



Reactive oxygen species production induced by pore opening in cardiac mitochondria: The role of complex II

Received for publication, November 16, 2016, and in revised form, April 14, 2017. Published, Papers in Press, April 27, 2017, DOI 10.1074/jbc.M116.768325

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Edited by John M. Denu

Succinate-driven reverse electron transport (RET) through complex I is hypothesized to be a major source of reactive oxygen species (ROS) that induces permeability transition pore (PTP) opening and damages the heart during ischemia/reperfusion. Because RET can only generate ROS when mitochondria are fully polarized, this mechanism is self-limiting once PTP opens during reperfusion. In the accompanying article (Korge, P., Calmettes, G., John, S. A., and Weiss, J. N. (2017) *J. Biol. Chem.* 292, 9882–9895), we showed that ROS production after PTP opening can be sustained when complex III is damaged (simulated by antimycin). Here we show that complex II can also contribute to sustained ROS production in isolated rabbit cardiac mitochondria following inner membrane pore formation induced by either alamethicin or calcium-induced PTP opening. Two conditions are required to maximize malonate-sensitive ROS production by complex II in isolated mitochondria: (a) complex II inhibition by atpenin A5 or complex III inhibition by stigmatellin that results in succinate-dependent reduction of the dicarboxylate-binding site of complex II (site II_f); (b) pore opening in the inner membrane resulting in rapid efflux of succinate/fumarate and other dicarboxylates capable of competitively binding to site II_f. The decrease in matrix [dicarboxylate] allows O₂ access to reduced site II_f, thereby making electron donation to O₂ possible, explaining the rapid increase in ROS production provided that site II_f is reduced. Because ischemia is known to inhibit complexes II and III and increase matrix succinate/fumarate levels, we hypothesize that by allowing dicarboxylate efflux from the matrix, PTP opening during reperfusion may activate sustained ROS production by this mechanism after RET-driven ROS production has ceased.

Following prolonged myocardial ischemia, reperfusion results in the generation of excessive reactive oxygen species

This work was supported by National Institutes of Health Grants R01 HL101228 and R01 HL117385 from the NHLBI, American Heart Association Western States Affiliate Post-doctoral Research Fellowship 11POST6110007, American Heart Association Scientist Development Grant 16SDG31180036, and the Laubisch and Kawata Endowments. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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(ROS)² from mitochondria that contribute to reperfusion injury. A recent metabolomics study demonstrated that succinate accumulation during ischemia correlated strongly with ROS production and injury during reperfusion (1). The authors proposed that the major source of damaging ROS under these conditions was succinate-driven reverse electron transport (RET) through complex I (2). Although RET is known to generate copious ROS, this is possible only when $\Delta\Psi$ is fully polarized (3–5). This raises the issue that mitochondria, which are depolarized during prolonged ischemia, would have to recover $\Delta\Psi$ fully during early reperfusion to generate ROS by this mechanism, which would then cease once ROS production induced PTP opening. Under conditions in which $\Delta\Psi$ either does not recover (6, 7) or recovers only slowly and partially (8–10) upon reperfusion as a result of PTP opening (11) and increased inner membrane leakiness (12–14), ROS production by RET would be self-limited. We therefore examined whether additional mechanisms might come into play to sustain succinate-driven ROS production after PTP opening has occurred, consistent with experimental evidence that ROS production increases simultaneously with PTP opening (15). In the accompanying paper (52), we showed that when ischemic injury to complex III is mimicked with antimycin, inner membrane permeabilization using either alamethicin or calcium-induced PTP opening stimulated robust ROS production by complex III by allowing Mg²⁺ influx into the matrix to activate malic enzyme 2 (ME2). Using malate derived from endogenous succinate and fumarate, ME2 reduced NAD⁺ to provide the reducing equivalents for ROS production by antimycin-inhibited complex III. In this study, we report that when the inner membrane is permeabilized, complex II can also serve as a robust source of ROS production after either inhibition of ubiquinone (Q) reduction in complex II or ubiquinol (QH₂) oxidation in complex III, providing another potential mechanism for succinate-driven ROS production during and after PTP opening.

Mitochondrial complex II (succinate dehydrogenase (SDH)) is responsible for succinate oxidation into fumarate in the TCA cycle, coupled to Q reduction to QH₂ and respiratory chain activity. Besides this main function, complex II is known to produce ROS directly from the reduced flavin site called site II_f

² The abbreviations used are: ROS, reactive oxygen species; AA5, atpenin A5; CoA, coenzyme A; CsA, cyclosporin A; ME2, malic enzyme; $\Delta\Psi$, mitochondrial membrane potential; PTP, permeability transition pore; RET, reverse electron transport; Q, ubiquinone; QH₂, ubiquinol; SDH, succinate dehydrogenase; Suc, succinate; TCA, tricarboxylic acid; OAA, oxaloacetate; SMP, submitochondrial particle; I/R, ischemia/reperfusion.

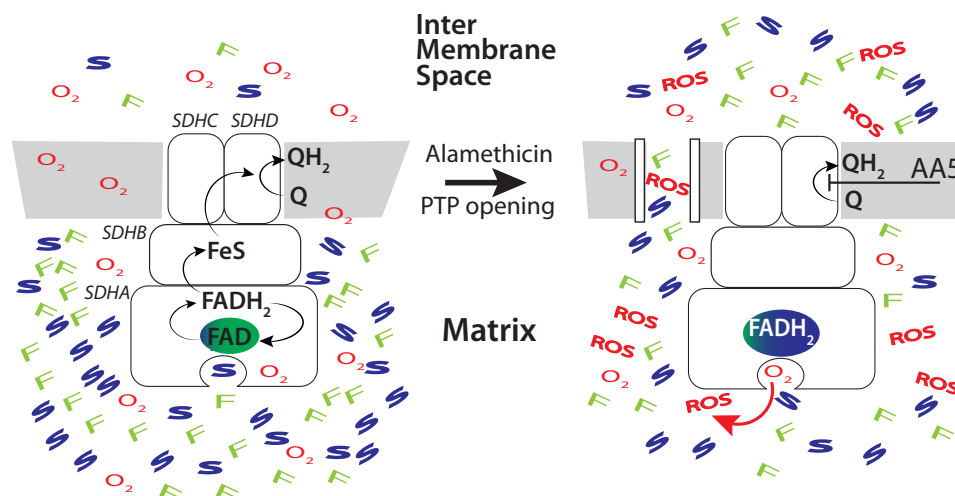


Figure 1. Proposed mechanism of increased ROS production by inhibited complex II following PTP opening. *Left panel*, under physiological conditions succinate (S) oxidation by complex II (succinate dehydrogenase composed of four subunits SDHA–D) results in flavin site reduction (FADH₂ generation) and further tunneling of electrons via Fe-S clusters to ubiquinone (Q) in the Q-binding site that generates ubiquinol (QH₂) by two sequential single electron transfers, requiring also two protons. QH₂ is further oxidized by complex III. No ROS are generated under those conditions because the flavin site is rapidly oxidized if there is no downstream inhibition, and dicarboxylates such as succinate and fumarate (F) shield access of O₂ to their binding site. *Right panel*, when electron transport is inhibited at the level of Q reduction by AA5 or further downstream, FADH₂ accumulates. After PTP opening in the inner membrane, matrix succinate/fumarate levels decrease, giving O₂ access to reduced flavin site to generate ROS.

(16) in the presence of low levels of dicarboxylates, as shown first by Imlay (17) in inverted respiratory vesicles from *Escherichia coli*. Subsequent studies by several groups have demonstrated ROS production by SDH-containing membranes prepared from *E. coli* strain KM5 (18), submitochondrial particles (SMP) from beef heart mitochondria (16), or mitochondria isolated from skeletal muscle (19). With electrons supplied by succinate, ROS production by complex II can be stimulated by the complex II inhibitor atpenin A5 (AA5) or by the complex III inhibitor stigmatellin, which blocks QH₂ oxidation by complex III. Conversely, ROS production by complex II is inhibited by malonate, consistent with ROS arising from the fully reduced flavin site II_f (16), although alternative ROS-generating sites have also been proposed (20). Brand and co-workers (19) have shown that H₂O₂ production increases with [succinate] up to 400 μM, but thereafter it significantly decreases at the millimolar [succinate] commonly used to energize mitochondria. This is because complex II produces ROS only when site II_f is unoccupied by dicarboxylates (16, 19). Succinate and other TCA intermediates (fumarate in particular) that bind to the dicarboxylate-binding site can restrict O₂ access to this site and thereby suppress ROS production by complex II. Because ischemia/hypoxia are known to significantly increase [succinate] and [fumarate] in the matrix (1, 21), this may prevent significant ROS production from complex II simply by preventing contact of reduced site II_f with O₂. Given that ROS production by inhibited complex II has been established by several groups, this study was designed to evaluate whether pore opening could create conditions promoting ROS production by complex II and, if so, determine what factors regulate it. We demonstrate that by accelerating succinate/fumarate efflux from the matrix and decreasing [dicarboxylate] in the vicinity of site II_f, pore formation in the inner mitochondrial membrane induced by the alamethicin or calcium-induced PTP opening allows O₂ access to reduced II_f sites with a resulting increase in

ROS production (Fig. 1). This mechanism of ROS production also requires either complex II inhibition at the level of Q reduction or inhibition of QH₂ oxidation downstream in complex III. These conditions may be met during I/R because the catalytic activities of complexes II and III are inhibited in mitochondria isolated from ischemic hearts after reperfusion (22–26). If so, then during reperfusion this mechanism may also contribute to continued damaging ROS production after PTP opening has occurred and succinate-fueled RET-driven ROS production has ceased.

Results

To examine conditions under which complex II can generate significant ROS during pore opening in the inner membrane, we exposed isolated cardiac mitochondria to the complex II inhibitor AA5 (0.5 μM) while measuring H₂O₂ in the buffer. AA5 binds to complex II with high affinity, and experiments with isolated cardiac mitochondria and submitochondrial particles have shown complete inhibition of complex II activity by 0.1 μM AA5 (27). As shown in Fig. 2A, H₂O₂ production was very low until AA5-inhibited mitochondria were permeabilized with alamethicin, at which point H₂O₂ production accelerated markedly and then leveled off. Addition of exogenous succinyl-CoA then restored H₂O₂ production to the initial level. The increase in ROS production after succinyl-CoA addition indicates that despite permeabilization, the matrix contained sufficient ADP/GDP and P_i to generate succinate from the added succinyl-CoA. The concentration dependence of the ability of AA5 to stimulate H₂O₂ production after alamethicin was practically unchanged between 0.1 and 1 μM (results not shown), and therefore we used 0.5 or 1 μM in all our experiments. Importantly, H₂O₂ production required pore formation by alamethicin and was proportional to the concentration of mitochondria.

Complex II and ROS production

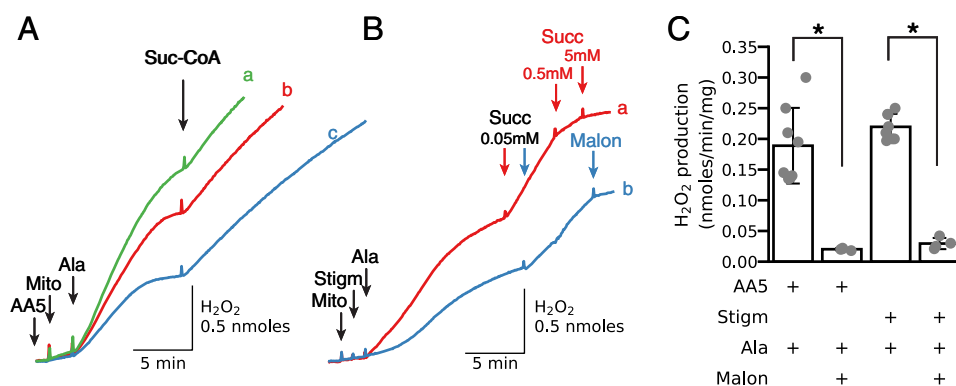


Figure 2. Alamethicin permeabilization promotes a rapid increase in H₂O₂ production when complex II is inhibited with atpenin A5 or complex III is inhibited with stigmatellin. *A*, isolated cardiac mitochondria (Mito) were added in concentrations of 1.0, 0.75, or 0.5 mg/ml (traces *a–c*, respectively) to buffer containing atpenin A5 (AA5, 0.5 μM). After permeabilization with alamethicin (Ala, 20 μg/ml), H₂O₂ production increased and plateaued as endogenous substrates were depleted and then reaccelerated after addition of succinyl-CoA (Succ-CoA) (0.15 mM). *B*, mitochondria were added at concentrations of 1.0 (trace *a*) and 0.5 (trace *b*) mg/ml to buffer containing stigmatellin (Stigm, 1 μM), followed by alamethicin and succinate (50 μM) as indicated. Decreased H₂O₂ production was reactivated with 50 μM succinate and inhibited in trace *a* with 0.5 and 5 mM succinate (Succ) and in trace *b* with 1 mM malonate (Malon). In this and other figures, additions to all traces are indicated by black arrows; additions to one trace only are indicated by the arrows of the same color as the trace. *C*, summary data (median ±95% confidence intervals, *, *p* < 0.05) for H₂O₂ production in AA5- and stigmatellin-inhibited mitochondria after alamethicin permeabilization and inhibition by malonate.

Fig. 2*B* demonstrates that in the absence of AA5, downstream electron transport inhibition at complex III with stigmatellin also resulted in increased ROS production after alamethicin. Stigmatellin effectively inhibits QH₂ oxidation by complex III and also prevents antimycin-induced ROS production by complex III. Because alamethicin eliminates Δψ and thereby stimulates NADH oxidation, endogenous substrates alone are unlikely to be able to increase the NADH/NAD ratio to the level required for ROS production during forward electron transport through complex I or ketoglutarate dehydrogenase. Moreover, without polarized Δψ, RET is not possible. By exclusion, complex II is the only plausible source of H₂O₂ production under these conditions. After the rapid initial increase in H₂O₂ production declined due to oxidation of II_f sites and depletion of endogenous succinate, addition of 50 μM succinate reactivated H₂O₂ production. Increasing [succinate] to 500 μM, however, suppressed H₂O₂ production, consistent with the previously mentioned data (16, 19) that higher [succinate] masks reduced II_f sites and prevents access to O₂. H₂O₂ production was also inhibited by malonate, which prevents succinate oxidation by complex II (Fig. 2*B*, blue trace *b*). Although complex II activity, determined in submitochondrial particles from cardiac mitochondria in the presence of 2.5 mM succinate was already 50% inhibited in the presence of 42 μM malonate (28), we used 1 mM malonate routinely and 5 mM for experiments with higher [succinate]. Summary findings are shown in Fig. 2*C*.

Because alamethicin dissipates Δψ, which increases respiratory activity and accelerates consumption of endogenous substrates, including succinate, and also promotes efflux of endogenous substrates through alamethicin pores, it was important to add AA5 before the inner membrane was permeabilized. In Fig. 3*A*, H₂O₂ production was highest (0.157 nmol/min/mg) when AA5 was added 2 min before alamethicin (purple trace), but it decreased to 22, 18, and 12% when AA5 was added 1, 2, or 3 min after alamethicin (green, red and blue traces, respectively). Decreased H₂O₂ production under these conditions could be rapidly reversed by adding exogenous succinate in low

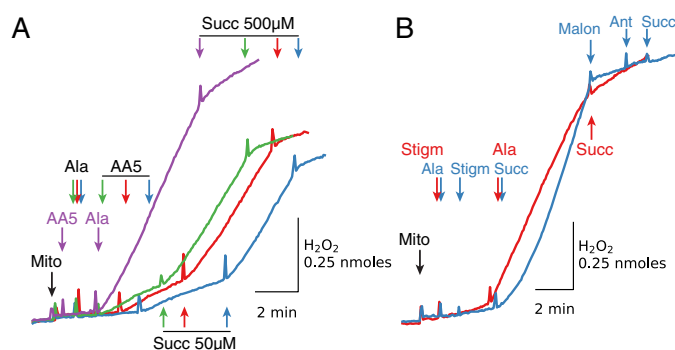


Figure 3. H₂O₂ production by inhibited complex II is maximized when mitochondria are permeabilized after AA5 (A) or stigmatellin (B) addition. *A*, compared with AA5 addition before alamethicin (Ala, purple trace), AA5 (0.5 μM) added 1 min (green trace), 2 min (red trace), and 3 min (blue trace) after alamethicin resulted in reduced H₂O₂ production. Low H₂O₂ production was enhanced by adding 50 μM succinate (Succ) but suppressed by 500 μM. *B*, alamethicin added after stigmatellin (Stigm) (2 μM) induced a rapid increase in H₂O₂ production that was inhibited with 5 mM succinate (red trace). Stigmatellin added after alamethicin (blue trace) had little effect, but H₂O₂ production was increased with a low concentration of succinate (75 μM) and inhibited with malonate (Malon) (5 mM). Note that ROS production by antimycin (Ant)-inhibited complex III after addition of 5 mM succinate was inhibited due to the presence of stigmatellin (blue trace). As in other figures, additions to all traces are indicated by black arrows; additions to one trace only are indicated by the arrows of the same color as the trace. Mito, mitochondria.

concentrations (50 μM). However, succinate in higher concentrations (500 μM) rapidly inhibited H₂O₂ production.

Similarly to increased H₂O₂ production induced by alamethicin after (but not before) AA5 (Fig. 3*A*), downstream inhibition of QH₂ oxidation with stigmatellin resulted in increased ROS production only when added before alamethicin (Fig. 3*B*, red trace). Stigmatellin added after alamethicin (Fig. 3*B*, blue trace) did not increase H₂O₂ production until exogenous succinate (75 μM) was added. ROS production induced by 75 μM succinate in the presence of stigmatellin was inhibited by malonate (5 mM) and not reactivated after sequential addition of antimycin and 5 mM succinate (Fig. 3*B*, blue trace). Note that under these conditions, stigmatellin inhibits ROS production by antimycin-inhibited complex III by blocking QH₂ oxidation.

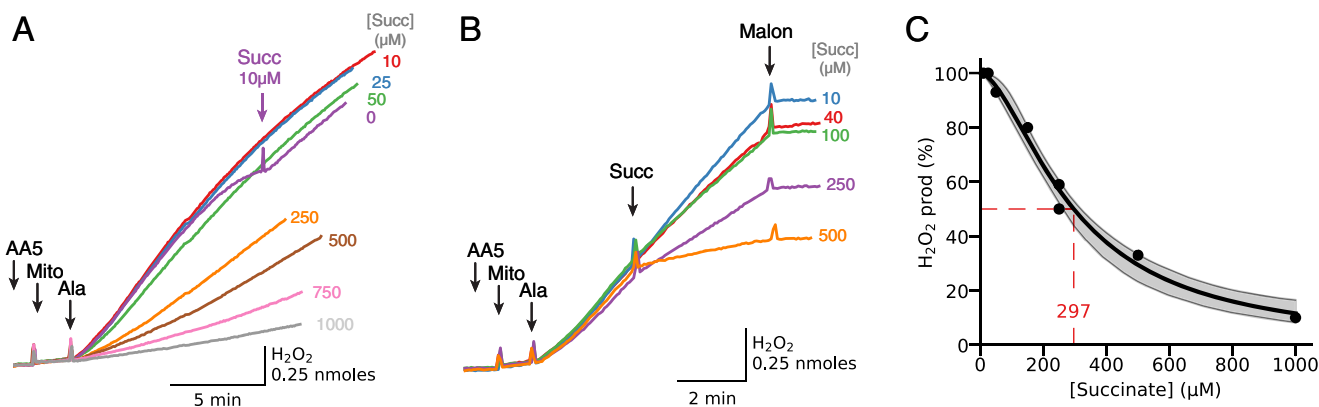


Figure 4. Concentration dependence of succinate-induced inhibition of H_2O_2 production by complex II-inhibited mitochondria. *A*, mitochondria (*Mito*) were incubated with AA5 (1 μM) and succinate (*Succ*) at the concentrations indicated at the end of trace. After permeabilization with alamethicin (*Ala*), H_2O_2 production was lower at higher [succinate]. The initial rate of H_2O_2 production with 10 μM succinate present (*red trace*) was similar to that generated by endogenous substrates in the absence of added succinate (*purple trace*). As in other figures, additions to all traces are indicated by *black arrows*; additions to one trace only are indicated by the *arrows of the same color as the trace*. *B*, H_2O_2 production in AA5 (0.5 μM)-inhibited mitochondria initiated by alamethicin was progressively suppressed by adding exogenous succinate in increasing concentrations, as indicated at the end of traces. At the end H_2O_2 production was inhibited with malonate (*Malon*) (5 mM). *C*, summary data for inhibition of H_2O_2 production as a function the [succinate], showing half-maximal inhibition at 297 μM . Shaded areas indicate 95% confidence intervals. *Ant*, antimycin; *Stigm*, stigmatellin.

The ability of high [succinate] to suppress H_2O_2 production by AA5-inhibited complex II (16, 19) is further illustrated in Fig. 4A. Compared with the case with only endogenous substrates present, addition of exogenous 10 μM succinate allowed AA5-inhibited mitochondria to maintain H_2O_2 production after permeabilization for a longer duration (Fig. 4A, compare *purple* and *red traces*). However, raising [succinate] to 25 μM did not further increase H_2O_2 production (*blue trace*), indicating that succinate-driven H_2O_2 production was already optimized at 10 μM succinate. Further increases in [succinate] suppressed H_2O_2 production, with half-maximal suppression at 297 μM succinate (Fig. 4C), consistent with succinate binding causing a progressive decrease in the concentration of reduced site II_f accessible to O_2 . The suppressant effect of succinate on H_2O_2 production was also evident when succinate was added after H_2O_2 production had been initiated by alamethicin (Fig. 4B).

SDH is a reversible enzyme and its dicarboxylate-binding site can bind a range of substrates and inhibitors, including succinate, fumarate, citrate, malate, malonate, and oxaloacetate (OAA). OAA is an intermediate of the TCA cycle that inhibits SDH by binding to the enzyme with high affinity ($K_d = 10^{-8}$ M), so that isolated SDH is obtained largely in deactivated form (29). Because of that tight binding, only a fraction of complex II in mitochondrial membranes isolated from cardiac tissue is found to be in the free active form (30), suggesting that the concentration of reduced II_f sites accessible to O_2 after inhibition of Q reduction or QH2 oxidation is decreased in our permeabilized mitochondria. To evaluate the importance of OAA binding on the ability of complex II to produce ROS, we followed a previously reported protocol (30) to remove bound OAA by incubating the preparation with malonate (see "Experimental procedures"). Fig. 5 shows that (AA5 + succinate)-induced H_2O_2 production by malonate-treated permeabilized mitochondria was about 65% higher compared with mitochondria incubated in the absence of malonate. Assuming that malonate treatment removed all bound OAA, the difference between H_2O_2 production with and without malonate treat-

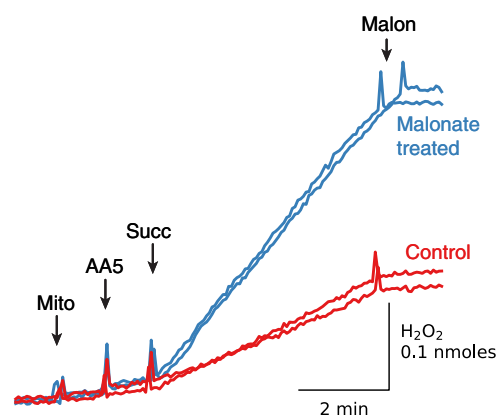


Figure 5. Malonate-induced removal of oxaloacetate bound to complex II enhances succinate-driven H_2O_2 production by AA5-inhibited permeabilized mitochondria. Permeabilized mitochondria were incubated with (*blue traces*) and without 1 mM malonate (*Malon*) (*red traces*), and the inhibitor was then removed by washing and centrifugation. The resuspended pellet was used for stimulation of ROS production by adding succinate (*Succ*) (50 μM) to AA5 (1 μM)-inhibited mitochondria (*Mito*). Dual traces are superimposed for each condition.

ment is due to OAA bound to SDH. OAA binding to complex II apparently lowers ROS production values in our experiments but is unlikely to change our main conclusion that PTP opening can increase ROS production by complex II under certain conditions. It is interesting that short cardiac ischemia resulted in a dramatic decrease in OAA in mouse heart, but it had no effect on the amount of OAA-free complex II (30).

In addition to strong inhibition by malonate (Fig. 4B) and high [succinate] (Fig. 4A), H_2O_2 production by AA5 inhibited complex II after permeabilization was even more sensitive to increases in [fumarate] (Fig. 6A), with half-maximal inhibition achieved at 11 μM fumarate (Fig. 6B).

As shown earlier in Figs. 2B and 3B, dicarboxylate- and malonate-sensitive H_2O_2 production could also be demonstrated in stigmatellin-inhibited mitochondria as a result of inhibition of QH2 oxidation by complex III. Fig. 7 illustrates the effects of stigmatellin in greater detail. When applied to intact isolated mitochondria energized with succinate, stigmatellin caused

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immediate almost full depolarization relative to alamethicin (Fig. 7A). The effects on H_2O_2 production are shown in Fig. 7B. When isolated normally polarized mitochondria were energized with 5 mM succinate (Fig. 7B, purple trace), H_2O_2 production due to RET increased significantly but was then suppressed by stigmatellin-induced depolarization. Addition of alamethicin at this point had no significant further effect on H_2O_2 production. In contrast, before alamethicin, 0.1 mM succinate (Fig. 7B, red trace) had no initial effect on H_2O_2 production either before or after stigmatellin-induced depolarization. At this point, however, alamethicin addition accelerated H_2O_2 production to a much higher rate than RET-induced H_2O_2 production. At 0.5 mM (Fig. 7B, blue trace), succinate had intermediate effects. These findings demonstrate that the rate of H_2O_2 production from complex II when [succinate] is optimal (<0.5 mM) exceeds that induced from complex I via RET at high [succinate] in fully polarized mitochondria. Here, we have to underline that ROS produced by complex I in the matrix is actively

neutralized by antioxidant enzymes, such that only a fraction of total H_2O_2 generated diffuses into the buffer. Also, we have shown previously that H_2O_2 efflux generated by complex I is more complete in alamethicin-permeabilized mitochondria (31) because of a significant decrease in matrix antioxidant activity. In addition, the inner membrane may have more limited permeability to H_2O_2 before alamethicin (32). These factors may underestimate the H_2O_2 production rate by RET.

The above experiments were performed in mitochondria permeabilized by alamethicin to create non-selective pores in the inner mitochondrial membrane. To determine whether PTPs have equivalent effects as alamethicin pores, mitochondria incubated with AA5 for 2 min were exposed to sequential addition of P_i (2.5 mM) and Ca^{2+} (50 μ M) to induce PTP opening (Fig. 8A, blue trace). H_2O_2 production increased significantly and was only mildly potentiated by addition of alamethicin but was suppressed by high [succinate] (1.5 mM). In contrast, if mitochondria were incubated in 250 μ M EGTA to

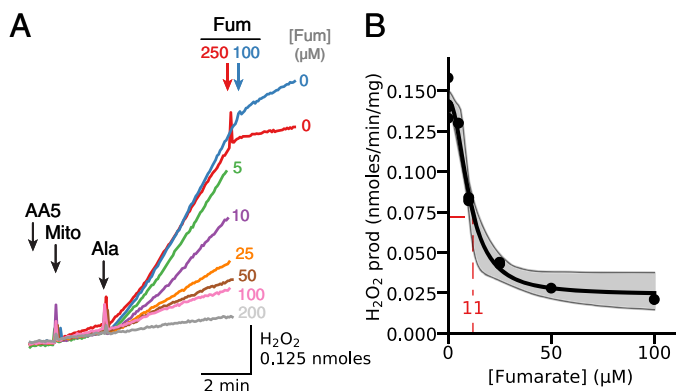


Figure 6. Concentration dependence of fumarate-induced inhibition of H_2O_2 production by complex II-inhibited mitochondria. A, mitochondria (Mito) were incubated with AA5 (1 μ M) and fumarate (Fum) at the concentrations indicated at the end of each trace. After permeabilization with alamethicin (Ala), H_2O_2 production progressively decreased with higher [fumarate]. As in other figures, additions to all traces are indicated by the arrows of the same color as the trace. B, summary data for inhibition of H_2O_2 production as a function [fumarate], showing half-maximal inhibition at 11 μ M. Shaded areas indicate 95% confidence intervals.

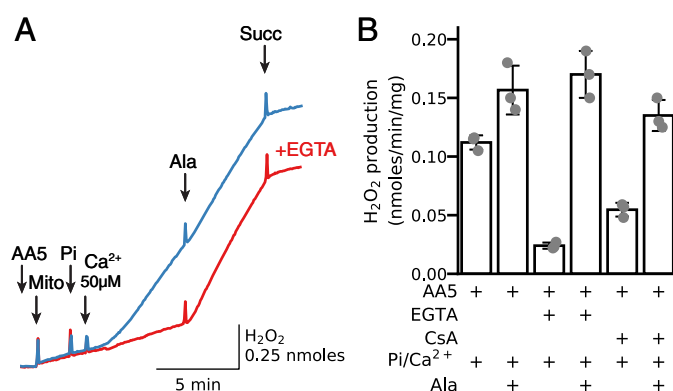


Figure 8. Calcium-induced PTP opening induces H_2O_2 production from complex II in AA5-inhibited mitochondria. A, in mitochondria (Mito) incubated with AA5 (0.5 μ M), addition of P_i (2.5 mM) and Ca^{2+} (50 μ M) to induce transient PTP opening caused a robust increase in H_2O_2 production (blue trace). Subsequent addition of alamethicin (Ala) had little additional effect. Pre-incubating with EGTA (0.25 mM) (red trace) abolished the increase in H_2O_2 production after Ca^{2+}/P_i addition, but alamethicin remained effective. B, summary data (median \pm 95% confidence intervals) of H_2O_2 production in AA5-inhibited mitochondria when PTP opening was triggered by Ca^{2+}/P_i , including the effects of the PTP inhibitor CsA (1.5 μ M). Succ, succinate.

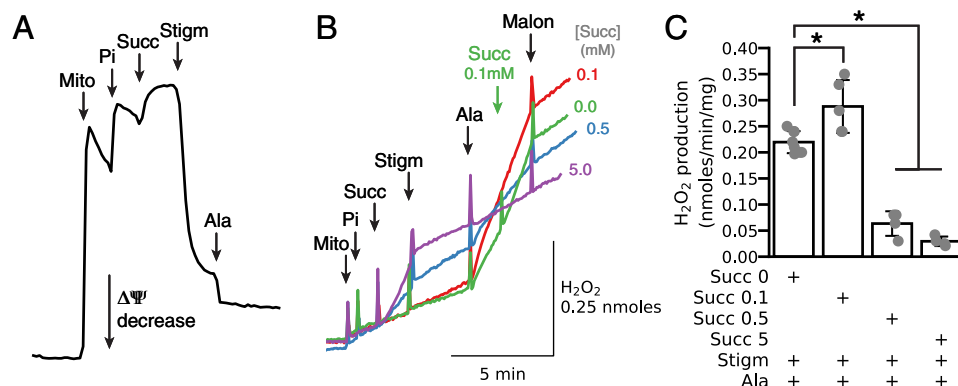


Figure 7. Comparison of succinate-driven H_2O_2 production by RET in intact fully polarized mitochondria with succinate-driven H_2O_2 production by complex II after permeabilization with alamethicin. A, demonstration that stigmatellin (Stigm, 1 μ M) dissipates $\Delta\Psi$ required for H_2O_2 production by RET in intact mitochondria (Mito) energized with succinate (Succ, 0.5 mM). P_i 2.5 mM. B, as expected, stigmatellin (1 μ M) inhibited succinate-induced H_2O_2 production by RET. The [succinate] is indicated at the end of each trace. Subsequent addition of alamethicin (Ala) robustly stimulated H_2O_2 production in the presence of endogenous or low exogenous [succinate], but it had no effect at 5 mM [succinate] (purple trace). Malonate (Malon, 1 mM) suppressed the increase H_2O_2 production induced by alamethicin. P_i 2.5 mM. As in other figures, additions to all traces are indicated by black arrows; additions to one trace only are indicated by the arrows of the same color as the trace. C, summary data (median \pm 95% confidence intervals, *, $p < 0.05$) of the effect of [succinate] on H_2O_2 production in stigmatellin-inhibited mitochondria after alamethicin permeabilization and inhibition by malonate.

prevent PTP opening, P_i and calcium addition had no significant effect on H_2O_2 production until alamethicin was also added (Fig. 8A, red trace). Similarly, incubation of mitochondria in the presence of CsA ($1.5 \mu M$) significantly inhibited the Ca^{2+}/P_i -induced increase in H_2O_2 production, although less effectively than EGTA (Fig. 8B). Thus, membrane permeabilization by alamethicin and calcium/ P_i -induced PTP opening had nearly equivalent effects on stimulating ROS production by complex II.

Discussion

Summary of major findings

The major finding of this study is that in the absence of exogenous substrates, inner membrane permeabilization for low-molecular-weight solutes achieved by exposing isolated cardiac mitochondria to either alamethicin or Ca^{2+}/P_i to induce PTP opening rapidly increased ROS production when electron flow through complex II was inhibited by AA5 prior to pore opening. Inner membrane permeabilization also increased ROS production by complex II in the absence of AA5 when downstream electron transport in complex III was inhibited with stigmatellin to prevent QH2 oxidation (or antimycin-stimulated ROS production by complex III). In these settings, ROS production declined gradually due to oxidation of reduced flavin sites and depletion of endogenous substrates, but it could be reactivated by adding succinate in low concentrations. Under these conditions, ROS could not originate from complex I or α -ketoglutarate dehydrogenase without a high NADH/NAD⁺ ratio (33–35), and RET was not possible due to $\Delta\psi$ dissipation. ROS production by complex III was also excluded by the experiments with stigmatellin, leaving complex II as the only plausible source of ROS under these conditions.

Most importantly, whereas low [succinate] maintained ROS production, higher [succinate] ($>100 \mu M$) inhibited ROS production, as did fumarate and malonate. These results demonstrate that although reduction of the dicarboxylate-binding site is required for ROS production by complex II, elevated dicarboxylate concentrations prevent O_2 access to reduced site II_f and thereby suppress ROS generation (Fig. 1), as described previously (16, 17, 19). Upon inner membrane permeabilization with either alamethicin or Ca^{2+} -induced PTP opening, dicarboxylate efflux from the matrix lowers their concentrations and exposes reduced site II_p , creating optimal conditions for ROS generation by complex II. The implication is that if either complex II or III is damaged during prolonged ischemia (22–26), then PTP opening during reperfusion could create similar conditions, promoting continued succinate-driven ROS production from reduced complex II after RET-driven ROS production has ceased.

Alamethicin pores versus PTP

PTP opening is known to increase inner membrane permeability at the start of cardiac reperfusion (11, 36). PTP opening can be also promoted *in vitro* by exposing isolated mitochondria to various inducers, including high $[Ca^{2+}]$, $[P_i]$, and/or oxidant chemicals. However, the presence of oxidant chemicals significantly complicates the evaluation of the role of PTP opening in ROS production. To avoid this complication, we

used either alamethicin or PTP opening induced by Ca^{2+}/P_i to mimic reperfusion-induced PTP opening. At low concentrations, exogenous pore-forming peptides like alamethicin and mastoparan have been shown to form regulated pores whose conductance properties are similar to PTP induced by low concentrations of oxidant chemicals (37). At higher concentrations, such as used in this study, alamethicin pores become unregulated, similar to PTP openings in the high conductance mode induced by chemicals (37). We emphasize that no direct comparison between PTP and alamethicin-generated pores is intended, except that both allow rapid equilibration of small molecules between matrix and extramitochondrial space. In this regard, any regulation of PTP open/closed states, including spontaneous reclosure of PTP such as observed in rat heart after ischemia/reperfusion (38), does not occur with alamethicin-generated pores, but it could modify distribution of solutes during ischemia/reperfusion. In addition, higher $[Ca^{2+}]$, often used to induce PTP opening, may promote H_2O_2 generation via activation of α -glycerophosphate dehydrogenase located in the outer surface of the inner membrane (39). Although ROS production by this enzyme is slow, it could modify ROS production by complex II in permeabilized mitochondria. With these caveats, however, alamethicin-generated pores appear to be a reasonable tool to study the importance of dicarboxylate redistribution on ROS production by complex II.

Dicarboxylate concentration dependence of ROS production by complex II

We found that H_2O_2 production by AA5-inhibited complex II was already optimized at 10–50 μM exogenous succinate and suppressed by higher concentrations. Brand and co-workers (19) reported peak H_2O_2 production at 400 μM succinate, with significant inhibition at higher [succinate] commonly used to energize mitochondria (5 mM). In these experiments using intact skeletal muscle mitochondria, ROS production by complexes I and III was inhibited with rotenone and myxothiazol, respectively, with the latter also providing downstream block for electron transport. Using SMP from beef heart mitochondria, maximal ROS production by AA5-inhibited complex II was observed at around 100 μM , closer to our findings. AA5-inhibited SMP also demonstrated progressive inhibition of ROS production in response to increased [succinate], similar to our results (16). Because the dicarboxylate carrier K_m for succinate is around 1 mM (16, 19), matrix [succinate] in non-permeabilized mitochondria may be significantly lower than the exogenous [succinate] in the extramitochondrial buffer (16) when succinate is being actively metabolized in the matrix. This may account for the requirement for higher [succinate] to reach maximum ROS production compared with the permeabilized mitochondria in our experiments. Indeed, more recent experiments with rotenone/myxothiazol-inhibited intact or permeabilized cardiac mitochondria have shown that H_2O_2 production is maximal at about 50 μM [succinate], followed by a gradual decrease at higher [succinate] (20). Furthermore, superoxide-generating activity of AA5-inhibited purified complex II is already high at very low [succinate] (2–5 μM) and decreases as the fumarate/succinate ratio increases (20).

Complex II and ROS production

A critically important finding from previous studies (16, 19) is that complex II can generate ROS only when site II_f is unoccupied by substrate. All TCA intermediates that bind to the dicarboxylate-binding site can therefore affect ROS production by complex II. We found that fumarate, a product of succinate oxidation that binds with comparable affinity to the dicarboxylate-binding site (40), is even more effective than succinate at inhibiting ROS production by AA5-inhibited complex II when added to permeabilized mitochondria, with only 11 μM fumarate required for 50% inhibition (Fig. 6). Attenuation of ROS generation by fumarate has been explained by two mechanisms as follows: (a) occupying the dicarboxylate-binding site, and (b) increasing the fumarate/succinate ratio which decreases complex II reduction (16). In alamethicin-permeabilized cardiac mitochondria incubated with myxothiazol, an increase in [fumarate] to modestly increase the fumarate/succinate ratio resulted in rapid inhibition of H₂O₂ production (41). However, antimycin-induced ROS production by cardiac submitochondrial particles was maximal at a [succinate]/[fumarate] ratio of 1:5 and was suppressed by either decreased or increased redox potential (42). Subsequent studies have confirmed a partially oxidized Q pool requirement for maximal ROS production by antimycin-inhibited complex III and also demonstrated that complex II can be a source as well as an inhibitor or enhancer of ROS production by complexes I and III (43). Together, these findings indicate that high matrix dicarboxylate concentrations inhibit ROS production by complex II, even when complex II or III become damaged during ischemia. Inner membrane permeabilization due to alamethicin or calcium-induced PTP opening allows dicarboxylate efflux from the matrix, depleting succinate, fumarate, and other dicarboxylates to levels that unmask reduced site II_p, allowing O₂ to interact and generate ROS. A somewhat different mechanism was recently proposed by Vinogradov and co-workers (20), who suggested that iron-sulfur clusters are involved in ROS production by complex II when Q reduction is inhibited. However, they also observed maximum ROS production at low [succinate] (50 μM). To explain the importance of the dicarboxylate-free II_f site for ROS production, the authors postulated long distance interaction between II_f and Q-reactive sites (20).

Relevance to I/R injury

Succinate accumulation during ischemia has been linked to excessive ROS production and enhanced cardiac injury during reperfusion (1). The hypothesized mechanism is that when ischemic depolarized mitochondria are reoxygenated, a subpopulation may recover $\Delta\psi$ sufficiently to generate ROS by RET through complex I using succinate as the electron donor. The ROS generated by RET may then trigger PTP openings promoting cardiac injury. However, once the repolarized mitochondria have been depolarized by PTP opening, further ROS production via succinate-driven RET ceases, introducing a negative feedback factor to self-limit ROS production. This purely RET-based mechanism, however, does not account for observations from Halestrap and co-workers (36), who found that in Langendorff-perfused rat hearts subjected to 30 min of global ischemia, increased ROS production during reperfusion primarily occurred after, rather than before, PTP opening. Both ischemic

preconditioning and CsA inhibited PTP opening during reperfusion, which might be expected to increase ROS production if succinate-driven RET were the primary mechanism. However, both interventions attenuated ROS production during reperfusion (36).

In contrast, if PTP opening induced by RET-derived ROS were to induce a secondary mechanism to sustain ROS production after RET ceased, this positive feedback mechanism would promote ROS-induced ROS release amplifying the levels of damaging ROS causing reperfusion injury. This study focusing on complex II and the accompanying article (52) focusing on complex III provide possible mechanisms for this positive feedback scenario in ROS production after PTP opening has occurred. In this case, PTP opening would promote succinate and fumarate efflux from the matrix, lowering their concentrations sufficiently to activate ROS production by complex II (Fig. 8). Here it is relevant to note that increases in cardiac fumarate levels due to knock-out of fumarate hydratase or administration of dimethyl fumarate for 5 days resulted in a robust cardioprotective effect against ischemia/reperfusion injury (44). Attenuation of reperfusion injury by increased [fumarate] was attributed to fumarate-induced activation of the Nrf2 pathway that confers cellular protection against oxidative stress by up-regulating antioxidant enzymes. Our results offer an additional explanation for fumarate's cardioprotective effect, namely that fumarate binding to site II_f suppresses amplification of damaging ROS production by complex II following PTP opening. In addition, as shown in the accompanying article (52), the entry of Mg²⁺ into the matrix would also activate malic enzyme, such that oxidation of low concentrations of malate arising from succinate and fumarate would provide electrons for ROS generation by damaged complex III. Thus, it is intriguing to speculate that when a population of mitochondria initially regenerates $\Delta\psi$ during reperfusion, succinate-driven ROS production by RET (1) triggers PTP opening, which then amplifies ROS production from complexes II and III by the mechanisms shown in these studies (Fig. 9). This scenario is consistent with recent experimental findings that SDH inhibition with malonate at the start of reperfusion inhibits both ROS production and PTP opening/cell damage (45). It is also consistent with the observation that the complex I inhibitor rotenone protects against I/R injury by suppressing RET and inhibiting the initial PTP opening that activates succinate-driven ROS production by complexes II and III. In addition rotenone blocks Q reduction by NADH, which, as we demonstrate in accompanying article (52), also inhibits NAD⁺/Mg²⁺-induced ROS production by antimycin-inhibited complex III. However, given the complexity of the rapidly changing cellular environment during I/R, it is unclear whether the conditions for robust ROS production by complex II and III would be met, and whether one pathway is more important quantitatively than the other. However, because PTP opening is necessary to achieve the appropriate conditions for activation of ROS production by these pathways, preventing succinate accumulation during ischemia seems a promising approach to both suppress initial PTP opening by RET-derived ROS and to inhibit further ROS production by inhibited complexes II and III. It is also intriguing to speculate that RET-derived ROS during brief ischemic episodes that are

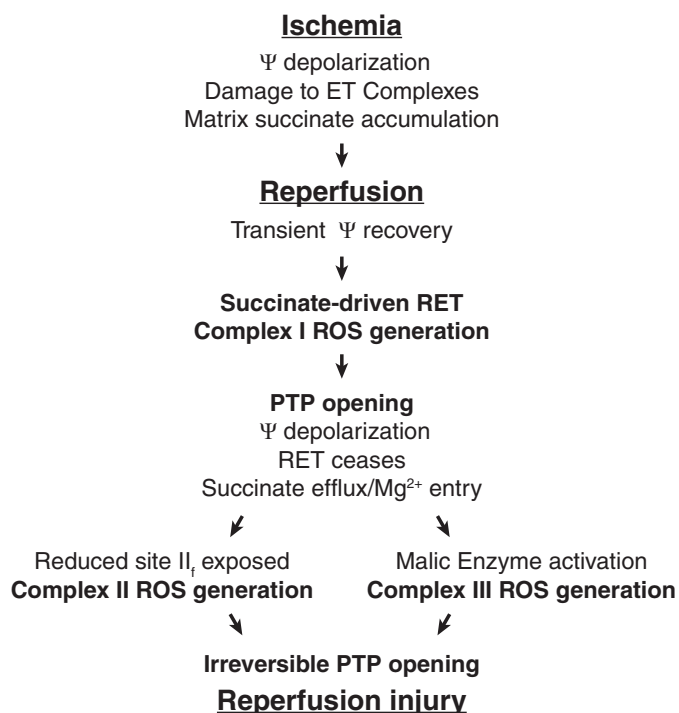


Figure 9. Proposed scenario of ROS generation during reperfusion after prolonged ischemia sufficient to damage complexes II and/or III. See text for description. *ET*, electron transport; *RET*, reverse electron transport through complex I; $\Delta\Psi$, membrane potential.

too short to damage electron transport complexes may play a role in ROS-dependent activation of cardioprotective signaling through the RISK and/or SAFE pathways, because succinate accumulation was significant after only 5 min of ischemia (1). Further studies will be necessary to investigate these possibilities.

Experimental procedures

Ethical approval

This study was approved by the UCLA Chancellor's Animal Research Committee (ARC 2003-063-23B) and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with UCLA Policy 990 on the Use of Laboratory Animal Subjects in Research (revised 2010).

Experimental techniques

All measurements were carried out using customized Fiber Optic Spectrofluorometer (Ocean Optics) in a partially open continuously stirred cuvette at room temperature (22–24 °C) (46). Mitochondria were isolated from rabbit hearts as described previously (47). Intact mitochondria were added (0.6 to 1.0 mg/ml) to the incubation buffer containing 250 mM sucrose, 10 mM Hepes, pH 7.4, with Tris.

Mitochondria were permeabilized with addition of alamethicin (20 $\mu\text{g/ml}$). Alamethicin creates pores allowing equilibration of low-molecular-weight components across the inner membrane, whereas high-molecular-weight proteins are retained in the matrix and intermembrane space (48). For stimulation of ROS production, it was important to add alamethicin

or Ca^{2+} after complex II inhibition with AA5 or downstream inhibition of QH2 oxidation in complex III with stigmatellin.

PTP opening was induced by adding 2.5 mM P_i and 50 μM Ca^{2+} to AA5-inhibited mitochondria. Selectivity of membrane permeabilization due to PTP opening was confirmed by adding EGTA (0.25 mM) or cyclosporin A (1.5 μM) to prevent PTP opening.

Isolated permeabilized mitochondria are known to contain OAA that is rather tightly bound to complex II (29) and therefore is expected to decrease free reduced flavin site concentration available for ROS generation. To remove OAA bound to complex II, permeabilized mitochondria were incubated with 1 mM malonate for 30 min at room temperature, a treatment demonstrated to reduce OAA bound to the enzyme (30). After dilution of the preparation with sucrose buffer, malonate is expected to rapidly dissociate from the enzyme, after which the essentially OAA/malonate-free preparation is separated by centrifugation. Control mitochondria were treated similarly in the absence of malonate.

H_2O_2 release from mitochondria was measured using 5 μM Amplex Red and 0.2 units of HRP in the buffer (excitation/emission, 540:590 nm). The increase in resorufin fluorescence was calibrated by adding H_2O_2 in known concentrations to the buffer containing permeabilized mitochondria.

Mitochondrial O_2 consumption was measured continuously by monitoring buffer O_2 content using a fiber optic oxygen sensor FOXY-AL300 (Ocean Optics) (46). Membrane potential ($\Delta\psi$) was determined by using tetramethylrhodamine as described previously (47). All chemicals were obtained from Sigma.

Statistical analysis

For each data set, the mean and accompanying 95% confidence intervals are reported. The conventional percentile bootstrap-resampling approach with 10,000 replications was used for estimating 95% confidence intervals as well as examining the significant difference between groups (effect size statistics) (49–51). A p value <0.05 was considered statistically significant. All analyses were performed by subroutines for bootstrapping developed in the Python programming language (51).

Author contributions—P. K. and J. N. W. conceived and coordinated the study. P. K., G. C., and S. J. A. performed and analyzed the experiments. P. K., J. N. W., G. C., and S. J. A. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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