macrophages. Although most of the existing work on Hig-2 is on human cell lines, fatty acid treatment of human THP-1, U937 and human monocyte derived macrophages (M1 and M2) did not result in increased Hig-2 expression levels, despite enhanced intracellular lipid accumulation, as manifested by Plin2 induction and confirmed by Oil red O staining. Basal expression of Hig-2 in THP-1 and U937 macrophages seems to be similar to its expression in mouse RAW 264.7 and primary mouse macrophages (Ct 24-27). Since cancer cell lines may have very different characteristics from primary cells, further studies on human primary cells such as human monocyte macrophages are necessary to study the differential regulation of Hig-2 in human and mouse macrophages. For that purpose, the high variation between human donors necessitates a big sample size in order to draw reliable conclusions. The data presented here on Hig-2 expression in M1 and M2 human monocyte derived macrophages are based on the average result of two different donors and cannot be considered conclusive.

Previously Gimm et al. visualized Hig-2 in HeLa cells using immunofluorescent staining. Hig-2 was found to partially colocalize with Plin2 to the periphery of lipid droplets, and after staining for organelle markers, its localization in other cellular organelles such as mitochondria, endoplasmic reticulum, Golgi, autophagosomes and peroxisomes was excluded. Lipid droplets in non-adipose tissues have very small size, often less than 1μm [16]. In addition, lipid droplets are often juxtaposed to the endoplasmic reticulun (ER), mitochondria and peroxisomes [17], which makes it difficult to distinguish them from other cellular organelles without using electron microscopy. Saturated fatty acids are known to induce endoplasmic reticulum

stress and promote the formation of foam cells [18]. The ER is a large organelle running throughout the cytoplasm and therefore it cannot be excluded that Hig-2 acts upon the lipid droplets formation via the ER. Although the intracellular localization of Hig-2 might be indicative of its function, further studies are necessary to elucidate how it may be involved in the formation of foam cells. Gimm et al. showed that overexpression of Hig-2 in HeLa cells clones increased lipid droplet formation. Currently, we are exploring siRNA mediated Hig-2 knockdown in macrophages, which we expect to reveal the potential involvement of Hig-2 in lipid droplets formation under baseline and conditions of hypoxia or Intralipid/fatty acids incubation. So far attempts to knock down Hig-2 in mouse RAW 264.7 macrophages have proven to be difficult.

In contrast to adipocytes, the primary function of macrophages is not to deposit lipids. Biogenesis of lipid bodies in macrophages is a process that happens in vivo during inflammatory reactions of different causes, such as bacterial infection, uptake of modified lipids or systemic inflammation. Lipid laden macrophages (foam cells) are sources of proinflammatory cytokines. Gimm et al. showed that Hig-2 overexpression is accompanied by lipid accumulation and cytokines expression in HeLa cells. Although it is likely that secretion of cytokines a is consequence of the lipid accumulation, we cannot exclude a direct role of Hig-2 in cytokine production by the macrophage.

In adipose tissue, which becomes enriched with macrophages during obesity, we observed no staining of Hig-2 at the periphery of adipocytes and thus around the lipid droplets. Instead, Hig-2 was present in the recruited macrophages. While both Hig-2 and Cd68 mRNA were increased in the adipose tissue, neither Hig-2 or Cd68 increased in the liver, despite steatosis as reflected by increased Cd36 mRNA levels. These data indicate that increase in Hig-2 mRNA is not necessarily connected with lipid droplet formation and might be a result of macrophage activation. This may be indirectly supported by the observation that Hig-2 is activated by hypoxia, which has been described to stimulate macrophage activation and phagocytic functions [19,20].

Plin2 has been also described to be induced during hypoxia [21]. In contrast to Hig-2, in mouse macrophages Plin2 was found to be a specific target of PPARs and was not induced by chemical hypoxia. This discrepancy might be due to a difference between DP-induced hypoxia and hypoxia induced by a decreased oxygen tension, which is the most common way to study hypoxia. However, based on our results we may speculate that Hig-2 is regulated

differently than a typical lipid droplet associated protein. In order to clarify the regulation of in vivo, it would be useful to measure its expression in the liver, adipose tissue and macrophages derived from PPARα-/- and PPARβ/δ -/- mice fed a high fat diet, as well as in Hif-1 KO mice.

This study provides a preliminary description of a newly identified target of fatty acids, Hig-2, in macrophages. Apart from what has already been suggested in the present discussion, the acquisition of a Hig-2 KO mouse would be extremely helpful to further characterize its function. Exposure of Hig-2 KO mice to high fat diet may reveal the potential involvement of Hig-2 in obesity and hepatic steatosis.

References

1. Galli, C. and P.C. Calder, Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review. Ann Nutr Metab, 2009. 55(1-3): p. 123-39.
2. Lee, J.Y., et al., Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem, 2001. 276(20): p. 16683-9.
3. Suganami, T., et al., Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol, 2007. 27(1): p. 84-91.
4. Weatherill, A.R., et al., Saturated and polyunsaturated fatty acids reciprocally modulate dendritic cell functions mediated through TLR4. J Immunol, 2005. 174(9): p. 5390-7.
5. Lee, J.Y., et al., Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. J Biol Chem, 2004. 279(17): p. 16971-9.
6. Lee, J.Y., et al., Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. J Lipid Res, 2003. 44(3): p. 479-86.
7. Oh, D.Y., et al., GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell, 2010. 142(5): p. 687-98.
8. Lichtenstein, L., et al., Angptl4 protects against severe proinflammatory effects of saturated fat by inhibiting fatty acid uptake into mesenteric lymph node macrophages. Cell Metab, 2010. 12(6): p. 580-92.
9. de Vogel-van den Bosch, H.M., et al., A cholesterol-free, high-fat diet suppresses gene expression of cholesterol transporters in murine small intestine. Am J Physiol Gastrointest Liver Physiol, 2008. 294(5): p. G1171-80.

10. Gentleman, R.C., et al., Bioconductor: open software development for computational biology and bioinformatics. Genome Biol, 2004. 5(10): p. R80.
11. Dai, M., et al., Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res, 2005. 33(20): p. e175.
12. Denko, N., et al., Epigenetic regulation of gene expression in cervical cancer cells by the tumor microenvironment. Clin Cancer Res, 2000. 6(2): p. 480-7.
13. Forman, B.M., J. Chen, and R.M. Evans, Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A, 1997. 94(9): p. 4312-7.
14. Kliewer, S.A., et al., Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A, 1997. 94(9): p. 4318-23.
15. Gimm, T., et al., Hypoxia-inducible protein 2 is a novel lipid droplet protein and a specific target gene of hypoxia-inducible factor-1. FASEB J, 2010. 24(11): p. 4443-58.
16. Suzuki, M., et al., Lipid droplets: size matters. J Electron Microsc (Tokyo), 2011. 60 Suppl 1: p. S101-16.
17. Lynes, E.M. and T. Simmen, Urban planning of the endoplasmic reticulum (ER): how diverse mechanisms segregate the many functions of the ER. Biochim Biophys Acta, 2011. 1813(10): p. 1893-905.
18. Ishiyama, J., et al., Unsaturated FAs prevent palmitate-induced LOX-1 induction via inhibition of ER stress in macrophages. J Lipid Res, 2011. 52(2): p. 299-307.
19. Acosta-Iborra, B., et al., Macrophage oxygen sensing modulates antigen presentation and phagocytic functions involving IFN-gamma production through the HIF-1 alpha transcription factor. J Immunol, 2009. 182(5): p. 3155-64.
20. Bostrom, P., et al., Hypoxia converts human macrophages into triglyceride-loaded foam cells. Arterioscler Thromb Vasc Biol, 2006. 26(8): p. 1871-6.
21. Saarikoski, S.T., S.P. Rivera, and O. Hankinson, Mitogen-inducible gene 6 (MIG-6), adipophilin and tuftelin are inducible by hypoxia. FEBS Lett, 2002. 530(1-3): p. 186-9

Chapter 7

General Discussion

Increased levels of plasma triglycerides is one of the features of the metabolic syndrome and is considered a risk factor for cardiovascular disease [1,2]. Metabolic syndrome is associated with delayed postprandial clearance of plasma triglycerides, leading to hyperlipidaemia. After a meal triglycerides are packaged into chylomicrons in the small intestine and via the lymph system reach the blood and the peripheral tissues. Triglyceride-rich chylomicrons deliver free fatty acids to the organs after undergoing lipolysis by lipoprotein lipase, which is anchored to the capillary endothelium [3]. Under physiological conditions, organs respond to the increased plasma levels of triglycerides, by increased uptake of lipids, increased mitochondrial fat oxidation, esterification and storage of fatty acids as intracellular lipid droplets [4-6]. In metabolic syndrome ectopic fat storage to tissues with limited fat deposition, such as liver, skeletal muscle and heart [7,8], concomitant with improper balance of fat uptake, intracellular lipid accumulation and fat oxidation in those organs, give rise to cellular lipotoxicity. Lipotoxicity is a general term used to describe the condition of cell dysfunction or cell death due to excess accumulation of fat [9]. Mitochondrial dysfunction, endoplasmic reticulum stress, apoptosis and inflammation are some of the cell dysfunctions observed in lipotoxicity [10] and have been reported to be common characteristics of lipid related diseases, such as myocardial dysfunction, atherosclerosis and liver steatosis. Apart from the intracellular levels of lipids, the composition of fatty acids has been recognized as a separate factor in lipotoxicity occurrence. Accordingly, saturated fatty acids have been described as more lipotoxic compared to unsaturated fatty acids. Despite the big effort to understand the functions of dietary fatty acids at the cellular level, still the mechanisms via which they may perturb cell function remain unclear. In the first part of this thesis, we explore direct target genes of different dietary polyunsaturated fatty acids (PUFAs), in the mouse heart and we investigate the role of Angptl4, a highly responsive gene to dietary fatty acids, in the prevention of cardiac lipotoxicity. In the second part of this thesis, we explore the role of Angptl4 in the context of atherosclerosis and inflammation. Finally, we present a preliminary characterization of Hig-2, which we found to respond potently to fatty acid uptake and hypoxia in macrophages and we further explore its expression under high fat diet in liver and adipose tissue.

Fatty acids differ in their lipotoxic potential

Fatty acids may affect lipotoxicity in several ways. They are substrates for production of lipotoxic compounds, they affect cell membrane organization and gene transcription [11-13]. Evidence suggest that fatty acids differ in their lipotoxic and inflammatory potential. Under conditions of increased influx of fatty acids, limited TG storage, or uncontrolled lipolysis, may direct fatty acids towards other metabolic pathways than lipid droplets, giving rise to a variety of lipotoxic derivatives, such as diacylglycerol, ceramides, C6-C22 acylcarnitines or other intermediates of impaired beta oxidation or lipid peroxide free radicals [14]. Increased levels of these intermediates have been associated with insulin resistance in skeletal muscle, cell apoptosis and myopathy in the cardiac muscle [15,16]. Long chain-saturated fatty acids, such as palmitate are precursors for ceramide synthesis. The latest is a highly bioactive lipid, which attacks cell membranes and promotes apoptosis. In addition, saturated fatty acids have been shown to induce endoplasmic reticulum stress, with subsequent effects on apoptosis, insulin sensitivity and uptake of modified lipids, such as oxidized LDL by macrophages [17,18]. In contrast n-3 PUFAs, DHA and EPA give rise to eicosanoids, such as resolvins and protectins, that have anti-inflammatory properties [19,20]. In turn, n-6 PUFAs have been described as pro-inflammatory, since they are precursors of arachidonic acid. Those differences have led to the hypothesis, that the ratio between n-3/n-6 PUFA in our diet is an important factor of inflammatory and immune response. In line with this hypothesis, it has been observed that diets which have been proven beneficial for health, such as the Mediterranean diet are characterized by increased consumption of n-3 PUFA, whereas Western diet, that has been associated with chronic disease developement is more enriched in n-6 PUFAs [21,22].

Fatty acids may differentially alter antigen presentasion and immune response via modification of cell membrabe organisation. Membrane lipid rafts require lipids with saturated acyl chains and cholesterol, which pack tightly to form a liquid ordered phase. This tight packing is conferred by favorable van der Waals forces between saturated acyl chains, in addition to hydrogen bonding between neighboring sphingolipids and between the variable sphingolipidamide and the 3-OH of cholesterol. Unsaturated acyl chains, including PUFAs, do not pack well with cholesterol molecules and therefore form a liquid disordered phase [23]. It has been speculated that through these

physical properties PUFA acyl chains can modify membrane lateral organization and protein appearance on the cell surface, which may alter antigen presentation, cytokine production, lymphocyte proliferation, surface molecule expression, phagocytosis, and apoptosis or inhibit the activity of Tcells, such as natural killer (NK) cells [24,25].

Finally fatty acids can directly affect gene transcription, via interaction with nuclear receptors. PPAR ligand dependent activation by PUFA has been the most well characterized nuclear receptors. So far, three different isotypes of PPARs have been identified PPARα, PPARβ/δ and PPARγ. PPARα has been previously described as a master regulator of lipid metabolism. Additionally, PPARα has been shown to have an anti-inflammatory affect, which is partly mediated via NF-κB inhibition [26]. Regards the role of saturated fat on inducing inflammation, several studies have provided compelling evidence that saturated fatty acids activate NF-κB and stimulate expression of NF-κB targets such as COX-2, iNOS and IL-1α in macrophages by activating TLR4 signaling in a MyD88, IRAK-1 and TRAF6 dependent manner [31,32]. In contrast, unsaturated fatty acids are ineffective or may even act as antagonists. It has been reported that saturated fatty acids activate TLR4 by promoting its recruitment to lipid rafts via a mechanism involving reactive oxygen species [33]. Furthermore, a recent study showed rpL13a snoRNAs U32a, U33, and U35a to mediate the effect of lipotoxic myristate, palmitate and stearate in respect to increased ROS production, ER stress and subsequent cell death, while the less lipotoxic plamitoleate and oleate didn't have the same effect [34].

Gene regulation by fatty acids in the focus of this thesis. In Chapter 3 we explore gene regulation by fatty acids in the mouse heart. Among heart's inbuilt metabolic characteristcs is increased fat oxidation rates and limited storage capacity. Therefore, increased formation of lipid droplets or lipotoxic derivatives in the myocardium results in myocardial metabolic inflexibility and cardiac failure. It has been suggested that cardiac increased fatty acid oxidation and uncoupling, occur more efficiently by oleic acid, compared to palmitic acid, preventing the accumulation of ROS and lipid derivatives in the cytoplasm. A small number of studies have investigated the differential effects of saturated and unsaturated fatty acids on cardiac metabolism. According to their findings, rodents that had received a diet rich in saturated fatty acids had increased ceramide production, apoptosis and cardiac damage after 8 weeks on diet. In contrast, those that were fed with unsaturated fatty acids were protected [27].

Furthemore, a transcriptomics analysis comparing the type of genes induced by either oleic acid or palmitic acid in the isolated rat cardiomyocytes showed increased expression of beta oxidation genes, such as Cpt1, Pdk4 and Ucp3 by oleic acid, whereas palmitic acid induced endoplasmic reticulum stress and ROS production [28]. In line with this, we were able to appreaciate a number of genes in our study that were robustly regulated by PPARα and they were involved in fatty acid oxidation, such as Acot1, Acot2 and Ucp3. In the present thesis, we were not able to investigate the direct effects of saturated fatty acids on cardiac gene transcription. The main reason for that is the fact that triglycerides composed of long chain saturated fatty acids are solid at room temperature and can not be administered as an oral gavage. Since long chain saturated fatty acids are the most lipotoxic for cardiomyocytes, it is of interest to investigate the type of genes regulated acutely by saturated fatty acid. For that purpose, triglycerides composed of saturated fatty acids could be delivered by infusing chylomicrons isolated from the lymph of rats fed a diet that contains exclusively tristearin or tripalmitin, although it would be quite difficult to determine the appropriate dose. Alternatively, VLDL-like particles could be prepared using tristearin or tripalmitin and subsequently infused to the mice.

In Chapter 3 we observed that after 6hours oral gavage of different PUFAs fed to WT and PPARα -/- mice, many target genes of PPARα were involved in inflammation and immunity. Immune related functions of PPARα have been less explored in the heart compared to other tissues, for example liver. Furthermore, we observed a substantial number of PUFA target genes to be PPARα independent. One gene that was markedly and consistently induced by all fatty acids was Zbtb16/PLZF, encoding a transcription factor that controls the development of effector functions in Natural Killer T (NKT) cells [29]. NKT cells represent a unique subset of lymphocytes that are reactive to so called lipid antigens, which include a broad range of microbial lipids that are unique structures of specific microorganisms and they are presented to them bound to Cd1d receptor. The latest is mainly expressed on antigen presenting cells, such as macrophages, dendritic cells, but it is also found in hepatocytes and according to public databases may be also present in white and brown adipose tissue. NKT cells comprise up to 2% of spleen, 20% of mononuclear cells in the liver and 40% of CD3+ cells in bone marrow in the mouse, making these cells a major component of the total T cell population. A recent study, interestingly showed that high fat diets enriched in saturated fatty acids (SFA)

and monounsaturated fatty acids (MUFA) caused a depletion in hepatic NKT cells in the liver leading to further activation of inflammatory signaling, insulin resistance, and hepatic steatosis, whereas high fat diet enriched with polyunsaturated fatty acids (PUFAs) didn't have this effect [30]. Cd1d expression in cardiomyocytes and functions of NKT cells in the heart haven't been explored. We have measured expression levels of Zbtb16 in several mouse tissues and we find heart to be the tissue with the highest expression of Zbtb16 followed by the white adipose tissue and skeletal muscle. Thus, we may even speculate that specific cardiac functions of Zbtb16 in cardiomyocytes may mediate the immune related effects of PUFAs, independently of their effect on PPAR activation.

Distinct functions of PPARα and PPARβ/δ in the heart

All three PPARs are expressed in the heart. PPARα and PPARβ/δ are highly expressed in the cardiomyocyte, while PPARγ is almost absent (Chapter 4) [35]. For long time, PPARβ/δ was believed to be redundant to PPARα functions in the skeletal muscle [36], mainly due to the observation, that in vitro activation studies with PPAR isotype specific agonists showed that both PPARα and PPARβ/δ are able to activate mitochondrial fat oxidation [37]. Although, in vivo data on PPARβ/δ activation with synthetic agonists have not been reported, mice lacking PPARβ/δ suffer from mild cardiac steatosis (normal cardiac triglycerides in 2-month-old mice and a twofold increase when they are 9 months old) and decreased OXPHOS in cardiac muscle [38]. Subsequent studies have suggested a distinct role for each of the PPAR isotypes in the heart and in the skeletal muscle [39,40], based on which PPARβ/δ has greater control over processes related to response to oxidative stress and adaptation to substrate availability. Accordingly, PPARβ/δ activation has been reported to reduce oxidative stress-induced apoptosis in cardiomyocytes [41] and to promote mitochondrial biogenesis, via PGC-1 activation [42]. Furthermore, Kelly et al.; have investigated the cardiomyocyte specific overexpression of PPARα and PPARβ/δ, showing the PPARβ/δ overexpression protected against cardiomyopathy. PPARβ/δ specifically regulated the expression of Glut4, hence PPARβ/δ overexpressing hearts had a higher capacity for glucose utilization compared to PPARα overexpressing ones. This resulted to reduced myocardial injury due to ischemia/reperfusion [43]. Finally, cardiomyocyte specific

deletion of PPARβ/δ, in PPARα null mice impaired mitochondrial biogenesis, but had no further effect of fat oxidation [44]. In chapter 4 we observed that upon activation of PPARs with physiological ligands, Angptl4, the most highly upregulated gene by all fatty acids, was a specific target of PPARβ/δ, but not PPARα, in mice with global deletion of PPARα or PPARβ/δ. Additionally, we found that PPARα didn't increase Angptl4 expression upon activation with fatty acids, in rat cardiomyocytes, that had been transfected with siRNA targeting PPARβ/δ. In the same chapter we describe that Angptl4 overexpression protects against cardiac lipotoxicity, while Angptl4 deletion leads to increase production of lipotoxic derivatives in the mouse heart. The same is probably true for the skeletal muscle. Staiger H. et al had showed that Angptl4 is a specific target of PPARβ/δ in the skeletal muscle, though expression of PPARβ/δ is much higher than PPARα in skeletal muscle than in the heart [45]. Overall these studies postulate a distinct function of PPARα and PPARβ/δ in the heart. Driven by the above described observations, we may speculate that PPARα secures ongoing of fat oxidation, which is crucial for the heart function, whereas PPARβ/δ may sense changes in substrate availability or even distinct lipotoxic derivatives and activate downstream mechanisms, which aim to protect the heart from lipotoxicity. Further, exploring the target genes and functions of PPARβ/δ in the heart would help to increase our understanding on the functions of these nuclear receptor. Although, initially we aimed to carry out a detailed comparison of gene regulation upon deletion of PPARα and PPARβ/δ after an oral gavage of dietary fatty acids great differences in mice genetic background made this comparison non feasible.

An intriguing question deriving from this thesis is how activation of PPARs by dietary PUFAs led to specific activation of Angptl4 by PPARβ/δ, while another well described PPAR target gene Ucp3 was specifically activated by PPARα. It is conceivable that in vivo metabolic processing of fatty acids give rise to compounds that may interact specifically with certain PPAR isotypes leading to differential gene regulation. Liver exhibits high and very similar rates of fat uptake with the heart, however long chain fatty acids effect on gene expression in the liver are mainly mediated by PPARα, with high overlap between different fatty acids, which is not the case in the heart. Possibly differences in the routes of uptake of fatty acids between the two tissues in the postprandial state may be the cause of differences in gene expression. Whereas the heart takes up dietary fatty acids as non-esterified fatty acids after LPL-

mediated hydrolysis, the liver internalizes dietary fatty acids as TGs within chylomicron remnant particles [46,47]. In the liver circulating FFAs, which primarily originate from adipose tissue lipolysis ("old fat"), do not seem to be able to activate PPARα [48,49], whereas endogenously synthesized fatty acids ("new fat") seem to mediate hepatic PPARα activation [48]. In contrast, hepatic PPARβ/δ can be activated by plasma FFA [49], and likely the same is true in skeletal muscle, as revealed by the stimulatory effect of elevated FFA on expression of PPARβ/δ target Angptl4 in skeletal muscle [45],[50]. Apart from lipolysis of chylomicrons via LPL, fatty acids may be delivered in the heart in the form on NEFAs bound to albumin, coming from the adipose tissue or from VLDL remnants, which has been shown to be internalized via the VLDL receptors in the heart [51]. Therefore, similar to the liver it would be interesting to investigate in what extent VLDL-derived fatty acids or NEFA may cause differential activation between PPARα and PPARβ/δ in the heart. Tissues differences in intracellular fatty acid processing, via which PPAR ligands become available may serve as a possible explanation. Recently, Zechner et al; described Atgl lipolysis as an essential intermediate process for intracellular formation and release of PPARα endogenous ligands in the heart [52]. AtglKO mice and cardiomyocyte specific AtglKO mice suffered from impaired fatty acid oxidation and cardiomyopathy, which could be corrected by PPARα synthetic ligand Wy 14643, but not by PPARβ/δ synthetic ligand GW 501516. Interestingly, it has been shown that in the liver Atgl promotes PPARα activity, independently of ligand-induced activation [53]. These observations suggest that synthetic agonists of PPARs follow different routes of PPAR activation, compared to fatty acids and serve as a plausible explanation for the little overlap that we observe in genes regulated by fatty acids and by Wy 141643 in the heart as opposed to the liver. However, still remains to be investigated whether different routes of lipids uptake from these tissues may differentially modify the Atgl dependent release of PPAR ligand formation or may even by pass it. Accordingly, future research on PPAR functions should be directed towards identifying the structure of endogenous PPAR ligands, in relation to their intracellular or tissue origin.

Anti-inflammatory functions of Angptl4

Angptl4 exhibits high sensitivity to lipids, responding acutely to fatty acid fluxes in heart (this thesis) and muscle [45], exercise, fasting [50] or high fat diet [54]. Angptl4 has been described to be a potent inhibitor of LPL activity and Angptl4 overexpressing animals are characterized by elevated plasma triglyceride levels, that are dramatically induced during fasting. In the present thesis we have shown that global overexpression of Angptl4 decreased the production of lipotoxic derivatives in the heart on mice that were on a high fat diet for 8weeks, while Angptl4 KO mice showed the opposite. In line with these findings cardiac specific Angptl4 transgenic animals are characterized by elevated plasma TG together with decreased cardiac LPL activity yet these mice do not exhibit any change in post-heparin plasma LPL activity or LPL activity in non-cardiac tissues [55]. Hence, Angptl4 produced in heart seems to primarily act locally to protect the heart from lipid overload and consequent lipotoxicity [56]. It is unclear what accounts for the primary autocrine/paracrine versus endocrine function of Angptl4 in tissues. Additionally, cardiac specific Angptl4 overexpression resulted in a cardiac dysfunction, due to decreased availability of fatty acids as a substrate. In the present study we didn't assess cardiac function in Angptl4 Tg or Angptl4 KO mice on a high fat. However, we plan to do this in the future.

Both Angptl4 and LPL are targets genes of PPARs. In the present thesis we have shown that Angptl4 is upregulated by PPARβ/δ in response to increased influx of fatty acids. In addition, we have previously shown that the same mechanism is employed by macrophages to prevent lipotoxocity and foam cell formation. In macrophages Angptl4 is specifically activated by PPARβ/δ agonist. Hence, we postulate that Angptl4 may serve as a lipid "gate" keeper, which responds to differences in intracellular levels of fatty acids and attenuates their increased uptake in order to prevent lipotoxicity. In line with this model, during hypoxia, that promotes the utilization of glucose instead of fatty acids by the cardiomyocytes, Angptl4 is also potently activated. We may speculate that via regulation of lipid fluxes Angptl4 may indirectly affect the activation status of antigen presenting cells, like macrophages and further influence systemic inflammation. In Chapter 5, we suggest another mechanism of Angptl4 on inflammatory response and chemotaxis. We were able to show that Angptl4 reduced atherosclerosis in mice on Western type diet with additional cholesterol

for 24 weeks, without causing any changes in plasma triglycerides and cholesterol compared to the control group. Angptl4 overexpression led to a significant decrease in monocyte adherence and macrophage accumulation in the plaque. In line with these findings, bone marrow derived macrophages (BMDMs) with higher expression of Angptl4 than the WT, migrated much less towards the chemoattractant protein MCP-1, compared to the WT BMDMs.

Our findings suggest an effect of macrophage produced Angptl4 on chemotaxis. Recent studies have showed that the C-terminal portion of Angptl4 protein interacts physically with integrin αVβ5 and affects keratinocyte migration, wound healing, and extracellular matrix remodelling [57,58]. Therefore, the role of the C-terminal may be more relevant in understanding the effect of Angptl4 in chemotaxis. Interestingly, we found that few TLR agonists increased Angptl4 expression in human THP-1 macrophages. TLRs are innate immune receptors, which recognize a wide range of bacterial proteins. Specifically TLR 4 has been shown to be activated by saturated fatty acids, while unsaturated fatty acids have been reported to have no effects or even may act as antagonists. Angptl4-/- mice on a high fat diet with saturated fatty acids, but not unsaturated fatty acids turn extremely sick after 12 weeks on a high fat diet, exhibiting inflammation and accumulation of foam cell macrophages in mesenteric lymph nodes. Our findings suggest a model of action of Angptl4 on inflammation, which probably involves TLR activation and thus, innate immunity, which doesn't necessarily exclude the effect of Angptl4 on LPL activity.

Several tissues have the ability to synthesize Angptl4 protein with the highest expression found in the liver, followed by the adipose tissue, thyroid, brain and small intestine. Recently, it has been suggested that non alcoholic fatty liver disease shares a common cause with atherosclerosis [59], the same might be is conceivable for adipose tissue inflammation that is often present in atherosclerosis. Therefore, we may speculate that Angptl4 might have a more systemic effect on atherosclerosis development, via local action in these tissues. Adipose tissue is a great pool of adipokines/cytokines and other lipophilic compounds. Several adipokines, such as adiponectin, visfatin, leptin and retinol binding protein-4 have been associated with systemic inflammation without necessarily having an effect of lipoproteins metabolism [60-65]. Angptl4 overexpression increases lipolysis in the adipose tissue. Hence we may speculate that Angptl4 function may affect the secretion of lipolysis released

proteins or other lipophilic molecules that may have an effect on systemic inflammation and thus atherosclerosis. Of course this is speculative and such compounds need to be identified. However, it is a suggestion for future research.

The role of Angptl4 in atherosclerosis in humans is uncertain. So far, there are inconsistent results regards the effects of a loss of function mutation of Angptl4 (E40K) on coronary atherosclerosis. In the study of Folsom et al E40K carriers exhibited lower levels of circulating triglycerides, higher HDL and overall a lower incidence of cardiovascular disease (CAD) incidence [66]. However, another study examining the CAD risk found that although E40K carriers had lower triglycerides and higher HDL, they showed a higher CAD risk independent of triglycerides and HDL-concentrations [67].

Hig-2

Transcriptomics data have been a treasury of valuable information, which allows discoveries of novel genes, much faster compared to the pre transcriptomics era. In chapter 6 we performed microarray analysis on macrophages treated with different fatty acids. Hig-2, a gene that has been previously described to be a target gene of hypoxia inducible factor 1 (HIF-1) was the highest upregulated gene by fatty acids in macrophages. HIF-1 is a transcription factor, which is sensitive to oxygen and levels of reactive oxygen species in the cells and it is upregulated during hypoxia. Accordingly, we found Hig-2 to be upregulated specifically by hypoxia in macrophages, but not by PPAR agonists. It is also upregulated in adipose tissue and liver during high fat diet, where we find it to colozalize with macrophages. Gimm T. et al described it as a lipid droplet associated protein [68]. Accumulating information on lipid droplets reveal that this cellular organelle have a rather complicated composition, which associates them with function beyond the triglyceride storage [69]. As it is discussed above they may host endogenous ligands of PPARs and other bioactive molecules. In relation to that, it is even conceivable that components of lipid droplets, such as lipid droplet-associated proteins, many of which are targets of PPARα and some are also able to promote fat utilization, such as Oxpat/pat-1 [70], may dissociate during lipolysis and regulate PPAR signalling. Indeed several lipid-associated droplets are regulated by ligand activation of PPARα, such as Mldp, Plin5. We didn't find Hig-2 to be

a PPAR target gene in macrophages, however its regulation by PPARs has to be explored in other tissues, such as liver and adipose tissue. In macrophages Hig-2 is upregulated by drug induced hypoxia, thus we may speculate that its role could be connected with substrate switch utilization (from fat to glucose), which is a characteric of hypoxia or inflammatory response, since hypoxia increases oxidative stress in macrophages and adipose tissue [71,72]. Lipid droplets are connected to the endoplasmic reticulum and also mitochondria and may affect the functions of these organs via proteins or lipophilic compounds exchanged among them. Future experiments in Hig-2-KO mice is expected to give more answers on the functions of Hig-2, which may serve as a link between high fat diet induced adipose tissue inflammation and liver steatosis and intracellular lipid induced inflammation.

Conclusions

In conclusion, this thesis contributes new information on gene regulation by dietary PUFA in the mammalian heart and provides mechanistic insight on their previous reported beneficial effects. We show that PUFA can activate transcriptional mechanisms that are able to sense very early increases in influx of fatty acids and upregulate Angptl4 in order to prevent excess uptake of fatty acids and protect the cardiomyocyte from lipotoxocity. The upregulation of Angptl4 was due to selective activation of PPARβ/δ, but not PPARα by linolenic acid. Furthermore, we provide new insight in the role of Angptl4 on lipid metabolism and inflammation by describing a potential anti-inflammatory role of Angptl4, which protects against atherosclerosis development, possibly in an LPL independent manner. Finally, this thesis provides novel information on target genes of fatty acid on macrophages, by exploring the case of Hig-2, the most highly upregulated gene by fatty acids in macrophages.

References:

1. Talayero, B.G. and F.M. Sacks, The role of triglycerides in atherosclerosis. Curr Cardiol Rep, 2011. 13(6): p. 544-52.
2. Franssen, R., et al., Obesity and dyslipidemia. Med Clin North Am, 2011. 95(5): p. 893-902.
3. Wang, H. and R.H. Eckel, Lipoprotein lipase: from gene to obesity. Am J Physiol Endocrinol Metab, 2009. 297(2): p. E271-88.
4. Goodpaster, B.H., et al., Intramuscular lipid content is increased in obesity and decreased by weight loss. Metabolism, 2000. 49(4): p. 467-72.
5. Sironi, A.M., et al., Ectopic fat storage, insulin resistance, and hypertension. Curr Pharm Des, 2011. 17(28): p. 3074-80.
6. Lelliott, C. and A.J. Vidal-Puig, Lipotoxicity, an imbalance between lipogenesis de novo and fatty acid oxidation. Int J Obes Relat Metab Disord, 2004. 28 Suppl 4: p. S22-8.
7. Montani, J.P., et al., Ectopic fat storage in heart, blood vessels and kidneys in the pathogenesis of cardiovascular diseases. Int J Obes Relat Metab Disord, 2004. 28 Suppl 4: p. S58-65.
8. Bugger, H. and E.D. Abel, Molecular mechanisms for myocardial mitochondrial dysfunction in the metabolic syndrome. Clin Sci (Lond), 2008. 114(3): p. 195-210.
9. Schaffer, J.E., Lipotoxicity: when tissues overeat. Curr Opin Lipidol, 2003. 14(3): p. 281-7.
10. Hotamisligil, G.S., Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell, 2010. 140(6): p. 900-17.
11. Stulnig, T.M., Immunomodulation by polyunsaturated fatty acids: mechanisms and effects. Int Arch Allergy Immunol, 2003. 132(4): p. 310-21.
12. Shaikh, S.R. and M. Edidin, Polyunsaturated fatty acids and membrane organization: elucidating mechanisms to balance immunotherapy and susceptibility to infection. Chem Phys Lipids, 2008. 153(1): p. 24-33.
13. Shaikh, S.R. and M. Edidin, Polyunsaturated fatty acids, membrane organization, T cells, and antigen presentation. Am J Clin Nutr, 2006. 84(6): p. 1277-89.
14. Bosma, M., et al., Re-evaluating lipotoxic triggers in skeletal muscle: Relating intramyocellular lipid metabolism to insulin sensitivity. Prog Lipid Res, 2012. 51(1): p. 36-49.
15. Wende, A.R. and E.D. Abel, Lipotoxicity in the heart. Biochim Biophys Acta, 2010. 1801(3): p. 311-9.
16. Iozzo, P., Myocardial, perivascular, and epicardial fat. Diabetes Care, 2011. 34 Suppl 2: p. S371-9.
17. Dyntar, D., et al., Glucose and palmitic acid induce degeneration of myofibrils and modulate apoptosis in rat adult cardiomyocytes. Diabetes, 2001. 50(9): p. 2105-13.

18. Chavez, J.A. and S.A. Summers, Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. Arch Biochem Biophys, 2003. 419(2): p. 101-9.
19. Lee, C.H., Resolvins as new fascinating drug candidates for inflammatory diseases. Arch Pharm Res, 2012. 35(1): p. 3-7.
20. Seki, H., Y. Tani, and M. Arita, Omega-3 PUFA derived anti-inflammatory lipid mediator resolvin E1. Prostaglandins Other Lipid Mediat, 2009. 89(3-4): p. 126-30.
21. Deckelbaum, R.J. and C. Torrejon, The Omega-3 Fatty Acid Nutritional Landscape: Health Benefits and Sources. J Nutr, 2012.
22. Margioris, A.N., Fatty acids and postprandial inflammation. Curr Opin Clin Nutr Metab Care, 2009. 12(2): p. 129-37.
23. Edidin, M., The state of lipid rafts: from model membranes to cells. Annu Rev Biophys Biomol Struct, 2003. 32: p. 257-83.
24. Costabile, M., et al., The immunomodulatory effects of novel beta-oxa, beta-thia, and gamma-thia polyunsaturated fatty acids on human T lymphocyte proliferation, cytokine production, and activation of protein kinase C and MAPKs. J Immunol, 2005. 174(1): p. 233-43.
25. Schroit, A.J. and R. Gallily, Macrophage fatty acid composition and phagocytosis: effect of unsaturation on cellular phagocytic activity. Immunology, 1979. 36(2): p. 199-205.
26. Genolet, R., W. Wahli, and L. Michalik, PPARs as drug targets to modulate inflammatory responses? Curr Drug Targets Inflamm Allergy, 2004. 3(4): p. 361-75.
27. Okere, I.C., et al., Differential effects of saturated and unsaturated fatty acid diets on cardiomyocyte apoptosis, adipose distribution, and serum leptin. Am J Physiol Heart Circ Physiol, 2006. 291(1): p. H38-44.
28. Lockridge, J.B., et al., Bioinformatic profiling of the transcriptional response of adult rat cardiomyocytes to distinct fatty acids. J Lipid Res, 2008. 49(7): p. 1395-408.
29. Alonzo, E.S. and D.B. Sant'Angelo, Development of PLZF-expressing innate T cells. Curr Opin Immunol, 2011. 23(2): p. 220-7.
30. Hua, J., et al., Dietary fatty acids modulate antigen presentation to hepatic NKT cells in nonalcoholic fatty liver disease. J Lipid Res, 2010. 51(7): p. 1696-703.
31. Lee, J.Y., et al., Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. J Lipid Res, 2003. 44(3): p. 479-86.
32. Lee, J.Y., et al., Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. J Biol Chem, 2003. 278(39): p. 37041-51.

33. Pushnova, E.A. and Y.S. Zhu, Quantitative restriction fragment length polymorphism: a procedure for quantitation of diphtheria toxin gene CRM197 allele. Anal Biochem, 1998. 260(1): p. 24-9.
34. Michel, C.I., et al., Small nucleolar RNAs U32a, U33, and U35a are critical mediators of metabolic stress. Cell Metab, 2011. 14(1): p. 33-44.
35. Gilde, A.J., et al., Peroxisome proliferator-activated receptor (PPAR) alpha and PPARbeta/delta, but not PPARgamma, modulate the expression of genes involved in cardiac lipid metabolism. Circ Res, 2003. 92(5): p. 518-24.
36. Muoio, D.M., et al., Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. J Biol Chem, 2002. 277(29): p. 26089-97.
37. Cheng, L., et al., Peroxisome proliferator-activated receptor delta activates fatty acid oxidation in cultured neonatal and adult cardiomyocytes. Biochem Biophys Res Commun, 2004. 313(2): p. 277-86.
38. Cheng, L., et al., Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. Nat Med, 2004. 10(11): p. 1245-50.
39. Barish, G.D., V.A. Narkar, and R.M. Evans, PPAR delta: a dagger in the heart of the metabolic syndrome. J Clin Invest, 2006. 116(3): p. 590-7.
40. Hondares, E., et al., PPARβ/δ, but not PPARα , activates PGC-1alpha gene transcription in muscle. Biochem Biophys Res Commun, 2007. 354(4): p. 1021-7.
41. Pesant, M., et al., Peroxisome proliferator-activated receptor delta (PPARβ/δ) activation protects H9c2 cardiomyoblasts from oxidative stress-induced apoptosis. Cardiovasc Res, 2006. 69(2): p. 440-9.
42. Wang, P., et al., Peroxisome proliferator-activated receptor {delta} is an essential transcriptional regulator for mitochondrial protection and biogenesis in adult heart. Circ Res, 2010. 106(5): p. 911-9.
43. Burkart, E.M., et al., Nuclear receptors PPARbeta/delta and PPARα direct distinct metabolic regulatory programs in the mouse heart. J Clin Invest, 2007. 117(12): p. 3930-9.
44. Liu, J., et al., Cardiomyocyte-Restricted Deletion of PPARbeta/delta in PPARα -Null Mice Causes Impaired Mitochondrial Biogenesis and Defense, but No Further Depression of Myocardial Fatty Acid Oxidation. PPAR Res, 2011. 2011: p. 372854.
45. Staiger, H., et al., Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator-activated receptor (PPAR)-delta and is of metabolic relevance in humans. Diabetes, 2009. 58(3): p. 579-89.
46. Augustus, A.S., et al., Routes of FA delivery to cardiac muscle: modulation of lipoprotein lipolysis alters uptake of TG-derived FA. Am J Physiol Endocrinol Metab, 2003. 284(2): p. E331-9.

47. Teusink, B., et al., Contribution of fatty acids released from lipolysis of plasma triglycerides to total plasma fatty acid flux and tissue-specific fatty acid uptake. Diabetes, 2003. 52(3): p. 614-20.
48. Chakravarthy, M.V., et al., "New" hepatic fat activates PPARα to maintain glucose, lipid, and cholesterol homeostasis. Cell Metab, 2005. 1(5): p. 309-22.
49. Sanderson, L.M., et al., Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) but not PPARα serves as a plasma free fatty acid sensor in liver. Mol Cell Biol, 2009. 29(23): p. 6257-67.
50. Kersten, S., et al., Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids. Arterioscler Thromb Vasc Biol, 2009. 29(6): p. 969-74.
51. Bharadwaj, K.G., et al., Chylomicron- and VLDL-derived lipids enter the heart through different pathways: in vivo evidence for receptor- and non-receptor-mediated fatty acid uptake. J Biol Chem, 2010. 285(49): p. 37976-86.
52. Haemmerle, G., et al., ATGL-mediated fat catabolism regulates cardiac mitochondrial function via PPAR-alpha and PGC-1. Nat Med, 2011. 17(9): p. 1076-85.
53. Ong, K.T., et al., Adipose triglyceride lipase is a major hepatic lipase that regulates triacylglycerol turnover and fatty acid signaling and partitioning. Hepatology, 2011. 53(1): p. 116-26.
54. Mandard, S., et al., The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. J Biol Chem, 2006. 281(2): p. 934-44.
55. Yu, X., et al., Inhibition of cardiac lipoprotein utilization by transgenic overexpression of Angptl4 in the heart. Proc Natl Acad Sci U S A, 2005. 102(5): p. 1767-72.
56. Georgiadi, A., et al., Induction of cardiac Angptl4 by dietary fatty acids is mediated by peroxisome proliferator-activated receptor beta/delta and protects against fatty acid-induced oxidative stress. Circ Res, 2010. 106(11): p. 1712-21.
57. Goh, Y.Y., et al., Angiopoietin-like 4 interacts with matrix proteins to modulate wound healing. J Biol Chem, 2010. 285(43): p. 32999-3009.
58. Goh, Y.Y., et al., Angiopoietin-like 4 interacts with integrins beta1 and beta5 to modulate keratinocyte migration. Am J Pathol, 2010. 177(6): p. 2791-803.
59. Bieghs, V., et al., NASH and atherosclerosis are two aspects of a shared disease: Central role for macrophages. Atherosclerosis, 2012. 220(2): p. 287-93.
60. Gerdes, S., et al., Leptin, adiponectin, visfatin and retinol-binding protein-4 - mediators of comorbidities in patients with psoriasis? Exp Dermatol, 2012. 21(1): p. 43-7.
61. Li, Z.Y., P. Wang, and C.Y. Miao, Adipokines in inflammation, insulin resistance and cardiovascular disease. Clin Exp Pharmacol Physiol, 2011. 38(12): p. 888-96.

62. Calabozo Raluy, M., et al., Fibromyalgia syndrome (fibrositis): as frequent as it is unknown]. Med Clin (Barc), 1990. 94(5): p. 173-5.
63. Hayashi, M., et al., Association of adiponectin with carotid arteriosclerosis in predialysis chronic kidney disease. Am J Nephrol, 2011. 34(3): p. 249-55.
64. Almer, G., et al., Adiponectin-coated nanoparticles for enhanced imaging of atherosclerotic plaques. Int J Nanomedicine, 2011. 6: p. 1279-90.
65. Gauvreau, D., et al., [Novel adipokines: links between obesity and atherosclerosis]. Ann Endocrinol (Paris), 2011. 72(3): p. 224-31.
66. Folsom, A.R., et al., Variation in ANGPTL4 and risk of coronary heart disease: the Atherosclerosis Risk in Communities Study. Metabolism, 2008. 57(11): p. 1591-6.
67. Talmud, P.J., et al., ANGPTL4 E40K and T266M: effects on plasma triglyceride and HDL levels, postprandial responses, and CHD risk. Arterioscler Thromb Vasc Biol, 2008. 28(12): p. 2319-25.
68. Gimm, T., et al., Hypoxia-inducible protein 2 is a novel lipid droplet protein and a specific target gene of hypoxia-inducible factor-1. FASEB J, 2010. 24(11): p. 4443-58.
69. Girousse, A. and D. Langin, Adipocyte lipases and lipid droplet-associated proteins: insight from transgenic mouse models. Int J Obes (Lond), 2011.
70. Wolins, N.E., et al., OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. Diabetes, 2006. 55(12): p. 3418-28.
71. Parathath, S., et al., Hypoxia is present in murine atherosclerotic plaques and has multiple adverse effects on macrophage lipid metabolism. Circ Res, 2011. 109(10): p. 1141-52.
72. Trayhurn, P., B. Wang, and I.S. Wood, Hypoxia in adipose tissue: a basis for the dysregulation of tissue function in obesity? Br J Nutr, 2008. 100(2): p. 227-35.

SUMMARY

Dietary fat is a strong predictor of chronic diseases, such as cardiovascular diseases, obesity, diabetes, dyslipidemia and metabolic syndrome. A great number of epidemiological and observational studies clearly show that in addition to the amount of fat consumed in a diet, fat composition is an equally important factor in the development of chronic diseases. Evidence abounds indicating that adherence to a diet with high content of polyunsaturated (PUFAs) and monounsaturated fatty acids (MUFAs) such as the Mediterranean diet has substantial health benefits, while diets with high content of saturated fatty acids (SFAs) such as the Western type diet increase the risk for the development of several chronic diseases.

Nutritional genomics or nutrigenomics investigates the interaction between nutrients and genes at the molecular level, by using genomic tools. Within the field of nutrigenomics, dietary fatty acids and their metabolites are seen as signaling molecules that target specific cellular response systems. Dietary fatty acids have been reported to bind physically to PPARs, a family of ligand activated transcription factors, that play a major role in metabolic homeostasis. Three PPAR isotypes have been identified, PPARα, PPARβ/δ and PPARγ. Their expression and target genes vary among different tissues and cell types.

After a meal triglycerides are packed into chylomicrons in the small intestine and via the lymph system, they reach the blood and the peripheral tissues. Triglyceride chylomicrons deliver free fatty acids to the organs after being lipolylised by lipoprotein lipase (LPL), which is anchored to the capillary endothelium. Among different organs, heart and liver show the highest uptake of dietary triglycerides, postprandialy. However, opposite to the liver, heart is a constant working muscle, which covers its demands on energy mainly by fatty acids, delivered to the heart via hydrolysis of circulating triglyceride-rich lipoproteins. Unbalanced fatty acid uptake and fatty acid oxidation is common in cardiac diseases, such as cardiac failure, myocardial ischemia and diabetes. Heart is characterized by decreased lipid storage capacity, therefore chronic elevated levels of lipids uptake and intracellular storage is considered harmful and may lead to lipotoxic cardiomyopathy.

Our first aim was to explore the whole genome effects of individual dietary fatty acids in the intact heart via transcriptional profiling. By conducting these experiments in wild-type and PPARα−/− mice, we aimed to determine the

specific contribution of PPARα, which has been previously described as a master regulator of lipid homeostasis in the heart. We took advantage of a unique experimental model, where mice were given a single oral bolus of synthetic triglycerides composed of a single fatty acid. We sacrificed the mice 6hours after the oral gavage and we compared the effects of different fatty acids on gene expression by microarray analysis in the total heart. Many genes were regulated by one particular treatment only and among those most of them showed large functional divergence. Although, the majority of genes responding to fatty acid treatment were regulated in a PPARα-dependent manner, emphasizing the importance of PPARα in mediating transcriptional regulation by fatty acids in the heart, we observed a substantial number of genes regulated in a PPARα-independent manner. Finally, we observed that deletion and activation of PPARα had a major effect on expression of numerous genes involved in metabolism and immunity.

We identified response to oxidative stress as the top upregulated process activated by all administered fatty acids in the heart. High rates of mitochondria oxidation, due to increased supply of substrate after the oral gavage are coupled with enzymatic and non-enzymatic mechanisms aiming to counterbalance the production of highly reactive secondary products of the respiratory chain, the reactive oxygen species (ROS) in the heart. Under conditions such as chronic high fat diet or insulin resistance, increased lipid influx in combination with uncontrolled production of ROS and lipid intermediates may result in mitochondrial malfunctioning and lipid accumulation. Myocardial lipotoxicity refers to the accumulation of intramyocardial lipids and is associated with contractile dysfunction and even myocytes death. We found Angptl4 to be the top upregulated gene, in all groups that received the fatty acids oral gavage. Angptl4 has been described as a target gene of PPARs and an endogenous inhibitor of the triglyceride hydrolyzing enzyme lipoprotein lipase (LPL), which catalyzes uptake of circulating lipids into tissues. We were able to show that the strong upregulation of Angptl4 by dietary fatty acids is mediated by PPARβ/δ and is part of a feedback mechanism aimed at protecting the heart against lipid overload and consequently fatty acid–induced oxidative stress, one of the hallmarks of lipotoxic cardiomyopathy.

Angptl4 has been shown to have a potent inhibitory effect in LPL activity and subsequent reduction in uptake of lipids by several tissues and cell types, including macrophages. Furthermore, Angptl4 was shown to prevent the

formation of foam cells in mesenteric lymph nodes upon high fat feeding. Accordingly, we hypothesized that Angptl4 may affect atherosclerosis development by reducing foam cell formation. Thus, our second aim was to investigate the role of Angptl4 on atherosclerosis development. We studied Angptl4 expression in atherosclerotic lesions and macrophages and determined the effect of Angptl4 transgenic overexpression in atherosclerosis prone ApoE3Leiden (E3L) mice fed a Western diet containing 0.4% cholesterol. We observed a decrease in atherosclerosis in Angptl4 overexpressing mice on an ApoE3L background. This effect was independent of the plasma cholesterol and triglyceride levels. Importantly, Angptl4Tg.E3L exhibited a less pro-inflammatory phenotype with decreased accumulation of monocytes/macrophages in the atherosclerotic plaque, suggesting an anti-inflammatory role of Angptl4 in atherosclerosis development.

Finally, we set out to identify transcriptional targets of fatty acids in macrophages, as part of a general goal to elucidate mechanisms through which fatty acids exhibit a direct role in modulating inflammatory processes in macrophages. We identified Hig-2 to be strongly upregulated by all treatments. We found expression of Hig-2 to be the highest in peritoneal macrophages and white adipose tissue. Chronic high fat feeding increased Hig-2 expression levels in adipose tissue but not in liver. Immunohistochemistry indicated colocalization of Hig-2 with Cd68 in infiltrating macrophages as part of crown-like structures. Based on these findings we propose that Hig-2 has a specific role in macrophages and may function as an interesting target in the study of obese adipose tissue.

In conclusion, this thesis contributes new information on gene regulation by dietary PUFA in the mammalian heart and provides mechanistic insight on their previous reported beneficial effects. Furthermore, we reveal a novel protective role of Angptl4 in atherosclerosis development. We propose that this effect is mediated by a mechanism, which is independent of inhibition of LPL-mediated systemic lipid clearance and it is probably related to the effect of Angptl4 on macrophage oxLDL uptake and chemotaxis. Finally, in the present thesis we start up an effort to identify fatty acid target genes in macrophages, which open new future research paths.

SAMENVATTING (Summary in Dutch)

De hoeveelheid vet in onze voeding draagt bij aan het risico voor diverse chronische ziekten, zoals hart en vaatziekten, obesitas, diabetes, dyslipidemie en metabool syndroom. Een groot aantal epidemiologische studies laten zien dat naast de hoeveelheid vet in de voeding ook de vetsamenstelling een belangrijke factor is in de ontwikkeling van chronische ziekten. Er zijn sterke aanwijzingen dat het volgen van een voeding die rijk is aan meervoudig onverzadigde vetzuren en enkelvoudig onverzadigde vetzuren, zoals een mediterrane voeding, aanzienlijke gezondheidsvoordelen oplevert, terwijl een voeding die rijk is aan verzadigde vetzuren, zoals een westerse voeding, het risico op diverse chronische ziekten verhoogt.

Nutritional genomics of nutrigenomcis onderzoekt de interactie tussen voeding en genen op moleculair niveau door gebruik te maken van genomics tools. Binnen het vakgebied van nutrigenomics worden vetzuren in onze voeding en de daaruit gevormde metabolieten gezien als signaal moleculen die aangrijpen op specifieke cellulaire respons systemen. Vetzuren binden aan een familie van ligand-geactiveerde transcriptiefactoren die een belangrijke rol spelen bij metabole homeostase: de zogenaamde PPARs. Er zijn drie verschillende PPARs bekend: PPARa, PPARb/d en PPARg. De expressie van de verschillende PPARs varieert aanzienlijk tussen verschillende weefsels en diverse celtypen.

Na een maaltijd worden triglyceriden verpakt in chylomicronen in de dunne darm en bereiken vervolgens via de lymfe het bloed en de perifere weefsels. De in chylomicronen aanwezige triglyceriden komen beschikbaar voor de organen als vrije vetzuren na lipolyse door het enzym lipoproteïne lipase dat verankerd zit in de wand van de capillairen. De organen die relatief de grootste hoeveelheid van het vet in de voeding opnemen zijn het hart en de lever. In tegenstelling tot de lever is het hart een orgaan dat veel fysieke arbeid verricht en voornamelijk in de energiebehoefte voorziet in de vorm van vetzuren die via hydrolyse van triglyceride rijke deeltjes beschikbaar komen. Een disbalans tussen opname van vetzuren en oxidatie van vetzuren is een veel voorkomend probleem bij hartziekten, zoals hartfalen, myocardiale ischemie en diabetes. Het hart heeft een zeer beperkte opslagcapaciteit aan vet. Indien als gevolg van een verhoogde vetopname er sprake is van een verhoogde vetopslag dan kan dit

schade veroorzaken en aanleiding geven tot zogenaamde lipotoxische cardiomyopathie.

Het eerste doel was om de individuele effecten van vetzuren op het intacte hart in kaart te brengen via transcriptionele profiling. Door deze experimenten uit te voeren in wildtype en PPARa-/- muizen zijn we in staat de specifieke bijdrage van PPARa vast te stellen. PPARa is eerder beschreven als master regulator van het vet metabolisme in het hart. In ons onderzoek is gebruik gemaakt van een uniek experimenteel model waarbij aan muizen een enkele orale dosis wordt toegediend van synthetische triglyceriden bestaande uit één specifiek vetzuur. De muizen werden 6 uur na de orale toediening opgeofferd en vervolgens werden de effecten van de verschillende vetzuren op gen expressie in het hart vergeleken door middel van microarray analyse. Een groot aantal genen reageerde alleen op één specifieke behandeling en binnen een set van vergelijkbaar reagerende genen bestond veel variatie qua functie. Het merendeel van de genen die op vetzuren reageerden vertoonden een afhankelijkheid van PPARa, wat het belang van PPARa in regulatie van gen expressie door vetzuren illustreert. Desondanks werden ook diverse genen onafhankelijk van PPARa door vetzuren gereguleerd. Tenslotte had de afwezigheid van PPARa en de activatie van PPARa een aanzienlijk effect op expressie van talloze genen die betrokken zijn bij metabolisme en immuniteit.

De respons op oxidatieve stress kwam naar voren als het proces dat het meest sterk door de diverse vetzuren geïnduceerd werd. Een intensieve mitochondriële oxidatie als gevolg van een verhoogde aanvoer van substraat door middel van de orale gavage brengt enzymatische en niet-enzymatische mechanismen in gang die erop gericht zijn de productie van reactive oxygen species (ROS) in de ademhalingsketen te neutraliseren. Onder condities zoals een chronisch hoog vet dieet of insuline resistentie kan de verhoogde opname van lipiden in combinatie met ongecontroleerde productie van ROS en lipide intermediairen leiden tot mitochondriële dysfunctie en zelfs het afsterven van myocyten. Angptl4 kwam naar boven als het gen dat het meest sterk geïnduceerd werd door de verschillende vetzuur behandelingen. Angptl4 is uitgebreid beschreven als target gen van PPARs en fungeert als endogene remmer van het enzyme lipoproteïne lipase, wat verantwoordelijk is voor de hydrolyse van circulerende triglyceride-rijke deeltjes en essentieel is voor de opname van vet in de weefsels. De resultaten lieten zijn dat de inductie van Angptl4 door vetzuren loopt via PPARb en deel uitmaakt van een feedback

mechanisme dat erop gericht is het hart te beschermen tegen een overdaad aan vet en de daarmee gepaard gaande oxidatieve stress, wat één van de kenmerken is van lipotoxische cardiomyopathie.

Van Angptl4 is bekend dat het een sterke remmer is van de LPL activiteit en de daarmee tevens de opname van lipiden door verschillende weefsels en cellen, waaronder macrofagen, onderdrukt. Onze groep heeft recentelijk aangetoond dat bij gebruik van een vetrijke voeding Angptl4 de vorming van schuimcellen in de mesenterische lymfknopen voorkomt. Bijgevolg ontstond de hypothese dat Angptl4 de ontwikkeling van atherosclerose zou kunnen remmen door de vorming van schuimcellen te onderdrukken. Het tweede doel was aldus om de rol van Angptl4 bij de ontwikkeling van atherosclerose te onderzoeken. Om dat te bereiken is de expressie van Angptl4 in atherosclerotische plaques bepaald en tevens het effect onderzocht van Angptl4 overexpressie in het ApoE3Leiden diermodel, wat gekenmerkt wordt door ontwikkeling van atherosclerose bij consumptie van voer dat rijk is aan vet en cholesterol. Transgene overexpressie van Angptl4 leidde tot een aanzienlijk afname van atherosclerose. Dit effect was onafhankelijk van de plasma cholesterol en triglyceriden gehalten. De Angptl4 transgene muizen hadden een minder inflammatoir fenotype met verminderde ophoping van monocyten/macrofagen in the atherosclerotische plaques, wat duidt op een anti-inflammatoire rol van Angptl4 bij de ontwikkeling van atherosclerose.

Het laatste deel was gericht op het proberen te identificeren van transcriptionele targets van vetzuren in macrofagen, wat deel uitmaakt van een poging om de mechanismen op te helderen waarbij vetzuren ontsteking in macrofagen kunnen beïnvloeden. Behandeling van macrofagen met diverse typen vet leidde tot een sterke toename van de mRNA expressie van het Hig2 gen. Expressie van Hig2 was het hoogst in peritoneale macrofagen en wit vetweefsel. Expressie van Hig2 ging omhoog in vetweefsel maar niet in lever van dieren die langdurig voer kregen dat rijk was aan vet. Immunohistochemie liet zien dat Hig2 in het vetweefsel op dezelfde plek aanwezig is als de macrofaag marker Cd68 en deel uitmaakt van zogenaamde crown-like structuren. Op grond van deze bevindingen stellen we voor dat Hig2 een specifieke rol heeft in macrofagen en zou kunnen dienen als belangrijke target bij de bestudering van vetweefsel.

Samengevat draagt dit proefschrift nieuwe informatie aan over regulatie van genen door vetzuren in het hart en tevens beter mechanistisch inzicht aanlevert

voor de beschreven gunstige effecten van onverzadigde vetzuren. Tevens toont het proefschrift een nieuwe beschermende rol van Angptl4 bij de ontwikkeling van atherosclerose, waarschijnlijk via een mechanisme dat onafhankelijk is van het effect van Angptl4 op vet klaring door LPL maar waarschijnlijk gerelateerd is aan effecten van Angptl4 op opname van geoxideerd LDL door LDL en op chemotaxis. Tenslotte laat het proefschrift de identificatie zien van nieuwe door vetzuren gereguleerde gene in macrofagen, wat nieuwe onderzoeksmogelijkheden biedt.

“ It was long, many things changed in my life in the last five years, I had to manage a great deal of new information and projects, I felt hopeful and hopeless about it several times, but I’ve walked down this long road and now I do feel a bit wiser than before.”
Great last words on my PhD experience!

Now enough about me! Acknowledgements follow!!!

Acknowledgements

I would like to dedicate this part of my PhD thesis book to all the people, that contributed to this book and most importantly made my scientific and personal life rich the last 5 years.

Professor Michael Müller i want to thank you from heart, for giving me the opportunity to do my PhD research in your group. You are a great manager and a true inspiration for the whole group. I really enjoyed your force and dynamism in getting your hands on new released information as soon as possible. I will continue following your Twitter.

My daily supervisor **Professor Sander Kersten**, there are not enough words to express my appreciation and gratitude for all the support and guidance you have offered to me all these years! Without your help this book wouldn't have been possible. I feel proud to have been your PhD student and I'll never forget you. I wish you many more fruitful years of research, in your new chair and of course the best for your sweet family. ☺ Thanks a lot for introducing me the avocado. This delicacy, whose edible value I was completely ignoring before I met you. ")

Professor Patrick Rensen, I would like to say many many thanks for your guidance and help the last two years. It was a great honour to have worked with you. I enjoyed a lot the Lipoprotein clubs and of course the unforgettable SSAR conference in Denmark. You are a great dancer ! (ok Sander is also a very good dancer, but honestly Patrick is smashing!) I wish you many more successful years in your research career and of course your artistic one!

Mijn paranimfen Guido and Frits. You are both great! **Guido**, I am very proud to have you, on my side during my defence. I want to thank you for all the advice and help all these years. I enjoyed our little talks at the corridor or during your visit for a candy in my office. Of course, I enjoyed a lot the Brazilian visit! I appreciate your honest, informative character and your real effort to invest true time in having things properly done. **Frits**, I am really happy that you accepted to be my paranimf. You have been a very motivating force for me. Your restless spirit and constant questions about everything made it nice to have you around. I enjoyed a lot our conversations and i always found your hyperactive moods very funny! I wish you all the best for the future!

Rinke, I would like to say many thanks for your help, especially the last year of my PhD. You would never refuse to help, even if you had a very heavy

schedule. I admire your ability to combine two jobs in two different departments, one in Nijmegen and one in Wageningen. I wish you all the best in your future career.

Diederik, thanks for the laugh, the beers, the dancing and of course for all the free chocolate with extra flavonoids!
Noortje and **Danielle,** you have been always nice and friendly to me. I wish to both of you good luck with finishing your PhD.
Jessica, Katja, Milene you can walk really really long!!! and that says a lot about you :-‘ . I still cannot believe that you walked for 26 hrs in a row.
Jessica thanks a lot for always being such a sweet and caring friend to me. I enjoyed our meetings in the town for beer or coffee. It was fun to go with you to a film festival and watch 2 days movies, with only 3 hour sleep in between. You are tough!
Katja, you have been a great roommate, a very positive force, and a great emotional support especially for the last months, when I was writing up my thesis! I really want to thank you for our interesting discussions, your motivating attitude and your humour. Take good care of the Anastasia fish and I will visit it some time later.
Milene, thanks for the dinners, your calm mood and the fun.
Sheril, you are very sweet and kind and I wish all the best for you and your family.
Jvalini and Nikkie, you moved into my room when I was quite tensed finishing up my book and research! I think I scared you at the beginning hehe**. Jvalini**, I enjoy your colourful mood and your positive view on people and situations. I wish you good luck with your project. **Nikkie**, you have been always encouraging me the last months. Thanks for caring for me and for being so friendly and funny! You were both a great boost for my spirit and I enjoyed to have you as roommates! All the best for the future!
Nicole, we have spent together all the years of my PhD. You are my roommate since the beginning. Your endless patience and your hard working attitude gave me lots of motivation all these years. We had some great laugh in the room, I’ll miss you! All the best to your family!
Susan, if you haven’t been my roommate, my life in the lab would have been very dull! Thanks for listening to my stories all these years. I am really happy for your new position in Australia and I am sure that you are going to make it a great adventure! Hope to visit you there. **Els,** I really enjoyed to have you as a roommate!
Laeticia, you have been a great friend, colleague and housemate! We shared work and house and managed both great! I will never forget the fun we had together, the parties, the excursions and much more! Thanks a lot for being next

to me in all cases, for your support and love. I am very proud of being your friend!!!

Maryam, thanks for your optimism and hopeful comments any time it was needed! I appreciate your friendship and I am still having lots of fun in the epic birthday Iranian style parties! All your family is amazing, with top of the top your mam, **Minoo**. It was a pleasure for me to get to know her!

Shorheh, you have been always taking care of me in your discrete and "invisible" way! Thanks a lot for being always very encouraging and supportive in the difficulties. I hope we see each other in the future!

Jenny and Mechteld, you were the magic hands behind my arrays projects! Thanks for all the help all these years and for always being good fun. **Karin, Mieke, Carolien, Wilma** you have been always very polite and friendly. Many thanks for all the help!

Lydia, I enjoyed our little talks. Thanks for all the interest you have been showing to me.

Mark and Philip, thank you for being always very helpful and nice to me!

Jan, i enjoyed a lot your stories from the land, where the sun always shines. I hope you will continue visiting Greece and enjoy the sea and the weather.

Rene, Wilma, Bert, Judith thank you very much for the help with the mouse epxeriments and for always being available for anything I needed.

Nancy (Yinxin Wang) I appreciated your help and kind character very much during the least two years of our collaboration. It has been a pleasure to meet you. I enjoyed our talks on science, on the dutch mentality and men with muscles. **Mariette** the same counts for you!:-)

Jimmy it was a pleasure to meet you. Thanks for all the help and advice.

Lidwien, you are not with us anymore. I would like to thank you for have been such a nice person, a helpful colleague and a dynamic lady, that always took personal care, of any problem that appeared to me. It was pleasure to have met you and I feel sad that you are gone.

Anand and Susan, thanks for being such sweet friends and neighbours for all these years! I am very grateful for all the help you have offered me, with the house, with Bobo, with everything! I feel more like home, since you are living close to me. I wish you all the best!

Jeremy, you have been a great friend for the last couple of years! I enjoyed a lot our talks in the pub. I want to thank you form heart for caring so much for me and for all the good advice and support I have received from you.

Dimitri (Mitsos), you were a terrible housemate ☺, but I love you a lot and I had a lot of fun, while we were sharing the house. I enjoyed a lot the dinners,

the billiard table in the middle of the living room and your cheerful and relaxed mood. I hope you get done with your PhD, soon.
Vlahakis Giorgos, I really enjoyed to have met you better and because of the boys it's impossible for me not to call you Vlahakis.
Andrea, you have been a very sweet friend and an inspiring figure in my life. I enjoyed a lot all our talks about life, universe and everything. You had always great answers to my existential crisis questions and you humour always made things nicer! I hope you'll find your way soon!
Alex, many thanks for all the fun we had together and all your love and caring.
Oriana and Maria, my Queens! You are both very special for me. **Oriana**, it was a pleasure and great experience to share the house with you for the last year. You'll do great, in whatever you do. I am sure about that. I feel lucky to have been experiencing your enthusiasm and passion for life, on a daily basis! **Maria**, I admire you kind and patient character and I enjoyed to have met you a bit better the last year.
Niko, thanks for having your house always open for dinners and gatherings. I will never forget the Eastern celebrations at your place. You have a really big heart and you are a special person. I always enjoyed hearing your stories!

To all the **MovieW people,** I would like to say that I am very happy that you're there to make my life interesting! We had a lot of adventure trying to save MovieW last year! I am really happy that we made it! We keep seeing each other.

Το μεγαλύτερο ευχαριστώ πάει **στην οικογένεια μου!** Χωρίς την βοήθεια σας, δεν θα είχα φτάσει εως εδώ! **Μαμά, μπαμπά** είμαι πολύ περίφανη που είστε γονείς μου. Και οι δύο μου μάθατε πάρα πολλά και θα είμαι για πάντα ευγνώμων για όλη την υποστήριξη και αγάπη που μου έχετε προσφέρει! Σας αγαπώ πολύ!
Στις αδερφές μου Μαριάνθη και Όλγα θέλω να πώ ότι σας αγαπώ πολύ και είμαι πού περίφανη για ότι έχετε καταφέρει έως τώρα! **Μαριάνθη**, να είσαι ευτυχισμένη με τον Γιώργο! **Όλγα**, καλή τύχη στις σπουδές σου και να έχεις τα μάτια σου ανοιχτά!
Στους αγαπημένους μου φίλους, **Βίκη και Νίκο** θέλω να πω ένα μεγάλο ευχαριστώ για την αγάπη και την πολύχρονη φιλία σας! Είμαι πολύ τυχερή να σας έχω φίλους και είμαι πολύ χαρούμενη, που η απόσταση μεταξύ μας δεν στάθηκε ποτέ εμπόδιο στις σχέσεις μας! Εύχομαι το καλύτερο για εσάς. Θα είμαι για πάντα δίπλα σας.

Last I want to say a big thanks to my♡**Bram**. I feel very lucky that you love me and it's great to be with you! I hope we'll have many years of joy! You are really in my heart.

Anastasia (Natasha)

A.G

About the author

Anastasia Georgiadi was born in June 20th, 1982 in Athens, Greece. She studied Nutrition and Dietetics in Harokopio University of Athens, Greece. After finishing her first degree she developed a greater interest in molecular biology and genomics and she enriched her previous studied in Nutrition, by following an MSc in Nutrition and Genomics, in Wageningen University, in the Netherlands. During her MSc studies she began working on her PhD project under the supervision of Prof. Sander Kersten and Prof . Michael R. Müller, in the Nutrition, Metabolism and Genomics group, in Wageningen University. In the first three years of her PhD (2006-2009), she worked as an early stage researcher (ESR) in the Marie Curie project, Systems Biology of Nuclear Receptors. During the last two years (2010-2011) she worked on atherosclerosis and lipid metabolism, for a project funded by the Netherlands heart foundation and with close collaboration with Prof. Patrick Rensen, in Leiden University Medical Centre (LUMC). This book is her PhD thesis.

List of publications

Georgiadi A, Lichtenstein L, Degenhardt T, Boekschoten MV, van Bilsen M, Desvergne B, Müller M, Kersten S. Induction of cardiac Angptl4 by dietary fatty acids is mediated by peroxisome proliferator-activated receptor beta/delta and protects against fatty acid-induced oxidative stress.; *Circ Res. 2010 Jun 11;106(11):1712-21*

Georgiadi A., Boekschoten MV., Müller M., Kersten S.; Detailed transcriptomics analysis of the effect of dietary fatty acids on gene expression in the heart. *Physiological Genomics 2012 Mar 19;44(6):352-61*

Georgiadi A and Kersten S. Mechanisms of gene regulation by fatty acids.; *Review Advances in Nutrition March 2012 3: 127-134*

Lichtenstein L, Mattijssen F, de Wit NJ, *Georgiadi A*, Hooiveld GJ, van der Meer R, He Y, Qi L, Köster A, Tamsma JT, Tan NS, Müller M, Kersten S. Angptl4 protects against severe proinflammatory effects of saturated fat by inhibiting fatty acid uptake into mesenteric lymph node macrophages. *Cell Metab. 2010 Dec 1 ;12(6):580-92.*

Buler M., Aatsinki S.M., Skoumal R., Komka Z, Toth M., Kerkela R., *Georgiadi A,* Kersten S., and Hakkola J. Energy-sensing factors coactivator peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) and AMP-activated protein kinase control expression of inflammatory mediators in liver: induction of interleukin 1 receptor antagonist. *J Biol Chem. 2012 Jan 13;287(3):1847-60.*

Georgiadi A., Wang Y., Stienstra R., Stalenhoef A., Tan N.S., Müller M., Rensen P.C.N., Kersten S. Angptl4 overexpression protects against development of atherosclerosis in mice. *(In preparation)*

Mattijssen F., *Georgiadi A.,* Kersten S. The hypoxia inducible gene (Hig-2) is a novel target gene of fatty acids in macrophages. *(In preparation)*

Overview of complete training activities

Discipline specific activities and courses

Systems biology: Statistical analysis of omics data, Wageningen 2006
FEBS-SysBio Austria, 2007
International Course On Laboratory Animal Science, Utrecht, 2007
Wageningen Institute of Animal Sciences course on Design of Animal Experiments,Wageningen 2008 (WGS course)
Masterclass Nutrigenomics, Wageningen 2007
Masterclass Nutrigenomics, Wageningen 2008
Masterclass Nutrigenomics, Wageningen 2009
1rtst International Advanced Course on Epigenesis and Epigenetics, Wageningen, 2008
International Atherosclerosis Research School, Hambourg, Germany, 2010
5th Annual Conference Society for Heart and Vascular Metabolism, Maastricht, 2007
49th International Conference on Lipids and Biosciences ICBL meeting Maastricht 2008
FEDERA symposiumon obesity,Leiden 2008
8th Endo-Neuro-Psycho Meeting, Doorwerth, 2008
Atherosclerosis Thrombosis and Vascular Biology (ATVB) Washighton U.S.A 2009
Wageningen Nutritional Sciences Forum : "Too much too little", Arhnem, 2009
World Pharma, Copenhagen 2010
International Society for the Study of Fatty Acids, Maastricht 2010
27th Ernst Klenk Symposium in Molecular Medicine, Cologne, Germany, 2011
8th NuGO week Wageningen 2011
17th Annual Scandinavian Atherosclerosis conference- An international meeting, Copenhagen, 2011
1rst Cardiovascular Dutch conference 2011
Systems Biology of Nuclear Receptors Marie-Curie meetings 2006-2009
Benelux Nuclear receptors meeting 2008-2010
Netherlands Lipoprotein Club, Leiden, 2008-2012
NWO-WGV meetings Deurne 2008-2011

General courses
NuGO PhD week 2007
Career Orientation Wageningen, 2009
Writing skills, NucSys
PhD Competence Assessment, Wagenigen, 2009

Optional

Human Nutrition PhD-study tour, East Coast U.S.A 2007

Organization and participation to the journal club, Division of Human nutrition, NMG Journal Club

NMG Scientific meetings

Gene Technology, MSc Course, Wageningen University, 2006

Bioinformatics, MSc Course, Wageningen University, 2006

Preparation of PhD proposal

Cover design: The cover was realized by Marc Lochs, http://www.lochs.nl/
marc@lochs.nl, Haarlem, The Netherlands

Author's picture created by Bram Damman, http://bramdamman.nl/

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Printing
CPI Koninklijke Wöhrmann B.V., www.wps.nl
Zutphen, The Netherlands