J Bioenerg Biomembr (2011) 43:359–366
DOI 10.1007/s10863-011-9363-6

The control of mitochondrial succinate-dependent H_2O_2 production

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Received: 6 April 2011 / Accepted: 25 May 2011 / Published online: 7 July 2011

Abstract In brain mitochondria succinate activates H_2O_2 release, concentration dependently (starting at 15 μM), and in the presence of NAD dependent substrates (glutamate, pyruvate, β-hydroxybutyrate). We report that TCA cycle metabolites (citrate, isocitrate, α-ketoglutarate, fumarate, malate) individually and quickly inhibit H_2O_2 release. When they are present together at physiological concentration (0.2, 0.01, 0.15, 0.12, 0.2 mM respectively) they decrease H_2O_2 production by over 60% at 0.1–0.2 mM succinate. The degree of inhibition depends on the concentration of each metabolite. Acetoacetate is a strong inhibitor of H_2O_2 release, starting at 10 μM and acting quickly. It potentiates the inhibition induced by TCA cycle metabolites. The action of acetoacetate is partially removed by β-hydroxybutyrate. Removal is minimal at 0.1 mM acetoacetate, and is higher at 0.5 mM acetoacetate. We conclude that several inhibitors of H_2O_2 release act jointly and concentration dependently to rapidly set the required level of H_2O_2 generation at each succinate concentration.

Keywords Brain mitochondria · Complex I · Complex II · Reactive oxygen species · H_2O_2 · Calorie restriction · Ketone bodies · Acetoacetate · Succinate · TCA cycle substrates

Abbreviations

ROS	Reactive oxygen species
$O_2^{\bullet -}$	Superoxide
HRP	Horseradish peroxidise
BSA	Bovine serum albumin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
AcAc	Acetoacetate
βOH	β-hydroxybutyrate
CR	Calorie restriction
IF	Intermittent fasting
AL	Ad libitum feeding
LDF	Limited daily feeding
OAA	Oxaloacetate
α-KG	α-ketoglutarate
MPP^+	1-methyl-4-phenylpyridinium
SOD	Superoxide dismutase
$NO^{\bullet}$	Nitric oxide
NOS	Nitric oxide synthase
PD	Parkinson disease
TCA (cycle)	Tricarboxylic acid (cycle)
NAC	N-acetyl-cysteine

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Introduction

The "free radical theory" of aging speculates that endogenous reactive oxygen species (ROS) are generated in cells and result in a pattern of cumulative damage leading to aging (Harman 1956). ROS are produced continuously and the signs of their presence (products of lipid peroxidation, protein oxidations, oxidative modifications of nuclear and mitochondrial DNA) increase with age. It is now recognized that low levels of these substances, particularly H_2O_2, are physiological messengers. Their effect is to increase cell proliferation, migration and survival. They promote the activation of transcription factors like NFkB and AP-1, inhibit protein tyrosine phosphatases (PTP1B) the inositol phosphatase PTEN, the SH2 domain containing inositol

phosphatase SHIP2, thus potentiating the action of tyrosine kinase dependent hormones. Furthermore the insulin receptor tyrosine kinase activity is increased by low concentrations of H_2O_2 and by an oxidative shift of the GSH redox state (Ma 2010; Dröge and Schipper 2007).

Mitochondria are a very important cellular producer of H_2O_2 (reviewed in Murphy 2009; Starkov 2008). The primary species produced is $O_2^{\bullet -}$, which is mostly generated in the mitochondrial matrix by NADH:CoQ-oxidoreductase (complex I) of the respiratory chain. It readily interacts with intramitochondrial Mn-SOD to generate H_2O_2 which can exit mitochondria (Murphy 2009; Starkov 2008). It is significant that animals lacking Mn-SOD do not survive long (Li et al. 1995). H_2O_2 is readily measured in the extramitochondrial space.

The complex I mediated production of H_2O_2 is however very low with NAD dependent substrates. Instead the succinate:CoQ-oxidoreductase (compex II) specific substrate succinate, also a reductant of CoQ, activates a concentration dependent high rate of H_2O_2 production. This process appears to depend on complex I, being rotenone-sensitive. It is generally believed that the succinate induced H_2O_2 generation depends on a reversed flux of electrons from complex II to complex I, which activates a process of one-electron transfer from a mitochondrial donor (possibly a CoQ semiquinone) to oxygen. However, succinate produces H_2O_2 in the presence of high concentrations of NAD dependent substrates (Zoccarato et al. 2007, 2009; Muller et al. 2008) and it does not stop the downhill flow of electrons in complex I. α- ketoglutarate production from glutamate is decreased but not abolished by a succinate concentration that produces H_2O_2, (Zoccarato et al. 2007). In brain mitochondria, in the presence of high concentrations of glutamate plus pyruvate, succinate activates a high rate of H_2O_2 production, which is apparent starting at very low succinate concentrations (below 15 μM), is sigmoidal with succinate and reaches the Vmax at less than 600 μM succinate (Zoccarato et al. 2009). Heart mitochondria behave similarly, but the succinate concentration curve is shifted to the right, i.e. to higher succinate concentrations (Bortolami et al. 2008). The Vmax of H_2O_2 production in brain mitochondria is very high, about 2.2 nmoles/mg/min. Some inhibitors of the succinate-dependent H_2O_2 generation were described: long chain acyl- CoA (Bortolami et al. 2008), propargylamine containing compounds (Zoccarato et al. 2008) and malate (Muller et al. 2008; Zoccarato et al. 2009).

In this study we investigated the action of TCA cycle substrates (beyond malate) on the succinate promoted H_2O_2 generation. We found that also citrate, isocitrate, α-ketoglutarate and fumarate at physiological concentrations act as inhibitors of H_2O_2 generation. Importantly, when mitochondria are incubated with a mixture of TCA substrates, each present at the concentration recently reported to be present in brain (Kashiwaya et al. 2010), a high overall inhibition level is rapidly obtained, sensitive to small variations of the concentration of each substrate. Ketone bodies, which have been reported to be beneficial in different pathological states, also decrease H_2O_2 production. Specifically, acetoacetate is a strong inhibitor acting rapidly, while βOH, which per se has no activity on H_2O_2 generation, partially removes the effect of acetoacetate. Importantly the inhibition by acetoacetate potentiates the inhibition induced by the TCA cycle substrates. We conclude that the high succinate-dependent production of H_2O_2 is rapidly decreased by other metabolites, each contributing with its concentration to the overall effect. In this way the cell rapidly adjusts H_2O_2 production rate based on changing needs.

Materials and methods

Chemicals and reagents Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was from Molecular Probes. HRP (horseradish peroxidise, grade I, EC 1.11.1.7) and all chemicals were from Sigma.

Animals Wistar albino rats were housed in temperature controlled cages (20–22 °C) fed ad libitum and maintained on a 12:12 light/dark cycle. Study design and experimental protocols of research project were in accord with animal welfare and protection and approved by Veterinary Control Services of Padova University.

Preparation of brain mitochondria Brain mitochondria were isolated from the cerebral cortices of rats (5 weeks to 15 month of age) by discontinuous Ficoll density gradient centrifugation as described in (Zoccarato et al. 2008).

Standard incubation medium Mitochondria (0.2–0.35 mg) were incubated at 35 °C in 125 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 500 mg/ml defatted BSA, 20 mM Na-HEPES pH 7.3 and 100 μM EGTA. Final volume 2.0 ml. Further additions as specified in the figure legends.

H_2O_2 measurement HRP (12.5 μg/ml, 3 Units) was included in the incubation mixture. At 30 s from the addition of mitochondria, Amplex Red (7 μM) was added to initiate the detection of H_2O_2. This was accomplished by monitoring the formation of resorufin, the Amplex Red oxidation product, using excitation and emission wavelengths of 563 and 587 nm, on a Shimadzu RL 5000 spectrofluorometer in a stirred cuvette (Zoccarato et al. 2009). The H_2O_2 calibration curve is linear in the 0–3.5 μM range, and at the end of the

essays, traces were calibrated by addition of 500 p moles of H_2O_2. The calibration was not influenced by the presence of TCA cycle metabolites or ketone bodies. The highest rates of H_2O_2 production were monitored soon after the addition of Amplex Red and continued essentially constant for 2–6 min. These rates were reported.

Mitochondrial membrane potential Mitochondrial membrane potential ($\Delta\psi$) was measured using fluorescence quenching of the cationic dye safranine (3 μM) at 495 nm excitation and 586 nm emission as in (Zoccarato et al. 2008).

Results

TCA cycle metabolites inhibit the succinate dependent H_2O_2 generation It has been reported that the succinate-activated production of H_2O_2 is inhibited by malate (Muller et al. 2008). The effect of malate was ascribed to the formation of OAA, a strong competitive inhibitor of succinate dehydrogenase (Muller et al. 2008). The inhibition was induced very rapidly upon addition of malate (Zoccarato et al. 2009). In the *absence* of malate, we observed that the succinate (0.1–0.2 mM)-dependent production of H_2O_2 was variably increased (by 5–30%), in different mitochondrial preparations, by glutamate (3–5 mM) or pyruvate (2–4 mM). The action of the two metabolites was identical and their effect was not additive. These results are consistent with glutamate and pyruvate acting by removing variable endogenous OAA (by transamination to aspartate or transformation into citrate). Also in the *presence* of added malate, glutamate and pyruvate increased the (lower) release rate, by about the same extent as without malate. We tentatively conclude that the malate inhibition of H_2O_2 release is partly mediated by OAA, and partly appears to be dependent on malate as such.

Succinate and malate are metabolites of the TCA cycle. The TCA cycle is the locus of convergence of the catabolism of glucose, fatty acid and some aminoacids, and of the biosynthesis of glucose (in liver), fatty acid, cholesterol, some aminoacids and heme. It is reasonable to hypothesize that the generation of H_2O_2 induced by succinate may be under the control of the concentration of other TCA cycle substrates beyond malate. Citrate was found to inhibit rapidly, concentration dependently the succinate dependent H_2O_2 production. Similarly behaved isocitrate and α-ketoglutarate. The experiment was performed in the presence of glutamate (3 or 5 mM) to remove interference by endogenous oxaloacetate (Fig. 1A). It is noticeable that the concentration of these three metabolites (i.e. citrate, isocitrate and α-ketoglutarate) was shown to increase jointly upon treatment of perfused heart with insulin or ketones while the succinate concentration decreased (Sato et al. 1995). Malate was the strongest inhibitor (Fig. 1A). Also fumarate gave some inhibition (about 23% at 0.5 mM and 35% at 1 mM) less than citrate (not shown). In vivo, the different substrates are present together, so we investigated the effect of the contemporary presence of the inhibitory metabolites, each present in the most likely in vivo concentration, as recently reported in Kashiwaya et al. (2010). We compared the effect of a mixture (citrate plus isocitrate plus α-ketoglutarate plus fumarate 0.2, 0.01, 0.15, 0,12 mM respectively), malate omitted, and of the complete mixture also containing malate (0.2 mM) (the physiological situation) (Fig. 1B). Then we monitored the effect of increasing to 0.7 mM the citrate and to 0.5 mM the α-ketoglutarate in the mixture (in the absence of malate). The most potent inhibition of H_2O_2 production was obtained with the complete mixture; removing malate decreased significantly the degree of inhibition, but a strong increase of the inhibition level was obtained in the latter condition by increasing citrate and α-KG (to 0.7 and 0.5 mM). It can be concluded that the contemporary presence of all substrates, each present at physiological concentration, inhibits maximally the succinate induced H_2O_2 release, and that increasing slightly the concentration of single metabolites potentiates the overall degree of inhibition. This experiment was performed with 0.2 mM succinate. We report next the effect of the complete of TCA cycle metabolites mixture (the physiological situation) on the succinate titration of H_2O_2 production (Fig. 2). The control succinate titration curve was identical to the one previously published (Zoccarato et al. 2009), only it was moved to slightly lower succinate concentrations, possibly due to more careful removal of endogenous substrates. The control curve of H_2O_2 generation was decreased at all points by the mixture and moved to higher succinate concentrations. The Vmax was also decreased by the mixture.

Acetoacetate is an inhibitor of H_2O_2 release Ketone bodies were repeatedly shown to have protective effects in different pathologies. Ketogenic diets have been since antiquity in the cure of refractory epilepsy. The cerebral symptoms of hypoglycemia in man are prevented by βOH and the post-traumatic metabolism was reported to improve. In rats and mice cerebral function in anorexic animals is improved and survival prolonged (Cahill and Veech 2003; Veech 2004; Maalouf et al. 2009). The toxicity of MPP^+ and β-amyloid protein in neurons was reported to be improved by ketone bodies (Kashiwaya et al. 2000). MPP^+ is a respiratory chain complex I inhibitor which is formed upon usage of bad heroin and causes Parkinson disease (PD)-like symptoms. Rotenone, the classical complex I inhibitor, induces the appearance of PD-like symptoms and oxidative stress (Betarbet et al. 2000). We

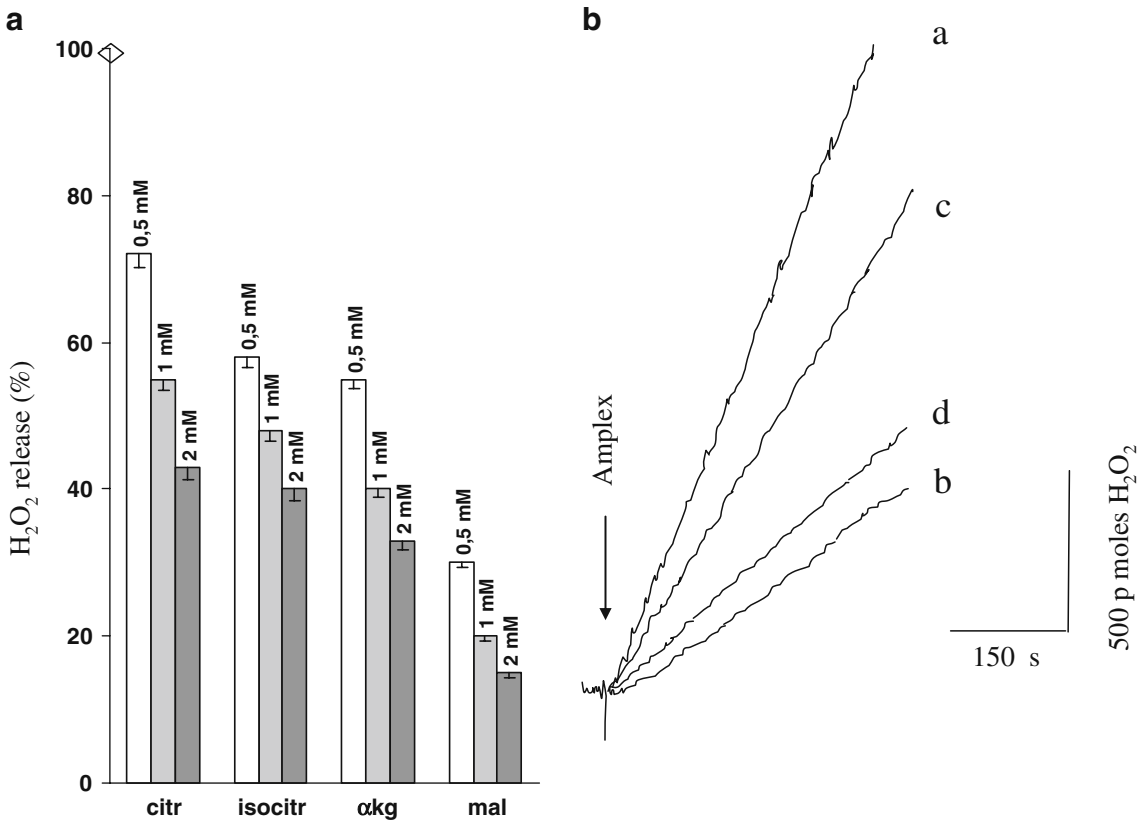

Fig. 1 **A** Inhibition of H_2O_2 release by TCA cycle metabolites. Mitochondria (0.3 mg) were incubated with glutamate (5 mM), HRP and succinate (0.2 mM) without (100% *lozenge*: 1,400±50 p moles H_2O_2/mg/min) or with the indicated concentrations of TCA cycle metabolites: citr, citrate; isocitr, isocitrate; αkg, α-ketoglutarate; mal, malate. Amplex Red was added at 30 s and H_2O_2 production was followed for 3–5 min. Data are the means ± SD (n=3). **B** Inhibition of H_2O_2 release by a physiological mixture of TCA cycle metabolites. The mixture contained (final concentrations) citrate 0.2 mM, isocitrate 0.01 mM, α-ketoglutarate 0.15 mM, fumarate 0.12 mM and malate 0.2 mM. Glutamate was 3 mM and succinate 0.2 mM. Mitochondria 0.2 mg. Amplex Red (Amplex) was added at 30 s. **a** Control, no mixture; **b** complete mixture; **c** mixture minus malate; **d** mixture minus malate, citrate 0.7 mM and α-ketoglutarate 0.5 mM. Traces are representative of duplicate traces from at least four independent experiments

have shown that a probable endogenous PD toxin, dopaminochrome, induces H_2O_2 production in brain mitochondria and the effect is strongly increased by very low rotenone concentrations (Zoccarato et al. 2005). These observations were suggestive of an oxidative origin of PD and were indicating that ketone bodies might have anti-oxidant effects. As mentioned, perfusing hearts with a mixture of AcAc and βOH was accompanied by the contemporary increase of citrate, isocitrate and αKG (but not succinate) three TCA cycle inhibitors of H_2O_2 release (Sato et al. 1995). These acute changes observed in heart were however not reproduced in the brain in a situation of chronic hyperketonemia as obtained upon feeding rats with the ketogenic compound D-βOH-1,3 butanediol mono ester (which also induced the production of malonyl-CoA and uncoupling proteins 4 and 5) (Kashiwaya et al. 2010). DL-βOH is oxidized by brain mitochondria, as monitored by the progressive increase of Δψ with increasing βOH (1–10 mM). βOH did not interfere with the succinate induced peroxide generation (a 10% decrease was observed at 8–10 mM βOH) and by itself it induced a very little H_2O_2 production (not shown). AcAc instead proved to be an inhibitor of H_2O_2 production. A strong inhibition was apparent at less than 0.1 mM AcAc, and the maximal effect was reached at about 0.5 mM. As with the other effectors the inhibition was not complete. Given that βOH is always present together with AcAc, we studied the effect of βOH on the AcAc induced inhibition. The ratio βOH/AcAc is variable, between 1 or less and 4, depending on mitochondrial NADH/NAD. The ratio is lower at low overall ketone bodies concentration, and increases with increasing ketones (Cahill and Veech 2003). βOH partially decreased the inhibition by AcAc, while per se having no effect on peroxide production (Fig. 3). Importantly, the inhibition by AcAc was evident also on top of the inhibition induced by the mixture of inhibitory TCA cycle metabolites. Figure 4A reports the curve of AcAc inhibition (0➔0.1 mM) in the presence of the physiological mixture of TCA metabolites. The inhibition by AcAc was evident at 10–20 μM. The effect of βOH on top of 0.1 mM AcAc is also reported. The reversal of AcAc inhibition was negligible

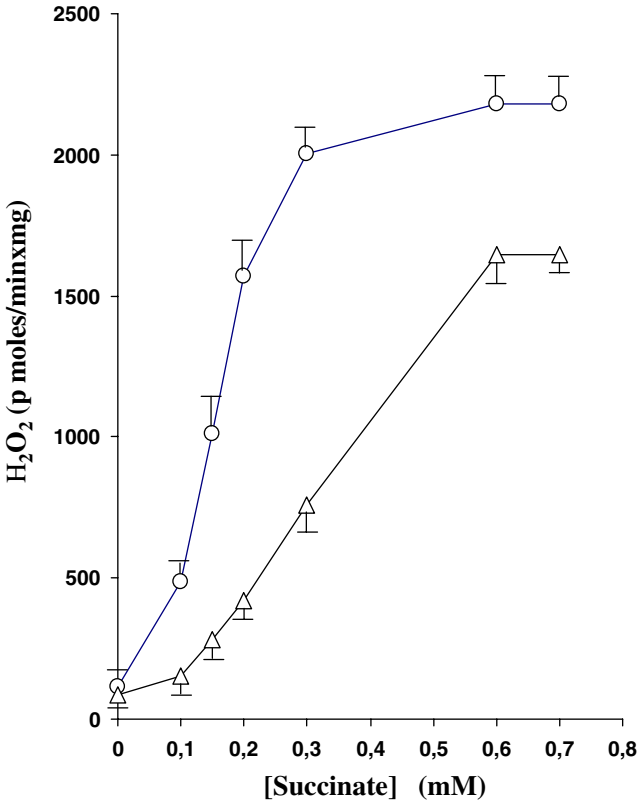

Fig. 2 Succinate titration of H_2O_2 release without and with the physiological mixture of TCA cycle metabolites. Glutamate (3 mM) was present. Succinate was variable as indicated (○). TCA cycle metabolites as in Fig. 1B (Δ). Mitochondria 0.3 mg protein. Data are taken from three independent experiments (two–three points per succinate concentration)

in these conditions. D-L-βOH was added at 1.6 mM, i.e. 8 times the AcAc concentration, given that the active species is presumably D-βOH and that the maximal βOH/AcAc ratio in vivo is 4. Figure 4B reports the inhibition by 0.5 mM AcAc on top of the physiological mixture of TCA cycle metabolites, and the effect of 4 mM D-L-βOH added together with AcAc and the mixture. At this higher AcAc (and βOH) concentration the reversal by βOH was more evident than at lower AcAc. βOH was without effect when added alone to the mixture.

It is remarkable that the steady state inhibition by AcAc, partial removal by βOH, as well as the inhibition by TCA cycle metabolites was attained rapidly, within 30 s, the time between the addition of mitochondria and that of Amplex Red.

Discussion

Much information is now available on how cells and organisms handle ROS. It is known that the respiratory chain dependent H_2O_2 production derives largely from intramitochondrially generated superoxide by the activity of Mn-SOD whose presence is necessary for survival (Li et al. 1995) and whose synthesis and removal are highly controlled (Pani et al. 2009; Ungvari et al. 2009; Sablina et al. 2005). Similarly the H_2O_2 removers GSH peroxidase and catalase are controlled and their activity is related to cell survival (Ungvari et al. 2009; Olmos et al. 2009). Furthermore antioxidant molecules as vitamins C and E, reducing compounds as N-acetyl-cysteine (NAC) are known to have a pro-survival activity, similarly to Nrf2 signalling (which is stimulated by NAC and by lipoic acid as well as by fasting-induced SIRT1 signalling) and whose action is apparently related to increasing GSH concentration (Suh et al. 2004; Ungvari et al. 2010).

On the other hand little is known about how ROS are produced, and how ROS generation is controlled. This process is very important, given that a low level of ROS production will lead to a low steady state ROS concentration, also independently of the removal activity.

Mitochondria are the main cellular site of $O_2^{\bullet -}/H_2O_2$ generation. The ability of succinate to promote H_2O_2 production in the presence of NAD dependent substrates, starting at very low concentrations identifies the succinate

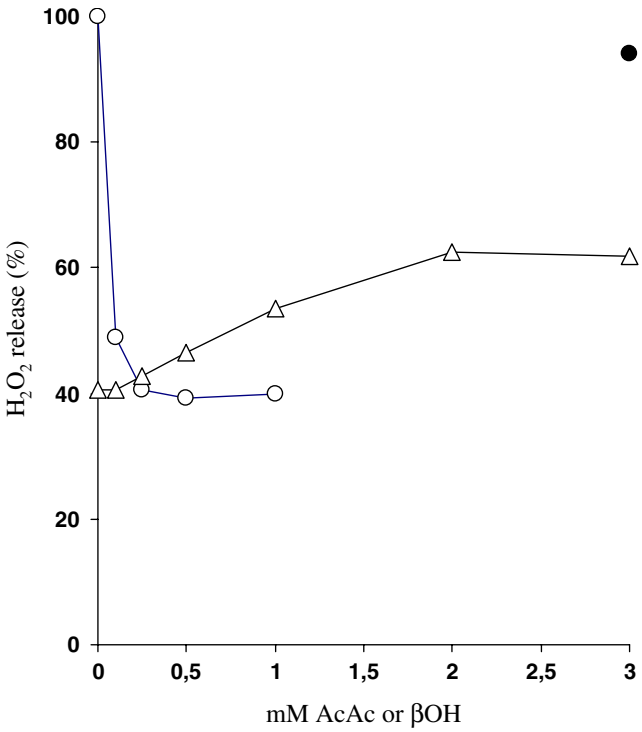

Fig. 3 Acetoacetate inhibition of the succinate induced H_2O_2 release. Effect of DL-β-hydroxybutyrate. Glutamate was 3 mM and succinate 0.2 mM. Mitochondria 0.25 mg protein. Amplex Red added at 30 s. (○) Acetoacetate (AcAc) titration; (Δ) DL-β-hydroxybutyrate (βOH) titration in the presence of 0.25 mM Acetoacetate; (●) βOH alone. 100% was 1,400 p moles H_2O_2/min/mg. Data are taken from one of three independent experiments

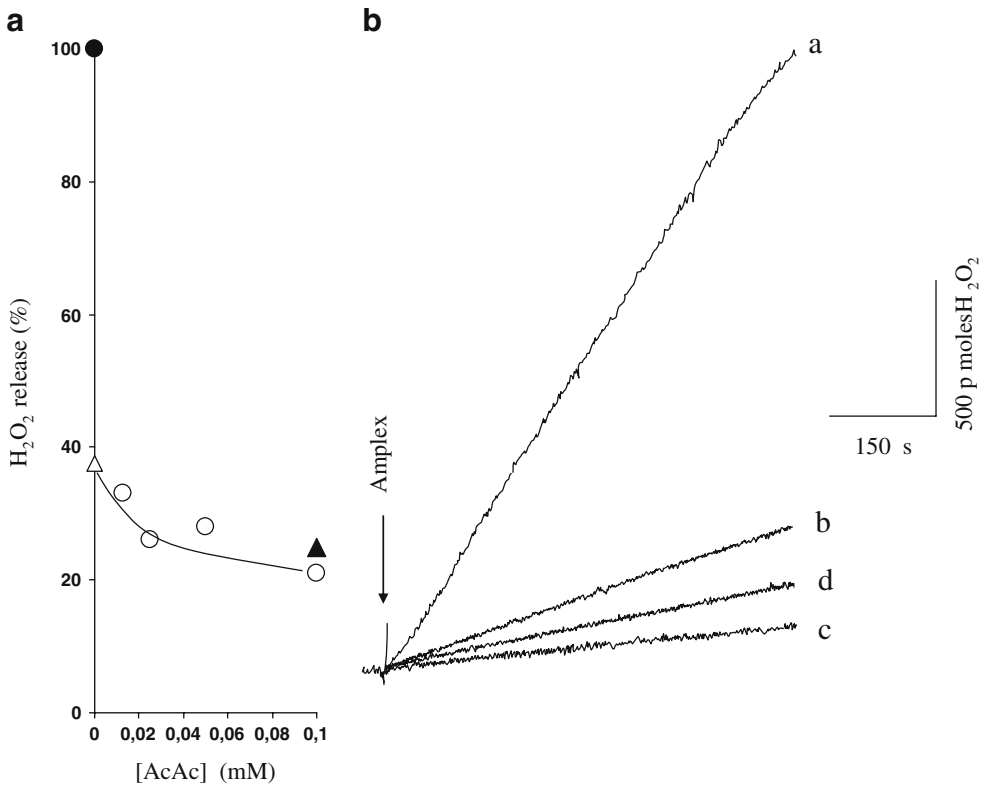

Fig. 4 Acetoacetate inhibition of the succinate induced H_2O_2 release in the presence of TCA cycle metabolites. **A** Glutamate was 3 mM, succinate 0.2 mM alone (●) (1,350 p moles H_2O_2/mg/min) or with the mixture of TCA cycle metabolites as in Fig. 1B (Δ), further containing acetoacetate as indicated (○). Mixture plus Acetoacetate 0.1 mM plus DL-β-hydroxybutyrate 1.6 mM (▲). Data from one of three similar independent experiments. Mitochondria 0.25 mg. **B** Traces: **a** glutamate (3 mM) and succinate (0.2 mM); **b** same as a), with the TCA cycle substrates mixture as in Fig. 1B; **c** mixture plus acetoacetate (0.5 mM); **d** mixture plus acetoacetate plus DL-β-hydroxybutyrate 4 mM. Traces are representative of duplicate traces from at least three independent experiments

induced peroxide generation as an intrinsic property of mitochondria. It is sigmoidal with succinate concentration, it reaches a high rate (2.0–2.2 nmoles H_2O_2/min/mg at 600 μM succinate) and the half maximal rate is at approximately 150 μM succinate. The higher half-maximal rate previously reported (Zoccarato et al. 2009) may be related to less careful removal of endogenous substrates. The physiological succinate concentration in brain has recently been reported to be approximately 100 μM (Kashiwaya et al. 2010). It may be questioned why mitochondria should release high amounts of H_2O_2 continuously and apparently without control, apart from the decreased rate observed upon addition of ADP (state 3 respiration) or coupled to the activity of uncoupling proteins, agents that decrease mitochondrial membrane potential. In this study we identified the inhibitory action on H_2O_2 release exerted by a number of cellular metabolites which are capable to concentration-dependently decrease the H_2O_2 output. Several TCA cycle metabolites rapidly induce the generation of an inhibited state of peroxide release at a given succinate concentration, each contributing by its concentration. In this way the cell can rapidly adjust the level of H_2O_2 production to changing necessities. We have shown previously that also long chain acyl-CoAs (non TCA metabolites) participate in this control (Bortolami et al. 2008). Another non TCA cycle inhibitor is AcAc. This substance acts at very low concentration and its effect is partially removed (at higher concentrations) by the other ketone body βOH. AcAc strongly and rapidly reinforces the inhibited state of H_2O_2 release induced by the physiological concentration of TCA substrates. It is significant that ketones have been recognized as probably a "good medicine" for a series of pathological states (Cahill and Veech 2003). Ketones counteract the toxicity of MPP^+, a toxin produced in bad heroin preparations which gives symptoms closely related to PD. MPP^+, similarly to rotenone, is an inhibitor of complex I of the mitochondrial respiratory chain. It was reported that rotenone induces a state of ROS hyperproduction which may be involved in the pathogenesis of PD (Betarbet et al. 2000). The endogenously produced toxin dopaminochrome activates the production of H_2O_2 in brain mitochondria, which is strongly stimulated by very low concentrations of rotenone (Zoccarato et al. 2005). For these reasons the inhibitory action of AcAc on H_2O_2 production may be the basis for pharmacological interventions in PD; and possibly also in Alzheimer's disease, which also appears to depend on excessive ROS production. It is remarkable that quite low (less than 20 μM) AcAc levels (approximating the levels present physiologically in blood in the fed state) appear sufficient to initiate the inhibition of H_2O_2 release.

It is to mention that an increase in ketone bodies is observed in man (particularly young men) after only a few hours of starvation (Cahill 2006), i.e. this is an early effect of food deprivation. CR is the only procedure available nowadays to delay aging, decrease neurodegenerative disease and cancer (Masoro 2000). Increased ketones were reported in limited daily feeding (LDF) (Mahoney et al.

2006) and intermittent fasting (IF) (i.e. 1 day of AL followed by 1 day of fasting) (Anson et al. 2003), the two CR regimens. In one study (Anson et al. 2003) the increase in ketones was however not reported in LDF, while in another a strong increase in βOH was found in the same strain of mice (Mahoney et al. 2006). The measurements were however performed in blood taken at different times after feeding. It may be concluded that during CR a state of increased ketonemia is probably invariably induced, that will decrease the mitochondrial H_2O_2 output. In both IF and LDF regimens a strong decrease of blood glucose and insulin is invariably observed (Anson et al. 2003). Insulin is an activator of $O_2^{\bullet -}/H_2O_2$ release, via activation of a cytosolic NADPH oxidase activity (Nox 4) in fat and endothelial cells (Goldstein et al. 2005). A decreased insulin concentration will determine lower H_2O_2 release. Furthermore it was recently reported that the putative Sirt 1 activator resveratrol inhibits NADPH oxidase activity (Akar et al. 2011) thus presumably decreasing insulin induced H_2O_2 production. Sirt 1 synthesis and activation are observed in the fasting state (Nisoli et al. 2005). High blood glucose concentrations induce ROS production in mitochondria (Brownlee 2001; Mukhopadyay et al. 2007). Interestingly it was shown that in diabetic rats hyperglycemia promotes a strong increase of cellular succinate in kidney (responsible for the diabetic increase of renin secretion) (Toma et al. 2008). The higher succinate will in turn increase mitochondrial H_2O_2 output. The latter situation will be less easily controlled by TCA cycle metabolites inhibitors (Fig. 2). This is likely the situation occurring in diabetes.

It is conceivable that the increase of ketone bodies and decrease of both glucose and insulin as observed in CR will determine a decrease of H_2O_2 production, probably responsible for the beneficial effects of CR. We are currently studying the properties of succinate induced H_2O_2 release in IF animals. After 15 weeks of treatment no differences with AL feed rats have appeared (data to be published elsewhere).

It is possible that other negative controllers of succinate dependent H_2O_2 production will be found. Succinate concentration appears to be variable physiologically (also independent of glucose concentration) and its concentration has been found to increase in hypoxic conditions (Taegtmeyer 1978) and during physical exercise (Hochachka and Dressendorfer 1976). A high succinate promotes high H_2O_2 release which is probably important in neovascularization and regeneration. A succinate specific 7TM receptor (GPR91) has recently been described, which seems implicated in these processes (He et al. 2004; Hakak et al. 2009).

We have previously identified also the existence of a positive control system of succinate-dependent H_2O_2 generation, linked to a decrease of electron flow in cytochrome c oxidase, as induced by $NO^{\bullet}$ or CO (Zoccarato et al. 2009).

The mechanistic explanation for these findings may be only tentative. Some facts are to be taken into account anyway: 1) low concentrations of succinate (about 15 μM) suffice to initiate H_2O_2 production in the presence of complex I substrates (glutamate plus pyruvate), i.e. in an apparently physiological situation (Zoccarato et al. 2009); 2) in similar conditions, during H_2O_2 production the downhill flow of electrons in complex I is slowed down, but not halted (Zoccarato et al. 2007); 3) in brain the ratio of mitochondrial NAD/NADH has been recently shown to be 1.4–1.6, values that do not support a situation of reverse electron transfer (Kashiwaya et al. 2010); 4) rotenone inhibits the succinate induced H_2O_2 release, implicating the intervention of complex I in the process. Succinate appears to activate electron transfer from a donor in complex I or complex II to O_2 (forming $O_2^{\bullet -}$). The nature of the electron donor is presently uncertain. One possibility is the autooxidation of a CoQ semiquinone. Another possibility is the auto-oxidation of an iron sulphur center. Center N1a of complex I has been shown to be capable of reducing O_2 (Kushnareva et al. 2002). It is so negative however (−392 mV) (Ohnishi 1975) no to be easily reduced by physiological NADH concentrations. It may be suggested that coupled succinate oxidation increases the reducibility of centre N1a (i.e. that succinate increases its redox potential). The regulation if complex II is complex. Several metabolites have been shown to interact with it. OAA was shown to be tightly bound to complex II. However the (almost irreversible) binding appears to take place during the process of isolation, linked to some conformational change (Maklashina et al. 2004). The site that binds OAA also binds other species (fumarate and malate) (Huang et al. 2006). Succinate oxidation is inhibited by citrate and βOH apparently by direct interaction with complex II (Hillar et al. 1975). αKG stimulates succinate oxidation in the presence of rotenone (Hillar et al. 1975). These interactions might mediate the inhibition by TCA cycle metabolites, and possibly by AcAc, of succinate-activated H_2O_2 release.

Acknowledgments This work was supported by "Finanziamenti di Ateneo", ex 60% funds.

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