We recently reported a previously unrecognized mitochondrial respiratory phenomenon. When [ADP] was held constant (“clamped”) at sequentially increasing concentrations in succinate-energized muscle mitochondria in the absence of rotenone (commonly used to block complex I), we observed a biphasic increase in succinate-energized respiration as a function of increasing [ADP]. With further increments in [ADP], respiration decreased associated with accumulation of OAA. Moreover, a low pyruvate concentration, that alone was not enough to drive respiration, was sufficient to metabolize OAA to citrate and completely reverse the loss of succinate-supported respiration at high [ADP]. Further, chemical or genetic inhibition of pyruvate uptake prevented OAA clearance and preserved respiration. In addition, we measured the effects of incremental [ADP] on NADH, superoxide, and H$_2$O$_2$ (a marker of reverse electron transport from complex II to I). In summary, our findings, taken together, support a mechanism (detailed within) wherein succinate-energized respiration as a function of increasing [ADP] is initially increased by [ADP]-dependent effects on membrane potential but subsequently decreased at higher [ADP] by inhibition of succinate dehydrogenase by OAA. The physiologic relevance is discussed.

Understanding mitochondrial electron transport is fundamental to our knowledge of a myriad of metabolic, neurologic, neoplastic, and other physiologic and pathophysiologic states.

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This article contains Figs. S1–S6.

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Complex II (succinate dehydrogenase, SDH)$^3$ is a major site for electron input into the mitochondrial respiratory chain. However, the control of complex II (succinate)-energized respiration is still not well-understood.

Mitochondrial respiration is commonly studied by adding substrates to generate electrons, most often using donors to complex I and/or complex II and under state 4 (no ATP production) and state 3 (ADP present in high amounts to generate maximal ATP production) conditions. However, when ADP is added to succinate-energized mitochondria in amounts expected to induce state 3 respiration, O$_2$ flux drops to low levels (1, 2). By adding incremental amounts of ADP rather than large amounts and by clamping the concentrations of added ADP, we recently observed a phenomenon not reported previously (3). The phenomenon is that respiration energized by succinate (without rotenone, commonly used with succinate to prevent reverse electron transport to complex I) actually increases over state 4 at low [ADP], reaches a peak at an intermediate concentration, and then sharply decreases to very low levels at high [ADP]. The loss of respiration at high ADP was clearly not because of simple loss of function over time of incubation, lower O$_2$ tension, or opening of the mitochondrial permeability transition pore, because the loss was rapidly reversible by complex I substrates or downstream electron donors and there was no evidence of pore opening (3).

We were surprised to find no prior report indicating that succinate-energized respiration peaks at low [ADP] before declining at higher [ADP]. One reason may be that, as stated above, most past studies of respiration by isolated mitochondria have been carried out in the absence and/or presence of substantial amounts of ADP rather than at intermediate concentrations. It is not physiologic to study respiration only under state 3 or state 4 conditions. Mitochondria in vivo do not function at either extreme, but rather in between (4). Another reason may be that the large portion of early studies of mitochondrial metabolism used liver mitochondria wherein we found that this biphasic succinate-energized effect is barely evi-

$^3$ The abbreviations used are: SDH, succinate dehydrogenase; OAA, oxaloacetic acid; RET, reverse electron transport; ROS, reactive oxygen species; $\Delta$Ψ, membrane potential; FCCP, p-trifluoromethoxyphenylhydrazone; EDL, extensor digitorum longus; 2DOG, 2-deoxyglucose; MPC, mitochondrial pyruvate carrier; OD, optical density; ANOVA, analysis of variance.

$^4$ For the purposes of this manuscript, we refer to state 4 as respiration in the presence of substrate but absence of added ADP, slightly at variance with the original description by Chance and Williams in 1955 (37) as respiration after depletion of added ADP.
dent (3). Moreover, an additional reason this phenomenon had not received past attention may be that, as above, it has become common practice to carry out studies of succinate-energized respiration in the presence of rotenone. However, rotenone is also not physiologic.

In the current report, we describe the mechanism underlying the above-mentioned phenomenon. To do this, we used ADP clamp methodology, 2-deoxyglucose plus hexokinase (5), combined with sensitive NMR technology to detect TCA cycle metabolites. In particular, this includes quantification of oxaloacetic acid (OAA), a metabolite that is very difficult to measure by MS because of instability (6, 7). Here we provide compelling evidence for the role of OAA in regulating biphasic complex II–supported respiration. Further, we describe how this process is related to changes in reverse electron transport (RET), reactive oxygen species (ROS), membrane potential (ΔΨ), and the oxidation/reduction state of NAD⁺ and NADH. These findings advance our understanding of complex II–energized respiration and, moreover, suggest that we may need to re-think how complex II respiration is studied, i.e. that we might best do this at intermediate degrees of ADP availability without rotenone. The physiologic relevance of our findings is discussed.

Results

O₂ flux and accumulation of TCA metabolites depend on [ADP]

Mitochondria were incubated for 20 min and the ADP concentration was clamped at the desired level (Fig. 1). On a given day separate 20-min incubations were carried out at all ADP concentrations shown on the x axis and the experiment was repeated on 6 different days. The O₂ tension in the Oxygraph drops with time but respiration is not affected until O₂ levels become very low. Nonetheless, because incubations were carried out for 20 min, it was necessary to periodically open the chamber to prevent marked deterioration in the oxygen content of the medium. O₂ flux (Fig. 1A) is depicted as the average respiration over time assuming that respiration, which cannot be measured while the chamber is open, remains at the average of the value between the time of chamber opening and closing (see “Experimental procedures”).

As shown in Fig. 1A, succinate-energized respiration was [ADP]-dependent, reaching a peak at 8 μM ADP before decreasing at higher concentrations. Metabolite and nucleotide concentrations were measured in the respiratory medium obtained at the end of each run (Fig. 1, B–G). Notably, the increase in OAA corresponded closely with the decrease in O₂ flux whereas precursors (malate and fumarate) paralleled O₂ flux and succinate utilization varied as expected dependent on consumption of the added substrate. NADH began to decrease as respiration peaked. Measurements of NAD⁺ were more variable but trended upward as NADH decreased.

**Pyruvate prevents the loss of O₂ flux at high [ADP] at a concentration too low to alter respiration when added alone**

In these experiments (Fig. 2), incubations were performed on succinate-energized mitochondria with or without a low (0.5 mM) concentration of pyruvate or carried out in the presence of pyruvate alone (no succinate). In each individual incubation, ADP was sequentially added at intervals to increase clamped [ADP] to the levels indicated. Respiration and ΔΨ were measured as the plateau values at each ADP concentration. These data show that pyruvate prevented the loss in succinate-energized respiration (Fig. 2A) and potential (Fig. 2B) at higher [ADP]. This was the case even though pyruvate was not sufficient to drive respiration on its own (Fig. 2A).

**Pyruvate clearance of OAA and rescue of succinate-energized respiration requires mitochondrial pyruvate uptake**

Succinate-energized mitochondria were incubated for 20 min in the presence of ADP clamped at 32 μM. Table 1 depicts respiration determined in the presence or absence of pyruvate.
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Figure 2. Respiration and \( \Delta \psi \) in mouse hind limb muscle mitochondria energized by 10 mM succinate, 10 mM succinate plus 0.5 mM pyruvate, or 0.5 mM pyruvate. Incubations were carried out in the presence of sequentially added and clamped ADP concentrations indicated on the x axis. A, respiration. B, membrane potential. Data represent mean ± S.E., n = 4 for each condition. *, p < 0.01; ††, p < 0.001 compared with succinate by two-way ANOVA with repeated measures for [ADP] and Tukey’s multiple comparison test.

Table 1
Respiration and metabolite and NADP concentrations in succinate (10 mM)-energized mouse hind limb skeletal muscle mitochondria incubated in the presence of 32 \( \mu \)M ADP

| Condition                  | Succinate alone | Succinate + pyruvate | Succinate + pyruvate + UK5099 |
|----------------------------|-----------------|----------------------|-------------------------------|
| Succinate (pmol O\(_2\)/mg/min) | 652 ± 19**††     | 6406 ± 238           | 1393 ± 37**                   |
| OAA (\(\mu\)M)             | 8.63 ± 0.65**†† | 0 ± 0                | 6.68 ± 0.29**                 |
| Citrate (\(\mu\)M)         | 0 ± 0**         | 5.60 ± 0.42          | 0 ± 0**                       |
| Malate (\(\mu\)M)          | 215.6 ± 6.2**   | 1641 ± 66            | 351.3 ± 7.2**                 |
| Fumarate (\(\mu\)M)        | 10.2 ± 0.40**†† | 40.0 ± 1.7           | 17.7 ± 0.19**                 |
| Succinate (mM)             | 9.53 ± 0.11**†† | 7.43 ± 0.20          | 8.87 ± 0.12**                 |
| NADH (mM)                  | 335 ± 30**††    | 1019 ± 44            | 541 ± 57**                    |
| NADH/NADH+                 | 0.083 ± 0.006** | 0.263 ± 0.026        | 0.146 ± 0.025*                |

and in the presence of pyruvate plus UK5099, a chemical inhibitor of the mitochondrial pyruvate carrier (8). Metabolite and NADH concentrations were measured in the respiratory medium obtained at the end of each run. These data show that pyruvate rescue of respiration (otherwise very low at 32 \( \mu \)M ADP) was blocked by UK5099 and the clearance of OAA to citrate was prevented. Changes in the OAA precursors (malate and fumarate) and succinate utilization were as expected based on respiration. The lower concentrations of NADH in the absence of pyruvate in the presence of pyruvate carrier inhibition are consistent with what was observed in Fig. 1F at high [ADP].

In further experiments (Table 2), succinate-energized mitochondria were isolated from mice deficient in the mitochondrial pyruvate carrier 1 (MPC1) or littermate controls in presence or absence of 0.5 mM pyruvate. Again, mitochondria were incubated for 20 min in the presence of ADP clamped at 32 \( \mu \)M. Consistent with the data for chemical inhibition of the pyruvate carrier (Table 1), pyruvate rescue of respiration required the carrier protein whereas the variations in metabolite concentrations were consistent with that observed for chemical inhibition.

OAA inhibits the extracted SDH complex

SDH activity of the extracted complex was first determined in the absence of ADP or in the presence of ADP at a low or high concentration (Fig. 3A). As opposed to the effect of ADP on succinate-driven respiration in the presence of functioning mitochondria (Fig. 1A), ADP, as expected, had no direct effect on the activity of the isolated complex. In further experiments, we found that OAA as low as 5 \( \mu \)M strongly inhibited SDH (Fig. 3B). To assess the specificity of the antibody employed in these assays, SDH was extracted by immune immobilization. The eluted protein was then run on a gradient gel and stained with Coomassie Blue (Fig. 3C). Visualization revealed bands at kDa values consistent with those expected for the four subunits of the SDH complex with little to suggest nonspecific extraction of other proteins and with a pattern very similar to that reported by the manufacturer of the antibody.

ADP impairs ROS production in succinate-energized mitochondria

Mitochondria were incubated in microplate wells with shaking within a plate reader for 20 min at different concentrations of clamped ADP in the presence or absence of pyruvate (0.5 mM). Mitochondria were energized by succinate under the same conditions used for the Oxygraph incubations of Fig. 1. \( \text{H}_2\text{O}_2 \) production under these conditions is largely generated at complex I through conversion from superoxide by MnSOD and serves as a marker for reverse electron transport (9–11). As shown in Fig. 4A, \( \text{H}_2\text{O}_2 \) production with or without pyruvate decreased markedly in incubations performed at higher [ADP]. In other experiments (Fig. 4, B–D), we measured ROS production by EPR spectroscopy, a specific signal for superoxide. Although superoxide may be generated at complex III or other sites beyond complex I, the marked decrease in superoxide at high [ADP] confirms the overall loss of ROS.

The dependence of \( \text{O}_2 \) flux on increments in [ADP] can be mimicked by increments in the concentrations of chemical uncouplers

In our prior report (3), we showed that the effect of decreasing potential with incremental ADP additions on \( \text{O}_2 \) flux could be mimicked using the chemical uncoupler, \( p \)-trifluoromethoxyphenylhydrazone (FCCP). However, we have become aware that the amount of incremental FCCP in our prior report was high compared with what has been used to titrate potential in other reports. Therefore, we reexamined this issue, using both FCCP and 2,4-dinitrophenol (DNP) to
titrate potential. As shown in Fig. S1, when these uncouplers were employed, the relationship between potential and O$_2$ flux was similar to that observed for ADP titration. We noted that the amount of FCCP needed to titrate potential to a given degree was less in this current study than in our above-mentioned past report.

Table 2  
Respiration and metabolite concentrations in succinate (10 mM) -energized mouse hind limb skeletal muscle mitochondria isolated from the mitochondrial pyruvate carrier 1 knock out (MCP1 KO) and littermate control mice incubated in the presence of 32 µM ADP

|                        | Control No pyruvate | Control 0.5 mM pyruvate | MCP1 KO No pyruvate | MCP1 KO 0.5 mM pyruvate | p value (interaction) |
|------------------------|---------------------|-------------------------|---------------------|-------------------------|-----------------------|
| Respiration (pmol O$_2$/mg/min) | 832 ± 87           | 6553 ± 185***           | 697 ± 41            | 1335 ± 45**             | p < 0.0001            |
| OAA (µM)               | 8.70 ± 0.53         | 0 ± 0***                | 10 ± 0.93           | 8.98 ± 1.2              | p < 0.0001            |
| Citrate (µM)           | 0 ± 0               | 6.58 ± 0.78***          | 0 ± 0              | 0 ± 0                   | p < 0.0001            |
| Malate (µM)            | 274 ± 33            | 1538 ± 51***           | 207 ± 10            | 342 ± 12*                | p < 0.0001            |
| Fumarate (µM)          | 14.2 ± 1.2          | 41.0 ± 1.3***          | 13.9 ± 1.3          | 18.6 ± 0.69*            | p < 0.0001            |
| Succinate (mM)         | 9.04 ± 0.16         | 7.74 ± 0.16***         | 8.97 ± 0.05         | 8.95 ± 0.09              | p < 0.0001            |

Figure 3. Immunoextraction of SDH and inhibition by OAA. A, SDH activity (∆A$_{600}$ nm) was determined after immunoextraction to immobilize the complex on the surface of microplate wells in the presence of ADP at the concentrations indicated on the x axis (mean ± S.E., n = 6 at each [ADP]). B, SDH activity determined as in panel A in the absence of ADP but in the presence of OAA at the concentrations indicated (mean ± S.E., n = 4 for each concentration). C, the SDH complex was immunoextracted on agarose beads using the same antibody (ab109908, Abcam) used for immobilization in panels A and B. The complex was eluted and run on a gradient gel stained with Coomassie Blue. E = eluate. *, p < 0.001 compared with zero OAA by one-way ANOVA.

Figure 4. ROS production by succinate-energized mitochondria. A, H$_2$O$_2$ production by mouse hind limb skeletal muscle mitochondria energized by succinate (10 mM) and incubated for 20 min at different concentrations of clamped ADP in the presence or absence of pyruvate (0.5 mM). Incubations were carried out with shaking in wells within a plate reader under the same conditions used for the Oxygraph incubations of Fig. 1. Data represent mean ± S.E., n = 5 for each point. H$_2$O$_2$ production decreased significantly and markedly with ADP addition by two-factor (treatment × [ADP]) ANOVA with repeated measures for [ADP]. There was no significant difference in H$_2$O$_2$ production at any given ADP. B-D, superoxide measured by EPR spectroscopy after incubation for 5 min, otherwise under the same conditions used for the Oxygraph incubations of Fig. 1, in the presence of ADP at the indicated concentrations.
The [ADP] dependence of succinate-supported respiration varies by muscle fiber type

Mitochondria isolated from extensor digitorum longus (EDL), soleus, and gastrocnemius muscle from normal Sprague-Dawley rats (rats were used instead of mice for ease in isolating specific muscle types) were energized with 10 mM succinate and incubated with sequential increments in clamped ADP. Fig. S2A shows that respiration reached much higher levels in mitochondria from gastrocnemius (mixed fiber type) compared with soleus (oxidative) or EDL (glycolytic). However, when respiration was expressed as a percent of state 4 (Fig. S2B), there was no difference in peak respiration between these muscle types. Interestingly, however, for the oxidative soleus mitochondria, respiration maintained a greater fractional increase over state 4 through higher concentrations of ADP. As expected, mitochondrial membrane potential decreased for all muscle types with sequential additions of ADP (Fig. S2C), while appearing initially higher in gastrocnemius mitochondria presumably because of the initial higher respiratory rate (Fig. S2A).

Discussion

Studies dating back decades describe OAA inhibition of SDH (1, 2, 12–15). However, those studies did not address the role of OAA in regulating O2 flux in intact respiring mitochondria. An older report showed that pyruvate prevented the loss of succinate-supported state 3 respiration, presumably by clearing OAA (16). That report did not actually demonstrate OAA clearance, however. Also, until our recent report (3), the biphasic dependence of succinate-supported respiration on [ADP] had not been reported.

Here we clarify the mechanism underlying this biphasic phenomenon (Fig. 5). The initial increase in respiration with incremental but lower amounts of ADP is easily understandable because of the reduction in ΔΨ as ADP phosphorylation con-

Figure 5. Schematic depicting the mechanism underlying the inhibitory effect of OAA and regulation of succinate-energized hind limb muscle mitochondrial respiration. Boxed italic text depicts relevant events. A, low ADP concentration. Membrane potential is reduced relative to state 4, so respiration is increased compared with state 4. RET is still active and ΔΨ is still high enough that NADH donation of electrons at complex I is prevented and [NADH] remains close to or only mildly lower than state 4. So, malate conversion to OAA will be minimal with little, if any, effect to inhibit SDH. Malate will accumulate and exit mitochondria through carrier proteins. B, high ADP concentration. When ADP is further incremented, the further drop in potential favors forward electron transport and RET is essentially abolished. NADH electron donation at complex I and, hence, NADH/NAD+ cycling is now enabled. Although the reaction is still kinetically to the left, some malate conversion to OAA occurs and enough OAA is generated to inhibit SDH, resulting in decreased respiration. *, only small amounts of OAA are needed to inhibit SDH. See text for further discussion.
sumes potential (4). It has long been known, that respiration increases with consumption of potential because of reduced downstream resistance to electron flow (17). In fact, in our past report we documented that ATP production, along with respiration, rises and falls with respiration as [ADP] is sequentially incremented and that the phenomenon can be mimicked by sequential addition of a chemical uncoupler (3).

On the other hand, it is more difficult to explain the decrease in respiration despite continued reduction in ΔΨ at higher ADP concentrations (Figs. 1A and 2, A and B). Our current data strongly imply that this is because of inhibition of SDH by OAA. First, OAA accumulation closely paralleled the decrease in respiration (Fig. 1B) as well as the decrease in malate and fumarate precursors (Fig. 1, C and D) consistent with OAA feedback inhibition of SDH. Second, we show that metabolism of OAA to citrate by a low concentration of added pyruvate, in uninhibited and genetically normal mitochondria, prevents the inhibition of respiration and the decline in the succinate metabolites, malate and fumarate (Tables 1 and 2). Further, this effect of pyruvate was not because of pyruvate-energized respiration because the concentration of pyruvate required for reversal was too low to generate meaningful O₂ flux on its own (Fig. 2A). Third, we show that the reversal effect of pyruvate was prevented by chemical or genetic inhibition of pyruvate uptake (Tables 1 and 2), indicating dependence on direct mitochondrial pyruvate utilization. Finally, we used modern methods to confirm decades-old reports that OAA, in fact, inhibits the extracted SDH complex and to add information indicating that it does so well within the range of the metabolite concentrations measured herein (Fig. 3B). Further, it is important to note that the activity of the extracted complex is not directly affected by ADP (Fig. 3A). This is as expected because any such effect would be dissociated from the effect of ADP to consume potential.

Although OAA inhibition can explain the post-peak decrease in succinate-supported respiration, other processes come into play. It is important to consider the following. ADP additions decrease ΔΨ. Higher ΔΨ impairs forward electron transport and favors RET (18, 19). These conditions maintain NADH in the reduced state (9, 20), in other words, impair electron donation at complex I. Conversely, lowering ΔΨ enhances forward electron transport and electron donation by NADH at complex I. Such electron donation allows NADH cycling back to NAD⁺, whereas the consequent reduction in [NADH] favors a shift in the malate < NADH equilibrium toward OAA. RET generates ROS at complex I, and H₂O₂ production is a known marker of RET (9, 21), although some degree of RET might remain in the absence of detectable ROS.

With these factors in mind, the sequence of events (over increasing [ADP]) would be as follows. As ADP is incremented to low concentrations ΔΨ drops mildly and, therefore, respiration increases. ΔΨ is still high and RET is active, however, so [NADH] is maintained, limiting electron donation by NADH at complex I and limiting NADH/NAD⁺ cycling. Hence, malate conversion to OAA will be impaired and SDH is not inhibited. As ADP is further incremented to intermediate concentrations, conditions begin to change. Although, RET (as detected by H₂O₂) decreases, OAA is still absent (or too low to detect) either because potential is still high enough or RET is still active enough that electron donation by NADH to CoQ at complex I is minimal. Given the leftward nature of the malate < OAA equilibrium (22), it is not surprising that these conditions need to change substantially to enable OAA formation. However, as ADP is further incremented to higher concentrations, the greater drop in ΔΨ will prevent RET and favor forward electron transport, now enabling NADH electron donation and NADH/ NAD⁺ cycling. OAA can now be generated in sufficient amounts to inhibit SDH and respiration. The concentrations of OAA are still small compared with malate, again consistent with the leftward nature of the malate dehydrogenase reaction, but only small concentrations of OAA are needed to inhibit SDH (Fig. 3B). The above processes at low and high [ADP] are depicted in Fig. 5.

Our data provide strong evidence for the above sequence of events. At low [ADP] potential drops (Fig. 2B), RET is active based on H₂O₂ production (Fig. 4), and NADH concentrations are maintained (Fig. 1F). At intermediate [ADP], NADH is maintained or drops only mildly (Fig. 1F). At higher [ADP], potential is further reduced (Fig. 2B), RET (H₂O₂) is low or absent (Fig. 4), NADH is lower (Fig. 1F), OAA is generated (Fig. 1B), and respiration is inhibited (Fig. 1A). Only small concentrations of OAA are needed to inhibit SDH (Fig. 3B).

Note that at high [ADP], NADH increases in response to pyruvate in genetically normal mice (Table 1). This is not the result of RET as membrane potential and ROS remain low at 32 μM ADP. However, this is easily explainable based on the clearance of OAA by pyruvate (Tables 1 and 2) because consumption of OAA would drive the kinetics of the malate/OAA reaction to the right leading to higher concentrations of NADH and because citrate, generated from OAA clearance, has been shown to exert a feed-forward effect on malate dehydrogenase (22).

Also of note is that an older report suggested that OAA may bind with extracted SDH in a long-acting fashion that is not easily reversible (23). This does not appear to hold for actively respiring muscle mitochondria, because our data show that restoration of succinate-supported respiration is rapid upon clearing OAA.

Although we previously reported the phenomenon of biphasic succinate-energized respiration (3), our current study, containing entirely new information, delineates the mechanism. Our past study allowed us to postulate a mechanism but lacked the detail needed for support. In that study, respirometry data were only obtained by sequential addition of ADP during individual runs as opposed to our current data obtained over multiple runs at sustained [ADP] throughout. Hence, we did obtain some metabolite data that was not obtained in the same mitochondrial incubates used for respiratory runs and was obtained over different time periods. In addition, in our current work we measured NADH and NAD⁺ which we did not directly determine in our past study. Further, we now show that pyruvate reversal requires only small amounts of the compound (too little to support respiration per se) and show both by chemical and genetic means that pyruvate carrier activity is essential, indicating direct dependence on its uptake. Also, we now thoroughly document changes in ROS including EPR assessment...
**Inhibition of mitochondrial complex II**

and show how they correspond to the time course of changes in respiration, potential, OAA, and citrate. Finally, we confirm, with modern technology, the effect of OAA on the activity of extracted the SDH complex.

Two aspects of our past study (3) deserve reiterating here. First, the biphasic relationship of succinate-energized respiration to [ADP] was not altered by exposure to a range of free calcium concentrations. Second, the decrease in succinate-supported respiration was not associated with opening of the mitochondrial permeability pore.

As the title of this work indicates, we use the phrase “ADP-dependent inhibition.” We could likewise call the phenomenon “ΔΨ-dependent inhibition” because potential is clamped at levels determined by [ADP] (see “Experimental procedures”) and because we were able to mimic the phenomenon with incremental amounts of the chemical uncouplers, 2,4-dinitrophenol, and FCCP (Fig. S1). However, we prefer the ADP terminology because ΔΨ is secondarily determined by ADP. To clarify, we do not prove that reduction in ΔΨ, per se, causes inhibition of complex II or rule out other effects of the chemical uncouplers. However, we provide a highly plausible scenario linking ADP effects on ΔΨ to events culminating in OAA accumulation and inhibition of SDH.

We also clarify that ADP reduces ΔΨ by providing substrate for ATP synthase, thereby utilizing ΔΨ (4). Note however that ΔΨ continues to drop at high [ADP] (Fig. 2B) even though, as shown in our previous report (3), ATP concentrations fall with respiration at high [ADP]. This is because the decrease in respiration limits electron transport and, thus, proton pumping needed to generate ΔΨ (4).

We must acknowledge that the amount of FCCP needed to create the same increments in potential in this current study was less than in our past report (3). However, the actual relationship between potential created and O2 flux is similar in the two reports (although we did not report potential in the past report). We can only speculate that somehow the FCCP in our past study was less effective, or had degraded, or conceivably that there was an error in reagent preparation.

An important question regards the physiologic relevance of the biphasic response of succinate-energized respiration to [ADP]. Although this still needs to be investigated in detail, we have reason to believe that there is, indeed, relevance. Here we show differences in this phenomenon between skeletal muscles of varying fiber type composition (Fig. S2). Moreover, in past reports, we showed tissue differences in the biphasic response to succinate (3) as well as a shift to the right in diabetic obese mice compared with controls (24). Regarding fiber type composition, it seems plausible that peak respiration in the more oxidative soleus muscle should be less than EDL or gastrocnemius (Fig. S2A) but more sustained (relative to state 4) at higher ADP (Fig. S2B). This plausibility is based on the need for sustained lower grade energy production by the oxidative muscle type.

Finally, we suggest that findings described here have important conceptual and methodological implications for future studies of respiration initiated at complex II. First assessing respiration at states 4 and 3, as commonly carried out, is not sufficient to determine peak succinate-energized mitochondrial function. Doing so will miss the peak effect at low ADP. Second, rather than use rote-processing to inhibit RET, respiration might be assessed at zero [ADP] (state 4) and at low ADP where RET, as assessed by H2O2 production, appears quite low (Fig. 4). Moreover, it is obvious that O2 flux in the presence of rotenone, or only at zero and high [ADP], is far from physiologic. Third, we emphasize that measuring respiration at clamped [ADP] is not difficult and might or should be carried out more often by methodology we employed herein (25) or by related techniques used by others (26–28). We also point out that, if desired, the 2DOG clamp procedure enables assessment of ATP production as 2DOG phosphate, a very sensitive and specific method as we have described (5, 25). Fourth, we show that OAA can effectively be measured by NMR using small amounts of isolated mitochondria avoiding the MS approach which suffers from OAA degradation (6, 7).

In summary, we provide compelling mechanistic evidence explaining a basic mitochondrial phenomenon, i.e. the biphasic dependence of succinate-energized O2 flux on ADP availability. We show that this is explained by [ADP]-dependent metabolite flow to OAA and feedback inhibition of SDH. This [ADP]-dependent action of OAA may represent a critical regulator of mitochondrial metabolism with implications toward our understanding of basic mitochondrial function.

**Experimental procedures**

**Reagents and supplies**

UK5099, ADP, and OAA were obtained from Millipore Sigma. According to the manufacturer, ADP was 99.5% pure. [13C6]succinate was purchased from Cambridge Isotope Laboratories. Otherwise, reagents, kits, and supplies were as specified or purchased from standard sources.

**Animal procedures**

Animals were maintained according to National Institutes of Health Guidelines and the protocol was approved by our Institutional Animal Care and Use Committee. Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were fed a normal rodent diet (13% kcal fat) (diet 7001, Teklad, Envigo, Indianapolis, IN) until sacrifice at age 5–9 weeks. Mice were euthanized by isoflurane overdose and cardiac puncture. Male Sprague-Dawley rats (Envigo, Indianapolis, IN) were fed a normal rodent diet until sacrifice at age 9 months. Rats were euthanized by pentobarbital.

Mice deficient in the skeletal muscle–specific mitochondrial pyruvate carrier (MPC), knockout mice (SkmKO), were generated by crossing mice with a floxed Mpc1 allele (Mpc1flfl) (29) with mice expressing Cre under control of the Myogenin promoter. Myogenin Cre selectively recombines floxed alleles in skeletal muscle (30). Confirmation of the MPC KO was documented by immunoblot analysis (Fig. S3).

**Preparation of mitochondria**

Total hind limb muscle mitochondria were prepared by differential centrifugation and purification on a Percoll gradient as we have described in the past (5). Mitochondrial integrity was assessed by cytochrome c release using a commercial kit (Cytochrome c Oxidase Assay Kit, Millipore Sigma), indicating a mean of 96% intact mitochondria over three assays, well within an acceptable range compared with mitochondrial preparations from several sources (31).
Respiration and membrane potential

All studies of mitochondrial respiration and inner membrane potential utilized freshly isolated and purified mitochondria on the day of preparation. Respiration was determined using an Oxygraph-2k high resolution respirometer (Oroboros Instruments, Innsbruck, Austria). In experiments wherein \( \Delta \Psi \) was also measured, this was carried out simultaneously with respiration using a potential sensitive tetraphenylphosphonium (TPP\(^+\)) electrode fitted into the Oxygraph incubation chamber. A TPP\(^+\) standard curve was performed in each run by adding tetraphenylphosphonium chloride at concentrations of 0.25, 0.5, 0.75, and 1 \( \mu \text{M} \) prior to the addition of mitochondria to the chamber. Mitochondria (0.1 mg/ml) were incubated at 37 °C in 2 ml of ionic respiratory buffer (105 mM KCl, 10 mM NaCl, 5 mM Na\(_2\)HPO\(_4\), 2 mm MgCl\(_2\), 10 mM HEPES, pH 7.2, 1 mM EGTA, 0.2% defatted BSA) with 5 units/ml hexokinase (Worthington Biochemical) and 5 mM 2DOG.

In all experiments, ADP was clamped (see below) at the desired concentration. In some experiments, ADP was maintained at a constant level throughout the 20-min incubation time. Although the \( O_2 \) tension in the Oxygraph drops with time, respiration is not affected until levels become very low. However, because incubations were carried out for 20 min, it was necessary to periodically open the chamber to prevent marked deterioration in the oxygen content of the medium. To illustrate this, an example Oxygraph tracing is shown in Fig. S4, wherein measurements were taken under conditions of high \( O_2 \) flux at high ADP (32 \( \mu \text{M} \)) and in the presence of pyruvate, which blocks the effect of high ADP to reduce respiration (as shown under “Results”).

In other experiments ADP was incrementally added to achieve desired sequential clamped concentrations with plateaus in respiration and potential achieved after each addition. An example of Oxygraph tracing is shown in Fig. S5.

ADP recycling and generation of the 2-deoxyglucose ATP energy clamp

We used a method we developed to carry out bioenergetic studies of isolated mitochondria under conditions of clamped ADP and membrane potential (5, 25). Mitochondrial incubations were carried out in the presence of hexokinase (HK), excess 2DOG, and varying amounts of added ADP. ATP generated from ADP under these conditions drives the conversion of 2DOG to 2DOG phosphate (2DOGDP) while regenerating ADP. The reaction occurs rapidly and irreversibly, thereby effectively clamping membrane potential determined by available ADP. This was in fact the case as we have demonstrated in the past for rat muscle (25), mouse liver (32), and mouse heart (32) mitochondria.

Immunoprecipitation and activity of SDH

We used a mitochondrial complex II immunocapture kit (Abcam, Cambridge, MA) to isolate intact complex II from mouse hind limb muscle mitochondria. The kit contains agarose beads covalently cross-linked to an mAb against SDH. Briefly, 1.4 mg of Percoll purified mouse hind limb muscle mitochondria were solubilized and centrifuged, and the supernatant applied to the agarose beads overnight at 4 °C in the presence of Halt™ protease inhibitor mixture. After washing three times, elution was carried out with 0.2 M glycine, pH 2.5, with 0.05% lauryl-maltoside, and the elution fraction was electrophoresed under reducing conditions on a Bio-Rad Criterion TGX 4–20% gradient gel at 100 V for 80 min and the gel stained with Coomassie Blue R-250.

SDH activity was determined using a kit designed for determining the complex II activity in human, mouse, rat, or bovine samples (Complex II Enzyme Activity Microplate Assay Kit, ab109908, Abcam, Cambridge, MA) which, according to the manufacturer, employs the same antibody utilized in the above-described complex II immunocapture kit. Detergent extracts were prepared from mouse hind limb muscle mitochondria previously frozen at \(-80\) °C in the presence of Halt™ protease inhibitor mixture (Thermo Fisher) and SDH captured in wells coated with mAb directed at complex II. Enzyme-driven production of ubiquinol coupled to the reduction of the dye, 2,6-dichlorophenolindophenol was monitored as the rate of decrease in absorbance at 600 nm (\( \Delta A_{600}\)) in milli-OD units/min over the linear range for 10 to 50 min.

Metabolite measurements

Metabolite measurements by NMR spectroscopy were carried out on the contents of the Oxygraph chamber after mitochondrial incubation with 10 mm \(^{13}\text{C}_4\)-succinate as substrate; hence the same media used for measuring respiration. Immediately after mitochondrial incubations, 1.5 ml of the chamber content was placed in tubes on ice and acidified with 91 \( \mu \text{L} \) of 70% perchloric acid. The solutions were then thoroughly mixed, sonicated for 30 s at a power setting of 4 watts on ice, and then stored at \(-80\) °C for up to 2 weeks. The sample tubes were then thawed on ice and centrifuged at 50,000 \( \times g \) for 20 min at 4 °C. Supernatants were removed from the centrifuge tube and 10 N KOH was added to bring the solution pH to 7.4, followed by centrifugation at 16,000 \( \times g \) for 15 min at 4 °C to remove precipitated salts. The cleared, neutralized supernatants were then stored at \(-80\) °C prior to NMR studies. For NMR sample preparations, 350 \( \mu \text{L} \) of the stored supernatant was added to 150 \( \mu \text{L} \) of 50 mm sodium phosphate, pH 7.4 in \( D_2\)O for metabolite measurement. \(^{13}\text{C}\) and \(^1\text{H}\) NMR assignments of succinate, malate, fumarate, OAA, and citrate were obtained by using standard compounds. OAA was found to be unstable with a half-life of about 14 h when tested at pH 7.4 and temperature at 25 °C. Therefore, after mitochondrial incubation, perchloric acid extraction was carried out as quickly as possible to destroy the mitochondrial enzymes and minimize the degradation of OAA. In addition, for determination of stability, known amounts of OAA were subjected to parallel incubation, perchloric acid extraction, neutralization, and storage.

Both \(^{13}\text{C}/^{1}\text{H}\) HSQC and HMQC spectra were collected at 25 °C on a Bruker Avance II 800 MHz NMR spectrometer equipped with a sensitive cryoprobe for the perchloric acid–extracted samples for quantification of metabolites of the mitochondrial incubations. All NMR spectra were processed using NMRPipe package (33) and analyzed using NMRView (34). Peak heights were used for quantification. Representative examples of the two-dimensional NMR spectra for OAA and citrate metabolite quantification are shown in Fig. S6.


**Mitochondrial ROS production as hydrogen peroxide or superoxide**

Mitochondria were incubated in microplate wells with shaking for 20 min in ionic respiratory buffer, mimicking the conditions utilized for our Oxygraph incubation. H₂O₂ production was assessed as we have described (5) using the fluorescent probe 10-acetyl-3,7-dihydroxyphenoxazine (DHPA or Amplex Red, Thermo Fisher), a highly sensitive and stable substrate for horseradish peroxidase and a well-established probe for isolated mitochondria (35). Fluorescence was measured and quantification carried out as we described previously (21).

Superoxide was determined by EPR spectroscopy with the help of our institution’s Electron Spin Resonance Facility. Purified total hind limb muscle mitochondria were prepared as described earlier. Reactions were assembled in 1.5 ml tubes containing 0.25 ml ionic respiratory buffer plus 5 units/ml superoxide dismutase (MnSOD, Sigma-Aldrich) and 0.25 ml ionic respiratory buffer plus 5 units/ml horseradish peroxidase (HRP, Sigma-Aldrich) to convert superoxide to hydrogen peroxide. Mitochondria were pre-incubated for 5 min in a 37 °C water bath. Reactions were transferred to a flat aqueous EPR cell, and the spectra were recorded at room temperature using the following instrument settings: microwave power 40 milliwatts, modulation amplitude 2 gauss, receiver gain 2×10⁵, conversion time 40.96 ms, time constant 81.92 ms, and sweep width 80 gauss/41.92 s. Spectra shown were the accumulation of seven scans. The recorded EPR signals can result from either superoxide or the hydroxyl radical. However, we have shown previously (21, 36) that the signal as we recorded from isolated mitochondria can be completely abolished by MnSOD, indicating specificity for superoxide.

**Quantification of NADH and NAD⁺**

The redox state of mitochondrial NADH (NAD) was measured using a commercially available NAD⁺/NADH Assay kit no. G9071 (Promega, Madison, WI). Samples were taken directly from the Oxygraph chamber after 20-min incubations to determine respiration. Duplicate 50 μl aliquots of chamber contents were processed according to the manufacturer’s instructions.

**Statistics**

Data were analyzed as indicated in the figure legends using GraphPad Prism (GraphPad Software, La Jolla, CA). Significance was considered at p < 0.05.

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