Cardioprotective effect of the PPAR ligand
tetradecylthioacetic acid in type 2 diabetic mice

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Running title: Protection of the diabetic heart

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ABSTRACT
Tetradecylthioacetic acid (TTA) is a novel PPAR ligand with marked hypolipidemic and
insulin-sensitizing effects in obese models. TTA has recently been shown to attenuate
dyslipidemia in patients with type 2 diabetes, corroborating the potential for TTA in anti-
diabetic therapy. In a recent study on normal mice, we showed that TTA increased
myocardial FA oxidation, which was associated with decreased cardiac efficiency and
impaired post-ischemic functional recovery. The aim of the present study was, therefore,
to elucidate the effects of TTA-treatment (0.5%, 8 days) on cardiac metabolism and
function in a hyperlipidemic type 2 diabetic model.

We found that TTA treatment increased myocardial FA oxidation, not only in non-
diabetic (db/+) mice but also in diabetic (db/db) mice, despite a clear lipid-lowering
effect. While TTA had deleterious effects in hearts from non-diabetic mice (decreased
efficiency and impaired mitochondrial respiratory capacity), these effects were not
observed in db/db hearts. In db/db hearts, TTA improved ischemic tolerance, an effect
that is most likely related to TTA’s antioxidant property. The present study strongly
advocates the need for investigation of the cardiac effects of PPAR ligands used in anti-
diabetic/hypolipidemic therapy, because of their pleiotropic properties.

Key words: diabetic cardiomyopathy, cardiac metabolism, ischemia-reperfusion, cardiac
function, cardiac efficiency
INTRODUCTION

Heart disease is the leading cause of death in diabetic patients. These patients have greater incidence of acute myocardial infarction (AMI), as well as higher mortality following AMI (17, 36). In addition, asymptomatic diabetic patients are predisposed to heart failure, suggesting a specific cardiomyopathy (13). Although the etiology of diabetic cardiomyopathy is not clear, there is strong evidence for a causal link to altered myocardial metabolism (5, 12).

The *db/db* mouse is a model of type 2 diabetes with cardiomyopathic features, notably reduced mechanical function and reduced functional recovery following ischemia-reperfusion (3, 16, 19). In addition, measurements of myocardial substrate utilization show a higher reliance on fatty acid (FA) oxidation for energy production (3), accompanied by reduced cardiac efficiency (8, 22). A causal link between altered metabolism, reduced efficiency and tolerance to ischemia in diabetes is supported by the fact that improving myocardial substrate utilization (inhibition of FA oxidation and stimulation of glucose oxidation), both acutely and following lipid-lowering treatment, can improve cardiac efficiency and post-ischemic functional recovery in obese/type 2 diabetic models (4, 19, 23).

Tetradecylthioacetic acid (TTA) is a non-β-oxidizable FA analogue that increases peroxisomal proliferation and FA oxidation in the liver via PPAR activation (7). Accordingly, TTA treatment decreases plasma lipids (7), prevents feeding-induced obesity and improves insulin sensitivity in rodent models of hyperlipidemia (26). In a recent open-labelled 4-week clinical study by Løvås *et al.*, TTA attenuated dyslipidemia
in type 2 diabetic patients (25), corroborating the potential for TTA in anti-diabetic therapy (31).

In a recent study from our group, it was found that TTA treatment of normal BalbC mice increased myocardial FA oxidation, decreased cardiac efficiency and reduced the myocardial tolerance to ischemia-reperfusion (18). The metabolic effect of TTA is in contrast to previous studies showing that lipid-lowering in hyperlipidemic models in response to \textit{in vivo} treatment with PPAR agonists is associated with decreased myocardial FA oxidation (2, 4, 23). Hence, further information about the cardiac effects of TTA treatment in type 2 diabetes is needed.
MATERIAL AND METHODS

Animals. C57BL/KsJ-lepr\textsuperscript{db}/lepr\textsuperscript{db} male diabetic mice (db/db) and their non-diabetic heterozygote littermates (db/+)(12-15 weeks old) were purchased from Harland (Bicester, England). All mice were housed in a room maintained at 23 °C and 55% humidity with a 12-h light/dark cycle. The mice were given ad libitum access to food and water. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Norwegian National Animal Research Committee. Tetradecylthioacetic acid (TTA) was given as a 0.5% (w/w) dietary supplement for 8 days (18).

Plasma parameters. Plasma glucose, free fatty acids (FA) and triacylglycerols (TG) 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), and ABX Diagnostics (Montpellier, France), respectively.

Cardiac metabolism. The hearts were perfused in working mode using a modified KHB buffer supplemented with 0.4 mM palmitate bound to 3% BSA (with endogenous FA concentration of 0.3 mM, resulting in a final FA concentration of 0.7 mM) and 5 mM glucose. All hearts were allowed to beat spontaneously. Glucose and palmitate oxidation were calculated by simultaneously measuring \(^{14}\text{CO}_2\) and \(^3\text{H}_2\text{O}\) released by the oxidation of [U-\(^{14}\text{C}\)]-glucose and [9,10-\(^3\text{H}\)]-palmitate, respectively (3).
Post-ischemic functional recovery. Hearts were perfused in working mode, and intra-ventricular pressure was obtained by inserting a 2 F micromanometer-tipped catheter (Millar, SPR 407) into the left ventricle via the atrial cannula (1.3 mm inner diameter). After 30 min pre-ischemic perfusion, hearts were subjected to 40 min low-flow ischemia (3.1 ml/g dry wt/ min), followed by 5 min reperfusion in Langendorff mode and 30 min in working mode. Hearts that did not produce pressure exceeding that of the afterload column were perfused in an “assisted” mode (19). Post-ischemic recovery of ventricular function was measured after 35 min reperfusion relative to baseline (pre-ischemic) values.

Cardiac efficiency and ventricular function. Cardiac efficiency was determined in a separate series of perfused hearts, by assessment of the relationship between cardiac work (pressure-volume area, PVA) and myocardial oxygen consumption (MVO$_2$) (19, 21). PVA was measured by means of a micromanometer-conductance catheter (1.4 F, Millar Instruments, Houston, TX) which was inserted through the apex into the left ventricle, and oxygen partial pressure was recorded by fibre-optic oxygen probes (FOXY-AL300, Ocean Optics ltd., Duiven, Netherlands), placed prior to the left atrial cannula and in the pulmonary trunk. Electrodes were connected to the right atrium for electrical pacing, and hearts were exposed to different workloads by changing preload and afterload. Steady-state values of PVA and MVO$_2$ were calculated at each workload, and regression analysis of the PVA:MVO$_2$ relationship allowed determination of unloaded MVO$_2$ (y-intercept) and contractile efficiency (inverse slope). MVO$_2$ was also measured in potassium-arrested hearts which were perfused in an unloaded retrograde mode in order to determine the oxygen cost of basal metabolism (MVO$_2$$_{BM}$), as described previously (8).
Transcriptional changes. Fresh samples from unperfused hearts were immersed in RNAlater (Qiagen, Hilden, Germany), and total RNA was extracted according to the RNeasy Fibrous Tissue Protocol kit (Qiagen Nordic, Norway). Real-time PCR (qPCR) was performed in an ABI PRISM 7900 HT Fast real-time thermal cycler (18). Primer/probe sequences for housekeeping genes, transcription regulators and PPAR\(\alpha\) target genes are given in Haftsad et al. (18). Primer sequences for antioxidant enzymes was as follows: catalase, Cat: forward: 5’-AGGAGGCGAAACTTTCCCAT-3’ and reverse: 5’-TCCGCTCTCTGTCAAAGTGTG-3’; superoxide dismutase, Sod: forward: 5’-CACCATTTTCTGGACAAACCT-G3’ and reverse: 5’-

TTAAACTTCTCAAAGACCCAAAAGTC-3’; glutathione peroxidase, Gshpx: forward: 5’-GGCTTCCCTTCCCAACCA-3’ and reverse: 5’-CCC-ACC-TGG-TCG-AAC-ATAC-CT-3’; and for the fatty acid transporters: CD36 antigen (also referred to as fatty acid translocase) Cd36: forward: 5’-ATGGGCTGTGATCGGAACTG-3’, and reverse: 5’-

GTCTTCCAATAAAGCATGTCTCC-3’; fatty acid transport protein, Fatp: forward: 5’-

TCCCTCCTACACTGCATCAA-3’ and reverse: 5’- ATGCTTCCAAGCAAATCTCC-3’; plasma membrane fatty acid binding protein, forward, Fabp: 5’-

ACCTGGAAGCTAGTGGACAG-3’ and reverse: 5’-

TGATGGTAGTAGGCTTGGTCAT-3’. Housekeeping genes were selected on the basis of the average expression stability values, determined with geNorm Normalisation kit (38) out of a selected pool of candidate genes.

Lipid content and lipid peroxidation in the heart. Lipids were extracted from samples of ventricular tissue from unperfused hearts by the method of Folch, dried and thereafter dissolved in a tert-butyl and Triton X-100/methyl alcohol mixture. The triglyceride (TG)
concentration of the extracts was measured with a Triglyceride 25 kit from ABX Diagnostics (Montpellier, France). Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) content, indicators of lipid peroxidation, was measured in ventricular tissue using a TBARS Assay kit (Cayman Chemical Company, MI, US), and a 4-HNE ELISA kit (Cell Biolabs, Inc. CA, US), respectively.

**Western blot analysis and protein activity measurements.** SDS-PAGE, 15%

polyacrylamide gels were loaded with 10 mg of protein and the proteins were electroblotted to nitrocellulose membranes. Membranes were probed overnight with anti-MnSOD (BD Transduction Laboratories) at 4°C and subsequently for 1 hour with HRP conjugated anti-mouse IgG (GE healthcare). Immunopositive bands were developed with Immobilon chemoluminescent reagent (Millipore, MA, USA), and detected with a Kodak Image Station 1000 (Kodak, Rochester, NY, USA). Ponceau S staining (Sigma, St. Louis, USA) confirmed equal loading. Total superoxide dismutase activity was measured using a Superoxide Dismutase Assay Kit (Cayman Chemical, Michigan, USA).

**Mitochondrial respiration and citrate synthase activity.** Mitochondrial respiration was measured in saponin-permeabilised cardiac fibres by high-resolution respirometry, using a two-chambered oxygraph (Oxygraph-2k, Oroboros Instruments GmbH, Innsbruck, Austria). Hearts were excised and rinsed in ice-cold relaxing solution (BIOPS, Oroboros, Innsbruck, Austria). A piece of myocardial tissue (~ 15 mg) was cut out from the endocardial free wall of the left ventricle with fine scissors and transferred to a Petri dish containing ice-cold BIOPS. Individual fibre bundles were separated with two pairs of sharp forceps, and pieces of bundles weighing 2-4 mg were permeabilised in BIOPS containing 50 µg/mL saponin (30 min at 4 °C), washed three times for 10 min in
respiration medium (MiR05, Oroboros Innsbruck, Austria) and blotted on filter paper. Individual bundles were weighed and transferred into each chamber containing air-saturated respiration medium at 37 °C, so that all measurements were made in duplicate. Respiration was assayed following addition of two different substrate mixtures: malate (2 mM) and pyruvate (10 mM) or malate (2 mM) and palmitoyl-carnitine (25 μM). Respiration was measured after addition of 2.5 mM ADP (V_{ADP}) and after addition of 1 μg/ml FoF1 ATPase inhibitor oligomycin (V_{Oligo}). O2 flux was calculated from the negative time derivative of the oxygen concentration signal, using DatLab 4 software from Oroboros Instruments GmbH (15). A sample of ventricular tissue from each heart (7-10 mg) was homogenized in phosphate buffer and the activity of citrate synthase, a mitochondrial enzyme, was measured spectrophotometrically according to the method of Srere (34).

**Statistical analysis.** Data are expressed as mean ± SE. Differences were determined by a two-way ANOVA followed by Holm-Sidak’s test or Fisher method. Student’s t-test were used when appropriate. The overall significance level was 0.05.
RESULTS

Biometric data. Diabetic db/db mice exhibited the typical characteristics of a severe diabetic phenotype, including marked obesity, hyperglycemia, and dyslipidemia (Table 1). Eight days treatment with TTA markedly reduced body weight in db/+ mice, but not in db/db mice. TTA significantly reduced plasma concentrations of FA, TG and glucose in both db/+ and db/db mice. The hypolipidemic effect of TTA is partly due to hepatic PPARα activation; in accordance with this we found a marked increase in hepatic pdk4 expression in both db/db and db/+ mice (3.9±0.8 and 10.5±2.4 fold, respectively). There were no changes in liver or heart weight following TTA treatment.

Myocardial substrate utilization and expression of metabolic genes. Hearts from untreated db/db mice exhibited reduced rates of glucose oxidation and increased rates of FA oxidation compared to db/+ hearts (Figure 1A). The increased FA oxidation was associated with a significant up-regulation of the mRNA content (untreated db/db versus control db/+) of several PPARα target genes: muscle carnitine palmitoyl transferase (Mctpl), PDH-kinase4 (Pdk4), uncoupling protein 3 (Ucp3), mitochondrial and cytosolic thioesterase I (Mte1 and Cte1), as well as FA transporters, fatty acid translocase (Cd36) and fatty acid transport protein (Fabp) (Table 2). Diabetic hearts also showed significant up-regulation of peroxisome-proliferator-activated receptor (PPAR) γ co-activator-α (Pgc1α) and Pparδ, while there was a small but significant down-regulation of Ppara (Table 2).

TTA treatment resulted in a 1.5 and 1.1 μmol/min/g dry wt increase in myocardial FA oxidation in db/+ and db/db hearts, respectively, and a concomitant decrease in glucose
oxidation (Figure 1A). In hearts from db/+ mice, the TTA-induced increase in FA oxidation was accompanied by an increased PPARα target gene expression (Mcpt1, Pdk4, Ucp3, Cte1 and Mte1; Table 2). In db/db hearts, TTA produced less marked changes in PPARα target genes; both increased, decreased and unchanged gene levels were observed (Table 2).

In accordance with previous findings in BalbC mice (unpublished), TTA treatment reduced the myocardial expression of Pgc1α, Ppara and Pparδ in both db/+ and db/db hearts (Table 2), which may suggest that persistent PPARα activation will down-regulate receptor and co-activator expression through negative feedback.

**Citrate synthase activity and mitochondrial respiration.** Diabetes has been associated with increased cardiac mitochondrial biogenesis, which is supported in the present study by a small increase in citrate synthase (CS) activity (145±4 vs. 123±3 U/mg wwt, in db/db and db/+, respectively, p<0.002,) and in pgc1α expression (Table 2) in db/db hearts. Although the significance of these changes is unclear, CS activity was used for normalization of mitochondrial respiration. Maximum ADP-stimulated respiration rates (V_{ADP}) were measured in saponin-permeabilised cardiac fibres from unperfused hearts, using pyruvate/malate or palmitoyl-carnitine/malate as substrates (Figure 1B). Cardiac fibers from db/db hearts showed significantly decreased V_{ADP} when measured with pyruvate, but not with palmitoyl-carnitine. The latter observation is in accordance with Boudina et al.(2007), who showed unchanged V_{ADP} in fibres isolated from palmitate and glucose-perfused db/db hearts (10). In contrast to their study, however, we did not observe any difference in the respiratory state between db/+ and db/db fibres in the presence of oligomycin (V_{oligo}) (data not shown).
TTA treatment did not alter citrate synthase activity in \(db/+\) (127±7 U/mg wwt) and
\(db/db\) (139±6 U/mg wwt) hearts. In myocardial fibers from TTA treated \(db/+\) mice, we
found reduced \(V_{\text{ADP}}\) both when using pyruvate and palmitoyl-carnitine as substrates
(Figure 1B). In cardiac fibers from TTA-treated \(db/db\) mice, however, there were no
changes in \(V_{\text{ADP}}\). TTA treatment did not change \(V_{\text{oligo}}\) in \(db/+\) or \(db/db\) fibers (data not
shown).

MVO\(_2\) and cardiac efficiency. Cardiac efficiency was examined by regression analysis
of the PVA-MVO\(_2\) relationship. The major advantage of this technique is that it allows
myocardial oxygen cost to be separated in two independent components: unloaded MVO\(_2\)
(the y-intercept) and contractile efficiency (the inverse slope). In accordance with
previous reports from our laboratory (19, 22), hearts from \(db/db\) mice showed decreased
cardiac efficiency due to an approximate 40% increase in unloaded MVO\(_2\) (Table 3). In
\(db/+\) mice TTA treatment resulted in a 1.5 fold increase in unloaded MVO\(_2\), while there
was no change in MVO\(_2\) following TTA treatment of \(db/db\) mice (Table 3). Contractile
efficiency was not altered by TTA, neither in \(db/+\) nor in \(db/db\) hearts. Measurements of
MVO\(_2\) in \(K^+\)-arrested retrogradely perfused hearts showed that the oxygen cost for basal
metabolism (MVO\(_2\)\(_{\text{BM}}\)) was increased following TTA treatment of \(db/+\) mice (0.78±0.10
vs. 0.27±0.09 J/min/g wet wt, \(p<0.001\), \(n=8\)), but not following treatment of \(db/db\) mice
(0.65±0.12 vs. 0.48±0.18 J/min/g wet wt, \(n=10\)).

Ventricular function and tolerance to ischemia-reperfusion. Functional parameters
assessed by pressure-volume (P-V) analysis showed impairment of both systolic and
diastolic ventricular function in hearts from \(db/db\) mice (Table 4), as indicated by
significant reductions in left ventricular end systolic and developed pressure, cardiac
output, stroke volume, $dP/dt_{\text{max}}$ and $dP/dt_{\text{min}}$, as well as an increase in the relaxation factor tau. TTA caused no change in the mechanical function of hearts from $db/+ \text{ or } db/db$ mice.

Ventricular function of hearts exposed to low-flow ischemia and reperfusion was evaluated by measurements of ventricular pressure (using a micromanometer-tipped catheter) and cardiac output. Ventricular dysfunction (reduced cardiac output and cardiac power) was observed in $db/db$ hearts during the pre-ischemic period, and was not influenced by TTA treatment (data not shown). In accordance with previous studies (3, 19), hearts from $db/db$ mice showed reduced tolerance to ischemia-reperfusion, as indicated by a reduced functional recovery (Figure 2). However, in contrast to what we have previously shown in non-diabetic mice (18), TTA treatment significantly improved post-ischemic functional recovery in $db/db$ hearts (Figure 2).

**Myocardial TG, lipid peroxidation and antioxidant enzymes.** Lipid accumulation and increased generation of reactive oxygen species (ROS) have been suggested to result in lipotoxicity and oxidative stress in the diabetic heart (10). We therefore explored the potential of TTA to reduce lipid accumulation and oxidative stress in $db/db$ hearts. Hearts from $db/db$ mice showed increased TG content with markedly increased levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-NHE) (Figure 3). Increased oxidative stress in $db/db$ hearts was supported by increased protein level and activity of SOD, as well as increased mRNA expression of catalase ($\text{Cat}$) and glutathione peroxidase ($\text{Gshpx}$) (Figure 4). TTA treatment decreased lipid peroxidation, as indicated by a reduction of MDA content in $db/db$ hearts, although 4-HNE did not show a similar reduction (Figure 3). TTA has been shown to exhibit antioxidant properties (7) by scavenging superoxide...
anions (6, 28), and in accordance with this notion TTA treatment reduced superoxide
dismutase (SOD) expression with a borderline reduction in SOD activity (p=0.67)
(Figure 4). TTA also decreased mRNA expression of glutathione peroxidase in db/db
hearts (Figure 4). The content of MDA or 4-HNE was not altered in db/+ hearts (data not
shown) in response to TTA treatment, despite a 25% reduction in myocardial TG content
(6.0±0.2 µmol/g vs. 4.6±0.2 µmol/g (n=8), p<0.001).
DISCUSSION

In the present study, we found that treatment with the PPAR agonist tetradecylthioacetic acid (TTA) increased fatty acid (FA) oxidation in hearts from both non-diabetic and type 2 diabetic mice. The TTA-induced increase in FA oxidation in non-diabetic hearts was accompanied by reduced cardiac efficiency and mitochondrial capacity. This was not the case for diabetic hearts; in contrast to the detrimental effect of TTA on ischemic tolerance in non-diabetic hearts (18), TTA improved functional recovery following ischemia-reperfusion and reduced oxidative stress in diabetic hearts.

The uptake and oxidation of FA in the heart is increased by elevated circulating FA concentration, due to Randle’s glucose-fatty acid cycle (32), as well as transcriptional changes caused by FA-induced PPARα activation (14). The increased myocardial FA uptake and oxidation in type 2 diabetes/obesity has therefore been regarded as the consequence of both of these processes. This is also supported in the present study where increased FA oxidation in \(db/db\) hearts was associated with increased expression of several PPARα target genes including genes encoding proteins important for FA uptake and oxidation. The accompanying increase in protein-mediated FA uptake and FA oxidation has been suggested to play a central role in the development of ventricular dysfunction due to cardiac steatosis and lipotoxicity (9, 20, 41). Another hallmark of diabetic hearts (also demonstrated in the present study) is decreased cardiac efficiency (22, 27), due to increased unloaded myocardial oxygen consumption (MVO₂) (8, 22). As a consequence of increased MVO₂, diabetic hearts show increased susceptibility to ischemia-reperfusion (3, 16, 19).
TTA is a sulphur-containing FA analogue which has been regarded as a pan-PPAR ligand, with the following rank order of activation: PPARalpha > PPARdelta > PPARgamma (24). TTA has hypolipidemic effects (7, 25, 26), which has been ascribed to PPAR-induced increase in hepatic β-oxidation, as well as actions on very-low-density-lipoprotein and TG metabolism (7). In accordance with this, we have found TTA treatment to reduce plasma lipids both in non-diabetic (18) and diabetic mice. We have in previous studies with db/db or diet-induced obese mice shown that the PPARα agonists fenofibrate and K-111 resulted in a reduction of the myocardial FA oxidation rate (2, 4), a finding attributed to their lipid-lowering effect. Although, the lack of comparison with other PPAR ligands and the exclusive use of db/db mice in this study as a single model of obesity and type 2 diabetes must be acknowledged as a limitation, it is obvious that the effect of TTA on the heart differs from the mentioned PPAR ligands in that the lipid-lowering effect of TTA is not only due to increased uptake and oxidation in the liver, but also in the heart. Taken together, these findings suggest that TTA exerts a direct transcriptional effect on the heart which overrides any effects related to the reduced lipid supply. Although the mechanism responsible for the increased FA oxidation rate was not investigated in the present study, the above notion is further supported by the finding that TTA-induced cardiometabolic effects are absent in PPARα KO mice (18). It should be noted that the increase in cardiac FA oxidation in TTA-treated db/db hearts was accompanied by no consistent change in PPARα target genes, showing that caution must be taken when mRNA expression is used as a substitute for direct measurements of metabolic rates (substrate flux). A limitation in the present study, however, is that the flux was measured *ex vivo* under fixed FA concentrations.
TTA treatment did not alter citrate synthase activity in hearts from non-diabetic or diabetic mice, suggesting that 8 days treatment did not induce cardiac mitochondrial proliferation. In non-diabetic $db/+$ mice, TTA treatment resulted in reduced maximum mitochondrial respiratory capacity, suggesting impaired mitochondrial function. In accordance with Hafstad et al., TTA also decreased cardiac efficiency in non-diabetic $db/+$ mice as a consequence of increased unloaded MVO$_2$ (18), which in turn was due to increased oxygen cost for basal metabolism. This increase in MVO$_2$ can only partly be explained by the fact that FA is a more O$_2$ consuming substrate, since the change in P/O ratio induced by a shift from 100% glucose to FA utilization only results in a 12% increase in MVO$_2$. Thus, it is clear that TTA treatment of non-diabetic hearts must induce additional oxygen consuming processes, e.g. various futile cycles (30) and/or mitochondrial uncoupling (33, 35). In contrast to its effect on normal hearts, TTA treatment did not alter mitochondrial respiratory capacity, cardiac efficiency or MVO$_2$ in hearts from $db/db$ mice.

Previous studies have demonstrated that acute and chronic administration of PPAR agonists limit myocardial ischemia-reperfusion injury in non-diabetic (37, 40), as well as obese/type 2 diabetic models (4, 11, 23). As studies from our laboratory have shown that improved post-ischemic functional recovery following PPAR treatment of obese/diabetic models is associated with improved myocardial substrate utilization (inhibition of FA oxidation and stimulation of glucose oxidation) (4, 23) and cardiac efficiency (23), we were surprised to find that hearts from TTA-treated $db/db$ mice showed improved
recovery of ventricular function after ischemia-reperfusion, despite elevated FA oxidation rates and unaltered cardiac efficiency. This shows that improved cardiac metabolism and efficiency are not the only predictors for increased ischemic tolerance in $db/db$ hearts and that TTA must provide additional cardioprotective effects. TTA was found to decrease myocardial TG content which may have provided cardioprotection as increased lipid accumulation and lipotoxicity is believed to be a contributing factor in the development of diabetic cardiomyopathy (1, 5, 41). TTA, however, is also a powerful anti-oxidant and has been shown to scavenge superoxide anions \textit{in vitro} (due to its sulfur ion which is a reducing agent) (6, 28). In line with this, TTA has been shown to decrease lipoprotein peroxidation in rats (29), and to improve the antioxidant status in obese Zucker ($fa/fa$) rats (39). The present study suggests that TTA also decreased oxidative stress in the diabetic heart, although an explanation for why TTA treatment reduced MDA in $db/db$ hearts but not 4-HNE is not apparent. The fact that TTA is a scavenger of superoxide anions (6, 28) was supported by the reduction in SOD expression and SOD activity. Thus, although measurement of oxidative stress markers after ischemia-reperfusion would have strengthened the study, we suggest that TTA provided cardiac protection during reperfusion in $db/db$ hearts due to reduced oxidative stress.

However, an explanation of the opposing cardioprotective effects of TTA in a diabetic and in a non-diabetic heart (18) is most likely multifactorial and complex; in non-diabetic hearts where oxidative stress is low, any potential cardioprotection (due to TTA’s antioxidant properties) will be overridden by its detrimental effect on cardiac efficiency, leading to reduced tolerance to ischemia-reperfusion (18). In $db/db$ hearts, however,
where TTA does not alter cardiac efficiency, it may, by a direct scavenging of superoxide anions, protect the hearts during ischemia-reperfusion.

In conclusion, TTA treatment of non-diabetic mice has deleterious effects on the heart in terms of reduced cardiac efficiency, impaired mitochondrial respiratory capacity and reduced functional recovery following ischemia-reperfusion (18). These effects of TTA were blunted or absent in diabetic hearts. Of particular importance was the finding that TTA offered cardioprotection in these hearts, most likely via reduced oxidative stress. Although this cardioprotective mechanism was not fully explored, the present study strongly advocates the need for investigating the cardiac effects of PPAR ligands used in antidiabetic/hypolipidemic therapy due to their pleiotropic effects.

Acknowledgements

The expert technical assistance of Knut Steinnes, Thomas Andreasen and Elisabeth Boerde is gratefully acknowledged.

Grants

This work was supported by operating grants from the Norwegian Diabetes Association, the Novo Nordisk Foundation, the Northern Norway Regional Health Authority (Helse Nord RHF) and the Norwegian Heart Foundation.

Disclosures
Thia Medica AS holds patents regarding effects of TTA. Rolf K Berge, University of Bergen and Haukeland University Hospital are shareholders of Thia Medica AS.


14. **Finck BN.** The PPAR regulatory system in cardiac physiology and disease. 


22. **How OJ, Aasum E, Severson DL, Chan WY, Essop MF and Larsen TS.**


24. **Liu HR, Tao L, Gao E, Lopez BL, Christopher TA, Willette RN, Ohlstein EH, Yue TL and Ma XL.**

   Tetradecylthioacetic acid attenuates dyslipidaemia in male patients with type 2 diabetes mellitus, possibly by dual PPAR-alpha/delta activation and increased mitochondrial fatty acid oxidation. *Diabetes Obes Metab* 11: 304-314, 2009.


FIGURE LEGENDS

Figure 1. Panel A. Myocardial fatty acid and glucose oxidation in hearts of db/+ (white bars) and db/db (gray bars) mice. Results are mean of 8-9 hearts in each group. Panel B. Mitochondrial respiratory maximal capacity measured in skinned cardiac fibres from db/+ and db/db mice. Results are obtained from 8-12 hearts in each group. Hatching indicates TTA-treatment. *, p<0.05 vs. untreated within the same group; #, p<0.05 vs. untreated db/+.

Figure 2. Post-ischemic functional recovery of cardiac output (CO), left ventricular developed pressure (LVDP) and cardiac power (CP) in percent of pre-ischemic function in isolated perfused working hearts from db/+ (white bars) and db/db (gray bars) and and TTA-treated db/db (gray hatched bars) mice. Hatching indicates TTA-treatment. Results are mean of 5-8 hearts in each group. *, p<0.05 vs. untreated db/db; #, p<0.05 vs. untreated db/+.

Figure 3. Triacylglycerols (TG), malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) content in hearts from db/+ (white bars), db/db (gray bars) and TTA-treated db/db (gray hatched bars) mice. Results are mean of 8 hearts in each group. *, p<0.05 vs. untreated db/db; #, p<0.05 vs. untreated db/+.

Figure 4. Superoxide dismutase mRNA (MnSOD) and protein (MnSOD) expression, as well as, activity (panel A), and mRNA expression of catalase (Cat) and glutathione peroxidise (Gshpx) (panel B) in heart tissue from db/+ (white bars), db/db (gray bars) and
TTA-treated db/db (gray hatched bars) mice. mRNA levels were expressed relative to the geometric mean of the housekeeping genes Hmbs, Gapdh and Hprt. Results are mean of 3-8 hearts in each group. * p<0.05 vs. untreated db/db; #, p<0.05 vs. untreated db/+.
Table 1. Effect of TTA on body, heart and liver weights, and on plasma concentrations of glucose, fatty acids (FA) and triacylglycerol (TG) in diabetic (db/db) and non-diabetic (db/+)_mice.

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<th>db/+</th>
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<td>+TTA</td>
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<tr>
<td>Body weight (g)</td>
<td>28.0±0.5</td>
<td>44.0±0.9</td>
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<td>Heart weight (dry, mg)</td>
<td>27.7±0.4</td>
<td>24.5±0.4</td>
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<td>Liver weight (wet, g)</td>
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<td>2.2±0.1</td>
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<td>Plasma glucose (mM)</td>
<td>15.6±0.8</td>
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<td>Plasma TG (mM)</td>
<td>0.74±0.05</td>
<td>0.96±0.08</td>
</tr>
<tr>
<td>Plasma FA (mM)</td>
<td>0.55±0.04</td>
<td>0.88±0.08</td>
</tr>
</tbody>
</table>

*, p<0.05 vs. untreated within the same group; #, p<0.05 vs. untreated db/+. Results are mean of 21-25 mice in each group.
Table 2. Effect of TTA on mRNA expression of PPARα targets and transcription regulators in hearts from diabetic (db/db) and non-diabetic (db/+). Results are mean of 6-8 hearts in each group. mRNA levels were expressed relative to the geometric mean of the housekeeping genes Hmbs, Gapdh, and Hprt, and is presented as fold expression as compared to untreated db/+. *, p<0.05 vs. untreated within the same group; #, p<0.05 vs. untreated db/+.

<table>
<thead>
<tr>
<th>Gene</th>
<th>db/+ +TTA</th>
<th>db/db +TTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mcpt1</td>
<td>1.00±0.07</td>
<td>1.85±0.13 *</td>
</tr>
<tr>
<td>Pdk4</td>
<td>1.00±0.14</td>
<td>2.63±0.51 *</td>
</tr>
<tr>
<td>Cd36</td>
<td>1.00±0.07</td>
<td>0.93±0.05</td>
</tr>
<tr>
<td>Fatp</td>
<td>1.00±0.09</td>
<td>0.69±0.08 *</td>
</tr>
<tr>
<td>Fabp</td>
<td>1.00±0.04</td>
<td>1.30±0.06 *</td>
</tr>
<tr>
<td>Ucp3</td>
<td>1.00±0.09</td>
<td>1.34±0.13 *</td>
</tr>
<tr>
<td>Mte1</td>
<td>1.00±0.07</td>
<td>13.38±1.06 *</td>
</tr>
<tr>
<td>Cte1</td>
<td>1.00±0.05</td>
<td>2.45±0.29 *</td>
</tr>
<tr>
<td>Pparα</td>
<td>1.00±0.06</td>
<td>0.47±0.05 *</td>
</tr>
<tr>
<td>Pparδ</td>
<td>1.00±0.08</td>
<td>0.90±0.17 *</td>
</tr>
<tr>
<td>Pgc1α</td>
<td>1.00±0.05</td>
<td>0.47±0.06 *</td>
</tr>
</tbody>
</table>
Table 3. Regression analysis of the MVO₂-PVA relationships in hearts from untreated and TTA-treated db/+ and db/db mice.

<table>
<thead>
<tr>
<th></th>
<th>db/+</th>
<th>+TTA</th>
<th>db/db</th>
<th>+TTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>y-intercept</td>
<td>4.80±0.46</td>
<td>7.37±0.63 *</td>
<td>7.18±0.75 #</td>
<td>6.44±0.46</td>
</tr>
<tr>
<td>Slope</td>
<td>2.34±0.18</td>
<td>1.76±0.17</td>
<td>2.86±0.56</td>
<td>3.06±0.28</td>
</tr>
<tr>
<td>r²</td>
<td>0.96±0.01</td>
<td>0.96±0.01</td>
<td>0.93±0.02</td>
<td>0.92±0.02</td>
</tr>
</tbody>
</table>

The y-intercept represents unloaded MVO₂ (expressed as Joule/beat/g wet wt · 10⁻³), the inverse of the slope represents contractile efficiency (dimensionless) and r² is the square of the regression coefficient. *, p<0.05 vs. untreated within the same group; #, p<0.05 vs. untreated db/+.
Table 4. Ventricular function in hearts from untreated and TTA-treated db/+ and db/db mice obtained by pressure-volume analysis.

<table>
<thead>
<tr>
<th></th>
<th>db/+</th>
<th>+TTA</th>
<th>db/db</th>
<th>+TTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats·min⁻¹)</td>
<td>448±11</td>
<td>442±10</td>
<td>431±11</td>
<td>397±15</td>
</tr>
<tr>
<td>Pₑₛ (mmHg)</td>
<td>67±0.8</td>
<td>70±1.5</td>
<td>63±1.0 #</td>
<td>64±0.6</td>
</tr>
<tr>
<td>Pₑ₃ (mmHg)</td>
<td>11.3±0.9</td>
<td>10.3±1.3</td>
<td>10.9±0.8</td>
<td>7.9±0.7</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>56.7±1.1</td>
<td>59.6±1.3</td>
<td>52.5±1.2 #</td>
<td>56.1±0.7</td>
</tr>
<tr>
<td>dP/dtₘₐₓ (mmHg·sec⁻¹)</td>
<td>4752±314</td>
<td>4843±253</td>
<td>3797±210 #</td>
<td>4500±249</td>
</tr>
<tr>
<td>dP/dtₘᵢₙ(mmHg·sec⁻¹)</td>
<td>-3800±290</td>
<td>-3941±195</td>
<td>-2737±159 #</td>
<td>-2915±093</td>
</tr>
<tr>
<td>Tau (g) msec Weiss</td>
<td>10.4±0.5</td>
<td>10.1±0.6</td>
<td>12.6±0.6 #</td>
<td>11.9±0.5</td>
</tr>
<tr>
<td>Aortic flow (ml·min⁻¹)</td>
<td>12.8±0.7</td>
<td>12.0±0.7</td>
<td>8.9±0.6 #</td>
<td>8.4±0.5</td>
</tr>
<tr>
<td>Coronary flow (ml·min⁻¹)</td>
<td>3.2±0.1</td>
<td>2.8±0.1</td>
<td>2.3±0.1 #</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>Cardiac output (ml·min⁻¹)</td>
<td>16.0±0.8</td>
<td>14.8±0.8</td>
<td>11.2±0.6 #</td>
<td>10.6±0.5</td>
</tr>
<tr>
<td>Stroke volume (µl)</td>
<td>36.2±2.1</td>
<td>33.4±1.5</td>
<td>26.4±1.9 #</td>
<td>26.9±1.5</td>
</tr>
</tbody>
</table>

Steady state parameters were obtained at pre- and after-loads of 10 and 50 mmHg, respectively. Pₑ₃, left ventricular end-diastolic pressure; Pₑₛ, left ventricular end-systolic pressure; LVDP, left ventricular developed pressure. #, p<0.05 vs. untreated db/+.

The data are obtained from 8-14 hearts in each group.
Figure 1

A

- Fat acid oxidation (umol/min/mg dry wt)
- Glucose oxidation (umol/min/mg dry wt)

B

- V_max
  - Succinate dehydrogenase (CS)
  - Oxidative phosphorylation (CS)

Figure 2
Figure 3
Figure 4

A

B

MnSod (normalized to db/μ)

MnSOD (normalized to db/μ)

MnSOD activity (U/mg protein)

Cat. (normalized to db/μ)

GSH/px (normalized to db/μ)