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Impairments in mitochondrial palmitoyl-CoA respiratory kinetics that precede development of diabetic cardiomyopathy are prevented by resveratrol in ZDF rats

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Key points

- Dysfunctional mitochondrial respiration may contribute to the establishment of diabetic cardiomyopathy, but this remains controversial; resveratrol, a polyphenol compound, has been shown to recover heart contractile function in rodent models of high-fat-diet-induced cardiac dysfunction.
- Therefore, we studied mitochondrial respiratory kinetic function in ZDF rats before overt diabetes and cardiac dysfunction manifested, and determined the efficacy of resveratrol to recover potential derangements in mitochondrial bioenergetics.
- We show that the electron transport chain functions normally in ZDF rats, as pyruvate and ADP respiratory kinetics were normal.
- In contrast, in ZDF rats, we show impairments in the sensitivity of mitochondria to lipids (palmitoyl-CoA) as well as the accumulation of reactive lipids and increased mitochondrial reactive oxygen species (ROS) emission rates.
- Supplementation with resveratrol improved palmitoyl-CoA respiratory kinetics and reactive lipid profiles, and normalized mitochondrial ROS emission rates.

Abstract Alterations in lipid metabolism within the heart may have a causal role in the establishment of diabetic cardiomyopathy; however, this remains equivocal. Therefore, in the current study we determined cardiac mitochondrial bioenergetics in ZDF rats before overt type 2 diabetes and diabetic cardiomyopathy developed. In addition, we utilized resveratrol, a compound previously shown to improve, prevent or reverse cardiac dysfunction in high-fat-fed rodents, as a tool to potentially recover dysfunctions within mitochondria. Fasting blood glucose and invasive left ventricular haemodynamic analysis confirmed the absence of type 2 diabetes and diabetic cardiomyopathy. However, fibrosis was already increased ($P < 0.05$) ~70% in ZDF rats at this early stage in disease progression. Assessments of mitochondrial ADP and pyruvate respiratory kinetics in permeabilized fibres from the left ventricle revealed normal electron transport chain function and content. In contrast, the apparent K_m to palmitoyl-CoA (P-CoA) was increased ($P < 0.05$) ~60%, which was associated with an accumulation of intracellular triacylgycerol, diacylglycerol and ceramide species. In addition, the capacity for mitochondrial reactive oxygen species emission was increased ($P < 0.05$) ~3-fold in ZDF rats. The provision of resveratrol reduced fibrosis, P-CoA respiratory sensitivity, reactive lipid accumulation and mitochondrial reactive oxygen species emission rates. Altogether the current data support the supposition that a chronic dysfunction within mitochondrial lipid-supported bioenergetics contributes to the development of diabetic cardiomyopathy, as this was present before overt diabetes or cardiac dysfunction. In addition, we show that resveratrol supplementation prevents these changes,

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supporting the belief that resveratrol is a potent therapeutic approach for preventing diabetic cardiomyopathy.

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Abbreviations ANT, adenine nucleotide translocase; CAT, catalase; CPTI, carnitine palmitoyltransferase I; DAG, diacylglycerol; $+dP/dt$, maximal rates of pressure development; $-dP/dt$, maximal rates of pressure decline; EDP, end-diastolic pressure; GSH, reduced glutathione; GSSG, oxidized glutathione; LC, lean control; LVP max, maximal left ventricular pressure; P-CoA, palmitoyl-CoA; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; TAG, triacylglycerol; ZDF rat, Zucker diabetic fatty rat; Z-Resv rat, Zucker diabetic fatty rat supplemented with resveratrol.

Introduction

Obesity, insulin resistance and dyslipidaemia are strong risk factors for the development of cardiovascular disease and the pathogenesis of atherosclerosis. While dyslipidaemia and elevated plasma lipid profiles have well-defined roles in the generation of atherosclerotic plaque formation, recent evidence also suggests that lipid accumulation within cardiac muscle may participate in the development of left ventricular dysfunction and diastolic heart failure in type 2 diabetes. Heart disease is a leading cause of death in type 2 diabetic patients, regardless of the presence of coronary artery disease and/or hypertension, a pathology termed diabetic cardiomyopathy (Stanley *et al.* 1997).

While the underlying cause of diabetic cardiomyopathy is probably multifactorial, one of the proposed mechanisms is impaired rates of ATP production. The heart produces more than 90% of its energy aerobically (Wisneski *et al.* 1987; Chandler *et al.* 2003), the majority of which is derived from aerobic lipid metabolism (Ventura-Clapier *et al.* 2011). Therefore, alterations in lipid metabolism within the heart may have a causal role in the establishment of diabetic cardiomyopathy. In support of this, cardiomyocyte-specific deletion of lipoprotein lipase (LpL) compromises lipid metabolism and the ejection fraction of hearts (Augustus *et al.* 2006). In addition, acipimox, an anti-lipolytic drug, reduced cardiac work in both healthy control and idiopathic dilated cardiomyopathy patients, supporting the belief that lipid metabolism is required for optimal cardiac performance (Tuunanen *et al.* 2006). While these studies suggest that increasing free fatty acid delivery to the heart improves contractile performance in some models of heart failure, increased plasma membrane transport of lipids, and the resultant accumulation of reactive lipids, have been strongly associated with diastolic dysfunction, fibrosis, left ventricular hypertrophy, and myocellular death in diabetic cardiomyopathy (Zhou *et al.* 2000; Finck *et al.* 2003; Yagyu *et al.* 2003; Stanley *et al.* 2005). Clearly, the balance of lipid transport and fatty acid oxidation within the heart is important for cardiac contractile performance.

Reactive lipid accumulation may also result from a dysfunction in mitochondrial fatty acid oxidation. Impaired mitochondrial respiratory function as a result of an attenuation in ADP sensitivity (increased apparent K_m for ADP) has been suggested to be a primary cause of pressure-induced diastolic heart failure (Ventura-Clapier *et al.* 2011). However, ADP sensitivity appears to be normal in the atria of human individuals with type 2 diabetes (Anderson *et al.* 2009*a*). Instead, a reduction in mitochondrial fatty acid oxidation, primarily as a result of a repressed peroxisome proliferator-activated receptor gamma coactivator 1-alpha-mediated gene expression has been associated with models of heart failure (Ventura-Clapier *et al.* 2011). In support of this, permeabilized muscle fibres from the atria of type 2 diabetic humans display repressed maximal palmitoylcarnitine respiration (Anderson *et al.* 2009*a*). However, the same research group has also shown in rodents that 12 weeks of a high-fat high-sucrose diet does not reduce palmitoylcarnitine respiration or apparent kinetics in the heart (Fisher-Wellman *et al.* 2013). Clearly, the role of impaired mitochondrial lipid metabolism in diabetic cardiomyopathy remains equivocal (Belke *et al.* 2000; Neitzel *et al.* 2003; Coort *et al.* 2004; Mazumder *et al.* 2004; Wang *et al.* 2005; Bugger *et al.* 2009). Adding further complexity is the observation that decreasing lipid transport into mitochondria through the administration of etomoxir and/or perhexiline improves cardiac function, even in the diabetic heart (Schmitz *et al.* 1995; Turcani & Rupp, 1997; Lee *et al.* 2005; Abozguia *et al.* 2010). These discrepancies highlight the likelihood that alterations in lipid metabolism could have divergent effects depending on the stage of heart failure, and therefore elucidating potential mitochondrial dysfunctional in lipid metabolism before the establishment of diabetic cardiomyopathy is of paramount importance in understanding the disease progression.

Therefore, in the current study we determined cardiac mitochondrial bioenergetics in Zucker diabetic fatty (ZDF) rats. The ZDF rat represents a modest model for diabetic cardiomyopathy and diastolic dysfunction (Schäfer *et al.* 2006; Marsh *et al.* 2007; Baynes & Murray, 2009; Radovits *et al.* 2009; van den Brom *et al.* 2009; Daniels *et al.* 2010, 2012), and we aimed to determine bioenergetics before overt type 2 diabetes and diabetic cardiomyopathy developed. Specifically we completed haemodynamic analysis of the left ventricle, and the Michaelis–Menten kinetic reponses of left ventricular permeabilized muscle fibres to various substrates, including ADP, pyruvate and palmitoyl-CoA (P-CoA) in animals at 11 weeks of age. While the function of the electron transport chain appeared normal, we show that the apparent K_m for P-CoA is increased ∼50% in ZDF rats, which was associated with intramyocellular lipid accumulation and an increase in mitochondrial reactive oxygen species (ROS) emission. In addition, administration of resveratrol, a compound previously shown to improve diastolic heart function in type 1 diabetic animals (Delucchi *et al.* 2012), and prevent or reverse cardiac dysfunction in high-fat-fed rodents (Louis *et al.* 2012; Qin *et al.* 2012), normalized P-CoA respiratory kinetics in the ZDF rat, while concomitantly improving intramyocellular lipid profiles and mitochondrial ROS emission rates. The current data strongly suggest that attenuations in mitochondrial respiratory sensitivity to P-CoA precedes the development of left ventricular dysfunction associated with diabetic cardiomyopathy, and that resveratrol treatment is able to normalize mitochondrial bioenergetic responses in pre-diabetic hearts.

Methods

Animals

Male ZDF rats (Charles River, St-Constant, QC, Canada) were housed in individual cages, with a reverse 12 h:12 h light–dark cycle, and were provided with food and water *ad libitum*. Lean control (LC) rats ($n = 13$) were fed a standard chow (Purina 5008 diet; Purina, St Louis, MO, USA). Twenty Six ZDF rats ($n = 13$ each group) were randomly assigned to either a stock diet (ZDF) or a stock diet supplemented with resveratrol (Cayman Chemicals; 200 mg (kg body weight)$^{-1}$), similar to previous publications (Lagouge *et al.* 2006) for 6 weeks (Z-Resv). Thereafter rats were anaesthetized with isoflurane and oxygen (2%:98%), left ventricular haemodynamics determined as outlined below, and then the left ventricle excised and either immediately used for bioenergetics assessments, fixed for histology or immediately frozen in liquid nitrogen. This study was approved by the University of Guelph Animal Care Committee, and conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health.

Measurement of *in vivo* left ventricular haemodynamics

Rats were anaesthetized with isoflurane and a catheter was inserted through the right carotid artery into the left ventricle. Recordings of haemodynamic signals were collected 15 min later. Maximal left ventricular pressure (LVP max), maximal rates of pressure development ($+dP/dt$), end-diastolic pressure (EDP) and maximal rates of pressure decline ($-dP/dt$) were collected.

Histochemical determination of left ventricular fibrosis

Left ventricle was fixed in 10% neutral buffered formalin (VWR, Mississauga, ON, Canada) dehydrated in xylene (Fisher Scientific, Ottawa, ON, Canada) and embedded in paraffin. Five micrometre sections were mounted on charged 1.2 mm Superfrost slides (Fisher Scientific) and stained using picrosirius red. Sections were subsequently imaged using an Olympus FSX 100 light microscope and images were acquired in Cell Sense software (Olympus, Tokyo, Japan). Using standard light microscopy, picrosirius red staining reveals collagen as red and muscle fibres and cytoplasm as yellow. For determination of fibrosis, Cell Sense software was used to threshold images, which isolated total area of red (fibrosis) and total area of yellow (cytoplasm/muscle fibre). Fibrosis was expressed as a percentage of total tissue area. For each animal, fibrosis was determined by averaging five different locations within the left ventricle.

Preparation of permeabilized muscle fibres

The preparation of permeabilized muscle fibre bundles was adopted from prior publications (Perry *et al.* 2011), as we have previously reported (Smith *et al.* 2012). Following dissection of the ventricle, fibre bundles (∼2 mg) were separated from the lumen wall in BIOPS buffer containing: CaK_2EGTA (2.77 mM), K_2EGTA (7.23 mM), Na_2ATP (5.77 mM), $MgCl_2.6H_2O$ (6.56 mM), sodium phosphocreatine (15 mM), imidazole (20 mM), dithiothreitol (0.5 mM) and MES (50 mM). Following separation, fibre bundles were placed in BIOPS containing 40 μg ml^{-1} saponin, agitated for 30 min and then fibres prepared for respiration were washed in respiration buffer (MiRO5) containing EGTA (0.5 mM), $MgCl_2.6H_2O$ (3 mM), potassium lactobionate (60 mM), KH_2PO_4 (10 mM), Hepes (20 mM), sucrose (110 mM) and fatty

acid-free bovine serum albumin (1 g l^{-1}). Fibres were left in cold MiRO5 until analysis.

Permeabilized muscle fibre respiration

Mitochondrial respiration was measured in permeabilized fibres by high-resolution respirometry (Oroboros Oxygraph-2k, Innsbruck, Austria) at 37°C and room air saturated oxygen tension in the presence of 25 μM blebbistatin (Perry *et al.* 2011, 2012). Separate permeabilized fibres from the same animal were used to determine the kinetic properties of ADP, pyruvate and palmitoyl-CoA (P-CoA). ADP (0, 100, 175, 250, 500, 1000, 2000, 4000, 6000 μM)-stimulated respiration was determined in the presence of 10 mM pyruvate and 5 mM malate. Pyruvate (0, 15, 30, 50, 75, 150, 500, 1000 μM)-stimulated respiration was determined in the presence of 5 mM ADP and 5 mM malate, while palmitoyl-CoA (P-CoA; 0, 10, 20, 40, 60, 80, 100, 125, 150, 250 μM)-supported respiration was determined in the presence of 5 mM ADP, 5 mM malate and 2 mM L-carnitine. Exogenous cytochrome *c* (10 μM) was added at the end of all respiration experiments to ensure outer mitochondrial membrane integrity. The addition of cytochrome *c* did not significantly elevate the respiration rate in any experiment.

Western blotting

Whole muscle was homogenized in lysis buffer and 5 μg of protein separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and quantified as previously reported (Smith *et al.* 2013). The following commercially available antibodies were used to detect α-tubulin (Abcam, Cambridge, MA, USA), oxidative phosphorylation (OXPHOS; Mitosciences), superoxide dismutase 2 (SOD2; Abcam) and catalase (CAT; Abcam) proteins. Bands were visualized using enhanced chemiluminescence (Western Lightning Plus-ECL, PerkinElmer), a FluorChem HD2 Alpha Innotech imager and quantified using the software provided.

Biochemical determination of intramuscular lipid content

Intramuscular levels of triacylglycerol, diacylglycerol and ceramide were measured as we have described previously by gas chromatography (Bonen *et al.* 2009).

Permeabilized muscle fibre mitochondrial H_2O_2 emission

Measurement of mitochondrial H_2O_2 emission was similar to previously described methodology (Anderson *et al.* 2009*b*). Briefly, mitochondrial H_2O_2 emission was determined fluorometrically (Lumina; Thermo Scientific, Fisher) in a constantly stirring cuvette at 37°C (Peltier controlled). Fibres were placed in a cuvette containing Buffer Z (Anderson *et al.* 2009*b*) supplemented with 25 μM blebbistatin, 40 U ml^{-1} of CuZnSOD, 10 μM Amplex Red (Invitrogen) and 0.5 U ml^{-1} horseradish peroxidase. Mitochondrial H_2O_2 emission was initiated by the addition of 10 mM succinate. The rate of H_2O_2 emission was calculated from the slope (fluorescence $second^{-1}$), after subtracting the background, from a standard curve established with the same reaction conditions and normalized to freeze-dried muscle weight.

Glutathione measurements

Reduced glutathione (GSH) and oxidized glutathione (GSSG) measurements were determined as previously described (Smith *et al.* 2013). Briefly, four small pieces of the left ventricle were separated while in liquid nitrogen. Two samples (in triplicate wells) were used to determine GSH and two samples (in triplicate wells) were used to determine GSSG in the presence of the scavenger methyl-2-vinylpyridinium triflate (M2VP). Total GSH and GSSG were measured as per manufacturer's instructions (Oxis International, Inc., Beverley Hills, CA, USA).

Statistics

The apparent K_m for ADP, glutamate, pyruvate and P-CoA were estimated in independent experiments with Michaelis–Menten kinetics utilizing Prism software (GraphPad Software, Inc., La Jolla, CA, USA), while the maximal respiration (V_{max}) was specified as the highest respiration value directly determined, as published previously (Perry *et al.* 2011). A one-way ANOVA with Newman–Keuls *post hoc* analysis was used for all comparisons. Statistical significance was accepted with a P value ≤ 0.05, and values represent means and standard error of the mean (SEM).

Results

Resveratrol improves systolic and diastolic function in the heart of ZDF rats

Diastolic dysfunction is a well-characterized phenotype of the ZDF rat (Schäfer *et al.* 2006; Marsh *et al.* 2007; Baynes & Murray, 2009; Radovits *et al.* 2009; van den Brom *et al.* 2009; Daniels *et al.* 2010, 2012). Previous reports have assessed cardiac function of ZDF rats at 14 weeks of age or later; however, we aimed to determine mitochondrial bioenergetics in animals before overt type 2 diabetes developed to determine potential mechanisms

that subsequently cause diabetic cardiomyopathy. The ZDF rat has a rapid onset of insulin resistance and type 2 diabetes, with average development of type 2 diabetes at 12 weeks of age. At 11 weeks of age only four ZDF animals displayed increased fasting blood glucose (data not shown), confirming the absence of overt type 2 diabetes. Therefore, we assessed cardiac function in 11-week-old rats. Specifically, we characterized heart function using invasive left ventricular haemodynamics to ensure overt diabetic cardiomyopathy had not already developed. While the ZDF rats displayed an increase in end-diastolic pressure (EDP), there were no alterations in maximal left ventricular pressure (LVP max), rates of relaxation ($-dP/dt$) and force development (Fig. 1). Combined, these data suggest that at 11 weeks of age ZDF animals begin to display the presence of marginal diastolic dysfunction, and therefore represent an ideal model/age to determine associations between changes in mitochondrial bioenergetics and the subsequent establishment of diabetic cardiomyopathy.

Resveratrol has previously been shown to delay mortality, improve ejection fraction, cardiac output, and prevent diastolic dysfunction in models of hypertension as well as type 1 diabetes and obesity (Rimbaud *et al.* 2011; Delucchi *et al.* 2012; Louis *et al.* 2012; Qin *et al.* 2012; Robich *et al.* 2012). In skeletal muscle, resveratrol has been shown to alter several aspects of mitochondrial bioenergetics, and therefore we used resveratrol as a potential modality to alter bioenergetics in the hearts of ZDF rats. With respect to haemodynamic analysis, resveratrol did not affect cardiac performance, which is not unexpected given the lack of profound diastolic dysfunction at this early age (Fig. 1). Three main mechanisms have been proposed to cause diabetic cardiomyopathy, including ventricle fibrosis, impaired ATP production and increased redox stress, and therefore we examined all in ZDF rats at 11 weeks of age.

Heart fibrosis is improved by resveratrol supplementation in ZDF rats

Cardiac remodelling, and in particular an increase in cardiac fibrosis, has been affiliated with diastolic dysfunction. In addition, resveratrol has been shown to

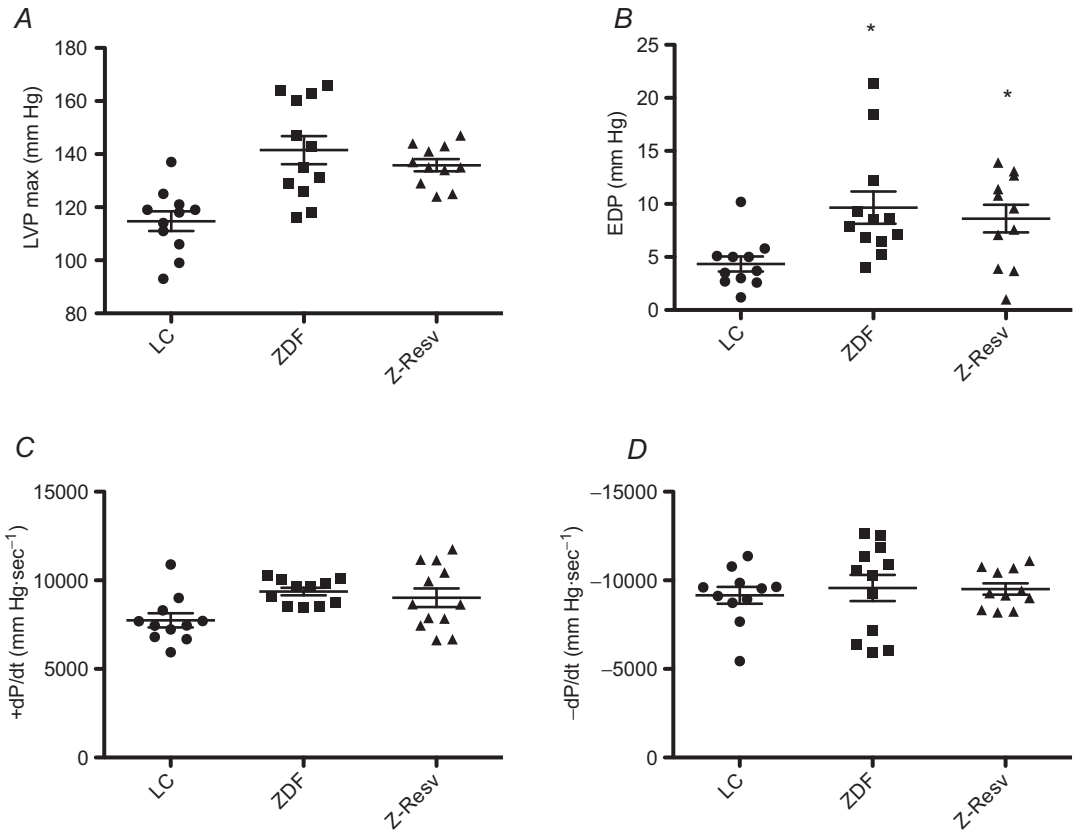

Figure 1. Left ventricular pressure characteristics were determined in lean (LC), Zucker diabetic fatty rats (ZDF) and ZDF rats supplemented with resveratrol (Z-Resv)
Maximal left ventricular pressure (*A*; LVP max); end-diastolic pressure (*B*; EDP); maximal rate of pressure development (*C*; $+dP/dt$) and maximal rate of pressure decline (*D*; $-dP/dt$) were determined in 11-week-old animals. Scatter plots depict the individual values as well as the means ± SEM. $n = 11$–12. ∗Significantly ($P < 0.05$) different from LC.

attenuate fibrosis in a model of type 1 diabetes (Delucchi *et al.* 2012) and in high-fat-fed mouse model of type 2 diabetes (Qin *et al.* 2012). We therefore examined myocardial fibrosis to determine if this was increased in ZDF animals or altered following resveratrol supplementation. ZDF rats displayed increased ($P < 0.05$) fibrosis (Fig. 2*A* and *B*), consistent with previous reports (Daniels *et al.* 2012). However, interestingly in the current study fibrosis was increased ∼65% at 11 weeks of age, comparable to the increase previously reported in 45-week-old ZDF rats (Daniels *et al.* 2012). It would therefore appear that fibrosis is an early event in the development of diabetic cardiomyopathy in the ZDF rat that is not exacerbated over time. In contrast, fibrosis was decreased ∼30% in ZDF rats supplemented with resveratrol and therefore, compared to lean control rats, cardiac fibrosis was not different in ZDF rats following resveratrol supplementation (Fig. 2*A* and *B*).

ADP respiratory sensitivity is impaired in ZDF rats and not recovered by resveratrol

Alterations in mitochondrial energy metabolism have repeatedly been implicated as a cause of diastolic dysfunction (reviewed in Ventura-Clapier *et al.* 2011). In particular, attenuated mitochondrial ADP-respiratory sensitivity in both the presence and absence of creatine is present in several models of heart failure (reviewed in Ventura-Clapier *et al.* 2011). Therefore, in the current study we first determined the apparent ADP-respiratory sensitivity in the presence of saturating pyruvate and malate. In all groups a typical Michaelis–Menten curve was observed (Fig. 3*A*), and therefore the apparent K_m and V_{max} were determined. However, in contrast to other models of heart failure, neither maximal ADP-stimulated respiration (Fig. 3*B*) nor the sensitivity to ADP (Fig. 3*C*) were altered in the ZDF rat or following resveratrol supplementation. The current working model predicts that the majority of ADP transport is associated with creatine kinase activity (Aliev *et al.* 2011) and therefore in a small subset of animals ($n = 5$) we also assessed ADP transport in the presence of 20 mM creatine. While the apparent K_m values were lower in the presence of creatine, they were not different in any group (LC, 102 ± 11; ZDF, 97 ± 14; Z-Resv, 135 ± 35). These data are consistent with the observation that permeabilized fibres from the atria of type 2 diabetic humans display normal ADP respiratory kinetics (Anderson *et al.* 2009*a*), further suggesting ADP transport is not affiliated with diabetic cardiomyopathy.

Complex I-supported substrate sensitivity in ZDF rats and following resveratrol supplementation

Given that ADP-respiratory sensitivity was not altered in the ZDF rat, we next determined the apparent respiratory

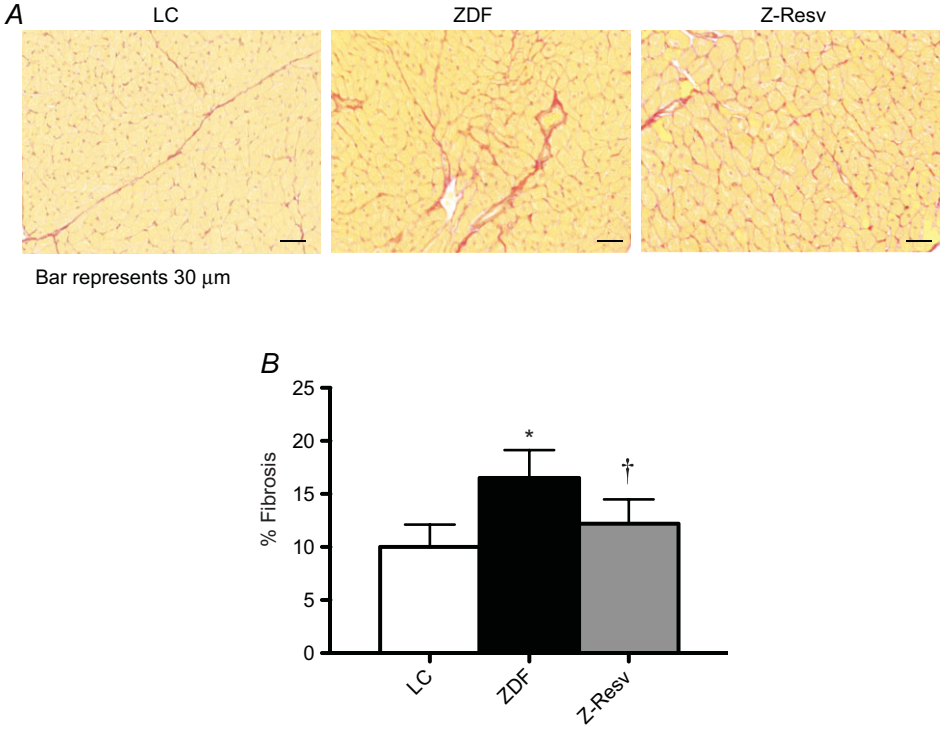

Figure 2. Left ventricular fibrosis in LC, ZDF and Z-Resv rats
Representative images are shown at ×4.2 magnification, and the black bar represents 30 μm. Values represent means ± SEM. $n = 4$–5. ∗Significantly ($P < 0.05$) different from LC. †Significantly ($P < 0.05$) different from ZDF.

sensitivity to pyruvate in the presence of 5 mM ADP. Similar to ADP titrations, substrate responses displayed typical Michaelis–Menten kinetics (Fig. 4*A*). Maximal pyruvate + malate-supported respiration was not different in any group studied (Fig. 4*B*), and was also similar to the V_{max} reported for the reverse experiment (titrating ADP in presence of pyruvate + malate; Fig. 3*B*). In addition, the apparent sensitivity to pyruvate was not altered in any group (Fig. 4*C*). The unaltered maximal respiration following ADP and pyruvate titrations suggests the absence of mitochondrial biogenesis. This is supported by the finding that protein content was not altered in the subunits of the electron transport chain in any group (Fig. 5*A*–*F*). Altogether, these data strongly suggest an absence of changes within the electron transport chain in ZDF rats, or following resveratrol supplementation.

P-CoA sensitivity is impaired in ZDF rats and improved following resveratrol supplementation

The heart produces more than 90% of its energy aerobically, the majority of which is derived from lipid metabolism within mitochondria (Ventura-Clapier *et al.* 2011). We therefore next determined mitochondrial respiratory sensitivity to P-CoA, a naturally occurring lipid that represents the majority of lipid available for

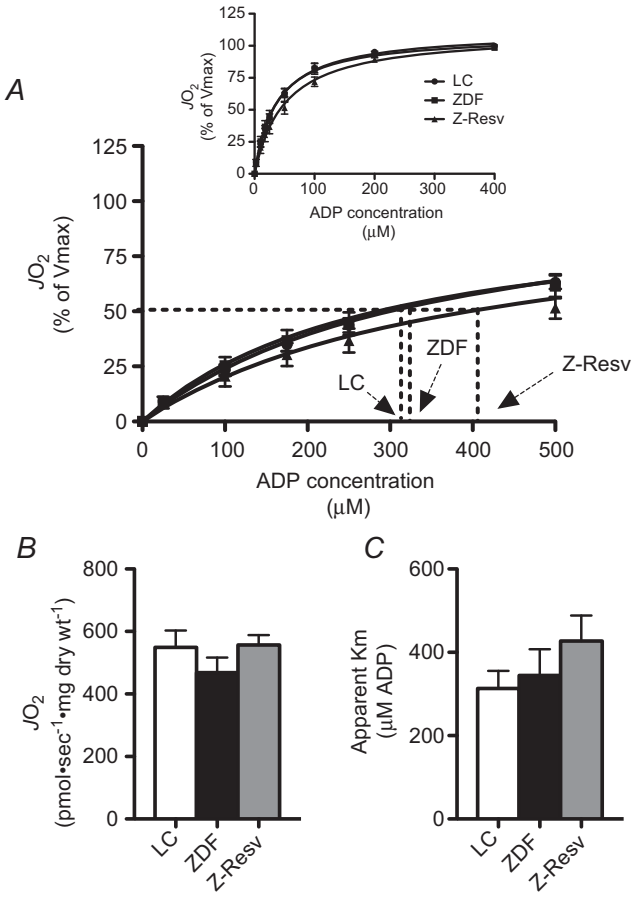

Figure 3. ADP respiratory kinetics in permeabilized muscle fibres from the left ventricle of LC, ZDF and Z-Resv rats
ADP titrated in the presence of 10 mM pyruvate and 2 mM malate revealed typical Michaelis–Menten kinetics (*A*). Maximal respiration (*B*; V_{max}) and the apparent K_m (*C*) were not different in any group. Values represent means ± SEM. n = 12–13 for determination of the apparent K_m. A few fibres were not adequately recovered to determine maximal respiration normalized to dry weight, and therefore n = 8 for V_{max}. JO_2 = rate of oxygen consumption.

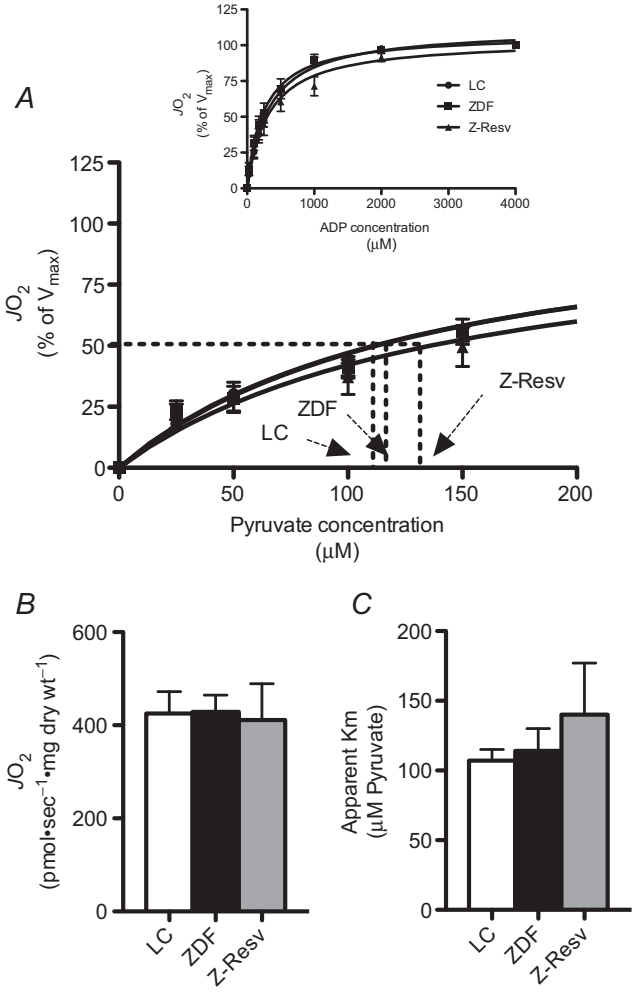

Figure 4. Pyruvate respiratory kinetics in permeabilized muscle fibres from the left ventricle of LC, ZDF and Z-Resv rats
Pyruvate was titrated in the presence of 5 mM ADP and 2 mM malate revealed typical Michaelis–Menten kinetics (*A*). Maximal respiration (*B*; V_{max}) and the apparent K_m (*C*) were not different in any group. Values represent means ± SEM. n = 9–12 for determination of the apparent K_m. A few fibres were not adequately recovered to determine maximal respiration normalized to dry weight, and therefore n = 6–7 for V_{max}.

oxidation *in vivo*. While maximal P-CoA-supported respiration was not altered in ZDF rats (Fig. 6*B*), in contrast, the ZDF rats displayed an attenuation in P-CoA sensitivity (apparent K_m increased ∼60%; Fig. 6*C*), supporting previous work showing a reduction in the expression of the liver isoform of carnitine palmitoyltransferase I (CPTI) at 7 weeks of age in ZDF rats (Zhou *et al.* 2000). Resveratrol supplementation increased maximal respiration ∼40% (Fig. 6*B*), while simultaneously recovering normal P-CoA respiratory sensitivity (Fig. 6*C*). Mitochondria in the hearts of ZDF rats therefore appear to have a specific dysfunction within either mitochondrial membrane lipid transport and/or β-oxidation that resveratrol fully recovers.

Lipid accumulation is reduced in the hearts of ZDF rats following resveratrol supplementation

In healthy hearts, approximately 70–90% of the fatty acids taken up are immediately oxidized (Wisneski *et al.* 1987; Chandler *et al.* 2003), while only a small proportion is esterified to triacylglycerol (TAG). Nevertheless, an accumulation of TAG within the heart has been shown to occur in 6-week-old ZDF rats (Bonen *et al.* 2009)

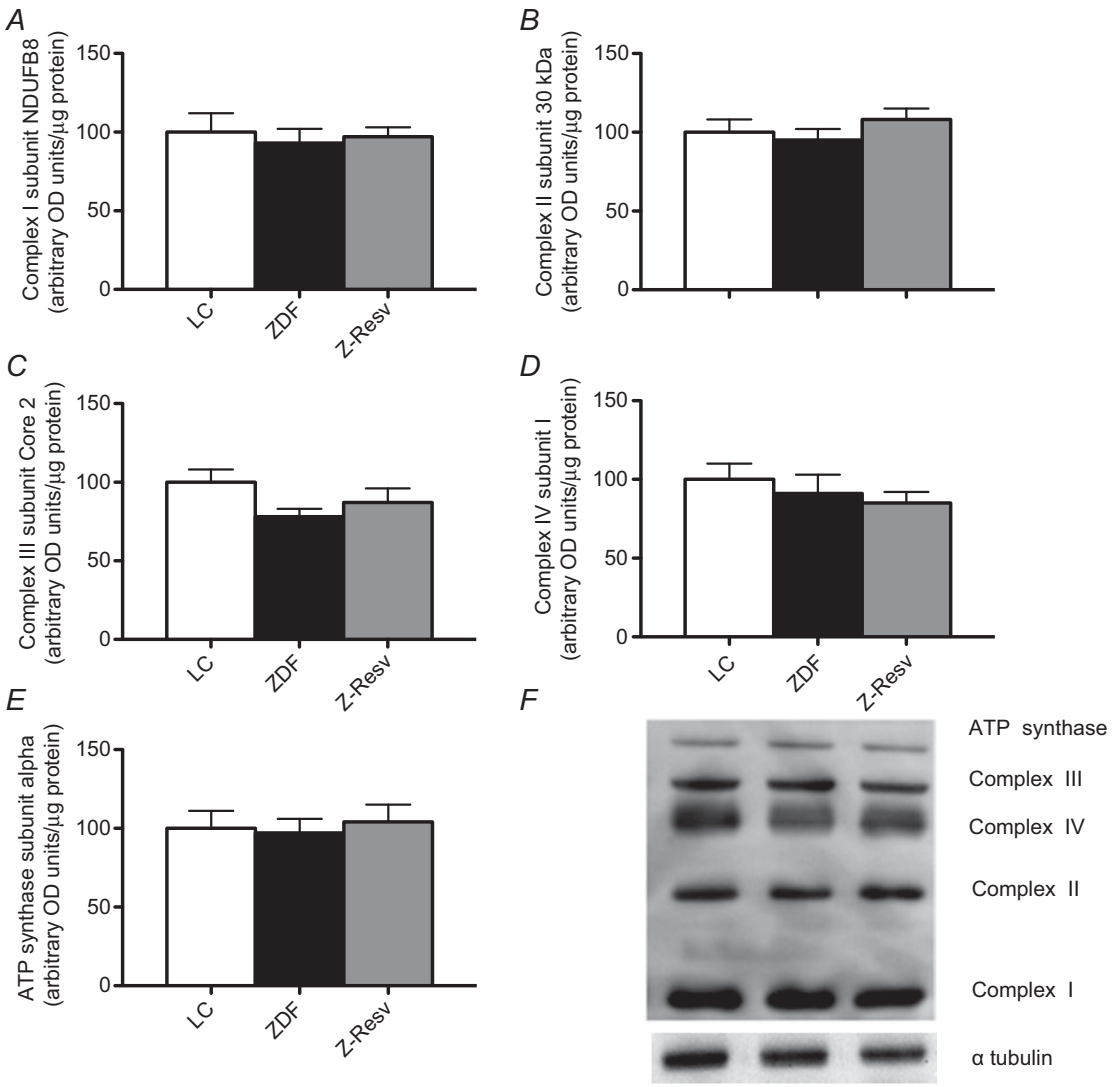

Figure 5. Markers of electron transport chain protein content in the left ventricle of LC, ZDF and Z-Resv rats
Complex I subunit NDUFB8 (*A*), complex II subunit 30 kDa (*B*), complex III subunit core 2 (*C*), complex IV subunit I (*D*), ATP synthase subunit α (*E*) were analysed and no significant differences were found. A representative OXPHOS blot, as well as α-tubulin which was used as a loading control, is shown in *F*. Values represent means ± SEM, $n = 7$ for all conditions, and 5 μg was loaded for all samples.

and is also associated with cardiac contractile dysfunction (Stanley *et al.* 2005). We therefore next aimed to determine if the observed improvements in mitochondrial P-CoA respiratory kinetics were associated with reductions in myocardial lipid species. Total heart TAG was increased ∼3.5-fold in the ZDF rat (Fig. 7*A*). While resveratrol decreased TAG content relative to untreated ZDF rats, it remained higher than lean control animals (Fig. 7*A*). The basic pattern of partial recovery of TAG following resveratrol supplementation was recapitulated in virtually all TAG species studied (Fig. 7*B*).

Similar to the response for TAG, total heart diacylglycerol (DAG) was increased in the ZDF rat (Fig. 7*A*), while resveratrol partially recovered total DAG content (Fig. 7*C*). The basic pattern of partial recovery of DAG following resveratrol was present for 16:0, 18:0 and 18:2n6c DAG species, which represented ∼62% of the total DAG species detected (Fig. 7*D*). In contrast, 18:1n9c and 16:1, which represent ∼4% of total DAG, were not altered by resveratrol (Fig. 7*D*).

Similar to both TAG and DAG, ZDF rats displayed increased total ceramide content within cardiac muscle (Fig. 7*E*). However, in contrast to TAG and DAG responses, resveratrol fully normalized total ceramide content to lean control values (Fig. 7*E*). In addition, the uniform increase in almost all TAG and DAG species determined was not recapitulated in ceramide species as only 16:0, 18:0 and 20:0 ceramide species were increased in ZDF animals (Fig. 7*F*). Of these, 16:0 and 18:0 ceramide contents, which represent ∼60% of total ceramide detected, were normalized following resveratrol treatment (Fig. 7*F*). Altogether, in ZDF rats an impairement in lipid-supported respiratory kinetics is associated with lipid accumulation, while resveratrol normalizes both of these responses, suggesting a strong association between mitochondrial respiratory lipid kinetics and lipid accumulation within the heart.

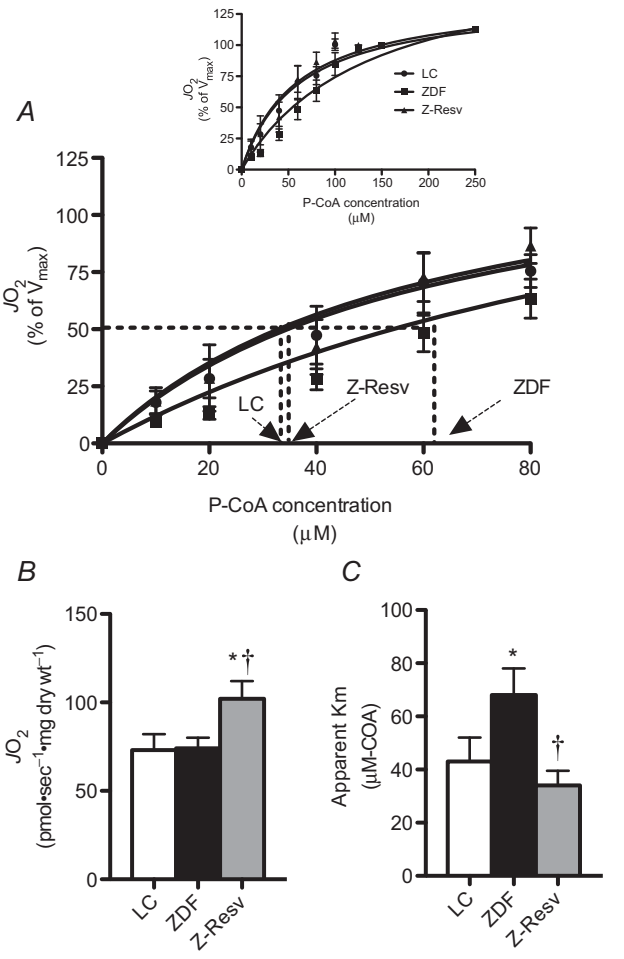

Figure 6. Palmitoyl-CoA (P-CoA) respiratory kinetics in permeabilized muscle fibres from the left ventricle of LC, ZDF and Z-Resv rats
P-CoA titrated in the presence of 5 mM ADP, 2 mM malate and 2 mM L-carnitine revealed typical Michaelis–Menten kinetics (*A*). Maximal respiration (*B*; V_{max}) and the apparent K_m (*C*) were not different in any group. Values represent means ± SEM. $n = 6$–7 for determination of the apparent K_m and V_{max}. *Significantly ($P < 0.05$) different from LC. †Significantly ($P < 0.05$) different from ZDF.

Increased mitochondrial reactive oxygen species emission in the hearts of ZDF rats is recovered following resveratrol supplementation

Mitochondrial ROS emission is increased in permeabilized muscle fibres from diabetic *db*/*db* mice and in the atria of type 2 diabetic humans (Boudina *et al.* 2007; Anderson *et al.* 2009*a*), and is thought to contribute to the diabetic cardiomyopathy phenotype (Nishikawa *et al.* 2000). We therefore also examined maximal rates of succinate-induced ROS emission, and similar to these previous reports show that mitochondrial ROS emission was increased ∼3–fold in a model of diabetes (ZDF rat; Fig. 8*A*). We also show that resveratrol supplementation fully normalized mitochondrial ROS emission (Fig. 8*A*), supporting previous work showing resveratrol improved oxidative stress in diabetic models (Thirunavukkarasu *et al.* 2007; Qin *et al.* 2012; Soufi *et al.* 2012). While maximal electron transport chain ROS capacity was increased in the ZDF rat, this was not associated with alterations in the ratio of reduced/oxidized glutathione (total GSH and GSSG not altered; data not shown), a sensitive barometer of the *in vivo* redox state (Fig. 8*B*), or in the expression of SOD2 or CAT proteins (Fig. 8*C* and *D*, respectively). While redox stress is apparent in the heart of humans with type 2 diabetes (Anderson *et al.* 2009*a*), in *db*/*db* mice (Boudina *et al.* 2007), and in ZDF rats at 15 weeks of age (Guleria *et al.* 2013), the current data suggest that overt oxidative stress had not developed at this early stage in the ZDF disease progression.

Discussion

The current data provide evidence that before overt diabetic cardiomyopathy develops in ZDF rats there is (1) increased fibrosis, (2) decreased mitochondrial P-CoA sensitivity, (3) accumulation of reactive lipids, and (4) an increased propensity for mitochondrial ROS emission. Interestingly, the provision of resveratrol (5) completely normalizes almost all of these metabolic derangements and therefore provides further understanding to the molecular benefits of resveratrol in improving cardiovascular health in the context of diabetes.

Fibrosis has a well-defined relationship with changes in rates of relaxation and compromised ventricular function. In contrast, the biological significance of impaired P-CoA sensitivity is currently unknown. Chronically, attenuated P-CoA sensitivity may result in lipid accumulation within the heart and diastolic dysfunction, and therefore be maladaptive and contribute to the development of diabetic cardiomyopathy. This is supported by the finding that

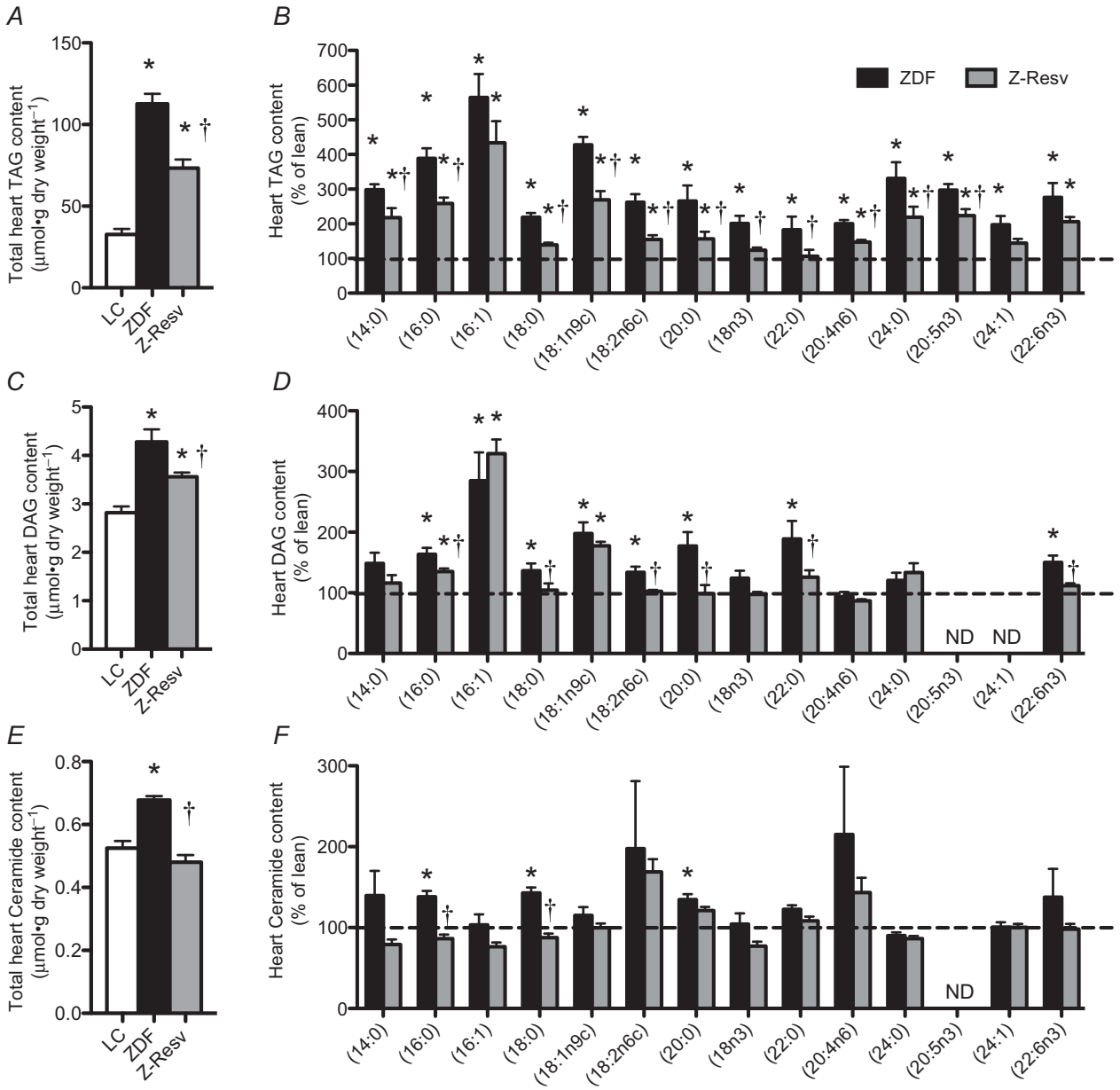

Figure 7. Left ventricular triacylglycerol (TAG), diacylglycerol (DAG) and ceramide contents in LC, ZDF and Z-Resv rats

Total and various lipid species of TAG (*A* and *B*), DAG (*C* and *D*) and ceramides (*E* and *F*) were determined using gas chromatography. Values represent means ± SEM. $n = 8$ for all analyses. ∗Significantly ($P < 0.05$) different from LC. †Significantly ($P < 0.05$) different from ZDF.

heterozygous CPTIb mice challenged with a pressure overload display lipotoxicity, impaired stroke volume and cardiac output, and increased fibrosis (He *et al.* 2012). However acutely, an attenuation in P-CoA sensitivity may be adaptive to prevent excessive fatty acid accumulation within mitochondria and the subsequent generation of ROS. This interpretation may help explain why pharmacological approaches that decrease lipid transport into mitochondria are beneficial in the context of diabetic cardiomyopathy (Schmitz *et al.* 1995; Turcani & Rupp, 1997; Lee *et al.* 2005; Abozguia *et al.* 2010).

While convenient to conclude that attenuated P-CoA respiratory kinetics would directly impair contractile function, ZDF hearts display an increased rate of fatty acid oxidation (Wang *et al.* 2005; van den Brom *et al.* 2009). However, in various models, including ZDF rats, rates of plasma membrane long-chain fatty acid transport are increased, and therefore lipid 'delivery' to mitochondria enhanced (Bonen *et al.* 2009). The transport of lipids across the plasma membrane is now recognized to be a protein-mediated process that is highly regulated, and the increase in lipid transport into the heart of ZDF rats appears to involve the translocation of fatty acid translocase (FAT/CD36) to the plasma membrane (Bonen *et al.* 2009). This molecular event occurs early in the disease progression, as the ZDF rat displays an increase in plasma membrane FAT/CD36 at 6 weeks of age (Bonen *et al.* 2009). Therefore, the attenuation in mitochondrial P-CoA sensitivity observed in the current study may not affect contractile function or impair rates of fatty acid oxidation at the whole heart level, and instead maintain the appropriate absolute quantity of lipid transported into mitochondria. In a high-fat environment mitochondrially derived ROS are primarily thought to result from lipid oxidation, as mitochondrial ROS emission occurs at a very low lipid concentration, an observation attributed to the fact that the electron transfer flavoprotein (ETF) involved in β-oxidation is membrane potential independent (Seifert *et al.* 2010). However, in a model known to have increased lipid delivery into cardiac muscle (Bonen *et al.* 2009), in the current study, despite an increase in the capacity of the electron transport chain for ROS emission and unaltered SOD2 and CAT protein content, a sensitive barometer of the *in vivo* redox state (GSH/GSSG) was not altered in the ZDF rat, suggesting the absence of overt oxidative stress. Altogether, these data suggest that limiting fatty acid transport into mitochondria may be beneficial in the context of maintaining redox balance. Interestingly, resveratrol, a polyphenol compound that has inherent antioxidant properties, decreased the capacity for mitochondrial ROS emission as well as normalized P-CoA respiratory sensitivity. It is possible that ROS mediates the observed attenuation in P-CoA sensitivity as a feedback mechanism to prevent excessive lipid accumulation within mitochondria

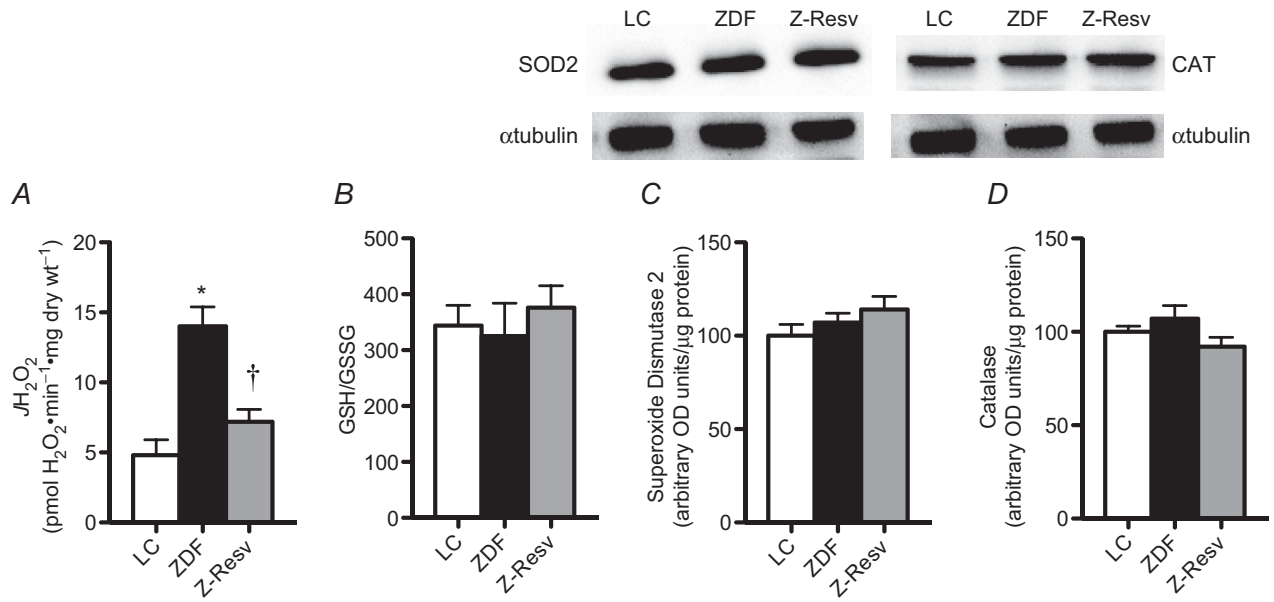

Figure 8. Rates of mitochondrial hydrogen peroxide emission ($J_{H_2O_2}$), the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) and antioxidant enzyme expression were determined in the left ventricle of LC, ZDF and Z-Resv rats

Rates of hydrogen peroxide were determine in permeabilized muscle fibres (*A*), while rapidly frozen whole muscle samples were used to determine GSH/GSSG (*B*) as well as the protein content of superoxide dismutase 2 (SOD2; *C*) and catalase (CAT; *D*) (OD, optical density). Values represent means ± SEM. $n = 5$–7. *Significantly ($P < 0.05$) different from LC. †Significantly ($P < 0.05$) different from ZDF.

and overt oxidative damage. Therefore, attenuating lipid transport into mitochondria may acutely maintain redox balance, and cardiac function. However, the obvious implications chronically to a reduction in P-CoA sensitivity involve impaired aerobic lipid metabolism, reactive lipid accumulation and compromised ventricular function.

Impaired ADP respiratory sensitivity is a common observation in various models of pressure-induced heart failure (Ventura-Clapier *et al.* 2011); however, this was not observed in the current model of early-stage diabetic cardiomyopathy. The attenuation in the apparent ADP respiratory K_m in other heart failure models appears to primarily occur as a result of a reduction in the expression of mitochondrial creatine kinase and adenine nucleotide translocase (ANT) (Ventura-Clapier *et al.* 2011). However, P-CoA has also been shown to interact with ANT, a required inner mitochondrial membrane ADP transporter. Specifically, long-chain acyl-CoA moieties have been shown to directly suppress ADP transport by competitively binding to the ADP binding site on ANT (Ho & Pande, 1974; Woldegiorgis & Shrago, 1979). Therefore, even in an absence of altered gene transcription of mitochondrial creatine kinase or ANT, a chronic attenuation in P-CoA respiratory sensitivity would be expected to impair ADP transport as a result of increased cytosolic and/or matrix P-CoA concentrations. This would be expected to further compromise aerobic respiration within the heart, and exaggerate an existing metabolic dysfunction. However, to date 'lipid'- and 'carbohydrate'-supported mitochondrial bioenergetics have been exclusively assessed independently, the current study included. Therefore, future work should consider assessing mitochondrial ADP-respiratory sensitivity in the presence of small quantities of P-CoA to better reflect the *in vivo* situation in hearts from diabetic animals.

Resveratrol, a polyphenol compound, has been shown to exert pleiotropic actions in several tissues that may be beneficial in the context of diabetic cardiomyopathy. Specifically, resveratrol has been shown to improve whole body glucose tolerance (Beaudoin *et al.* 2013), skeletal muscle glucose uptake (Smith *et al.* 2013), white adipose tissue metabolism (Beaudoin *et al.* 2013), and several derangements associated with hypertension (Rimbaud *et al.* 2011), myocardial infarction (Chen *et al.* 2008), ischaemia/reperfusion (Lin *et al.* 2008) and high-fat-diet-induced diabetic cardiomyopathy (Louis *et al.* 2012; Qin *et al.* 2012). In the context of diet-induced cardiac pathologies, resveratrol has been shown to prevent fibrosis, cellular oxidative stress and diastolic dysfunction (Louis *et al.* 2012; Qin *et al.* 2012). We extend these findings by suggesting resveratrol partially mediates these effects by improving mitochondrial bioenergetics. Specifically, we show that resveratrol normalized P-CoA respiratory sensitivity, increased maximal rates of P-CoA respiration and decreased maximal succinate-induced mitochondrial ROS emission. In addition, the previous observations that resveratrol improved cardiac redox balance were somewhat ambiguous (Louis *et al.* 2012; Qin *et al.* 2012), as it was not clear if this protection manifested from changes within cardiac tissue, or simply a response to systemic adaptations. However, the current study clearly shows that the increased propensity for mitochondrial ROS emission in ZDF rats is recovered following resveratrol supplementation, suggesting intrinsic changes within the heart contributes to the previously observed resveratrol-induced improvement in redox balance, although systemic adaptions are probably involved as well. The reduction in mitochondrial H_2O_2 emission in the current study, in combination with the antioxidant properties of resveratrol, probably contribute to the improvement in cardiac fibrosis, as decreasing cellular ROS production prevents cardiac fibrosis (Liu *et al.* 2010), while decreasing the antioxidant capacity of the heart (SOD1-deficient mice) induces gene expression patterns associated with fibrosis (Hagler *et al.* 2013).

In addition, in the current study, the recovery of mitochondrial P-CoA respiratory sensitivity may contribute to the normalization of reactive lipids in ZDF animals. Importantly, the cytotoxic effects of ceramides are well characterized in cardiomyocytes (Park *et al.* 2008), as ceramide induces apoptosis (Okere *et al.* 2006), probably aggravating the pathological development of diabetic cardiomyopathy, which is known to manifest with a decrease in mitochondrial content chronically (Ventura-Clapier *et al.* 2011). In the current study, ZDF rats primarily displayed an increase in 16:0 and 18:0 derived ceramides, which have also been reported to be elevated in CPTI heterozygous-induced cardiac pathologies (He *et al.* 2012). Therefore, there appears to be a clear association between impaired lipid transport into mitochondria and the accumulation of specific ceramide species. In combination with normalizing P-CoA respiratory kinetics, resveratrol fully normalized total ceramide content as well as 16:0 and 18:0 derived ceramide species. Therefore, resveratrol improved all aspects of mitochondrial bioenergetics and reactive lipid profiles in hearts from the ZDF rat.

Altogether the current data support the supposition that both cardiac fibrosis and a chronic dysfunction within mitochondrial lipid-supported bioenergetics contribute to the development of diabetic cardiomyopathy. Specifically, we show that P-CoA respiratory sensitivity is impaired in ZDF rats, which coincides with an accumulation of intramyocellular lipids and cardiac fibrosis. In addition, we show resveratrol supplementation prevents these changes, supporting the belief that resveratrol is a potent therapeutic approach for preventing diabetic cardiomyopathy.

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Additional information

Competing interests

The authors have no conflict of interest to disclose.

Author contributions

G.P.H. designed experiments, performed experiments, analysed and interpreted data and wrote the manuscript. M.-S.B., C.G.R.P., D.C.W., J.A.S., A.A. and A.C. performed experiments, analysed data and edited the manuscript. All authors have read and approved the final version of the manuscript. All experiments were conducted at the University of Guelph, with the exception of the GC lipid analyses which were conducted at the Medical University of Bialystok.

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