Succinate modulation of H_2O_2 release at NADH:ubiquinone oxidoreductase (Complex I) in brain mitochondria

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Complex I (NADH:ubiquinone oxidoreductase) is responsible for most of the mitochondrial H_2O_2 release, both during the oxidation of NAD-linked substrates and during succinate oxidation. The much faster succinate-dependent H_2O_2 production is ascribed to Complex I, being rotenone-sensitive. In the present paper, we report high-affinity succinate-supported H_2O_2 generation in the absence as well as in the presence of GM (glutamate/malate) (1 or 2 mM of each). In brain mitochondria, their only effect was to increase from 0.35 to 0.5 or to 0.65 mM the succinate concentration evoking the semi-maximal H_2O_2 release. GM are still oxidized in the presence of succinate, as indicated by the oxygen-consumption rates, which are intermediate between those of GM and of succinate alone when all substrates are present

together. This effect is removed by rotenone, showing that it is not due to inhibition of succinate influx. Moreover, α-oxoglutarate production from GM, a measure of the activity of Complex I, is decreased, but not stopped, by succinate. It is concluded that succinate-induced H_2O_2 production occurs under conditions of regular downward electron flow in Complex I. Succinate concentration appears to modulate the rate of H_2O_2 release, probably by controlling the hydroquinone/quinone ratio.

Key words: brain mitochondrion, electron transfer, hydrogen peroxide production, NADH:ubiquinone oxidoreductase (Complex I), reactive oxygen species, succinate.

INTRODUCTION

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

Mitochondrial Complex I (NADH:ubiquinone oxidoreductase) is the most complicated and least understood electron carrier in the respiratory chain. Its primary function is to pump protons across the inner membrane. A second property of Complex I is a highly modulated O_2^- production. O_2^- from Complex I is generated on the matrix side of the inner membrane [1] and is transformed into H_2O_2 by mitochondrial MnSOD (manganese superoxide dismutase). The highly permeable H_2O_2 is the measured species in the extramitochondrial space. The importance of ROS removal from the mitochondrial matrix is demonstrated by the poor survival of MnSOD-null mice [2] and by the increased lifespan of mice expressing mitochondrial catalase [3]. Complex III is also known to produce O_2^- (mainly on the cytosolic space). However this O_2^- release is largely dependent on the presence of the electron transfer inhibitor antimycin A [4].

ROS have been considered important in various pathologies [5] and aging [6]. More recently, however, it has been recognized that ROS have important physiological roles as signalling molecules whose functions are just emerging [7]. Complex I is now considered to be the major site of O_2^-/H_2O_2 production.

H_2O_2 is generated during coupled respiration of NAD-linked substrates (the release is increased by the electron transfer inhibitor rotenone); it is also generated, at a much higher rate, during coupled succinate respiration (production is strongly inhibited by rotenone and by decreasing membrane potential). These properties are taken as evidence that succinate-dependent

H_2O_2 generation occurs in Complex I upstream of the rotenone inhibition site (i.e. via energy-dependent reverse electron transfer from Complex II to Complex I [8–13]). We have shown that the Parkinson's disease toxin dopaminochrome (derived from dopamine) promotes a strong increase of H_2O_2 production both with GM (glutamate/malate) and in the further presence of succinate [14], and that H_2O_2 is removed by GSH peroxidase [15]. The succinate-dependent H_2O_2 production is controlled by ΔpH (mitochondrial pH gradient) across the inner membrane and is depressed when such gradient is abolished [16]. The physiological importance of succinate-sustained H_2O_2 generation, being linked to a situation of reverse electron transfer, has been considered dubious [9–13,16,17].

In the present study, we analysed the production of H_2O_2 in brain mitochondria in the presence of both the NAD-linked substrates GM (or pyruvate/malate, which gave the same results) and succinate, i.e. close to *in vivo* conditions. We show that succinate-dependent H_2O_2 production initiates at low physiological concentrations and is only marginally modified by the presence of GM. Furthermore, GM oxidation is progressively decreased, but not abolished, by succinate and the two substrates appear to compete for electron transfer.

We propose that the sharp dependence of the H_2O_2-generation rate on succinate concentration is an important modulator in the overall cellular ROS production.

MATERIALS AND METHODS

Reagents

Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was from Molecular Probes. HRP (horseradish peroxidase) (grade I; EC
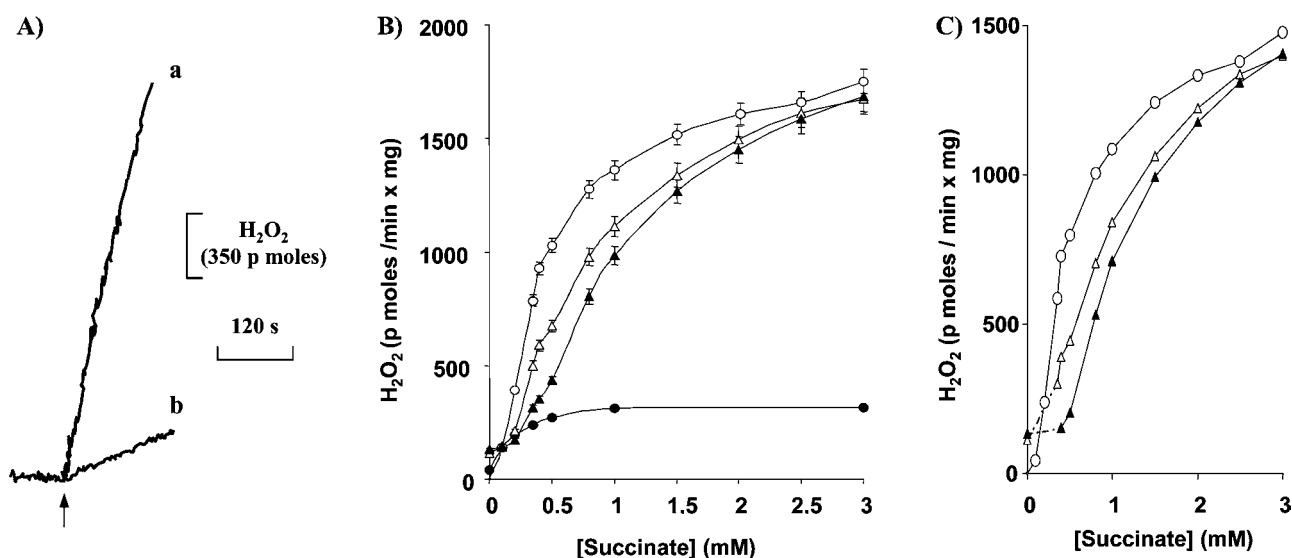

Figure 1   Titration of succinate-promoted H_2O_2 generation

(**A**) Different rates of H_2O_2 production are elicited by GM and succinate. Succinate (trace a) and GM (trace b) were 1 mM. Mitochondria (0.75 mg/1.6 ml) were incubated in standard incubation medium containing HRP. Amplex Red was added at the time indicated by the arrow. (**B**) H_2O_2 production was measured in the absence (○) and presence of GM, 1 mM (△) or 2 mM (▲) each. Where present, rotenone was 2.5 μM (●) without GM. (**C**) Net H_2O_2 releases calculated by subtracting the rotenone rates from the rates in the absence of rotenone in (**B**). Traces in (**A**) are representative of duplicate traces from at least seven independent experiments. Results in (**B**) are means $\pm$ S.D. for at least five independent experiments.

1.11.1.7), catalase from bovine liver (EC 1.11.1.6) and α-KG [α-oxoglutarate (α-ketoglutarate)] dehydrogenase from pig heart (EC 1.2.4.2) were supplied from Sigma. All other reagents were of analytical grade.

Preparation of rat brain mitochondria

Brain mitochondria were isolated from cerebral cortices of 6–7-week-old rats as described in [15].

Standard incubation method

Mitochondria (0.4–0.6 mg/ml) were incubated at 30 °C in 125 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 500 μg/ml defatted BSA, 20 mM Mops, pH 7.2 (adjusted with KOH), and 100 μM EGTA. Further additions were as specified in the Figure legends.

H_2O_2 measurements

H_2O_2 was measured with 7 μM Amplex Red and 15 μg/ml HRP (3.75 units) included in the incubations. H_2O_2 was detected by the formation of the fluorescent Amplex Red oxidation product resorufin using excitation and emission wavelengths of 563 and 587 nm respectively on a Shimadzu RL-5000 spectrofluorimeter in a stirred cuvette [14]. H_2O_2 release was linear for at least 6–8 min. The H_2O_2 calibration scale is linear in the 0–5 μM range, and, at the end of each assay, traces were calibrated by the addition of H_2O_2 (500 pmol).

Assay of α-KG

The α-KG assay was performed as in [18,19]. Briefly, mitochondria (4 mg/ml) were incubated in the standard incubation medium with agitation for 10 min with the indicated amount of substrates. During incubation, substrate concentrations of glutamate, malate and succinate were kept constant by addition of 0.3 mM GM and 0.5 mM succinate at 5 min. Reactions were stopped by addition of 12 % cold PCA (perchloric acid), and samples were left on ice for 20 min before centrifugation at 5000 g for 5 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 sodium phosphate buffer, pH 7.3, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM EGTA, 1 mM NAD^+ and 0.2 mg/ml (160 m-units) of α-KG dehydrogenase in a Fluoroskan plate-reader at 355 nm excitation and 460 nm emission. 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 nmol/min per mg of protein. Similar results were obtained by spinning-down mitochondria at the end of incubation and measuring α-KG in the PCA-treated supernatant. Also, including catalase (5 μg/ml) in the incubation medium was without any significant effect on α-KG accumulation.

Other assays

$\Delta\Psi$ (mitochondrial membrane potential) was measured using fluorescence quenching of the cationic dye safranine (3 μM) at 495 nm excitation and 586 nm emission as in [15]. NAD(P) reduction was monitored fluorimetrically at 348 nm excitation and 464 nm emission as in [15]. Mitochondrial respiration was monitored in the presence of HRP and Amplex Red with a Clark-type oxygen electrode in a 1.6 ml closed chamber with continuous stirring.

RESULTS AND DISCUSSION

Complex I-dependent H_2O_2 release with succinate and GM

Figure 1(A) shows the high H_2O_2 production rate in brain mitochondria oxidizing succinate (trace a) and the much slower H_2O_2 generation with GM as the substrates (trace b). The production of H_2O_2 as a function of succinate concentration, in the absence and presence of GM, is shown in Figure 1(B). The maximal H_2O_2 release (with 3 mM succinate) was unaffected by GM. The half-maximal H_2O_2 production with succinate alone was at approx. 0.35 mM. With GM (1 mM each), a concentration

**Table 1   O$_2$ consumption rate measured in mitochondria oxidizing different respiratory substrates**

Mitochondria (0.9 mg/1.6 ml) were incubated in standard medium supplemented with Amplex Red and HRP, and respiration rates were measured. GM (1 mM each), succinate (0.5 mM) and rotenone (1 μM) were present or added as indicated. Results are means $\pm$ S.D. for at least seven independent experiments.

Substrate	Rate of O$_2$ consumption (n-atoms/min per mg)
GM	10.5 $\pm$ 2.5
GM + succinate	16.8 $\pm$ 2.0
Succinate	28.0 $\pm$ 3.0
Succinate + GM	17.5 $\pm$ 1.2
Rotenone/GM/succinate	27.5 $\pm$ 2.0

Figure 2   Succinate does not prevent GM oxidation

Mitochondria (0.9 mg/1.6 ml) were incubated as in Table 1 with variable succinate and constant GM (1 mM each). Results are for succinate alone ($\bigcirc$) and succinate in the presence of GM ($\square$). Results are means $\pm$ S.D. for at least five independent experiments.

which produced maximal Δp (mitochondrial protonmotive force), the H$_2$O$_2$ release at zero succinate was approx. 0.15 nmol/min per mg of protein, and the further addition of succinate promoted a sharp increase of H$_2$O$_2$ production, with a half-maximal stimulation at approx. 0.5 mM. A similar behaviour was obtained at higher (2 mM) GM. In this situation, the half-maximal stimulation of H$_2$O$_2$ release by succinate was increased to 0.6–0.65 mM. The rotenone-inhibited H$_2$O$_2$-production rates with succinate are also shown in Figure 1(B). It is likely that this residual H$_2$O$_2$ release also originated from Complex I, since succinate promoted a large NAD(P) reduction in the presence of rotenone, probably via malate dehydrogenase. Similar results were obtained with piericidin (results not shown). According to the hypothesis that H$_2$O$_2$ generation with succinate and rotenone does not originate from Complex I, these rates were subtracted from the succinate and succinate/GM values of Figure 1(B) and are reported in Figure 1(C). They certainly represent Complex I-dependent H$_2$O$_2$ release with succinate and GM.

Succinate does not prevent electron flow from GM in Complex I

The generally accepted view is that succinate promotes H$_2$O$_2$ release by pushing electrons back into Complex I in a reverse mode of electron transfer. With NAD-linked substrates and in the absence of succinate, H$_2$O$_2$ release is slow and can be increased in a non-physiological situation, i.e. in the presence of rotenone-type electron transfer inhibitors and high ΔpH [17]. Alternatively, H$_2$O$_2$ release was reported to be increased in cytochrome c-depleted mitochondria [20]. The findings reported in the present study show that low succinate promotes H$_2$O$_2$ release also in the presence of high NAD-linked substrates. It can be questioned whether succinate in these conditions operates a 'reverse' mode of electron flux, i.e. whether the oxidation of GM is prevented. To solve this problem, first we analysed the rates of O$_2$ consumption with GM (1 mM each), succinate and a mixture. Typical results are reported in Table 1, which shows the experiment with 0.5 mM succinate (a concentration that promotes a large H$_2$O$_2$ release). The oxidation rate was higher with succinate than with GM, probably reflecting the lower number of protons pumped by succinate, which does not feed electrons to Complex I. However, the O$_2$ consumption rate with the mixture was intermediate between that of the two constituents alone, a possible indication that both succinate and GM were contributing to O$_2$ consumption when they were present together. In the presence of rotenone, the O$_2$-consumption rate with succinate was not significantly affected by GM, showing that there was no limitation to succinate availability. The overall titration of O$_2$ consumption with increasing succinate, in the absence and presence of constant (1 mM) GM
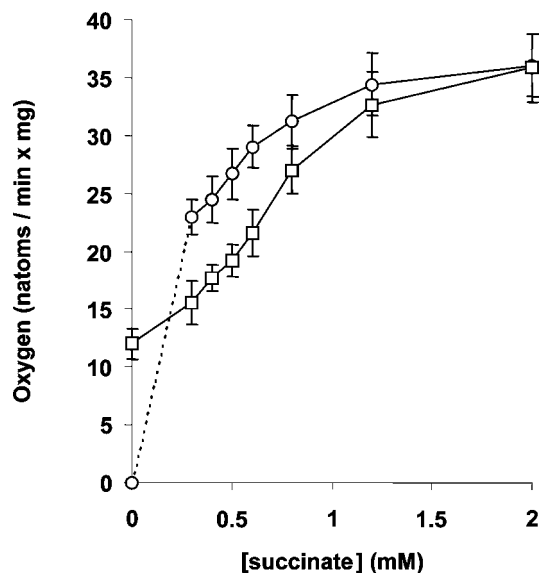
is reported in Figure 2. Above 0.2 mM, the succinate oxidation rate was higher than that of GM, and the addition of GM induced a decrease of O$_2$ consumption to intermediate values. Only above approx. 1.5 mM succinate, GM became incapable of decreasing the succinate oxidation rate. These results confirm that a fraction of the overall oxidation rate was indeed dependent on GM when both GM and succinate were supplied to mitochondria.

Although these results provide strong evidence in favour of the hypothesis that electrons actually flow downwards in Complex I also during the succinate-promoted H$_2$O$_2$ release in the presence of GM, they are not a direct demonstration that this really occurs. As a second approach to prove this point, we measured the generation of α-KG with GM in the absence and presence of succinate. The accumulation of α-KG is connected directly to the oxidation of GM, via glutamate dehydrogenase, or via glutamate/OAA (oxaloacetete) transaminase, where OAA originates from malate oxidation. As shown in Figure 3, α-KG accumulated during the oxidation of GM, but not during succinate oxidation. With GM, the further presence of succinate progressively decreased, but did not abolish, α-KG production. At 1 mM succinate, α-KG production was still 30% that of the control without succinate. These results are in agreement with the data on respiration presented above.

These experiments demonstrate conclusively that electrons keep flowing down from Complex I in the presence of succinate and during the process of succinate-supported H$_2$O$_2$ production.

H$_2$O$_2$ release is controlled by ΔpH

The succinate-induced H$_2$O$_2$ release is dependent on Δp across the inner mitochondrial membrane. However, Δp is the sum of two components, $\Delta\Psi$ and ΔpH. It has been shown previously that ΔpH is a major controller of succinate-dependent H$_2$O$_2$ production [16]. In fact, the inclusion of the K$^+$/H$^+$ ionophore nigericin (in a high K$^+$ medium) allows K$^+$ equilibration and hence H$^+$ equilibration (ΔpH = 0); this results in a corresponding equivalent increase of $\Delta\Psi$ to maintain Δp constant ([16], and results not shown). Under these conditions, the succinate
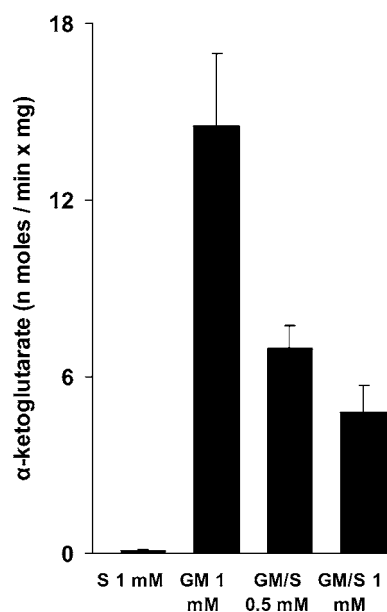
Figure 3 α-KG is produced during coupled oxidation of GM and succinate

Mitochondria (4 mg/ml) were incubated for 10 min with 1 mM GM and succinate (S) as indicated. Extra GM (0.3 mM each) and succinate (0.5 mM) were added at 5 min. Results are means ± S.D. for duplicate incubations from five preparations.
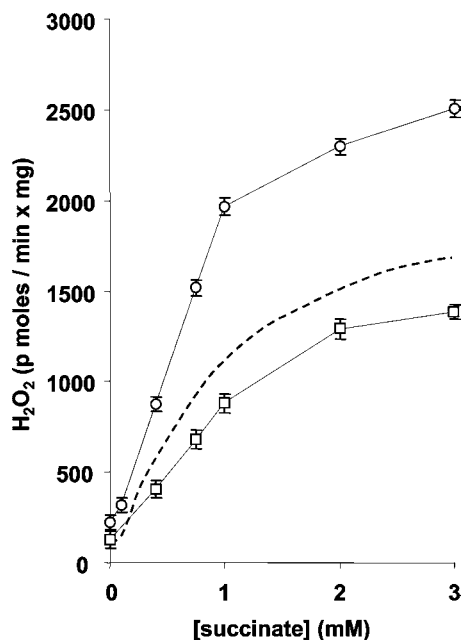

Figure 4 H_2O_2 release rates are controlled by ΔpH

Conditions as in Figure 1 with 1 mM GM. The titration was performed in the presence of 100 nM nigericin (□) or in the absence of phosphate (○). The broken line represent values from Figure 1(B) (trace in the presence of 1 mM GM). Results are means ± S.D. for at least four independent experiments.

promoted H_2O_2 release is at its lowest. The succinate titration of H_2O_2 release in the presence of nigericin and GM (Figure 4) shows that the overall production rate was depressed relative to that shown in Figure 1(B) (broken line), but that the pattern was unchanged. On the contrary, increasing ΔpH by omitting

phosphate (whose influx via the P_i–H^+ symporter decreases ΔpH) promoted a higher succinate-dependent H_2O_2 release [16], without changing the general behaviour in the presence of GM (Figure 4).

Conclusions

The results of the present study show that the succinate stimulation of mitochondrial H_2O_2 release occurs at low concentrations and that the presence of GM modifies only marginally the succinate promotion of H_2O_2 release. Furthermore, GM oxidation is decreased, but not prevented, by succinate.

When both NADH and succinate are present (the *in vivo* situation), it appears that they compete for Complex I (or a part of it), the first pushing electrons down and the second pushing electrons up into the complex. The two processes appear to occur at the same time, possibly due to competition between regular quinone reduction from NADH and re-binding of the reduced species due to its higher concentration during the operation of Complex II. Electron backflow with succinate could mean just binding of QH_2 (hydroquinone) originating from Complex II to the Q (quinone)-binding site on Complex I with O_2^- generation occurring right there (as speculated in [17]), instead of electrons going uphill to a different site of O_2^- production. In this view, the fact that GM are still oxidized in the presence of succinate does not contradict any conclusions drawn previously.

The high mitochondrial QH_2/Q ratio induced by succinate appears to be the major determinant of H_2O_2 release. Similarly, GM alone induce high H_2O_2 release rates in cytochrome *c*-depleted mitochondria [20]. In the latter situation, the removal of cytochrome *c* decreases the rate of electron flow and consequently increases QH_2/Q. A similar effect could occur following NO^- inhibition of cytochrome oxidase [21,22].

Succinate concentration *in vivo* is controlled by the activity of the other dehydrogenases, which are NAD-dependent. It is significant that *in vivo* succinate concentration oscillates between 0.3 and 1 mM, as determined by Sato et al. [23] in perfused heart. Preliminary results appear to indicate a similar organization of H_2O_2 release in heart mitochondria.

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