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Long chain fatty acyl-CoA modulation of H_2O_2 release at mitochondrial complex I

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Abstract Complex I is responsible for most of the mitochondrial H_2O_2 release, low during the oxidation of the NAD linked substrates and high during succinate oxidation, via reverse electron flow. This H_2O_2 production appear physiological since it occurs at submillimolar concentrations of succinate also in the presence of NAD substrates in heart (present work) and rat brain mitochondria (Zoccarato et al., *Biochem J*, 406:125–129, 2007). Long chain fatty acyl-CoAs, but not fatty acids, act as strong inhibitors of succinate dependent H_2O_2 release. The inhibitory effect of acyl-CoAs is independent of their oxidation, being relieved by carnitine and unaffected or potentiated by malonyl-CoA. The inhibition appears to depend on the unbound form since the acyl-CoA effect decreases at BSA concentrations higher than 2 mg/ml; it is not dependent on ΔpH or Δp and could depend on the inhibition of reverse electron transfer at complex I, since palmitoyl-CoA inhibits the succinate dependent NAD(P) or acetoacetate reduction.

Keywords Succinate · NAD substrates · Reverse electron flow · NAD(P) reductive state · Fatty acids · Short chain acyl-CoAs · Carnitine · Malonyl-CoA · Membrane potential

Abbreviations

ROS	reactive oxygen species
G/M	glutamate/malate
α-KG	α-ketoglutarate
SOD	superoxide dismutase
Hepes	Piperazine etan sulfonic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horse-radish peroxidase
PCA	perchloric acid
$\Delta\psi$	mitochondrial membrane potential
ΔpH	mitochondrial pH gradient
Δp	the mitochondrial protonmotive force
palmitoyl CoenzimeA	palm-CoA
Bovine Serum Albumine	BSA
$O_2^{\cdot-}$	superoxide
Acyl-CoA Binding Proteins	ACBP
Fatty Acid Binding Proteins	FABP

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Introduction

Mitochondria are the major site of reactive oxygen species (ROS) production in mammalian cells and superoxide $\left(O_2^{\cdot-}\right)$ appears to be the primary ROS produced as the result of single electron reduction of O_2.

The primary function of mitochondrial complex I is to pump protons across the inner membrane. A second property of the complex is a highly modulated production of $O_2^{\cdot-}$. The latter is released on the matrix side of the inner

membrane (St-Pierre et al. 2002) and is transformed into H_2O_2 by intramitochondrial Mn-SOD; the permeable H_2O_2 is measured extramitochondrially by HRP and a suitable electron donor. $O_2^{\cdot-}$ is produced also by complex III (mainly on the cytosolic side; St-Pierre et al. 2002) however this release is largely dependent on the electron transfer inhibitor Antymycin-A (Turrens et al. 1985). H_2O_2 production is activated by NAD-linked substrates and is stimulated by the complex I inhibitor rotenone (Turrens and Boveris 1980). Alternatively, a much higher production rate is induced in the presence of BSA by succinate oxidation. Succinate feeds electrons to CoQ via complex II, downhill of complex I. In this system, rotenone functions as an inhibitor (Han et al. 2003; Hansford et al. 1997; Korshunov et al. 1997; Lambert and Brand 2004a, b; Liu et al. 2002; Votyakova and Reynolds 2001). The rotenone sensitivity is a strong indication that complex I is responsible also for such a production. It was concluded that succinate generates H_2O_2 by pushing electrons back from QH_2 into complex I in an energy dependent reverse mode of electron transfer. The succinate effect was considered not relevant physiologically, in the assumption that when low succinate is present with a large excess of NAD-linked substrates (the physiological situation) this would impede reverse electron transfer. We reported instead, in brain mitochondria, that succinate activates H_2O_2 production with high affinity (half maximal stimulation at 0.3 mM succinate) and that the behaviour is essentially unchanged in the presence of high (1 or 2 mM) glutamate/malate (G/M), whose effect was only to slightly increase the succinate concentration evoking the semi-maximal H_2O_2 release (Zoccarato et al. 2007). Furthermore, succinate was found to decrease but not to inhibit fully G/M oxidation at complex I (measured as the production of α-KG; Zoccarato et al. 2007). These results show that H_2O_2 generation is induced by succinate without changing the regular down-flow of electrons at complex I. We have shown previously that the Parkinson disease toxin dopaminochrome (derived from dopamine) induces a strong increase of H_2O_2 production both with G/M and in the further presence of succinate (Zoccarato et al. 2005) and that this H_2O_2 is removed by GSH peroxidase (Zoccarato et al. 2004).

We proposed that $O_2^{\cdot-}$ is produced by the auto-oxidation of a CoQ ubisemiquinone in equilibrium with the QH_2/Q couple, whose ratio is increase by succinate (Zoccarato et al. 2007). Indeed the ratio, as well as the peroxide production, can be increased also with NAD-linked substrates alone, provided that electron flow is slowed by cytochrome c removal (Kushnareva et al. 2002). Other FAD-dependent substrates could modulate complex I $O_2^{\cdot-}$ production, such as glycerol-3-phosphate via the glycerol-3-phosphate dehydrogenase (Tretter et al. 2007b) or fatty acids. Several specific effects were indeed attributed to long chain acyl-CoAs, which are reported to deeply affect cellular metabolism and mitochondrial functions (Faergeman and Knudsen 1997) acting also as inhibitors of the transhydrogenase (Kozlov et al. 1984; Rydstrom 1972) and of the adenine nucleotide translocator (Veerkamp et al. 2000).

Fatty acids are essentially not oxidized in brain mitochondria due to the low activity of 3-ketoacyl-CoA thiolase (Yang et al. 1987), while they are the major energy substrate for cardiac muscle, generating 70% ATP by their mitochondrial β-oxidation (Taegtmeyer et al. 1980). In this study we report an organization of H_2O_2 production in heart mitochondria similar to that described in brain mitochondria. Furthermore, we show that the fatty acids oxidation does not promote per se high H_2O_2 release while the succinate-stimulated H_2O_2 generation appears deeply depressed by long chain acyl-CoAs independently of their mitochondrial oxidation. This effect appears active also in brain mitochondria that do not oxidize fatty acids.

Experimental procedures

Reagents Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was from Molecular Probes, Horseradish peroxidase (HRP; grade I;EC1.11.1.7), α-ketoglutarate (α-KG) dehydrogenase from pig heart (EC 1.2.4.2) and β-hydroxybutyrate dehydrogenase (from *Pseudomonas lemoignei* EC 1.1.1.30), long and short chain acyl CoAs were from Sigma. All other reagents were of analytical grade.

Preparation of mitochondria

Rat heart mitochondria were isolated from 6–7 weeks old rats with the Polytron procedure essentially as described (Palmer et al. 1977). BSA 0.5 mg/ml was included in all isolation media. Mitochondria suspending medium was composed of 0.25 M, 0.2 mM EGTA and 10 mM Hepes pH 7.3. After protein determination BSA 10 mg/ml was added to the concentrated suspension (30–40 mg/ml determined by the Gornall procedure, Gornall et al. 1949).

Brain mitochondria were isolated from cerebral cortices of 6–7 weeks old rats as described (Zoccarato et al. 2004).

Standard Incubation Unless otherwise indicated (0.3 mg/ml) were incubated at 30°C in the standard incubation medium: 125 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 100 μM EGTA, 20 mM HEPES, pH 7.2 containing 1 mg/ml defatted bovine serum albumin (BSA).

Hydrogen peroxide measurement Hydrogen peroxide was measured essentially as in (Mohanty et al. 1997; Zoccarato

et al. 2005) with 40 μM Amplex Red and 6 μg/ml HRP (1 U) on a Fluoroskan Ascent FL plate reader in 24 wells plates with the indicated concentration of substrates. The reaction was started with addition of the Amplex red/HRP mixture. H_2O_2 was detected by the formation of the fluorescent product resorufin (544/590 nm ex/em wavelengths). Internal standard of 1 nmol H_2O_2 was added at the end of each assay. The wells were read every minute and mean reading of every well was considered. The first 10 min rates were calculated. Values are reported as picomoles per minute per milligram protein.

Assay of α-Ketoglutarate The α-KG assay was performed as in (McCormack and Denton 1979). Mitochondria (4 mg/ml) were incubated in the standard incubation medium under agitation for 10 min with the indicated amount of substrates. During incubation substrate concentrations of glutamate, malate and succinate were maintained constant by addition of 0.3 mM glutamate and malate and 0.5 mM succinate at 5 min. Reactions were stopped by addition of cold PCA (12%) and samples left in ice 20 min before centrifugation (15,000×*g*, 10 min). Enzymatic fluorimetric titration of NADH formed by α-KG oxidation was carried out in 100 μl aliquots of the supernatant neutralized with KOH in 200 μl (final volume) of medium containing 100 mM Na-phosphate (pH 7.3), $CaCl_2$ 5 mM, $MgCl_2$ 1 mM, EGTA 5 mM, NAD^+ 1 mM, and 0.2 mg/ml (160 mU) of α-KG dehydrogenase in the plate reader Fluoroskan at 355/460 nm ex/em. The reaction was started by addition of 100 μM CoASH. α-KG concentrations were calculated from the titration curve of the rates obtained with known comparable amounts of standard α-KG. Values are reported as nanomoles per minute per milligram protein. Similar results were obtained by spinning down mitochondria at the end of incubation and measuring α-KG in the PCA-treated supernatant.

Assay of β-hydroxybutyrate The β-hydroxybutyrate assay was performed as in Williamson et al. (1962). The incubation was for 10 min at 2 mg/ml mitochondrial suspension. During incubation the substrate concentrations were: 5 mM acetoacetate, 1 mM succinate, 10 and 20 μM palm-CoA, 1 μg/ml rotenone and succinate concentration was maintained constant by addition of 0.3 mM succinate at the fifth minute. Reactions were stopped by addition of cold PCA (12%) and samples left in ice 20 min before centrifugation (15,000×*g*, 10 min). Enzymatic fluorimetric titration of NADH formed by β-hydroxybutyrate oxidation was carried out in 100 μl aliquots of the supernatant neutralized with KOH at pH 8.5 in 200 μl (final volume) of 0.1 M Tris–HCl (pH 8.5) containing 1.5 mM EDTA 0.5 mM NAD^+, 100 mM hydrazine-HCl. The reaction was started by additions of 20 mU of β-hydroxybutyrate dehydrogenase in the plate reader Fluoroskan (ex/em 355/460 nm). β-hydroxybutyrate concentrations (reported as micromolar) were calculated from the titration curve of the rates obtained with known comparable amounts of standard β-hydroxybutyrate.

Measurement of mitochondrial membrane potential Membrane potential (Δψ) was measured using fluorescence quenching of the cationic dye safranine (3 μM) at ex/em 495/586 nm in 0.3 mg/ml of mitochondrial suspension in a Shimatzu spectrofluorimeter as in Petit et al. (1990).

Measurement of mitochondrial NAD(P)H redox state Fluorescence of NAD(P)H redox state was followed fluorimetrically at ex/em 340/460 nm in Shimatzu Spectrofluorimeter in a 3 ml quartz cuvette under stirring.

Measurement of mitochondrial respiration Oxygen consumption was monitored with a Clark-type oxygen electrode in a 1.6 ml closed chamber with continuous stirring.

Results

General properties of H_2O_2 release at complex I

Rat heart mitochondria, similarly to brain mitochondria (Zoccarato et al. 2007) exhibited a low production rate of H_2O_2 during the oxidation of the NAD-dependent substrates G/M. This is shown in Fig. 1, where the maximal peroxide release with G/M accounted for only 10% of the production with 1 mM succinate. Succinate induced a strong dose dependent increase of H_2O_2 output, which was evident below 0.5 mM and reached the maximum at 2–3 mM. When G/M (1 mM each) were co-incubated with increasing succinate concentrations, the curve of H_2O_2 release was moved to higher succinate concentrations, but still reached approximately the same maximum at 2–3 mM. As reported for muscle and brain mitochondria, also in heart mitochondria rotenone stimulated (from 20 to 40 pmol/min/mg protein) the low G/M dependent H_2O_2 release and powerfully inhibited (90% inhibition) the succinate-dependent H_2O_2 production confirming the involvement of complex I. Also the residual production of H_2O_2 with rotenone and succinate is likely from complex I, originating from the NAD reduction by succinate-derived malate. Again, like in brain mitochondria, succinate did not prevent NAD-dependent substrate oxidation since α-KG production from G/M (via glutamate dehydrogenase or via transamination with malate-derived oxaloacetete) was only decreased by 1 mM succinate by 42±17% (data±SD in duplicate from three different preparations). Also the ΔpH

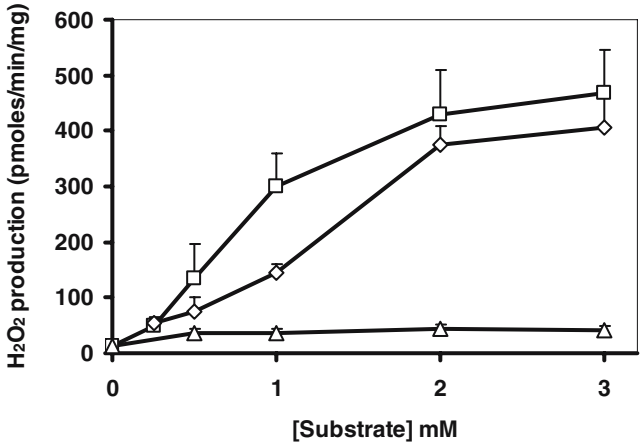

Fig. 1 Dose dependent H_2O_2 generation in heart mitochondria by G/M and succinate. RHM (0.30 mg/ml) were incubated in standard incubation medium in the presence of the indicated substrate concentrations and the reactions started by addition of the mixture Amplex Red and HRP as indicated in Experimental procedures. The calculated H_2O_2 rates are reported for increasing succinate concentrations with (*empty diamond*) or without (*empty square*) G/M (1 mM each) and for increasing G/M concentrations (*empty triangle*). Data represent the mean±SD of at least three different preparation in duplicates

and Δp dependence was essentially the same as described (Lambert and Brand 2004a; Zoccarato et al. 2007): in the presence of the K^+/H^+ ionophore nigericin and in high K^+ medium the ΔpH component of Δp is converted into $\Delta\psi$; loss of the ΔpH component inhibited the succinate (and slightly increased the G/M) dependent H_2O_2 production.

Succinate induced H_2O_2 generation is inhibited by long chain Acyl-CoAs In an attempt to investigate the property of the other major FAD-dependent process in heart mitochondria, i.e. fatty acid degradation, we studied the effect of different free fatty acids on H_2O_2 production. Free fatty acids per se did not produce nor modify the succinate dependent H_2O_2 production (Table 1), independently of their oxidation. Medium chain fatty acids were oxidized per se without the help of carnitine and ATP. They generated a high $\Delta\psi$ (Fig. 2 trace a) and produced low H_2O_2. Long chain fatty acids require activation to CoA thioesters [via the Acyl CoA synhetase present in intact heart mitochondrial (Sayed-Ahmed et al. 1999)] and in the presence of carnitine are transformed into fatty acyl-carnitine (by means of carnitine palmityl transferase I) to be transported into the matrix and oxidized. This is reported in Fig. 2 trace b showing that oleate, in the presence of ATP, CoA and oligomycin (to prevent ATP-dependent energization), was unable to increase membrane potential significantly, until carnitine was also added. Figure 3 reports the succinate titration of H_2O_2 production, with and without carnitine, in the same conditions. Oleyl-CoA, which was generated in the absence of carnitine, acted as an inhibitor of succinate

Table 1 Effect of fatty acid on H_2O_2 induced generation

	None (pmol min^{-1} mg^{-1})	Succinate 1 mM (pmol min^{-1} mg^{-1})
None	10±3	300±100
Palmitate 50 μM	12±1	303±53
Decanoate 50 μM	34±17	293±160
Eptanoate 50 μM	34±16	381±88
Oleate 50 μM	12±5	445±87

Different rates of H_2O_2 production in RHM are elicited by the indicated amount of fatty acid alone or added on succinate (1 and 2 mM). All other condition as in Fig. 1.

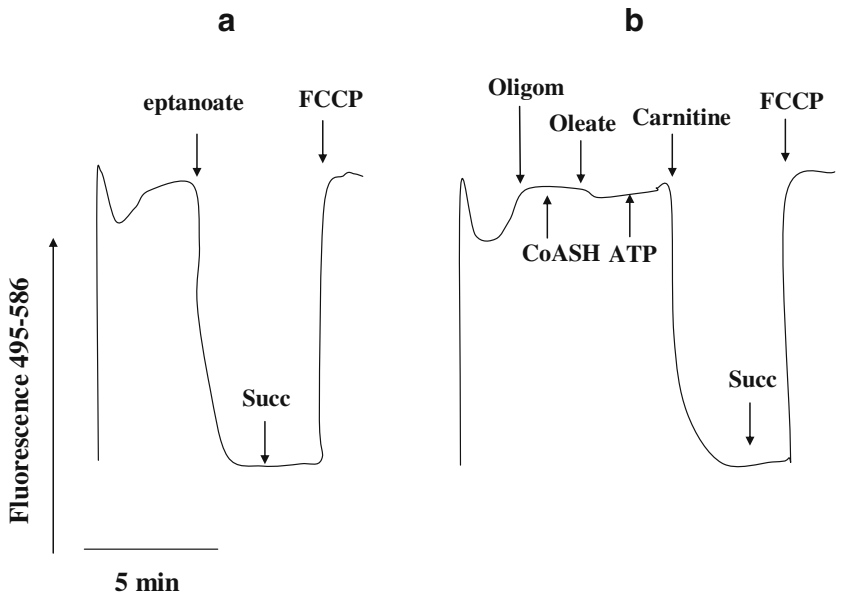

Fig. 2 Effect of long or short fatty acid on safranine (3 μM) measured $\Delta\psi$ When indicated eptanoate or oleate (40 μM), oligomycin (1 μM), CoASH (100 μM), ATP (2 mM), carnitine (2 mM) and succinate (2 mM) were added. All other condition as in Fig. 1

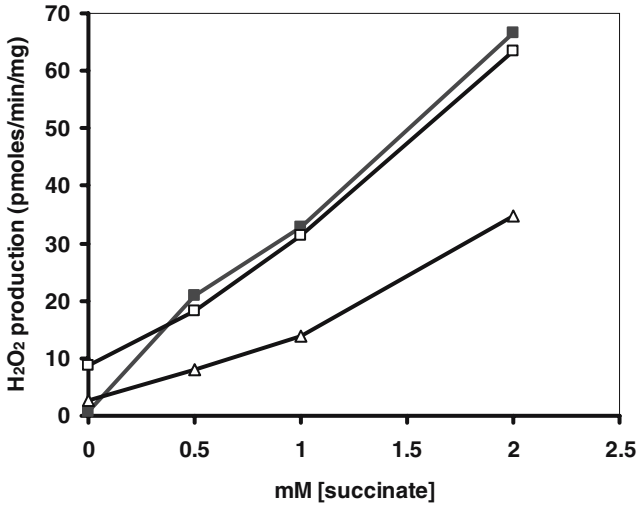

Fig. 3 Inhibition of H_2O_2 generation by Oleyl CoA. H_2O_2 production rates in RHM are reported for increasing concentration of succinate alone (*filled square*), or in the presence of oleate 40 μM plus CoASH 100 μM and ATP 2 mM with (*empty square*) or without (*empty triangle*) carnitine 2 mM. To prevent ATP hydrolysis oligomicyn (1 μM) was always present. All other condition as in Fig. 1

dependent H_2O_2 production. Such inhibition was readily removed by carnitine. These results suggest an inhibition by acyl-CoA of succinate-dependent H_2O_2 release. An inhibition of dicarboxylate transporter by long chain acyl-CoAs (Halperin et al. 1972) has been excluded since in the presence of BSA (our experimental conditions and ref (Ventura et al. 2005)), only palm-CoA concentration higher than 20 μM slightly affect succinate oxidation (0±5% inhibition by 10 μM and 18±2% by 20 μM palm-CoA). It has to be recalled that intracellular acyl-CoAs are linked to the acyl-CoA binding proteins (ACBP), and that the binding activity of BSA and ACBP are superimposable as reported in liposomal models (Rasmussen et al. 1990; Rasmussen et al. 1993).

We performed titrations of the palm-CoA inhibition of succinate induced H_2O_2 generation (Fig. 4a). These indicated an apparent IC_{50} value of 5 μM for palm-CoA, a concentration well below the minimal required for interfering with succinate oxidation. A similar curve was obtained with the less inhibitory palmitoleoyl-CoA (Fig. 4a) exhibiting an IC_{50} of 12 μM. Short chain acyl-CoAs were instead without activity on succinate-induced peroxide as shown in Fig. 5, where short medium and long chain acyl-CoA are compared. Only long chain acyl-CoAs appeared to be inhibitors, and the inhibition depended strictly on the chain length of the CoA thioesters which act at the outer face of the inner membrane. This is shown in Fig. 4b where the inhibition by palm-CoA was progressively removed by carnitine which induces the production of the non inhibitory palmitoyl-carnitine; and in Fig. 4c where the inhibition by 0.5 mM carnitine on palm-CoA effect was dose-dependently removed by malonyl-CoA, the physiological inhibitor of

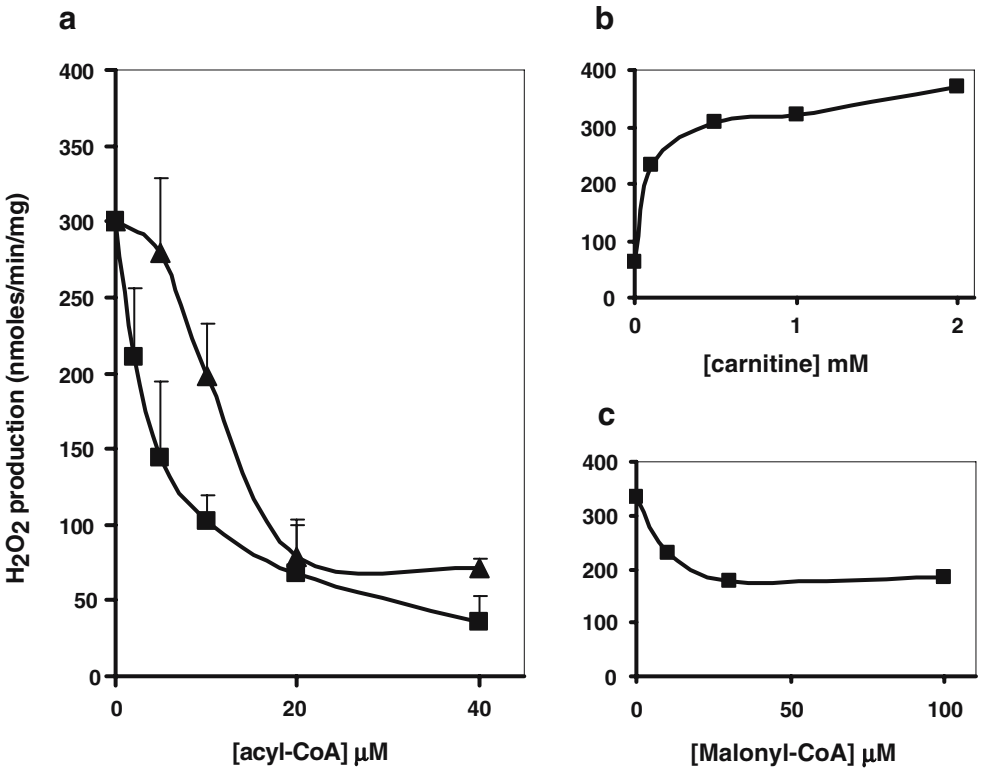

Fig. 4 Inhibition of succinate dependent H_2O_2 generation by palm-CoA (**a**). Effect of carnitine (**b**) and malonyl-CoA (**c**). **a** The rates of H_2O_2 production by 1 mM succinate in the presence of increasing concentration of palm-CoA (*filled square*) or palmitoleoyl-CoA (*filled triangle*) are reported. **b** The rates obtained with increasing concentration of carnitine added to 10 μM palm-CoA and 1 mM succinate. **c** The rates obtained with increasing concentration of malonyl-CoA added to 10 μM palm-CoA, 1 mM succinate, 0.5 mM carnitine. All other conditions are as in Fig. 1

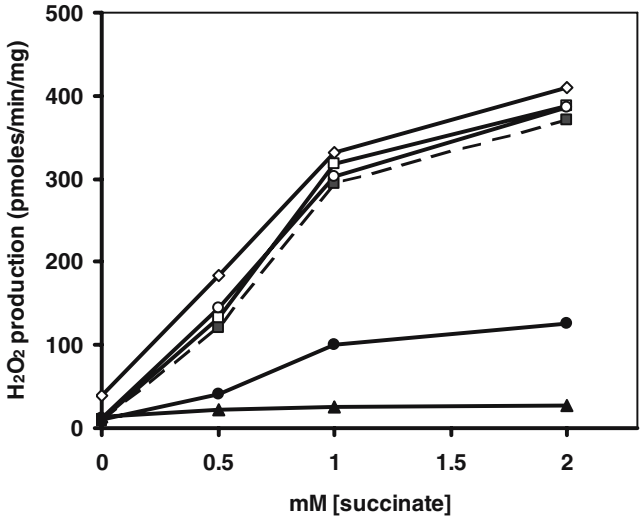

Fig. 5 The effect of acyl-CoA on succinate dependent H_2O_2 generation. The H_2O_2 production rates induced by increasing concentration of succinate alone (*filled square*), plus 30 μM acetoacetyl-CoA (*empty square*), plus 30 μM octanoyl-CoA (*empty diamond*), plus 30 μM malonyl-CoA (*empty circle*), plus 30 μM palm-CoA (*filled triangle*) and plus 10 μM palm-CoA (*filled circle*) are reported. All other condition as in Fig. 1

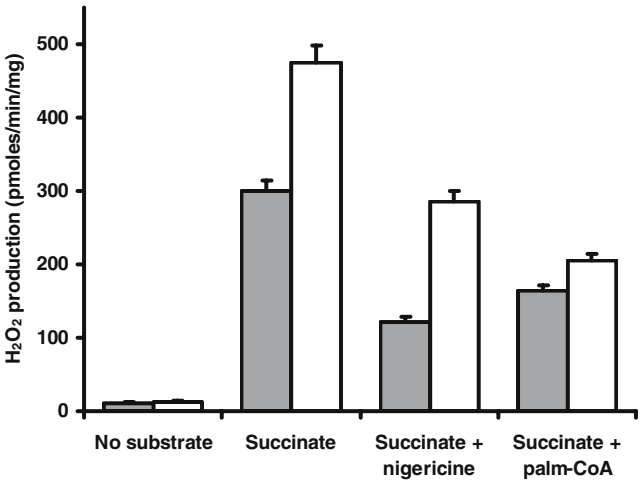

Fig. 7 H_2O_2 production dependence on ΔpH. The H_2O_2 production rates in standard incubation medium with 1.2 mM Pi (*filled square*) or without Pi (*empty square*) are reported as indicated. Where present the additions were: succinate 1 mM, nigericin 100 nM and palm-CoA 10 μM. All other condition as in Fig. 1. The values (means±SD of triplicates measurements) are all significantly different from the respective succinate control $P<0.01$(Student's *t* test)

carnitine palmitoyl transferase I; in this condition palmitoyl-carnitine was not formed and the H_2O_2 release was again 80% maximal.

Palm-CoA did not decrease the succinate-generated membrane potential, ruling out the possibility that its effect depended on a partial uncoupling (Fig. 6). The palm-CoA inhibition was also independent of ΔpH. In fact 10 μM palm-CoA inhibited H_2O_2 generation similarly to H^+/K^+ exchanger nigericin both in the absence of phosphate (maximal ΔpH) and in the presence of phosphate (whose influx via the Pi/H^+ symporter decreases ΔpH; Fig. 7). The effect of palm-CoA was unaffected by the presence of G/M on top of succinate (not shown).

We can conclude that external long chain fatty acyl-CoAs appear as the most probable physiological modulators of the succinate stimulated H_2O_2 production. Palm-CoA slightly stimulated the low G/M dependent H_2O_2 production, both at high and low ΔpH.

Increasing BSA removes the palm-CoA inhibition of H_2O_2 release In cells fatty acids and long chain acyl-CoAs are not free but are largely bound to binding proteins FABP and ACBP respectively. BSA acts in the same way binding acyl-CoAs and fatty acids although with different affinities. It is well known that succinate induced H_2O_2 production requires the presence of BSA. The requirement for BSA has recently been studied in detail (Tretter et al. 2007a) and

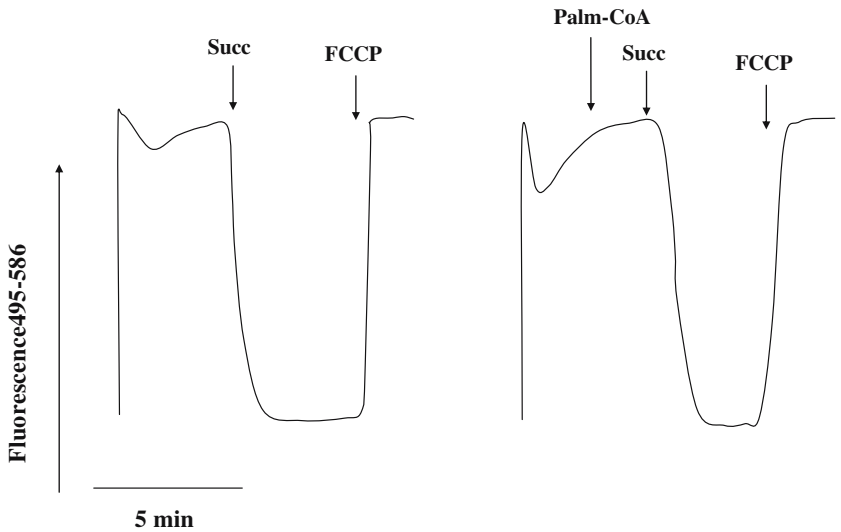

Fig. 6 Effect of palm-CoA on succinate dependent Δψ. Δψ was measured by safranine as in Fig. 3. When indicated 1 mM succinate, 10 μM palm-CoA and 1 μM FCCP were added

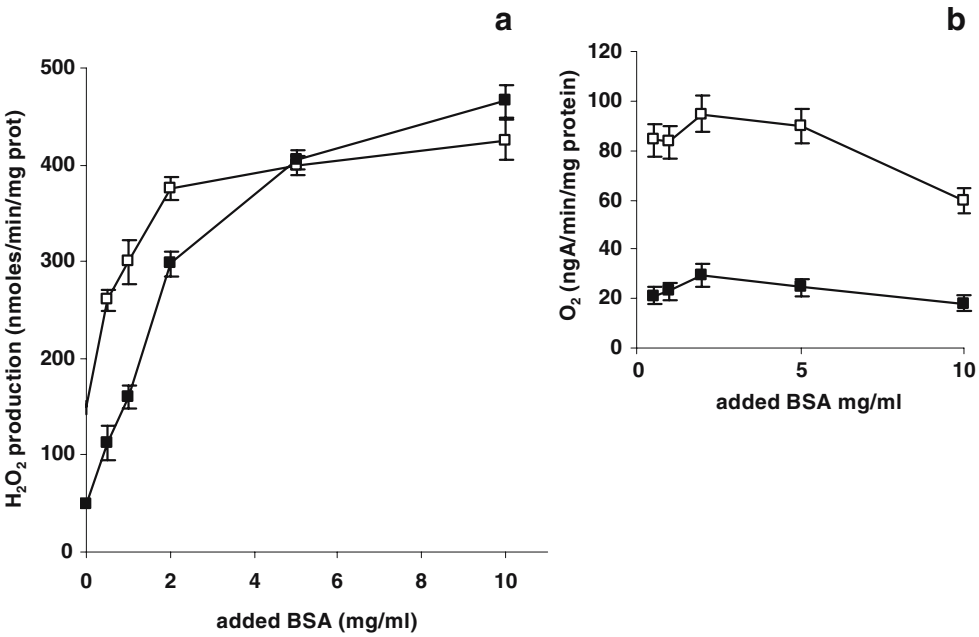

Fig. 8 Effect of added BSA on Succinate dependent H_2O_2 production and its inhibition by palm-CoA (**a**) and on palm-CoA respiratory rates (**b**). In **a** the H_2O_2 production rates with increasing concentration of added BSA and 1 mM succinate are reported in the absence (*filled square*) or in the presence (*empty square*) of 10 μM palm-CoA. In **b** the state 3 (*empty square*), induced by ADP, and state 4 (*filled square*) respiratory rates of 10 μM palm-CoA in the presence of 0.5 mM carnitine are also reported at increasing BSA concentrations. All other conditions are as in Fig. 1

attributed to a small decrease of membrane potential in the absence of BSA. We analysed the inhibition by palm-CoA of succinate-induced H_2O_2 generation as a function of BSA concentration added to the incubation medium. Figure 8a reports that increasing BSA above 2 mg/ml progressively removes the inhibitory effect of palm-CoA without affecting the succinate-induced H_2O_2 production. The H_2O_2 production at zero added BSA is likely the consequence of BSA being present in the mitochondrial preparation allowing a minor succinate dependent H_2O_2 release. Instead palm-CoA oxidation (Fig. 8b), measured at the Clark electrode in the presence of carnitine, was not affected until BSA was increased above 5 mg/ml. These data indicate that it is the fraction of the palm-CoA not bound to BSA (or likely in vivo to ACBP or FABP) that effectively acts as inhibitor of succinate stimulated H_2O_2 release.

Long chain acyl-CoAs inhibit NAD(P) reduction and reverse electron flow at complex I

Long chain acyl-CoAs are known to be powerful inhibitors of mitochondrial energy dependent transhydrogenase (Kozlov et al. 1984; Rydstrom 1972). This enzyme is the principal one responsible for NADP reduction in mitochondria. In turn, H_2O_2 removal depends largely on NADPH (via glutathione or thioredoxin peroxidase; Bjornstedt et al. 1995; Chance et al. 1979; Zoccarato et al. 2004). However heart mitochondria also contain catalase (Phung et al. 1994; Radi et al. 1991).

We decided to investigate the effect of palm-CoA on mitochondrial NAD(P) reduction. Figure 9 shows that during coupled succinate oxidation a large NAD(P) reduction was observed (trace a), which was completely removed upon uncoupling. The subsequent addition of rotenone promoted a partial reduction (originating from the oxidation of succinate-derived malate). Due to the lack of energy-dependent transhydrogenase activity in this condition the reduced specie is mostly NADH. In the presence of palm-CoA the succinate-dependent pyridine nucleotide

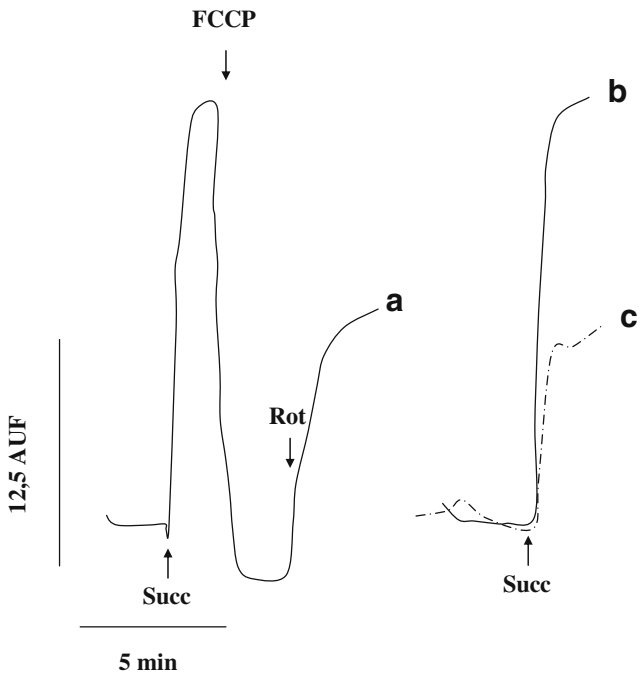

Fig. 9 Effect of FCCP, rotenone and palm-CoA on NAD(P)H reduction by succinate. Trace *a*: total NAD(P)H was reduced by 1 mM succinate. FCCP and rotenone were 1 μM e 1 μg/ml. 50 μM malonyl-CoA (trace *b*) or malonyl-CoA plus palm-CoA 10 μM (trace *c*) were added before succinate

reduction was strongly decreased (Fig. 9 trace c), to a level close to that of NADH determined in the previous trace (Fig. 9 trace a) and in trace b where malonyl CoA is added before succinate. In these experiments malonyl-CoA was also included to prevent slow palm-CoA oxidation. The strong inhibition by palm-CoA of NAD(P) reduction tentatively reflects the inhibition of transhydrogenase but may involve also the inhibition of reverse electron flow from succinate. To confirm the last effect a direct measurement of the rate of NADH formation from succinate in coupled mitochondria was performed by measuring succinate supported acetoacetate reduction in the presence and absence of palm-CoA. The amount of β-hydroxybutyrate formed was slightly inhibited by 10 μM palm-CoA and much more strongly by 20 μM palm-CoA, lowered to a level similar to that reached with rotenone and attributable to the activity of malate dehydrogenase (Fig. 10).

These results suggest that palm-CoA partially inhibited the reverse electron transfer from succinate in heart mitochondria in conditions where succinate oxidation per se was largely unaffected. The acyl-CoA effects appear to represent a metabolic control of the mitochondrial respiratory chain-dependent H_2O_2 release that could be effective also in non fatty acids oxidizing tissues.

Fatty acid oxidation is reported to be essentially absent in brain mitochondria, due to the low activity of 3-ketoacyl-CoA thiolase (Yang et al. 1987). We tested the effect of acyl-CoAs on H_2O_2 production also in brain mitochondria,

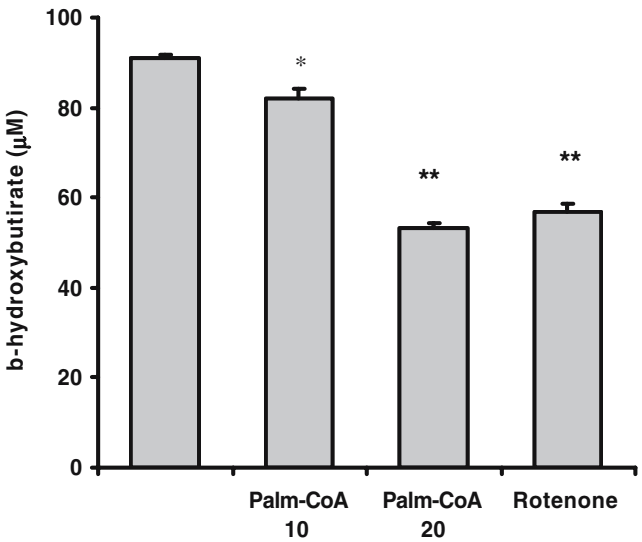

Fig. 10 Succinate dependent acetoacetate reduction. Effect of palm-CoA and rotenone. β-hydroxybutirate formation (μM) is reported in the indicated experimental conditions. Succinate and acetoacetate were 1 and 5 mM respectively. When indicated rotenone (1 μg/ml) and palm-CoA (10 or 20 μM) were added. Values are means±SD of at least three different preparation in duplicates. Significantly different from control *Single asterisk* $P<0.05$; *double asterisk* $P<0.01$(Student's *t* test)

to analyze if the inhibition extends to systems which do not utilize fatty acids significantly as oxidizable substrates. We confirmed that 10 μM palm-CoA inhibited succinate-dependent H_2O_2 production as in heart mitochondria. Also in rat muscle mitochondria palm-CoA appears to inhibit succinate supported H_2O_2 generation (work in progress).

Discussion

Mitochondrial $\left(O^{\cdot}_{2}^{-}\right)$ production is largely dependent on complex I. $\left(O^{\cdot}_{2}^{-}\right)$ is generated intra-mitochondrially and is readily converted to H_2O_2 by intramitochondrial Mn-SOD. The highly permeable H_2O_2 is monitored extra-mitochondrially by the sensitive HRP/Amplex Red method. A deficient $O_2^{\cdot-}$ removal, by genetic removal of Mn-SOD is incompatible with extended life span (Li et al. 1995; Melov et al. 1999) The process of mitochondrial $\left(O^{\cdot}_{2}^{-}\right)$ generation is highly variable and strongly controlled. NAD-linked substrates are poor H_2O_2 producers. However succinate is a strong H_2O_2 generator. Succinate-dependent H_2O_2 release is largely sensitive to the complex I inhibitor rotenone (and to decreasing Δp) and it was concluded that succinate operates via reverse electron flow from complex II to complex I (Han et al. 2003; Hansford et al. 1997; Korshunov et al. 1997; Lambert and Brand 2004a, b; Liu et al. 2002; Votyakova and Reynolds 2001). Thus, the succinate effect was considered to be linked to a non physiological backward electron flow and it was assumed that the physiological situation where low succinate is present together with a large excess of NAD-linked substrates would impede reversal-dependent H_2O_2 generation. We have shown that these assumptions are incorrect since 1) co-presence of G/M does not impede H_2O_2 generation by low succinate and only increases the concentrations of the succinate-dependent H_2O_2 production curve, and 2) when G/M and succinate are oxidized together, electrons from G/M still partly move down in complex I (i.e. α-KG is still generated) and yet succinate promotes H_2O_2 production. It is likely that the succinate-dependent H_2O_2 release is a consequence of the succinate-dependent elevation of the mitochondria QH_2/Q ratio. In fact a high rate of H_2O_2 release has been reported also with G/M alone, provided that the re-oxidation of QH_2 was slowed by cytochrome *c* removal (Kushnareva et al. 2002). These observations indicate that complex I is a highly modulated H_2O_2 generator in physiological conditions, with succinate concentration [together with NADH/NAD and Δp, particularly its ΔpH component (Lambert and Brand 2004a, b)] acting as effector. Analysing the behaviour of fatty acids, the other major class of heart mitochondrial substrates, we detected a function of long chain CoA esters, described in this study.

Long chain acyl-CoAs are reported to have several controlling activities in cells (Faergeman and Knudsen 1997) in the presence of relevant concentration of ACBP and FABP. BSA was found to bind acyl-CoAs in a similar way (Rasmussen et al. 1990). In the presence of BSA succinate oxidation was not affected by low acyl-CoA concentrations (10 μM). In these conditions, as shown in this study, succinate stimulated H_2O_2 generation was strongly inhibited by 10 μM palm-CoA. The effect was removed by carnitine which transforms acyl-CoAs into acyl-carnitines; it was ripristinated by malonyl-CoA which inhibits carnitine palmitoyl transferase I. Given the impermeability of acyl-CoAs across the inner mitochondrial membrane the results are consistent with long chain fatty acyl-CoAs acting from outside to inhibit H_2O_2 generation. The presence of BSA is necessary for succinate induced H_2O_2 production (Han et al. 2003; Hansford et al. 1997; Korshunov et al. 1997; Lambert and Brand 2004a, b; Liu et al. 2002; Votyakova and Reynolds 2001). This requirement has recently been analysed in detail by the group of Adam Vizi (Tretter et al. 2007a). The effect of BSA on H_2O_2 production *in vitro* is likely mimicked by the effect of FABP *in vitro*, supporting the possibility that this is a real physiological phenomenon. Acyl-CoAs partition in phospholipid vesicles by insertion of the hydrophobic acyl chains into the bilayers. It is likely that ACBP (and to a lesser extent FABP) that bind acyl-CoAs with high affinity are the physiological carriers of acyl-CoAs in the cell. This means that palm-CoA is contended between the ACBP and the membranes or sites in membranes that compete for its binding. The fact that the long chain acyl-CoAs do not inhibit succinate oxidation but inhibit the succinate-supported H_2O_2 release suggests the presence of distinct acyl-CoA binding sites. Total long chain acyl-CoA concentration has been reported to rise to rather high values in vivo (Ciapaite et al. 2007). This supports the possibility that acyl-CoAs are physiological negative modulators of succinate-dependent peroxide release and this role may be exerted also in tissues incapable of fatty acid oxidation suggesting a more general modulatory effect by long chain acyl-CoAs on $O_2^{\cdot-}$ production by mitochondria. Acyl-CoAs are known inhibitors of energy dependent transhydrogenase and also appear to modulate negatively succinate supported reverse electron transfer as shown by the finding that the reduction of acetoacetate supported by succinate oxidation (a measure of electron backflow) is sensitive to the presence of palm-CoA. This may be rationalized if long chain acyl-CoAs decrease the affinity of $CoQH_2$ binding to complex I. This leads to a strong decrease of NAD(P) reduction from succinate in the presence of palm-CoA. The decreased NADPH concentration in turn acts as an inhibitor of H_2O_2 removal via GSH (or thioredoxin) peroxidase. So long chain acyl-CoAs on one hand inhibit the succinate supported $\left(O_2^{\cdot-}\right)$ formation but on the other they decrease the H_2O_2 removing ability of mitochondria. The opposite effects could depend on the metabolic cellular conditions, i.e. on the level of succinate or other substrates that determine the rate of mitochondrial peroxide release.

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