

Mitochondrial reactive oxygen species generation in obese non-diabetic and type 2 diabetic participants

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Abstract

Aims/hypothesis The aim of this study was to measure mitochondrial reactive oxygen species (ROS) production directly from skeletal muscle biopsies obtained from obese insulin-resistant non-diabetic and type 2 diabetic participants.

Methods Ten lean healthy, ten obese non-diabetic and ten type 2 diabetic participants received a euglycaemic–hyperinsulinaemic clamp to measure whole body insulin sensitivity. Mitochondria were isolated from skeletal muscle biopsies, and mitochondrial ATP synthesis and hydrogen peroxide production were measured *ex vivo* under conditions that maximally stimulate ATP synthesis and ROS production using chemiluminescent and fluorescent techniques, respectively.

Results Compared with lean controls, both obese non-diabetic and type 2 diabetic participants were resistant to insulin, and had a reduced rate of mitochondrial ATP production. Obese insulin-resistant participants had a decreased rate of mitochondrial ROS production, while ROS production rate in participants with type 2 diabetes was similar to that in lean healthy participants. In non-diabetic participants, the rate of ROS production was strongly correlated with the rate of ATP synthesis and the glucose disposal rate measured with the euglycaemic–hyperinsulinaemic clamp. The ROS/ATP ratio in obese insulin-resistant participants was similar to that in lean insulin-sensitive participants, while the ratio was significantly elevated in type 2 diabetes participants.

Conclusions/interpretation Since, in absolute terms, the maximal capacity for mitochondrial ROS production was not increased in either obese insulin-resistant participants or in type 2 diabetic participants, these results do not favour a role for increased mitochondrial ROS production in the pathogenesis of insulin resistance in human skeletal muscle. However, care should be taken in extrapolating these *ex vivo* observations to the *in vivo* situation.

Keywords ATP synthesis · Insulin resistance · Mitochondria · Reactive oxygen species · Type 2 diabetes

Abbreviations

EGP endogenous glucose production
MRS magnetic resonance spectroscopy
ROS reactive oxygen species
TGD total glucose disposal

Introduction

Insulin resistance in skeletal muscle is a characteristic feature of type 2 diabetes [1] and the insulin resistance (metabolic) syndrome [2, 3]. Impaired insulin signalling, as well as post-signalling defects, contribute to muscle insulin resistance in type 2 diabetes (see review by DeFronzo [4]).

Increased oxidative stress has been implicated in the pathogenesis of type 2 diabetes [5–9] and in the development of diabetic micro- and macrovascular complications [10]. Increased oxidative stress has been hypothesised to activate a variety of stress kinases, e.g. c-Jun-NH[2]-kinase (JNK), which through serine phosphorylation of insulin receptor substrate 1 (IRS-1) impair the insulin signalling cascade and lead to insulin resistance in skeletal muscle

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[5, 9]. Although this hypothesis has been widely promulgated to explain the development of insulin resistance in skeletal muscle, convincing evidence for increased oxidative stress in skeletal muscle in insulin-resistant participants has yet to be provided. The assumption that oxidative stress is increased in the skeletal muscle of insulin-resistant participants is based upon indirect evidence, including: [1] increased plasma and urinary levels of oxidised lipid and protein products in obese insulin-resistant and type 2 diabetic participants [11–15]; and [2] improved whole body insulin sensitivity following treatment of type 2 diabetic patients with antioxidants [16, 17]. No previous study has reported reactive oxygen species (ROS) production measured directly in skeletal muscle in insulin-resistant participants. The aim of this study was to measure ROS generation directly in skeletal muscle and to relate ROS generation to insulin sensitivity measured with the euglycaemic–hyperinsulinaemic clamp technique. Since most of the ROS in the cell originate from the mitochondria [18], we quantitated the rate of ROS production in mitochondria isolated from insulin-sensitive lean participants, insulin-resistant obese non-diabetic participants and type 2 diabetic participants.

Methods

Participants Ten lean (BMI < 27.5 kg/m²), ten obese (BMI > 30 kg/m²) and ten type 2 diabetic participants were studied (Table 1). Lean and obese participants had a normal 75 g OGTT. Diabetic participants were treated with diet alone, or with metformin and/or sulfonylurea. No participants had ever received thiazolidinediones or insulin. All participants had normal liver, cardiopulmonary and kidney functions as determined by medical history, physical examination, screening blood tests, electrocardiogram and urinalysis. None of the non-diabetic participants was tak-

ing any medication and no participant participated in a regular exercise programme. Body weight had been stable (± 2 kg) for at least 3 months before the study in all participants. The study protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio. Informed written consent was obtained from all participants before their participation. All studies were performed at the General Clinical Research Center (GCRC) of the University of Texas Health Science Center at 08:00 hours following a 10–12 h overnight fast.

OGTT Prior to the OGTT, a catheter was placed into an antecubital vein. Blood samples were collected at –30, –15, 0, 30, 60, 90 and 120 min for the measurement of plasma glucose and insulin concentrations.

Euglycaemic–hyperinsulinaemic clamp Before the start of the insulin clamp [19], a catheter was placed into an antecubital vein for the infusion of all test substances. A second catheter was inserted retrogradely into a vein on the dorsum of the hand, and the hand was placed into a thermoregulated box heated to 70°C. At 08:00 hours, all participants received a prime (0.925 MBq \times fasting plasma glucose [mmol/l]/5)–continuous (9.25 kBq/min) infusion of [³-³H]glucose (DuPont NEN Life Science Products, Boston, MA, USA). After a 2 h (3 h in type 2 diabetic participants) basal tracer equilibration period, participants received a prime–continuous insulin infusion at the rate of 240 pmol min⁻¹ m⁻² (80 mU min⁻¹ m⁻²) for 240 min. During the last 30 min of the basal equilibration period, plasma samples were taken at 5–10 min intervals for determination of plasma glucose and insulin concentrations, and [³H]glucose radioactivity. During the insulin infusion, plasma glucose concentration was measured every 5 min, and a variable infusion of 20% (wt/vol.) glucose was adjusted, based on the negative feedback principle, to

Table 1 Metabolic characteristics of the study groups

Characteristic	Lean	Obese	T2DM	<i>p</i> value (ANOVA)
<i>n</i>	10	10	10	
Sex (male/female)	7/3	6/4	8/2	NS ^a
Age (year)	43 \pm 3	44 \pm 3	44 \pm 3	NS
BMI (kg/m ²)	25.7 \pm 0.6	39.7 \pm 2.7	38.9 \pm 2.1	<0.0001
FPG (mmol/l)	5.3 \pm 0.1	5.4 \pm 0.2	7.7 \pm 0.6	<0.01
HbA _{1c} (%)	5.2 \pm 0.1	5.5 \pm 0.2	6.9 \pm 0.5	<0.05
FPI (pmol/l)	14 \pm 7	70 \pm 14	139 \pm 14	<0.0001
TGD (μ mol kg ⁻¹ min ⁻¹)	54 \pm 4	32 \pm 3	26 \pm 2	<0.001
SSPI (pmol/l)	660 \pm 35	910 \pm 63	799 \pm 35	<0.0001
TGD/SSPI	83 \pm 6	37 \pm 4	32 \pm 3	<0.0001

^a χ^2 test

FPG, fasting plasma glucose; FPI, fasting plasma insulin; SSPI, steady-state plasma insulin concentration; T2DM, type 2 diabetes mellitus

maintain the plasma glucose concentration at each participant's fasting plasma glucose level in non-diabetic participants and at ~ 5.6 mmol/l in participants with type 2 diabetes, with a coefficient of variation of $<5\%$. Plasma samples were collected every 15 min from 0 to 180 min and every 5–10 min from 180 to 240 min for the determination of plasma glucose and insulin concentrations and [^3H] glucose specific activity.

Muscle biopsies Sixty minutes before the start of the insulin clamp, when participants were at their fasting plasma glucose level, a vastus lateralis muscle biopsy (~ 300 mg) was obtained under local anaesthesia (1% lidocaine) with a percutaneous needle, as previously described [20]. The muscle biopsy was placed in buffer on ice for mitochondrial isolation and measurement of mitochondrial ATP synthesis and ROS production rate.

Analytical techniques Plasma glucose was measured by the glucose oxidase reaction (Glucose Oxidase Analyzer; Beckman, Fullerton, CA, USA). Plasma insulin concentration was measured by radioimmunoassay (Coat A Count; Diagnostic Products, Los Angeles, CA, USA). Plasma NEFA was measured by an enzymatic colorimetric method (Wako Chemicals, Neuss, Germany).

Mitochondrial purification Mitochondria were purified from muscle tissue as previously described [21]. Mitochondrial integrity was assessed by respiratory control ratio (>6 with pyruvate) at the end of each experiment. All procedures were performed on ice and the entire isolation procedure lasted about 60–70 min. The final mitochondrial solution was kept on ice and used immediately following isolation.

Mitochondrial ATP production Aliquots of the final mitochondrial suspension were used for measurement of mitochondrial ATP production rate with a chemiluminescent technique. The reaction mixture included a luciferin–luciferase ATP-monitoring reagent (Roche, Berlin, Germany), substrates for oxidation and 75 $\mu\text{mol/l}$ ADP. Substrates added were as follows: 5 mmol/l glutamate plus 5 mmol/l malate; 10 mmol/l succinate plus 1 $\mu\text{mol/l}$ rotenone. These conditions allow the measurement of the maximal mitochondrial production rate. Initially, mitochondrial aliquots were incubated at 37°C for 5 min in buffer containing (mmol/l): 124 KCl, 5 MgCl_2 , 2 K_2HPO_4 , 10 HEPES at pH 7.44. Substrates were then added and the reaction was started with the addition of luciferin–luciferase with ADP. The reaction was performed in a 96-well plate and plate readings were made with Fluoroskan Ascent instrument (Thermo, Boston, MA, USA). A blank well (mitochondria without substrate) was used to measure

background and its value was subtracted from all other wells. All reactions for a given sample were monitored simultaneously and calibrated with addition of an ATP standard (Roche).

Mitochondrial ROS production The maximal rate of mitochondrial ROS production was measured indirectly as H_2O_2 release from the mitochondria. H_2O_2 was determined with Amplex Red (Molecular Probes, Eugene, OR, USA), as described previously [22]. Horseradish peroxidase (1 unit/ml) (HRP), which catalyses the H_2O_2 -dependent oxidation of non-fluorescent Amplex Red (80 $\mu\text{mol/l}$) to fluorescent Resorufin Red superoxide dismutase (Sigma, St Louis, MI, USA), was added at 30 U/ml to convert all superoxide into H_2O_2 . This is necessary since superoxide produced outside the mitochondria reacts very rapidly with HRP and HRP–compound (I). Compound (I) is an intermediate state of peroxidases, formed by the reaction of the enzyme with H_2O_2 . We monitored resorufin formation (Amplex Red oxidation by H_2O_2) at a $\lambda_{\text{excitation}}$ of 545 nm and a $\lambda_{\text{emission}}$ of 590 nm using a Fluoroskan-FL Ascent Type 374 multi-well plate reader (Labsystems, Helsinki, Finland). The slope of resorufin formation was converted into the rate of H_2O_2 production using a standard curve. Fluorescence remained linear, with a H_2O_2 concentration ranging from 0 to 2 $\mu\text{mol/l}$. The lowest H_2O_2 concentration detected was <1 nmol/l. Since the volume of the reaction mixture is 100 μl , the assay detects the production of 0.1 pmol H_2O_2 . The assay was performed in 96-well plates at 100 μl per well with a measuring duration of 20 ms, every 2 s for 6 min. All samples were run in duplicate and the CV for complex I substrate was 5.4% and for succinate was 7.5%. We performed all assays at 37°C in 125 mmol/l KCl, 10 mmol/l HEPES, 5 mmol/l MgCl_2 and 2 mmol/l K_2HPO_4 (pH 7.44), with 20–40 μg of mitochondrial protein per 100 μl of reaction buffer. H_2O_2 generation was completely inhibited with the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 1 $\mu\text{mol/l}$) indicating that H_2O_2 originated from the mitochondria.

Statistical analysis and calculations Following an overnight fast, steady-state conditions prevail; endogenous glucose production (EGP) was calculated as the [^3H] glucose infusion rate (dpm/min) divided by the plasma [^3H] glucose specific activity (dpm/mmol). During the insulin clamp, non-steady-state conditions for [^3H] glucose specific activity prevail; the rate of glucose appearance (R_a) was calculated with Steele's equation [23]. The rate of residual EGP during the insulin clamp was calculated by subtracting the exogenous glucose infusion rate from the tracer-derived rate of glucose appearance. The insulin-stimulated total glucose disposal (TGD) rate was calculated

by adding the rate of residual EGP to the exogenous glucose infusion rate and dividing by the steady-state plasma insulin concentration (SSPI) during the last hour of the insulin clamp (TGD/SSPI).

ATP synthesis rate was measured as the slope of chemiluminescence over time. The slope of the blank well (mitochondria without substrate) was subtracted from the slope obtained with any given substrate, and then normalised per mg mitochondrial protein per min. Similarly, the rate of H_2O_2 production was measured as the slope of fluorescence over time. The slope of the blank well was subtracted from the slope obtained with any given substrate and normalised per mg mitochondrial protein per min. All values are presented as the mean \pm standard error. For comparison between groups, ANOVA was used. Statistically significant differences were confirmed by the Bonferroni test. Simple Pearson correlation was used to assess correlation between variables. All statistical analyses were performed with SPSS (version 14) (SPSS, Chicago, IL, USA) and statistical significance was considered at $p < 0.05$.

Results

Table 1 presents the anthropometric and metabolic characteristics of the three study groups. Participants were well matched for age and sex. Obese and type 2 diabetic participants had significantly greater BMI and lower TGD rate than lean participants. Obese and type 2 diabetic participants had similar BMI and TGD rates.

Compared with lean healthy controls, obese and type 2 diabetic participants had significantly lower rates of mitochondrial ATP synthesis with both complex I substrates (glutamate, pyruvate and palmitoyl carnitine) and complex II substrate (succinate). Figure 1 presents the rate of mitochondrial ATP synthesis with glutamate and succinate. Compared with lean healthy controls, obese and type 2 diabetic participants had significantly lower rates of mitochondrial ATP synthesis with both substrates. The rate of ATP synthesis with complex I substrate was decreased by 33%, 42% and 32%, respectively ($p = 0.02$) in obese participants, and by 39%, 33% and 31%, respectively ($p = 0.02$) in type 2 diabetic participants. ATP synthesis with succinate was decreased by 46% and 45% in obese and type 2 diabetic participants, respectively ($p < 0.002$).

Mitochondrial ROS production was greater with complex I substrate compared with complex II substrate (Fig. 2a,b). In lean healthy individuals, the rate of ROS production with glutamate was 263 ± 55 pmol [mg protein] $^{-1}$ min $^{-1}$ compared with 81 ± 11 pmol [mg protein] $^{-1}$ min $^{-1}$ with succinate ($p < 0.002$). Obese participants had a significantly lower rate of ROS production with both substrates compared with lean and type 2 diabetic partic-

ipants: 106 ± 16 , 263 ± 56 and 216 ± 56 pmol (mg protein) $^{-1}$ min $^{-1}$ with glutamate and 24 ± 6 , 81 ± 11 and 70 ± 18 pmol (mg protein) $^{-1}$ min $^{-1}$ with succinate, respectively ($p < 0.05$ with ANOVA, for glutamate and succinate).

When non-diabetic participants (lean and obese) were pooled, ROS production with both substrates strongly correlated with the rate of mitochondrial ATP production ($r = 0.69$, $p < 0.001$ for glutamate [Fig. 3a] and $r = 0.80$, $p < 0.0001$ for succinate [Fig. 3b]) and with TGD rate ($r = 0.71$, $p < 0.0004$ for glutamate [Fig. 4a] and $r = 0.63$, $p < 0.002$ for succinate [Fig. 4b]).

Since mitochondrial metabolism is driven by the energy demand of the cell, and ROS generation is a by-product of normal metabolism, we calculated the ratio of ROS generation to ATP production rate. This ratio reflects the number of ROS molecules generated during the production of one ATP molecule. The ratio between mitochondrial H_2O_2 generation rate and mitochondrial ATP synthesis rate was 3.6×10^{-3} , 2.7×10^{-3} and 6.5×10^{-3} with complex I substrate and 1.0×10^{-3} , 0.8×10^{-3} and 2.6×10^{-3} with complex II substrate in lean, obese, and type 2 diabetic

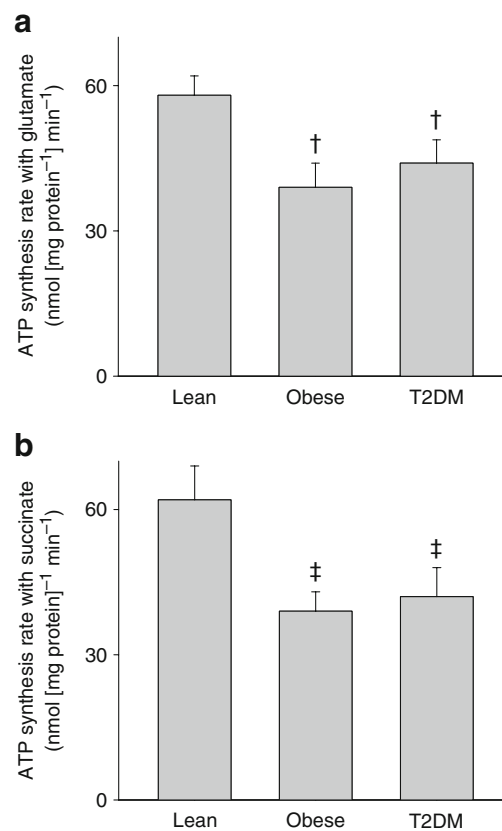


Fig. 1 Rate of mitochondrial ATP production in lean healthy participants, obese non-diabetic participants and type 2 diabetic (T2DM) participants with glutamate (5 mmol/l) plus malate (5 mmol/l) (a) and succinate (10 mmol/l) plus rotenone (1 μ mol/l) (b). † $p = 0.02$, ‡ $p = 0.002$ vs lean group

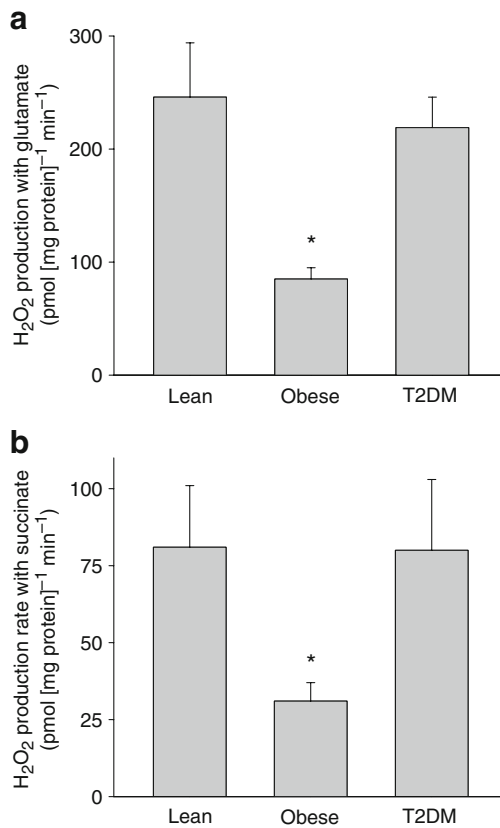


Fig. 2 Rate of mitochondrial hydrogen peroxide production in lean healthy participants, obese non-diabetic participants and type 2 diabetic (T2DM) participants with glutamate (5 mmol/l) plus malate (5 mmol/l) (a) and succinate (10 mmol/l) plus rotenone (1 μ mol/l) (b). * $p < 0.05$ vs lean group

participants, respectively (Fig. 5) ($p < 0.001$ for glutamate and $p < 0.05$ for succinate with ANOVA).

The relationship between ROS production and HbA_{1c} with both glutamate and succinate was divergent in diabetic and non-diabetic individuals. In non-diabetic participants increased HbA_{1c} levels were associated with a decrease in mitochondrial ROS production rate, while in type 2 diabetic individuals increased HbA_{1c} levels were associated with increase in ROS production rate. Thus, the relationship between mitochondrial ROS production rate and HbA_{1c} was described by a U-shaped curve (Fig. 6a, b).

Discussion

Increased oxidative stress has been implicated in the pathogenesis of type 2 diabetes [6], diabetic complications [10], cardiovascular disease [24], cancer and the normal ageing process [25]. Increased oxidative stress has been suggested to cause insulin resistance in skeletal muscle by activating stress kinases, e.g. JNK and nuclear factor kappa B (NF κ B), that impair insulin signalling via serine

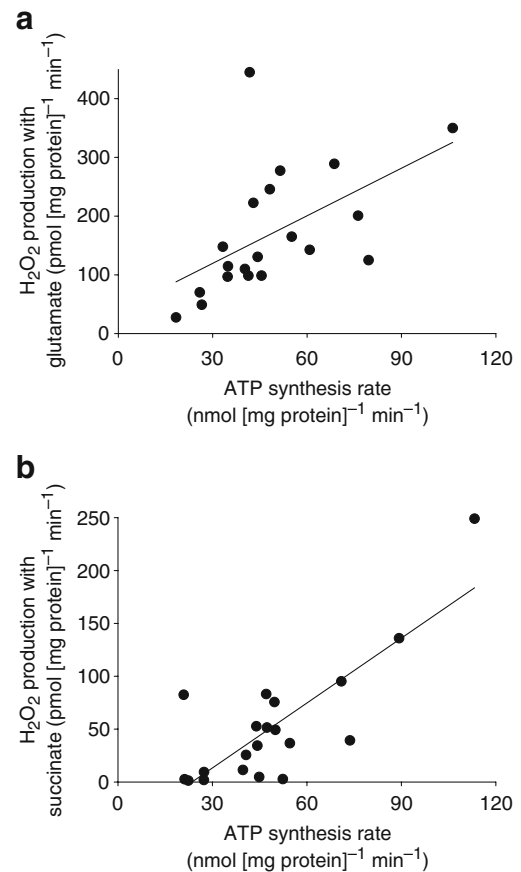


Fig. 3 Relationship between mitochondrial ATP production and ROS generation with complex I (glutamate plus malate) (a) ($r = 0.69$, $p < 0.001$) and complex II (succinate plus rotenone) (b) substrates in non-diabetic participants ($r = 0.80$, $p < 0.0001$)

phosphorylation of the insulin receptor and IRS-1 [5, 7]. Although this hypothesis has been suggested by many authors, direct evidence for increased ROS production in skeletal muscle in insulin-resistant individuals has yet to be provided. In this study we demonstrate that the maximal mitochondrial ROS production capacity in mitochondria isolated from obese non-diabetic, insulin-resistant individuals is lower than in lean normal-glucose-tolerant insulin-sensitive individuals. We also demonstrate that, in non-diabetic participants, the maximal mitochondrial ROS production capacity of both complex I and complex II is strongly correlated with the maximal rate of mitochondrial ATP production. Since obese insulin-resistant individuals have a significant decrease in mitochondrial ATP synthesis rate, and since ROS production is a by-product of the normal oxidative phosphorylation process, the decrease in mitochondrial ROS production observed in this study probably reflects the reduced rate of mitochondrial ATP synthesis associated with increased insulin resistance and decreased insulin-stimulated glucose disposal. The comparable ROS/ATP ratio in lean healthy insulin-sensitive and

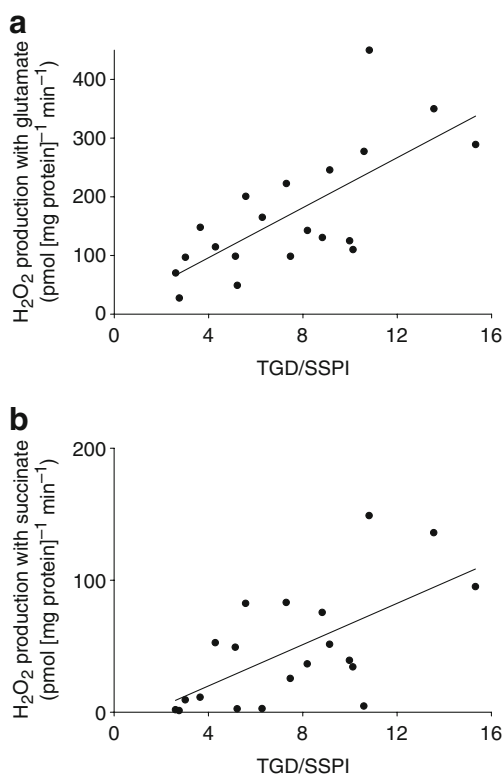


Fig. 4 Relationship between ROS generation with complex I (glutamate plus malate) ($r=0.71$, $p=0.0004$) (a) and complex II (succinate plus rotenone) ($r=0.63$, $p<0.002$) (b) substrates vs insulin-stimulated TGD rate during the last hour of the euglycaemic–hyperinsulinaemic clamp in non-diabetic (lean plus obese) participants. SSPI, steady-state plasma insulin concentration during the last hour of the euglycaemic–hyperinsulinaemic clamp

obese insulin-resistant individuals supports this hypothesis. Although these results demonstrate that the maximal ROS production capacity measured *ex vivo* is diminished, caution should be employed when extrapolating to the *in vivo* situation. The ratio of ROS/ATP production indicates the number of ROS molecules generated per 1000 ATP molecules synthesised. Our results demonstrate that in lean insulin-sensitive individuals, skeletal muscle mitochondria generate ~3.5 molecules of H₂O₂ with complex I substrate per 1,000 ATP molecules synthesised, while, with complex II substrate, approximately one molecule of H₂O₂ is generated per 1000 ATP molecules synthesised. The ROS/ATP ratio in obese insulin-resistant individuals is similar to that in lean insulin-sensitive participants for both complex I substrate and complex II substrate, suggesting that the decrease in mitochondrial ROS production in obese insulin-resistant individuals is the result of decreased oxidative phosphorylation, rather than a functional change in mitochondrial activity in obese insulin-resistant participants.

Decreased mitochondrial oxidative phosphorylation has been reported *in vivo* using magnetic resonance spectroscopy (MRS) in a variety of insulin resistance states,

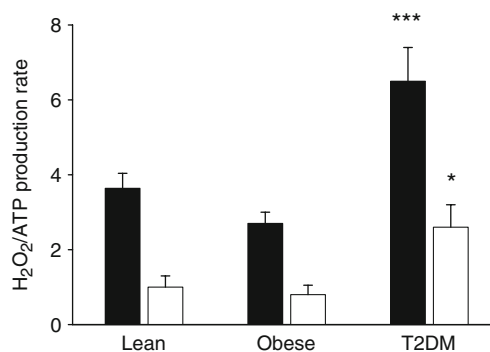


Fig. 5 Ratio between hydrogen peroxide generation and ATP production rate in lean insulin-sensitive participants, obese non-diabetic participants and type 2 diabetic participants (T2DM). Black bars, ratio with complex I substrate (glutamate plus malate); white bars, ratio with complex II substrate (succinate plus rotenone). * $p<0.05$, *** $p<0.001$ vs corresponding lean group

including participants with a positive family history for type 2 diabetes, elderly participants and type 2 diabetic participants (see review by Schrauwen-Hinderling et al. [26]). However, the precise relationship between decreased mitochondrial oxidative phosphorylation and insulin resistance remains unclear [26–29]. Whether decreased mitochondrial function is the cause or the effect of insulin

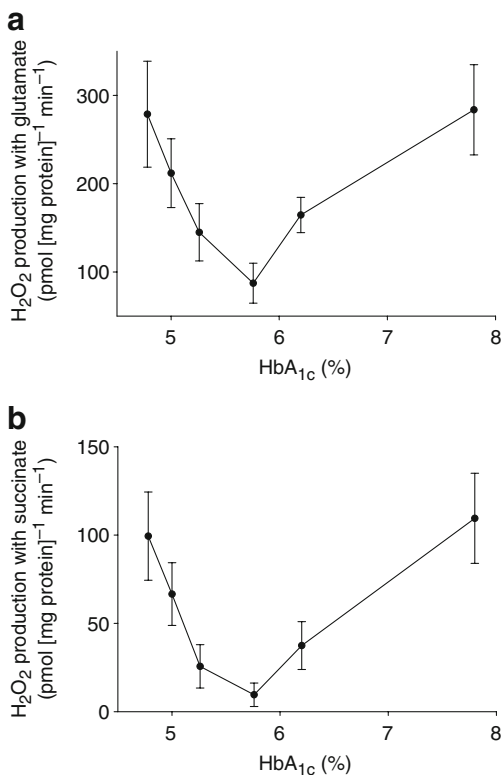


Fig. 6 Relationship between mitochondrial hydrogen peroxide production with glutamate plus malate (a) and succinate plus rotenone (b) vs HbA_{1c}. Participants were divided into six equal groups based upon their HbA_{1c} level

resistance or not, our results indicate that reduced oxidative phosphorylation, when measured *ex vivo*, is associated with a decrease in the rate of mitochondrial ROS production in skeletal muscle. If a similar decrease in mitochondrial ROS production occurred *in vivo* in association with the well-documented decrease in oxidative phosphorylation reported in insulin-resistant participants with MRS, this would not support a role for increased mitochondrial ROS production in the pathogenesis of muscle insulin resistance in obesity. However, even if a decrease in mitochondrial ROS production were to be demonstrated *in vivo*, this would not exclude a causal role of increased oxidative stress in the development of insulin resistance. Previous studies have reported increased plasma and urinary levels of products of fat and protein oxidation in obese insulin-resistant individuals [5–9]. In this regard, ROS can be generated from other sources in the body, e.g. adipocytes [30]. NADPH oxidase [31] also may contribute to the generalised increased level of oxidative stress in obese insulin-resistant individuals. Alternatively, decreased levels of defence mechanism, with an unchanged level of ROS production, could also lead to an increase in oxidative stress markers in obese insulin-resistant individuals [32].

As for obese insulin-resistant non-diabetic individuals, participants with type 2 diabetes also had a decrease in the maximal rate of mitochondrial ATP synthesis compared with lean insulin-sensitive individuals. Our observation of decreased mitochondrial ATP synthesis in diabetic participants is consistent with the reduced oxidative phosphorylation demonstrated in type 2 diabetic participants *in vivo* using MRS [33], and it is probably related to the insulin resistance (decreased rate of TGD) rather than to the diabetic state, since a similar decrease was observed in the obese insulin-resistant non-diabetic participants. Because our study is cross-sectional, we can not determine what is the cause and what is the effect. However, unlike the obese participants, the maximum rate of ROS production *ex vivo* in type 2 diabetes was normal when viewed in absolute terms, and, when expressed relative to the rate of ATP production, ROS generation was increased twofold compared with obese individuals (6.5-fold for complex I and 2.6-fold for complex II in type 2 diabetic participants compared with 3.6- and 1.0-fold in lean and 2.7- and 0.8-fold in obese individuals, respectively). These results indicate that, relative to their metabolic rate, type 2 diabetic participants had an increased rate of mitochondrial ROS production *ex vivo* compared with age- and BMI-matched non-diabetic participants. However, it should be emphasised that the present studies were carried out *ex vivo*, and muscle ROS production has yet to be measured *in vivo*. It also should be noted that under our experimental conditions, which used saturating substrate concentrations, mitochondrial ATP synthesis rate and ROS generation are

maximally stimulated. However, under physiological conditions *in vivo*, the mitochondria operate under sub-maximal conditions and mitochondrial metabolism is driven by the cellular energy demand (ATP/ADP ratio). Thus, our data indicate that for the production of the same amount of ATP, type 2 diabetes participants generated twice the amount of ROS than did non-diabetic participants, both insulin-sensitive (lean) and insulin-resistant (obese). Since type 2 diabetic participants have rates of ATP production and insulin-stimulated glucose disposal rates that are reduced similarly to those in obese insulin-resistant non-diabetic individuals, our results indicate that the increased ROS/ATP production ratio in type 2 diabetic participants is probably the result of some aspect of the diabetic state. Moreover, the increase in ROS production relative to the rate of total body glucose disposal and ATP synthesis is consistent with the hypothesis that oxidative stress is increased in diabetic participants and may play a role in the development of diabetic vascular complications [10].

Although the molecular defect that could be responsible for ATP synthesis rate and increased ROS production in participants with type 2 diabetes has yet to be defined, a defect in complex V could explain the results. Thus, a decrease in complex V activity in participants with type 2 diabetes would result in a decreased rate of mitochondrial ATP synthesis accompanied by an increased proton gradient across the inner mitochondrial membrane, leading to an increase in the rate of ROS production. Consistent with this hypothesis, a recent study [34] has reported an increased rate of phosphorylation of mitochondrial complex V in diabetic participants compared with lean healthy controls. However, complex V activity was not reported in that study. It remains to be seen whether a change in the phosphorylation state of mitochondrial complex V could explain the current results (increased ROS production and decreased ATP synthesis) in type 2 diabetic participants.

A limitation of the present study is that the measurement of mitochondrial function was performed *ex vivo*. Removal of the mitochondria from their physiological environment could alter their metabolic characteristics. In addition to the change in substrate availability, which is an important regulator of mitochondrial activity, removal of intracellular metabolites that could influence mitochondrial activity could also alter mitochondrial metabolism. For example, we have shown previously that elevated concentrations of fatty acyl Co-A inhibit oxidative phosphorylation in skeletal muscle mitochondria, and an increased level of intracellular fatty acyl Co-A has been reported in both type 2 diabetic and obese non-diabetic insulin-resistant individuals compared with lean insulin-sensitive participants [21]. Thus, mitochondrial metabolism measured *in vivo* may differ from that measured *ex vivo*. However, at present there is no *in vivo* method to measure ROS production.

It is noteworthy that in humans the rate of ROS production in complex I is about threefold greater than that of complex II. This rate of ROS production is in marked contrast to that observed in rodents, where the rate of ROS production in complex II is about twice that of complex I [22]. Since complex II produces superoxide to both sides of the inner mitochondrial membrane [35] while complex I produces superoxide only toward the inner mitochondrial matrix, this suggests that, for the same level of metabolism, the level of cytosolic oxidative stress would be greatly (five- to sixfold) increased in rodents compared with humans.

In conclusion, we have demonstrated in this study that the maximal mitochondrial ROS production capacity measured *ex vivo* in obese and type 2 diabetic participants is not increased, in absolute terms, in mitochondria isolated from skeletal muscle. However, the ratio of ROS/ATP production in obese non-diabetic participants is similar to that in lean healthy individuals, and increased in participants with type 2 diabetes. Taken together, these results do not favour a role for increased mitochondrial ROS production in the development of insulin resistance. However, the present results were obtained under *ex vivo* conditions. *In vivo* measurement of mitochondrial ROS production will be necessary to validate the relevance of the present observations. Unfortunately, the measurement of muscle ROS production *in vivo* is not currently feasible.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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