Original Paper

Palmitate-Induced Cell Death and Mitochondrial Respiratory Dysfunction in Myoblasts are Not Prevented by Mitochondria-Targeted Antioxidants

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Key Words

Palmitate • Mitochondrial respiration • mtDNA integrity • Mitochondria-targeted antioxidants • MitoQ • MitoTEMPOL • Skeletal muscle • Myoblasts • Myotubes • Spare respiratory capacity

Abstract

Background/Aims: Deleterious effects of saturated fatty acids in skeletal muscle cells are well known but their impact on mitochondrial respiration has not been well studied. Mitochondrial oxidative damage has been implicated to play a role in their effect. The purpose of this study was to evaluate viability, mtDNA integrity and mitochondrial respiration in C2C12 myoblasts and myotubes exposed to palmitate and to test the effect of mitochondria-targeted antioxidants MitoQ and MitoTEMPOL in preventing palmitate-induced damage. **Methods:** Cells were treated with tested compounds, mtDNA damage was detected by quantitative PCR and mitochondrial respiration was measured using an extracellular flux analyzer XF24. **Results:** Palmitate caused mtDNA damage, which was associated with reduced mitochondrial respiration and cell death in myoblasts but not in myotubes. MitoTEMPOL was able to prevent palmitate-induced mtDNA damage in myoblasts but failed to prevent cell death. MitoQ did not show any protective effect and both compounds markedly inhibited mitochondrial respiration. **Conclusion:** Our results indicate that skeletal muscle progenitor cells could be the first target of the deleterious action of palmitate, as myoblasts appeared to be more sensitive to its effects than myotubes possibly in part due to a lower spare respiratory capacity in the former. Only MitoTEMPOL prevented palmitate-induced mtDNA damage but neither antioxidant was able to prevent cell death and both antioxidants had a marked negative effect on respiration.

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Introduction

Elevated levels of plasma free fatty acids (FFA), which are characteristic for obesity and type 2 diabetes [1, 2], are associated with various pathophysiological consequences in skeletal muscle, although recently this simple association has been put into question [3]. Development of insulin resistance and changes in mitochondrial function in skeletal muscle have been found in healthy humans and animals after lipid infusion [4, 5] or after prolonged fasting [6]. Experiments on cultured skeletal muscle cells show that exposure to long-chain saturated FFA but not unsaturated FFA leads to the induction of insulin resistance, inflammation, some features of mitochondrial dysfunction and apoptosis [7-9]. Although information has accumulated regarding the role of FFA in the induction of insulin resistance in skeletal muscle and cultured skeletal muscle cells [4, 5, 7, 10] less data is available about their effect on mitochondrial function and specifically mitochondrial respiration.

Metabolic states characterized by hyperglycemia and high levels of circulating free fatty acids have been shown to be associated with a chronic oxidative stress and increased mitochondrial reactive oxygen species (ROS) production [11, 12]. Increased production of hydrogen peroxide associated with insulin resistance was observed also in rodents on high fat diet with no change in mitochondrial respiratory function [13]. There are several studies in cultured skeletal muscle cells showing an increase of mitochondrial ROS production induced by palmitate [14-16] accompanied by mtDNA damage and some features of mitochondrial dysfunction [17, 18]. Mitochondrial DNA has an important role in the regulation of mitochondrial respiration and oxidative damage of mtDNA was postulated as an initial event leading to mitochondrial dysfunction [18].

Therapy that can decrease mitochondrial oxidative damage could prevent damage associated with excess of nutrients such as saturated fatty acids. Mitochondria-targeted antioxidants accumulate in mitochondria and have been shown to be more effective in preventing mitochondrial oxidative damage than untargeted antioxidants [19, 20]. MitoQ and MitoTEMPOL are two promising compounds, whose targeting to mitochondria is achieved by the covalent attachment of an antioxidant molecule to the lipophilic triphenylphosphonium cation (TPP⁺) [21]. The shielded positive charge of these lipophilic cations enables them to permeate lipid bilayers easily and to accumulate several hundred-fold within mitochondria, due to the large membrane potential [22]. MitoQ is a ubiquinone derivative which exhibited both antioxidant and anti-apoptotic properties in a large number of *in vitro* as well as *in vivo* studies [19, 23-26]. MitoTEMPOL consists of the piperidine nitroxide TEMPOL conjugated with a TPP⁺ moiety. It has been shown to be effective in preventing lipid peroxidation and mtDNA damage in skeletal muscle cells [27] or in protecting pancreatic β -cells against oxidative stress under glucolipotoxic conditions [24].

The purpose of the present study was to clarify the effect of palmitate on viability, mtDNA integrity and mitochondrial respiration in C2C12 skeletal muscle cells. We tested the effect of palmitate both on differentiated myotubes and undifferentiated myoblasts. We found palmitate-induced mtDNA damage which was associated with reduced mitochondrial respiration, increased production of hydrogen peroxide and cell death in myoblasts, but not in myotubes. Following on the hypothesis that palmitate caused damage through an induction of oxidative stress, we hypothesized that treatment with mitochondria-targeted antioxidants might prevent this damage in myoblasts and we tested two such antioxidants, MitoQ and MitoTEMPOL.

Materials and Methods

Materials

Mitochondria-targeted antioxidants MitoQ and MitoTEMPOL were kindly provided by Dr. Michael P. Murphy (MRC Mitochondrial Biology Unit, Cambridge, UK). Propyltriphenylphosphonium bromide, palmitate and bovine serum albumin (fatty acid free) were purchased from Sigma-Aldrich. Fetal bovine

serum for cell cultures was from Life Technologies, penicillin and streptomycin were from Sigma-Aldrich. Blood & Cell Culture DNA Mini Kit for DNA isolation was obtained from Qiagen and reagents for QPCR from Applied Biosystems.

Cell culture and treatment

As a model of skeletal muscle we used murine C2C12 myoblasts. Cells were obtained from the European Collection of Cell Cultures and were used as myoblasts, which served as a model of satellite cells, and as myotubes, an *in vitro* model of muscle fibres. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere of 95% humidity and 5% CO₂ and maintained below a maximal confluence of 70-80%. To initiate differentiation, cells were allowed to reach 100% confluence and the medium was changed to the differentiation medium (DMEM containing 2% fetal bovine serum), which was replaced every 48 h. Myotubes were used for experiments after 7 days, when full differentiation was observed.

A stock concentration of palmitate was prepared by conjugating palmitate with fatty acid-free bovine serum albumin (BSA), using a modified method described by Cousin [28]. Briefly, palmitate was dissolved in 0.1 M sodium hydroxide at 70°C to a final concentration of 100 mM. Palmitate was then mixed with 15% fatty acid-free BSA at 50°C for 30 min, yielding a final stock solution of 5 mM. The molar ratio of FFA/BSA was 2:1 in this preparation which is close to the ratio observed in human serum [29]. A control BSA solution was prepared by mixing a volume of 0.1 M sodium hydroxide equal to that of palmitate in the palmitate-BSA solution with 15% fatty acid-free BSA. The pH of both solutions was adjusted to 7.4. After sterilization by filtration the actual concentration of palmitate was determined using the NEFA (non-esterified fatty acids) kit (Randox Laboratories, UK).

Cells were incubated for 18 h in DMEM containing palmitate-BSA conjugate or DMEM with BSA only (control). For experiments with antioxidants MitoQ or MitoTEMPOL were added to cells 1 h prior to the addition of palmitate and then incubated for 18 h. All compounds were washed away before measurements. We also treated cells with corresponding concentrations of propylTPP (pTPP) to control for the effect of the cation moiety itself [30].

Cell viability assay

Cell viability after the exposure to various concentrations of palmitate and antioxidants was assessed using the MTS assay (Promega). The incubation medium with tested compounds was changed to a medium with the MTS reagent at the end of exposure and cells were incubated for another 2 h at 37°C. The absorbance of dissolved formazan was measured at 490 nm in a microplate reader. Data are displayed as a percentage of cells with no additions.

Detection of extracellular hydrogen peroxide

Extracellular hydrogen peroxide (H₂O₂) was measured using the Amplex Red reagent (Life Technologies) by monitoring its oxidation to fluorescent resorufin in the presence of horseradish peroxidase (HRP). Cells were grown in 96-well plates and treated as described above. The incubation medium was then removed and cells were incubated with the reaction mixture at 37°C for 30 min protected from light. The reaction mixture consisted of 50 µM Amplex Red reagent and 0.1 U/ml HRP in Krebs-Ringer phosphate (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl₂, 1.22 mM MgSO₄, 5.5 mM glucose, pH 7.35). Fluorescence was detected at 590 nm with excitation at 540 nm. Protein content in each well was then determined by the bicinchoninic acid kit (Sigma). Data are expressed as relative fluorescence units (RFU) of resorufin (H₂O₂ emission) per microgram of protein.

Analysis of mtDNA damage by quantitative PCR

The integrity of mtDNA was assessed following treatments, using the quantitative polymerase chain reaction (QPCR) methodology [31, 32] with some modifications. The QPCR assay is based on the fact that any damage to DNA blocks the progression of the DNA polymerase on a template and will result in a decreased amplification. Amplification is thus inversely proportional to the amount of DNA damage on a given template.

Cells were incubated with tested compounds in 6 cm Petri dishes and high molecular weight DNA was then isolated with the Blood & Cell Culture DNA Mini Kit (Qiagen) as described in the manufacturer's protocol with some modifications. The tissue protocol was used, since the protocol for DNA extraction of cultured cells involves an isolation of nuclei and hence a loss of mtDNA [31]. The concentration of total cellular DNA was determined fluorometrically using Qubit dsDNA HS Assay Kit and Qubit fluorometer (Life Technologies). Stock DNA was diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to a final concentration of 3 µg/ml, which was used as a template for PCR. Two targets of mtDNA were amplified: a long target to quantify levels of mtDNA lesions and a short target to control for changes in mtDNA copy number. The long target (≈ 10 kbp) spanned ND1 and ND5 genes and the small target (127 bp) was part of ND1 gene. The primers were synthesized by Sigma and the primer nucleotide sequences were as follows: 5'-GCC AGC CTG ACC CAT AGC CAT AAT-3' and 5'-GCC GGC TGC GTA TTC TAC GTT A-3' for short target, 5'-GCC AGC CTG ACC CAT AGC CAT AAT-3' and 5'-GAG AGA TTT TAT GGG TGT AAT GCG G-3' for long target. The total volume for each PCR reaction was 20 µl, consisting of 15 ng of DNA template, 1 U rTth DNA polymerase XL (Applied Biosystems), 1× buffer XL (provided with the rTth polymerase), 200 µM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, 100 µg/ml of BSA, 1 mM Mg(OAc)₂ for long target and 1.5 mM Mg(OAc)₂ for short target. To ensure quantitative conditions, a control reaction containing 50% of template DNA was included during each set of amplification and only amplification ranging from 40 to 60% was considered acceptable. Automated hot-start using AmpliWax PCR Gems 50 (Applied Biosystems) was used to increase sensitivity and specificity of PCR. The parameters for the short mitochondrial target were 1 min at 94°C, then 20 cycles of 30 s at 94°C, 45 s at 64°C, 45 s at 72°C, followed by a final extension step of 10 min at 72°C. The parameters for the long mitochondrial target were 1 min at 94°C, then 18 cycles of 15 s at 94°C, 12 min at 64°C, followed by a final extension step of 10 min at 72°C. The PCR products were then quantified fluorometrically and the results were expressed as amplification relative to the control, which was defined as undamaged for the purposes of this assay.

Analysis of mitochondrial respiration

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using an extracellular flux analyzer XF24 (Seahorse Bioscience). The assay medium consisted of modified DMEM lacking sodium bicarbonate (XF Assay medium, Seahorse Bioscience) supplemented with 4 mM L-glutamine, 1 mM pyruvate and 5.5 mM glucose. The pH was adjusted to 7.4 at 37°C on the day of the experiment.

For experiments with myoblasts cells were seeded at a density of 13,000 cells per well in 24-well plates designed for this instrument and left to attach for at least 2 h. Cells were then treated with tested compounds as described above. For experiments with myotubes cells were differentiated for 7 days in assay plates prior to treatment. The culture medium was replaced with the assay medium 1 h prior to measurement and cells were kept at 37 °C in an atmosphere without CO₂. Mitochondrial stress test was then performed to assess the bioenergetic status of the cells [33]. OCR measurements were obtained before and after sequential additions of the ATPase inhibitor oligomycin (1 µM), inner membrane uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 µM) and inhibitors of complex I and III rotenone (1 µM) and antimycin A (1 µM) in myoblasts. For myotubes the concentration of FCCP was increased to 1.5 µM to obtain a maximal effect. After completion of the measurement, assay medium was removed and well contents were lysed in CellLytic MT lysis buffer (Sigma). Protein content in each well was determined by bicinchoninic acid kit (Sigma). The following parameters were then calculated: basal respiration (baseline respiration before addition of compounds), ATP turnover-linked respiration (oligomycin-sensitive respiration), maximal respiration (maximal uncoupled respiration), spare respiratory capacity (maximal uncoupled respiration minus basal respiration) and proton leak (oligomycin-insensitive respiration). Basal respiration is controlled strongly by ATP turnover, which alters mainly in response to ATP demand in the cell, and by changes in proton leak [34]. Maximal respiration after the addition of an uncoupler then reflects the maximal capacity of the electron transport chain. Non-mitochondrial respiration (after the addition of rotenone/antimycin A) was subtracted from basal and maximal respiration and proton leak. Data are expressed as pmol O₂/min/µg protein. Coupling efficiency (CE) was calculated as the ratio of ATP turnover-linked respiration and basal respiration [33].

Glycolytic activity (ECAR) was measured in parallel with respiration and the following parameters were calculated: basal ECAR (before addition of compounds), oligomycin-stimulated ECAR, and glycolytic reserve capacity (oligomycin-stimulated minus basal ECAR). Data are expressed as mpH/min/µg protein.

Fig. 1. Palmitate decreased viability of myoblasts (A) and myotubes (B) in a dose-dependent manner. Cells were treated with palmitate (PA) in concentrations varying from 0.01 to 1 mM or corresponding concentrations of BSA (C) for 18 h and cell viability was assessed using MTS assay. The effect of palmitate was statistically significant from 0.05 mM PA in myoblasts and 0.3 mM PA in myotubes. Only the highest concentration of BSA (corresponding to 1 mM palmitate) significantly decreased viability of myoblasts. Data are displayed as a percentage of untreated cells and presented as means and 95% CI from five independent experiments.

Citrate synthase activity measurement

The citrate synthase activity was determined in whole cell extracts using the Citrate Synthase Assay Kit (Sigma). The assay mixture contained 0.3 mM acetyl coenzyme A, 0.5 mM oxaloacetate and 0.1 mM 5,5-dithiobis(2-nitrobenzoic acid). Protein content of samples was determined by the bicinchoninic acid kit. The enzyme activity was measured spectrophotometrically at 30°C at 412 nm and expressed in nmol/min/mg protein.

Statistical analysis

All data represent at least three experiments and are expressed as means and 95% confidence intervals (CI, in square brackets). Mixed-effects model ANOVA with treatment as the fixed effect and set of experiment as the random effect was used followed by Dunnett post-hoc test for multiple comparisons. All analyses were performed in Statistica 9.1 (StatSoft, Inc.). P values less than 0.05 were considered statistically significant.

Results

Palmitate decreased cell viability and caused mtDNA damage in myoblasts and myotubes

We evaluated the cytotoxic effect of palmitate on myoblasts and myotubes in the concentration range of 0.01 - 1 mM. Cells were exposed to palmitate-BSA conjugate or to corresponding concentrations of BSA only. The results of MTS assays show a dose-dependent reduction of viability in both myoblasts and myotubes after an 18 h exposure to palmitate (Fig. 1A and B). Myoblasts showed a higher sensitivity to the cytotoxic effect of palmitate than differentiated myotubes. Only the highest concentration of BSA (corresponding to 1 mM palmitate) led to significantly decreased viability of myoblasts (Fig. 1A).

Based on toxicity assays 100 μ M concentration of palmitate was chosen and its effect on mtDNA integrity was tested. We consider this concentration of palmitate as physiologically relevant: although total plasma fatty acids can be as high as 1 mM or even higher under some physiological and pathological conditions (prolonged fasting, obesity, type 2 diabetes) [4], the concentration of palmitate under most conditions is generally much lower [35]. We used the QPCR assay to monitor damage to mtDNA by comparing the relative amplification of a very long fragment of mtDNA from treated and non-treated samples. As shown in Fig. 2A the relative amplification of the long mtDNA fragment was decreased both in myoblasts (difference of means C vs. PA -15.18 [-27.78, -2.59]) and myotubes (-16.84 [-29.68, -4.01]) after palmitate exposure suggesting that palmitate caused similar mtDNA damage.

Fig. 2. Palmitate-induced mtDNA damage in myoblasts (PA-MB) and myotubes (PA-MT) and increased H₂O₂ production in myoblasts. Cells were treated with 100 μ M palmitate or 0.3% BSA for 18 h. A) integrity of mtDNA was assessed by QPCR, where mtDNA of controls was defined as undamaged (100% amplification). Palmitate significantly decreased relative amplification of 10 kb fragment of mtDNA both in myoblasts and myotubes. B) extracellular H₂O₂ was measured by monitoring Amplex Red oxidation to fluorescent resorufin. Palmitate significantly increased H₂O₂ production only in myoblasts. Results are presented as means and 95% CI from at least four independent experiments. ** $p < 0.01$, *** $p < 0.001$ compared to controls.

Palmitate increased reactive oxygen species (ROS) production in myoblasts but not in myotubes

Extracellular hydrogen peroxide was measured to evaluate the role of ROS in the effect of palmitate. As shown in Fig. 2B myoblasts exposed to 100 μ M palmitate showed significantly higher hydrogen peroxide production compared to controls (difference of means 39.54 [15.10, 63.97] RFU/ μ g protein). Myotubes showed higher basal levels of hydrogen peroxide in controls, which were not further increased after the treatment with palmitate (-9.03 [-60.73, 42.66]).

Palmitate affected mitochondrial respiration more in myoblasts than in myotubes

After treatment with 100 μ M palmitate for 18 h, the bioenergetic status of cells was assessed as shown in Fig. 3A. Palmitate exposure led to a changed bioenergetic profile of both myoblasts and myotubes but in a different manner, as shown in Fig. 3B and C. In myoblasts palmitate exposure led to a decreased basal respiration (difference of means -2.88 [-5.98, 0.22] pmol O₂/min/ μ g protein), ATP turnover-linked respiration (-2.86 [-5.47, -0.25]), maximal respiration (-10.59 [-17.68, -3.50]) and spare respiratory capacity (SRC) (-7.71 [-11.94, -3.47]) compared to controls while there was virtually no difference in proton leak (-0.02 [-0.58, 0.53], Fig. 3B).

In myotubes exposed to palmitate basal respiration was significantly increased compared to controls (difference of means 1.16 [0.15, 2.16]) due to a higher proton leak (0.60 [0.31, 0.90]) but other parameters were not significantly affected (difference of means for ATP-turnover linked respiration 0.55 [-0.22, 1.32], maximal respiration 2.44 [-2.74, 7.62] and SRC 1.29 [-3.25, 5.82], Fig. 3C). We found a decrease in coupling efficiency (CE) in both myoblasts (difference of means C vs. PA -0.04 [-0.06, -0.02]) and myotubes (-0.05 [-0.06, -0.03]) exposed to palmitate (Fig. 3D). There was no difference in non-mitochondrial respiration between palmitate-treated and control cells in either group (data not shown). Extracellular acidification rates did not differ significantly between palmitate-treated and control myoblasts or myotubes suggesting no difference in glycolytic activity (Table 1).

Citrate synthase activity was decreased in palmitate-treated myoblasts but not in myotubes

Citrate synthase (CS) activity was measured as a mitochondrial mass marker. CS activity decreased significantly in myoblasts after the treatment with palmitate (difference of means

Fig. 3. Effect of palmitate exposure on mitochondrial respiration (oxygen consumption rates, OCR) in myoblasts and myotubes measured under different respiratory conditions. Cells were treated with 100 μ M palmitate (PA) or 0.3% BSA (C) for 18 h and then the growth media with palmitate was removed and replaced with assay media 1 h prior the assessment of mitochondrial function. OCR was measured before and after sequential injections of the indicated compounds. A) mitochondrial stress test experiment showing times of mitochondrial inhibitors addition to myoblasts. B,C) individual mitochondrial respiratory parameters (basal, ATP turnover-linked and maximal respiration; proton leak and spare respiratory capacity - SRC) calculated for myoblasts (B) and myotubes (C) as described in methods section. D) palmitate-induced decrease in coupling efficiency both in myoblasts (MB) and myotubes (MT). Results are presented as means and 95% CI from four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to controls.

Table 1. Glycolytic activity (ECAR) in myoblasts and myotubes after the treatment with palmitate. Data are expressed as mpH/min/ μ g protein and results are presented as means and 95% CI from four independent experiments

	Basal	Oligomycin-stimulated	Glycolytic reserve capacity
Myoblasts			
Control	1.83 [1.44, 2.23]	2.85 [2.26, 3.44]	1.02 [0.78, 1.25]
PA	2.04 [1.77, 2.32]	3.16 [2.69, 3.62]	1.11 [0.90, 1.33]
Myotubes			
Control	0.38 [0.29, 0.48]	0.73 [0.60, 0.87]	0.35 [0.29, 0.41]
PA	0.43 [0.30, 0.55]	0.74 [0.56, 0.92]	0.32 [0.24, 0.39]

C vs. PA -15.63 [-29.97, -1.30] nmol/min/mg protein), whereas no significant difference was observed between palmitate-treated and control myotubes (-1.44 [-39.45, 36.57], Table 2).

Since the effects of palmitate on respiratory and mitochondrial parameters in myotubes were negligible further experiments using mitochondria-targeted antioxidants were performed only in myoblasts.

Table 2. Citrate synthase activity in myoblasts and myotubes after the treatment with palmitate. Data are expressed as nmol/min/mg protein and results are presented as means and 95% CI from four independent experiments. * $p < 0.05$ compared to controls

	Control	PA
Myoblasts	147.36 [135.86, 158.87]	131.73 [122.08, 141.37] *
Myotubes	283.77 [255.66, 311.88]	282.33 [253.40, 311.26]

Fig. 4. Neither MitoQ nor MitoTEMPOL prevented palmitate-induced cell death in myoblasts. Cells were pre-treated with MitoQ or MitoTEMPOL at the indicated concentrations for 1 h and then co-incubated with palmitate for 18 h. Cell viability was then assessed using MTS assay. Data are displayed as a percentage of untreated cells. Results are presented as means and 95%CI from four independent experiments, n.s. – not significantly different from palmitate.

Fig. 5. MitoTEMPOL but not MitoQ decreased palmitate-induced mtDNA damage and hydrogen peroxide production in myoblasts. Cells were pre-treated with 0.5 μ M MitoQ, 10 μ M MitoTEMPOL or corresponding concentrations of pTPP for 1 h and then co-incubated with palmitate for 18 h. A) integrity of mtDNA was assessed by QPCR. MitoTEMPOL prevented a palmitate-induced decrease in the relative amplification of 10 kb fragment of mtDNA. B) extracellular H_2O_2 was measured by monitoring Amplex Red oxidation to fluorescent resorufin. MitoTEMPOL decreased palmitate-induced H_2O_2 production but the difference did not reach statistical significance. MitoQ treatment led to a significantly increased H_2O_2 production. Results are presented as means and 95%CI from three (A) or four (B) independent experiments. ## $p < 0.01$ compared to controls, *** $p < 0.001$ compared to palmitate.

Mitochondria-targeted antioxidants had no effect on palmitate cytotoxicity

If antioxidants are to be used in cells it is important to ensure that doses used are not toxic to a given cell type. We tested the cytotoxicity of both antioxidants in myoblasts in a broad concentration range. MitoQ significantly decreased cell viability at concentrations over 0.5 μ M and MitoTEMPOL for concentrations over 10 μ M (data not shown). Based on these results we pre-treated cells with 0.1 or 0.5 μ M MitoQ, 5 or 10 μ M MitoTEMPOL or

Fig. 6. Effect of MitoQ, MitoTEMPOL and pTPP compounds on palmitate-affected mitochondrial respiration of myoblasts. Cells were pre-treated with 0.5 μ M MitoQ, 10 μ M MitoTEMPOL or corresponding concentrations of pTPP for 1 h and then co-incubated with palmitate for 18 h. The growth medium with tested compounds was removed and replaced with the assay medium 1 h prior the assessment of mitochondrial function. Oxygen consumption rates (OCR) were measured before and after sequential injections of the indicated compounds. Both MitoQ and MitoTEMPOL markedly inhibited mitochondrial respiration of myoblasts compared to controls or cells exposed only to palmitate. The effect was observed also for higher concentration of pTPP (10 μ M). Results are presented as means and 95%CI from three independent experiments.

corresponding concentrations of propylTPP (pTPP) for 1 h and then co-incubated with palmitate for 18 h. We found no difference in the viability of cells treated only with palmitate and cells pre-treated with two different concentrations of MitoQ, MitoTEMPOL or pTPP (Fig. 4, data for pTPP not shown). Mitochondria-targeted antioxidants MitoQ and MitoTEMPOL were therefore unable to prevent palmitate-induced cell death.

MitoTEMPOL but not MitoQ prevented palmitate-induced mtDNA damage and both antioxidants inhibited mitochondrial respiration

Based on toxicity assays and the literature [23, 27, 36, 37] we used 0.5 μ M concentration of MitoQ and 10 μ M concentration of MitoTEMPOL for subsequent experiments testing their ability to prevent palmitate-induced mtDNA damage and mitochondrial respiratory dysfunction. Treatment with MitoTEMPOL prevented palmitate-induced mtDNA damage as shown by the relative amplification of the long mtDNA fragment (difference of means C vs. PA -15.67 [-28.24, -3.11], C vs. PA + 10 μ M MitoTEMPOL -4.72 [-17.29, 7.84], Fig. 5A). No effect was observed for MitoQ and for either concentration of pTPP (difference of means C vs. PA + 0.5 μ M MitoQ -18.31 [-31.27, -5.36], C vs. PA + 10 μ M pTPP -20.14 [-32.71, -7.57], C vs. PA + 0.5 μ M pTPP -13.93 [-26.50, -1.37], Fig. 5A).

Both antioxidants had a profound negative effect on mitochondrial respiration (Fig. 6). They markedly inhibited basal respiration, ATP turnover-linked respiration, maximal respiration and spare respiratory capacity compared to controls or cells exposed only to palmitate. Only proton leak and non-mitochondrial respiration were not affected. Inhibition of respiration was not exclusive for antioxidants themselves but was observed also for the 10 μ M concentration of pTPP pointing to a deleterious effect of the cations themselves.

The effect of MitoQ and MitoTEMPOL on reactive oxygen species production in palmitate-treated myoblasts

MitoTEMPOL had a promising antioxidant potential as it significantly decreased hydrogen peroxide production in non-treated control myoblasts (difference of means C vs. 10 μ M MitoTEMPOL -22.1 [-41.54, -2.647] RFU/ μ g protein, data not shown). In myoblasts exposed to palmitate MitoTEMPOL decreased palmitate-induced production of hydrogen peroxide but the difference did not reach statistical significance (C vs. PA 44.62 [8.13, 81.11], PA vs. PA + 10 μ M MitoTEMPOL -17.52 [-54.03, 18.99], Fig. 5B). MitoQ did not prevent palmitate-induced production of hydrogen peroxide and caused even increased production of this ROS (PA vs. PA + 0.5 μ M MitoQ 38.76 [2.23, 75.25], Fig. 5B). This increase was observed

also in non-treated control cells exposed to MitoQ (C vs. 0.5 μ M MitoQ 38.97 [15.12, 62.82], data not shown). Control pTPP compounds only slightly increased the production of ROS in palmitate-treated myoblasts (not statistically significant, PA vs. PA + 10 μ M pTPP 12.08 [-24.41, 48.57], PA vs. PA + 0.5 μ M pTPP 16.48 [-20.83, 53.79], Fig. 5B).

Discussion

Increased oxidative stress associated with some features of mitochondrial dysfunction was reported in skeletal muscle cells after the exposure to palmitate [17, 18]. However, the effects of saturated fatty acids on mitochondrial respiration have not been well studied, particularly in undifferentiated skeletal muscle cells. The present study was designed to clarify the effect of palmitate on viability and mitochondrial respiratory function in differentiated and undifferentiated C2C12 skeletal muscle cells and to test the ability of mitochondria-targeted antioxidants MitoQ and MitoTEMPOL to prevent palmitate-induced damage.

Palmitate appeared to be cytotoxic for myoblasts as well as myotubes in a dose-dependent manner although myoblasts were much more sensitive. We have chosen a physiologically relevant concentration of palmitate (100 μ M) [35] to study further whether mtDNA integrity is affected in palmitate-treated cells. Mitochondrial DNA is one of the main targets for reactive oxygen species and its damage has been suggested as a marker of oxidative damage [38]. We found mtDNA damage after the treatment with palmitate in both myoblasts and myotubes using the QPCR method, which is able to detect a wide range of types of DNA lesions. Our observation is in accordance with another study [18] where mtDNA damage was detected in L6 myotubes exposed to palmitate using quantitative alkaline Southern blot analysis. Authors of this study postulated that mtDNA damage is a critical event leading to mitochondrial dysfunction with consequent increase in oxidative stress and finally to apoptosis [18]. In our experiments this was true only for myoblasts, as mtDNA damage after the exposure to palmitate was similar in myoblasts and myotubes but we detected mitochondrial dysfunction (discussed below) with increased hydrogen peroxide production (by about 50%) and decreased viability (by about 35%) only in myoblasts. It should be noted that the concentration of palmitate used in our study was lower than in most previous studies showing its deleterious effects in myotubes [14, 17, 18].

Mitochondrial respiration after the treatment with palmitate was affected in different ways in myoblasts and myotubes. We observed decreased basal mitochondrial respiration, uncoupler-induced maximal respiration, spare respiratory capacity (SRC) and coupling efficiency in palmitate-treated myoblasts. A decrease in all parameters of mitochondrial respiration except the proton leak in palmitate-treated myoblasts could be explained by a reduction in mitochondrial mass as the activity of citrate synthase was significantly lower in palmitate-treated myoblasts. In myotubes, uncoupler-induced maximal respiration, SRC and the citrate synthase activity were not affected after the exposure to palmitate suggesting no change in mitochondrial respiratory capacity or mitochondrial density. Palmitate caused higher basal respiration because of an increased proton leak showing a slight uncoupling of mitochondrial respiration from oxidative phosphorylation, an effect already reported for long-chain fatty acids in skeletal muscle [39]. This effect could be, at least in part, mediated through the uncoupling protein-3 (UCP-3) which is specifically expressed in differentiated muscle cells [40] since we did not observe an increase in proton leak in undifferentiated myoblasts. Although the coupling efficiency, a portion of basal respiration used for ATP synthesis, was decreased after the treatment with palmitate; the rate of ATP turnover-linked respiration itself was not affected.

The presented results show that palmitate caused similar mtDNA damage in myoblasts and myotubes, which was associated with mitochondrial respiratory dysfunction and decreased viability only in myoblasts. We hypothesize that this higher resistance of myotubes to the effect of palmitate compared to myoblasts could be in part explained by their higher

spare respiratory capacity (SRC, 75% of maximal OCR in myotubes vs. 53% in myoblasts) and therefore an increased ability to face the stress caused by palmitate. SRC is critical for survival and function of cells as it reflects the maximum ability of mitochondria to maintain energy production in response to an increase in energy demand, like in acute or chronic stress [41, 42].

Mitochondria-targeted antioxidants MitoQ and MitoTEMPOL used in this study have been previously shown to protect pancreatic β -cells against oxidative stress under glucolipotoxic conditions [24], inhibit peroxide-induced mitochondrial oxidative damage in endothelial cells [23] or prevent oxidative damage of mtDNA in myoblasts [27]. In our study, MitoTEMPOL prevented palmitate-induced mtDNA damage in C2C12 myoblasts and decreased production of hydrogen peroxide in non-treated cells confirming its antioxidant potential. In palmitate-treated myoblasts there was a trend towards a reduction of hydrogen peroxide emission but the difference did not reach statistical significance ($p=0.06$). However, we did not observe a protective effect of MitoTEMPOL against palmitate-induced cell death or mitochondrial respiratory dysfunction. MitoQ had no significant effect on palmitate-induced mtDNA damage or decreased viability of myoblasts and caused even increased hydrogen peroxide production. Pro-oxidant properties of a higher dose of MitoQ (1 μ M) have been reported in certain conditions in isolated mitochondria [43, 44] while simultaneously noting its antioxidant potential. We tested even lower concentrations of antioxidants in toxicity assays, but they were also not able to prevent palmitate-induced cell death of myoblasts (Fig. 4). When comparing with previous studies showing protective effects of antioxidants it should be taken into consideration that the effects will most probably depend on cell type, number of mitochondria in cells or the degree of reliance on oxidative phosphorylation.

Furthermore we found a strong inhibition of mitochondrial respiration after the treatment with both antioxidants. The functional impact of mitochondria-targeted antioxidants on cellular bioenergetics was shown recently for micromolar concentrations of MitoQ and MitoTEMPOL in mouse kidney mesangial cells [45]. We observed an inhibition of mitochondrial respiration also for 10 μ M concentration of propylTPP showing that the TPP⁺ moiety itself could be the cause of this effect. This is again in agreement with the above mentioned work of Reily et al. [45] where the acute toxicity of TPP-conjugated compounds was tested and it was found that the TPP⁺ moiety itself has a negative impact on cellular bioenergetics. Concentrations of targeted antioxidants should therefore be carefully selected and tested for potential bioenergetics effects and an "inactive" TPP⁺ compound should always be included as a control.

We conclude that palmitate in physiologically relevant doses affected mainly myoblasts, which were more sensitive to its effects than differentiated myotubes, possibly due to a lower spare respiratory capacity. The results suggest that skeletal muscle progenitor cells may be the first target of the deleterious action of palmitate. Mitochondria-targeted antioxidants were unable to prevent palmitate-induced cell death in myoblasts although MitoTEMPOL prevented mtDNA damage. Both antioxidants as well as propylTPP markedly inhibited mitochondrial respiration in myoblasts. These results show that the effects of mitochondria-targeted antioxidants as well as TPP⁺ compounds on cellular bioenergetics need to be further studied and taken into account when used in cell culture studies or for therapeutic purposes.

Disclosure Statement

The authors confirm that there are no conflicts of interest.

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