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Brown adipose tissue mitochondria oxidizing fatty acids generate high levels of reactive oxygen species irrespective of the uncoupling protein-1 activity state

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ABSTRACT

Mitochondria from brown adipose tissue (BATM) have a high enzymatic capacity for fatty acid oxidation and therefore are an ideal model to examine the sites of reactive oxygen species (ROS) generation during fatty acid oxidation. ROS generation by BATM (isolated from 3-week-old rats) was measured during acylcarnitine oxidation as release of H_2O_2 into the medium and as inactivation of the matrix enzyme aconitase. The following results were obtained: (1) BATM release large amounts of H_2O_2 in the coupled as well as in the uncoupled states, several times more than skeletal muscle mitochondria. (2) H_2O_2 release is especially large with acylcarnitines of medium-chain fatty acids (e.g. octanoylcarnitine). (3) Reverse electron transport does not contribute in a significant extent to the overall ROS generation. (4) Despite the large release of H_2O_2, the ROS-sensitive matrix enzyme aconitase is not inactivated during acylcarnitine oxidation. (5) In contrast to acylcarnitines, oxidation of α-glycerophosphate by BATM is characterized by large H_2O_2 release and a pronounced aconitase inactivation. We hypothesize that acylcarnitine-supported ROS generation in BATM may be mainly associated with acyl-CoA dehydrogenase and electron transferring flavoprotein-ubiquinone reductase rather than with complexes of the respiratory chain.

1. Introduction

Brown adipose tissue (BAT) is well-known for its inducible thermogenic mitochondrial uncoupling [1]. This thermogenic function is under strict control of hormonal and sympathetic nervous systems. The main hydrogen sources, at least under active thermogenesis, are long-chain fatty acids [1], which are oxidized along with glucose [2]. Fatty acids are liberated from fat stores in white adipose tissue by activated lipases under hormonal signal [1]. In this process, long-chain fatty acids play a dual function: (i) in the non-esterified form, as activators of the uncoupling protein (UCP1), and (ii) in form of CoA or carnitine esters, as electron suppliers for mitochondrial respiration. BAT mitochondria (BATM) are equipped with high enzymatic capacities for the degradation of acyl-CoAs, thereby liberating large amounts of NADH and $FADH_2$ by β-oxidation. NADH is oxidized by complex I of the electron transfer chain (ETC), whereas $FADH_2$ feeds electrons into ETC via the electron-transferring flavoprotein (ETF). Furthermore, it is known since about five decades that reactive oxygen species (ROS) are formed as by-products during ETC operation [3,4]. The initial reaction of mitochondrial ROS generation is one-electron transfer from certain sites within the ETC to molecular oxygen, thereby forming superoxide ($O_2^{\bullet -}$). During β-oxidation of activated fatty acids, such "electron leakage" can theoretically occur at the levels of acyl-CoA dehydrogenase, ETF, ETF-ubiquinone oxidoreductase and complex III [5–7].

ROS production during oxidation of non-fatty acid substrates by BATM has recently been studied in several laboratories [8–14]. There is, however, little information on ROS generation accompanying oxidation of fatty acids. Since BAT is an extremely potent fatty acid-oxidizing tissue, the use of mitochondria from this tissue may provide a deeper insight into the mechanism and sites of $O_2^{\bullet -}$ generation during fatty acid degradation.

The subject of the present investigation was mitochondria isolated from BAT of infant rats. For comparison, mitochondria from skeletal muscles of these animals (RSMM) were also used, as fatty acids are also known to be important respiratory substrates for this tissue. In addition, brown fat and skeletal muscle cells can share the same ancestral cellular lineage ([14] and references therein). Mitochondria from muscle tissue also contribute significantly to thermogenesis [15]. We studied ROS generation during oxidation of short-, medium- and long-chain fatty acids supplied in form of carnitine esters. Apart from comparing the rates of ROS production by BATM and RSMM we expected to clarify, whether the intriguing observation

Abbreviations: AR, Amplex Red; BAT, brown adipose tissue; BATM, brown adipose tissue mitochondria; ButC, butyrylcarnitine; DecC, decanoylcarnitine; DPI, diphenyleneiodonium; ETF, electron transferring flavoprotein; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl-hydrazone; GP, α-glycerophosphate; HRP, horseradish peroxidase; MyrC, myristoylcarnitine; OctC, octanoylcarnitine; PalC, palmitoylcarnitine; RET, reverse electron transfer; ROS, reactive oxygen species; RSMM, rat skeletal muscle mitochondria; $\Delta\psi$, mitochondrial transmembrane potential

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made for heart mitochondria [16] that oxidation of carnitine esters of medium- and long-chain fatty acids was not linked to the reverse electron transfer (RET), nor was it accompanied by RET-related high ROS generation comparable to that associated with succinate oxidation, also holds for mitochondria from other tissues. Since succinate is a very poor substrate for BATM [17], α-glycerophosphate (GP) was applied as source of $FADH_2$ generation for comparison. GP is oxidized at the outer surface of the mitochondrial inner membrane by FAD-containing glycerol-3-phosphate dehydrogenase, utilizing coenzyme Q as electron acceptor [18,19]. RET can proceed from GP instead of succinate [8,20,21].

The present investigation shows that BATM release large amounts of ROS when oxidizing carnitine esters of medium- and long-chain fatty acids. In analogy to rat heart mitochondria [16], this process is not related to RET. Interestingly, high production of ROS during fatty acid oxidation by BATM seems to be associated with the external leaflet of the inner mitochondrial membrane rather than with the matrix-oriented leaflet. This observation may indicate that sites such as acyl-CoA dehydrogenase and ETF-ubiquinone oxidoreductase rather than complexes of the respiratory chain are involved in this process.

2. Materials and methods

2.1. Biological material

Brown adipose tissue (mostly interscapular) was isolated from 3-week-old rats housed together with mothers at room temperature [22]. Pups were still suckling. The animals were killed by decapitation and mitochondria were isolated as described by Cannon and Lindberg [23]. Tissue homogenization was performed in 250 mM sucrose plus 10 mM Tris–HCl (pH 7.4) supplemented with 1 mM EDTA and 1% fatty acid-free bovine serum albumin (BSA). The final pellet was resuspended in BSA-free homogenization medium. Skeletal muscle mitochondria were isolated mostly from *musculus gastrocnemius* of adult rats essentially as described in [24] with modifications. For digestion of the tissue, nagarse was used instead of trypsin. Thereafter, tissue homogenization was done as described for isolation of BATM, including supplementation with of BSA. Resuspension of the final mitochondrial pellet was as described for BATM. Protein content in the mitochondrial stock suspensions was determined by the biuret method using BSA as standard.

2.2. Incubations

All incubations were performed at 37 °C using a medium composed of 110 mM mannitol, 60 mM KCl, 60 mM Tris–HCl, 10 mM KH_2PO_4, 0.5 mM EGTA and 1% fatty acid-free BSA (pH 7.4). For skeletal muscle mitochondria the same medium without BSA was used. Additions of low- and medium-chain acylcarnitines are expressed in concentration units (μM), those of long-chain esters, because of their hydrophobicity, in nmol per mg mitochondrial protein.

2.3. Oxygen consumption

Oxygen uptake by mitochondria was measured using an Oroboros oxygraph (Oxygraph®, Bioenergetics and Biomedical Instruments, Innsbruck, Austria).

2.4. Membrane potential and NAD(P) reduction

Mitochondrial transmembrane potential ($\Delta\psi$) was measured fluorimetrically with safranin O as described previously [16]. Since GDP was found to react with safranin and slightly to diminish its fluorescence, the order of additions was as follows: safranin + GDP → mitochondria → substrate → FCCP.

2.5. ROS generation

Release of H_2O_2 from mitochondria was measured with the Amplex Red/horseradish peroxidase (AR/HRP) assay. Briefly, mitochondria (usually 0.2 mg protein/ml) were incubated in the incubation medium supplemented with 5 μM AR, HRP (2 U/ml) and Cu, Zn-superoxide dismutase (2 U/ml). Resorufin (peroxidation product of AR) fluorescence was monitored by Perkin-Elmer Luminescence spectrometer LS 50B at 560 nm excitation and 590 nm emission wavelengths. Resorufin fluorescence was calibrated with H_2O_2. Since the response of the AR/HRP assay to H_2O_2 is diminished by both GDP and mitochondria (see also [25]), rates of H_2O_2 release were calculated using appropriate calibration factors.

ROS release to the matrix compartment was evaluated by inactivation of the mitochondrial matrix enzyme aconitase [26]. This was measured spectrophotometrically by recording the rate of $NADP^+$ reduction as described in [27]. Briefly, mitochondria (0.2 mg protein/ml) were preincubated in a spectrophotometer cuvette for 10 min at 37 °C in the standard incubation medium (from which EGTA was omitted) without additions (controls) or with the indicated substrates. Thereafter, lauroyl maltoside (0.05% v/v) was added to permeabilize mitochondria, followed by citrate (5 mM), $NADP^+$ (0.8 mM), $MnCl_2$ (0.6 mM) and isocitrate dehydrogenase (2 U/ml).

2.6. Enzyme assays

Before measuring enzymatic activity, mitochondria were permeabilized by three freezing/thawing cycles. Absorption changes were measured in the standard incubation medium thermostated to 30 °C using a Cary 3E UV–Visible Spectrophotometer.

Activity of acyl-CoA dehydrogenase in mitochondria was measured by following the reduction of ferricenium hexafluorophosphate to ferrocene [28,29]. In this assay ferricenium mimics ETF as electron acceptor.

Ubiquinol-cytochrome c oxidoreductase (complex III) was assayed spectrophotometrically by monitoring the initial rate of reduction of oxidized cytochrome c by using the ubiquinol analog decylubiquinol [30]. In brief, all components (3 mM NaN_3, 60 μM cytochrome c, 0.1 mM EDTA) dissolved in 50 mM phosphate buffer plus 10 μg protein of the mitochondrial suspension (permeabilized by freezing/thawing cycles) were added to both the sample and the reference cuvettes. The assay was started by addition of decylubiquinol (50 μM) and reduction of cytochrome c was followed at 550 nm ($\varepsilon = 19.2\ cm^2/\mu mol$). Decylubiquinol was reduced by adding a small crystal of $NaBH_4$ to a solution of commercial decylubiquinone in ethanol. When the reaction mixture became colorless, the liquid was transferred to another tube (omitting the crystal), acidified by addition of 0.1 volume of 1 M HCl and stored at −20 °C [31].

2.7. Chemicals

Acylcarnitines were from Larodan Fine Chemicals (Malmö, Sweden). Diphenyleneiodonium, malate, succinate, α-glycerophosphate, FCCP, ferricenium hexafluorophosphate, isocitrate dehydrogenase, decylubiquinone, cytochrome c, lauroyl maltoside, GDP, rotenone, antimycin A, Cu,Zn-superoxide dismutase, nigericin and horseradish peroxidase (Type VI-A) were from Sigma-Aldrich Chemie GmbH (Sternheim, Germany). Amplex Red was obtained from Invitrogen (Eugene, Oregon, USA).

2.8. Statistical analysis

Data are represented as means ± SD for 4–10 different incubations using separate mitochondrial preparations. Multiple comparisons

were done using one-way ANOVA plus Tukey multiple comparison test (SigmaPlot 11.0 software) for Figs. 2, 3, 4 and 7 ($P<0.05$).

3. Results

3.1. Oxidative capacity with acylcarnitines

It has been reported that palmitoylcarnitine (PalC) is a very good substrate for BATM [32]. Here we have confirmed this observation (Fig. 1) and shown that this also applies to other long- and medium-chain acylcarnitines (Fig. 2). Oxygen electrode traces (Fig. 1) clearly show that the rate of O_2 uptake with octanoylcarnitine (OctC) as substrate is over twice as high for BAT mitochondria (BATM, trace b) than for uncoupled skeletal muscle mitochondria (RSMM, trace d). Also the respiration with α-glycerophosphate (GP), presented for comparison, was higher for BATM (trace a) than for RSMM (trace c). These traces as well as the summarized results presented in Fig. 2 also show that BATM are uncoupled and become recoupled by addition of GDP, the known blocker of UCP1. A lag in attaining full rate of the uncoupled respiration by BATM oxidizing acylcarnitines can be noted (Fig. 1, trace b). Mean value of the respiratory control ratio (uncoupled vs. recoupled with GDP) for our BATM preparations oxidizing OctC was 4.0 ± 0.5 ($n = 6$).

Similar high rates of oxygen uptake were found with other carnitine esters of both long-, medium- and short-chain fatty acids (Fig. 2, panel A). With all these substrates, as well as with GP, oxygen uptake was strongly decreased by 2 mM GDP, this effect being fully reversed by the chemical uncoupler FCCP (illustrated only for PalC and OctC). Fig. 2, panel B, shows that RSMM exhibit similar respiratory control ratios (around 5) with acylcarnitines, although absolute values of the oxygen uptake rates are by about 50% lower.

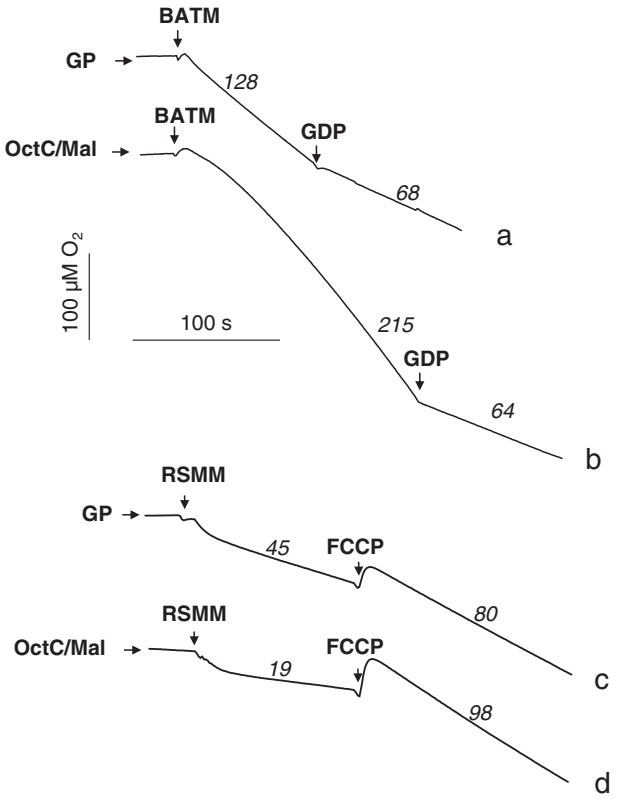

Fig. 1. Oxygen uptake by mitochondria from brown adipose tissue and skeletal muscle oxidizing octanolycarnitine and α-glycerophosphate. The incubation medium contained either 0.2 mM octanoylcarnitine plus 5 mM malate (OctC/Mal) or 15 mM α-glycerophosphate (GP). Mitochondria from brown adipose tissue (BATM, traces a and b) or skeletal muscle (RSMM, traces c and d) were added at the amount of 0.5 mg protein/ml. GDP (2 mM) and FCCP (0.15 µM) were added where indicated. The rates of respiration (nmol O_2/min per mg protein) are given at the traces.

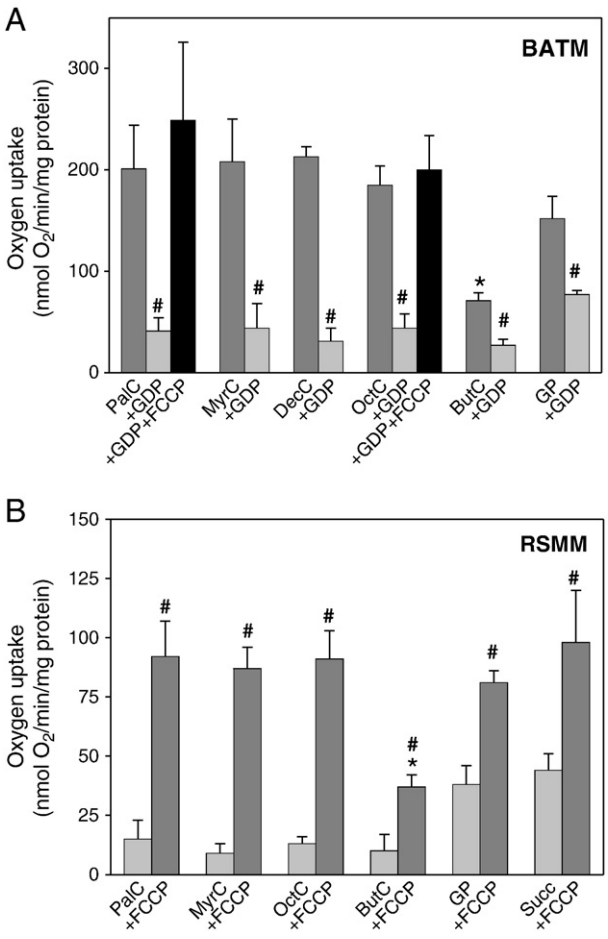

Fig. 2. Oxygen uptake by mitochondria from brown adipose tissue (A) and skeletal muscle (B) oxidizing carnitine esters of fatty acids of different chain length. The media contained mitochondria corresponding to 0.5 mg protein/ml and the following substrates as indicated: PalC (30 nmol/mg protein), MyrC (30 nmol/mg protein), DecC (0.2 mM), OctC (0.2 mM), ButC (0.2 mM), GP (15 mM) and succinate (Succ, 5 mM). Incubations of BATM (panel A) oxidizing acylcarnitines also contained 5 mM malate, whereas those of RSMM (panel B) oxidizing acylcarnitines contained 0.1 mM malate. GDP (2 mM) and FCCP (0.15 µM) were added where indicated in order to recouple or fully to uncouple the mitochondria, respectively. Data shown are means ± SD for 3–9 preparations in A and for 5 preparations in B. Statistical significance: A, *ButC uncoupled vs. uncoupled PalC or MyrC or DecC or OctC or GP; #recoupled (+GDP) vs. uncoupled. B, *ButC uncoupled vs. uncoupled PalC or MyrC, or OctC or GP or Succ; #uncoupled (+FCCP) vs. coupled.

The high rates of acylcarnitine oxidation by BATM required the presence of a rather high (5 mM) concentration of malate. This can be explained by a very low permeability of isolated BATM to di- and tricarboxylate substrate anions [33], while they exhibit a highly condensed matrix after preparation from the tissue [34].

3.2. Release of H_2O_2 from mitochondria

ROS generation measured with the AR/HRP assay reflects the amount of H_2O_2 released to the incubation medium. The rates of the H_2O_2 release by freshly isolated (uncoupled) BATM oxidizing acylcarnitines of various chain lengths are shown in Fig. 3, panel A. It can be seen that the rates were the highest for medium-chain esters (OctC and DecC), whereas they were much lower for long-chain (PalC and MyrC) and short-chain (ButC) esters. Recoupling of the mitochondria with GDP enhanced ROS generation, which was statistically significant for OctC. For comparison, ROS production with GP as the sole substrate is also shown.

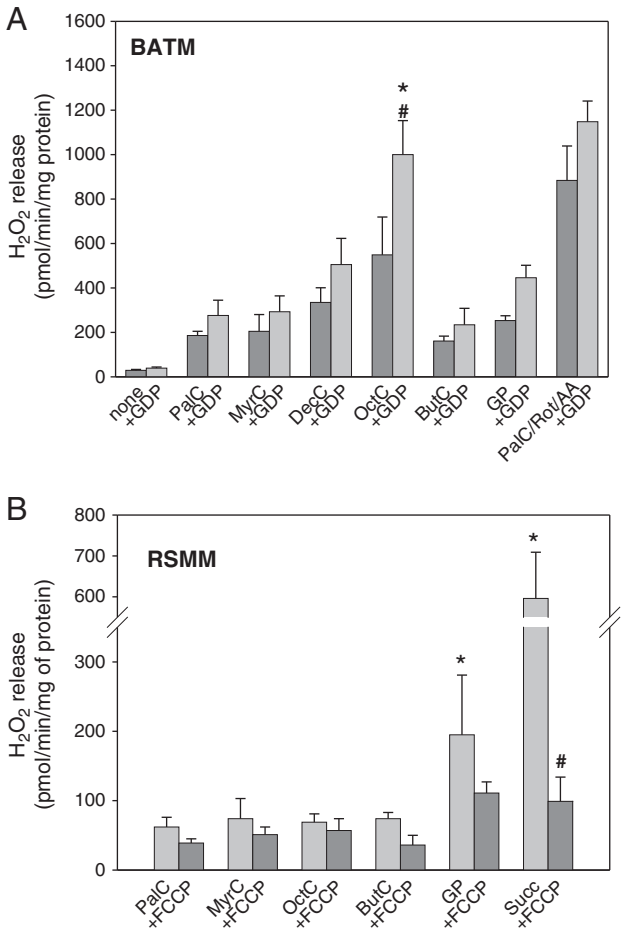

Fig. 3. H_2O_2 release by BATM and RSMM. Incubation conditions and substrates were the same as in Fig. 2 except that the medium contained 0.2 mg mitochondrial protein/ml. Other additions were as follows: rotenone (Rot, 2 μM) and antimycin A (AA, 2 μM). Data shown are means ± SD for 4–8 preparations. Statistical significance: A, *OctC recoupled (+GDP) vs. recoupled with all other carnitine esters or GP, #OctC recoupled (+GDP) vs. OctC uncoupled. B, *coupled GP or Succ vs. PalC or MyrC or OctC or ButC; #uncoupled Succ (+FCCP) vs. coupled Succ.

Fig. 3, panel A, also shows that double inhibition of the ETC with rotenone and antimycin A dramatically increased ROS formation by BATM with PalC as substrate.

In the coupled state, RSMM oxidizing acylcarnitine esters of various fatty acid chain length released H_2O_2 into the medium at a much lesser rate than BATM (Fig. 3, panel B). This difference, about 4- (PalC) to 13-fold (OctC), correlates with a 5- to 10-fold higher activity of acyl-CoA dehydrogenase activity in BATM, whereas the activity of complex III was twice as high in RSMM than in BATM (Table 1). In contrast to acylcarnitines, succinate oxidation by RSMM gave rise to high ROS generation (Fig. 3, panel B). Since succinate-supported ROS production by RSMM is mostly due to RET, it was sharply decreased, as expected, by the chemical uncoupler FCCP. In contrast, ROS generation by RSMM oxidizing either acylcarnitines or GP was only moderately decreased.

Table 1
Comparison of the activities of acyl-CoA dehydrogenase and complex III in mitochondria from brown adipose tissue (BATM) and skeletal muscle (RSMM).

Mitochondria	Acyl-CoA dehydrogenase (μmol/min/mg)	Complex III (nmol/min/mg)
BATM	135 ± 21 (OctCoA; n = 9) 237 ± 85 (PalCoA; n = 8)	665 ± 220 (n = 6)
RSMM	14 ± 5 (OctCoA; n = 3) 46 ± 14 (PalCoA; n = 3)	1183 ± 382 (n = 4)

Comparison of data from Figs. 2 and 3 allows to calculate the proportion of oxygen reduced in one-electron process to the total oxygen uptake (the H_2O_2/O_2 ratio). For BATM oxidizing OctC this ratio amounts to 0.003 (= 0.3%) in the uncoupled state and 0.02 (= 2%) after recoupling with GDP. For RSMM the respective values with the same substrate are about one order of magnitude lower: 0.06% in the uncoupled state (+FCCP) and 0.5% in the coupled state (for comparison see [35]).

3.3. ROS release into the mitochondrial matrix

ROS release to the matrix compartment is responsible for the inactivation of the matrix enzyme aconitase, which is very sensitive to $O_2^{\bullet -}$ and, in a much lesser extent, to H_2O_2 [26]. Comparing the AR/HRP assay and aconitase activity measurement, one can assess the proportion of ROS directly released at the external side of the inner mitochondrial membrane and those primarily released at the internal side. Relation between these two types of measurement can provide an insight into the mechanisms and/or loci of ROS generation. As shown in Fig. 4, there was no significant inactivation of aconitase activity during oxidation of acylcarnitines, in contrast to a moderate inactivation when GP was the oxidized substrate. However, high degree of aconitase inactivation with PalC was observed when the ETC was blocked with rotenone plus antimycin A.

3.4. Transmembrane potential and reduction of the mitochondrial NAD(P) pool

Since RET-associated ROS generation strongly depends on energization of the inner mitochondrial membrane, Δψ built up by BATM recoupled with GDP and oxidizing acylcarnitines was estimated with safranin O. Representative traces for OctC, PalC and GP presented in Fig. 5 show that they are excellent substrates for energization of BATM, although Δψ formed in the presence of PalC seems slightly lower than that in the presence of OctC or GP. It is also evident that the rate of Δψ formation is distinctly lower with these two acylcarnitines than with GP. This experiment also showed that succinate failed to energize BATM. The latter finding confirms that succinate is unsuited as substrate for BATM [17].

Apart from high Δψ, high reduction of mitochondrial NAD(P) is the second requirement for RET-dependent ROS generation. To estimate

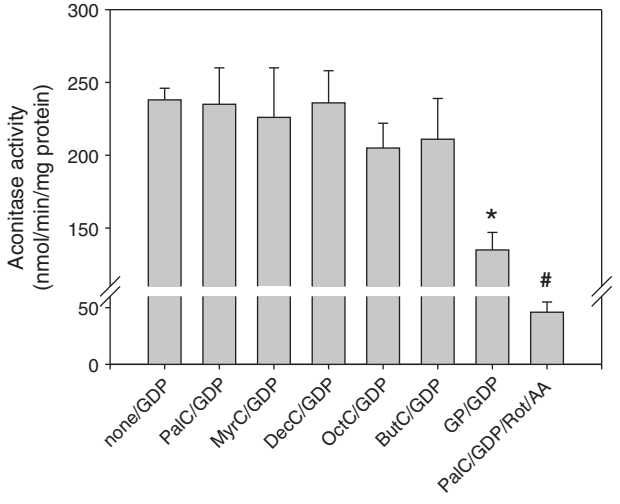

Fig. 4. ROS generation by BATM assessed as aconitase inactivation. Conditions and additions were as in Fig. 2A. Data are means ± SD for 4–8 preparations. Significance: *GP/GDP vs. all acylcarnitines/GDP; #PalC/GDP/Rot/AA vs. PalC/GDP.

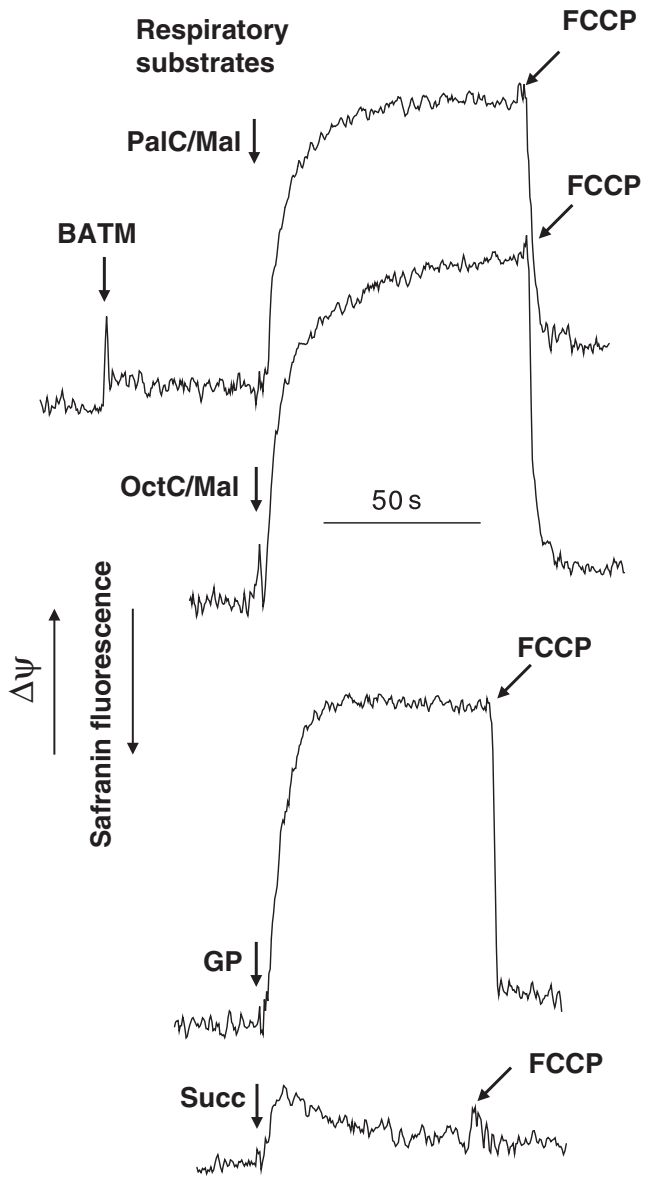

Fig. 5. Membrane potential of BATM oxidizing different substrates. Mitochondria (1 mg protein/ml) were suspended in the incubation medium supplemented with 5 μM safranin O, 2 mM GDP and the indicated substrates. FCCP (1 μM) was added where indicated. Concentrations of the respiratory substrates were as in Fig. 2. Decrease of safranin fluorescence corresponds to the increase of the membrane potential Δψ. Representative traces.

the reduction of the NAD(P) pool, BATM were first incubated in the absence of added substrate to attain a complete oxidation of nicotinamide nucleotides. Thereafter, GDP was added to recouple the mitochondria, followed by the respiratory substrates OctC, PalC or GP. Fig. 6 shows that the highest reduction of NAD(P) was found with OctC, whereas that by PalC was distinctly slower (reproducible results). The reduction by GP, apparently reflecting RET, was lower than by both aforementioned acylcarnitines. When the incubation mixture became deficient in oxygen, the NAD(P)H level with acylcarnitines further increased. In contrast, GP-associated NAD(P) reduction declined. It is also noteworthy that the rate of NAD(P) reduction by GP was slowed down when rotenone was added (not shown).

3.5. Effect of rotenone, diphenyleneiodonium and nigericin

Rotenone, inhibitor of CoQ binding to complex I, is well known to inhibit RET that accompanies succinate oxidation and, consequently, RET-dependent ROS production during succinate oxidation (see e.g. [16]). It was therefore of interest to see the effect of rotenone on ROS production during fatty acid oxidation by BATM. As shown in Fig. 7, rotenone strongly activated low ROS production with long-chain MyrC and was without effect in case of large ROS generation with medium-chain OctC. In no case any inhibition by rotenone of ROS generation by BATM oxidizing acylcarnitines could be observed.

Diphenyleneiodonium (DPI) has been proposed specifically to abolish succinate-dependent ROS generation associated with RET in skeletal muscle mitochondria [36]. Therefore we checked this long-known [37] hypoglycemic agent as a probe to identify a possible contribution of RET in fatty acid-linked ROS generation in BATM. It appeared (Fig. 7) that this compound decreased H_2O_2 release by BATM by over 50% when mitochondria were oxidizing medium-chain OctC, whereas the inhibitory effect in case of long-chain MyrC was much smaller. This experiment was carried out in chloride-free medium (KCl was substituted by potassium gluconate), because DPI is also known to catalyze chloride-hydroxide exchange across membranes [37].

Nigericin, a known H^+/K^+ antiporter, has often been used to collapse the pH gradient across the inner mitochondrial membrane (ΔpH) and thus to increase the electric component (Δψ) of the protonmotive force. Therefore, it might alter the rate of ROS production depending on whether it is affected by either Δψ or ΔpH. It appeared,

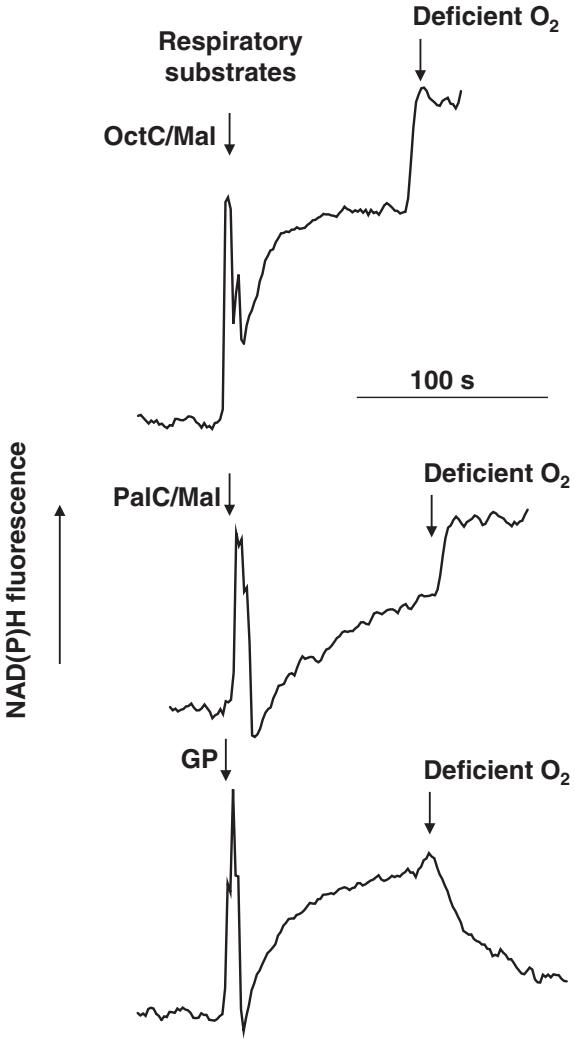

Fig. 6. Substrate-induced reduction of the endogenous NAD(P) pool in BATM. Mitochondria (1 mg protein/ml) were suspended in the substrate-free incubation medium. After oxidation of endogenous substrates, acylcarnitines plus malate or α-glycerophosphate (GP) were added at concentrations as in Fig. 2. Representative traces.

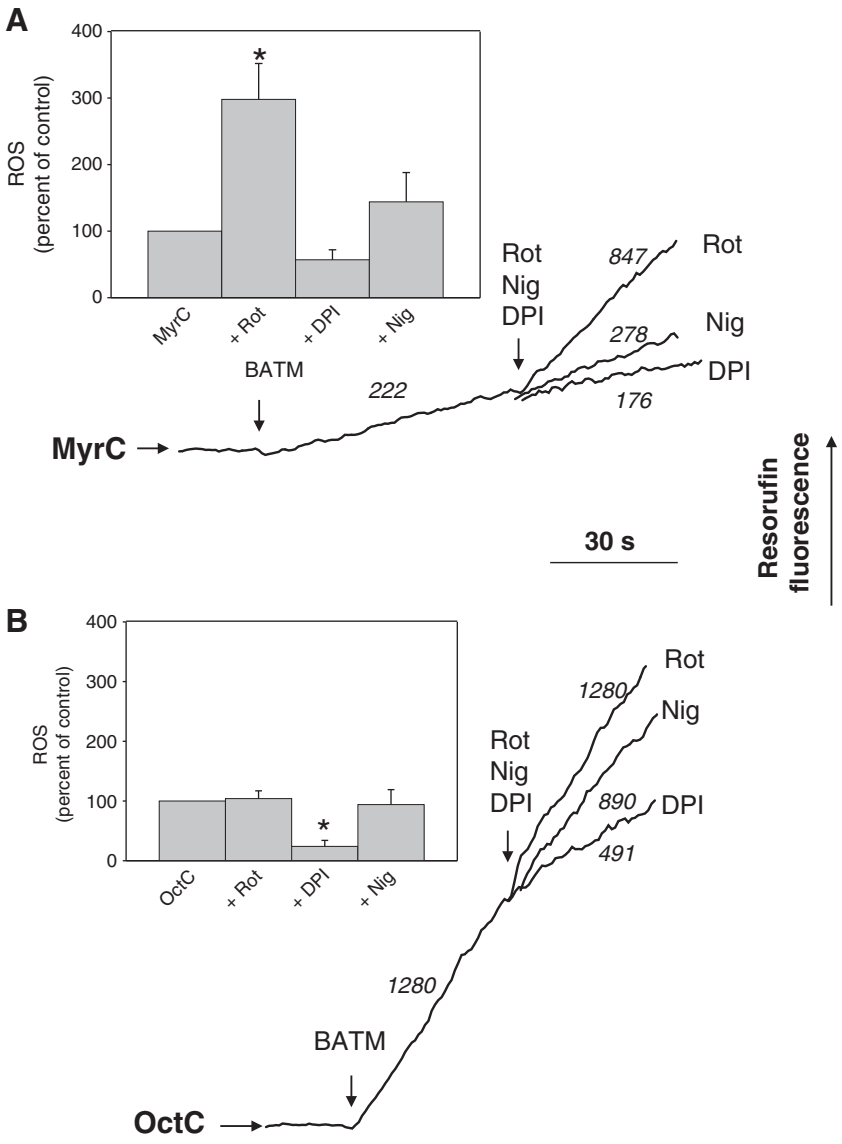

Fig. 7. Effect of rotenone, diphenyleneiodonium and nigericin on H_2O_2 release by BATM oxidizing myristoyl- or octanolycarnitine. Mitochondria (0.2 mg protein/ml) were suspended either in the normal incubation medium (for rotenone and nigericin effects) or in the medium in which KCl was substituted by potassium gluconate (for diphenyleneiodonium effect). Both media were supplemented with 2 mM GDP. The additions were as follows: rotenone (Rot), 2 μM; diphenyleneiodonium (DPI), 5 μM; and nigericin (Nig), 0.5 μM. Other conditions were as in Fig. 3. A, Typical spectrofluorometer traces. The numbers at the traces indicate the rates of H_2O_2 release (pmol/min per mg protein). B, Mean values ± SD for 4 experiments. Control data (100%) are from Fig. 3A. Statistical significance with respect to MyrC or OctC alone is indicated (*).

however, that this rate was only slightly affected with both MyrC and OctC (plus malate) in BATM (Fig. 7).

4. Discussion

The present study has shown that oxidation of activated fatty acids (supplied in the form of carnitine esters) by BATM from infant rats is a potent source of ROS, much better than oxidation of these substrates by RSMM. Furthermore, ROS generation by BATM is also much higher than that by heart and liver mitochondria [16]. This finding is in line with a much higher ROS generation by BATM than by RSMM as very recently reported in a study on mouse mitochondria oxidizing pyruvate plus malate or succinate [14]. Although ROS production by mitochondrial preparations oxidizing fatty acids may partly be associated with peroxisomal contamination [38], it is unlikely that high ROS generation described in the present study was due to peroxisomes, as the latter organelles have been found to oxidize acyl-CoA esters rather than carnitine esters [38]. The higher ROS generation by BATM than by RSMM could be attributed in part to a higher capacity of the former mitochondria to oxidize fatty acids (Fig. 2). In addition, it has been reported that RSMM and BATM differ considerably by their redox capacity, in particular by the matrix GSH/GSSG ratio that amounts to 46 and to 13 in RSMM and in BATM, respectively [14]. Consequently, a smaller amount of H_2O_2 is likely to be generated in and to escape from RSMM than from BATM. In this context it is worthy to remember that depletion of matrix glutathione with 1-chloro-2,4-dinitrobenzene strongly stimulates the release of H_2O_2 from mitochondria (e.g. [39]).

The unusually high rate of ROS production by BATM oxidizing fatty acids as observed in the present investigation was only comparable to the rate of ROS generation by brain, heart and skeletal muscle mitochondria oxidizing succinate, where most of this process occurred due to RET [35,40]. This succinate-linked ROS generation becomes abolished by uncoupling or treatment with rotenone, an inhibitor of coenzyme Q binding site at complex I, features generally considered as characteristic for RET-driven ROS generation. It was, therefore, intriguing to examine whether RET may also contribute to ROS generation by BATM oxidizing carnitine esters of fatty acids.

As it has been discussed previously [16], RET could theoretically occur during fatty acid oxidation, since it feeds electrons to the respiratory chain by two pathways, one directly to complex I via β-hydroxyacyl-CoA dehydrogenase [41] and another one via acyl-CoA dehydrogenase to complex III via ETF and ETF dehydrogenase. We have, however, shown [16] that in rat liver and heart mitochondria this is not accompanied by RET from complex III to complex I. The present study shows that, in contrast to heart mitochondria, BATM after recoupling with GDP are able to (i) build a high $\Delta\psi$ (Fig. 5) and (ii) rapidly to reduce the mitochondrial NAD(P) pool (Fig. 6) when oxidizing OctC and, at a lower rate, PalC. Interestingly, with GP as the sole substrate, the NAD(P)H pool becomes re-oxidized when the O_2 concentration in the incubation medium falls almost to zero, so that the mitochondria can no longer be energized by the respiratory chain. This clearly shows that, in that case, the reduction state of NAD(P) is exclusively maintained due to RET. In contrast, with acylcarnitines, reduction of the NAD(P) pool further increases upon anaerobiosis (Fig. 6).

At first glance, high $\Delta\psi$ and high reduction of the NAD(P) pool might argue for RET to occur during fatty acid oxidation by these mitochondria. In fact, Oelkrug et al. [12] have recently proposed that $O_2\bullet^-$ generation during β-oxidation in BATM from UCP1-ablated mice could be linked to RET. However, our study on ROS production does not support the concept that this may result from reverse rather than from forward electron transfer. First, the high ROS generation by BATM oxidizing acylcarnitine esters is not inhibited by rotenone, as it should be in case of RET. On the contrary, ROS generation is either unaffected (when it is very high as in case of OctC) or is increased (when it is lower as in case of MyrC) by rotenone (Fig. 7). Second, nigericin, known to collapse ΔpH across the inner mitochondrial membrane, has no or only a slight and variable effect on the rate of ROS generation by BATM oxidizing acylcarnitines (Fig. 7), whereas it is known to decrease RET-supported ROS generation by mitochondria oxidizing succinate [42]. In this context a somewhat intriguing effect was obtained with DPI. This hypoglycemic agent has been proposed specifically to inhibit RET-driven $O_2\bullet^-$ generation accompanying succinate oxidation in skeletal muscle and brain mitochondria [36,43], the effect also occurring in chloride anion-free media and therefore being not due to changes in matrix pH (DPI is a Cl^-/OH^- antiporter). The observed strong inhibition by DPI of ROS generation by BATM oxidizing OctC observed in the present study (Fig. 7) cannot be, however, indicative for RET, as this high ROS production with the same substrate was insensitive to rotenone. It has to be, therefore, assumed that in this case DPI perhaps reacted with some flavin groups not related to RET but involved in fatty acid oxidation. DPI has also been found to inhibit H_2O_2 production by brain mitochondria oxidizing glutamate plus malate [43]. Finally, since $O_2\bullet^-$ generated by complex I is exclusively released into the matrix compartment, it should be expected that this RET-associated $O_2\bullet^-$ release partly inactivates aconitase. In fact, a significant aconitase inactivation during succinate oxidation by rat heart mitochondria has been shown [16]. In contrast, oxidation of acylcarnitines by BATM is generally not accompanied by a significant aconitase inactivation (Fig. 4). Consequently, this lack of aconitase inactivation can be taken as a further argument against a significant contribution of RET-supported superoxide production to the total ROS generation during acylcarnitine oxidation by BATM.

Some more information on the site and mechanism of $O_2\bullet^-$ generation during fatty acid oxidation by BATM can be deduced from the comparison of ROS release measured with the AR/HRP assay and that evaluated from aconitase inactivation. As mentioned, the latter process reflects $O_2\bullet^-$ primarily released into the matrix compartment, whereas the former one results from both $O_2\bullet^-$ and H_2O_2 released directly at the outer face of the inner membrane as well as from H_2O_2 that diffuses secondarily from the inner mitochondrial compartment to the outer space. In this context it is interesting to note that oxidation of all acylcarnitine esters tested in the present investigation was accompanied by no or only a small (in case of OctC) inactivation of mitochondrial aconitase (Fig. 4), indicating release of all, or most of, $O_2\bullet^-$ formed directly to the outer face of the inner membrane. This was in sharp contrast to an approximately 50% inactivation with GP as substrate. With the latter substrate, the superoxide radical could be generated (i) at the active site of glycerophosphate dehydrogenase, located on the outer side of the inner membrane [18,19], (ii) at complex III and (iii) at complex I due to RET. In fact, Miwa and Brand [44] observed a pronounced inactivation of aconitase in mitochondria from *Drosophila* oxidizing GP, supporting the view that $O_2\bullet^-$ was generated on both sides of the membrane at about equal rates. In the case of complex I, this sidedness of $O_2\bullet^-$ release fits well with the proposed site of electron leak from the hydrophilic arm of complex I [45,46]. It is also worthy to note that in case of double-inhibited respiratory chain, with rotenone and antimycin A, and PalC as substrate, aconitase became considerably inhibited pointing to a large release of $O_2\bullet^-$ into the matrix compartment.

The relation between ROS release to both sides of the inner mitochondrial membrane depending on the respiratory substrate being oxidized is best illustrated by plotting ROS production evaluated by the two approaches applied, i.e. the AR/HRP assay against the aconitase inactivation assay. It clearly shows (Fig. 8) that points for acylcarnitine esters, independently of the fatty acid chain length, are grouped in the right side of the plot (low or no aconitase inactivation), independently of the intensity of ROS released estimated with the AR/HRP assay. These results taken together allow us to speculate that the high ROS generation accompanying fatty acid oxidation by rat BATM does not occur within the respiratory chain itself but, rather, on distinct enzymes of fatty acid oxidation like acyl-CoA dehydrogenase and ETF-ubiquinone oxidoreductase. Such hypothesis has also been addressed to the β-oxidation-linked ROS generation in muscle and liver mitochondria [5,6]. Only upon inhibition of the respiratory chain by rotenone and antimycin A, some sites located deep within the inner membrane or on its internal side become active in one-electron transfer to molecular oxygen. It has to be noticed that acyl-CoA dehydrogenase in rat BATM was found to be five to ten times more active, depending on the fatty acid chain length, than in rat skeletal muscle mitochondria, whereas complex III activity was twice as high in the latter than in the former (Table 1). The observed large ROS generation together with a large enzymatic capacity of acyl-CoA dehydrogenase may suggest that the first step of the β-oxidation pathway is an important source of $O_2\bullet^-$ generation. Reduced FAD prosthetic groups of acyl-CoA dehydrogenases exert high reactivity towards molecular oxygen, thereby exhibiting

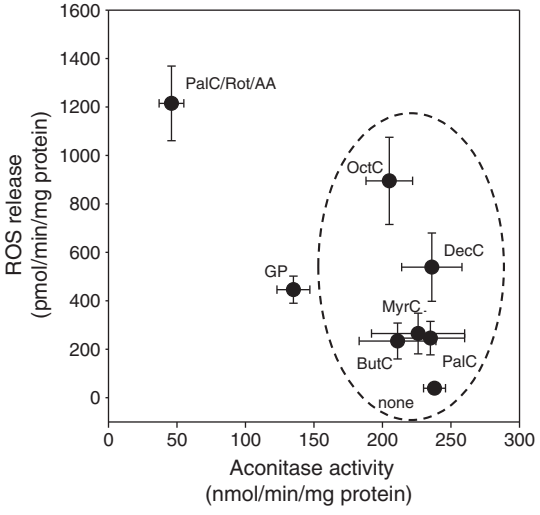

Fig. 8. Correlation between H_2O_2 release and aconitase activity in BATM. Data for H_2O_2 release (taken from Fig. 3) are plotted against data for aconitase activity (taken from Fig. 4).

tendency to form H_2O_2 [47]. In addition, a different reactivity of short-, medium- and long-chain acyl-CoA dehydrogenases towards molecular oxygen is a likely explanation of the observed variation of H_2O_2 release with different acylcarnitines (Fig. 3A). Indeed, despite significant sequence homology of mitochondrial acyl-CoA dehydrogenases and peroxisomal acyl-CoA oxidases, their reactivity towards molecular oxygen is quite different (for review see [48]). Moreover, electrons liberated from acyl-CoA dehydrogenase are transmitted by ETF-ubiquinone oxidoreductase to complex III. ETF-ubiquinone oxidoreductase is a flavoprotein located in the inner mitochondrial membrane, which contains three potential sites for one-electron transfer to oxygen, namely FAD, a 4Fe4S cluster and a ubiquinone-binding site [49]. Furthermore, the binding pocket for ubiquinone is located within a hydrophobic environment [50], a likely condition for an oxygen-rich environment supporting one-electron transfer from semiubiquinone to molecular oxygen.

In conclusion, in this study we report that isolated BATM oxidizing acylcarnitines release far more H_2O_2 than RSMM. Surprisingly, despite a pronounced ROS production, no remarkable inactivation of aconitase occurred, suggesting that superoxide was mostly released from the outer side of the inner mitochondrial membrane. The physiological importance of this large ROS production is not clear and requires further investigation. According to a recent proposal, the large ROS generation by BATM might be related in some way to UCP1 [14]. In fact, already a decade ago Echtay et al. [51] have proposed that uncoupling proteins may be activated not only by non-esterified fatty acids but also by superoxide. It can be therefore speculated that, by this means, UCP1 might mediate high proton conductance of the inner mitochondrial membrane under thermogenic conditions even in the presence of physiologically high levels of purine nucleotides. Thus, the high production of ROS by BATM might be of crucial importance for the physiological function of BAT.

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