J Physiol 591.23 (2013) pp 6089–6101

The Journal of Physiology

Submaximal ADP-stimulated respiration is impaired in ZDF rats and recovered by resveratrol

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Key points

- Disparity exists within the literature surrounding mitochondrial dysfunction and insulin resistance and previous reports have primarily examined mitochondrial function as a capacity measurement.
- We show that submaximal ADP-stimulated respiration rates are lower in ZDF rats, which coincides with decreased adenine nucleotide translocase 2 (ANT2) protein content.
- Supplementation of ZDF rats with resveratrol improves skeletal muscle insulin sensitivity, increases submaximal ADP-stimulated respiration rates and increases ANT2 protein content.
- Improvements in the ability of ADP to attenuate mitochondrial reactive oxygen species (ROS) emission and cellular redox balance were also observed following resveratrol supplementation.
- These data suggest that mitochondrial dysfunction is present in skeletal muscle insulin resistance when assessed at submaximal ADP concentrations and that ADP dynamics may influence skeletal muscle insulin sensitivity through alterations in the propensity for ROS formation.

Abstract Mitochondrial dysfunction and reactive oxygen species (ROS) have been implicated in the aetiology of skeletal muscle insulin resistance, although there is considerable controversy regarding these concepts. Mitochondrial function has been traditionally assessed in the presence of saturating ADP, but ATP turnover and the resultant ADP is thought to limit respiration *in vivo*. Therefore, we investigated the potential link between submaximal ADP-stimulated respiration rates, ROS generation and skeletal muscle insulin sensitivity in a model of type 2 diabetes mellitus, the ZDF rat. Utilizing permeabilized muscle fibres we observed that submaximal ADP-stimulated respiration rates (250–2000 μM ADP) were lower in ZDF rats than in lean controls, which coincided with decreased adenine nucleotide translocase 2 (ANT2) protein content. This decrease in submaximal ADP-stimulated respiration occurred in the absence of a decrease in electron transport chain function. Treating ZDF rats with resveratrol improved skeletal muscle insulin resistance and this was associated with elevated submaximal ADP-stimulated respiration rates as well as an increase in ANT2 protein content. These results coincided with a greater ability of ADP to attenuate mitochondrial ROS emission and an improvement in cellular redox balance. Together, these data suggest that mitochondrial dysfunction is present in skeletal muscle insulin resistance when assessed at submaximal ADP concentrations and that ADP dynamics may influence skeletal muscle insulin sensitivity through alterations in the propensity for mitochondrial ROS emission.

DOI: 10.1113/jphysiol.2013.259226

(Received 23 May 2013; accepted after revision 27 September 2013; first published online 30 September 2013)
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Abbreviations ANT, adenine nucleotide translocase; CPT-I, carnitine palmitoyltransferase I; FFA, free fatty acid; LC, lean control; P-CoA, palmitoyl-coenzyme A; PmFB, permeabilzed muscle fibre bundle; ROS, reactive oxygen species; T2DM, type 2 diabetes mellitus; ZDF, Zucker diabetic fatty.

Introduction

Skeletal muscle, by virtue of its mass and overall rate of insulin-stimulated glucose disposal, is a highly important tissue in the aetiology of insulin resistance and type II diabetes mellitus (T2DM). The mechanisms that result in skeletal muscle insulin resistance remain poorly elucidated and controversial, although a number of hypotheses have been proposed. For example, mitochondrial dysfunction has been suggested to be a cause of skeletal muscle insulin resistance, and is classically defined as a reduction in mitochondrial content and/or an intrinsic impairment within mitochondria (Kelley *et al.* 2002; Lowell & Shulman, 2005), but there is significant discrepancy within the literature surrounding this concept (Kelley *et al.* 2002; Lowell & Shulman, 2005; Mogensen *et al.* 2007; Boushel *et al.* 2007; Phielix *et al.* 2008; de Feyter *et al.* 2008*a*,*b*; Holloszy, 2009; Holloway *et al.* 2010; Lenaers *et al.* 2010; Hoeks & Schrauwen, 2012).

Of potential importance, mitochondrial function has been traditionally assessed as a capacity measurement as maximal ADP concentrations have been utilized to promote mitochondrial respiration (Boushel *et al.* 2007; Mogensen *et al.* 2007; Phielix *et al.* 2008). However, these previous examinations of mitochondrial function may not reflect the *in vivo* situation as under most physiological conditions ATP turnover and subsequent ADP provision is thought to limit respiration (Wilson, 1994). Therefore, from a physiological standpoint, it may be more appropriate to examine mitochondrial function in the presence of submaximal concentrations of ADP, although this has yet to be considered within the context of skeletal muscle insulin resistance.

In addition to mitochondrial dysfunction, a causal role of mitochondrial reactive oxygen species (ROS) production has been proposed to explain the progression of insulin resistance and T2DM in animals and humans (Brownlee, 2001; Houstis *et al.* 2006; Anderson *et al.* 2009). In support of this concept, mitochondrial ROS generation is elevated in the over-fed and insulin-resistant states, and attenuating mitochondrial ROS emission using mitochondrial-targeted antioxidant approaches prevents diet-induced insulin resistance (Anderson *et al.* 2009; Boden *et al.* 2012). Therefore, numerous studies (Houstis *et al.* 2006; Chen *et al.* 2008; Anderson *et al.* 2009; Hoehn *et al.* 2009; Lee *et al.* 2010; Boden *et al.* 2012) have suggested that by decreasing mitochondrial-derived ROS, insulin sensitivity can be maintained or improved. However, a relationship between mitochondrial ROS and insulin resistance is not universally found, indicating there is still some disparity (Paglialunga *et al.* 2012).

Importantly, the supply of ADP to mitochondria decreases the propensity for ROS emission (Korshunov *et al.* 1997). Therefore, the ability of the mitochondria to transport ADP into the mitochondrial matrix to subsequently decrease ROS emission may have a role in the protection of insulin sensitivity. In this regard, adenine nucleotide translocase (ANT) has an obligatory role in counter-transporting ADP and ATP across the inner mitochondrial membrane (Klingenberg, 2008). Skeletal muscle expresses two primary ANT isoforms, ANT1 and ANT2, but ANT2 possesses a higher transport efficiency and has a higher V_{max} (Dorner *et al.* 2006). The transport of ADP is also the governing factor for oxidative phosphorylation (Pfaff *et al.* 1965), indicating that any alteration in ADP transport can directly impact mitochondrial function (Dorner *et al.* 2006). Therefore, ADP transport may sit as a nexus between mitochondrial function/dysfunction and mitochondrial ROS production. Intriguingly, the polyphenolic compound resveratrol has been previously shown to improve the oxidative status of the muscle (Chen *et al.* 2011) concomitantly with improving insulin sensitivity (Baur *et al.* 2006) and may increase ANT expression (Seymour *et al.* 2010; Rimbaud *et al.* 2011).

Therefore, we investigated the potential link between submaximal ADP-stimulated respiration rates, ROS generation and skeletal muscle insulin sensitivity in a widely used model of T2DM, the ZDF rat. It is hypothesized that submaximal ADP-stimulated respiration will be repressed in the ZDF rat and that resveratrol will recover this decrement back to lean control values.

Methods

Animals

Male ZDF rats (Charles River, Wilmington, MA, USA) were housed in individual cages, with a reverse 12:12 h light–dark cycle, and were provided with food and water *ad libitum*. Lean control (LC) rats ($n = 13$) were fed a

stock diet. Twenty-six ZDF (Zucker diabetic fatty) rats ($n = 13$ each group) were randomly assigned to either a stock diet (ZDF) or a stock diet supplemented with resveratrol (Cayman Chemicals, Ann Harbor, MI, USA; 200 mg kg^{-1} body weight similar to previous publications (Lagouge *et al.* 2006)) for 6 weeks (ZDF+RESV). Previous work has shown that ZDF rats are insulin resistant at 6 weeks and become type II diabetic by 12 weeks, so we attempted to delay the onset of skeletal muscle insulin resistance by treating with resveratrol from 5 weeks until 11 weeks of age (Suh *et al.* 2005). Soleus (for glucose uptake) and red gastrocnemius (all other experimental procedures) muscles were removed under isoflurane. Originally, this study was planned with $n = 8$ for all experiments. However, upon completing the first set of experiments additional animals ($n = 5$) were acquired to determine a link between decreased ADP transport and mitochondrial ROS emission (i.e. ROS in the presence of ADP (see Fig. 6*B*)). Additionally, ADP titrations (see Fig. 3) and maximal ROS (see Fig. 6*A*) measurements were repeated as these are key findings and we aimed to ensure repeatability ($n = 13$). 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 circulating variables

Fasting blood glucose (Freestyle lite, Abbott Laboratories, St-Laurent, QC, Canada), insulin (ELISA: Millipore, Billerica, MA, USA), free fatty acids (FFAs; colorimetric assay: Wako Diagnostics, Richmond, VA, USA) and triglycerides (colorimetric assay: Sigma-Aldrich, Oakville, ON, Canada) we all measured from commercially available kits.

Basal and insulin-stimulated glucose transport

Glucose transport was measured as previously described (Ritchie *et al.* 2011). Briefly, pregassed (95% O_2/5% CO_2) Medium 199 containing 0.1% bovine serum albumin (BSA) was warmed to 30°C and used as a base for all glucose uptake buffers. Insulin (10 mU ml^{-1}) (Humulin R; Eli Lilly, Toronto, Ontario, Canada) was added to all buffers for the insulin-stimulated condition. Excised soleus strips were placed in glass vials containing pre-incubation buffer for 30 min in the presence or absence of insulin (10 mU ml^{-1}; maintained in all subsequent buffers). Soleus strips were then weighed and digested for 10 min in 1 ml of 1 M NaOH at 95°C. Muscle from each sample was sampled in duplicate and analysed by liquid scintillation counting, from which glucose transport was calculated.

Preparation of permeabilized muscle fibres

The preparation of permeabilized muscle fibre bundles (PmFBs) was adopted from Perry *et al.* (2011), as we have previously reported (Smith *et al.* 2011). Following dissection of red gastrocnemius, fibre bundles (~2 mg) were separated in BIOPS buffer containing, CaK_2-EGTA (2.77 mM), K_2-EGTA (7.23 mM), Na_2-ATP (5.77 mM), $MgCl_2.6H_2O$ (6.56 mM), Na_2-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 BSA (1 g l^{-1}). Fibres prepared for H_2O_2 emission were washed in Buffer Z containing K-MES (110 mM), KCl (30 mM), EGTA (1 mM), K_2HPO_4 (10 mM) and $MgCl_2.6H_2O$ (10 mM). PmFBs were left in cold MIRO5 or Buffer Z until analysis.

Permeabilized muscle fibre respiration

Mitochondrial respiration was measured in PmFBs by high-resolution respirometry (Oroboros Oxygraph-2 k, Innsbruck, Austria) at 37°C and room air saturated oxygen tension in the presence of 25 μM blebbistatin to ensure PmFB relaxation (Perry *et al.* 2011). In the presence of 5 mM ADP and 2 mM malate, separate PmFBs from the same animal were used to determine complex I (pyruvate) and complex I+II (pyruvate+succinate) respiration rates. Separate PmFBs from the same animal in the presence of 5 mM ADP and 2 mM malate were then used to determine glutamate (0, 100, 175, 250, 500, 2000, 4000 μM) and pyruvate (0, 15, 30, 50, 75, 150, 500, 1000 μM) stimulated respiration across a range of substrate concentrations. ADP (0, 100, 175, 250, 500, 1000, 2000, 4000, 6000 μM)-stimulated respiration was determined in the presence of 10 mM pyruvate and 2 mM malate. To measure palmitoyl-coenzyme A (P-CoA)-supported respiration, MIRO5 + 5 mM ADP, 2 mM malate and 2 mM L-carnitine was used as the respiration medium. P-CoA (150 μM) was added to initiate respiration. To measure palmitate-supported respiration, MIRO5 + 1 mM ATP + 5 mM ADP, 2 mM malate, and 2 mM L-carnitine + 1 mM CoA was used as the respiration medium. Palmitate (250 μM) was added to initiate fatty acid-supported respiration. 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.

Permeabilized muscle fibre mitochondrial H_2O_2 emission

Measurement of mitochondrial H_2O_2 emission was similar to a previously described method (Anderson *et al.* 2009). Briefly, mitochondrial H_2O_2 emission was determined fluorometrically (Lumina, Thermo Scientific, Waltham, MA, USA) in a constantly stirring cuvette at 37°C (peltier controlled). PmFBs were placed in a cuvette containing Buffer Z supplemented with 25 μM blebbistatin, 40 U ml^{-1} CuZnSOD, 10 μM Amplex Red (Invitrogen, Carlsbad, CA, USA), 0.5 U ml^{-1} horseradish peroxidase with or without 100 μM ADP. 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/min), after subtracting the background from a standard curve established with the same reaction conditions and normalized to freeze-dried muscle weight.

Glutathione measurements

GSH and GSSG measurements were determined as previously described (Anderson *et al.* 2009). Briefly, two muscle chips (in triplicate wells) were used to determine GSH and two separate muscle chips (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 the manufacturer's instructions (Oxis International, Inc. Beverly Hills, CA, USA).

Western blotting

All samples were analysed for total protein (BCA protein assay), and samples were separated by electrophoresis on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes as previously reported (Smith *et al.* 2011). Commercially available antibodies were used to detect OXPHOS cocktail (ab110413, Abcam, Mitosciences, Cambridge, MA, USA), ANT1 (ab110322, Abcam), ANT2 (ab118125, Abcam) and cytochrome *c* oxidase complex IV (A21347, Invitrogen).

Statistics

The apparent K_m for glutamate, pyruvate and ADP was estimated on independent experiments utilizing 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 previously described (Perry *et al.* 2012). A one-way analysis of variance (ANOVA) with a Newman–Keuls *post hoc* analysis was utilized for all comparisons except for Fig. 1*A* where a Student's two-tailed *t* test was used. Statistical significance was accepted at $P \leq 0.05$.

Results

Resveratrol recovers skeletal muscle insulin sensitivity in ZDF rats

The body weights of the ZDF and ZDF+RESV groups were significantly elevated ($P < 0.05$) compared to LC (Table 1). Importantly, fasting blood glucose and insulin concentrations were significantly increased in the ZDF group compared to LC. Resveratrol increased blood insulin concentrations and normalized blood glucose values. Circulating FFAs and triglyceride were also significantly elevated in the ZDF group, but these values were not altered with resveratrol supplementation (Table 1).

To investigate insulin-stimulated glucose uptake in skeletal muscle, we utilized an incubated soleus muscle preparation. Insulin increased glucose uptake in LC animals (~40%), but did not stimulate glucose uptake in untreated ZDF rats ($P > 0.05$). In contrast, administration of resveratrol in ZDF rats increased insulin-stimulated glucose uptake ~40%. As a result, insulin-stimulated glucose uptake was only repressed in the soleus muscle of untreated ZDF animals (Fig. 1*A*).

Submaximal ADP-stimulated respiration is impaired in ZDF rats

Skeletal muscle insulin sensitivity was not associated with alterations in the maximal capacity of the electron transport chain (complex I and complex I+II) as the LC, ZDF and ZDF+RESV groups displayed similar maximal rates (Fig. 1*B*). To examine mitochondrial respiratory characteristics, we determined respiration rates in the presence of various concentrations of glutamate or pyruvate, which supply reducing equivalents to complex I. Maximal respiration, the apparent K_m and respiration rates at submaximal substrate concentrations for glutamate and pyruvate were not different between groups (Figs. 2 and 3), indicating that electron transport chain function was similar. In contrast, while the ADP apparent K_m and V_{max} values were not significantly different between groups (Fig. 4*B* and *C*) submaximal ADP-stimulated respiration rates (250, 500, 2000 μM ADP; Fig. 4*A*) in ZDF rats were significantly lower than in both LC and resveratrol-treated ZDF rats.

These data are the first reporting submaximal ADP-stimulated respiration in a model of T2DM and suggest that mitochondrial dysfunction is present when assessed in this manner (Fig. 4), while electron transport capacity is not altered.

Table 1. Basic characteristics

	Lean control	ZDF	ZDF+RESV
Body weight (g)	288 ± 9.0	384 ± 5.2*	371 ± 7.5*
Blood glucose (mmol l^{-1})	4.1 ± 0.8	11.0 ± 1.8*†	5.4 ± 0.3
Insulin (pmol l^{-1})	257.7 ± 73.3	960.7 ± 118.4*†	1536.1 ± 155.4*
FFA (mmol l^{-1})	0.6 ± 0.05	0.9 ± 0.15*	1.0 ± 0.15*
Triglycerides (mmol l^{-1})	0.2 ± 0.02	4.8 ± 0.6*	4.3 ± 0.6*

Values represent means ± SEM; n = 8. *Significantly ($P < 0.05$) different from LC. †Significantly ($P < 0.05$) different from ZDF+RESV.

Lipid-supported respiration is elevated with resveratrol treatment

P-CoA- and palmitate-supported state III (5 mM ADP) respiration rates were not different between LC and ZDF (Fig. 5*A* and *B*). In contrast, state III P-CoA- ($P = 0.08$) and palmitate- ($P < 0.05$) supported respiration rates were higher in resveratrol-treated animals in the face of unchanged respiratory capacity (Fig. 5*A* and *B*).

Mitochondrial and ANT content

In ZDF rats treated with resveratrol there was no induction of mitochondrial biogenesis (representative blots shown in Fig. 6*A*) to account for the increased submaximal ADP-stimulated respiration observed in the current study. Considering submaximal ADP-stimulated respiration appears to be depressed in the ZDF rat and recovered with resveratrol supplementation, we next measured the protein expression of ANT1 and 2 to investigate if these differences in ADP-stimulated respiration were affiliated with the content of ADP transport proteins. ANT1 protein abundance was not different across the three groups (Fig. 6*B*). In contrast, ANT2 protein content was significantly reduced in the ZDF group compared to LC (−34%; Fig. 6*C*) and following resveratrol supplementation, ANT2 protein content was not different from LC values. The increase in ANT2 protein content may help account for the improved submaximal

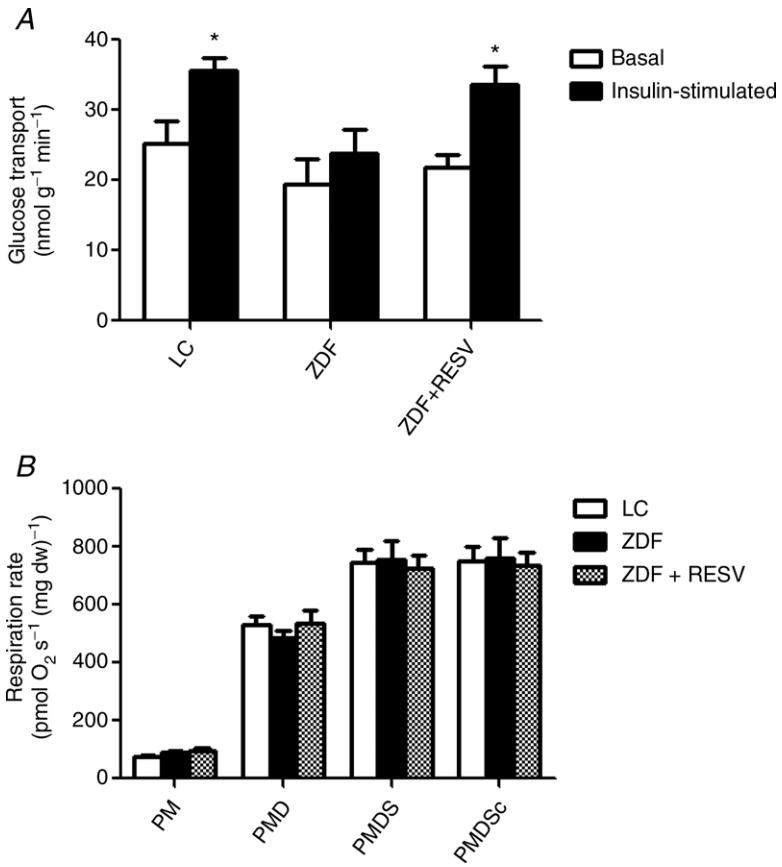

Figure 1. Depressed insulin-stimulated glucose uptake is not associated with decreased mitochondrial respiratory capacity
A, soleus strips from control ZDF rats (ZDF) were unable to significantly promote glucose (3-*O*-methyl-glucose) uptake in response to 10 mU ml^{-1} insulin, and resveratrol treatment (ZDF+RESV) recovers this decrement. *B*, mitochondrial complex I and complex I+II supported respiratory capacity is not different between lean control (LC), ZDF and ZDF+RESV. Respiratory control ratios were also unchanged: LC = 7.59 ± 0.63, ZDF = 7.65 ± 0.63 and ZDF+RESV = 8.08 ± 0.27. Values represent means ± SEM; n = 8. *Significantly different ($P < 0.05$) from basal value. PM, pyruvate+malate; PMD, pyruvate+malate+ADP; PMDS, pyruvate+malate+ADP+succinate; PMDSc, pyruvate+malate+ADP+cytochrome *c*.

ADP-stimulated respiration observed with resveratrol supplementation.

Mitochondrial H_2O_2 emission and cellular redox state

We next investigated a potential mechanism of action to account for how submaximal ADP-stimulated respiration could be related to skeletal muscle insulin sensitivity. Mitochondrial-derived ROS have been linked to insulin resistance and the provision of ADP to mitochondria can decrease ROS production (Korshunov *et al.* 1997; Anderson *et al.* 2009). Therefore, we examined mitochondrial H_2O_2 emission rates (a marker of mitochondrial ROS production) in the presence and absence of a submaximal ADP concentration (100 μM). Between groups, the maximal capacity of mitochondria to emit H_2O_2 was not different (Fig. 7*A*). In contrast, in the presence of a submaximal ADP concentration, H_2O_2 emission rates were ∼34% higher in the ZDF group compared to LC ($P = 0.06$; Fig. 7*B*). Following treatment with resveratrol, H_2O_2 emission rates were ∼40% lower ($P < 0.05$) in the ZDF+RESV group compared to ZDF (Fig. 7*B*). These data suggest that resveratrol treatment may improve skeletal muscle insulin sensitivity by enhancing ADP transport, which results in a decrease in mitochondrial ROS production. To determine if these changes in mitochondrial H_2O_2 emission rates were associated with the cellular redox state, we measured the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG ratio). The LC animals surprisingly had the lowest GSH/GSSG ratios, suggesting they had the greatest oxidative stress despite not being overfed (Table 2). It may be that ZDF animals have evolved compensatory adaptations to accommodate the chronic fuel oversupply. Nonetheless, resveratrol treatment increased the GSH/GSSG ratio, independent of altering total GSH content within the ZDF animal, suggesting that the skeletal muscle of the ZDF+RESV group was less oxidized than that of the ZDF group, supporting our functional PmFB data. Together, these data suggest a potential link between mitochondrial function and mitochondrial ROS production in the aetiology of skeletal muscle insulin resistance.

Discussion

In the current study we provide evidence for a potential link between mitochondrial function and ROS emission in the context of skeletal muscle insulin sensitivity. Specifically, we show that mitochondrial respiration at submaximal ADP concentrations is impaired in a model of T2DM, an observation that coincided with decreased ANT2 protein content and a decreased ability of ADP to attenuate mitochondrial ROS emission. We further show that resveratrol treatment in ZDF rats recovers skeletal muscle insulin sensitivity, submaximal ADP-stimulated respiration and ANT2 protein content in concert with improving ADP attenuation of mitochondrial ROS and cellular redox balance (GSH/GSSG ratio). Together, our data suggest a novel link between mitochondrial function,

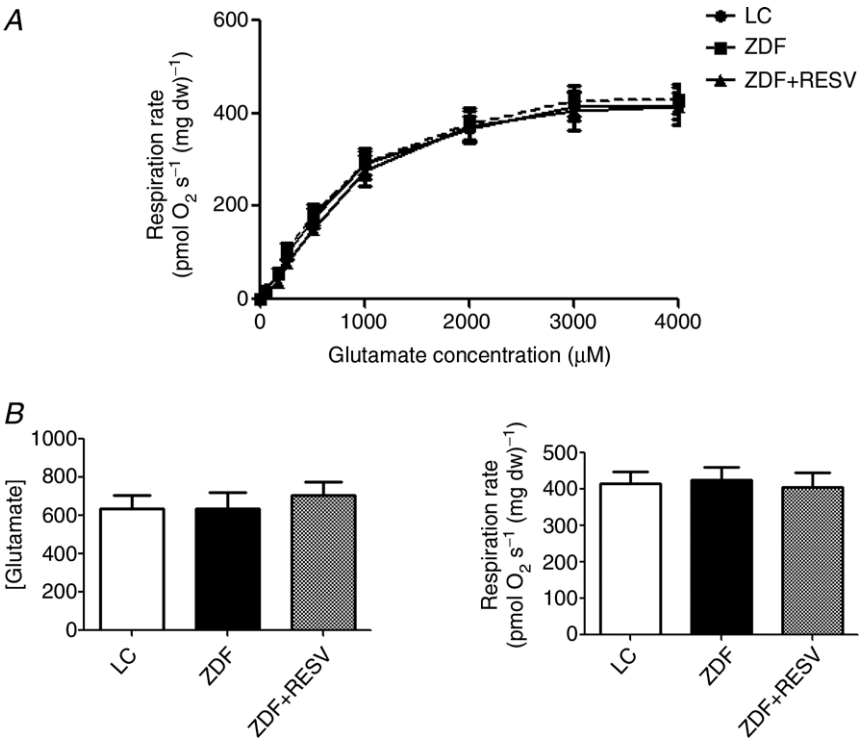

Figure 2. Glutamate respiratory kinetics are not different between groups
A, Glutamate respiration rates across all concentrations examined were not different between groups. *B* and *C*, apparent glutamate K_m (*B*) and V_{max} (*C*) were also not significantly different. Values represent means ± SEM; $n = 8$.

ROS emission and skeletal muscle insulin sensitivity, and places a new emphasis on ANT2 protein content as a potential regulator of insulin sensitivity.

Mitochondrial dysfunction in T2DM

There is considerable controversy surrounding the notion of mitochondrial dysfunction as a cause of insulin resistance (Kelley *et al.* 2002; Boushel *et al.* 2007; Mogensen *et al.* 2007; Phielix *et al.* 2008; de Feyter *et al.* 2008*a*,*b*; Holloszy, 2009; Holloway *et al.* 2010; Lenaers *et al.* 2010; Hoeks & Schrauwen, 2012), which may reflect differences in methodologies and experimental approaches. In the current study, there were no differences in maximal ADP-stimulated respiration, supporting three previous studies in ZDF rats that utilized *in vivo* phosphorous magnetic resonance spectroscopy (P-MRS) and *ex vivo* determinations of mitochondrial function (de Feyter *et al.* 2008*a*; Lenaers *et al.* 2010; Holloway *et al.* 2010). In contrast, titrating ADP revealed attenuated respiration rates in ZDF animals at submaximal ADP concentrations. Therefore, we suggest that mitochondrial dysfunction, as defined by a decrease in submaximal ADP-stimulated respiration, is associated with the progression of T2DM. While this method of ADP titration has not previously been utilized to study skeletal muscle mitochondrial function in insulin-resistant muscle, another research group has utilized titrations of various substrates in human muscle from type II diabetics (Larsen *et al.* 2011). In their study, Larsen *et al.* (2011) unexpectedly found that complex I and complex II sensitivity were increased in humans with T2DM. Within the context of mitochondrial ROS production, increased mitochondrial substrate sensitivity may be detrimental as proton motive force would be generated more rapidly per reducing equivalent supplied, which would be exacerbated in the presence of attenuated submaximal ADP transport. Clearly, future work should examine submaximal ADP-stimulated respiration in humans with T2DM to address this speculation.

Resveratrol and mitochondrial fatty acid-supported respiration

Following resveratrol supplementation, we observed an increase in maximal fatty acid-supported respiration (both P-CoA and palmitate) in the face of unchanged respiratory capacity. These data match previous work in resveratrol-supplemented humans examining mitochondrial bioenergetics utilizing PmFBs (Timmers *et al.* 2011). They observed that octanoylcarnitine-supported respiration and citrate synthase activity were elevated while respiratory capacity was unchanged in obese humans following 30 days of resveratrol supplementation (Timmers *et al.* 2011). Octanoylcarnitine bypasses carnitine palmitoyltransferase I (CPT-I) and therefore has direct access to the mitochondrial matrix where beta oxidation takes place (Noland *et al.* 2007). Considering that all fatty acid-supported (palmitate, P-CoA and

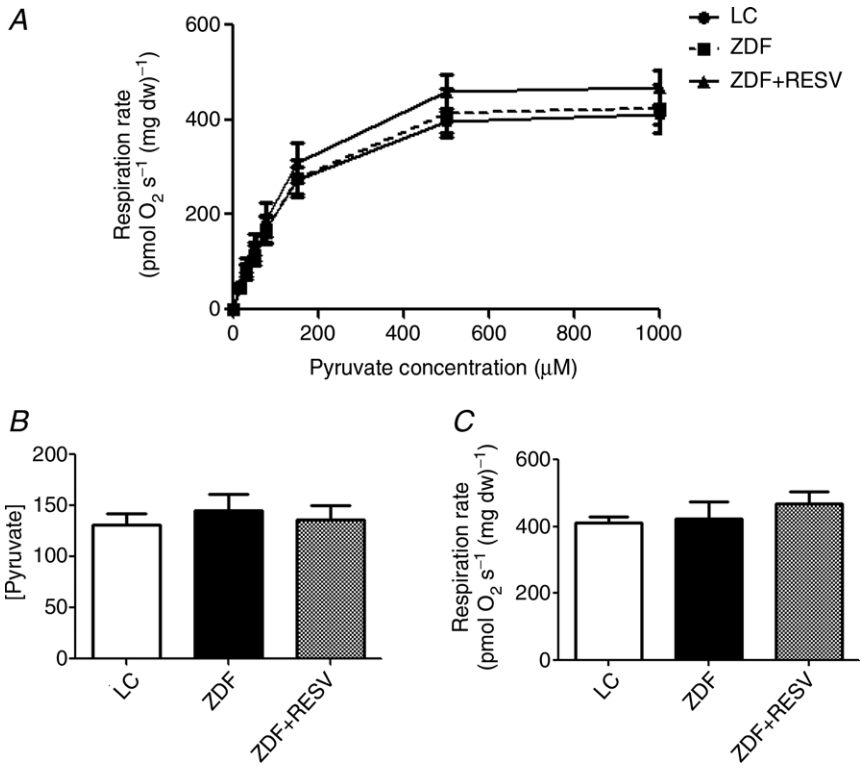

Figure 3. Pyruvate respiratory kinetics are not different between groups
A, Pyruvate respiration rates across all concentrations examined were not different between groups. *B* and *C*, apparent glutamate K_m (*B*) and V_{max} (*C*) were also not significantly different. Values represent means ± SEM; $n = 8$.

octanoylcarnitine) respiration rates increase with resveratrol supplementation, resveratrol appears to influence fatty acid oxidation independent of CPT-I. Although CPT-I has traditionally been viewed as the primary rate-limiting step for mitochondrial fatty acid oxidation (Shepherd *et al.* 1966), an earlier study pointed out that tissue acylcarnitine levels are increased under conditions known to involve increased rates of fatty acid oxidation (Bremer & Norum, 1967). The increase in acylcarnitine concentration suggests that the rate-limiting step must be downstream of CPT-I because if CPT-I was rate-limiting there would be no excess substrate to undergo the carnitine acetyltransferase (CrAT) reaction to produce the acylcarnitines. Therefore, altered regulation of fatty acid oxidation downstream of CPT-I (potentially beta oxidation) is likely with resveratrol supplementation and has been previously observed (Chen *et al.* 2011). Classically, the regulation of beta oxidation has been attributed to the balance of substrates and products, although beta oxidation can also be regulated by the cellular redox state (Eaton, 2002), suggesting a possible mechanism by which resveratrol could promote fatty acid oxidation independent of CPT-I.

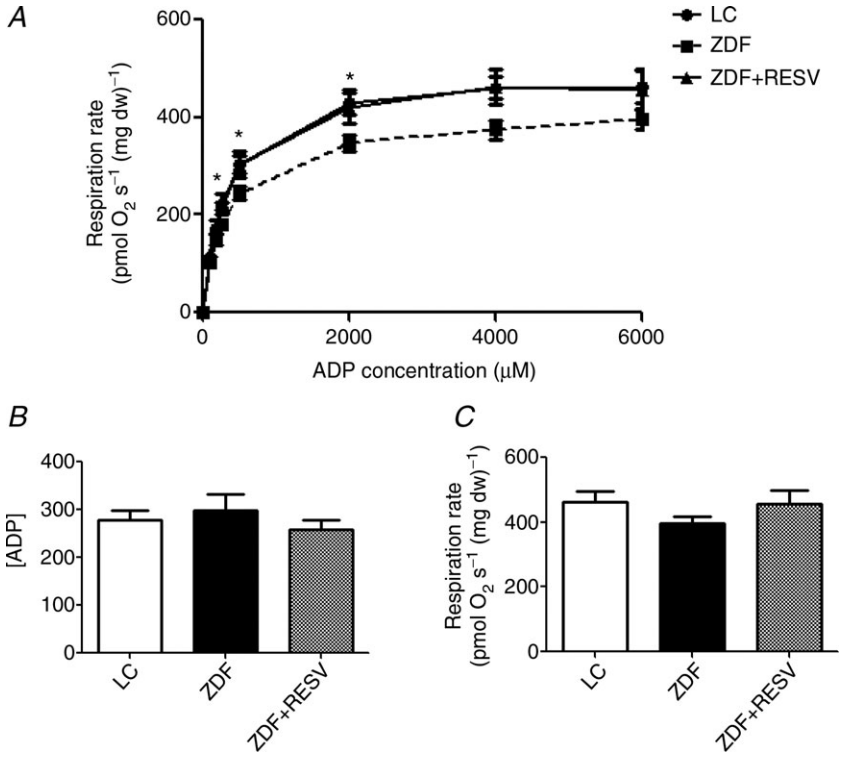

Figure 4. Submaximal ADP-stimulated respiration is significantly lower in ZDF animals and resveratrol recovers this decrease
A, At ADP concentrations of 250, 500 and 2000 μM, ZDF respiration rates are significantly lower than both lean control (LC) and resveratrol-supplemented (ZDF+RESV) groups. *B* and *C*, apparent ADP K_m (*B*) and V_{max} (*C*) were not significantly different. Values represent means ± SEM; $n = 13$.*Significantly ($P < 0.05$) different from LC and ZDF+RESV.

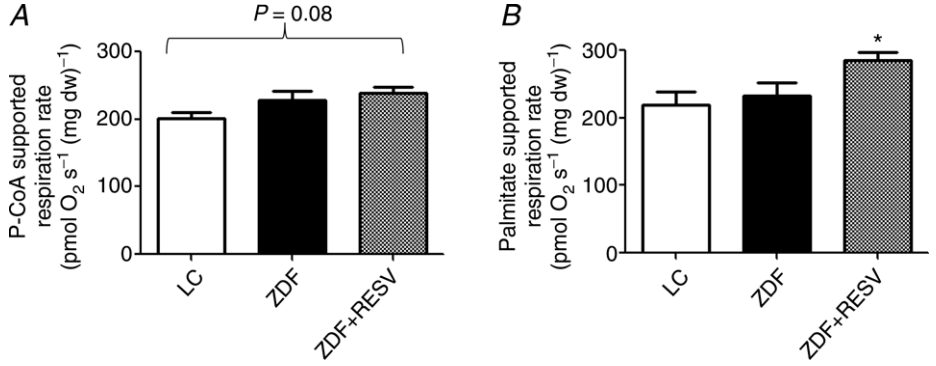

Figure 5. State III fatty acid-supported respiration rates are elevated with resveratrol treatment
Resveratrol supplementation increased state III (5 mM ADP) palmitoyl-CoA (P-CoA, 150 μM) (*A*) and palmitate (250 μM) (*B*) supported respiration rates. Values represent means ± SEM; $n = 8$ (A), $n = 5$ (B). *Significantly ($P < 0.05$) different from LC.

Proton leak and post-translational modifications of ANT

ANT has previously been suggested to account for 50–66% of basal proton leak and it has been speculated that an increase in ANT protein could lower ROS emission through the dissipation of proton motive force (Brand *et al.* 2005). However, in the current study, non ADP-stimulated respiration (state IV) was unchanged following resveratrol administration despite increased ANT2 protein content. These data suggest that ANT protein does not independently determine basal proton leak. To explain this discrepancy, we hypothesize that post-translational regulation of ANT may induce the uncoupling function of ANT similar to the mechanism surrounding the induction of UCP2- and UCP3-mediated uncoupling (Mailloux *et al.* 2011). Indeed, considering the critical function ANT has in regulating energy homeostasis, it is not surprising that numerous studies have observed extensive post-translational regulation of ANT function. Evidence now exists that ANT1 can be phosphorylated on tyrosine 194 and phosphorylation is associated with an increase in activity (Feng *et al.* 2008). Exposure to high oxygen tension or oxidative stress-induced glutathionylation result in decreased ADP transport into the mitochondrial matrix (Yan & Sohal, 1998; Queiroga *et al.* 2010). Additionally, long-chain fatty acyl-CoA moieties can directly suppress ANT function (Ho & Pande, 1974). While these data indicate that ANT function can be regulated, this does not diminish the physiological implications of increasing ANT2 protein in the current study. Instead, the improvements in cellular

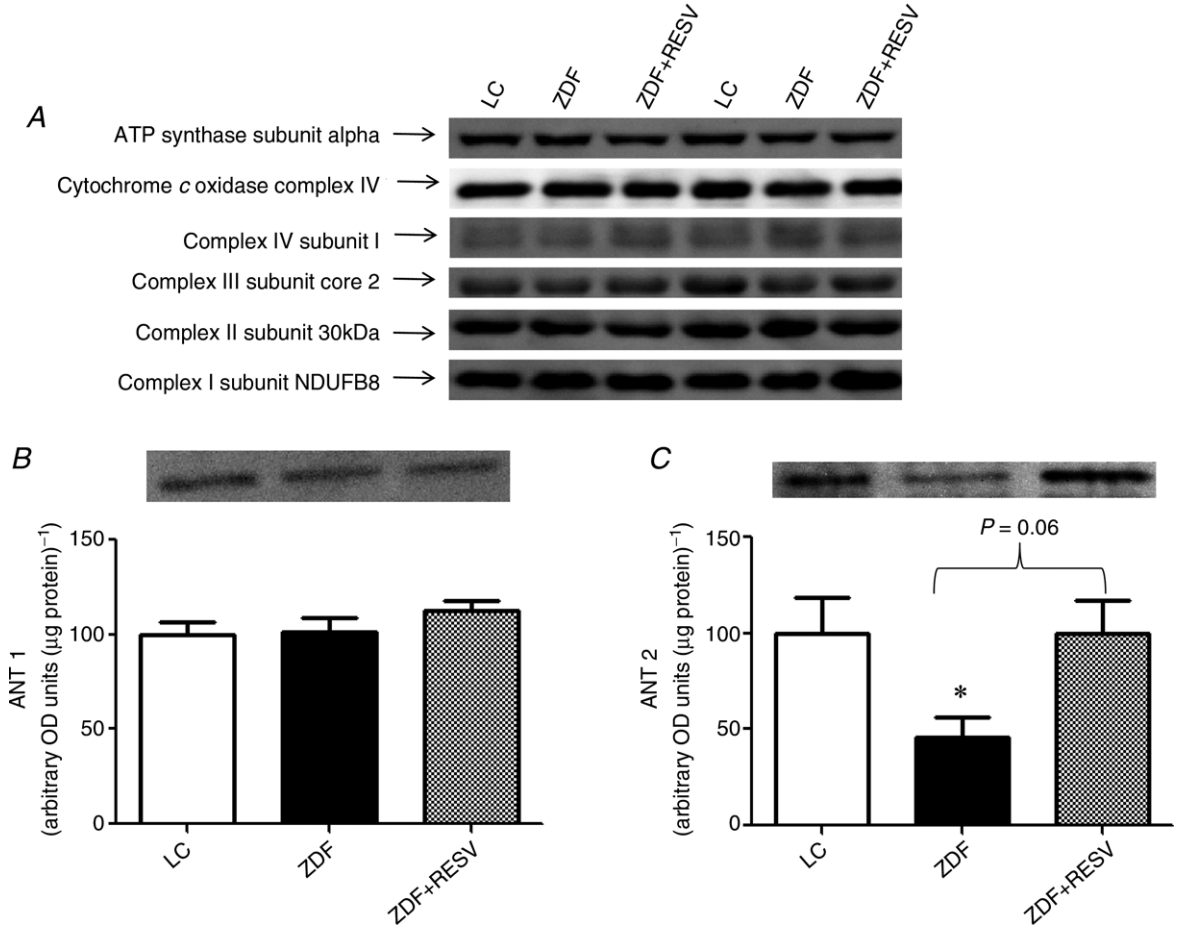

Figure 6. Mitochondrial content and adenine nucleotide translocase isoform content
A, muscle homogenates from lean control (LC), ZDF control rats (ZDF) and ZDF rats supplemented with resveratrol (ZDF+RESV) were analysed for protein content of mitochondrial markers (30 μg loaded): complex I subunit NDUFB8 (Mitosciences), complex II subunit 30 kDa (Mitosciences), complex III subunit core 2 (Mitosciences), complex IV subunit I (Mitosciences), ATP synthase subunit alpha (Mitosciences). No significant differences were found. Two representative blots are shown. *B*, there were no differences in ANT1 content between groups (20 μg protein loaded). *C*, ZDF rats showed a decrease in ANT2 expression and supplementation with resveratrol increased ANT2 expression up to LC values (40 μg protein loaded). Values represent means ± SEM; $n = 8$. *Significantly ($P < 0.05$) different from LC.

redox balance and rates of fatty acid-supported respiration following resveratrol supplementation probably further improve ANT function as resveratrol has been linked to reducing oxidative stress and increasing fatty acid metabolism (current study and others (Chen *et al.* 2011; Timmers *et al.* 2011)).

ANT content, oxidative stress and insulin resistance

Taking into account our finding that submaximal ADP-stimulated respiration is depressed in ZDF rats, we attempted to identify a mechanism by which ADP dynamics could be related to insulin resistance. Previous work has highlighted a link between skeletal muscle oxidative stress and insulin sensitivity, as mitochondrial-targeted antioxidant interventions and genetic approaches that over-express antioxidant enzymes within mitochondria protect against diet-induced skeletal muscle insulin resistance (Anderson *et al.* 2009; Boden *et al.* 2012). Our work extends these findings by demonstrating that improved ADP dynamics are associated with decreased H_2O_2 emission and improved cellular redox state, which are all associated with improved skeletal muscle insulin sensitivity. Our speculative interpretation is as follows: increasing ANT2 expression generates an environment whereby mitochondrial ROS emission is mitigated at a faster rate in the presence of low ADP concentrations. Considering ADP levels in skeletal muscle are low in the absence of exercise, an increase in ANT content and the resultant augmented ADP sensitivity would protect against ROS-induced insulin resistance (Brownlee, 2001; Houstis *et al.* 2006; Anderson *et al.* 2009). To further support our hypothesis that ADP dynamics are linked to ROS production and therefore insulin resistance, previous work in a liver cell line has shown that over-expression of ANT2 decreases oxidative stress and enhances mitochondrial function (Kim *et al.* 2012). Additionally, mitochondria isolated from ANT1-deficient mice produce greater amounts of ROS, resulting in damaged mtDNA and cellular dysfunction (Graham *et al.* 1997), while in cardiac tissue, over-expression of ANT1 protects against the development of diabetes-induced cardiomyopathy (Wang *et al.* 2009). Together, we speculate a mechanism whereby improved ADP dynamics decrease mitochondrial ROS emission and maintain cellular redox balance, resulting in enhanced insulin sensitivity. While ZDF animals share a number of similar traits with human T2DM, including hyperglycaemia, insulin resistance, hyperlipidaemia (Greene *et al.* 1994) and elevated acylcarnitines (Mihalik *et al.* 2010), we recognize that the ZDF animal model is not fully representative. This is apparent as the GSH/GSSG ratio is higher in ZDF animals than in LC animals while in insulin-resistant and type II diabetic humans a reduction in this ratio has been reported (Anderson *et al.* 2009). Previous studies showing a protection of insulin sensitivity by mitigating mitochondrial ROS production have utilized acute (6 h; Hoehn *et al.* 2009) or short-term (4–6 weeks; Houstis *et al.* 2006; Anderson *et al.* 2009) antioxidant interventions. However, a recent study has divorced the relationship between the oxidative state of the muscle and insulin sensitivity when treating with a mitochondrial-targeted antioxidant chronically (16 weeks; Paglialunga *et al.* 2012). Therefore, our work, in combination with others (Houstis *et al.* 2006; Anderson *et al.* 2009; Hoehn *et al.* 2009) may suggest that ROS is important as an early (4–6 weeks) sensor of fuel oversupply to induce insulin resistance, however this mechanism becomes disconnected from insulin sensitivity by 16 weeks (Paglialunga *et al.* 2012).

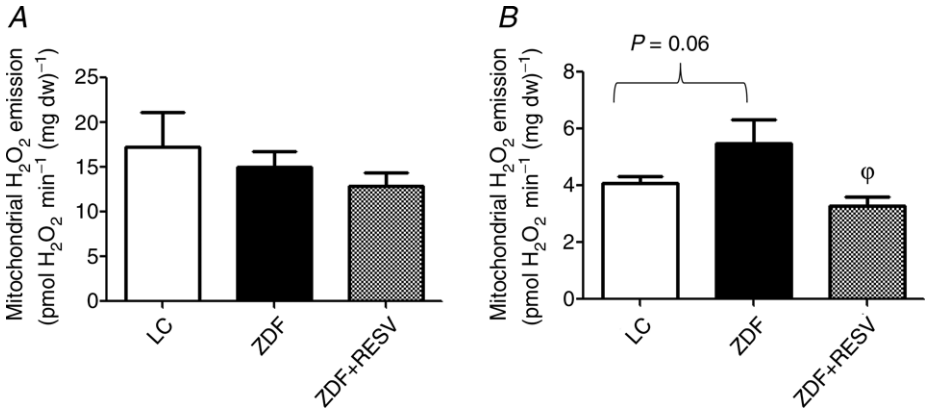

Figure 7. In ZDF rats, supplementation with resveratrol decreases the propensity for H_2O_2 emission in the presence of 100 μM ADP

A, the maximal capacity of the mitochondria to emit H_2O_2 was not different between lean controls (LC), ZDF control (ZDF) and ZDF supplemented with resveratrol (ZDF+RESV). *B*, in the presence of 100 μM ADP, the capacity of the mitochondria to emit H_2O_2 was significantly lower in the ZDF+RESV group compared to ZDF. $n = 13$ (A) and $n = 5$ (*B*). All H_2O_2 emission measurements were done in duplicate. $^{\varphi}$Significantly ($P < 0.05$) different from ZDF.

Table 2. Cellular redox status

	GSH	GSSG	GSH/GSSG
LC	6.03 ± 0.44	0.15 ± 0.01	40.6 ± 2.51
ZDF	6.46 ± 0.53	0.11 ± 0.02	64.1 ± 7.95
ZDF+RESV	6.72 ± 0.62	0.08 ± 0.01	86.1 ± 13.3*$^{\varphi}$

ZDF rats supplemented with resveratrol (ZDF+RESV) had a higher reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio compared to ZDF and LC. Values represent means ± SEM; $n = 8$. *Significantly ($P < 0.05$) different from LC. $^{\varphi}$Significantly ($P < 0.05$) different from ZDF.

Nonetheless, resveratrol improved the redox state within the ZDF animal concomitantly with improved skeletal muscle glucose uptake. Therefore, it will be important to discern whether our current observations regarding ADP dynamics in ZDF animals extend to human type II diabetic individuals.

Conclusion

In summary, our data suggest that mitochondrial dysfunction is present in skeletal muscle insulin resistance when assessed at submaximal ADP concentrations and that ADP dynamics may influence skeletal muscle insulin sensitivity through alterations in the propensity for mitochondrial ROS emission. We found that submaximal ADP-stimulated respiration is impaired in ZDF animals and resveratrol supplementation improved submaximal ADP-stimulated respiration concomitantly with enhancing insulin sensitivity. The favourable effects of resveratrol may be mediated through an improved ability of ADP to attenuate mitochondrial ROS emission.

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Additional information

Competing interests

None declared.

Author contributions

B.K.S., C.G.R.P., D.C.W. and G.P.H. designed experiments, performed experiments, analysed and interpreted data and wrote the manuscript. E.A.F.H., I.R.R. and M.S.B. performed experiments and edited the manuscript. P.D.N. and J.C.S. interpreted data and edited the manuscript.

Funding

This work was funded by The Natural Sciences and Engineering Research Council of Canada (NSERC; G.P.H. and J.C.S.) and CIHR (D.C.W.) and infrastructure was purchased with the assistance of the Canadian Foundation for Innovation (G.P.H., J.C.S. and D.C.W.) as well as the Ontario Research Fund (G.P.H., J.C.S. and D.C.W.). B.K.S. is supported by an NSERC graduate scholarship and D.C.W. is a Canada Research Chair. Funding from Carleton University (J.C.S.) is acknowledged. Additional support was provided by grants from the United States Public Health Service: [R01 DK073488] and [DK074825] (P.D.N.).

Acknowledgements

None declared.