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ARTICLE

Targeting of mitochondrial reactive oxygen species production does not avert lipid-induced insulin resistance in muscle tissue from mice

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Abstract
Aims/hypothesis High-fat, high-sucrose diet (HF)-induced reactive oxygen species (ROS) levels are implicated in skeletal muscle insulin resistance and mitochondrial dysfunction. Here we investigated whether mitochondrial ROS sequestering can circumvent HF-induced oxidative stress; we also determined the impact of any reduced oxidative stress on muscle insulin sensitivity and mitochondrial function.
Methods The Skulachev ion (plastoquinonyl decyltriphenylphosphonium) (SkQ), a mitochondria-specific antioxidant, was used to target ROS production in C2C12 muscle cells as well as in HF-fed (16 weeks old) male C57Bl/6 mice, compared with mice on low-fat chow diet (LF) or HF alone. Oxidative stress was measured as protein carbonylation levels. Glucose tolerance tests, glucose uptake assays and insulin-stimulated signalling were determined to assess muscle insulin sensitivity. Mitochondrial function was determined by high-resolution respirometry.
Results SkQ treatment reduced oxidative stress in muscle cells (−23% $p<0.05$), but did not improve insulin sensitivity and glucose uptake under insulin-resistant conditions. In HF mice, oxidative stress was elevated (56% vs LF $p<0.05$), an effect completely blunted by SkQ. However, HF and HF+SkQ mice displayed impaired glucose tolerance (AUC HF up 33%, $p<0.001$; HF+SkQ up 22%; $p<0.01$ vs LF) and disrupted skeletal muscle insulin signalling. ROS sequestering did not improve mitochondrial function.
Conclusions/interpretation SkQ treatment reduced muscle mitochondrial ROS production and prevented HF-induced oxidative stress. Nonetheless, whole-body glucose tolerance, insulin-stimulated glucose uptake, muscle insulin signalling and mitochondrial function were not improved. These results suggest that HF-induced oxidative stress is not a prerequisite for the development of muscle insulin resistance.

Keywords Antioxidant treatment · High-fat high-sucrose diet · Insulin resistance · Oxidative stress · Reactive oxygen species · SkQ

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Abbreviations

AU	Arbitrary units
CoA	Coenzyme A
CS	Citrate synthase
FCCP	Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
GPx1	Glutathione peroxidase 1
GSK3	Glycogen synthase kinase 3
HADH	Hydroxylacyl dehydrogenase
HF	High-fat, high-sucrose diet
LF	Low-fat chow diet
MCAT	Mitochondrial catalase
OXPHOS	Oxidative phosphorylation
pAkt	Phosphorylated Akt
pGSK3	Phosphorylated GSK3
ROS	Reactive oxygen species
SkQ	Skulachev ion (plastoquinonyl decyltriphenylphosphonium)
SOD2	Superoxide dismutase 2

Introduction

Type 2 diabetes has been associated with oxidative stress, a condition that arises when production of reactive oxygen species (ROS) exceeds the antioxidant defence system's capacity. Several studies have shown that skeletal muscle mitochondrial ROS production is increased in insulin-resistant conditions [1, 2], which are an early hallmark in the development of type 2 diabetes. Moreover, it has been suggested that ROS and oxidative stress are causal factors of skeletal muscle insulin resistance [3, 4].

Lee et al [5] recently showed that age-induced development of insulin resistance, which is associated with increases in ROS production and oxidative stress in mice, could be prevented when mitochondrial H_2O_2 release was attenuated by overexpression of the gene encoding mitochondrial catalase (MCAT), which also prevented the development of mitochondrial dysfunction observed upon ageing. Interestingly, type 2 diabetic patients also have mitochondrial dysfunction, as exemplified by smaller and damaged mitochondria [6], reduced in vivo muscle ATP production capacity [7, 8] and lowered mitochondrial density [9, 10]. Furthermore, we have previously demonstrated that ex vivo intrinsic skeletal muscle mitochondrial respiration [11] and in vivo mitochondrial oxidative capacity [8] are reduced in patients with type 2 diabetes. Taken together, these findings suggest that elevated ROS and oxidative stress in skeletal muscle are implicated in insulin resistance and may be the underlying cause of the mitochondrial dysfunction observed in type 2 diabetes.

Therefore, we investigated whether the targeting of mitochondrial ROS production in an in vitro and in vivo model of lipid-induced insulin resistance could alleviate insulin resistance by lowering muscle oxidative stress. For this purpose, we used a novel oral mitochondria-specific antioxidant, the Skulachev ion (plastoquinonyl decyltriphenylphosphonium) (SkQ). SkQ is a small cationic molecule that targets and accumulates in the inner mitochondrial membrane [12]. SkQ mainly scavenges superoxide radicals arising from complex I, but can also sequester ROS generated from complex III [13], thus preventing excessive production of mitochondria-generated ROS. In vitro studies have shown that SkQ has a wide concentration range of antioxidant capacity [13], and in vivo studies have demonstrated that SkQ treatment effectively reduced oxidative damage in several models of oxidative stress [12–14].

In the present study we used the SkQ compound to: (1) determine whether sequestering of mitochondrial ROS production can prevent increased ROS production and oxidative stress in skeletal muscle under high-fat conditions; and (2) examine the impact of the anticipated lowering of oxidative stress on skeletal muscle insulin sensitivity and mitochondrial function.

Methods

Chemicals SkQ was generously donated by O. Fedorkin (Mitotech, Moscow, Russia). All other chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise stated.

C2C12 cell glucose uptake assay C2C12 cells (LGC standards, Teddington, UK) were maintained in DMEM (GlutaMAX low-glucose DMEM; Invitrogen, Breda, the Netherlands) supplemented with 10% FCS (vol./vol.) and grown on ECM (extracellular matrix) matrigel-coated culture plates. Cells were differentiated towards multinucleated myotubes over the course of a week in αMEM supplemented with 2% FCS (vol./vol.). Differentiated myotubes were pretreated with 20 nmol/l SkQ or vehicle (control) during the last 3 days of differentiation before collection. For the oxidative stress assay, myotubes were lysed in RIPA buffer and protein carbonyls were determined as described below. Prior to the glucose uptake assays, the cells were treated for 24 h with 500 μmol/l palmitate conjugated to BSA (essential NEFA-free) (ratio BSA:palmitate 1:2.5) or solely BSA (control). Deoxyglucose uptake was performed as previously described [15].

In vivo mouse study Male C57Bl/6 mice (8 weeks old; $n=10$ per group) were purchased from Charles River (Maastricht, the Netherlands) and housed individually on a 12 h light: 12 h dark cycle. The mice were placed on either a low-fat chow diet (LF) (Ssniff, Soest, Germany) or a

high-fat, high-sucrose diet (HF) (Research Diets, New Brunswick, NJ, USA) for 16 weeks. A third group of mice received the HF plus the SkQ antioxidant (HF+SkQ). Based on previous studies, an SkQ dose of 250 nmol/kg body weight [16] was supplemented throughout the study via the drinking water [17]. Water bottles for all mice were refreshed three times per week, and body weight and food intake were measured weekly. After the 16 week dietary intervention period, mice were fasted for 3 h and killed by cervical dislocation under basal control (n=5) conditions or after 10 min of insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) stimulation (10 U/kg i.p.; n=5). Skeletal muscles were excised, frozen in liquid nitrogen-cooled isopentane (2-methyl-butane; Fluka, Zwijndrecht, the Netherlands) and stored at −80°C until further analysis. All protocols were approved and conducted in accordance with Maastricht University Animal Ethics Committee guidelines.

Glucose tolerance tests and measurements from plasma Glucose tolerance tests were performed after week 15 of the intervention, following a 5 h fast as previously described [18]. Blood glucose was measured using a glucose meter (LifeScan, Milpitas, CA, USA) and samples were collected in microtitre tubes (BD, Franklin Lakes, NJ, USA). Plasma was separated by centrifugation for 5 min at 5,000 *g* and stored at −80°C. Plasma insulin was measured using a commercially available RIA kit (Millipore, Billerica, MA, USA). Plasma triacylglycerol and NEFA levels were determined with commercially available kits from Roche (Schlieren, Switzerland) and Wako Chemicals (Neuss, Germany), respectively.

Western blot To determine (phosphorylated)Akt and oxidative phosphorylation (OXPHOS) proteins, gastrocnemius muscle was homogenised in lysis buffer (10% NP40 [vol./vol.], 10% SDS [vol./vol.], 100 mmol/l PMSF in PBS) and cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche). Equal amounts of protein were loaded on to SDS-PAGE gel, and standard running, blocking and incubation protocols were followed using primary antibodies against Akt, phosphorylated Akt (pAkt) (S473) (Cell Signaling Technology, Danvers, MA, USA), OXPHOS proteins (Mitosciences, Eugene, OR, USA), sarcomeric actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin. The appropriate secondary antibodies (Invitrogen, Paisley, UK) were applied and blots were visualised using the Odyssey Near Infrared Imager (Licor, Leusden, the Netherlands) or with a maximum sensitivity substrate (SuperSignal West Femto; Thermo Fisher Scientific, Waltham, MA, USA) in a reader (ChemiDoc XRS; BioRad, Hercules, CA, USA). For glycogen synthase kinase 3 (GSK3) and JUN N-terminal kinase (JNK) phosphorylation, and IRS1 content levels, muscle samples were homogenised in Bioplex lysis buffer containing protease and phosphatase inhibitors (Biorad), after which protein content was determined using a BCA-kit (Thermo Scientific, Rockford, IL, USA). Protein (10 μg) was loaded on to SDS-PAGE gels, and standard blocking and incubation protocols were followed using primary antibodies against phospho-GSK3α/β-Ser21/9 and phospho-JNK-Thr183/Tyr185 (Cell Signaling Technology), or against IRS1 [19]. Loading corrections for phosphorylation levels were performed by reprobing with antibodies recognising α-tubulin (Calbiochem, Darmstadt, Germany) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology). Bound antibodies were detected with horseradish-peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence, and visualised using the Versadoc system (Biorad). Blots were quantified using a software package (Quantity One, version 4.6.9; Biorad). Protein carbonyl levels were assessed with a protein oxidation detection kit (Oxyblot; Millipore) as described elsewhere [20].

Mitochondrial DNA copy number The mitochondrial DNA copy number was determined by the ratio of *Cox2* expression (mitochondrial gene) over *Ucp2* expression (nuclear gene) as previously described [21]. Briefly, isolated DNA (Nucleospin Tissue kit; Macherey Nagel, Düren, Germany) from gastrocnemius muscle samples was analysed by real-time PCR using a sequence detector (ABI 7900; Applied Biosystems, Branchburg, NJ, USA) and the following program: one cycle at 50°C for 2 min then at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The absolute quantification of each gene was determined by a standard curve.

Enzyme activities The activity of superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPx1) was measured in quadriceps muscle homogenates. SOD2 activity was determined according to Oberley and Spitz [22] and distinguished from total SOD activity by pre-incubating the samples with NaCN (5 mmol/l) for 30 min. GPx1 activity was determined according to Paglia and Valentine [23] with reduced glutathione at a concentration of 10 mmol/l. The activity of hydroxylacyl dehydrogenase (HADH) and citrate synthase (CS) was measured in gastrocnemius muscle homogenates as previously described [24].

Mitochondrial isolation, respiration and ROS measurements In a second set of mice (n=7 per group) on the same diet and intervention regimen as that described above, skeletal muscle mitochondria were isolated and respiration was measured as previously described [25, 26]. Respiration rates in isolated mitochondria (0.1 mg/ml) were determined at 37°C by polarographic oxygen sensors in a two-chamber Oxygraph (Oroboros Instruments, Innsbruck, Austria) using pyruvate (5 mmol/l) plus malate (3 mmol/l), or palmitoyl-coenzyme A

(CoA) (50 μmol/l) plus carnitine (2 mmol/l) as substrates. For mitochondrial ROS production, we evaluated H_2O_2 release from isolated mitochondria over 15 min at 37°C using Amplex Red fluorescence quantification (Invitrogen). Briefly, mitochondria (50 μg) were added to respiration buffer (100 mmol/l sucrose, 50 mmol/l KCl, 20 mmol/l TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid), 1 mmol/l EDTA, 4 mmol/l KH_2PO_4, 2 mmol/l $MgCl_2$, 3 mmol/l malic acid and 0.1% [wt/vol.] fatty acid-free BSA, pH 7.2) fuelled by succinate (10 mmol/l) in the presence of complex III inhibitor antimycin (1 μmol/l) to maximise ROS production [27]. The reaction started upon the addition of Amplex Red reagent (100 μmol/l) and horseradish peroxidase (2 U/ml). Superoxide dismutase (100 U/ml) was added to the buffer to ensure complete conversion of superoxide to H_2O_2 and to prevent the superoxide radical from interacting with the horseradish peroxidase [28].

Statistics Results are expressed as means±SEM and were analysed by one-way ANOVA followed by Newman–Keuls post-hoc test, or by two-way ANOVA followed by Bonferroni's post-hoc test where appropriate. Basal and insulin-stimulated conditions in muscle were analysed by two-tailed Student's *t* test. Significance was set at $p<0.05$. All graphs and statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

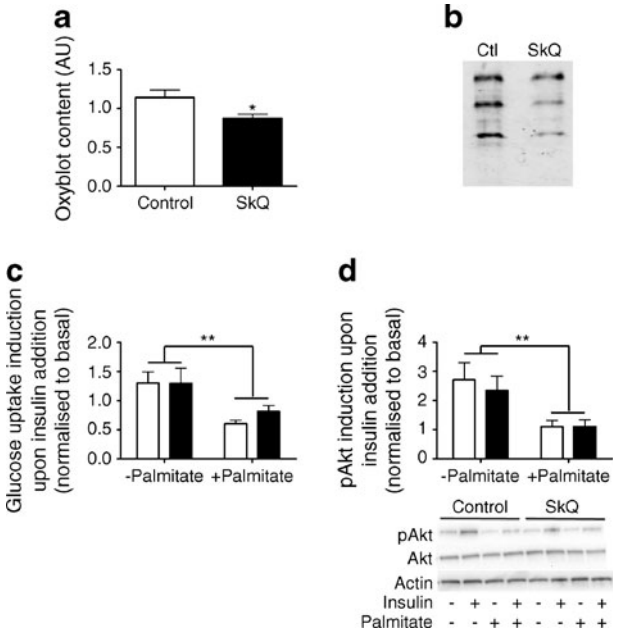

Fig. 1 ROS sequestering and insulin sensitivity in C2C12 cells. (**a**) Oxyblot content and (**b**) representative blot in differentiated C2C12 cells without (control [Ctrl]) or with SkQ treatment; $n=6$ per condition. Glucose uptake (**c**) in control (white bars) and SkQ-pretreated (black bars) cells upon insulin stimulation in the absence or presence of palmitate as indicated; $n=5$–8 per condition. (**d**) pAkt abundance under the same conditions as above (**c**), with representative blot of pAkt, total Akt and actin (loading control) under basal, and insulin- and palmitate-stimulated conditions. Data (**a**, **c**, **d**) are presented as mean±SEM; *$p<0.05$ and **$p<0.01$

Results

SkQ treatment does not improve palmitate-induced insulin resistance in vitro SkQ treatment significantly lowered oxidative stress by 23% ($p<0.05$) (Fig. 1a, b) as determined by protein carbonyl content, a marker of oxidative damage, in C2C12 myotubes under basal conditions. We investigated whether this reduced level of oxidative stress affected cellular glucose uptake under basal, insulin-stimulated and palmitate-induced insulin-resistant conditions. Basal glucose uptake was similar between control and SkQ-treated myotubes (control 1.00±0.09, SkQ 0.94±0.06 arbitrary units [AU], $n=5$–7, p=NS). Insulin-stimulated glucose uptake was comparable between control and SkQ-treated myotubes (Fig. 1c). The incubation of C2C12 muscle cells with palmitate induced insulin resistance, as seen in the significant reduction of glucose uptake upon insulin stimulation in control and SkQ-treated cells ($p<0.001$, two-way ANOVA). There was no significant difference between palmitate-induced insulin resistance in control and SkQ-treated myotubes (Fig. 1c). Furthermore, pAkt, a marker of insulin signalling, revealed comparable results. Basal pAkt levels (control 0.22±0.06, SkQ 0.18±0.04 AU, $n=8$, p=NS) and insulin induction of pAkt levels were similar in both cell groups (Fig. 1d). Palmitate also reduced the insulin-stimulated pAkt response by about half in control myotubes, a reduction not restored by SkQ treatment ($p<0.001$, two-way ANOVA) (Fig. 1d). No significant difference was seen in pAkt levels between control and SkQ myotubes in the presence of palmitate. Total Akt levels were comparable between control and SkQ myotubes under all test conditions (Fig. 1d). Thus the SkQ-induced lowering of oxidative stress did not suffice to ameliorate lipid-induced insulin resistance in muscle cells.

SkQ treatment in vivo does not influence weight gain or plasma lipid levels To extend our findings from the cell model to the in vivo situation, we examined the effects of SkQ treatment in mice. Male mice (8 weeks old) were maintained on an LF or placed on an HF for 16 weeks. A second group of HF-fed mice received drinking water supplemented with SkQ (HF+SkQ). HF mice weighed 31% ($p<0.0001$) more than LF mice and had significantly elevated NEFA, glucose and insulin levels (Table 1). The HF+SkQ group also had significantly more weight gain and elevated plasma values compared with LF controls, with changes similar to those observed in HF mice. Food intake was comparable in all three groups (Table 1), suggesting no taste aversion to the SkQ treatment and normal eating behaviour.

Table 1 Body weight, plasma values and skeletal muscle enzyme activity

Variable	LF	HF	HF+SkQ	*p* value
Body weight (g)	27.6±0.4	40.1±1.2***	40.3±1.3***	<0.0001
Food intake (kJ/week)	489.9±19.2	494.1±34.7	584.9±64.0	NS
Triacylglycerol (mmol/l)	1.99±0.30	2.67±0.12	2.94±0.34	0.07
NEFA (mmol/l)	0.53±0.09	1.24±0.15***	1.30±0.06***	0.0003
Glucose (mmol/l)	5.87±0.25	7.98±0.69**	8.13±0.33**	0.0027
Insulin (pmol/l)	61.2±14.3	218.7±28.9***	212.1±20.5***	0.0003
GPx1 activity (mU/mg)	5.83±0.37	7.01±0.37	6.65±0.24	0.07
HADH activity (U/mg)	38.54±6.57	62.07±8.31	63.47±9.37	0.07

Values are expressed as mean±SEM; n=5–10

**p<0.01 and ***p<0.001 vs LF

SkQ treatment reduces HF-induced oxidative damage Oxidative stress measured as protein carbonyl content was increased by 56% upon HF feeding (p<0.05 vs LF) (Fig. 2a, b). Consistent with our findings in C2C12 muscle cells, SkQ treatment in vivo effectively prevented the diet-induced increase in skeletal muscle oxidative damage (p<0.5 vs HF) (Fig. 2a, b). Since SkQ specifically targets mitochondrial ROS production, we also measured H_2O_2 release from freshly isolated skeletal muscle mitochondria. H_2O_2 emission was significantly increased in the HF group compared with LF (54%, p<0.05) (Fig. 2c). SkQ treatment normalised H_2O_2 levels in HF+SkQ mice to those of the LF controls (Fig. 2c), thus confirming the efficacy of the mitochondrial-specific ROS scavenger as shown above and previously reported [12–14]. In addition, SOD2 (the mitochondrial SOD) activity was significantly elevated in HF mice (twofold vs LF, p<0.01) (Fig. 2d), suggesting a state of increased ROS production. SkQ treatment significantly blunted the HF-induced increase in SOD2 activity (p<0.05) (Fig. 2d). Finally, a trend was observed for increased activity of the cytosolic antioxidant GPx1 in both HF-fed groups (Table 1).

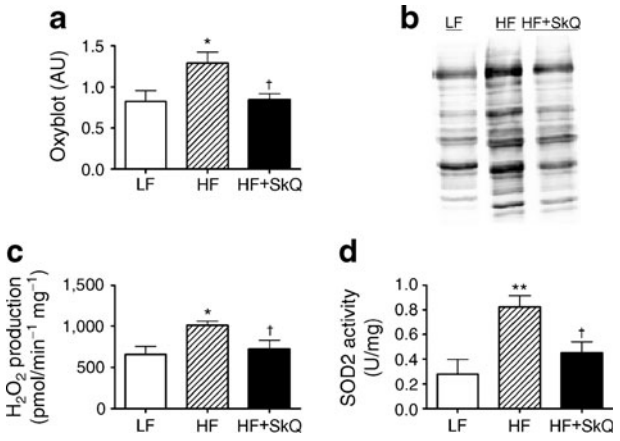

Fig. 2 SkQ treatment effectively blunts HF-induced oxidative stress and ROS production. (**a**) Quantification of muscle protein carbonyl in LF, HF and HF+SkQ mice, with (**b**) a representative protein Oxyblot. (**c**) Isolated mitochondria hydrogen peroxide production and (**d**) SOD2 activity assay in muscle homogenates. Data are presented as mean± SEM; n=7 per group for isolated mitochondrial assays and n=5 per group for activity and oxyblot assays; *p<0.05 vs LF, **p<0.01 vs LF and $^{\dagger}p$<0.05 vs HF

ROS sequestering does not improve insulin sensitivity Both HF and HF+SkQ mice displayed exacerbated glucose metabolism compared with LF mice, as illustrated by decreased glucose tolerance after 15 weeks of the intervention (Fig. 3a). The AUC for glucose levels during the glucose tolerance test was significantly increased by 33% (p<0.001) in HF and by 22% (p<0.01) in HF+SkQ mice (Fig. 3b). Insulin levels measured during the glucose tolerance test were also significantly higher in both groups (Fig. 3c). Insulin AUC levels were increased twofold (p<0.05) and fourfold (p<0.01) in HF and HF+SkQ, respectively, compared with LF mice (Fig. 3d), indicative of the hyperinsulinaemia associated with insulin resistance. No statistical difference between HF and HF+SkQ mice was observed for any of the variables mentioned above. In addition, muscle-specific insulin signalling was evaluated by examining levels of pAkt and its downstream target, phosphorylated GSK3 (pGSK3), in gastrocnemius muscle under basal and insulin-stimulated conditions (Fig. 4). Although insulin significantly stimulated pAkt levels in LF, HF and HF+SkQ mice (p<0.001, basal vs insulin) (Fig. 4a), the absolute increase in insulin-stimulated pAkt was blunted in HF and HF+SkQ mice (HF −35%, p<0.01; HF+SkQ −62%, p<0.001 compared with LF). In addition, pGSK3 was significantly increased upon insulin stimulation in LF mice (74% vs basal, p<0.05) (Fig. 4b), while insulin signalling was severely disrupted in HF and HF+SkQ mice, as there was no insulin-mediated increase in pGSK3 over basal levels (p=NS) (Fig. 4b). Total Akt was similar for all three groups under basal and insulin-stimulated conditions (Fig. 4a); moreover, no change was observed in IRS1 protein abundance (Fig. 4c). Phosphorylation levels of JNK, an inflammation marker of insulin resistance, were unaltered in all three groups (Fig. 4d), suggesting that HF and HF+SkQ mice have disruptions in insulin signalling, with no effect on inflammatory stress signalling.

The effect of ROS sequestering on mitochondrial content and respiration Mitochondrial density was estimated by mitochondrial DNA copy number, CS activity and the protein

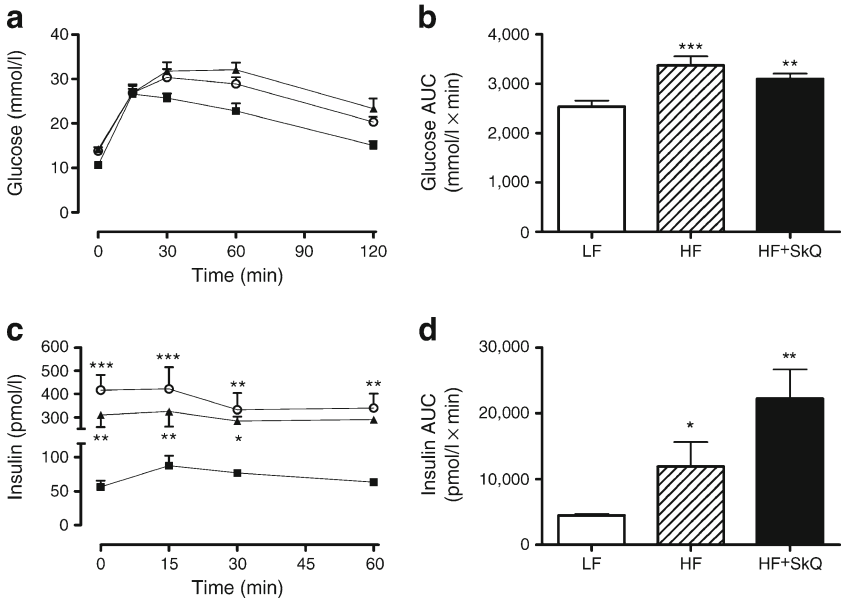

Fig. 3 ROS sequestering does not improve glucose tolerance in HF-fed mice. (**a**) Glucose tolerance curves after 15 weeks of intervention in LF- (black squares), HF- (black triangles) and HF+SkQ-fed (white circles) mice, with (**b**) AUC analysis in groups as indicated. (**c**) Insulin levels and (**d**) AUC during the glucose tolerance test in the same groups. Data are presented as mean±SEM; n=6–10 mice per group; *p<0.05, **p<0.01 and ***p<0.001 vs LF. Two-way ANOVA analysis revealed group (p<0.0001) and time (p<0.0001) effects for glucose, and a group (p<0.0001) effect for insulin during the glucose tolerance test

content of structural components of the respiratory chain complexes (OXPHOS). Whereas no significant difference was detected in the mitochondria DNA copy number (Fig. 5a), CS activity was significantly increased in HF mice (65%, p<0.01) (Fig. 5b) compared with LF. Similarly, the OXPHOS complexes were increased in HF mice (57% vs LF, p<0.01) (Fig. 5c, d). Interestingly, these HF-induced increases in CS activity and OXPHOS content were normalised to LF levels in HF+SkQ mice (Fig. 5b, c). Finally, we investigated the impact of ROS sequestering on mitochondrial respiration, fuelled by either a carbohydrate-derived (pyruvate) or a fatty acid-derived (palmitoyl-CoA in the presence of carnitine) substrate in isolated mitochondria (Table 2). Under the HF condition, ADP-stimulated (state 3) pyruvate-supported respiration rates tended to decrease (p=0.06) compared with LF mice. The maximum carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)-induced (state U) respiration, indicating the maximum capacity of the electron transport chain on a given substrate, was significantly decreased by 30% vs LF mice (p<0.05) (Table 2). At the same time, there was no change in the oligomycin-insensitive (state 4) respiration rate, a marker of mitochondrial proton leak. SkQ supplementation did not restore the HF-induced reduction in pyruvate-supported respiration, as HF+SkQ mice displayed

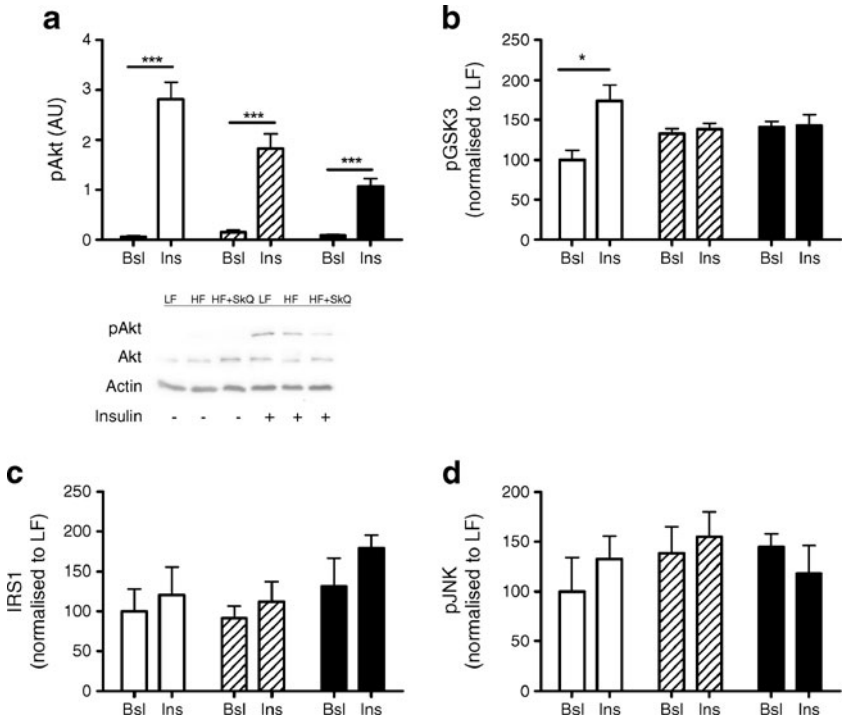

Fig. 4 Skeletal muscle insulin signalling. (**a**) Basal control (Bsl) and insulin-stimulated (Ins) pAkt in LF (white bars), HF (hatched bars) and HF+SkQ (black bars) gastrocnemius samples, with representative pAkt, total Akt and actin (loading control) blots. (**b**) Levels of pGSK3, (**c**) total IRS1 content and (**d**) pJNK in groups as above (**a**). Data (**a**, **c**–**e**) are presented as mean±SEM; n=5 mice per group per condition; *p<0.05 and ***p<0.001 for basal vs insulin-stimulated conditions

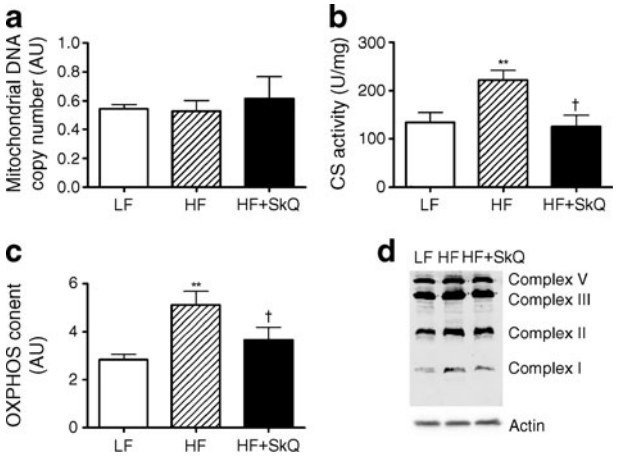

Fig. 5 Mitochondrial density markers. (**a**) Mitochondrial DNA copy number, (**b**) gastrocnemius muscle CS activity, (**c**) total OXPHOS complex quantification and (**d**) representative blots with sarcomeric actin as loading control for mouse groups as shown. Data are presented as mean±SEM; $n=5$ for mtDNA and western blot and $n=10$ for CS assay; $^{**}p<0.01$ vs LF and $^{\dagger}p<0.05$ vs HF

similar decreases in pyruvate-supported state 3 and state U ($p<0.05$) respiration rates. We did not observe any change in fatty acid-supported mitochondrial respiration under ADP- or maximal-stimulated conditions in any of the groups (Table 2). Furthermore, skeletal muscle HADH activity (a beta-oxidation enzyme) showed a tendency to be upregulated under both high-fat conditions (Table 1).

Discussion

In the present study, we tested whether mitochondria-targeted ROS sequestering (SkQ treatment) was able to preserve skeletal muscle insulin sensitivity in a muscle cell model of fatty acid-induced and in a mouse model of diet-induced insulin resistance. In vitro, SkQ treatment effectively reduced oxidative stress in C2C12 myotubes, but did not improve glucose uptake or insulin signalling under lipid-induced insulin resistance. In vivo, SkQ treatment successfully normalised the level of HF-induced oxidative stress in mouse muscle, but this did not reverse the reduction in glucose tolerance and skeletal muscle insulin sensitivity upon HF feeding.

Several studies have shown a link between insulin resistance, ROS production and oxidative stress in in vitro and in vivo models. Palmitate exposure in L6 myotubes resulted in a dose-dependent decrease in insulin-mediated glucose uptake, paralleled by increased superoxide production [29] and mitochondrial DNA damage [30]. In addition, cultured cells incubated with H_2O_2 had greater insulin resistance [31], and ROS production was identified as the common denominator and causal factor in several cellular models of insulin resistance [4]. Many studies have also reported beneficial effects on insulin sensitivity when oxidative stress was blunted. As mentioned previously, MCAT overabundance in mice improved insulin sensitivity and attenuated H_2O_2 emission in [5] ageing- and [1] high-fat diet-induced models of insulin resistance. Similarly, rats treated with a small mitochondrial antioxidant peptide were protected from high-fat diet-induced mitochondrial H_2O_2 emission, impaired glucose uptake and reduced skeletal muscle insulin sensitivity [1]. In contrast to these studies [1, 5], we were unable to confirm that interfering with mitochondrial ROS production, which leads to reduced oxidative stress levels, averts HF-induced insulin resistance in skeletal muscle. These discrepancies may be due to the technical approach used (genetic overexpression vs oral antioxidant), the induction of oxidative stress (age- vs diet-induced) and/or the duration of the intervention (6 vs 16 weeks). In addition, the diet composition may also have led to differences

Table 2 Intrinsic mitochondrial respiration levels

	Respiration pmol $(s\times mg)^{-1}$			
Respiration state	LF	HF	HF+SkQ	*p* value
Pyruvate				
State 3	5,356±441.3	3,928±515.1	3,680±481.2	0.0621
State 4	442.1±20.3	384.6±56.7	345.4±40.3	NS
State U	9,188±650.8	6,348±886.8*	6,436±771.3*	0.0291
Palmitoyl-CoA + carnitine				
State 3	1,413±341.4	1,348±212.5	1,467±244.9	NS
State 4	446.9±40.1	392.2±40.5	395.0±36.5	NS
State U	1,781±472.4	1,654±285.5	1,859±331.8	NS

Values expressed as mean±SEM; $n=6$–7

ADP-stimulated (state 3) respiration was induced by the addition of ADP (450 μmol/l), state 4 respiration was attained upon addition of the ATP-synthase inhibitor oligomycin (1 μg/ml) and maximum oxygen flux (state uncoupled, U) was achieved by titrating the chemical uncoupler FCCP

*$p<0.05$ vs LF

between the studies. In the present study, we used a diet high in fat and sucrose, which is known to induce oxidative stress, mitochondrial dysfunction and insulin resistance [32]. This may be relevant since the source of fat has been implicated as a major contributor to lipid-induced ROS and insulin resistance [30], but high sucrose levels also increase ROS production and oxidative stress [33].

However, not all studies support the notion that ROS production is a causal factor in insulin resistance. Thus it has been shown that insulin stimulates the generation of ROS, since when insulin binds to its receptor, a short burst of cellular ROS is produced, acting as second messenger that mediates insulin signalling [34, 35]. Furthermore, Loh et al [36] demonstrated that subtle increases in ROS production were in fact associated with improved insulin sensitivity in mice deficient in the cytosolic antioxidant GPx1, suggesting that ROS may even help enhance insulin sensitivity. At the same time, overabundance of GPx1 resulted in hyperglycaemia, hyperinsulinaemia and insulin resistance in mice [37], indicating that H_2O_2 quenching disrupts insulin action. Moreover, Yokota et al [38] demonstrated that mice fed a high-fat diet and treated with apocynin, an NAD(P)H oxidase inhibitor, had blunted skeletal muscle superoxide production, yet with no effect on glucose uptake or other markers of insulin sensitivity. Finally, Abdul-Ghani et al [39] failed to show a difference in skeletal muscle mitochondrial H_2O_2 levels between type 2 diabetic participants and age-matched controls, suggesting that increased mitochondrial ROS is not a mediator of insulin resistance. Here, we demonstrated, using a novel mitochondria-specific antioxidant, that in vitro reductions of oxidative stress did not avert lipid-induced disruptions of insulin-stimulated glucose uptake and insulin signalling, although it remains possible that palmitate exposure in these cells leads to insulin resistance via mechanisms that are independent of oxidative stress. On the other hand, in vivo whole-body glucose tolerance, as well as insulin-mediated pAkt in skeletal muscle remained impaired in mice on an HF supplemented with SkQ, a mitochondria-targeted antioxidant, despite reduced skeletal muscle oxidative stress. Although the slightly increased insulin levels during the glucose tolerance test in our HF+SkQ mice suggest that SkQ treatment causes an even higher degree of HF-induced insulin resistance, this is not supported by the muscle pAkt and in vitro glucose uptake results. However, as we did not perform clamp studies, it is possible that effects on skeletal muscle insulin-stimulated glucose uptake in vivo remained undetected. Moreover, alterations in muscle glucose transport subsequent to lipid-induced insulin resistance may exist, regardless of an effect on Akt signalling [40].

Bonnard et al [32] demonstrated that when mice were fed a high-fat high-sucrose diet, similar to that used in the present study, oxidative stress was coupled to reduced mitochondrial biogenesis and dysfunction. In contrast, several studies have observed upregulated mitochondria biogenesis in response to a high-fat insult [41, 42]; however, this response seems to be dependent on the intervention time [21, 25] and/or the method of determining mitochondrial density [32]. In the present study, we measured several markers of mitochondrial density, and observed that OXPHOS content and CS activity revealed increased mitochondrial density in the HF-fed condition, in agreement with previous reports [41]. Interestingly, these two markers were normalised to LF levels upon ROS sequestering. This finding is in line with other reports showing that antioxidant supplementation suppresses exercise-induced mitochondrial biogenesis [43, 44], suggesting a potential role of ROS in mitochondrial biogenesis, although not all studies support this conclusion [45].

We also observed a substrate-specific reduction upon pyruvate (plus malate)-supported mitochondrial respiration. These results are consistent with those of Mogensen et al [46], who also demonstrated reduced pyruvate-supported respiration rates with no change in lipid-supported respiration rates in isolated muscle mitochondria from type 2 diabetes participants compared with age- and BMI-matched controls. Thus SkQ treatment effectively reduced skeletal muscle oxidative damage, but did not reverse the HF-induced decrease in pyruvate-supported respiration. Therefore we conclude that this reduction in pyruvate-supported mitochondrial respiration does not occur as a result of oxidative stress. It is tempting to speculate that increased markers of mitochondrial content (OXPHOS proteins and CS activity) in HF-fed mice may have compensated for the reduced intrinsic function. Since this response appeared to be blunted in HF+SkQ mice, the latter are likely to have had lower total mitochondrial oxidative capacity than HF-fed mice, despite similar levels of insulin resistance. These findings also argue against mitochondrial dysfunction playing a causal role in the development of HF-induced insulin resistance, which has been suggested previously [6, 7].

A potential limitation of the present study is that only the 16 week endpoint variables were examined, making it impossible to comment on the favourable short-term effects of antioxidant treatment on insulin sensitivity that have been reported by others [47]. Nor can we exclude putative beneficial effects of SkQ treatment upon prolonged exposure. Furthermore, we only tested one dose. In this respect, other studies have clearly shown the dose used by us to be optimal in inbred and outbred rodents in longevity studies [16, 48]. However, it is possible that the full blockade of HF-induced ROS production was too rigid, dissipating putative beneficial effects of ROS, such as stimulation of endogenous antioxidant capacity or mitochondrial biogenesis, which themselves might in turn affect insulin sensitivity. Finally,

oxidative stress is one of several putative mechanisms associated with lipid-induced insulin resistance. If lipid-induced insulin resistance is independent of increased ROS production, then an intervention designed to suppress oxidative stress clearly will not improve insulin sensitivity.

In conclusion, an antioxidant specific to the sequestering of mitochondrial ROS production (SkQ) successfully suppressed oxidative damage in vitro and under HF conditions in vivo. Despite this relief of oxidative stress, SkQ treatment did not ameliorate lipid-induced insulin resistance in the two study models. Therefore, our results suggest that diet-induced oxidative stress is not a prerequisite for the development of skeletal muscle insulin resistance.

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Contribution statement SP designed the study, researched data, carried out data analysis and wrote the article. BVB, MB, MPV, EAC, JAJ, DVB, GDH, and DMO researched data and reviewed/edited the manuscript. JJB contributed to data interpretation and reviewed/edited the manuscript. PS contributed to the discussion and critically revised the article. JH designed the study, contributed to the discussion and critically revised the article. All authors approved the final version of the manuscript.

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

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