THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 6, Issue of February 6, pp. 4166–4174, 2004

Printed in U.S.A.

Respiration-dependent Removal of Exogenous H_2O_2 in Brain Mitochondria

INHIBITION BY Ca^{2+}*

Received for publication, July 25, 2003, and in revised form, November 19, 2003
Published, JBC Papers in Press, November 20, 2003, DOI 10.1074/jbc.M308143200

Franco Zoccarato‡, Lucia Cavallini, and Adolfo Alexandre

From the Department of Biological Chemistry and Consiglio Nazionale delle Ricerche, Unit for the Study of Biomembranes, University of Padova, Viale G. Colombo 3, Padova 35121, Italy

In brain mitochondria, state 4 respiration supported by the NAD-linked substrates glutamate/malate in the presence of EGTA promotes a high rate of exogenous H_2O_2 removal. Omitting EGTA decreases the H_2O_2 removal rate by almost 80%. The decrease depends on the influx of contaminating Ca^{2+}, being prevented by the Ca^{2+} uniporter inhibitor ruthenium red. Arsenite is also an inhibitor (maximal effect ~40%, IC_{50}, 12 μM). The H_2O_2 removal rate (EGTA present) is decreased by 20% during state 3 respiration and by 60–70% in fully uncoupled conditions. H_2O_2 removal in mitochondria is largely dependent on glutathione peroxidase and glutathione reductase. Both enzyme activities, as studied in disrupted mitochondria, are inhibited by Ca^{2+}. Glutathione reductase is decreased by 70% with an IC_{50} of about 0.9 μM, and glutathione peroxidase is decreased by 38% with a similar IC_{50}. The highest Ca^{2+} effect with glutathione reductase is observed in the presence of low concentrations of H_2O_2. With succinate as substrate, the removal is 50% less than with glutamate/malate. This appears to depend on succinate-supported production of H_2O_2 by reverse electron flow at NADH dehydrogenase competing with exogenous H_2O_2 for removal. Succinate-dependent H_2O_2 is inhibited by rotenone, decreased $\Delta\Psi$, as described previously, and by ruthenium red and glutamate/malate. These agents also increase the measured rate of exogenous H_2O_2 removal with succinate. Succinate-dependent H_2O_2 generation is also inhibited by contaminating Ca^{2+}. Therefore, Ca^{2+} acts as an inhibitor of both H_2O_2 removal and the succinate-supported H_2O_2 production. It is concluded that mitochondria function as intracellular Ca^{2+}-modulated peroxide sinks.

It is generally believed that the mitochondrial electron transfer chain is one of the major cellular generators of reactive oxygen species (ROS),[1] which include superoxide ($O_2^{\cdot-}$), H_2O_2, and the hydroxyl free radical $OH^{\cdot}$ (1–3). The mitochondrial production of ROS is supposed to be important in the aging process and in the pathogenesis of neurodegenerative diseases such as Parkinson's disease (4, 5). Furthermore, evidence has been presented recently that diabetic complications may be secondary to hyperglycemia-induced generation of ROS by mitochondria (6). It was found that some electrons leak out from accumulating unstable intermediates of the respiratory chain, performing a partial reduction of molecular oxygen, generating $O_2^{\cdot-}$, which is in turn rapidly dismutated by Mn-superoxide dismutase to H_2O_2 (7, 8). There is, however, some controversy as to whether mitochondria are an important source of ROS under physiological and pathological conditions (9, 10). H_2O_2 production has been ascribed to Complex I and Complex III of the respiratory chain, being induced in deenergized mitochondria by succinate in the presence of antimycin or by NAD-linked substrates in the presence of rotenone (11–14). Some studies were conducted more recently in coupled mitochondria in the absence of respiration inhibitors. In these, it was shown that H_2O_2 is produced during controlled (state 4) succinate oxidation (succinate feeds electrons to Complex II, which in turn reduces Complex III) and that such production is abolished by decreasing $\Delta\psi$ (*i.e.* during ADP-stimulated respiration, state 3), and oddly, also in the presence of the Complex I inhibitor rotenone (15–20). These results are a strong evidence that succinate-supported H_2O_2 production in coupled mitochondria does not derive from Complex III (as with antimycin) but rather from energy-dependent reverse electron transfer to Complex I and autooxidation of some carrier therein, either iron-sulfur centers (21–23) or the active site flavin (18) whose reduction is prevented by rotenone when the electrons originate from succinate. Some experiments were reported using NAD-dependent substrates, and it appeared that little or no H_2O_2 was produced in these conditions during state 4 respiration (19, 20).

In this study, we analyze a previously undescribed aspect of H_2O_2 handling by mitochondria, namely their ability to remove exogenously supplied H_2O_2. We demonstrate that H_2O_2 is actively removed by respiring mitochondria and identify a physiologically significant inhibitory control exerted by Ca^{2+} on such removal. We also report on the properties and controls of mitochondrial production of H_2O_2 and figure out the balance between H_2O_2-removing and H_2O_2-producing processes in non-inhibited respiring mitochondria. The possibility that mitochondria may be engaged in active removal of H_2O_2 has been suggested some years ago (24).

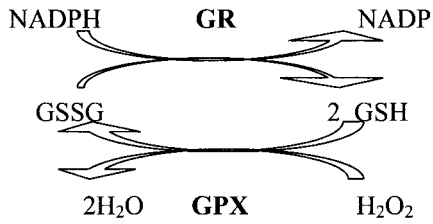
EXPERIMENTAL PROCEDURES

Reagents—Scopoletin (7-hydroxy-6-methoxy-2H-1-benzopyran-2-one), bovine serum albumin (essentially fatty acid free), the Ca^{2+} indicator Fura-2, pentapotassium salt, and glutathione reductase (EC 1.6.4.2) were supplied from Sigma. Peroxidase from horseradish (HRP) (grade I, EC 1.11.1.7) was from Roche Applied Science. The Ca^{2+} fluorescent dye

* This work was supported by 60% Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) funds and MURST Grant 2001-063932. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 39-049-8276147/6; Fax: 39-049-8073310; E-mail: franco.zoccarato@unipd.it.

[1] The abbreviations used are: ROS, reactive oxygen species; HRP, horseradish peroxidase; MOPS, 4-morpholinepropanesulfonic acid; GR, glutathione reductase; GPX, glutathione peroxidase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; RR, ruthenium red.



SCHEME 1. GR activity was monitored as detailed in the legend for Fig. 8. GPX activity was measured by following NADPH oxidation with GSH and H_2O_2 and the further presence of commercial dialyzed GR (0.4 μg/1.6 ml:0.4 units/1.6 ml).

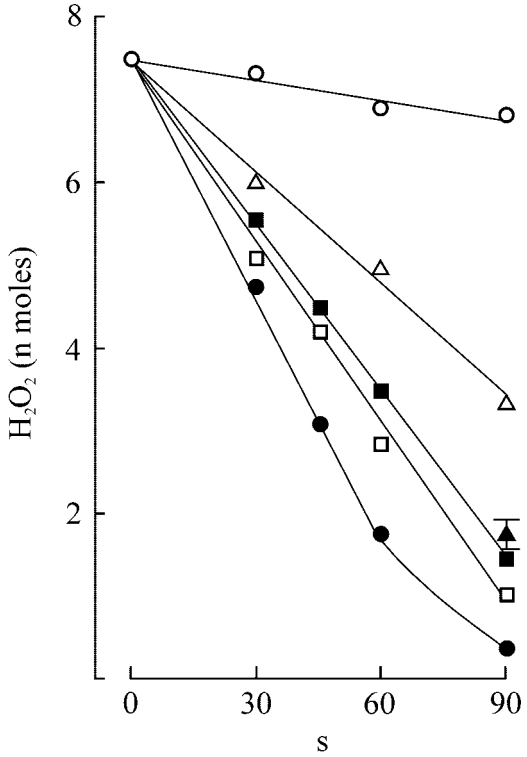


FIG. 1. **Respiration-supported removal of H_2O_2.** Mitochondria (0.85 mg/1.6 ml) were incubated in standard incubation medium containing EGTA (375 μM). H_2O_2 (8 nmol) was added at 2 min. Residual H_2O_2 was determined at selected times as detailed under "Experimental Procedures." Further additions are as follows: malonate (0.3 mM) and FCCP (3 μM) (○); glutamate (2 mM) and malate (2 mM) (●); succinate (2 mM) (△); succinate followed by rotenone (1.5 μM) added 20 s prior to H_2O_2 (□); succinate, glutamate, and malate (■); succinate followed by ruthenium red (1 μM) added 20 s prior to H_2O_2 (▲). Data points from a single experiment are representative of at least seven independent experiments.

TABLE I
H_2O_2 removal by respiring mitochondria in different metabolic conditions

Incubations are as shown in Fig. 2. The results are taken from 10 mitochondrial preparations. Data are means ± S.E. derived from at least five independent experiments.

	H_2O_2 removal
	nmol/mg × min
Malonate and FCCP	0.3 ± 0.2
Glutamate/malate and EGTA	6.7 ± 0.6
Glutamate/malate, EGTA, and ADP (1.5 mM)	5.6 ± 0.5
Glutamate/malate, EGTA, and FCCP	2.6 ± 0.6
Glutamate/malate	1.4 ± 0.4
Glutamate/malate, EGTA; ruthenium red at 45 s, $CaCl_2$ at 1 min	6.5 ± 0.5
Succinate, EGTA	3.2 ± 0.4
Succinate, EGTA, and ADP	3.5 ± 0.4
Succinate, EGTA, and FCCP	0.9 ± 0.4
Succinate, EGTA, and rotenone	5.4 ± 0.5
Succinate, EGTA, and glutamate/malate	4.8 ± 0.5
Succinate	1.2 ± 0.4

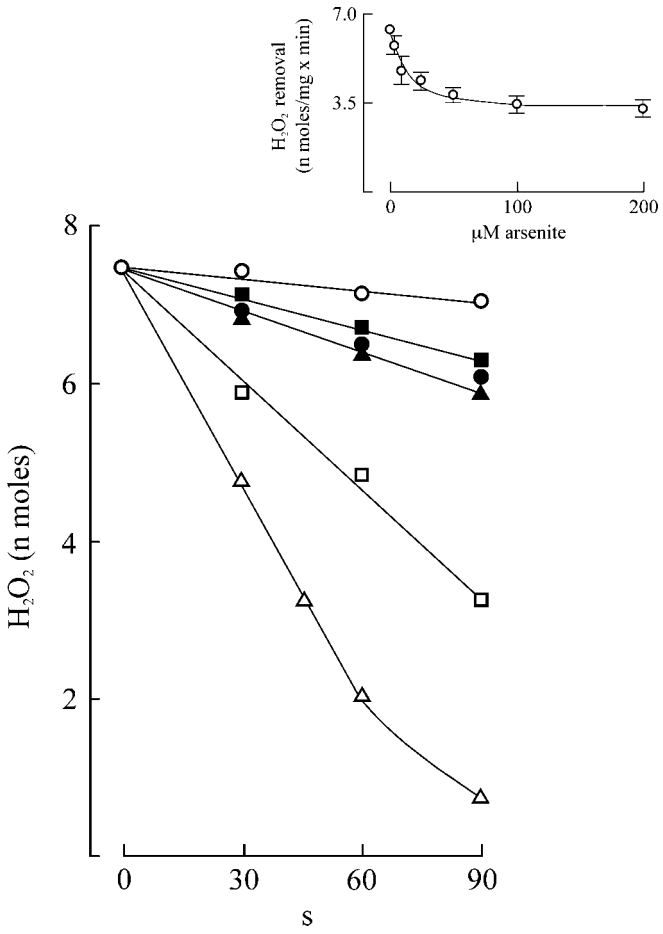


FIG. 2. **Ca^{2+} and arsenite inhibit the respiration-supported removal of H_2O_2.** Mitochondria were incubated in standard incubation medium. Further additions are as follows: malonate and FCCP (○); glutamate and malate (●); glutamate/malate, EGTA, and $CaCl_2$ (375 μM) (▲); glutamate/malate and EGTA followed by ruthenium red at 45 s and $CaCl_2$ at 1 min(△); glutamate/malate, EGTA, and arsenite (50 μM) (□); glutamate/malate, arsenite, EGTA, and $CaCl_2$ (■). Data points from a single experiment are representative of at least five independent experiments. *Inset*, dose-response effect of the arsenite inhibition of glutamate/malate-supported H_2O_2 removal. EGTA is present. The data ± S.E. are collected from three preparations, with at least four determinations per each arsenite concentration.

Calcium-Green-5N was from Molecular Probes. All other reagents were of analytical grade.

Preparation of Rat Brain Mitochondria—The cerebral cortices of two 6–7-week-old rats were rapidly removed into 20 ml if ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, 0.5 mM EDTA, 0.05 mM EGTA, pH 7.3) and homogenized. The homogenate was centrifuged at 900 × *g* for 5 min at 4 °C. The supernatant was centrifuged at 8500 × *g* for 10 min, and the resulting pellet was resuspended in 1 ml of isolation medium. This was layered on a discontinuous gradient consisting of 4 ml of 6% Ficoll, 1.5 ml of 9% Ficoll, and 4 ml of 12% Ficoll (all prepared in isolation medium) and centrifuged at 75,000 × *g* for 30 min. The myelin, synaptosomal, and free mitochondrial fractions formed respectively above the 6% layer, as a doublet within the 9% layer and as a pellet. The pellet was resuspended in 250 mM sucrose, 10 mM K-HEPES, pH 7.2, and centrifuged at 8500 × *g* for 15 min before being resuspended in this last medium to 10–20 mg of protein/ml by the Gornall protein assay. The mitochondria were well coupled as judged by the increment of the oxygen consumption rate upon the addition of ADP (respiratory control ratio), which was between 3.5 and 7.0 with glutamate/malate as substrates. Oxygen consumption was monitored with a Clark-type oxygen electrode.

Standard Incubation Procedure—Mitochondria (0.4–0.6 mg/ml)

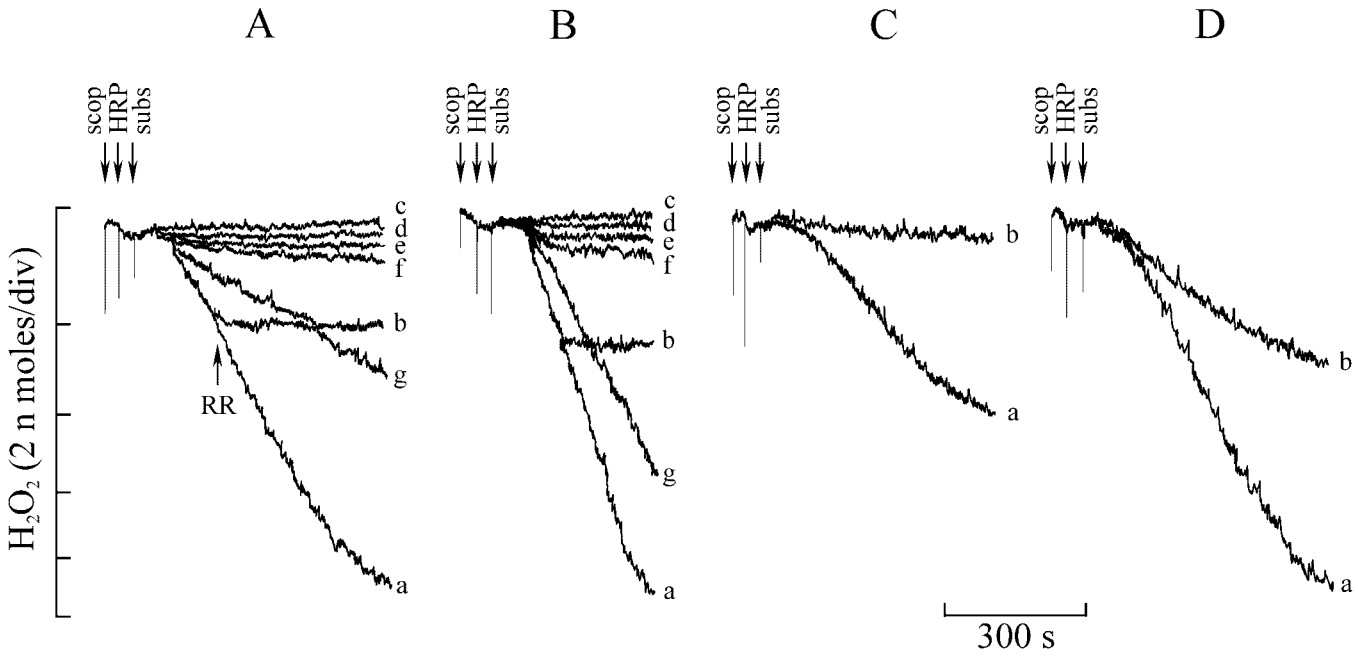


FIG. 3. **H_2O_2 production by respiring mitochondria.** Mitochondria (0.7 mg/1.6 ml) were incubated in standard medium. Scopoletin (*scop*), HRP, and substrates (*subs*) were added as indicated. *A*, succinate (2 mM) and EGTA (*trace a*); succinate and EGTA, RR added at the *arrow* (*trace b*); succinate, no EGTA (*trace c*); glutamate/malate, EGTA (*trace d*); succinate, EGTA, and ADP (*trace e*); succinate, EGTA, and rotenone (*trace f*); succinate, glutamate/malate, EGTA (*trace g*). *B*, same as *panel A*, with arsenite (50 μM). *C*, succinate (0.6 mM) EGTA (*trace a*); succinate, EGTA, and glutamate/malate (*trace b*). *D*, same as *panel C*, with arsenite. Traces are representative of duplicate traces from at least three independent experiments.

were incubated at 30 °C in a medium containing 125 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 500 μg/ml defatted bovine serum albumin, 20 mM MOPS (pH 7.2, adjusted with KOH).

Hydrogen Peroxide Measurements—Mitochondrial H_2O_2 removal and H_2O_2 release were assessed by the oxidation of scopoletin by horseradish peroxidase in the presence of H_2O_2 (1, 25). Scopoletin fluorescence was monitored at excitation and emission wavelengths of 365 and 450 nm, respectively, on a Shimadzu RL-5000 spectrofluorometer in a stirred cuvette thermostatted at 30 °C. For measurements of H_2O_2 consumption, H_2O_2 was added to respiring mitochondria generally at 3 min of incubation, and residual H_2O_2 was determined by the addition of scopoletin (5 μM) followed 20 s later by HRP (15 μg/ml, 3.75 units). The interval between scopoletin and HRP was necessary to allow stabilization of the fluorescence signal after scopoletin. In the absence of HRP, the scopoletin fluorescence remained unmodified for over a minute. HRP promoted an immediate fluorescence decrease, which was proportional to H_2O_2. After reaching equilibrium, each trace was calibrated with standard H_2O_2 additions. The H_2O_2 calibration scale is slightly non-linear, and the overall fluorescence decrease in each trace was compared with a calibration scale obtained by the addition of known amounts of H_2O_2 to mitochondria that were treated with HRP plus scopoletin. The addition of HRP to a scopoletin containing medium in the absence of H_2O_2 gave a small non-specific fluorescence variation, which was subtracted from each curve to obtain the correct measurements. The zero time H_2O_2 concentration was determined by adding H_2O_2 to mitochondria that were already treated with HRP and scopoletin. Alternatively, H_2O_2 was supplemented to uncoupled scopoletin-treated mitochondria in the presence of malonate, and HRP was added few s later. No fluorescence decrease was detected in the absence of HRP.

For measurements of H_2O_2 production, mitochondria were supplemented with scopoletin and HRP at 2 min of incubation followed by the addition of substrates and inhibitors as detailed in the figures. At the end of the experiment, each trace was calibrated with standard H_2O_2.

Measurement of Mitochondrial NAD(P)H—The mitochondrial NADP reduction was monitored by recording its relative fluorescence intensity at 348 nm (excitation) and 464 nm (emission) with a Shimadzu RL-5000 spectrofluorometer in a stirred cuvette thermostatted at 30 °C. Mitochondria were suspended at 0.4 mg/ml in sucrose-containing medium (250 mM mannitol, 75 mM sucrose, 5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 500 μg/ml defatted bovine serum albumin, 5 mM MOPS, pH 7.3, adjusted with KOH). Substrates were added at 90 s of incubation. Since the baseline fluorescence trace without substrates exhibited a decline with time, which was also more accentuated in the absence of EGTA, the fluorescence traces reported in Fig. 7 represent the difference fluorescence between the trace with substrate and a parallel trace (with or without EGTA) without substrate

Glutathione Reductase (GR) and Glutathione Peroxidase (GPX) Activities—For measurements of GR and GPX activities, mitochondrial pellets were resuspended in a small volume of H_2O and subjected to 2–3 cycles of freeze-thawing. Aliquots of disrupted mitochondria (~0.9 mg) were suspended in 1.6 ml of incubation medium (pH 7.35). NADPH was supplemented at 125 μM. To monitor the combined GR and GPX activities, NADPH oxidation, measured at 345 nm (excitation) and 450 nm (emission), was initiated by the addition of GSH (1 mM) and H_2O_2 (as detailed in the figures) (Scheme 1).

Other Assays—The mitochondrial glutathione was determined in mitochondrial pellets after incubation, by the method of Tietze *et al.* (26) as modified by Xia *et al.* (27) as in Ref. 28. Free Ca^{2+} concentrations in the incubation medium were calculated using the Ca^{2+}-sensitive fluorescent dye fura-2, pentapotassium salt (with excitation and emission wavelengths set at 340 and 505 nm, respectively; K_D 320 nM) and the fluorescent dye Calcium-Green-5N (with excitation and emission wavelengths set at 505 and 535 nm, respectively; K_D 14 μM). The use of the low affinity Ca^{2+} indicator Calcium-Green-5N and the high affinity Ca^{2+} indicator fura-2 (and pentapotassium salt) can give an indication of the magnitude of free Ca^{2+} concentration in the incubation medium. $\Delta\psi$ was estimated using fluorescence quenching of the cationic dye safranine O, which is accumulated and quenched inside energized mitochondria (29). The excitation wavelength was 495, and the emission wavelength was 586 nm, and the dye concentration used was 2 μM.

RESULTS

In the first group of experiments, we investigated the fate of exogenous H_2O_2, supplied in small amounts to brain mitochondria during controlled (state 4) or ADP-stimulated (state 3) respiration. As shown in Fig. 1, exogenous H_2O_2 was removed slowly in the absence of respiratory substrates. In these experiments, malonate, which by inhibiting succinate dehydrogenase limits the cycling of endogenous substrates, and an uncoupler to minimize NADPH generation via energy-dependent transhydrogenase, were generally also included (Fig. 1). This background removal may depend on contaminating catalase or the non-specific action of heme proteins. When controlled (state 4) respiration was activated with glutamate/malate, which feed electrons to Complex I (NADH dehydrogenase) of the respira-

tory chain, the rate of exogenous H_2O_2 removal was substantially increased. In a series of experiments, the net removal rate was 6.4 ± 0.6 nmol × min^{-1} × mg^{-1}, mean ± S.E., $n = 7$. Coupled respiration leads to extensive NADP reduction via energy-dependent transhydrogenase; in turn, NADPH promotes GSSG reduction via GR, and the GSH thus formed activates the GPX-mediated H_2O_2 removal. The GR/GPX system is considered to account for most of the mitochondrial peroxidase activity. These results show that respiring mitochondria are net removers, rather than producers, of H_2O_2 (see Scheme 2).

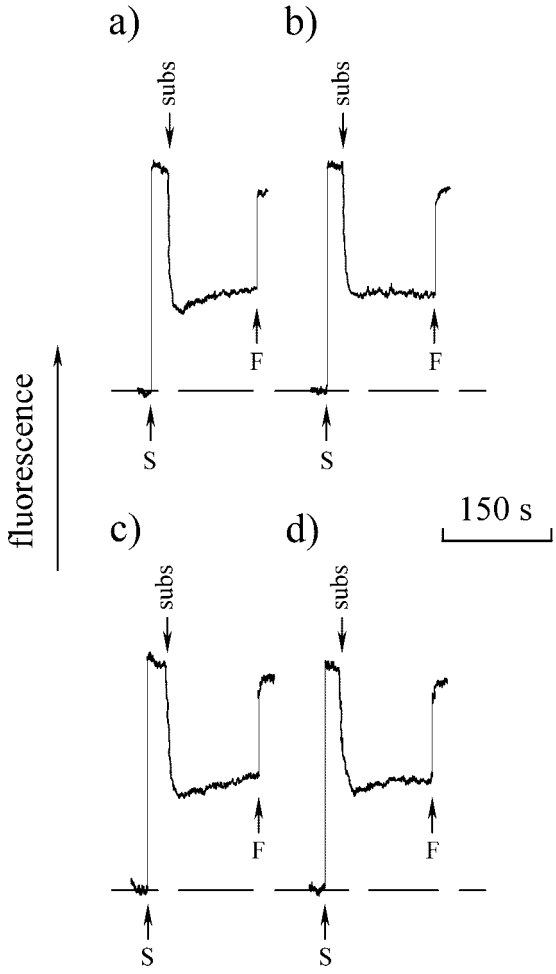


FIG. 4. **The mitochondrial membrane potential in the absence and presence of EGTA.** Mitochondria (0.7 mg/1.6 ml) were treated with 3 μM safranin (*S*) as indicated and supplemented where indicated (*subs*) with glutamate/malate (*traces a* and *b*) or succinate (*traces c* and *d*) in the absence (*traces a* and *c*) and presence (*traces b* and *d*) of EGTA. FCCP (*F*) was 3 μM. Typical traces are reported, representative of duplicate experiments from two independent preparations.

We next studied H_2O_2 removal using succinate as the substrate. Succinate feeds electrons to CoQ of the inner mitochondrial membrane, via Complex II. Also, with succinate, NAD(P) is highly reduced, due to reverse electron transfer through Complex I to NAD followed by NADP reduction via energy-dependent transhydrogenase (Refs. 16 and 18 and see below). Despite the similarity of the redox state of the NADP pool with succinate and glutamate/malate, the removal of exogenous H_2O_2 was ~50% slower with succinate (6.4 ± 0.6 (with glutamate/malate) *versus* 3.3 ± 0.4 (with succinate) nmol of H_2O_2 removed/mg × min, mean ± S.E., $n = 5$). However, if rotenone was supplied immediately prior to H_2O_2 during succinate oxidation, the succinate-supported H_2O_2 removal increased, to a level comparable with that with glutamate/malate (Fig. 1). Thus, manipulating the activity of Complex I, which lies uphill of the entry point of electrons from succinate, affects the activity of mitochondrial H_2O_2 removal. This result is reminiscent of the finding that succinate-supported H_2O_2 production is inhibited by rotenone (15–20).

When the experiments were performed under state 3 conditions, *i.e.* in the presence of ADP, the net glutamate/malate-supported peroxide removal was slightly slower than in state 4 (5.3 ± 0.5 nmol × min^{-1} × mg^{-1}, mean ± S.E., $n = 5$). The succinate-supported removal in state 3 was just about the same as in state 4. In the presence of the uncoupler FCCP, H_2O_2 removal was strongly decreased. Some residual activity was, however, still clearly evident with glutamate/malate (Table I).

Ca^{2+} and Arsenite Inhibit the Glutamate-Malate-supported H_2O_2 Removal—The experiments of Fig. 1 were performed in the presence of EGTA. Surprisingly, omitting EGTA from the incubation medium reduced by about 80% the glutamate/malate-supported net rate of measured exogenous H_2O_2 removal (Fig. 2). Such inhibition was due to Ca^{2+} contamination of the incubation medium (~6 μM). In fact, a similar level of inhibition was observed also in EGTA-containing media supplied with enough Ca^{2+} to increase its free concentration to the low μM range, thus ruling out an effect of EGTA *per se* or of some other contaminating ion. Furthermore, Ca^{2+} influx into

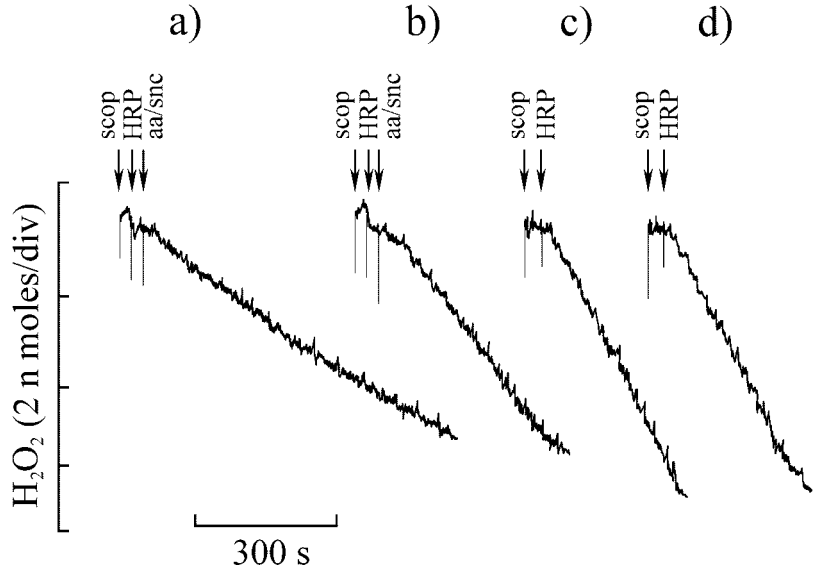


FIG. 5. **Succinate-induced H_2O_2 production in the absence of antimycin is not stimulated by superoxide dismutase.** Mitochondria (0.8 mg/1.6 ml) were incubated in standard medium with EGTA. Scopoletin (*scop*) and HRP were added as indicated. Succinate (2 mM) (*suc*) and antimycin A (1 μg) (*aa*) supplemented 5 s prior to succinate were added as indicated (*traces a* and *b*). Succinate was present in the incubation medium (*traces c* and *d*). Superoxide dismutase (18 μg/1.6 ml) was included in *traces b* and *d*. Typical traces are reported, representative of duplicate experiments from three independent preparations.

the mitochondria was required for inhibition to occur since the Ca^{2+} inhibition of exogenous H_2O_2 removal was prevented by ruthenium red (RR), an inhibitor of the Ca^{2+} uniporter, supplemented in the presence of EGTA and prior to Ca^{2+} addition. As will be reported below, the Ca^{2+} effect depends on the Ca^{2+} inhibition of the GR/GPX system and represents a novel unanticipated control of the mitochondrial handling of ROS.

The glutamate/malate-supported removal of exogenous H_2O_2 in the presence of EGTA was partially inhibited by the vicinal thiol reagent arsenite (Fig. 2). The arsenite-sensitive component represents about 40% of the overall peroxidase activity. The half-maximal inhibitory concentration of arsenite was ~12 μM (Fig. 2, *inset*). Arsenite was without effect on the rate of glutamate/malate oxidation. The highest inhibition (over 90%) of mitochondria-associated peroxidase activity was obtained when Ca^{2+} and arsenite were present together (Fig. 2). An overall picture of the H_2O_2 removal activity and of some relevant controls is reported in Table I.

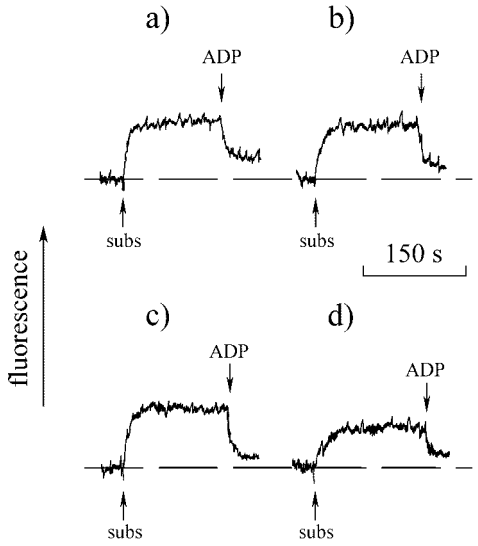


FIG. 6. **NAD(P) reduction in the absence and presence of EGTA.** Mitochondria (0.9 mg/1.6 ml) were incubated as described under "Experimental Procedures." The substrates (*subs*) were glutamate/malate (*traces a* and *b*) or succinate (*traces c* and *d*). EGTA was present in *traces a* and *c* and omitted in *traces b* and *d*. Where indicated, ADP (1.2 mM) was added. Typical traces are reported, representative of duplicate experiments from five independent preparations.

Succinate-supported H_2O_2 Production and Its Controls—We investigated the possibility that the different rates of exogenous H_2O_2 removal with glutamate/malate and succinate in the presence of EGTA as described above could be related to different rates of H_2O_2 production by the mitochondria with different substrates and in different metabolic situations, leading to a competition between external and internal H_2O_2 for the mitochondrial peroxidase(s). Peroxide production in coupled mitochondria was studied in the presence of HRP and scopoletin, providing a trap system that removes H_2O_2 immediately upon reaching the extramitochondrial space. In the absence of a suitable trap, no H_2O_2 accumulation took place. This may be expected in view of the new finding that mitochondria are net removers of H_2O_2. As shown in Fig. 3*A* (*trace a*), coupled mitochondria respiring on succinate in the presence of EGTA released H_2O_2. On the contrary, H_2O_2 release with glutamate/malate was hardly detectable (*trace d*). The succinate-dependent H_2O_2 was removed by rotenone and during state 3 respiration, which decreases $\Delta\psi$ (*traces f* and *e*) as already described by others. Furthermore, H_2O_2 release was prevented if EGTA was omitted (or in the presence of EGTA and enough Ca^{2+} to reach a low μM free Ca^{2+} concentration) (*trace c*). Such Ca^{2+} inhibition occurred without affecting $\Delta\Psi$, as monitored following safranine fluorescence (Fig. 4), and appears to repre-

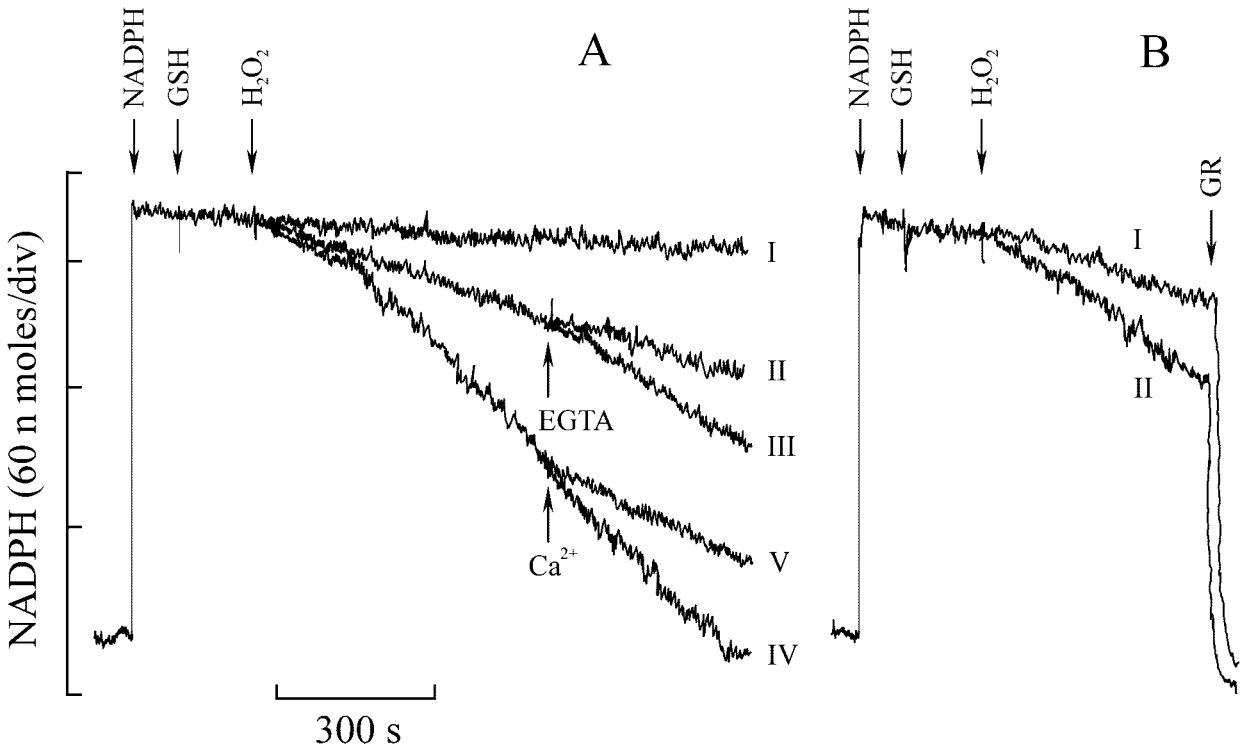


FIG. 7. **The combined activity of GR and GPX in freeze-thawed mitochondria is reversibly inhibited by Ca^{2+}.** In *A*, freeze-thawed mitochondria (1 mg/1.6 ml) were incubated in standard medium (pH 7.35). Further additions are as follows: NADPH (200 nmol), GSH (1 mM), and H_2O_2 (250 nmol). *Trace I*, EGTA present, GSH (or H_2O_2) omitted; *trace II*, no EGTA; *trace III*, EGTA added at the *arrow*; *trace IV*, EGTA present; *trace V*, EGTA present, Ca^{2+} (375 μM) added at the *arrow*. When necessary, the pH was adjusted to compensate for the H^+ release upon metal binding by EGTA. *B*, same as *panel A*, with EGTA in the presence (*trace I*) and absence (*trace II*) of 200 μM Ca^{2+}. Commercial GR (0.4 μg/1.6 ml:0.4 units/1.6 ml) was added where indicated. Typical traces are reported, representative of duplicate traces from at least five independent experiments.

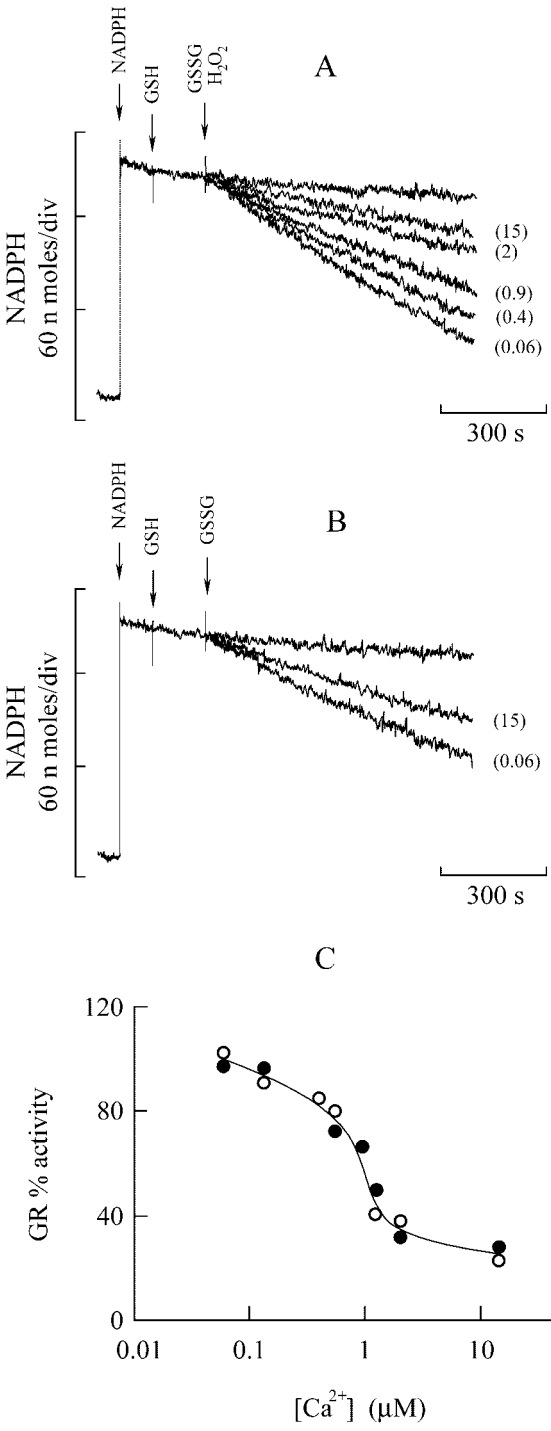


FIG. 8. **Ca^{2+} inhibition of GR activity and the effect of H_2O_2.** *A*, freeze-thawed mitochondria (0.8 mg/1.6 ml) incubated as in described in the legend for Fig. 6, in the presence of GSH (1 mM) and NADPH (150 nmol) as indicated. NADPH oxidation was initiated by the combined addition of GSSG (25 μM) and H_2O_2 (50 μM). EGTA was 375 μM. The free Ca^{2+} concentration was adjusted by the addition of $CaCl_2$ to reach the micromolar values indicated at the side of each trace. *B*, experimental conditions as in *panel A*. NADPH oxidation was initiated by the addition of GSSG (75 μM) and H_2O_2 was omitted. *C*, dependence of GR activity on the free Ca^{2+} concentration. Data are taken from two representative experiments.

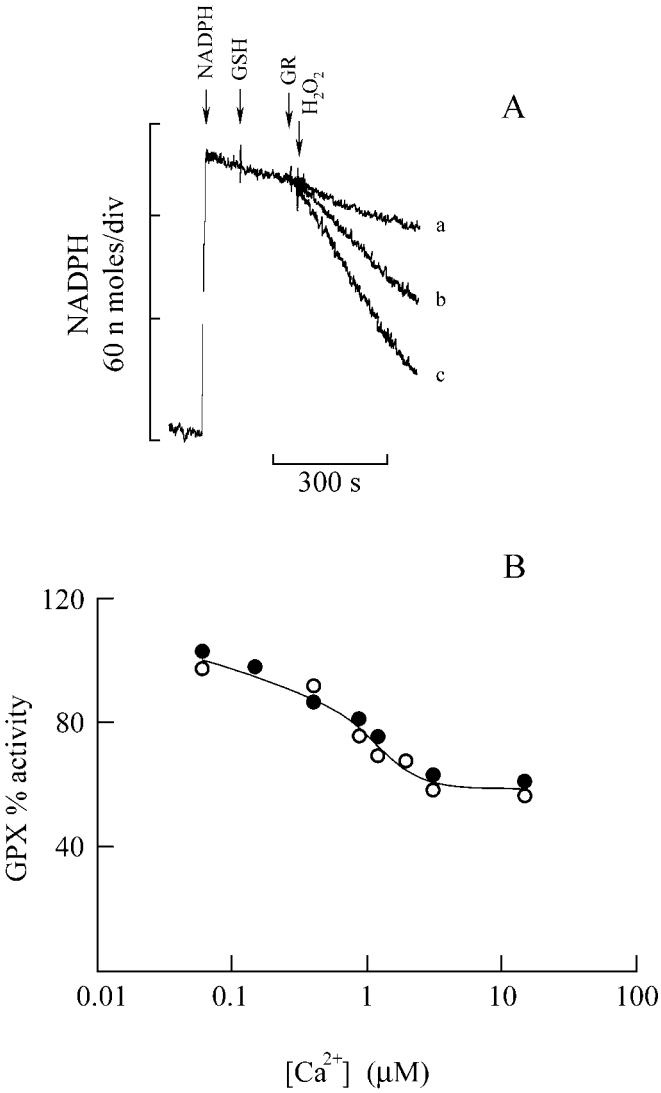
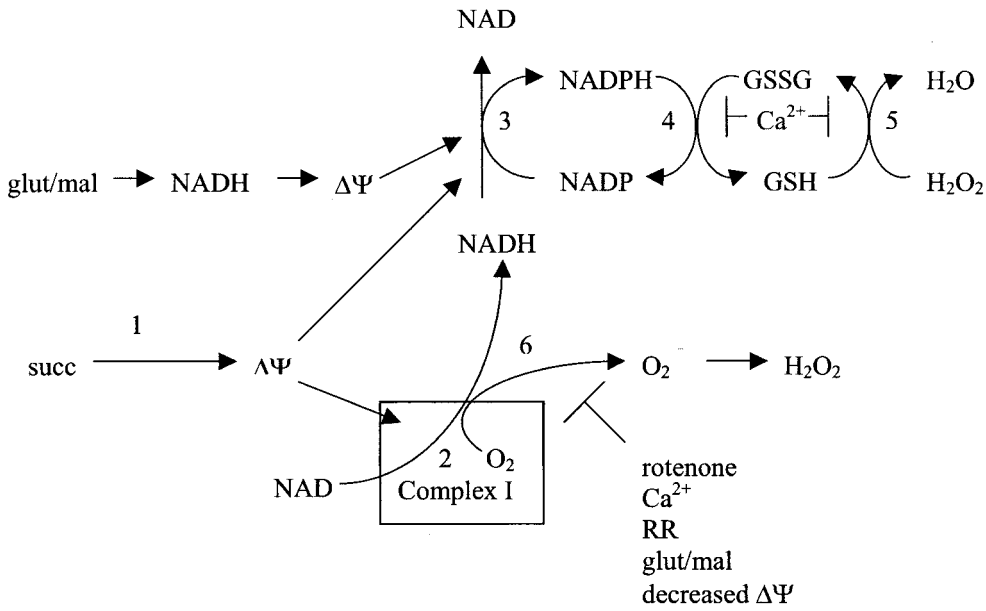


FIG. 9. **Ca^{2+} inhibition of GPX.** Experimental conditions are as described in the legend for Fig. 7. A further addition of commercial GR was made. In *trace a*, mitochondria were omitted to determine the non-enzymatic peroxidase activity. The rate of GSH oxidation was unmodified in the presence and absence of EGTA or of EGTA plus Ca^{2+}. In *A*, *traces b* and *c*, freeze-thawed mitochondria were 0.9 mg/1.6 ml. EGTA was present, together with $CaCl_2$, to raise the free Ca^{2+} concentration to 3 μM (*trace b*) or with no added $CaCl_2$ (*trace c*). *B*, dependence of GPX activity on the free Ca^{2+} concentration. Data are taken from two representative experiments.

sent a direct action of Ca^{2+} on the site of superoxide production. Also RR inhibited H_2O_2 release, in the presence of EGTA, apparently via a direct interaction with the superoxide production site at Complex I (Fig. 3*A*, *trace b*). Furthermore, H_2O_2 release was strongly depressed by glutamate/malate, probably because they decrease reverse electron flow from succinate (*trace g*). The latter effect increased with decreasing succinate concentration, Fig. 3*C*.

It is noticeable that exogenous H_2O_2 removal is highest with glutamate/malate, which are poor H_2O_2 producers, and that rotenone, RR, and glutamate/malate, which decrease the succinate-supported peroxide production, at the same time increase the net succinate-supported rate of exogenous H_2O_2 removal (Fig. 1 and Table I). The finding suggests that exogenous H_2O_2 and the endogenous production compete for the removing system(s). The Ca^{2+} effect is more complex, as Ca^{2+} inhibits both the exogenous peroxide removal and the endogenous production. These results suggested that Ca^{2+} may have a direct inhibition effect on H_2O_2 removal (see below and see Scheme 2).

We have shown in the preceding section that arsenite inhibits partially the respiration-dependent mitochondrial peroxidase activity. As shown in Fig. 3, *B* and *D*, arsenite did not



SCHEME 2. Succinate (*succ*) oxidation (*1*) generates $\Delta\psi$, which drives reverse electron flow from NAD to NADH at Complex I (*2*) and NADH-supported NADP reduction via energy dependent transhydrogenase (*3*). Glutamate plus malate (*glut/mal*) generate NADH, whose oxidation also generates $\Delta\psi$ and NADPH. NADPH promotes GSSG reduction via GR (*4*); in ,turn GSH promotes H_2O_2 removal via GPX (*5*). *Reactions 4* and *5* are Ca^{2+}-inhibited. Succinate-induced reverse electron flow at Complex I is accompanied by intramitochondrial generation of O_2^-. O_2^- production is inhibited by rotenone, Ca^{2+}, RR, glutamate plus malate, and decreased $\Delta\psi$.

increase H_2O_2 release with glutamate/malate and, unlike Ca^{2+}, increased the rate of succinate-dependent H_2O_2 production, without modifying the inhibitory controls as described above. Since arsenite has no discernible effect on the H_2O_2 detection system, its primary effect appears to be the direct inhibition of a component of H_2O_2 removal, allowing a higher fraction of endogenous peroxide to reach HRP/scopoletin.

Unlike the antimycin-A induced release of H_2O_2, which was recently shown to be stimulated by exogenous superoxide dismutase (14), the H_2O_2 produced by succinate during coupled respiration was not stimulated by superoxide dismutase (Fig. 5). This shows that the antimycin-promoted O_2^- production at Complex III is released toward the cytosolic face of the inner membrane, whereas the superoxide generated by succinate via reverse electron transfer at Complex I is released toward the matrix side, accessible to mitochondrial Mn-superoxide dismutase but not to extramitochondrial superoxide dismutase.

The Redox State of NAD(P) during Respiration-driven H_2O_2 Removal—As shown in Fig. 6, state 4 respiration with both glutamate/malate and succinate (EGTA present) promoted a high reduction of mitochondrial NAD(P). During state 3 respiration, there was a partial oxidation with glutamate/malate, which was more pronounced with succinate. This is expected since reverse electron transfer from succinate is impaired as a consequence of ADP-induced decrease of the protonmotive force. If the experiments were performed omitting EGTA, NAD(P) reduction was still high during state 4 respiration with glutamate/malate. However, with succinate as substrate, NAD(P) reduction was generally less extensive, although membrane potential was unaffected (see above). RR had no influence on succinate promoted NAD(P) reduction (not shown).

Glutathione Reductase and Glutathione Peroxidase are Ca^{2+}-inhibited—The finding that Ca^{2+} influx decreases the mitochondrial ability to remove exogenous H_2O_2 prompted us to study whether the GR/GPX system may be Ca^{2+}-inhibited. The experiments were performed in mitochondria whose integrity was disrupted by 2–3 cycles of freeze-thawing. In this system, it is possible to measure the combined activity of the two enzymes by monitoring the oxidation of added NADPH in the presence of GSH and H_2O_2 (Scheme 1). As shown in Fig. 7, NADPH oxidation required both GSH and H_2O_2. Furthermore, the activity was higher in the presence of EGTA and decreased by 60–70% if EGTA was omitted or if some Ca^{2+} was included in the EGTA trace to increase the free Ca^{2+} to 2.5 μM. The Ca^{2+} effect was readily reversible, *e.g.* removing Ca^{2+} by the addition of excess EGTA reactivated the Ca^{2+}-inhibited NADPH oxidation, and alternatively, increasing free Ca^{2+} decreased the NADPH oxidation rate observed at zero free Ca^{2+}.

The NADPH oxidation patterns of Fig. 7 depend on the combined activity of the two mitochondrial enzymes, GR and GPX, and do not allow us to determine the site of the Ca^{2+} sensitivity. However, as shown in Fig. 7*B*, the addition of excess commercial GR some time after the initiation, by GSH plus H_2O_2, of NADPH oxidation induced an immediate large oxidation of NADPH, indicative of the accumulation of GSSG during the progress of the reaction. This demonstrates that the rate of GSH oxidation by H_2O_2 in broken mitochondria was faster than that of GSSG reduction by the endogenous reductase activity. It may be concluded that the experiments of Fig. 7 mostly monitored GR and that the latter is Ca^{2+}-sensitive. This is more clearly shown in Fig. 8*A*, in which, to make sure that enough GSSG was present from the beginning at the time of H_2O_2 addition, some GSSG was supplemented together with H_2O_2. A family of traces was performed in these conditions, in which the concentration of EGTA was kept constant, and free Ca^{2+} was adjusted to the concentrations reported in parentheses by additions of $CaCl_2$. From a series of such experiments, the half-maximal inhibition by Ca^{2+} was calculated to be 0.9 μM (Fig. 8*C*). Although not a substrate of GR, H_2O_2 (50 μM) was included in the experiments of Fig. 8*A*. In fact, the Ca^{2+} sensitivity was less evident if H_2O_2 was omitted. This is shown in Fig. 8*B*, in which H_2O_2 was substituted by 50 μM GSSG. Specifically, without H_2O_2, the NADPH oxidation rate with no Ca^{2+} was lower and that with 15 μM free Ca^{2+} was higher than in the corresponding traces in which H_2O_2 was included. Thus, the inhibition by Ca^{2+} is promoted by H_2O_2. It is noticeable that the effect of H_2O_2 is visible at concentrations less than 10 μM (not shown). Further experiments will clarify this point. One consequence of the finding that low Ca^{2+} concentrations are inhibitors of GR should be that when mitochondria are

presented with H_2O_2 in the presence of glutamate/malate but in the absence of EGTA, GSSG should accumulate in the mitochondria. Indeed, in experiments in which we monitored the redox state of GSH during glutamate/malate-supported H_2O_2 (20 μM) removal, we found that the GSH/GSSG ratio decreased from 4.5 ± 0.4 to 1.5 ± 0.5 (n = 3) after 2 min of H_2O_2 treatment in the absence of EGTA, as compared with the ratio with EGTA.

We investigated whether GPX is also negatively controlled by Ca^{2+}. To this end we supplemented the test system with excess commercial GR to render GPX rate limiting and monitored the H_2O_2-dependent NADPH oxidation in the presence of GSH. As shown in Fig. 9, the non-enzymatic peroxidase activity (as measured without mitochondria) was unmodified by Ca^{2+}. However, the mitochondria-dependent activity was partially Ca^{2+}-inhibited. The inhibition was about 40%, and the half-inhibitory concentration of Ca^{2+} was about 0.9 μM (Fig. 9*B*). Similar results were obtained in experiments in which the disappearance of H_2O_2 was measured directly. In this setup, 20 μM H_2O_2 was supplemented to GSH-containing cuvettes in the presence and absence of free Ca^{2+}(15 μM). Scopoletin, followed 20 s later by HRP, was added at 1 min to measure the residual H_2O_2. The non-enzymatic peroxidase activity, as measured without mitochondria, was unaffected by Ca^{2+}. The net H_2O_2 consumption in the presence of mitochondria was 6 ± 0.5 and 3.5 ± 0.4 (n = 6) nmol × mg^{-1} × min^{-1} in the absence and presence of Ca^{2+}.

The finding that mitochondria are active scavengers of external peroxide and that Ca^{2+} negatively controls removal via inhibition of GR/GPX suggests that mitochondria operate in cells as a Ca^{2+}-modulated peroxide sink. In fact, after 4 min of incubation with 200 μM tyramine, a substrate for monoamine oxidase of the outer mitochondrial membrane, the H_2O_2 accumulated in the suspending medium was 12.6 ± 1.0 nmol/mg (n = 3) and 13.2 ± 1.5 nmol/mg (n = 3) with no substrate (with and without EGTA), 10.7 ± 1.0 nmol/mg (n = 3) with glutamate/malate (minus EGTA), and only 2.6 ± 1.0 nmol/mg (n = 3) with glutamate/malate and EGTA.

DISCUSSION

There is some controversy as to whether mitochondria are an important source of ROS under physiological and pathological conditions, despite numerous studies reporting that mitochondria release H_2O_2. Most of the studies were performed in non-physiological situations in the presence of respiratory chain inhibitors such as rotenone and antimycin. Relatively few data are available on the mitochondrial H_2O_2 production in physiological coupled conditions in the absence of unnatural effectors. The results of these studies show that H_2O_2 production is mainly supported by succinate oxidation in the absence of ADP (state 4 respiration) via reverse electron transfer to Complex I (15–20).

In the present study, we tried to approach the mitochondrial handling of ROS from a different perspective. We questioned how mitochondria deal with exogenous H_2O_2. It is clear that if mitochondria are net producers of H_2O_2, as suggested by the large majority of the studies, they should be incapable of removing H_2O_2. We found instead that respiring brain mitochondria are strong removers of exogenous peroxide, both during state 4 and during state 3 respiration. However, some intramitochondrially produced H_2O_2 is capable to reach the extramitochondrial space since it is monitored by the HRP/scopoletin trap in the suspending medium.

The measured peroxidase activity (generally considered to be largely represented by the GR/GPX system, but thioredoxin peroxidase activity may also be present, Refs. 30 and 31) was maximal with NAD-linked substrates (glutamate plus malate) and about 50% lower with succinate, which feeds electrons to the respiratory chain via Complex II. Peroxidase activity turned out to be a regulated process. In fact, exogenous H_2O_2 consumption was partially inhibited (~40%, semimaximal effect at 12 μM) by the vicinal dithiol reagent arsenite. More importantly, the H_2O_2 removal was largely prevented if small amounts of Ca^{2+} were taken up by the mitochondria. Contaminating Ca^{2+} in the incubation medium was sufficient for maximal inhibition (~80%). Ca^{2+} influx was necessary since the effect was prevented by the Ca^{2+} uniporter inhibitor RR. The decreased net H_2O_2 removal rate with Ca^{2+} could in principle depend on an increased peroxide production, supplying a higher amount of endogenous H_2O_2 in competition with exogenous H_2O_2 for the peroxidase(s), or on a decreased activity of the H_2O_2-removing system. To distinguish between these possibilities and to provide an explanation for the lower rate of external H_2O_2 removal with succinate, we monitored H_2O_2 production.

The main findings were that glutamate/malate are very poor H_2O_2 producers, whereas succinate releases H_2O_2 during state 4 respiration in the presence of EGTA in a process that depends on reverse electron transfer at Complex I. The succinate-dependent H_2O_2 release is itself regulated, being inhibited by Complex I inhibitor rotenone or by decreasing membrane potential (15–20), and furthermore, by low Ca^{2+} (acting without decreasing $\Delta\Psi$), by RR, and by glutamate/malate. In turn, rotenone, RR, and glutamate/malate, which depress H_2O_2 production, increase the ability of succinate to remove exogenous H_2O_2, thus favoring the idea that endogenously produced H_2O_2 competes with exogenous H_2O_2 for removal. The finding that Ca^{2+} strongly inhibits exogenous H_2O_2 removal without increasing, and actually inhibiting (with succinate), the mitochondrial peroxide production suggested that the H_2O_2-removing system itself may be Ca^{2+}-inhibited.

The direct analysis of GR/GPX activity in disrupted mitochondria demonstrated that Ca^{2+} is an inhibitor, acting at low concentrations both on GR (maximal inhibition, about 70%) and on GPX (maximal inhibition, about 40%). In both cases, the half-maximal effect is at ~0.9 μM Ca^{2+}. These intramitochondrial Ca^{2+} concentrations are readily achieved physiologically and are similar to the concentrations required for the activation of the pyruvate, isocitrate, and an oxoglutarate dehydrogenases (32). The Ca^{2+} effect on GR is more evident in the presence of H_2O_2, which acts rapidly at concentrations as low as 10 μM. Modulation of enzyme activity by peroxide and other ROS is not uncommon. Arsenite, which inhibits a significant fraction of the H_2O_2-scavenging activity of mitochondria, has no effect on GR/GPX. This may be an indication that the arsenite-sensitive component may in fact represent an independent peroxidase system (30, 31).

The finding that the extramitochondrial trap competes with the mitochondrial peroxidase(s) for the removal of mitochondrially produced H_2O_2 is puzzling. H_2O_2 appears to derive from internally produced superoxide, its production rate being not stimulated by exogenous superoxide dismutase. This means that a significant fraction of this H_2O_2 must exit mitochondria escaping removal, in order to re-enter and be processed only at a second time. One possibility is that in a mixed mitochondria population, some mitochondria that are H_2O_2 producers have a poor peroxidase activity, and their H_2O_2 is released, to be taken up by mitochondria with higher peroxidase. It may also be argued that the active site of a fraction of mitochondrial peroxidases may face the outer face of the inner membrane, thus primarily reacting with external H_2O_2. In this case, H_2O_2 in excess of internal peroxidase capacity could diffuse to the intermembrane space, being metabolized immediately only if

released close to the peroxidase and otherwise diffusing outside. The possibility could be considered that arsenite-sensitive peroxidase may face the intermembrane space. It could also be argued that since the affinity for H_2O_2 of the exogenous trap is presumably much high than that of GR/GPX, internal H_2O_2 may diffuse outside before reaching a sufficient concentration to be efficiently removed by the endogenous system.

Our results show that under the conditions tested, the H_2O_2-removing ability of mitochondria is in excess over the mitochondrial peroxide production rate, with glutamate/malate as well as with succinate, in the absence and presence of Ca^{2+}. This leads to the new concept that mitochondria function as intracellular Ca^{2+}-modulated peroxide sinks, in which a modest increase of mitochondrial Ca^{2+} favors a prooxidant state. Indeed, H_2O_2 produced at the outer mitochondrial membrane by monoamine oxidase activity accumulates in the suspending medium in the absence of respiratory substrates or with glutamate/malate and no EGTA, but is almost completely removed by glutamate/malate in the presence of EGTA.

Increasing evidence suggests that the production of H_2O_2 might be an integral component of membrane receptor signaling (33). In mammalian cells, a variety of extracellular stimuli, including peptide growth factors and cytokines, induce a transient increase of the intracellular concentration of H_2O_2, which appears to be required for the protein tyrosine phosphorylation induced by growth factors (34, 35). Frequently, the increase of H_2O_2 is accompanied by the increase of cytosolic Ca^{2+}. Such increased cytosolic Ca^{2+} may secondarily promote the increase of mitochondrial Ca^{2+} (36) followed by the inhibition of H_2O_2 removal as described here, thus favoring the accumulation of H_2O_2 and potentiating the agonist-dependent signal transduction. Scheme 2 summarizes most of the properties and controls of H_2O_2-removing and -generating systems in mitochondria.

In conclusion, we have shown that mitochondria are strong removers of H_2O_2 and that removal is Ca^{2+}-inhibited. H_2O_2 is also produced by mitochondria, but only during succinate oxidation, and then in highly controlled conditions. The results show that mitochondria behave as an intracellular Ca^{2+}-controlled peroxide sink.

REFERENCES

1. Loschen, G., Flohe, L., and Chance, B. (1971) *FEBS Lett.* **18,** 261–264
2. Boveris, A., Oshino, N., and Chance, B. (1972) *Biochem. J.* **128,** 617–630
3. Chance, B., Sies, H., and Boveris, A. (1979) *Physiol. Rev.* **59,** 527–605
4. Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000) *Nat. Neurosci.* **3,** 1301–1306
5. Jenner, P. (2001) *Trends Neurosci.* **24,** 245–247
6. Brownlee, M. (2001) *Nature* **414,** 813–820
7. Loschen, G., Azzi, A., and Flohe, L. (1974) *FEBS Lett.* **42,** 68–72
8. Boveris, A., and Cadenas, E. (1975) *FEBS Lett.* **54,** 311–314
9. Forman, H. J., and Azzi, A. (1997) *FASEB J.* **11,** 374–375
10. Staniek, K., and Nohl, H. (2000) *Biochim. Biophys. Acta* **1460,** 268–275
11. Turrens, J. F., and Boveris, A. (1980) *Biochem. J.* **191,** 421–427
12. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) *Arch. Biochem. Biophys.* **237,** 408–414
13. Zoccarato, F., Cavallini, L., Deana, R., and Alexandre, A. (1988) *Biochem. Biophys. Res. Comm.* **154,** 727–734
14. St-Pierre, J., Buckingham, J. A., Roebuck, S. J., and Brand, M. D. (2002) *J. Biol. Chem.* **277,** 44784–44790
15. Cino, M., and Del Maestro, R. F. (1989) *Arch. Biochem. Biophys.* **269,** 623–638
16. Hansford, R. G., Hogue, B. A., and Mildaziene, V. (1997) J. *Bioenerg. Biomembr.* **29,** 89–95
17. Korshunov, S. S., Skulachev, V. P., and Starkov, A. A. (1997) *FEBS Lett.* **416,** 15–18
18. Liu, Y., Fiskum, G., and Schubert, D. (2002) *J. Neurochem.* **80,** 780–787
19. Starkov, A. A., Polster, B. M., and Fiskum, G. (2002) *J. Neurochem.* **83,** 220–228
20. Votyakova, T. V., and Reynolds, I. J. (2001) *J. Neurochem.* **79,** 266–277
21. Herrero, A., and Barja, G. (1997) *Mech. Ageing Dev.* **98,** 95–111
22. Genova, M. L., Ventura, B., Giuliano, G., Bovina, C., Formiggini, G., Parenti-Castelli, G., and Lenaz, G. (2001) *FEBS Lett.* **505,** 364–368
23. Lenaz, G., Bovina, C., D'Aurelio, M., Fato, R., Formiggini, G., Genova, M. L., Giuliano, G., Pich, M. M., Paolucci, U., Castelli, G. P., and Ventura, B. (2002) *Ann. N. Y. Acad. Sci.* **959,** 199–213
24. Sandri, G., Panfili, E., and Ernster, L. (1990) *Biochim. Biophys. Acta* **1035,** 300–305
25. Boveris, A., Nartino, E., and Stoppani, A. O. M. (1977) *Anal. Biochem.* **80,** 145–158
26. Tietze, F. (1969) *Anal. Biochem.* **27,** 502–522
27. Xia, Y., Hill, K. E., and Burk, R. F. (1985) *J. Nutr.* **115,** 733–742
28. Zoccarato, F., Deana, R., Cavallini, L., and Alexandre, A. (1989) *Eur. J. Biochem.* **180,** 473–478
29. Akerman, K. E. O., and Wikstrom, M. K. F. (1976) *FEBS Lett.* **68,** 191–197
30. Watabe, S., Hiroi, T., Yamamoto, Y., Fujioka, Y., Hasegawa, H., Yago, N., and Takahashi, S. Y. (1997) *Eur. J. Biochem.* **249,** 52–60
31. Rhee, S. G., Kang, S. W., Chang, T. S., Jeong, W., and Kim, K. (2001) *IUBMB Life* **52,** 35–41
32. McCormack, J. G., Halestrup, A. P., and Denton, R. M. (1990) *Physiol. Rev.* **70,** 391–425
33. Rhee, S. G. (2000) *Science's STKE* http://stke.org/cgi/content/full/OC_sigtrans; 2000/53/pe
34. Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997) *J. Biol. Chem.* **272,** 217–221
35. Sundaresan, M., Yu, X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) *Science* **270,** 296–299
36. Rizzuto, R., Bernardi, P., and Pozzan, T. (2000) *J. Physiol.* (*Lond.*) **529,** 37–47

Respiration-dependent Removal of Exogenous H_2O_2 in Brain Mitochondria: INHIBITION BY Ca^{2+}

Franco Zoccarato, Lucia Cavallini and Adolfo Alexandre

J. Biol. Chem. 2004, 279:4166-4174.
doi: 10.1074/jbc.M308143200 originally published online November 20, 2003
