Effects of Short Term Adiponectin Receptor Agonism on Cardiac Function and Energetics in Diabetic db/db Mice

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ABSTRACT

Objective: Impaired cardiac efficiency is a hallmark of diabetic cardiomyopathy in models of type 2 diabetes. Adiponectin receptor 1 (AdipoR1) deficiency impairs cardiac efficiency in non-diabetic mice, suggesting that hypoadiponectinemia in type 2 diabetes may contribute to impaired cardiac efficiency due to compromised AdipoR1 signaling. Thus, we investigated whether targeting cardiac adiponectin receptors may improve cardiac function and energetics, and attenuate diabetic cardiomyopathy in type 2 diabetic mice.

Methods: A non-selective adiponectin receptor agonist, AdipoRon, and vehicle were injected intraperitoneally into Eight-week-old db/db or C57BLKS/J mice for 10 days. Cardiac morphology and function were evaluated by echocardiography and working heart perfusions.

Results: Based on echocardiography, AdipoRon treatment did not alter ejection fraction, left ventricular diameters or left ventricular wall thickness in db/db mice compared to vehicle-treated mice. In isolated working hearts, an impairment in cardiac output and efficiency in db/db mice was not improved by AdipoRon. Mitochondrial respiratory capacity, respiration in the presence of oligomycin, and 4-hydroxynonenal levels were similar among all groups. However, AdipoRon induced a marked shift in the substrate oxidation pattern in db/db mice towards increased reliance on glucose utilization. In parallel, the diabetes-associated increase in serum triglyceride levels in vehicle-treated db/db mice was blunted by AdipoRon treatment, while an increase in myocardial triglycerides in vehicle-treated db/db mice was not altered by AdipoRon treatment.

Conclusion: AdipoRon treatment shifts myocardial substrate preference towards increased glucose utilization, likely by decreasing fatty acid delivery to the heart, but was not sufficient to improve cardiac output and efficiency in db/db mice.

Keywords: Adiponectin; Mitochondria; Metabolism; Myocardial contraction; Adiponectin receptor
INTRODUCTION

Adiponectin is an adipocyte-derived cytokine that regulates whole body glucose and lipid metabolism. Plasma adiponectin levels are decreased in obesity, insulin resistance and type 2 diabetes, and are thought to contribute to the development of insulin resistance in obesity, which can be reversed by replenishment of adiponectin. Adiponectin may attenuate insulin resistance by increasing fatty acid (FA) oxidation, which reduces the triglyceride content in skeletal muscle and liver in obese and type 2 diabetic mice. Adiponectin exerts its cellular functions by activation of G-protein coupled adiponectin receptors (AdipoRs), AdipoR1 and AdipoR2. AdipoR1 is ubiquitously expressed in metabolically active organs, including the heart, and has been shown to activate AMP kinase (AMPK) signaling. As such, AdipoR1 increases mitochondrial biogenesis and oxidative capacity via increased expression and posttranslational activation of the master regulator of mitochondrial biogenesis, pparg coactivator 1 alpha (PGC-1α). AdipoR2 is predominantly expressed in the liver, and its activation increases the expression of peroxisome proliferator-activated receptor α (PPARα) to promote FA oxidation. Studies focusing on adiponectin signaling revealed that adenosine-mediated expression of either AdipoR1 or AdipoR2 in the liver ameliorated obesity-linked insulin resistance and diabetes. Conversely, simultaneous disruption of AdipoR1 and AdipoR2 abrogated adiponectin-specific binding and the glucose-lowering effect of adiponectin, and resulted in marked glucose intolerance and insulin resistance. Thus, beneficial systemic and organ-specific metabolic effects of adiponectin seem to be mediated by adiponectin receptors.

Adiponectin is required to maintain cardiac function and integrity, in particular in response to various stressors. Adiponectin deficiency leads to increased hypertrophy and accelerated development of heart failure following transverse aortic constriction. Lack of adiponectin also increases myocardial infarct size, and exogenous recombinant adiponectin not only blunted increased infarct size but even protected against ischemia reperfusion injury. Furthermore, pressure overload-induced hypertrophy was exaggerated in type 2 diabetic db/db mice, which was attenuated by restoration of normal serum adiponectin levels, suggesting that diabetes-associated hypoadiponectinemia may contribute to cardiac complications in diabetes, and that adiponectin supplementation may represent a useful therapeutic approach to attenuate cardiovascular disease in conditions of hypoadiponectinemia.

However, the application of adiponectin in clinical practice is limited by the complex quaternary structure of adiponectin, its rapid turnover, and the high costs of recombinant adiponectin production. These limitations have been overcome by the recently discovered AdipoR agonist, AdipoRon, a synthetic molecule that is orally active and binds to both AdipoR1 and AdipoR2. AdipoRon treatment has been shown to attenuate post-ischemic cardiac injury and to alleviate cardiac fibrosis and hypertrophy upon chronic pressure overload, suggesting that increasing AdipoR signaling bears therapeutic potential. Of note, AdipoRon administration also ameliorates insulin resistance and type 2 diabetes, and attenuates diabetic nephropathy and mesenteric vascular dysfunction in type 2 diabetic db/db mice with concomitant hypoadiponectinemia. Thus, it is tempting to speculate that AdipoRon treatment may be capable of attenuating diabetes-associated cardiovascular complications, which are also related to impaired adiponectin signaling.

A typical trait of diabetic cardiomyopathy observed in models of type 2 diabetes is impaired cardiac efficiency, related to increased myocardial FA utilization.
of impaired cardiac efficiency may include FA-induced reactive oxygen species (ROS)-mediated activation of mitochondrial uncoupling proteins, and/or oxygen waste for non-contractile processes.\textsuperscript{20,21} We previously reported that lack of AdipoR1 but not AdipoR2 impairs cardiac efficiency in non-diabetic animals, which was attenuated by mitochondria-targeted antioxidant treatment, potentially by reversing ROS-induced mitochondrial uncoupling.\textsuperscript{22} These findings led us to speculate that decreased AdipoR signaling due to hypoadiponectinemia in the setting of type 2 diabetes may contribute to impaired cardiac efficiency in diabetic cardiomyopathy. In this study, we hypothesized that activation of AdipoRs by AdipoRon treatment may improve cardiac efficiency and energetics in mice with obesity, insulin resistance and type 2 diabetes.

\section*{MATERIALS AND METHODS}

\subsection*{1. Animals and treatments}
Male \textit{db/db} mice (homozygous \textit{Lepr}\textsuperscript{db} mice) and respective control mice (C57BLKS/J mice) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed in individually ventilated cages in a specific pathogen free facility, with 12 hours daylight/dark cycles at 22°C. Animals were fed a laboratory standard chow and water ad libitum. At the age of 8 weeks, mice received daily intraperitoneal injections of 10 mg/kg (in 2% dimethyl sulfoxide [DMSO]) of the non-selective adiponectin receptor agonist, AdipoRon, Adiponectin receptor agonist (ab144867), CAS-number 924416-43, Abcam plc; Cambridge), for 10 days. Control mice received vehicle injections. The study conformed to the \textit{Guide for the Care and Use of Laboratory Animals} published by the US National Institutes of Health and was performed after securing approval by the Regierungspräsidium Freiburg, Germany (G-16-135).

\subsection*{2. Metabolite and adiponectin measurements}
Serum free FAs, serum triglycerides, and myocardial triglyceride levels were measured using the Free Fatty Acid Quantification kit, the Serum Triglyceride Determination kit, or the Triglycerides Quantification kit (all Merck, Darmstadt, Germany), respectively. Serum adiponectin levels were determined using the Mouse High Molecular Weight Adiponectin ELISA kit (Kendall Scientific, Lincolnshire, IL, USA).

\subsection*{3. Transthoracic echocardiography}
Transthoracic echocardiography was performed before start of treatment and after 7 days of treatment, as described previously.\textsuperscript{23} Mice were anesthetized using isoflurane inhalation (3% for induction and 2% for maintenance of anesthesia). M-Mode images and 2D parasternal short axis images were taken using a Vivid 7 Dimension (GE Healthcare, Munich, Germany) micro-imaging system equipped with an i13L transducer (14 MHz). Ejection fraction and LV geometry were quantified as described before.\textsuperscript{23}

\subsection*{4. Isolated working heart perfusion}
Hearts were excised and examined by isolated working mouse heart perfusion, as described previously.\textsuperscript{24} In brief, hearts were perfused using Krebs-Henseleit Buffer containing (in mmol/L) 128 NaCl, 5 KCl, 1 KH\textsubscript{2}PO\textsubscript{4}, 1.3 MgSO\textsubscript{4}, 15 NaHCO\textsubscript{3}, 2.5 CaCl\textsubscript{2}, 0.8 palmitate (bound to 3\% bovine serum albumin), and 11 glucose at 37°C, with 50 mmHg afterload and 15 mmHg preload. Aortic pressure changes were measured using a pressure catheter placed inside the aortic cannula (Millar Micro-Tip; Millar Instruments, Houston, TX, USA). Cardiac output and cardiac power were quantified to evaluate contractile function. Myocardial oxygen

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consumption (MVO$_2$) was measured as difference of percent oxygen concentration in pre- and postcardial buffer samples using a fiber-optic oxygen sensor (Ocean Optics, Orlando, FL, USA). Cardiac efficiency was calculated as ratio of hydraulic work to MVO$_2$. Glucose oxidation and glycolytic flux were determined in the same perfusion using [U-$^{14}$C] glucose and [5-$^3$H] glucose, and palmitate oxidation was measured in separately perfused hearts by determining the amount of $^3$H$_2$O released from [9,10-$^3$H] palmitate.

5. Mitochondrial function
Mitochondria were isolated by differential centrifugation, and rates of oxygen consumption and adenosine triphosphate (ATP) synthesis were determined as described before. In brief, respiratory rates were measured using a Clark-type oxygen electrode (Strathkelvin, North Lanarkshire, Scotland) at 25°C in 500 µL KCl-rich buffer containing 120 mmol/L KCl, 5 mmol/L KH$_2$PO$_4$, 1 mmol/L EGTA, 1 mg/mL BSA and 3 mmol/L HEPES, supplemented with 20 µmol/L palmitoyl-carnitine and 2 mmol/L malate, and containing 0.6 mg/mL mitochondria. Respiration was measured in the presence of substrates alone (state II), after addition of 0.6 mmol/L ADP (state III), after complete turnover of ADP to ATP (state IV), and after ADP turnover in the presence of oligomycin. Respiratory control index (RCI) was calculated as the ratio of state III to state IV respiration.

6. RNA extraction and quantitative reverse transcription polymerase chain reaction
Total RNA was isolated from hearts with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA), purified using the RNeasy Kit (Qiagen, Hilden, Germany), and reverse transcribed using the SuperScriptIII Reverse Transcriptase Kit (Invitrogen Corp.), as described before. SYBR Green (Bio Rad, Munich, Germany) was used as a probe, and amplification was measured using the CF X96 Real-Time PCR system (Bio Rad). Data were normalized to the levels of the invariant transcripts of 60S acidic ribosomal protein P0 and are presented as arbitrary units normalized to expression levels of vehicle-treated C57BLKS/J mice. Primer sequences are presented in Supplementary Table 1.

7. Western blot analysis
Proteins of whole heart homogenates or isolated mitochondria were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA), incubated with primary antibodies overnight at 4°C, washed with TBS-T and incubated with secondary antibodies for 1 hour at room temperature, as reported previously. The following primary antibodies were used: Anti-AMPK 1:1,000 (#2532; Cell Signaling, Boston, MA, USA), Anti-phospho AMPK (Thr172) 1:1,000 (#2531; Cell signaling), Anti-calcium/calmodulin-dependent protein kinase 2 (CaMKK2) 1:1,000 (#16810; Cell Signaling), Anti-phospho-CaMKK2 (Ser495) 1:1,000 (#16737; Cell Signaling), Anti-c-Jun N-terminal kinase (JNK) 1:2,000 (#ab179461; Abcam, Cambridge, UK), Anti-phospho-JNK (Tyr185, Tyr185, Tyr223) 1:2,000 (#ab76572; Abcam), Anti-liver kinase B1 (LKB1) 1:1,000 (#3050; Cell Signaling), Anti-phospho-LKB1 (Ser428) 1:1,000 (#3482; Cell Signaling), Anti-pyruvate dehydrogenase (PDH) 1:1,000 (#3205S; Cell Signaling), Anti-phospho-PDH-E1α (Ser293) 1:5,000 (#ABS204; Millipore Sigma), Anti-4-hydroxynonenal-Michael Adducts 1:1,000 (#393207; Merck). Secondary antibodies: Anti-Mouse IgG Fab2 Alexa Fluor, anti-Rabbit IgG (H+L) Fab2 Alexa Fluor (both Cell Signaling). Detection and quantification of fluorescence bands were performed using the Bio-Rad Western Blot Imaging system ChemiDoc MP. Loading control for total protein was performed using Anti-alpha-Tubulin antibodies 1:2,000 (#T9026; Sigma-Aldrich, St. Louis, MO, USA) or staining with Coomassie Blue R-250 (Bio-Rad, Hercules, CA, USA).
8. Statistical analysis
Data are presented as means±SEM. Groups were compared using a 2-way analysis of variance (2-way ANOVA) using SPSS statistical software (IBM, Armonk, NY, USA). Post-hoc analyses were performed using a Bonferroni test. Significant difference was accepted when \( p<0.05 \).

RESULTS

1. Decreased adiponectin levels in \( \text{db/db} \) mice
Eight-week-old \( \text{db/db} \) mice and C57BLKS/J control mice received daily intraperitoneal injections of AdipoRon (10 mg/kg) or vehicle (2% DMSO) for 10 consecutive days. Body weight was markedly increased in \( \text{db/db} \) mice compared to C57BLKS/J mice (Fig. 1A). After AdipoRon treatment, body weight was mildly reduced compared to vehicle-treated mice. Levels of high molecular weight (HMW) adiponectin were lower in \( \text{db/db} \) mice compared to C57BLKS/J mice (Fig. 1B). AdipoRon did not affect adiponectin levels in \( \text{db/db} \) or C57BLKS/J mice. Thus, decreased levels of serum adiponectin in a murine model of obesity and type 2 diabetes, which also remained unaffected by AdipoRon treatment, was in line with our hypothesis and corroborated previous findings. Consistent with previous studies,\(^{25}\) heart weight-to-body weight ratio was reduced in \( \text{db/db} \) mice compared to C57BLKS/J mice (Fig. 1C). However, we observed no difference in heart weight-to-tibia length ratio (Fig. 1D), or upon AdipoRon treatment of either C57BLKS/J or \( \text{db/db} \) mice.

Fig. 1. Body weight and adiponectin levels. Body weight (A, \( n=12–22 \)), serum high-molecular-weight adiponectin (B, \( n=4 \)), heart weight-to-body weight ratio (C, \( n=14–21 \)) and heart weight-to-tibia length ratio (D, \( p=8–9 \)) in C57BLKS/J and \( \text{db/db} \) mice treated with Veh or AdipoRon for 10 days. Veh, vehicle.

\(^* p<0.05\) using post-hoc test; 2-way ANOVA: \(^\dagger\) effect of genotype; \(^\ddagger\) effect of treatment; \(^\S\) interaction effect.
2. Negligible effect of AdipoRon on cardiac function and dimensions in db/db mice

Cardiac function was evaluated following AdipoRon or vehicle treatment using echocardiography. Compared to C57BLKS/J mice, db/db mice showed an increase in systolic interventricular septum thickness and a decrease in left ventricular internal diameters and volumes (Table 1). Cardiac output was unchanged, and ejection fraction was increased in db/db mice. Left ventricular wall thickness was not different among groups. These observations would be consistent with the development of concentric cardiac hypertrophy in diabetic db/db mice. Except for a further reduction of left ventricular internal diameter and volume at systole, AdipoRon treatment did not differentially affect left ventricular or septal wall thickness, left ventricular diameter and volume at diastole, ejection fraction, stroke volume, or cardiac output (Table 1).

Next, cardiac function, efficiency and energetics were investigated in isolated working heart perfusions. Cardiac output and cardiac power were reduced in db/db mice irrespective of the treatment (Fig. 2A and B). Despite a decrease in MVO₂, cardiac efficiency was also reduced in db/db mice treated either with AdipoRon or vehicle (Fig. 2C and D). While AdipoRon treatment increased cardiac power in C57BLKS/J and db/db mice, AdipoRon treatment did not affect cardiac output or efficiency (Fig. 2A, B and D). Taken together, both using in vivo and ex vivo evaluation of cardiac function and/or cardiac dimensions, short term AdipoRon treatment did not significantly improve overall cardiac function and dimensions in diabetic db/db mice.

3. AdipoRon induces shift towards increased glucose utilization in db/db mice

Increased cardiac FA oxidation is a well-known trait of models of type 1 and type 2 diabetes. We analyzed myocardial substrate oxidation in isolated working hearts. Mean values of palmitate oxidation were increased by 2.7-fold in vehicle-treated db/db mice compared to vehicle-treated C57BLKS/J mice; statistical significance was however not achieved (Fig. 2E). Rates of glucose oxidation and glycolysis were not different between vehicle-treated db/db and C57BLKS/J mice, although mean values of glucose oxidation were lower in db/db mice (Fig. 2F and H). AdipoRon-treated C57BLKS/J mice showed an increase in both palmitate and glucose oxidation, whereas glycolysis remained unaffected compared to vehicle-treated C57BLKS/J mice (Fig. 2E, F and H). Unexpectedly, myocardial palmitate oxidation rates were not increased in AdipoRon-treated db/db mice compared to vehicle-treated C57BLKS/J mice.

Table 1. Echocardiographic parameters 10 days following treatment with vehicle or AdipoRon (n=4–7)

| Parameter | C57BLKS/J (vehicle) | db/db (vehicle) | C57BLKS/J (AdipoRon) | db/db (AdipoRon) | Two-way ANOVA |
|-----------|----------------------|-----------------|----------------------|------------------|---------------|
| IVSd (mm) | 0.67±0.03            | 0.75±0.06       | 0.70±0.11            | 0.71±0.04        | NS            |
| IVSs (mm) | 0.86±0.04            | 1.14±0.05*      | 0.94±0.12            | 1.09±0.02*       | §             |
| LVPWd (mm) | 0.77±0.06            | 0.76±0.05       | 0.78±0.07            | 0.78±0.05        | NS            |
| LVPWs (mm) | 0.94±0.06            | 0.94±0.07*      | 1.03±0.06            | 1.02±0.05        | NS            |
| LVIDd (mm) | 4.29±0.08            | 3.67±0.04*      | 4.00±0.19            | 3.63±0.05*       | §             |
| LVIDs (mm) | 3.45±0.12            | 2.59±0.08*      | 3.03±0.19            | 2.36±0.07*       | §             |
| EDV (mL)  | 82.1±2.4             | 57.1±1.3*       | 71.0±4.6             | 56.5±1.3*        | §             |
| ESV (mL)  | 49.1±2.4             | 24.5±1.4*       | 37.0±3.4             | 18.8±1.0*        | §             |
| EF (%)    | 47.1±3.6             | 63.2±2.2        | 54.4±5.6             | 65.6±4           | §             |
| SV (mL)   | 35±1.9               | 33.0±9.9        | 34.6±4.6             | 36.2±2.0         | NS            |
| CO (mL/min) | 13.8±0.9             | 13.4±0.4        | 13.0±1.2             | 13.3±0.5         | NS            |
| HR (bpm)  | 419±16               | 408±13          | 383±23               | 362±21           | NS            |

Data are shown as mean±SD.
IVS, interventricular septum; LVPW, left ventricular posterior wall; LVID, left ventricular internal diameter; EDV, enddiastolic volume; ESV, endsystolic volume; EF, ejection fraction; SV, stroke volume; CO, cardiac output; HR, heart rate; s, systolic; d, diastolic; NS, not significant.
Post-hoc test: *p<0.05 vs. C57BLKS/J (vehicle), †p<0.05 vs. C57BLKS/J (AdipoRon); 2-way ANOVA: §Genotype effect; $Treatment effect.
In addition, rates of glucose oxidation (+131%) and glycolysis (+73%) were markedly increased in AdipoRon-treated \textit{db/db} mice compared to vehicle-treated \textit{db/db} mice (Fig. 2F and H). As a consequence, the ratio of glucose oxidation to palmitate oxidation was markedly increased in AdipoRon-treated \textit{db/db} mice compared to vehicle-treated C57BLKS/J or vehicle-treated \textit{db/db} mice (Fig. 2G). Thus, AdipoRon treatment induced an obvious switch in substrate preference towards increased glucose utilization in \textit{db/db} mice.

**4. Effect of AdipoRon on mitochondrial function and 4-Hydroxynonenal (4-HNE) levels**

Mitochondrial function was evaluated by determining oxygen consumption rates in mitochondria isolated from hearts that were preperfused in the isolated working mode...
using 0.8 mM palmitate and 11 mM glucose. State 3 respiration using palmitoyl-carnitine as a substrate was not different among groups (Fig. 3A). While respiration in the presence of oligomycin was increased in AdipoRon-treated C57BLKS/J mice compared to vehicle-treated C57BLKS/J mice, there were no significant changes of state 4 respiration, the RCI, or respiration in the presence of oligomycin among groups, indicating no relevant overall effects on mitochondrial coupling (Fig. 3A and B). Since AdipoRon treatment may lower oxidative stress, we measured whole heart and mitochondrial 4-HNE levels. Mean values for whole heart or mitochondrial 4-HNE levels were increased in vehicle-treated db/db mice, however significant differences among groups were not observed (Fig. 3C and D).

5. Effects of AdipoRon on AdipoR signaling and metabolic gene expression

AdipoRon treatment has been shown to increase AMPK phosphorylation, and the expression of PGC-1α, PPARα, and of genes of mitochondrial biogenesis and FA oxidation. While AMPK phosphorylation was not reduced in db/db mice compared to C57BLKS/J mice irrespective of the treatment, AdipoRon treatment did not increase but instead decrease AMPK phosphorylation (Fig. 4A, Supplementary Fig. 1A). In line with this, phosphorylation of the upstream regulators of AMPK, CaMKK2 and LKB1, were significantly reduced upon

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**Fig. 3.** Myocardial mitochondrial function and oxidative stress. Oxygen consumption rates (A) and RCI (B) of mitochondria isolated from hearts of C57BLKS/J and db/db mice treated with Veh or AdipoRon for 10 days. 4-Hydroxynonenal (4-HNE) levels determined in whole heart tissue (C) or in isolated cardiac mitochondria (D) of C57BLKS/J and db/db mice treated with Veh or AdipoRon for 10 days.

Veh, vehicle; RCI, respiratory control index.

*p < 0.05 using post-hoc test; 2-way ANOVA: †interaction effect.
AdipoRon treatment of db/db mice (Fig. 4B and C, Supplementary Fig. 1B). Expression of PP2A, another upstream regulator of AMPK, was not changed in db/db mice or by AdipoRon treatment (Fig. 4D). Interestingly, phosphorylation of JNK was reduced in the heart of db/db mice compared to C57BLKS/J treated with AdipoRon, but was not affected by AdipoRon treatment in either group of mice (Fig. 4E, Supplementary Fig. 1C). Lastly, phosphorylation of PDH was significantly increased in both C57BLKS/J and db/db mice upon treatment with AdipoRon (Fig. 4F, Supplementary Fig. 1D).

PPARα expression was markedly induced by AdipoRon treatment in C57BLKS/J mice, accompanied by increased expression of PPARα targets that facilitate FA oxidation (Hadhβ,
Acadl), but inhibit glucose oxidation (pyruvate dehydrogenase kinase, isoenzyme 4), or regulate mitochondrial uncoupling (uncoupling protein isoform [Ucp] 2, Ucp3) (Fig. 5A and B). Expression of PPARα and above mentioned PPARα targets was also increased in vehicle-treated db/db mice, but was not further increased by AdipoRon treatment (Fig. 5A and B). Furthermore, this was accompanied by reduced expression of genes related to glycolysis (phosphofructokinase [muscle type], pyruvate kinase muscle isozyme) in both db/db mice compared to C57BLKS/J mice, as well as following AdipoRon treatment of C57BLKS/J mice (Fig. 5C). While expression of AdipoR1 was not different among groups, expression of AdipoR2 was markedly increased by AdipoRon treatment in C57BLKS/J mice (Fig. 5D). Expression of AdipoR2 was also increased in vehicle-treated db/db mice but was not further increased in AdipoRon-treated db/db mice (Fig. 5D). Expression of the AdipoR signaling targets PGC-1α and Sirt1 was not different among groups. Thus, a mismatch between metabolic gene expression and actual substrate oxidation rates measured in isolated working hearts, and lack of increased AMPK phosphorylation in response to AdipoRon, suggests that rates of myocardial substrate oxidation may not primarily be attributable to AdipoRon-mediated effects on myocardial gene expression or AMPK phosphorylation.

6. AdipRon attenuates hyperlipidemia in db/db mice

To further evaluate the mechanism that may limit myocardial FA oxidation and drive the shift towards increased glucose utilization in AdipoRon-treated db/db mice, we analyzed...
serum lipid levels. Indeed, adiponectin has been shown to lower serum triglyceride levels by increasing expression and activity of lipoprotein lipase (LPL) and very low density lipoprotein receptor (VLDLr) activity in skeletal muscle and white adipose tissue.\textsuperscript{27} Serum triglyceride levels were increased in vehicle-treated \textit{db/db} mice, and this increase was blunted by AdipoRon treatment (\textit{Fig. 6A}). A similar trend was observed for free FAs which showed increased mean levels in vehicle-treated but not AdipoRon-treated \textit{db/db} mice (\textit{Fig. 6B}).

AdipoRon treatment did not affect triglyceride or free FA levels in C57BLKS/J mice. Myocardial expression of LPL and VLDLr was not different between vehicle-treated and AdipoRon-treated \textit{db/db} mice (\textit{Fig. 6C}). Since intramyocellular triglycerides have been identified to be a dynamic source of intracellular long-chain FAs that contribute to FA oxidation and ligand-mediated PPAR\textsubscript{a} activation,\textsuperscript{28} myocardial triglyceride levels were analyzed. Myocardial triglyceride levels were increased in \textit{db/db} mice, but AdipoRon treatment had no additional effect on triglyceride levels (\textit{Fig. 6D}). AdipoRon also did not affect myocardial triglyceride levels in C57BLKS/J mice. Thus, systemic effects of AdipoRon treatment on serum lipid levels may limit myocardial FA oxidation in \textit{db/db} mice by attenuating delivery of FAs to the heart.

\textbf{Fig. 6.} Serum and myocardial lipid levels. Serum triglycerides (A), serum free fatty acids (B), myocardial mRNA expression of LPL and VLDLr (C), and myocardial triglycerides (D) in C57BLKS/J and \textit{db/db} mice treated with Veh or AdipoRon for 10 days (n=3–9). Veh, vehicle; LPL, lipoprotein lipase; VLDLr, very low density lipoprotein receptor; mRNA, messenger RNA. \textsuperscript{*}p<0.05 using post-hoc test; 2-way ANOVA: \textsuperscript{†}effect of genotype; \textsuperscript{‡}effect of treatment; \textsuperscript{§}interaction effect.

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DISCUSSION

This study investigated whether adiponectin receptor agonism may improve cardiac efficiency and function in a model of obesity and type 2 diabetes associated with hypoadiponectinemia. Echocardiography revealed a phenotype consistent with mild concentric hypertrophy in \( \text{db/db} \) mice, and isolated working heart perfusions revealed an intrinsic defect in cardiac contractility in \( \text{db/db} \) mice. The observation of decreased cardiac function in working hearts but not by echocardiography may be related to the absence of neurohumoral mechanisms and the evaluation of cardiac function under conditions of defined loading in isolated working hearts, thereby unmasking existing intrinsic defects in contractility in diabetic hearts not detectable by echocardiography. Importantly though, none of these effects were improved by treatment with AdipoRon, suggesting that short-term treatment is not sufficient to attenuate defects in cardiac function in type 2 diabetic \( \text{db/db} \) mice. Interestingly, Kim et al.\(^{16}\) recently showed that AdipoRon treatment prevented a mild decrease in fractional shortening, and blunted an increase in fibrosis and apoptosis in \( \text{db/db} \) mice. However, these animals were 16 weeks old at the start of treatment, received a higher dose of AdipoRon (30 mg/kg), and the duration of therapy was longer (4 weeks). In light of these findings, we cannot exclude beneficial effects of AdipoRon on diabetic cardiomyopathy and thus speculate that adjustments of the dosage and treatment period may be necessary to achieve significant improvement of function and efficiency in the myocardium. We acknowledge that potential beneficial effects of AdipoRon on diastolic function previously described in other studies were not evaluated.\(^{16}\)

It is important to note that the dose of AdipoRon used in our study was sufficient to cause normalization of serum triglyceride levels and to decrease mean levels of serum FAs. This observation is consistent with previous studies showing that increasing serum adiponectin levels by adenovirus-mediated overexpression of adiponectin is capable of lowering serum triglycerides, and that AdipoRon treatment is capable of lowering both serum triglycerides and free FAs in \( \text{db/db} \) mice.\(^{13,27}\) Although we did not determine the glucose-lowering potential of AdipoRon in the current study, previous studies have shown that AdipoRon significantly reduces fasting plasma glucose and insulin levels following oral administration of high AdipoRon doses (50 mg/kg),\(^{13}\) but does not alter plasma glucose or HbA1c at lower doses (30 mg/kg).\(^{13}\) Importantly, AdipoRon treatment also ameliorated glucose intolerance and insulin resistance in \( \text{db/db} \) mice at a higher dose.\(^{13}\) Thus, AdipoRon treatment may be a promising and, in contrast to recombinant adiponectin, clinically feasible therapeutic approach to control hyperlipidemia and reverse unfavourable metabolic traits of diabetes. The mechanism of systemic lipid lowering observed in our study may be related to increased expression and/or activity of LPL and VLDLr in skeletal muscle and/or adipose tissue, resulting in increased VLDL catabolism, as observed in mice with a 12-fold increase in serum adiponectin levels.\(^{27,29}\) In addition, lipid lowering may be related to AdipoRon-induced expression of PGC-1\(\alpha\) and enzymes of FA oxidation in skeletal muscle, and increased expression of PPAR\(\alpha\) and FA oxidation genes in liver, thereby increasing mitochondrial oxidative capacity for FAs derived from circulating triglycerides.\(^{13}\) AdipoRon also increased expression of PPAR\(\alpha\) in the heart, which correlated with increased expression of the PPAR\(\alpha\) target gene, UCP2, and enhanced rates of uncoupled respiration, thereby confirming previous studies that reported induction of UCP2 expression by adiponectin and suggesting that AdipoRon promoted mitochondrial uncoupling by increasing UCP2 expression.\(^{30}\) The functional significance of this observation remains to be elucidated.
Importantly, the capability of AdipoRon to normalize triglyceride levels in db/db mice suggests that our AdipoRon treatment was indeed effective, at least in extramyocardial tissues. Of note, we did also observe a strong induction of myocardial PPARα expression in C57BLKS/J mice in response to AdipoRon, although systemic lipid levels were unchanged in response to AdipoRon in C57BLKS/J mice. Since increased expression of AdipoR2 is sufficient to increase PPARα expression, we speculate that AdipoRon was sufficient to activate cardiac AdipoR2 receptors. In addition, increased expression of AdipoR2 in AdipoRon-treated C57BLKS/J mice suggests that AdipoRon may have induced AdipoR2 expression via a feed-forward mechanism. Similar observations have been made in human keratinocytes where adiponectin treatment increased the expression of AdipoR1. Such a feed-forward mechanism could have amplified myocardial AdipoR2 signaling in our study and could thereby explain the strong induction of PPARα expression in C57BLKS/J mice upon AdipoRon treatment. The existence of such a feed-forward mechanism in cardiac tissue however remains to be confirmed experimentally. Interestingly, AMPK phosphorylation, an early downstream target in AdipoR1 signaling, was not increased in response to AdipoRon in C57BLKS/J mice, as similarly observed in control groups of other studies. In fact, AMPK phosphorylation was even decreased in response to AdipoRon, potentially mediated by decreased activity of its upstream regulators, CaMKK2 and/or LKB1. In line with these results, we also observed unchanged phosphorylation of the adiponectin downstream target, JNK. Collectively, it is therefore tempting to speculate that AdipoRon may have preferentially activated the myocardial AdipoR2 subpopulation of the adiponectin receptor family.

Functional evaluation of myocardial substrate oxidation revealed that AdipoRon treatment induced a marked shift towards increased reliance on glucose-derived ATP production in db/db mice. Given the mismatch of increased expression of PPARα and downstream targets of beta oxidation with normal rates of FA oxidation in AdipoRon-treated db/db mice, we propose that AdipoRon-mediated normalization of increased serum lipid levels may have limited FA oxidation in db/db mice despite increased myocardial PPARα levels and enzymatic capacity for FA oxidation. Indeed, delivery of energy metabolic substrates to the heart is an essential regulatory mechanism that determines the pattern of myocardial substrate oxidation, and increased myocardial FA delivery is considered the major driver of increased myocardial FA oxidation in models of diabetes, including the db/db mouse model. As a consequence, rates of glucose utilization may be increased to maintain energy homeostasis via the reverse Randle cycle. However, inhibition of PDH (indicated by increased phosphorylation status) was observed as a result of AdipoRon treatment in both C57BLKS/J and db/db mice, suggesting that increased rates of glucose oxidation are not mediated by this enzyme. We speculate that an alternative route involving pyruvate carboxylase, which may increase TCA cycle anaplerosis by conversion of pyruvate to oxaloacetate, may increase oxidation of glucose, although this was not measured in the current study. While expression and activity of LPL and VLDLr can be driven by adiponectin, lack of a decrease in LPL and VLDLr expression in hearts of AdipoRon-treated db/db mice compared to vehicle-treated db/db mice suggests that LPL and VLDLr were not involved in limiting myocardial FA oxidation in AdipoRon-treated db/db mice. Furthermore, unchanged myocardial triglyceride levels between the groups may argue against a decreased utilization of FAs derived from myocardial triglyceride stores, although effects of AdipoRon on the dynamic turnover of FAs in the triglyceride pool has not been evaluated. Thus, we propose that the shift towards increased reliance on glucose-derived ATP production in db/db hearts in response to AdipoRon may be a consequence of systemic lipid lowering.
The therapeutic value of shifting myocardial substrate oxidation towards preferential glucose utilization to improve cardiac function in the failing heart is an ongoing debate. While some studies in animals and humans suggest that treatment with metabolic modulators that inhibit FA oxidation and/or stimulate glucose utilization may improve cardiac function and/or NYHA state, other studies demonstrate similar improvements without concomitant alterations in the myocardial substrate oxidation pattern or suggest other mechanisms that mediate beneficial effects. In diabetes, excess myocardial utilization of FA may have detrimental consequences, including increased oxygen consumption and impaired cardiac efficiency, accumulation of lipotoxic intermediates, and increased ROS production. Thus, attenuation or even prevention of increased FA oxidation and thus inducing a substrate switch towards increased glucose utilization in the diabetic heart could be anticipated to ameliorate diabetic cardiomyopathy. Consistent with this hypothesis, inhibition of myocardial mitochondrial import and oxidation of FAs using etomoxir improved cardiac function in rat models of acute and chronic diabetes. The same group also reported that normalization of cardiac substrate oxidation by administration of a high glucose/high insulin regime during perfusion of/db/db mouse hearts did not attenuate cardiac dysfunction, although an impairment in cardiac efficiency was restored by attenuating an increase in unloaded MVO$_2$. Interestingly, impaired cardiac efficiency was also normalized in sheep with STZ-induced type 1 diabetes in response to glucose-insulin-potassium infusion, which resulted from improved stroke work efficiency and decreased unloaded MVO$_2$. In our study, the shift towards increased reliance on glucose as an energy substrate neither attenuated cardiac dysfunction nor improved cardiac efficiency. Collectively, beneficial effects of shifting myocardial substrate preference towards glucose utilization in the diabetic heart may depend on the temporal application and the specific substance used to induce a substrate shift. Certainly, metabolism-independent effects cannot be excluded for any of these substances. We therefore propose that treatment strategies solely aiming at restoration of a physiological substrate preference in the diabetic heart may not necessarily be a successful approach to attenuate defects in cardiac function. Beneficial effects of AdipoRon treatment in a previous study may be linked to signaling effects not primarily related to modulation of cardiac energy substrate oxidation.

In conclusion, short term AdipoRon treatment shifts myocardial substrate preference towards increased glucose utilization, likely by decreasing FA delivery to the heart. However, this substrate shift was unable to improve cardiac output and efficiency in/db/db mice, or to reverse signs of concentric hypertrophy. These findings suggest that longer treatment duration and higher dosage of AdipoRon treatment may be necessary to achieve beneficial effects on myocardial function, and that shifting substrate metabolism towards increased glucose utilization may not necessarily be sufficient to improve cardiac function in the diabetic heart.

SUPPLEMENTARY MATERIALS

Supplementary Table 1
Sequences of forward and reverse primers used for reverse transcription quantitative polymerase chain reaction

Click here to view
Supplementary Fig. 1
Myocardial protein expression. p-AMPK (Thr172) and AMPK (A), p-LKB1 (Ser428) and LKB1 (B), p-JNK (Tyr185/185/223) and JNK (C), and p-PDH (Ser293) and PDH (D) in hearts of C57BLKS/J and db/db mice treated with Veh or AdipoRon for 10 days (n=5–6).

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