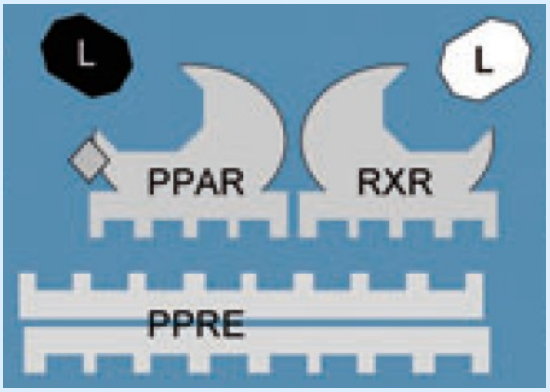
Treatment of Obesity and Type 2 Diabetes with PPARα Agonists – Effects on Myocardial Metabolism, Gene Expression and Ventricular Function

Ahmed Murtaz Khalid



A dissertation for the degree of Philosophiae Doctor

UNIVERSITY OF TROMSØ
Faculty of Medicine
Department of Medical Physiology

2009

Contents

In the loving memory of my grandparents…

Acknowledgements

This study was carried out at the Department of Medical Physiology, University of Tromsø from January 2005 until December 2008 and was mainly supported by the University of Tromsø. I wish to express my heartiest appreciation and gratitude to my supervisor Professor Terje S. Larsen for introducing me into the field of molecular cardiovascular physiology. I am thankful for enthusiastic support, intellectual guidance, encouragement, helping in preparing the thesis and never ending patience throughout all these years without which I don't think I could have made it.

I would also like to thank my co-supervisor Ellen Aasum for helping me out with the project planning, laboratory work and above all for her enthusiastic guidance throughout these years. I also want to thank Professor David L. Severson for sharing his critical thoughts and useful feedback during the preparation of manuscripts. Here I will also like to thank Trine Lund for introducing me into the exciting world of gene expression.

In addition I am highly grateful to all my colleagues, Ole Jakob How, Neoma Boardman, Martin Hagve and Anne Dragøy Hafstad for their beneficial collaboration and exchange of useful thoughts. I am highly indebted to the skillful technical support provided by Elisabeth Børde, Knut Steinnes, Fredrik Bergheim, Thomas Andreasen and Karin Akselsen. I also want to thank the staff of animal department for their technical support.

Finally, my warm thanks go to my wife Tanzeela and my beautiful little world, Wishma and Wahab for their love, support and patience. Last but not the least I want to thank my beloved parents and sisters for their support, guidance and endless love.

Ahmed Murtaz Khalid
Tromsø, November 2008

Abbreviations

ACC	Acetyl CoA Carboxylase
ACO	Acyl CoA Oxidase
ACS	Acyl CoA Synthase
AGE	Advanced Glycation Endproducts
AMPK	AMP-Activated Protein Kinase
ATP	Adenosine Triphosphate
BMI	Body Mass Index
CPT	Carnitine Palmitoyl Transferase
Ct	Threshold Cycle
DIO	Diet Induced Obese
FA	Fatty Acids
FABP	Fatty Acid Binding Protein
FAO	Fatty Acid Oxidation
FAT/CD36	Fatty Acid Translocase
FATP	Fatty Acid Transport Protein
GLUT	Glucose Transporter
HDL	High Density Lipoproteins
IKK	Inhibitory KappaB Kinase
IL	Interleukin
IRS-1	Insulin receptor substrate 1
JNK	c-Jun N-terminal Kinase
LCAD	Long Chain Acyl CoA Dehydrogenase
LCAS	Long Chain Acyl CoA Synthase
LTB	Leukotriene B
MCAD	Medium Chain Acyl CoA Synthase
MCD	Malonyl CoA Decarboxylase
MMP	Matrix Metalloproteinase
MTE1	Mitochondrial Thioestrase 1
MVO_2	Myocardial Oxygen Consumption
NF-κB	Nuclear Factor Kappa B
PDH	Pyruvate Dehydrogenase
PDK4	Pyruvate Dehydrogenase Kinase-4
PFK1	Phosphofructokinase 1
PI3-K	Phospho-inositol-3-Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator Activated Receptor
PPRE	Peroxisome Proliferator Response Element
PVA	Pressure Volume Area
q PCR	Quantitative Real Time PCR
RAS	Renin-angiotensin System
ROS	Reactive Oxygen Species
RXR	Retinoid X Receptor
TG	Triglyceride
TNFα	Tumor Necrosis Factor Alpha
TTA	Tetradecylthioacetic Acid
UCP	Uncoupling Protein
VCAM-1	Vascular Cell Adhesion Molecule-1

List of papers

Paper I Aasum E, Khalid AM, Gudbrandsen OA, How OJ, Berge RK, Larsen TS. Fenofibrate modulates cardiac and hepatic metabolism and increases ischemic tolerance in diet-induced obese mice. J Mol Cell Cardiol. 2008; 44: 201-209.

Paper II Khalid AM, Aasum E, Hafstad AD, Lund T, Severson DL, Larsen TS. Improved cardiac metabolism following in vivo treatment of type 2 diabetic mice with fenofibrate depends on reduction of plasma lipids, as well as glucose (manuscript).

Paper III Hafstad AD, Khalid AM, Hagve M, Lund T, Larsen TS, Severson DL, Clarke K, Berge RK, Aasum E. Administration of tetradecylthioacetic acid (TTA) stimulates myocardial fatty acid oxidation despite having a lipid-lowering effect (submitted manuscript).

Paper IV Khalid AM, Hafstad AD, Larsen TS, Severson DL, Boardman N, Hagve M, Lund T, Berge RK, Aasum E. Cardioprotective effect of the pan PPAR ligand tetradechylthioacetic acid (TTA) in type 2 diabetic hearts (manuscript).

(Khalid and Hafstad have made equal contributions to paper III and IV)

INTRODUCTION

Obesity – a major health concern

Obesity has turned up as one of the major health concerns in the 21st century and is one of the leading causes of preventable death (11). Obesity is a term applied to excess body weight with an abnormally high proportion of body fat. Thermodynamically speaking, imbalance between energy intake (feeding) and energy expenditure (physical activity) leads to obesity (113) . Development of obesity is, however, more complicated than that; sedentary life style, genetic factors, medical illness, microbiological aspects, social factors and neurobiological mechanisms are also involved (20; 33)

Measurement of obesity. Obesity is commonly defined as body mass index (BMI, weight in kilograms divided by height in meters squared) of 30 kg/m^2 or higher (92). BMI (or Quetelet index) was introduced between 1830 and 1850 by the Belgian polymath Adolphe Quetelet (60). The classification of body weight according to WHO is as follows:

BMI < 18.5	underweight
BMI 18.5 - 24.9	normal weight
BMI 25 - 29.9	overweight
BMI 30 - 39.9	obese
BMI 40 or higher	severely obese

Although there is a correlation between obesity and cardiac disease, BMI is not a precise predictor of cardiovascular disease; absolute waist circumference or waist to hip ratio are more precise measures of central obesity and correlate better to health risks than BMI (69).

Obesity, metabolic syndrome and insulin resistance. One of the obesity-associated health risks is development of metabolic syndrome. According to the WHO metabolic syndrome is characterized by the presence of central obesity, dyslipidemia (elevated triglycerides and decreased HDL cholesterol), impaired glucose tolerance or insulin resistance, type 2 diabetes and high blood pressure. In order to be diagnosed with metabolic syndrome an individual must be obese (BMI > 30 kg/m^2) plus at least 2 additional risk factors, e.g. dyslipidemia and hypertension or dyslipidemia and impaired fasting glucose (127).

More than 40 years ago Randle proposed that fatty acid (FA) compete with glucose as an energy substrate in rat heart and diaphragm muscle, and speculated that increased fatty acid oxidation was responsible for the obesity-induced insulin resistance (118; 119). The proposed mechanism was that an increased FA uptake ultimately resulted in an increase in the intramitochondrial acetyl CoA/CoA and NADH/NAD+ ratios with subsequent inactivation of the pyruvate dehydrogenase. The high uptake of FA will lead to an increase in the intracellular concentration of citrate, leading to inhibition of phosphofruktokinase (Figure 1) (a rate limiting enzyme of glycolysis) and as a consequence, glucose 6 phosphate will accumulate and inhibit glucose uptake. Roden (122) also indicated an inhibitory effect of fatty acids on glucose transport and phosphorylation. More recently, it has been shown that an increase in the intracellular concentration of FA metabolites (diacylglycerol, fatty acyl CoA) leads to activation of protein kinase C (PKC), c-Jun N-terminal kinase (JNK)

and inhibitory kappaB kinase (IKK), which ultimately block the insulin signaling pathway through functional inhibition of insulin receptor substrate -1 (IRS-1), (19; 67). Last, but not least, release of endocrine factors from adipose tissue, alone or in combination with other factors like tumor necrosis factor alpha (TNFα), can lead to insulin resistance (116).

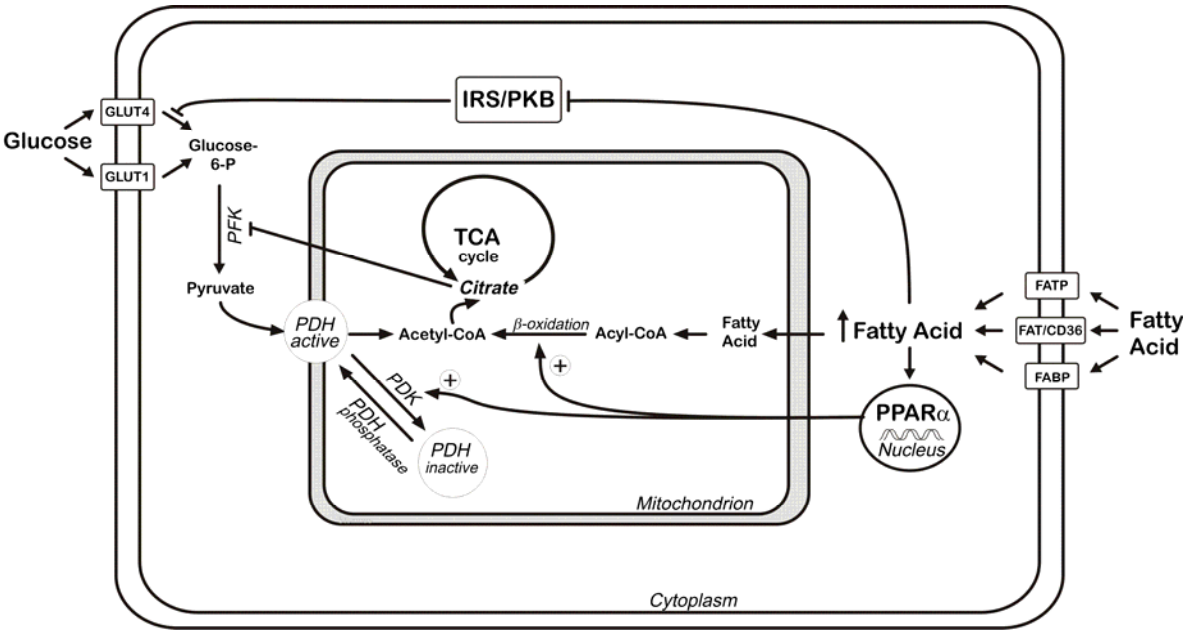


Fig. 1: Inhibition of glucose oxidation by increased FA supply. The uptake and oxidation of FA lead to citrate accumulation and inhibition of glucose utilization via inhibition of PFK. In addition FA inhibits glucose utilization via PDK-mediated inhibition of PDH via the PPARα pathway, as well as via inhibition of insulin signaling. PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; IRS, insulin receptor substrate; PKB, protein kinase B; FA, fatty acid; PPARα, peroxisome proliferator-activated receptor alpha.

Diabetes and cardiac disease

Diabetes mellitus is classified into type 1 and type 2 diabetes. Hereditary factors, diet and lifestyle, obesity and fat distribution, gestational predisposition and age are the main etiological factors of type 2 diabetes (123). Type1 diabetes is most often of genetic origin, although a combination of genetic predisposition with viral infection and autoimmune

disorders cannot be ruled out. The increased prevalance of type 2 diabetes worldwide is associated with significant increase in cardiovascular morbidity and mortality. Diabetes is presently estimated to affect more than 150 million patients worldwide, and this number is expected to double by 2025 (75; 143). Type 2 diabetic patients are more prone to develop angina and acute myocardial infarction, and the mortality rate is much higher among diabetics compared to non-diabetics suffering from acute myocardial infarction (57). The estimated four year survival rate is approximately 50% (37). There are two pathophysiological processes leading to type 2 diabetes-related cardiac complications: ischemic heart disease as a result of accelerated atherosclerosis and a specific diabetic cardiomyopathy, characterized initially as diastolic dysfunction which progresses to symptomatic heart failure (43). Although the pathogenesis of diabetic cardiomyopathy is multi-factorial and complex (Figure 2), metabolic disturbances related to hyper-lipidemia, hyper-glycemia and insulin resistance, as well as myocardial fibrosis, oxidative stress, and pro-inflammatory cytokines are proposed to be contributing factors (42).

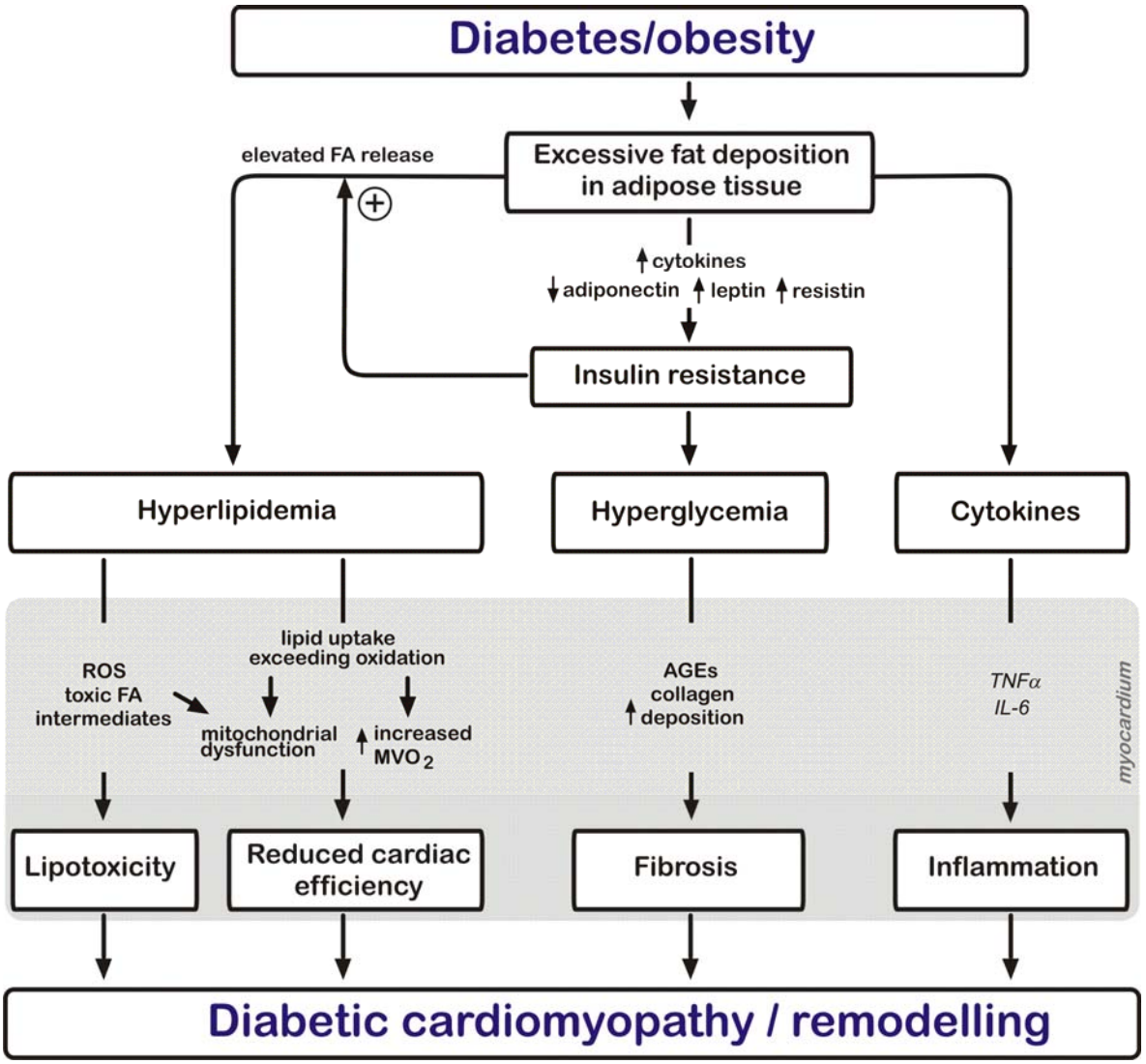


Fig. 2: Potential mechanisms for the development of diabetic cardiomyopathy: ROS, reactive oxygen species; TNFα, tumor necrosis factor alpha; IL-6, interleukin-6; AGE, advanced glycation end products.

Myocardial substrate utilization

The cardiac muscle needs a continuous supply of fuel in order to keep pumping. The daily ATP requirement of the heart is between 3.5 and 5 kg ATP (109).The heart demonstrates a great deal of flexibility with respect to its substrate selection, relying mainly on carbohydrates in the fed state, while lipids are the main source of energy during fasting, as suggested by Bing in the early 1950s(17). Long chain FA and glucose are the predominant sources of energy for the myocardium during normal resting conditions (104), but lactate

can also contribute to energy production, especially during periods of increased workload (38).

Fatty acids. Oxidation of FA (unesterified, albumin-bound FA from adipose tissue or FA produced by lipoprotein lipase-mediated hydrolysis of triglyceride-rich lipoproteins) accounts for 60-70% of cardiac energy production. Fatty acid uptake in cardiac myocytes is mediated by FA transport proteins (FATP), FA translocase (FAT/CD36) and FA binding protein (FABP) (85). In the cytosol FA is activated by acyl-CoA synthase (ACS) to form acyl-CoA, which can either be transported into the mitochondria for β-oxidation or undergo esterification to form intracellular TG (82). Before entering into the mitochondria acyl CoA is converted by carnitine palmitoyl transferase1 (CPT-I) to acyl carnitine (100) which in turn is reconverted to fatty acyl-CoA by CPT-II. Malonyl-CoA (produced by acetyl-CoA carboxylase) inhibits the activity of CPT1 and is considered a major inhibitor of FA oxidation (91). On the other hand, degradation of malonyl-CoA by malonyl CoA decarboxylase results in increased mitochondrial FA uptake. Once taken up by the mitochondria long chain fatty acyl CoA undergoes β-oxidation to yield acetyl CoA (Figure 3) which is further oxidized in the krebs cycle to CO_2, resulting in generation of NADH and FADH2 which ultimately provide reducing equivalents to the electron transport chain in oxidative phosphorylation (95).

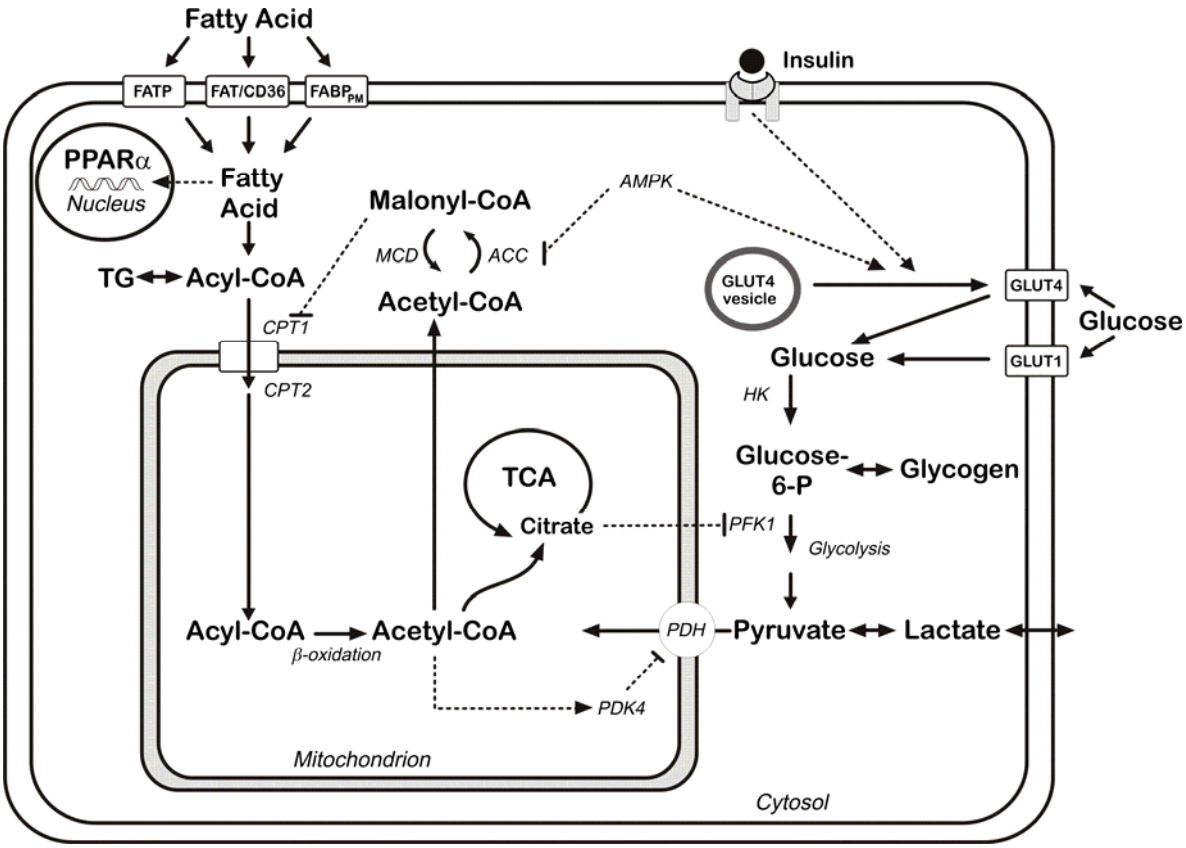


Fig. 3: Fatty acid and glucose uptake and metabolism. Intracellular FA uptake is mediated by passive diffusion or via transporters (CD36, FABP). Mitochondrial FA uptake is mediated by CPT-I and CPT-II, followed by entry into the β-oxidation, yielding acetyl-CoA and reducing equivalents which are channelled through the electron transport chain. Glucose is taken up by the transporters GLUT1/4, undergoes glycolysis to yield pyruvate, which thereafter is converted to acetyl CoA by PDH and further processed in the TCA cycle. FABP, Fatty acid binding protein; CPT-I, carnitine palmitoyl transferase 1; CPT-II, carnitine palmitoyl transferase-II; GLUT1, glucose transporter 1; GLUT4, (insulin-regulated) glucose transporter 4; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid cycle.

Glucose. Glucose transport into cardiomyocytes is facilitated by GLUT1 and GLUT 4 (96) members of glucose transporter (GLUT) family. Myocardial glucose transport in basal conditions is mainly regulated by GLUT1 because of its more pronounced localization in the sarcolemma. GLUT4 is predominantly stored intracellularly, and its sarcolemmal translocation is mainly regulated by insulin-mediated phospho-inositol-3-kinase (PI3-K) pathway (85). However contraction induced myocardial glucose uptake is regulated by AMP-dependent kinase (AMPK)-mediated translocation of GLUT4 (28). Inside the cell,

glucose combines with a phosphate radical (glucose trapping) in the presence of hexokinase to form glucose 6-phosphate. Thereafter activated glucose 6-phosphate can enter into three different pathways: a catabolic pathway (glycolysis), glycogenesis or the pentose phosphate pathway. Pyruvate being the end product of glycolysis can be converted into acetyl CoA which enters into TCA cycle in the mitochondria. This key metabolic step is controlled by the pyruvate dehydrogenase (PDH) complex, which in turn is inhibited by pyruvate dehydrogenase kinase 4 (PDK4).

Transcriptional regulation of myocardial metabolism

Peroxisome proliferator activated receptors are ligand-activated transcription factors known to control many physiological functions, including glucose and lipid metabolism, cell growth and differentiation and vascular inflammatory processes (13; 47; 71). Thus, PPARs are important regulators of nutrient-gene interactions and potential targets for treatment and prevention of metabolic disturbances (124).

PPAR isotypes. Peroxisome proliferator-activated receptors (PPARs) are subclassified into three types on the basis of their chromosomal localization and variation in tissue expression: PPARα, PPARβ/δ and PPARγ. These three isoforms were first discovered in *Xenopus laevis* (African clawed frog) (39), and later they have been identified in several mammalian species. Contrary to the PPARα and γ, the mammalian PPAR β isoform was only partly homologous to the Xenopus isoform, and for that reason the mammalian PPAR β was given an alternative name, PPAR δ (31). PPAR α is mainly expressed in liver, heart, kidney, skeletal muscle, endothelium, vascular smooth muscle, monocytes and macrophages (9). PPAR γ is expressed in white and brown adipose tissue, muscle, colon and liver (9), while the expression of PPAR δ is ubiquitous. Like other nuclear factors,

PPARs contain both a ligand-binding domain and a DNA-binding domain. Upon binding of ligand PPARs undergo conformational changes and form a heterodimer with the retinoid-X receptor (RXR, ligand-activated receptor) (72). Gene transcription is initiated following binding of the PPAR:RXR complex to the PPRE element in the enhancer site of the regulated gene (Figure 4). In addition, interaction of co-activators/co-repressors with the PPAR:RXR complex plays an important role in determining the physiological response to PPAR ligands. Finally, the activity of PPARs is regulated by their phosphorylation status, which is controlled by multiple kinase pathways, e.g. AMPK, JNK and mitogen-activated protein kinase (MAPK) (35).

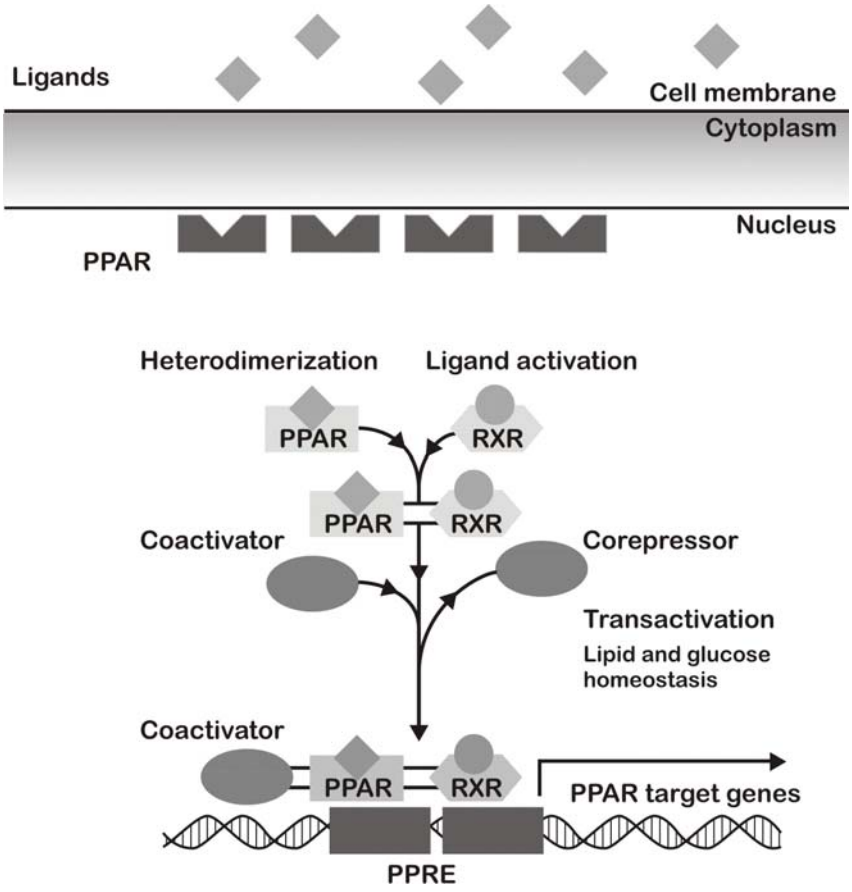


Fig. 4: Ligand-induced conformational change in PPARs results in heterodimer formation with RXR. This heterodimer, in association with co-activators, alters gene transcription by binding to specific PPREs in the promoter region of the target gene. PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; RXR, retinoid X receptor (modified from Libby and Plutzky 2007) (83).

PPAR ligands. In 1997 Krey and colleagues (77) screened several natural and synthetic candidate ligands (using a co-activator-dependent receptor ligand assay) and identified that naturally occurring FA (palmitic, oleic, linoleic and arachidonic acid) and their metabolites, as well as lipid-lowering drugs, were real ligands of the three PPAR subtypes. A number of synthetic ligands with different receptor specificity have become available over the last few years (Table 1).

Table 1: PPAR ligands and their receptor specificity

Ligand	PPAR specificity	Reference
Fibrates	α	(76; 132)
WY-14643	α	(24)
GW7647	α	(148)
GW9578	α	(53)
LY-518674	α	(107)
K-111 (previously called BM-17.0744)	α	(1; 93)
Tetradecylthioacetic acid (TTA)	pan	(16; 88)
Thiazoldinediones (glitazones)	γ	(128)
GW 501516	δ	(131)
GW 0742	δ	(24; 34)
L-165041	δ	(117)

Diabetes-induced changes in myocardial fuel selection

Type 2 diabetes is associated with dramatic changes in the concentration of energy substrates in the circulation, which in turn will influence cardiac metabolism. Plasma levels of both glucose and FA are increased in diabetes, and the rate of substrate uptake and oxidation in the cardiomyocytes are greatly influenced by the plasma supply. Impaired glucose uptake in the diabetic heart is a consequence of FA-induced inhibition of sarcolemmal GLUT4 expression (6). Besides, the increased FA supply will activate cardiac PPARα and promote expression of genes involved in FA uptake (84) and oxidation (144). As a consequence, cytosolic citrate levels will increase and inhibit glycolysis via inhibition

of PFK-1 (49; 106) so that the heart depends mainly on FA as energy source. It has been suggested that the increased plasma availability of FA in diabetes results in increased myocardial FA uptake which might exceed the oxidative capacity of the heart (45). This imbalance between FA uptake and oxidation leads to accumulation of triacylglycerols, FA intermediates and ceramide within the cardiomyocyte, causing a state of lipotoxicity (150). Ceramide and diacylglycerol accumulation will further aggravate the condition by activating PKC isoforms, thereby increasing CD36-mediated FA uptake. In addition, these kinases induce inhibitory phosphorylations of IRS-1 resulting in insulin resistance (111). Studies have shown that there is a strong correlation between development of contractile dysfunction and myocardial lipotoxicity, which might be reversed by altering myocardial metabolism by use of PPAR ligands (26; 52; 110; 150).

Prevention of myocardial lipotoxicity by administration of PPAR agonists

PPARs are thought to play a vital role in preventing dyslipidemia by regulating transport, oxidation, storage and synthesis of FA (74). As mentioned above, PPARα is mainly expressed in tissues expressing high rates of mitochondrial FA oxidation, such as heart, liver and kidney (73), regulating transcription of genes involved in FA uptake and oxidation (146).

PPARα: It has been shown that incubation of cardiomyocytes with PPARα ligands results in increased expression of PPARα target genes and increased FA oxidation (51; 140). The expression of PPARα target genes involved in cardiac FA uptake and oxidation pathways was increased in mice over-expressing PPARα (MHC-PPAR mice) (45). In addition, feedback induction of PPARα target genes, secondary to inhibition of fatty acid oxidative flux was lost in PPARα null mice (36). It is therefore generally accepted that increased FA oxidation in diabetic hearts is due to upregulation of PPARα (22; 147). On the other hand,

when diabetic and obese mice were chronically treated with synthetic a PPARα ligands (K-111), myocardial FA oxidation was reduced in perfused hearts from these animals (1; 2). This finding was surprising to many researchers in the field, but realizing that *in vivo* administration of the ligand also caused a profound stimulation of FA oxidation in the liver, implying an important mechanism for reduction of the plasma concentration of circulating lipids (FA and triglycerides), it became apparent that the reduction of myocardial FA oxidation in response to *in vivo* administration PPARα agonists could be a secondary effect of the reduced lipid burden.

PPARγ: In contrast to PPARα, little is known about how PPARγ affects myocardial metabolism. However, experiments on type 2 diabetic mice performed in our own laboratory show that *in vivo* administration of rosiglitazone (an anti-diabetic drug in the thiazolidinedione class of drugs) results in a marked reduction of myocardial FA oxidation (with a simultaneous increase in glucose oxidation) in perfused hearts from these animals (65). Again, we have suggested that this response is secondary to the lipid-lowering action of the drug, in which PPARγ activation in adipose tissue leads to increased uptake and storage of FA, so that the adipose tissue serves as a sink for circulating FA.

PPAR β/δ: Despite predominant expression of PPARβ/δ in cardiomyocytes (51), little is known about their role in the regulation of myocardial lipid metabolism. Cheng has however documented that PPARβ/δ overexpression in cardiomyocytes, as well as treatment of cardiomyocytes with a highly specific PPARδ ligand (GW0742) was associated with elevation of FA oxidation genes (25). In support of this observation this author found that myocardial FA oxidation was decreased in a cardiomyocyte-restricted PPAR β/δ knockout mouse model (24). This decrease in FAO was further associated with

increased myocardial triglyceride content, indicating that PPARδ is involved in regulating myocardial substrate utilization.

Anti-inflammatory properties of PPAR agonists

Early evidence that PPARα is involved in mediating anti-inflammatory responses came from a study by Devchand *et al*. (32), in which it was documented that LTB4, a chemotactic agent of inflammation was more efficiently cleared from plasma of PPARα over-expressing mice, when compared with PPARα null mice. The postulated mechanism was that LTB-4, being a PPARα ligand itself, will result in activation of PPARα and induce LTB-4 catabolism in a feedback manner (32). Subsequent studies have documented anti-inflammatory effects of PPARα via inhibition of various pro-inflammatory molecules, e.g. tissue factor (89; 105) and matrix metalloproteinase 9 (MMP9) by interfering with NF-κB pathway (126). Recently PPARα has been shown to inhibit a pro-inflammatory cytokine (osteopontin) involved in the development of atherosclerosis (102). It has also been documented that PPARα, γ and δ reduce the cytokine-induced endothelial expression of adhesion molecules, e.g. VCAM-1 (21; 68; 121) along with reduction in macrophage infiltration within atherosclerotic plaques.

AIMS OF STUDY

The general purpose of this thesis work was to examine the effects of chronic administration of PPARα agonists on myocardial metabolism and ventricular function in obese/type 2 diabetic mice. Two different agonists were used; *fenofibrate*, which is used clinically to prevent hyperlipidemia and *tetradecylthioacetic acid (TTA)*, a sulphur-containing FA with PPARα-activating potency. Both compounds were administered to the animals via the diet.

The specific aims were to find out:

(i) if fenofibrate and TTA were able to improve blood chemistry and reduce the predominant FA oxidation (and recover the capacity for glucose oxidation) in hearts from obese/diabetic mice

(ii) if changes in metabolism following treatment with the compounds were associated with altered gene expression, as evaluated by real-time PCR measurements of the mRNA expression of selected PPARα-regulated genes

(iii) if the two agonists had the potential to reduce myocardial oxygen consumption and improve functional recovery following ischemia-reperfusion

METHODOLOGICAL CONSIDERATIONS

Real-time quantitative PCR

Gene expression analysis is of prime importance in the field of biological research, and among the methods which are being used for this purpose real-time qPCR has gained a lot of popularity because of its rapidity and sensitivity as compared to the traditional method for quantitative measurement of gene expression such as Northern blotting. Real-time qPCR is employed for both absolute and relative quantification of gene expression. We have used a relative quantification method by measuring the changes in Ct values for the specific genes of interest. Ct is fractional cycle number at which the PCR product crosses threshold of detection, which happens in the exponential phase of the amplification plot (see below). In relative mRNA quantification data is normalized against a housekeeping gene. This technique is especially suitable when small amounts of tissue or cells are available (46). The theory behind the mode of action of PCR was outlined by Kerry Mullis in 1983 (97; 98), although some pioneering work was also done by Gobind Khorana in 1971, who described the basic principle of replicating DNA by using two primers. In the initial step complimentary DNA, synthesized from isolated mRNA by reverse transcription was channelled through different phases of real-time qPCR (denaturation, annealing and extension) along with primers and probes of the genes of interest, leading to an exponential increase in the number of copies of the gene of interest. The fluorescence signal in the exponential phase was captured by the qPCR detector and displayed by the software as an amplification plot. In gene expression analysis several parameters need to be controlled, such as the amount of starting material, sample handling, variation in the reverse transcription efficiencies and differences between tissues or cells in overall transcriptional activity (5). Various strategies have been employed to normalize these variations. For instance, in northern blot analysis RNA mass is a frequently used normalization scale, but it

is not always representative of the mRNA fraction, because it consists predominantly of rRNA. In addition, it has been documented that various biological factors and drugs could effect the transcription of rRNA (130). Normalization of the expression of the target genes is a reliable method (108). When performing relative quantification of the expression of a target gene it is important to choose a suitable gene for use as a reference or housekeeping gene. Ideally, housekeeping genes should not vary in response to the experimental intervention but, unfortunately, despite being constant in a given cell-type or experimental condition, housekeeping genes can still vary (141). Therefore, instead of using one reference gene, we have used the geometric mean of the three best out of a selection of 5-6 housekeeping genes, as recommended by Vandesompele *et al.* (141).

Isolated working mouse heart model

Hearts from small rodents are perfused *ex vivo* in mainly two different modes: (i) the Langendorff mode, in which the myocardium is perfused with a crystalloid buffer provided retrogradely to the aorta from a constant pressure head or a pump (constant flow) and (ii) the working heart mode, in which the left ventricle receives oxygenated buffer from a preload reservoir and ejects its content into an afterload column, which in turn creates the myocardial perfusion pressure (103). The Langendorff perfusion is named after Oskar Langendorff, who already in 1895 (80) described a method for measuring mechanical function of the isolated mammalian heart. In 1967 Neely developed the isolated working rat heart model (103) which is widely used for studying mechanical function and metabolic status of the rodent heart. Grupp *et al.* (54) for the first time examined functional parameters in an isolated, working mouse heart. These authors used mechanical resistance clamps to achieve a mean arterial pressure of 50 mmHg. Larsen *et al*. (81) developed the technique further, using a hydrostatic column to maintain similar or higher afterload

pressures, thereby achieving a stable working heart preparation. Functional and metabolic aspects of the working murine heart was also evaluated by de Windt *et al* (29). The major advantage with this model is that a number of experimental parameters like preload, afterload and heart rate, as well as the energy substrate supply, can be easily controlled (81). However it is important to remember that the *ex vivo* heart is devoid of any neurohumoral influence. In most studies the heart is perfused with a buffer containing only a few substrates (most often glucose ± FA). Despite these shortcomings, the isolated working mouse heart model has proven to be very useful, in particular because it allows simultaneous measurements of cardiac function and metabolism (103) in various transgenic models and mouse models of disease (90).

Measurement of myocardial substrate utilization

We measured rates of myocardial glucose and fatty acid oxidation by including trace amounts of [U-^{14}C] glucose and [9, 10-^{3}H] palmitate in the perfusion medium. $^{14}CO_2$ and $^{3}H_20$, which were released by the oxidation of these substrates were trapped and used for calculation of the oxidation rates (3; 12; 14). However we did not measure the contribution of other energy substrates like lactate and ketone bodies, which can contribute to energy metabolism during exercise and starvation/diabetes, respectively (109).

Measurement of myocardial oxygen consumption and cardiac work

The oxygen tension of freshly oxygenated perfusion buffer, as well as of the coronary drainage, was measured by fibre-optic oxygen probes placed in the preload line and pulmonary trunck, respectively. Myocardial oxygen consumption (MVO_2) was calculated as the product of this "arterio-venous" difference in oxygen content and the coronary flow.

Cardiac work was measured as pressure-volume area (PVA), using a miniaturized (1.2 and 1.4 French) pressure-volume catheter, a technology which was introduced in our laboratory by Dr. Ole-Jakob How (62-64). Steady-state PVA and MVO_2 values were obtained at different workloads by changing the pre- and afterload settings. Unloaded MVO_2 is the myocardial oxygen consumption at zero work, as determined by the y-intercept of the PVA:MVO_2 relationship, and represents oxygen used for basal metabolism and excitation – contraction coupling. Contractile efficiency is the inverse of the slope of the relationship (i.e. PVA-dependent increase in MVO_2 divided by the PVA) (135; 136)

PPAR ligands of interest

Tetradecylthioacetic acid: TTA is a non-beta oxidizable fatty acid analogue with substitution of sulfur atom at position 3 in the carbon chain (Figure 5) and that is the reason why TTA cannot be oxidized itself (16). However, TTA leads to an increase in mitochondrial β-oxidation of FA (40; 55), probably related to the finding that 3-thia fatty acids caused production of megaperoxisomes and micromitochondria (15; 78) and ultimately stimulate peroxisomal mitochondrial β-oxidation of fatty acids (15).

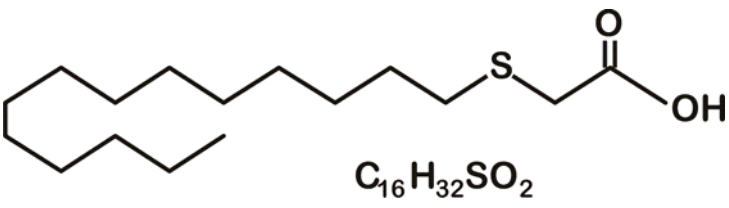


Fig 5: Chemical structure of Tetradecylthioacetic acid (TTA) showing substitution of sulfur atom at position 3 in the carbon chain.

Raspe has demonstrated that TTA can activate both PPAR α and PPAR γ in a concentration dependent manner (120). TTA feeding has caused proliferation of mitochondria not only in

the liver, (15; 78; 138) but also in skeletal muscle and heart (138). In NIH-3T3 cells from rodents it activates PPARα, PPARδ and PPARγ in a descending order (88). Short-term administration of TTA was associated with induction of hepatic CPT-I and CPT-II activities and FA oxidation (86; 87; 139). Thus, TTA by increasing the expression of genes involved in FA catabolism in liver resulted in decreased production of lipids from the liver and decreased plasma lipid levels (120). Besides its effects on lipid metabolism Muna *et al*. has documented antioxidant effects of TTA (99). Anti-inflammatory effects of TTA are also evident by its ability to attenuate TNFα-mediated endothelial cell activation (41).

Fenofibrate: This compound (Figure 6) is a derivative of fibric acid and has been used in the treatment of hyperlipidemia for more than two decades (94).

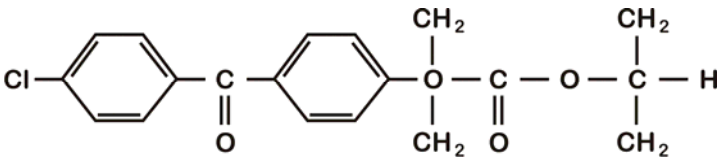


Fig. 6: Molecular structure of fenofibrate

Clofibrate and gemfibrozil became available in the United States in 1967 and1982 respectively. Fenofibrate, the third generation fibric acid derivative was synthesized in 1975 (129) and was introduced in clinical practice the same year (18). Today, fenofibrate is one of the most commonly used lipid-lowering drug worldwide with a wide range of effects on cholesterol and TG metabolism (10). The mechanism of action involves PPARα-mediated increase in hepatic FA oxidation, which consequently reduces the plasma concentration of

lipids (56). In studies on type 2 diabetic (*db/db*) mice, long term fenofibrate administration improved hyperglycemia, insulin resistance, albuminuria and glomerular lesions (112). Fenofibrate treatment resulted in significant increases of plasma adiponectin levels in normal mice, as well as in cultured 3T3-L1 adipocytes (61), indicating that adipose tissue might also be a target organ for fibrates.

K-111 (previously called BM 17.0744): K-111 (2,2-dicholoro-12-(p-chlorophenyl)-dodecanoic acid) (Figure 7) belongs to a group of ω-substituted alkyl carboxylic acid and is structurally not related to thiazolidinediones.

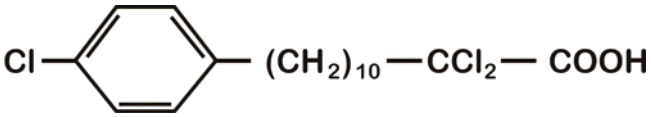


Fig.7: Molecular structure of K-111.

K-111 is a newly developed insulin sensitizer and its anti-hyperinsulinemic and lipid-lowering effects have been observed in rodents (115). It is a potent activator of PPARα with no activity against PPARγ (93; 145). A study comparing troglitazone with K-111 has revealed that the latter exerts a more potent hypoglycemic effect at lower doses (114). A similar hypoglycemic effect was associated with increased myocardial glucose oxidation along with a concomitant reduction in FA acid utilization was observed by our group in diabetic (db/db) mice treated with K-111 (1).

SUMMARY OF RESULTS

The peroxisome proliferator-activated receptors (PPARs) are a family of three ligand-activated transcription factors, PPARα, PPARβ, and PPARγ. The clinical importance of the pathways controlled by these receptors has been demonstrated by the use of synthetic ligands for the receptors, e.g. fibrates (PPARα agonists) is used as a lipid-lowering agent in hyperlipidemic patients, while thiazolidinediones (PPARγ agonists) have beneficial effects on insulin sensitivity, as well as the lipid profile, in type 2 diabetes. The potential clinical benefit of PPARs on improving myocardial metabolism in type 2 diabetes is, however, less known.

In **paper I** we examined the effects of long-term administration of the anti-lipidemic drug, fenofibrate, using diet-induced obese (DIO) mice. The plasma concentrations of fatty acids (FA), triacylglycerol (TG) and glucose was measured following four weeks treatment, as well as myocardial FA and glucose oxidation in isolated perfused hearts from these animals. In addition, we measured hepatic FA oxidation and biochemical activity and gene expression of metabolic enzymes in both liver and heart tissue. DIO mice, which were produced by feeding normal NMRI mice a sucrose-enriched diet, are considered a model of obesity or pre-diabetes. In line with this notion, the results showed that they have enlarged adipose tissue depots and exhibit increased plasma concentrations of lipids (FA and TG) and glucose, as well as increased myocardial FA oxidation and reduced glucose oxidation.

Eleven to twelve weeks treatment with fenofibrate (0.1 % via the diet, w/w), however, led to reductions in the adipose tissue mass and plasma concentrations of lipids and glucose. The reduced lipid supply to the heart was associated with reduced rates of myocardial FA oxidation (and increased glucose oxidation), while hepatic FA oxidation was increased.

These changes in FA oxidation were accompanied by a significant decrease and increase in the activity of CPT-II and ACO activity in heart and liver, respectively. Moreover, examination of PPARα-regulated target genes showed increased expression of PDK4, CPT-I, CPT-II, ACO and UCP2 in the liver, but no significant changes in cardiac tissue. Finally, hearts from DIO mice showed mechanical dysfunction, as indicated by lower baseline aortic flow and cardiac power than lean control hearts, and less functional recovery following ischemia-reperfusion. These defects were reversed by fenofibrate treatment.

In conclusion, chronic treatment of DIO mice with fenofibrate resulted in normalization of the fuel supply to the heart and a concomitant improvement of the myocardial fuel utilization. The improvement in cardiac metabolism was associated with improved cardiac function and increased tolerance to ischemic stress.

In **paper II** we examined the ability of fenofibrate to modify cardiac metabolism in diabetic (db/db) mice. Because the db/db mouse represents a rather severe degree of diabetes, we decided to use two different doses of the drug, a low dose of 0.1% and a high dose of 0.2%. Diabetic mice treated with K-111 (a PPARα agonist) served as positive controls, since previous studies from our own laboratory have demonstrated marked improvements in cardiac metabolism following treatment with this compound.

Low-dose fenofibrate treatment resulted in reduced plasma concentration of FA and TG, but the concentration of glucose was not affected. On the other hand, both high-dose fenofibrate and K-111 treatment produced a drop in the plasma concentration of both lipids and glucose. Liver tissue from mice treated with fenofibrate and K-111 showed increased PPARα target gene expression in comparison to non-treated db/db mice, supporting the

notion that the lipid-lowering effect of the compound was due to increased hepatic FA oxidation. As expected, the myocardial expression of PPARα target genes was up-regulated in hearts from db/db mice, but this response was prevented in hearts treated with fenofibrate and K-111. In line with previous results (1), treatment with K-111 caused a shift in myocardial metabolism in db/db hearts in favor of increased glucose oxidation. The same response was obtained after high-dose fenofibrate treatment, while myocardial substrate oxidation was unaffected by low-dose fenofibrate treatment. We did not observe any effect on the ventricular function following treatment, neither with (low or high-dose) fenofibrate nor K-111 (data not shown).

In **paper III** we examined the effect of tetradecylthioacetic acid (TTA, a sulfur-containing fatty acid which has been characterized as a pan PPAR agonist) on plasma concentrations of energy substrates and myocardial metabolism in normal Balb/c mice. Animals were treated with TTA (0.5% w/w added to the diet) for 8 days. Thereafter, heart and liver were excised and used for measurement of mRNA expression of PPARα target genes. Another group of animals was used to study the effect of TTA on myocardial oxidation of FA and glucose, as well as its effect on the mechanical performance of the heart. Samples for measurement of plasma concentrations of lipids and glucose were obtained from all experimental groups.

In line with previous results in rodents (59), the concentration of lipids was reduced in plasma from TTA-treated mice. At the same time, the expression of PPARα target genes (mRNA) was up-regulated both in liver and heart. TTA-treatment caused a 2.5-fold increase in myocardial FA oxidation (with a concomitant reduction in glucose oxidation), which matches the increased expression of PPARα target genes in the heart. It should be

noted, however, that the increase in myocardial FA oxidation occurred in the face of decreased levels of plasma lipids. Furthermore, there was no increase in myocardial FA oxidation in PPARα-null mice treated with TTA. Thus, it appears that the metabolic effects of TTA on the heart must be due to a direct stimulatory effect on cardiac PPARα, while the plasma lipid-lowering effect is most likely linked to activation of PPARα in the liver. TTA had no clear effect on the ventricular function. The TTA-induced increase in myocardial FA oxidation rate was associated with a near two-fold increase in unloaded MVO_2, as well as decreased recovery of ventricular function following low-flow ischemia.

We conclude that TTA, unlike other PPARα agonists, resulted in increased FA oxidation at the expense of glucose oxidation, increased expression of genes encoding FA metabolizing enzymes, increased myocardial oxygen consumption and reduced tolerance to ischemia-reperfusion. To our knowledge, TTA is the only compound having this effect on cardiac metabolism.

In the last paper (**paper IV**) we tested the effect of chronic TTA treatment in the diabetic db/db mouse, which is a well accepted model of type 2 diabetes. We (4; 64) and others (22; 23) have shown that hearts from db/db mice exhibit elevated rates of FA oxidation and reduced rates of glucose oxidation. Hearts from db/db mice also show increased unloaded MVO_2 and reduced tolerance to ischemia (58; 64). Based on the findings of a direct stimulatory effect of TTA on FA oxidation in normal mouse hearts (paper III), however, we wondered whether TTA treatment would increase myocardial FA oxidation further in db/db hearts. Such an effect could have a dual outcome; it could compromise cardiac function by further increasing unloaded MVO_2, but if TTA accelerates FA oxidation to a greater extent than FA uptake it could probably also correct the imbalance between fatty acid uptake and

oxidation and prevent myocardial lipotoxicity. Indeed, this assumption was supported by the fact that myocardial TG content was significantly reduced following TTA treatment.

Type 2 diabetic (db/db) and non-diabetic (db/+) mice were treated with TTA for 8 days, and the same set of experiments as described for paper III were performed. Again we observed significant reductions in circulating FA, TG and glucose in db/db mice and increased mRNA expression of enzymes involved in the FA oxidation pathway in the liver. Myocardial FA oxidation was significantly increased in hearts from TTA-treated db/db mice, although the relative increase was considerably less than in hearts from control mice (75% vs 300%), and was not reflected in a further increase in unloaded MVO_2. Surprisingly, TTA-mediated increase in myocardial fatty acid oxidation was not associated with increased expression of PPARα target genes in the diabetic mouse hearts.

The most intriguing finding, however, was that hearts from TTA-treated db/db mice, in contrast to hearts from non-diabetic mice, showed improved functional recovery following ischemia-reperfusion.

DISCUSSION

The cardiac metabolic phenotype of obese/diabetic mice is characterized by elevated FA oxidation and reduced glucose oxidation. In this thesis we have shown that chronic fenofibrate treatment of diet-induced obese (paper I) and type 2 diabetic mice (paper II) reduced plasma lipids and glucose and caused a switch in myocardial fuel selection towards higher glucose utilization at the expense of fatty acids. This change in myocardial metabolism was associated with improved ventricular function and improved tolerance to ischemia-reperfusion in DIO mice. Treatment of non-diabetic (paper III), as well as type 2 diabetic mice (paper IV), with TTA was associated with accelerated myocardial FA oxidation, in spite of a reduced plasma supply of lipids. TTA also caused increased oxygen consumption and reduced tolerance to ischemia in hearts from non-diabetic mice. To our surprise, however, TTA treatment seemed to be protective in hearts from type 2 diabetic mice.

Effect of fenofibrate on cardiac metabolism in obese mice and type 2 diabetic mice

In accordance with other studies (56; 70) , we found that fenofibrate treatment had a marked hypo-lipidemic effect and improved glucose homeostasis in diet induced obese (DIO) mice (paper I) and type 2 diabetic mice (paper II). Fenofibrate treatment also improved myocardial metabolism in these mice with increased oxidation of glucose at the expense of fatty acids.

At a first glance, the changes in cardiac metabolism in response to fenofibrate were surprising, since PPARα regulates the expression of genes controlling both FA uptake and oxidation in the heart (44; 134). Thus, it is well documented that isolated cardiomyocytes

incubated with PPARα ligands, including endogenous ligands such as FA, show increased expression of PPARα target genes and increased FA oxidation (51; 140). Also, transgenic mice with cardiac-specific over-expression of PPARα show increased expression of PPARα target genes and increased FA oxidation (45). In line with this view one might argue that administration of a synthetic PPARα agonist should increase cardiac fatty acid oxidation further. On the other hand, we have previously shown that chronic treatment of type 2 diabetic mice (1; 2) with the PPARα ligand K-111 (previously called BM-17.0744) counteracts the high rates of myocardial palmitate oxidation which is otherwise found in these animals, a finding which was confirmed in paper II. We suggest that *in vivo* treatment with PPARα ligands, by draining plasma lipids to the liver (increased hepatic β-oxidation, paper I), will relieve the lipid load on the heart and result in adaptational changes in myocardial metabolism that are also maintained in the *ex vivo* setting. The lipid-lowering effect of fenofibrate could also be related to increased hepatic catabolism of VLDL triglyceride (mediated by lipoprotein lipase) and inhibition of the hepatic synthesis and secretion of VLDL (8). In support of this view fenofibrate treatment was found to markedly increase PPARα target genes in the liver, while the cardiac expression of these genes were either unchanged (paper I) or decreased (paper II). This is in accordance with Cook *et al*. (27), showing minor changes in PPARα target gene expression in extrahepatic tissues following administration of different PPARα ligands. Based on these findings, we suggest that the effects of fenofibrate on the heart should be regarded as a secondary response to the lipid-lowering effect of the compound.

In paper II we found that the high dose of fenofibrate, but not the low dose, was able to shift the energy metabolism in hearts from db/db mice. The failure to improve myocardial metabolism following low-dose fenofibrate treatment was surprising, since the treatment

resulted in reduced plasma FA and TG concentrations, without affecting the plasma glucose concentration. This combination of a low lipid and high glucose supply should by itself favour increased glucose oxidation in the heart, in line with the concept that the plasma supply is determining the metabolic phenotype of the heart. Apparently, the lack of effect on cardiac metabolism of low-dose fenofibrate is related to the persisting elevated glucose concentration. The high plasma glucose concentration during low-dose fenofibrate treatment will contribute to a near normal myocardial glucose uptake in the db/db mice despite insulin resistance (133). However, the glucose taken up is not properly utilized, and accumulated intracardiomyocyte glucose may increase flux through the hexosamine biosynthetic pathway (with increased N-acetyl-glucosamine production) (48) and influence gene transcription and post-translational alterations of proteins. These mechanisms could probably override the influence of substrate supply and prevent repair of cardiac metabolism during low-dose fenofibrate treatment. With high-dose fenofibrate both plasma FA and glucose concentrations were reduced, and in this case we also obtained a switch in myocardial fuel selection.

Effect of fenofibrate treatment on cardiac function in obese and type 2 diabetic mice

Tabernero (137) found that chronic treatment of normal mice with fenofibrate reduced myocardial infarct size and improved post-ischemic contractile function in perfused hearts from these animals. In line with these results we found improved post-ischemic cardiac recovery in hearts from DIO mice (paper I). This response could possibly be a result of the fenofibrate-mediated metabolic switch in favor of glucose (and possibly reduced MVO_2), although PPARα mediated anti-inflammatory effects, including suppression of vascular inflammation and oxidative stress cannot be ruled out (30).

In hearts from DIO mice fenofibrate reversed the diet-induced ventricular dysfunction observed during normoxic/pre-ischemic conditions (paper I). However, we did not find any improvement in ventricular function in db/db mice (paper IV), despite hypo-lipidemic and hypoglycemic effects of the drug and normalization of myocardial metabolism (highest dose of fenofibrate). The db/db mouse represents a severe degree of diabetes, and irreversible structural changes (50) and a high oxidative stress (125) in this diabetic model might have prevented any functional benefit. In addition, the db/db mice were treated for only 4 weeks, compared to 10 weeks treatment of the DIO mice.

Effects of TTA treatment on myocardial metabolism in normal and diabetic mice

As mentioned above, the diabetic heart relies mainly on FA for energy production, and PPARs play a central role in the regulation of myocardial FA utilization. In accordance with previous studies, (88) we found that chronic TTA-treatment resulted in a significant reduction in the plasma concentration of glucose and lipids. The lipid-lowering effect was most likely secondary to PPARα-mediated hepatic uptake and oxidation of FA, as indicated a significant up-regulation of hepatic PPARα target genes. TTA-induced hepatic mitochondrial proliferation has also been suggested to play a central role in the regulation of plasma triglyceride concentration (15; 16; 139).

Based on our previous results obtained with fenofibrate (paper I) and K-111 (1; 2) , a plasma profile with low lipid values should favor low rates of myocardial fatty acid oxidation. Again we were surprised to find the opposite, namely a markedly increased myocardial FA oxidation plus up-regulation of cardiac PPARα target genes (paper III and

IV). One possible explanation for this phenomenon could be related to the uptake mechanism of TTA; during intestinal absorption TTA is probably incorporated in complex lipids during the reconversion of the products of lipid digestion, entering the lymph as very low density lipoproteins (VLDL) and chylomicrons. These particles are subsequently taken up in the blood and transported to the heart where the lipoprotein lipase at the coronary endothelium will provide for an efficient uptake of TTA into the myocardial cells, allowing direct PPARα-mediated effects on myocardial metabolism. In support of this view Asiedu *et al* (7) have reported higher concentrations of TTA in heart than in liver of TTA-treated Wistar rats. In addition, gene expression analysis showed significant up-regulation of PPARα target genes in hearts from TTA-treated Balb/c and *db/+* mice (paper III and IV). On the other hand, there was no up-regulation of these genes in hearts from TTA- treated *db/db* mice. This observation could be related to the fact that PPAR α target genes are already up-regulated in diabetic myocardium, due to the increased supply of plasma lipids (44). In addition, any direct activation of PPARα by TTA was probably counteracted by the reduced supply of FA (the endogenous ligands of PPARα) as a result of the lipid-lowering effect of TTA.

In this respect TTA was different from fenofibrate (and other PPARα agonists used in our group), for which the liver was the main target organ.

Effect of TTA treatment on cardiac function in diabetic mice and non-diabetic mice

Increased myocardial FA oxidation in hearts from TTA-treated normal Balb/c and db/+ mice (papers III and IV) was associated with increased myocardial oxygen consumption (unloaded MVO_2). In hearts from TTA-treated Balb/c mice (paper III) we also observed

reduced ischemic tolerance, while it was unchanged in db/+ hearts (paper IV). Hearts from TTA-treated db/db mice, on the other hand, showed increased post-ischemic functional recovery, despite increased FA oxidation. It is not difficult to understand that the strong increase in MVO_2 in normal hearts in response to TTA will compromise the mechanical function of the heart, particularly in the reperfusion phase. The diabetic hearts, however, are probably adapted to a high FA utilization, and so the relatively moderate increase in myocardial FA oxidation in hearts from db/db mice in response to TTA treatment did not cause any further increase in unloaded MVO_2. Probably, any TTA-mediated elevation of MVO_2 in these hearts was masked by the inherently high MVO_2 (64).

The improved post-ischemic function in hearts from TTA-treated mice could probably be explained by TTA-mediated prevention of lipotoxicity. In the diabetic heart FA uptake exceeds the rate of oxidation, leading to accumulation of (toxic) lipid metabolites (147). Apparently, the increase in myocardial FA oxidation plus the decreased lipid supply in TTA-treated mice prevented this process. Thus, we observed a marked reduction in myocardial triglyceride content in TTA-treated diabetic hearts as compared to the non-treated controls. Interestingly, Yu (149) has documented that PPARδ-mediated reduction in myocardial adiposity is vital for preventing diabetes-related myocardial dysfunction. In addition, TTA-mediated cardioprotection could be related to the reported antioxidant and anti-inflammatory effect of the compound (79); studies have shown that *in vivo* activation of PPARα is associated with induction of antioxidant enzymes such as catalase and superoxide dismutase (30; 66), which might be able to counteract the detrimental effects of oxidative stress during reperfusion. Finally, Wayman (142) reported that both PPARα and γ are involved in reducing myocardial infarct size via inactivation of the NF-κB pathway in ischemic heart.

CONCLUSION

This thesis has demonstrated that fenofibrate can improve myocardial metabolism (slow down FA oxidation and increase glucose oxidation) in hearts from obese and type 2 diabetic mice by altering the plasma substrate supply to the heart. In hearts from obese mice this metabolic improvement left the heart less vulnerable to ischemic stress. TTA treatment caused a marked increase in myocardial PPARα activity and FA oxidation. In non-diabetic hearts this increase in FA oxidation was associated with increased MVO_2 and decreased tolerance to ischemia. In diabetic hearts, on the other hand, TTA treatment was cardioprotective. Taken together, these results show that PPARα agonists can be useful tools for modifying cardiac metabolism, with potential improvements in cardiac function, in obesity and type 2 diabetes.

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APPENDIX

Primer and probe sequences used in real-time quantitative RT-PCR

Gene name	Primer/probe sequence (5'-3')	
PPARα target genes		
Muscle carnitine palmityol transferase I		
mcpt1	Forward	ATG-ATG-TAT-CGC-CGC-AAA-CT
	Reverse	ATC-TGG-TAG-GAG-CAC-ATGG-GT
	Probe	TCA-AGC-CGG-TAA-TGG-CAC-TGG-CAC-TGG-G
Liver carnitine palmityol transferase 1		
lcpt1	Forward	AAA-CCC-ACC-AGG-CTA-CAG-TG
	Reverse	GGC-ACT-GCT-TAG-GGA-TGT-GT
	Probe	# 2 in Roche Universal ProbeLibrary
PDH-kinase		
pdk4	Forward	TTC-ACA-CCT-TCA-CCA-CAT-GC
	Reverse	AAA-GGG-CGG-TTT-TCT-TGA-TG
	Probe	CGT-GGC-CCT-CAT-GGC-ATG-GCA-TTC-TTG
Uncoupling protein 3		
ucp3	Forward	TAC-CCA-ACC-TTG-GCT-AGA-CG
	Reverse	GCC-TGG-CAA-TCT-TTT-GCT-T
	Probe	# 69 in Roche Universal ProbeLibrary
Uncoupling protein 2		
ucp2	Forward	ACA-GCC-TTC-TGC-ACT-CCT-G
	Reverse	GGC-TGG-GAG-ACG-AAA-CAC-T
	Probe	# 2 in Roche Universal ProbeLibrary
Cytosolic thioesterase I		
cte1	Forward	ACC-CCC-TGT-GAC-TAT-CCT-GAG
	Reverse	TTC-TAC-CAG-AGG-GCT-TTG-CA
	Probe	AGC-ATC-TAC-AAC-ATC-CTT-GAG-GCC-ATC-CT
Mitochonsdrial thioesterase I		
mte1	Forward	TGG-GAA-CAC-CAT-CTC-CTA-CAA
	Reverse	CCA-CGA-CAT-CCA-AGA-GAC-CA
	Probe	AGA-CTA-TAC-CCC-CTG-TGT-CCC-TTG-TGA-GAA
Medium-chain acyl CoA dehydrogenase		
mcad	Forward	TGG-CAT-ATG-GGT-GTA-CAG-GG
	Reverse	CCA-AAT-ACT-TCT-TCT-TCT-GTT-GAT-CA
	Probe	AGG-CAT-TTG-CCC-CAA-AGA-ATT-TGC-TTC

Gene name		Primer/probe sequence (5'-3')
Housekeeping genes		
Glyceraldehyd-3-phosphate dehydrogenase		
gapdh	Forward	TCA-CCA-CCA-TGG-AGA-AGG-C
	Reverse	GCT-AAG-CAG-TTG-GTG-GTG-CA
	Probe	ATG-CCC-CCA-TGT-TTG-TGA-TGG-GTG-T
Cyclophilin		
cyclo	Forward	CTG-ATG-GCG-AGC-CCT-TG
	Reverse	TCT-GCT-GTC-TTT-GGA-ACT-TTG-TC
	Probe	CGC-GTC-TGC-TTC-GAG-CTG-TTT-GCA
Hypoxanthine-guanine phosphoribosyl transferase		
hprt	Forward	TCC-TCC-TCA-GAC-CGC-TTT-T
	Reverse	CCT-GGT-TCA-TCA-TCG-CTA-ATC
	Probe	# 95 in Roche Universal ProbeLibrary
Succinate dehydrogenase complex subunit A		
sdha	Forward	TGT-TCA-GTT-CCA-CCC-CAC-A
	Reverse	CAC-GAC-ACC-CTT-CTG-TGA-TG
	Probe	# 71 in Roche Universal ProbeLibrary
*A*cidic ribosomal phosphoprotein		
arbp	Forward	CGA-GAA-GAC-CTC-CTT-CTT-CCA
	Reverse	TTA-TCA-GCT-GCA-CAT-CAC-TCA-G
	Probe	# 105 Roche
Hydroxymethylbilane synthase		
hmbs	Forward	GCT-CAG-ATA-GCA-TGC-AAG-AGA-CTA
	Reverse	TCT-GGA-CCA-TCT-TCT-TGC-TGA
	Probe	# 49 in Roche Universal ProbeLibrary