# Paper II

# Improved Cardiac Metabolism Following in Vivo Treatment of Type 2 Diabetic Mice with Fenofibrate Depends on Reduction of Plasma Lipids, as Well as Glucose

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**Abstract**

The plasma supply of energy substrates plays a key role in determining the cardiac metabolic phenotype. In diabetes, a high plasma supply of fatty acids (FA) leads to a predominant oxidation of FA for energy production, while glucose oxidation is markedly suppressed. The *db/db* mouse is a well accepted model of type 2 diabetes, showing hyperglycemia, hyperlipidemia, and hyperinsulinemia. Hearts from these mice exhibit altered substrate metabolism, characterized by an over-reliance on FA for energy production and low contribution of glucose. In the present study we tested whether the capacity for glucose utilization could be recovered in isolated working hearts from *db/db* mice following long-term (4 weeks) treatment with fenofibrate, using two different doses of the compound (0.1% and 0.2%, given as admixture to the diet). Mice treated with K-111 (a PPARα agonist, previously known as BM 17.0744) served as positive controls. In line with previous results, treatment with K-111 resulted in a significant reduction of the plasma concentrations of FA, triacylglycerol (TG) and glucose. Low-dose (0.1 %) fenofibrate treatment resulted in reduced plasma concentration of FA and TG, whereas the concentration of glucose was unaffected. With high-dose (0.2 %) fenofibrate, however, significant reductions of both lipids and glucose were obtained. Hearts from K-111-treated *db/db* mice showed a 74% decrease in FA oxidation and a near 2-fold increase in glucose oxidation. Treatment with low-dose fenofibrate failed to improve cardiac metabolism, whereas high-dose fenofibrate caused a similar shift in cardiac metabolism as seen with K-111. The alterations in cardiac metabolism were associated with changes in the myocardial and hepatic expression of PPARα-regulated target genes. These results indicate that reduction of plasma lipids alone is not sufficient for improving cardiac metabolism in diabetes, and that reduction of plasma glucose is also required.

## Introduction

The plasma supply of energy substrates, mainly fatty acids (FA) and glucose plays a key role in determining the cardiac metabolic phenotype. In diabetes, a high supply of FA leads to a predominant oxidation of FA for energy production according to the Randle cycle, while glucose oxidation is markedly suppressed. Circulating FA are also endogenous activators of the nuclear peroxisome proliferator-activated receptors (PPARs), which are nuclear ligand-activated transcription factors regulating lipid metabolism and homeostasis (14). In the heart FA activation of PPARα up-regulates the expression of key proteins in the FA utilization pathway, including FA uptake, FA esterification to triacylglycerol (TG), as well as mitochondrial transport and β-oxidation of FA (12; 19), Thus, FA-induced PPARα activation also plays an important role, leading to the predominant use of FA for energy production in the diabetic heart (9; 14).

Synthetic PPAR agonists have become attractive tools in the treatment of hyperlipidemia occurring in diabetes and insulin resistance. The PPARα agonist fenofibrate is used to reduce cholesterol levels in patients at risk of cardiovascular disease, and it is also reported to reduce plasma lipids in diabetic/obese animals (3). K-111 (previously named BM 17.0744) is another synthetic PPARα agonist which has been shown to exert hypolipidemic as well as hypoglycemic action in diabetic animals (16). In the type 2 diabetic (*db/db*) mouse, chronic treatment with the K-111 compound led to normalization of plasma lipids (FA and triacylglycerol) and glucose (1). In addition, it caused a switch in the fuel selection of *ex vivo* working hearts from these animals towards a higher utilization of glucose at the expense of FA (1). More recently, we have reported that treatment of diet-induced obese (DIO) mice with 0.1% fenofibrate resulted in normalization of the fuel supply to the heart (i.e. reduced plasma triacylglycerol and glucose) and a concomitant improvement of the myocardial fuel utilization (3).

In the present study we examined the ability of fenofibrate to modify cardiac metabolism in diabetic (*db/db*) mice, which is a well accepted model of type 2 diabetes, showing hyperglycemia, hyperlipdemia, and hyperinsulinemia. In accordance with other researchers (5; 8), we have demonstrated repeatedly that hearts from these mice exhibit altered substrate metabolism, characterized by an over-reliance on FA for energy production and low contribution of glucose (1; 2), as well as development of ventricular dysfunction (2). Two different doses of the drug were used; a low dose of 0.1 % (similar to what we previously has used for DIO mice) and a high dose of 0.2%. The latter dose has previously been shown to

reduce the plasma concentration of glucose and insulin in *db/db* mice (15). Diabetic mice treated with K-111 served as positive controls.

## MATERIAL AND METHODS

**Animals**. C57BL/KsJ-leprdb/leprdb male diabetic (*db/db*) mice and their non-diabetic heterozygote littermates (*db/+*) were purchased from Harlan (Bicester, England). All mice were housed in a room maintained at 23 ºC and 55 % humidity with a 12-h light/dark cycle. The mice were given ad libitum access to food and water and treated in accordance to the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes. Diabetic mice were randomly divided in four groups and treated for 28 days with fenofibrate (which was given as admixture to the diet, 0.1% or 0.2%, w/w) or for 21 days with K-111 (24 mg/L, given via the drinking water). The animals were 8-9 weeks old at the start of the treatment period. Age-matched untreated diabetic and non-diabetic (*db/+*) mice were included as controls.

**Plasma parameters**. Plasma glucose, free fatty acid and triacylglycerol were determined in blood samples taken from the cavity of the animals at the day of sacrifice, using commercial kits from Boehringer Mannheim (Mannheim, Germany), Wako Chemicals (Neuss, Germany), ABX Diagnostics (Montpellier, France), and DRG Diagnostics (GmbH, Germany), respectively.

**Real-time quantitative RT-PCR.** Fresh tissue samples were immersed in RNAlater (Qiagen, Hilden, Germany) and stored at 4°C until RNA extraction. Total RNA was extracted according to the recommendations in the RNeasyFibrous Tissue protocol (Qiagen, Hilden, Germany). The RNA concentration was measured spectrophotometrically (NanoDrop, Witec, Switzerland), and stored at -80°C before use. cDNA was obtained from 1 μg total RNA according to the iScript cDNA Synthesis Kit (BioRad, Sundbyberg, Sweden). Real-time PCR (qPCR) was performed in an ABI PRISM 7900 HT Fast real-time thermal cycler using a 1:4 dilution of the cDNA and the TaqMan Fast Universal PCR master mix (Applied Biosystems, Foster City, CA). The primer/probe sequences for the genes studied (see appendix) were obtained from Eurogentec Ltd (Seraing, Belgium). Primers and TaqMan probes (2 μl of cDNA) were used in a 20 μl final volume. A negative control without cDNA template was included in every assay. The PCR efficiency for all genes was determined by performing a dilution series of a pool of all samples. Housekeeping genes were selected on the basis of the average expression stability values determined with geNorm Normalisation kit (20) out of a selected pool of candidate genes. In the heart the expression of target gene mRNAs were

normalised to the geometric mean of the three best housekeeping genes, whereas *cyclo* was used for quantifying hepatic mRNA content.

**Heart perfusion and measurements of ventricular function and cardiac metabolism**

Following injection of heparin (100 U, i.p.) and anesthetic (10 mg sodium pentobarbital, i.p.), hearts were excised and perfused in the working mode as previously described (2), using a modified Krebs-Henseleit-bicarbonate (KHB) buffer supplemented with 0.4 mM palmitate bound to 3% BSA and 5 mM glucose (the actual FA concentration was 0.7 mM due to the presence of endogenous FA in the BSA batch). All hearts were allowed to beat spontaneously. Glucose and palmitate oxidation were determined by simultaneous measurements of $^{14}CO_2$ and $^{3}H_2O$ released by the oxidation of [U-$^{14}$C]-glucose and [9,10-$^{3}$H]-palmitate, respectively (2; 5).

**Statistical analysis.** Data are expressed as mean ± SEM. Differences between treated and untreated *db/db* mice with respect to biometrics, blood chemistry and myocardial metabolism were analysed by Student's t-test for unpaired data. The corresponding data for the *db/+* mice included in the various experimental series were pooled and used for comparison. Values of cardiac and hepatic mRNA expression were expressed relative to values from untreated *db/db*, and differences between the groups were analysed by one-way ANOVA followed by Holm Sidak's test for multiple comparisons. The overall significance level was 0.05.

## RESULTS

**Biometric data.** Treatment of *db/db* mice with either low or high dose fenofibrate was associated with a slight additional increase in body weight, but this effect was not seen after treatment with K-111. (Table 1) There was no difference in heart weight, neither between diabetic and non-diabetic, nor between treated and non-treated diabetic mice. On the other hand, the liver was clearly enlarged in *db/db* mice, and both fenofibrate and K-111 treatment led to a further increase in the liver weight (Table 1), which is a well known effect of PPARα ligands in rodents (6).

**Plasma parameters.** The plasma concentrations of glucose, non-esterified FA, triacylglycerol and (TG) of untreated *db/db* were significantly increased, compared to the values obtained for lean control mice (Table 2). Chronic treatment of *db/db* mice with the PPARα ligand K-111 reduced plasma glucose to levels that were not different from those measured in non-diabetic (*db/+*) mice, and it also significantly reduced plasma FA and TG levels (Table 2). Treatment with both low- and high-dose fenofibrate significantly reduced the plasma concentrations of FA and TG. On the other hand, only the highest dose (0.2%) of fenofibrate was able to significantly reduce plasma glucose levels.

**Myocardial metabolism.** In accordance with previous results, we found that hearts from *db/db* mice expressed lower rates of glucose oxidation and higher rates of FA oxidation, compared to those from non-diabetic *db/+* mice (Fig. 1). Treatment of *db/db* mice with K-111 resulted in a significant (2.2 fold) increase in glucose oxidation, while at the same time FA oxidation declined by 33% (Fig. 1, lower panel). Treatment with low-dose fenofibrate did not lead to any change in myocardial glucose or FA oxidation, while high-dose fenofibrate, in the same way as K-111, resulted in an altered cardiac phenotype with improved capacity for glucose oxidation. (Fig. 1, upper and middle panels)

**Ventricular function.** In accordance with previous observations, ventricular function of the hearts from adult *db/db* mice was clearly impaired. Moreover, treatment with fenofibrate or K-111 had no significant effect on ventricular function in *db/db* mice (data not shown).

**Transcriptional changes in heart and liver.** Alterations in mRNA expression of PPARα-regulated genes were investigated both in heart and liver. Liver is the main target organ for PPARα, in line with this notion the results revealed more pronounced responses to administration of fenofibrate and K-111 in the liver (Table 3). The cardiac expression of *mcpt1* (carnitine palmitoyl transferease 1, controlling the FA transport into the mitochondria) and *mte1* (mitochondrial thioesterase 1, most likely regulating the concentration of long-chain fatty acyl CoA in the mitochondrial matrix) were increased in non-treated *db/db* hearts,

relative to non-treated *db/+*. Treatment of the *db/db* mice with K-111, as well as the high-dose fenofibrate, suppressed the cardiac expression of PPARα-regulated genes, while the effect was less marked following low-dose fenofibrate treatment.

In liver tissue of *db/db* mice, increased expression of *lcpt1*, *pdk4*, *mcad* and *ucp2* (relative to *db/+*) was indicative of increased fatty acid uptake and oxidation. The expression of *pdk4* and *ucp2* was markedly increased in response to administration of the PPARα agonists (especially K-111), whereas the mRNA expression of mcad and *lcpt1*was not influenced.

**Discussion**

In the present study we have documented a significant shift in fuel selection in favor of glucose in hearts from diabetic (*db/db*) mice treated with (high-dose) fenofibrate, which correlated with marked improvements of the plasma chemistry (reduced concentrations of lipids and glucose). Amelioration of the excessive myocardial fatty acid (FA) oxidation, which otherwise characterizes the diabetic heart, was accompanied by reduced cardiac expression of several PPARα-regulated genes involved in fatty acid oxidation.
Fenofibrate has been reported to increase hepatic FA uptake and oxidation (3; 10) and decrease the synthesis of VLDL triglyceride (4), which would explain the reduced plasma triglyceride level observed in the present study. There is also evidence that fenofibrate increases the catabolism of VLDL (10). The reduced plasma glucose concentrations, as well as the marked shift in cardiac fuel selection in favor of glucose in *db/db* mice treated with high-dose fenofibrate, are both indications of improved glucose homeostasis. This metabolic effect of fenofibrate could in part be explained by increased catabolism of FA in the liver, which will drain lipids from the plasma (3) and thereby relieve FA-mediated increase in FA oxidation in cardiac muscle (7; 9; 17). The fact that mRNA expression was reduced in heart and increased in liver in response to fenofibrate (as well as K-111) supports the notion that the liver is the main target (ligand-induced PPARα activation), while the effect on the heart is a secondary effect (reduced FA-induced PPARα activation)..

Interestingly, the present study demonstrated that treatment with both low- and high-dose fenofibrate reduced circulating lipids, but only the high dose resulted in a switch in cardiac substrate metabolism (reduced myocardial FA oxidation and a concomitant increase in glucose oxidation). Thus, reduced myocardial FA supply cannot be the sole explanation for the shift in myocardial metabolism in *db/db* mice following high-dose fenofibrate treatment and additional mechanisms must be involved.

One possible explanation may be related to the dose-dependency of fenofibrate with regard to improvement of insulin sensitivity. Fenofibrate has been reported to improve insulin resistance via alterations in the production of adipose tissue-derived cytokines (interleukin-6, TNFα and adiponectin) (13; 15). Probably, the low dose of fenofibrate was insufficient to produce the anti-inflammatory effects of the compound, and therefore also insufficient to produce the switch in cardiac substrate metabolism towards glucose. Another factor which may explain why the low-dose of fenofibate failed to provide the cardiometabolic effect in

*db/db* mice, is the elevated plasma concentration of glucose. The sustained high plasma glucose concentration may contribute to a near normal myocardial glucose uptake, despite the prevailing insulin resistance (18). However, since the glucose taken up is not properly utilized, accumulated intracardiomyocyte glucose may increase flux through the hexosamine biosynthetic pathway (with increased N-acetyl-glucosamine production), which again has been suggested to influence gene transcription and post-translational alterations of proteins (11). These mechanisms could probably override the influence of the low FA and TG supply and prevent repair of cardiac metabolism in the presence of low-dose fenofibrate. This finding is in contrast to a previous study from our group on diet-induced obese mice (3) where we found that a similar dose (0.1%) as the low-dose of fenofibrate used in the present study, clearly increased glucose oxidation and reduced FA oxidation following long term treatment (10 wk) with fenofibrate. Thus, pharmacological remodeling of diabetes-induced alterations in cardiac metabolism seems to depend not only on the pharmacological dose in question, but also on the severity of the diabetic state, and/or the duration of the treatment.

In conclusion the present study shows that chronic administration of fenofibrate, when given in sufficiently high doses, can improve myocardial metabolism by alleviating the excessive FA oxidation and recovering glucose oxidation in hearts from type 2 diabetic mice. This improvement in metabolism was not, however, associated with any improvement in post-ischemic myocardial function.

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**TABLES**

**Table 1:** Body, heart, liver, and adipose tissue weights in lean control (*db/+*) mice, untreated diabetic (*db/db*) mice, and *db/db* mice treated with fenofibrate (FF) or K-111.

| | n | **Body wt (mg)** | **Heart dry wt (mg)** | **Liver wt (g)** |
|---|---|---|---|---|
| *db/+* | 17 | 27.7 ± 0.3 | 26.0 ± 0.8 | 1.25 ± 0.04 |
| *db/db* | 16 | 43.9 ± 0.7 | 23.6± 0.5 | 2.13 ± 0.08 |
| *db/db* FF (0.1%) | 11 | 47.5 ± 1.2 * | 25.7 ± 1.0 | 4.13 ± 0.23 * |
| *db/db* | 10 | 44.8 ± 1.5 | 24.0 ± 0.5 | 2.14 ± 0.11 |
| *db/db* FF (0.2%) | 10 | 47.1 ± 0.7 * | 25.8 ± 0.7 | 4.18 ± 0.26 * |
| *db/db* | 13 | 44.6 ± 0.7 | 23.5± 0.5 | 2.29 ± 0.10 |
| *db/db* K-111 | 12 | 44.8 ± 1.1 | 25.5 ± 0.6 | 4.34 ± 0.18 * |

Body and organ weights were measured at the end of the treatment when the animals had reached an age of 12-13 weeks. n = number of animals in each group. Data from the lean control (*db/+*) mice were pooled and included for comparison. *, significantly different from untreated diabetic (*db/db*) mice.

**Table 2:** Plasma concentrations of glucose, fatty acids (FA) and triacylglycerol (TG) in lean control (*db/+*) mice, untreated diabetic (*db/db*) mice, and *db/db* mice treated with fenofibrate (FF) or K-111.

| | **Glucose (mmol/L)** | **FA (mmol/L)** | **TG (mmol/L)** |
|---|---|---|---|
| *db/+* | 15.5 ± 1.0 | 0.53 ± 0.03 | 0.67 ± 0.05 |
| *db/db* | 52.4 ± 3.6 | 1.06 ± 0.08 | 1.09 ± 0.13 |
| *db/db* FF (0.1%) | 41.2 ± 3.6 | 0.54 ± 0.07 * | 0.78 ± 0.07 * |
| *db/db* | 55.8 ± 2.5 | 0.73 ± 0.12 | 0.74 ± 0.09 |
| *db/db* FF (0.2%) | 25.6 ± 2.3 * | 0.32 ± 0.23 * | 0.54 ± 0.04 * |
| *db/db* | 43.5 ± 3.3 | 1.12 ± 0.13 | 0.84 ± 0.10 |
| *db/db* K-111 | 18.1 ± 1.4 * | 0.57 ± 0.06 * | 0.72 ± 0.11 |

Plasma samples from the animals included in Table 2 were taken at the day of sacrifice. Data from the lean control (*db/+*) mice were pooled and included for comparison. *, significantly different from untreated diabetic (*db/db*) mice.

**Table 3:** mRNA expression of PPARα-regulated genes in heart and liver tissue from lean control (*db/+*) mice, untreated diabetic (*db/db*) mice, and *db/db* mice treated with fenofibrate (FF) or K-111.

| **HEART** | ***pdk4*** | ***mcpt1*** | ***mcad*** | ***ucp3*** | ***mte1*** |
|---|---|---|---|---|---|
| *db/+* | 1.01±0.15 | 0.75±0.04* | 0.92±0.15 | 1.00±0.25 | 0.69±0.05* |
| *db/db* | 1.00±0.13 | 1.00±0.04 | 1.00±0.08 | 1.00±0.10 | 1.00±0.08 |
| *db/db* FF (0.1%) | 0.45±0.13* | 0.87±0.03* | 0.91±0.05 | 0.93±0.14 | 0.77±0.06* |
| *db/db* FF (0.2%) | 0.33±0.03* | 0.79±0.05* | 0.72±0.06* | 0.47±0.05* | 0.58±0.04* |
| *db/db* K-111 | 0.52±0.05* | 0.79±0.04* | 0.82±0.04* | 0.71±0.05 | 0.70±0.05* |

| **LIVER** | ***pdk4*** | ***lctp1*** | ***mcad*** | ***ucp2*** |
|---|---|---|---|---|
| *db/+* | 0.23±0.07* | 0.76±0.08* | 0.36±0.02 | 0.68±0.04* |
| *db/db* | 1.00±0.12 | 1.00±0.07 | 1.00±0.10 | 1.00±0.10 |
| *db/db* FF (0.1%) | 3.50±0.63* | 0.97±0.13 | 1.04±0.10 | 6.87±0.46* |
| *db/db* FF (0.2%) | 6.04±1.47* | 1.22±0.10 | 0.89±0.04 | 4.81±0.99* |
| *db/db* K-111 | 22.87±1.38* | 1.16±0.09 | 1.67±0.19 | 8.19±0.57* |

In the heart the genes were normalised to the geometric mean of the housekeeping genes *hprt, gapdh* and *hmbs*. In liver the genes were normalized to *cyclo*. The data are expressed relative to values from untreated *db/db*. Five animals were included in each group. *, significantly different from untreated diabetic (*db/db*) mice.

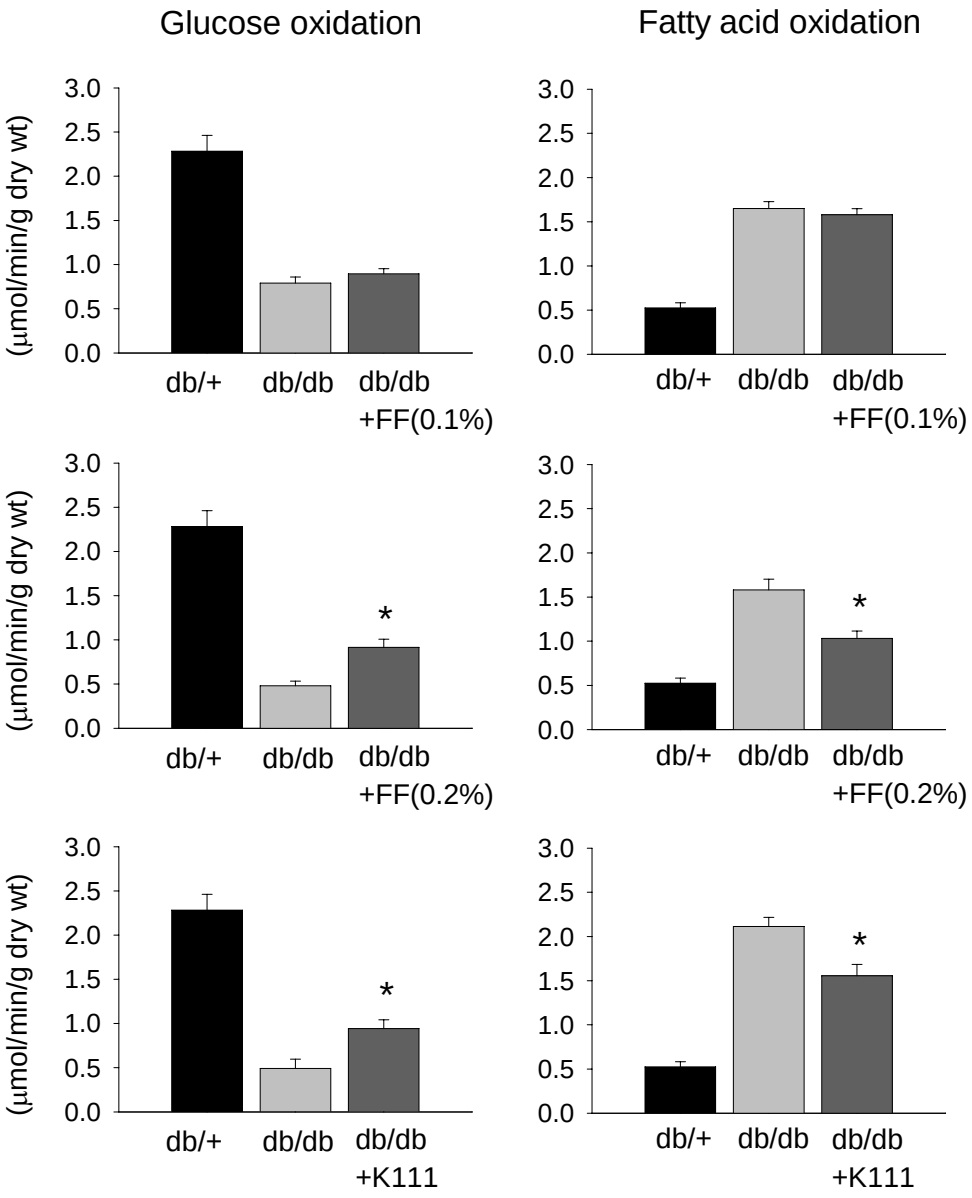


**Figure 1.** Rates of glucose and fatty acid oxidation in isolated perfused hearts from untreated *db/db* mice and from *db/db* mice treated with fenofibrate (FF) and K-111. The data from the lean control (*db/+*) mice were pooled and included for comparison. *, significantly different from untreated diabetic (*db/db*) mice. The number of hearts included in each group varied between 6 and 11.