Redox regulation of endogenous substrate oxidation by cardiac mitochondria

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Korge, Paavo, and James N. Weiss. Redox regulation of endogenous substrate oxidation by cardiac mitochondria. Am J Physiol Heart Circ Physiol 291: H1436–H1445, 2006. First published April 14, 2006; doi:10.1152/ajpheart.01292.2005.—Reactive oxygen species (ROS) play important roles in regulating mitochondrial function, as well as in ischemia-reperfusion injury and cardioprotection. Here we show that, in the absence of exogenous substrates, cardiac mitochondria have a surprisingly large capacity to phosphorylate ADP by oxidizing endogenous substrates, provided that H$_2$O$_2$ is removed from the extramitochondrial environment and a reduced environment is maintained in the matrix. In isolated mitochondria without exogenous substrates, addition of catalase and the membrane-permeant reducing agent N-acetylcysteine (Nac) or the ROS scavenger mercaptopropionylglycine significantly increased the ability to phosphorylate added ADP, as demonstrated by 1) full recovery of membrane potential ($\Delta$ψ) and matrix volume from ADP-induced dissipation and shrinkage, 2) ADP-dependent increase in $\Delta$ψ consumption, and 3) enhanced rate of ATP synthesis. Removal of extramitochondrial H$_2$O$_2$ by catalase was required to stimulate endogenous substrate oxidation, as shown by the increase in $\Delta$ψ consumption and $\Delta$ψ. This effect was greatly enhanced by addition of Nac or mercaptopropionylglycine to suppress oxidation-induced ROS increases in the matrix. Theoretical considerations, as well as reversible inhibition of $\Delta$ψ consumption with 3-mercaptopropionic acid and pyruvate in state 3, indicate that these substrates are fatty acids. Under in vivo conditions in which powerful antioxidant conditions are maintained, this mechanism may be important in stimulation of $\beta$-oxidation and ATP production at low levels of extramitochondrial fatty acids. Incapacitation of this mechanism may potentially contribute to mitochondrial dysfunction during oxidative stress.

isolated mitochondria; redox environment; endogenous substrates; adenosine diphosphate phosphorylation

AS ENERGY PRODUCERS, the central and most important function of mitochondria is generation of a proton electrochemical gradient by respiratory chain activity, which is utilized to synthesize ATP and regulate other mitochondrial functions such as Ca$^{2+}$ uptake and matrix volume, which depend on the proton gradient. Altered ability of mitochondria to support this gradient can have a dramatic influence on cellular function and survival. The majority of available information on the regulation of the proton electrochemical gradient, which in the presence of inorganic phosphate (P$_i$) is mostly reflected by the value of membrane potential ($\Delta$ψ), has been obtained from isolated mitochondria. In vitro studies permit more rigorous control of the environment and allow function to be followed with greater accuracy than in mitochondria in vivo. However, limitations in accurate replication of the in vivo extramitochondrial environment complicate extrapolation to the in vivo situation. These difficulties include quantitative, as well as qualitative, indexes. In cardiac myocytes, for example, mitochondrial volume is about four times cytoplasmic volume, whereas isolated mitochondria in a cuvette are suspended in an extramitochondrial volume at least three orders of magnitude larger. As a result, in vitro mitochondria are typically exposed to a vast reservoir of substrates and various regulatory factors that may not be characteristic of in vivo conditions. The relatively small cytoplasmic pool of substrates immediately accessible to mitochondria, coupled with more restricted diffusion and inhibited transport under certain conditions in vivo, may lead to situations in which substrate availability becomes transiently limited and ATP production must rely on more complete $\beta$-oxidation of fatty acids already available in the matrix. Qualitatively, in vitro mitochondria are generally studied in an oxidizing environment, whereas in vivo mitochondria are generally well protected by powerful antioxidant systems.

Mimicking these conditions by limiting substrate availability and protecting the redox environment allowed us to demonstrate a surprisingly large capacity of in vitro heart mitochondria to enhance electron transport and support $\Delta$ψ during ADP phosphorylation in the absence of added substrates. These results also suggest that, under oxidizing conditions, ROS produced by fatty acid oxidation do not allow complete oxidation of fatty acids in the matrix and $\Delta$ψ consumption for ATP synthesis. Collectively, our findings show that a reduced redox environment in the cardiac cell could be an important condition for completion of fatty acid oxidation and, at the same time, for efficient consumption of the proton gradient for ATP synthesis, possibly due to inhibition of ROS-induced proton leak.

METHODS

Mitochondrial Isolation

Mitochondria were isolated from rabbit hearts by homogenization and differential centrifugation as described previously (11, 29). The final pellet was resuspended in EGTA-free buffer to yield 20–30 mg/ml of mitochondrial protein by Lowry protein assay. Freshly isolated mitochondria were characterized by coupling ratios >8 measured after they were energized with 1.5 mM pyruvate, malate, and glutamate in the presence of 5 mM P$_i$ and 0.4 mM ADP. Mitochondria were kept on ice and used within 5 h after isolation.

Experimental Conditions

All measurements were carried out using a fiber-optic spectrofluorometer (Ocean Optics) in a closed continuously stirred cuvette at room temperature (22–24°C). Mitochondria (0.4–0.6 mg/ml) and then 2.5 mM P$_i$, ADP, catalase, and Nac were added to the incubation buffer (100 mM KCl, 10 mM HEPES, pH 7.4, with Tris) containing...
Mitochondria and substrates (100%) relative to that after addition of 0.5 mM caproic acid, pyruvate, and/or citric acid cycle intermediates (5 mM each) were added. To determine the ability of mitochondria to sustain ADP phosphorylation, we added ADP in the presence or absence of exogenous substrates.

**Mitochondrial O₂ consumption.** For continuous measurement of mitochondrial O₂ consumption, the decrease in buffer O₂ content was monitored via a fiber-optic O₂ sensor inserted through a hole in the cuvette cover. This method allows simultaneous determination of O₂ consumption, Δψ, matrix volume changes, and Ca²⁺ uptake. The tip of the O₂ sensor fiber was positioned in the center of the cuvette, where it reacted to changes in PO₂. In most experiments, except those with N₂ for calibration, the surface of the buffer was exposed to room air. This may have decreased O₂ consumption rates to some extent, but because the stirring speed was constant, this small decrease was always a constant value. Also, under these conditions, addition of ADP accelerated O₂ consumption significantly, indicating that the small O₂ flux into the buffer had no major impact. Zero PO₂ was obtained by direction of a stream of N₂ through the hole in the cuvette cover, so that the stirred buffer had no contact with the air (11).

**Mitochondrial Δψ.** Tetramethylrhodamine methyl ester (TMRM, 200 nM) was included in the cuvette solution, and mitochondrial Δψ was estimated from TMRM fluorescence at 580 nm as described previously (11, 22). When used at low concentrations, TMRM does not suppress respiration (22). Mitochondrial Δψ is expressed as percentage of the TMRM fluorescence in the presence of coupled mitochondria and substrates (100%) relative to that after addition of 0.5 μM cyanide p-trifluoromethoxyphenylhydrazone or alamethicin (5 μg/ml) to fully depolarize mitochondria (0%)

**Mitochondrial swelling.** Changes in matrix volume were estimated according to a standard procedure by measurement of 90° light scattering with excitation and emission wavelengths set at 520 nm. Changes in matrix volume were compared with maximum (100%) swelling induced by addition of alamethicin at the end of the experiment.

**Mitochondrial ATP production.** Mitochondrial ATP production was evaluated by continuous monitoring of NADPH fluorescence in a coupled hexokinase-glucose-6-phosphate dehydrogenase assay as described elsewhere (2). Basic conditions for this assay were identical to those used to evaluate recovery of Δψ and matrix volume from ADP-induced dissipation and shrinkage, except enzymes/chemicals for ATP determination were present.

**ROS production.** ROS production was measured by the increase in fluorescence resulting from oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) to fluorescent dichlorofluorescein (DCF). Membrane-permeant DCFH diacetate or a chloromethyl derivative of DCFH diacetate (10 μM) was incubated with mitochondria for 20 min at room temperature and then for 1 h on ice. DCFH-loaded mitochondria were pelleted by brief centrifugation, washed, and used for evaluation of ROS production as determined by an increased rate of DCF fluorescence. The precise ROS that directly mediates DCFH oxidation is difficult to define. Our experiments showing that DCFH oxidation in mitochondria requires catalytic iron support the role of hydroxyl radicals in DCFH oxidation, as reported by several groups (27 and references therein). However, hydroxyl radical generation in the Fenton reaction requires superoxide and subsequent H₂O₂ production; therefore, an increase in DCF fluorescence in mitochondria should also reflect an increase in production of these species.

**Mitochondrial Ca²⁺ uptake.** Mitochondrial Ca²⁺ uptake was measured with a Ca²⁺-selective minielectrode (World Precision Instruments) in conjunction with a reference electrode.

**Chemicals and Data Analysis**

TMRM and DCFH diacetate or its chloromethyl derivative were obtained from Molecular Probes, 3-mercaptopropionic acid from Fluka, manganase(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP) from A. G. Scientific, and all other chemicals from Sigma. Catalase, Nac, mercaptopropionyl glycine (MPG), and MnTMPyP stock solutions were prepared in incubation buffer before each experiment. Nac and MPG solutions were adjusted to pH 7.4 with Tris. Results are presented as original traces, with summary data shown as means ± SD. Student’s t-test was used to assess statistical significance, with Bonferroni’s correction for more than two groups.

**RESULTS**

**Catalase and Nac Enhance ADP Phosphorylation From Endogenous Substrate Oxidation**

Freshly isolated mitochondria were added to KCl buffer containing BSA to reduce membrane leakiness (proton leak), and 2.5 mM P_i was added, as required for ADP phosphorylation (Fig. 1A). Under these conditions, mitochondria respiring on endogenous substrates were unable to support Δψ during repeated ADP additions, a condition used throughout this study to test the readiness of mitochondria for sustained ADP phosphorylation. Full recovery of Δψ after ADP-induced dissipation signals that >99% of ADP has been phosphorylated; i.e., the ATP-to-ADP ratio is expected to be ~100 under such conditions (15). Δψ recovery also allows matrix volume, which shrinks in proportion to Δψ dissipation, to recover due to K⁺/P_i uptake accompanied by osmotically driven water influx (9). After several additions of 10 μM ADP, however, Δψ remained dissipated, and the matrix contracted. Addition of 0.5 μM catalase had no effect, but subsequent addition of 5 mM Nac, a cell-permeant antioxidant (1, 18, 25) that can enter the matrix, resulted in a significant increase in Δψ, in parallel with acceleration of O₂ consumption and recovery of matrix volume. Subsequently, these mitochondria were able to recover from Δψ dissipation and matrix shrinkage induced by repeated 10- to 20-fold higher ADP additions. At higher ADP loads, the recovery time from Δψ dissipation and matrix volume became prolonged, with less increase in O₂ consumption. Rapid recovery of Δψ after oligomycin indicated that the inability to recover Δψ was related to consumption of the protonmotive force during ADP phosphorylation (Fig. 1A).

In separate experiments, addition of catalase alone enhanced mitochondrial uptake of contaminant Ca²⁺, in a manner independent of exogenous substrates and P_i (see below). To exclude the possibility that the effects of catalase and Nac were due to stimulation of respiration by enhanced uptake of Ca²⁺ (13, 28), in Fig. 1B we show how mitochondria from the same preparation in Fig. 1A responded to the identical protocol in the presence of EGTA. After addition of catalase and 5 mM Nac, the mitochondrial response to ADP was even more accelerated (note the more rapid increase in O₂ consumption and recovery of Δψ and matrix volume after ADP-induced dissipation than in Fig. 1A). These data indicate that the contaminant Ca²⁺ significantly decreased, rather than increased, mitochondrial ability to support Δψ during ADP phosphorylation.

The ability of mitochondria to support ADP phosphorylation with only endogenous substrates in the presence of catalase and Nac was sensitive to Nac concentration. Figure 1C shows that, in EGTA buffer, addition of 50 μM Nac after catalase promoted less rapid mitochondrial recovery in response to ADP than addition of 2.5 mM Nac.

We speculated that the endogenous mitochondrial substrates supporting ADP phosphorylation under these conditions are
Fatty acids. In this case, addition of pyruvate, which is known to suppress fatty acid oxidation and vice versa (17), might be expected to inhibit the effects of catalase and Nac, as shown in Fig. 1C. After the ability of mitochondria to recover Δψ and matrix volume had been restored by catalase and Nac, addition of pyruvate inhibited the subsequent mitochondrial response to a second 150 μM ADP challenge. After addition of pyruvate, there was no increase in O₂ consumption after addition of ADP, Δψ remained dissipated, and the matrix contracted. At this point, addition of the citric acid cycle intermediate malate (5 mM) to boost respiratory power led to full recovery. Indeed, when mitochondria were energized by citric acid cycle intermediates such as malate, fumarate, α-ketoglutarate, and succinate in addition to endogenous substrates, recovery of Δψ and matrix volume after ADP additions was robust and even more enhanced by the presence of pyruvate.

The effects of catalase and Nac on the ability of mitochondria to recover from ADP loads in seven different mitochondrial preparations are summarized in Fig. 1D. The cumulative ADP load that mitochondria could phosphorylate using endogenous substrates was defined by their ability to recover Δψ from ADP-induced dissipation after successive ADP additions. Addition of 0.5 μM catalase and 2.5 mM Nac increased cumulative ADP load dramatically from 25 ± 8 to 1,461 ± 399 nmol/mg protein (P < 0.001). Attempts to demonstrate this effect in liver mitochondria under similar conditions, however, were unsuccessful (3 preparations, data not shown).

Catalase and Nac were both required to support Δψ during ADP phosphorylation (Fig. 2), but the order of addition was unimportant. In Fig. 2A, addition of 2.5 mM Nac alone had no effect, but subsequent addition of catalase promoted Δψ recovery, matrix volume recovery, and increased O₂ consumption, allowing mitochondria to tolerate further ADP additions, similar to Fig. 1A. Once again, subsequent addition of pyruvate inhibited the ability of mitochondria to recover from ADP, unless a citric acid cycle intermediate, such as α-ketoglutarate, was also added. The inhibitory effect of pyruvate was reversed with all citric acid cycle intermediates tested: malate and succinate were the most potent, followed by α-ketoglutarate and fumarate.

To confirm further that pyruvate was acting by inhibiting fatty acid oxidation, we also tested its effect during oxidation of exogenous caproic acid, a short-chain fatty acid that rapidly enters the matrix (bypassing the highly regulated carnitine-
dependent transport of long-chain fatty acids). In the presence of exogenous caproic acid, mitochondria showed an improved ADP-phosphorylating capacity (Fig. 2B) compared with endogenous substrates alone (Fig. 2A). After addition of pyruvate, mitochondria failed to restore Δψ and matrix volume or to increase O2 consumption in response to the same amount of ADP (200 μM) until malate was also added. In contrast, in the absence of caproic acid, pyruvate oxidation supported Δψ recovery reasonably well when relatively small concentrations (<100 μM) of ADP were added, but not at higher concentrations (Fig. 2C). The latter failure depended on the amount of mitochondria added and was rapidly corrected with malate (Fig. 2C), which accounts for why isolated mitochondria have almost always been energized with a combination of pyruvate and a citric acid cycle intermediate (most frequently malate).

Figure 2D confirms directly that Nac and catalase enhanced the rate of ATP synthesis. Mitochondria responded to the first addition of 10 μM ADP with a rapid increase in ATP synthesis, consistent with their ability to recover Δψ after the first ADP addition (Figs. 1A and 2A). However, ATP synthesis after subsequent ADP additions was depressed, corresponding to the incomplete recovery of Δψ and matrix volume. Addition of 2.5 mM Nac + 0.5 μM catalase then rapidly increased ATP synthesis rate (~4-fold). Subsequent addition of 5 mM pyruvate slowed, and 5 mM malate then partly restored, ATP synthesis rate. Inset: rate of ATP production immediately before and after addition of Nac + catalase. Values are means ± SD (n = 6).

Inhibition of Fatty Acid β-Oxidation by 3-Mercaptopropionoic Acid Suppresses Effects of Nac and Catalase on State 3 Respiration

To further establish that the endogenous substrate utilization stimulated by Nac and catalase was endogenous fatty acid, we...
studied the effects of 3-mercaptopropionic acid. 3-Mercapto-
propionic acid is known to inhibit β-oxidation in rat heart
mitochondria in state 3, because its metabolites reversibly
inhibit acyl-CoA dehydrogenase, but it has no significant effect
on the citric acid cycle or oxidative phosphorylation (21). In
mitochondria incubated in the presence of BSA and EGTA, the
ability of catalase and Nac to enhance Δψ recovery and O2
consumption from ADP-induced dissipation, 5 mM Nac and then 1
μM 3-mercaptopropionic acid (Fig. 3B). The summary data in
Fig. 3C show that the average O2 consumption after addition of
150 μM ADP in the presence of 3-mercaptopropionic acid was
only <10% of control. Under those conditions, respiratory
power was insufficient to restore Δψ, which remained dissipated
(Fig. 3B). After addition of pyruvate, malate, and glutamate,
Δψ rapidly recovered, with a parallel increase in O2
consumption. The response of energized mitochondria to ad-
dition of ADP was similar to that of controls (Fig. 3B),
confirming previous findings that inhibitor has no significant
effect on the citric acid cycle, electron transport, or ADP
phosphorylation (21).

**Catalase Accelerates Electron Transport and Increases O2
Consumption Through a Mechanism That Is Further
Stimulated by Nac**

In the experimental settings described above (Figs. 1 and 2),
catalase and Nac were required to restore Δψ after dissipation
by repeated ADP additions. The relative role of these two
antioxidants in the overall mobilization of endogenous sub-
strates, however, remains unclear. To investigate this issue
further, the effects of catalase and/or Nac on Δψ, O2 consum-
tion, and matrix Ca2+ uptake were recorded under the condi-
tions described in Figs. 1 and 2, except Pi was omitted, because
Pi transiently is known to increase O2 consumption, which
accounts for the ability of mitochondria to restore Δψ during
subsequent Ca2+ pulses. Nac alone had no major effect on mitochondrial O2 consump-
tion or the ability to support Δψ during Ca2+ accumulation
(Fig. 4C). Δψ dissipation increased further with addition of 3
μM Ca2+, which apparently promoted some release of already
accumulated Ca2+. However, subsequent addition of catalase
resulted in activation of electron transport, recovery of Δψ,
and enhancement of Ca2+ uptake. Collectively these results
showed that catalase is responsible for activating electron
transport and that its effect is significantly enhanced by mem-
brane-permeable Nac, which by itself is not able to enhance
electron transport and support Δψ.

**Possible Role of ROS**

We next explored the mechanisms by which catalase and
Nac allowed increased β-oxidation of endogenous fatty acids
to support Δψ and ADP phosphorylation. Addition of mem-
brane-impermeant catalase to isolated mitochondria is ex-
pected to scavenge H2O2 outside mitochondria, whereas mem-
brane-permeant Nac is expected to prevent oxidation of im-
portant sulfhydryl (SH) groups in the matrix. In isolated
mitochondria, the matrix of which was loaded with the fluo-
rescent ROS sensor DCFH, catalase increased DCFH oxida-

![Fig. 3. 3-Mercaptopropionic acid (3-Merc) inhibits ADP-stimulated O2 consumption in the presence of Nac and catalase. Mitochondria (0.5 mg/ml) were added to KCl buffer containing 0.5 mg/ml BSA and 0.1 mM EGTA, 2.5 mM Pi was added, and then ADP was added in 10 μM increments. After Δψ failed to recover from ADP-induced dissipation, 5 mM Nac and then 1 μM catalase were added. Subsequent additions of ADP induced increases in O2 consumption that are characteristic of coupled, energized mitochondria. B: experiment described in A repeated in the presence of the β-oxidation inhibitor 3-mercaptopropionic acid (200 μM), which severely depressed ADP-stimulated O2 consumption. Addition of pyruvate, malate, and glutamate (Glu) normalized mitochondrial response to ADP. C: rate of O2 consumption after addition of 150 μM ADP in the absence (control, taken for 100%) or presence of 200 μM 3-mercaptopropionic acid. Values are ± means ± SD (n = 5). Dashed lines in A and B show O2 consumption rates after 150 μM ADP (used for statistical analysis). Note that O2 consumption traces are inverted.](http://ajpheart.physiology.org/)

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tion, indicating increased ROS production in the matrix. We determined ROS production by mitochondria with an ROS sensor located inside the mitochondrial matrix. A sensor outside the matrix would be expected to indicate reduced ROS from H$_2$O$_2$ depletion by catalase. Matrix DCFH oxidation increased further after addition of caproic acid (Fig. 5A). When the order was reversed, caproic acid increased DCFH oxidation, which was further potentiated by catalase (Fig. 5B). If it is assumed that DCFH oxidation accurately reflects ROS production, these results indicate that catalase and caproic acid increased matrix ROS production and that their effects were additive. A possible interpretation of these findings is that removal of H$_2$O$_2$ by catalase stimulates H$_2$O$_2$ efflux, decreasing matrix H$_2$O$_2$ and relieving its inhibition of electron transport chain/fatty acid oxidation. With increased electron transport, however, ROS production in the matrix increased. However, we cannot exclude direct effects of the probe itself on respiratory chain activity and enzymes in the matrix connected with ROS generation. Interestingly, the effect of catalase and caproic acid on matrix DCFH oxidation rate was significantly less when pyruvate and citric acid cycle intermediates were present, indicating increased ROS production in the matrix. We determined ROS production by mitochondria with an ROS sensor located inside the mitochondrial matrix. A sensor outside the matrix would be expected to indicate reduced ROS from H$_2$O$_2$ depletion by catalase. Matrix DCFH oxidation increased further after addition of caproic acid (Fig. 5A). When the order was reversed, caproic acid increased DCFH oxidation, which was further potentiated by catalase (Fig. 5B). If it is assumed that DCFH oxidation accurately reflects ROS production, these results indicate that catalase and caproic acid increased matrix ROS production and that their effects were additive. A possible interpretation of these findings is that removal of H$_2$O$_2$ by catalase stimulates H$_2$O$_2$ efflux, decreasing matrix H$_2$O$_2$ and relieving its inhibition of electron transport chain/fatty acid oxidation. With increased electron transport, however, ROS production in the matrix increased. However, we cannot exclude direct effects of the probe itself on respiratory chain activity and enzymes in the matrix connected with ROS generation. Interestingly, the effect of catalase and caproic acid on matrix DCFH oxidation rate was significantly less when pyruvate and citric acid cycle intermediates were present (Fig. 5C).

In mitochondria utilizing endogenous fatty acids, Nac (Fig. 5D) decreased catalase-stimulated DCFH oxidation and also blunted the further increase after caproic acid addition. We take these data to indicate that the catalase-induced increase in ROS production subsequently inhibits oxidation of endogenous substrates, thereby limiting Δψ recovery in the presence of ADP, unless the effects of ROS are also neutralized (e.g., by Nac). In contrast, when ample exogenous substrates are present, Δψ is more firmly supported and less sensitive to ROS accumulation.

To confirm the importance of ROS, we also tested the membrane-permeant ROS scavenger MPG. MPG (5 mM) was as effective as Nac in suppressing increased matrix DCFH oxidation induced by catalase or caproic acid (Fig. 5E) but less effective than Nac in restoring Δψ and matrix volume after an ADP load (Fig. 6). Once mitochondria failed to respond to ADP additions, Nac further improved their ability to enhance substrate oxidation in state 3 required for Δψ and matrix volume recovery (Fig. 6). Superoxide dismutase (SOD) was ineffective in replacing catalase in combination with Nac (data not shown), as might be expected, because SOD activity is high in the matrix already. It has been estimated that the steady-state matrix concentration of superoxide is about two orders of magnitude lower than that of H$_2$O$_2$ (5).

We also tested the cell-permeable SOD mimetic MnTMPyP, which increases superoxide dismutation. Preloading of mitochondria with 10–50 μM MnTMPyP had little effect on catalase-induced DCFH oxidation (results not shown). This finding is not surprising, if we consider the chemistry of DCFH oxidation (see below).

To investigate further the cause of the catalase-induced increase in DCF fluorescence, additional experiments were performed in isolated mitochondria loaded with a chloromethyl derivate of DCFH diacetate, which is better retained than DCFH and is more suitable for long incubations. Figure 7A shows that when mitochondria were energized with caproic acid, the subsequent addition of catalase increased DCF fluorescence, as shown in Fig. 5. Mitochondria were then subjected to anoxia by infusion of N$_2$ into the tightly closed cuvette, resulting in a decrease in buffer O$_2$ content (Fig. 7A, bottom trace). After O$_2$ fell below the sensitivity of the O$_2$ electrode, the DCF fluorescence rate decreased, consistent with O$_2$ levels...
that are inadequate to support ROS generation (5, 8). With reoxygenation, the DCFH oxidation rate increased again (Fig. 7A). Similarly, in Fig. 7B, caproic acid-energized mitochondria were subjected to anoxia and then energized with catalase. In the O2-depleted state, catalase had a small effect on DCF fluorescence, which increased significantly once buffer O2 was increased. These findings further validate the belief that the changes in DCF fluorescence track ROS production and also indicate a modest nonspecific effect (4, 12, 16). Figure 7B also shows that, in the presence of O2, addition of the complex I inhibitor rotenone increased DCF fluorescence, suggesting that complex I was the source of increased ROS production. Further addition of complex IV substrates tetramethyl-p-phenylenediamine and ascorbic acid rapidly increased O2 consumption and inhibited further DCFH oxidation. DCFH oxidation remained inhibited after NaN3 addition, suggesting that a change in the redox environment after addition of exogenous reducing substrates was responsible.

Figure 7C shows that H2O2 and catalytic metal are required to promote the increase in DCF fluorescence. H2O2 increased DCF fluorescence in a concentration-dependent manner, supporting the conclusion that DCFH oxidation is >80% sensitive to H2O2 (6). However, in the presence of the iron chelator desferoxamine, the rapid increase in DCF fluorescence induced by H2O2 was severely depressed. Involvement of iron-H2O2 in DCFH oxidation, first demonstrated in a cell-free cuvette assay 15 years ago (19) but subsequently not always acknowledged, suggests a complex chemistry for mitochondrial DCFH oxidation and explains how an increased rate of H2O2 production could enhance DCF fluorescence in the matrix.

**DISCUSSION**

In this study, we demonstrate for the first time that isolated heart mitochondria have a rather significant intrinsic ability to support ROS generation during ADP phosphorylation in the absence of exogenous substrates. This ability to oxidize endogenous substrates in response to ADP loading can be demonstrated only under specific conditions, i.e., after addition of catalase and Nac, which are expected to reduce H2O2 accumulation and preserve a reducing environment on both sides of the inner membrane. Although not investigated in the present study, a decrease in ROS production is expected to decrease also proton leak of the inner membrane (3), which could have a significant effect on ADP phosphorylation. Under normal resting conditions, heart mitochondria are known to oxidize primarily fatty acids, such that termination of respiration during rapid heart extraction and cooling is likely to leave significant amounts of activated fatty acids trapped in the matrix. Total intramitochondrial CoA is ~3 mM, and during maximal β-oxidation of fatty acids, 90–95% of CoA is acylated (7). If we assume a matrix volume of 1 μl/mg, 1 mg of mitochondria may contain ~2
nmol (1.2 × 10^{15} molecules) of activated long-chain fatty acids. Complete oxidation of this amount would allow phosphorylation of ~1.5 × 10^{17} ADP molecules, which is reasonably close to the amount we observed after addition of catalase and Nac (phosphorylation of 1.4 μmol of ADP translates to 8 × 10^{17} molecules of ADP; Fig. 1D). It seems plausible that the explanation for the effect of catalase + Nac (Fig. 1) is activation of intramitochondrial substrate oxidation related to addition of ADP. Apparently, redox conditions generated by catalase and Nac allow much more complete fatty acid oxidation in response to ADP, so that phosphorylation is inhibited in parallel with depletion of fatty acids trapped in the matrix. Although it is not possible to extrapolate our results directly to in vivo conditions, it is possible that a similar protection of functionally important SH groups exists inside healthy cardiac myocytes, in which powerful antioxidant systems such as catalase, glutathione peroxidase, and glutathione reductase maintain low H_{2}O_{2} levels and a reduced environment. This could mean that fatty acid oxidation in cardiac cells is redox dependent, such that, in a reduced environment, low concentrations of activated fatty acids can be efficiently utilized for ATP production, whereas in an oxidized environment, fatty acid transport into the mitochondria is required to support the same ATP production rate.

One caveat is that in vivo cytoplasmic O_{2} concentration is only a few micromoles (10); i.e., heart mitochondria are protected from the high O_{2} concentrations to which isolated mitochondria are typically exposed. On the basis of in vitro experiments, it is also difficult to understand how such high rates of respiration are maintained at low-micromolar O_{2} concentrations.

**Mechanism of Catalase + Nac Effects**

The finding that catalase + Nac was required to enhance ADP phosphorylation in mitochondria without exogenous sub-

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**Fig. 6.** Effects of catalase + MPG (in place of Nac) on ability of mitochondria to oxidize endogenous substrates in response to ADP loads. Mitochondria (0.6 mg/ml) were incubated in KCl buffer containing 0.5 mg/ml BSA. In the presence of 2.5 mM Pi, Δψ and matrix volume failed to recover after 4 additions of 10 μM ADP. Addition of 1 μM catalase had no effect on Δψ, matrix volume, or O_{2} consumption, but subsequent addition of 5 mM MPG led to Δψ and matrix volume recovery and enhanced subsequent recovery after 50 μM ADP additions. Recovery from even higher (50–200 μM) ADP loads was more effectively supported by subsequent addition of Nac than MPG. Experiment was repeated with 2 different preparations.

**Fig. 7.** Effects of O_{2} depletion on DCFH oxidation rates in caproic acid-energized mitochondria exposed to catalase. DCFH chloromethyl derivate-loaded mitochondria (0.5 mg/ml) were energized with caproic acid and incubated under conditions described in Fig. 4 legend, and changes in DCF fluorescence and buffer O_{2} content were simultaneously recorded. A: addition of 1 μM catalase before O_{2} depletion of mitochondria. B: addition of catalase after O_{2} depletion of buffer. O_{2} was depleted by gassing the cuvette with N_{2} and relieved by exposing the cuvette to air. Other additions are as follows: 2 mM ascorbic acid (AA), 30 μM tetramethyl-p-phenylenediamine (TMPD), 10 μM rotenone (Rot), and 1 mM NaN_{3}. C: DCFH oxidation by H_{2}O_{2} depends on availability of chelatable iron. DCFH-loaded mitochondria were incubated in KCl buffer and exposed to 500 μM H_{2}O_{2}, which resulted in rapid increase of DCF fluorescence. This H_{2}O_{2}-induced increase in fluorescence was avoided in KCl buffer containing 5 mM desferroxamine mesylate (Desf).
strates implies that removal of H$_2$O$_2$ and preservation of a reduced redox state were required. Catalase enhanced matrix DCFH fluorescence associated with endogenous or exogenous fatty acid oxidation (Fig. 5). This was a rather unexpected finding. We confirmed that the major component of DCFH oxidation was directly related to ROS production, but we also found that DCFH oxidation in the matrix was somewhat sensitive to nonspecific factors (Fig. 7). Consistent with its effect on increasing DCFH oxidation, catalase also increased $\Delta$$\psi$, which can be explained by relief of electron transport inhibition due to increased H$_2$O$_2$ efflux. St.-Pierre et al. (26) showed that isolated heart mitochondria respiring on palmitoyl carnitine generated H$_2$O$_2$ at a much higher rate than mitochondria respiring on pyruvate and malate. ROS production was enhanced without addition of any inhibitors and originated from the matrix, because addition of SOD had no significant suppressant effect. To account for enhanced ROS production during fatty acid oxidation, St.-Pierre et al. proposed greater steady-state reduction of complex I or additional superoxide production by electron transfer flavoprotein and electron transfer flavoprotein quinone oxidoreductase, which could act as potential sources of ROS production. Our findings are consistent with the belief that complex I is the site of increased ROS production by endogenous fatty acids in the presence of catalase, because rotenone enhanced the rate of DCFH oxidation by catalase (Fig. 7B). Because catalase is too large to enter the intermembrane space or matrix, it presumably acts indirectly, by lowering extramitochondrial H$_2$O$_2$ concentration and creating a sink for H$_2$O$_2$ efflux from the matrix. Lowering matrix H$_2$O$_2$ per se would be expected to reduce DCFH oxidation, but because the opposite occurred, we hypothesize that the decrease in H$_2$O$_2$ in the vicinity of mitochondria accelerates ROS production in mitochondria.

Nac, on the other hand, is known to penetrate membranes (1, 18, 25) and can inhibit matrix DCFH oxidation (Fig. 5C). As demonstrated in Fig. 4, catalase by itself activated endogenous fatty acid oxidation, but Nac significantly enhanced the effect of catalase but had little effect on its own. One of the expected effects of Nac would be an increase in the amount of free CoA (CoA-SH) in the matrix, which would accelerate $\beta$-oxidation flux and also acetyl-CoA oxidation in the citric acid cycle. A decrease in the available CoA-SH pool, coupled with insufficient concentration of citric acid cycle intermediates, would suppress fatty acid oxidation. The dramatic additive increase in matrix ROS by fatty acid oxidation and catalase would further decrease CoA-SH. As a reducing agent, Nac may relieve this inhibition by regenerating CoA-SH. It is interesting that the membrane-permeant ROS scavenger MPG was unable to fully substitute for Nac when catalase was present (Fig. 6). This may favor the CoA-SH explanation, because Nac directly regenerates CoA-SH, whereas MPG only indirectly inhibits CoA-SH oxidation by decreasing ROS and, therefore, may be less efficient.

A highly reduced state could also inhibit permeability transition pore flickering, such that the membrane would hyperpolarize and superoxide production would increase as proposed recently to explain the role of glutathione in the modulation of H$_2$O$_2$ production (24). Flickering would be stimulated by uptake of the contaminant Ca$^{2+}$ (30), which could explain the effect of EGTA (Fig. 1B). However, cyclosporin A, which is expected to inhibit pore flickering, did not mimic the effect of catalase + Nac.

Inhibition of fatty acid oxidation by pyruvate, and vice versa, can be explained by the well-known reciprocal relation between these oxidation pathways, inasmuch as an increase in the mitochondrial ratio of acetyl-CoA to CoA-SH inhibits the pyruvate dehydrogenase complex (17, 20) and also $\beta$-oxidation (7).

Physiological Implications

We found that endogenous fatty acids were unable to maintain $\Delta$$\psi$ and matrix volume required to support vigorous ADP phosphorylation unless a reduced redox environment was protected by catalase and Nac. A similar protection of functionally important SH groups inside healthy cardiac myocytes is normally provided by powerful antioxidant systems, such as catalase, glutathione, glutathione peroxidase, and glutathione reductase, to maintain low H$_2$O$_2$ levels and a reduced environment. Interestingly, a decrease in H$_2$O$_2$ production by overexpression of catalase targeted to mitochondria has recently been shown to increase the life span of mice (23). The stimulation of $\beta$-oxidation of endogenous fatty acids in phosphorylating mitochondria by catalase and Nac suggests that $\beta$-oxidation flux is regulated by the redox environment. Moreover, in an oxidizing environment, much higher intramitochondrial fatty acid levels are required to phosphorylate the same amount of ADP. The extent to which this redox-dependent control is physiologically important, however, remains a matter of speculation, because fatty acid channeling and $\beta$-oxidation regulation are complex.

It is interesting that, in isolated mitochondria, pyruvate alone had a limited ability to support $\Delta$$\psi$ during sustained ADP phosphorylation, which could be overcome by providing citric acid cycle intermediates, such as malate (Fig. 2C). Recently, studies in pyruvate-energized skeletal muscle mitochondria showed that the ADP-induced increase in O$_2$ consumption was only 2% of the increase in mitochondria energized with pyruvate and malate (1 mM each). These findings suggest that isolated muscle mitochondria may contain little or no citric acid cycle intermediates (14). In cardiac cells, relative loss of citric acid cycle intermediates has shown to limit contractile function (20).

In summary, in the absence of exogenous substrates, cardiac mitochondria have a surprisingly large capacity to oxidize endogenous substrates in response to a decrease in $\Delta$$\psi$, provided that the extramitochondrial environment removes H$_2$O$_2$ and a reduced environment is maintained in the matrix. This mechanism may be important for maintaining $\beta$-oxidation flux and ATP production if mitochondrial uptake of fatty acids is acutely inadequate, which might occur during sudden increases in cardiac work or during ischemia. On the other hand, prooxidant conditions during ischemia-reperfusion are expected to disable this mechanism and, potentially, contribute to mitochondrial dysfunction in this setting.

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