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Original Contribution

Enhanced hydrogen peroxide generation accompanies the beneficial bioenergetic effects of methylene blue in isolated brain mitochondria

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ABSTRACT

The redox dye methylene blue (MB) is proven to have beneficial effects in various models of neurodegenerative diseases. Here we investigated the effects of MB (100 nM, 300 nM, and 1 μM) on key bioenergetic parameters and on H_2O_2 production/elimination in isolated guinea pig brain mitochondria under normal as well as respiration-impaired conditions. As measured by high-resolution Oxygraph the rate of resting oxygen consumption was increased, but the ADP-stimulated respiration was unaffected by MB with any of the substrates (glutamate malate, succinate, or α-glycerophosphate) used for supporting mitochondrial respiration. In mitochondria treated with inhibitors of complex I or complex III MB moderately but significantly increased the rate of ATP production, restored $\Delta\Psi_m$, and increased the rate of Ca^{2+} uptake. The effects of MB are consistent with transferring electrons from upstream components of the electron transport chain to cytochrome *c*, which is energetically favorable when the flow of electrons in the respiratory chain is compromised. On the other hand, MB significantly increased the production of H_2O_2 measured by Amplex UltraRed fluorimetry under all conditions, in resting, ATP-synthesizing, and respiration-impaired mitochondria, with each substrate combination supporting respiration. Furthermore, it also decreased the elimination of H_2O_2. Generation of H_2O_2 without superoxide formation, observed in the presence of MB, is interpreted as a result of reduction of molecular oxygen to H_2O_2 by the reduced MB. The elevated generation and impaired elimination of H_2O_2 should be considered for the overall oxidative state of mitochondria treated with MB.

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Methylene blue (MB)[1] has been used with multiple therapeutical purposes in the treatment of malaria [3,30,41], carbon monoxide or cyanide poisoning [23], methemoglobinemia [47], and neurodegenerative diseases [16,21,25,66,72,73,87,96] and in photodynamic therapy [58]. MB is able to take electrons on its aromatic thiazine ring to be reduced to leukomethylene blue (MBH_2) and transfer electrons to other compounds depending on the redox states and the concentration of MB (see [65]). It is a lipophilic compound that accumulates in the mitochondria driven by the mitochondrial membrane potential ($\Delta\Psi_m$), and its major cellular target associated with the neuroprotective and cognitive-enhancing effect is assumed to be the mitochondrial metabolism ([8–10,93]; for review see [65]). Owing to its redox-cycling property MB in mitochondria can be reduced to MBH_2 primarily by NADH at complex I of the respiratory chain and shuttle electrons to cytochrome *c* and oxygen to form water (see [65]), therefore increasing oxygen consumption, which has been described in various cell types [10,44,93]. It has been described that MB depolarizes mitochondria in HeLa cells [58], but is protective against glutamate-induced mitochondrial depolarization in HT-22 cells [60].

Data concerning the effects of MB on reactive oxygen species (ROS) homeostasis are somewhat controversial. Pro-oxidant effects of MB (in >1 μM) have been demonstrated by 2′,7′-dichlorofluorescin oxidation [10,58], but MB proved to be protective against H_2O_2-induced cell death [10,87] and exhibited antioxidant effects in various systems [5,60,93].

Considering that mitochondrial mechanisms are assumed to underlie the beneficial effects of MB, studies addressing specific properties reflecting the bioenergetic competence of mitochondria treated with MB are scarce [88,89,93]. In this work we studied in detail the effects of MB on the mitochondrial bioenergetics and H_2O_2 release in isolated brain mitochondria. In most of the previous studies with MB, ROS formation was detected with dichlorofluorescin diacetate (DCF-DA), which has the disadvantage of reacting with ROS slowly and with uncharacterized stoichiometry, lacking correct calibration, and reacting also with non-ROS compounds [37]. Here we used the Amplex UltraRed fluorescent dye, which enables a

Abbreviations: $\Delta\Psi\mu$, mitochondrial membrane potential; PTP, permeability transition pore; ROS, reactive oxygen species; RET, reverse electron transport; α-GPDH, α-glycerophosphate dehydrogenase; α-GP, α-glycerophosphate; MB, methylene blue.

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fast, sensitive, and well-calibrated detection of H_2O_2 released from mitochondria to the medium [59,71,91]. To identify the specific effects of MB on mitochondrial bioenergetics respiration, ATP synthesis, $\Delta\Psi_m$, and Ca^{2+} uptake were investigated in both normal and respiration-impaired isolated brain mitochondria energized by various respiratory substrates.

Materials and methods

Isolation of mitochondria

Mitochondria of synaptic and nonsynaptic origin were isolated from guinea pig brain using a discontinuous Percoll gradient as detailed earlier [67,81]. Animals were decapitated by a process in accordance with the International Guiding Principles for Biomedical Research Involving Animals and Guidelines for Animal Experiments at Semmelweis University. Brains were quickly removed and homogenized in buffer A (in mM: 225 mannitol, 75 sucrose, 5 Hepes, 1 EGTA, pH 7.4 (KOH)) and then centrifuged for 3 min at 1300*g*. The supernatant was centrifuged for 10 min at 20,000*g*, and then the pellet was suspended in 15% Percoll and layered on a discontinuous gradient consisting of 40 and 23% Percoll layers, which was then centrifuged for 8 min at 30,700*g*. After resuspension of the lowermost fraction in buffer A, it was centrifuged at 16,600*g* for 10 min, and then the pellet was resuspended in buffer A and centrifuged again at 6300*g* for 10 min. After the supernatant was discharged, the pellet was resuspended in buffer B (in mM: 225 mannitol, 75 sucrose, 5 Hepes, pH 7.4 (KOH)).

Incubation medium

Experiments were carried out in a standard assay medium containing (in mM) 125 KCl, 20 Hepes, 2 K_2HPO_4, 1 $MgCl_2$, 0.1 EGTA, pH 7.0 (KOH), supplemented with 0.025% fatty-acid-free bovine serum albumin.

Mitochondrial respiration

Mitochondrial oxygen consumption assays were performed using the high-resolution respirometry system Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) [28] at 37 °C in 2-ml chambers. Data were digitally recorded and analyzed; oxygen flux was calculated as the negative time derivative of the oxygen concentration, $cO_2(t)$. Oxygen sensors were calibrated routinely at air saturation and in oxygen-depleted medium. Mitochondria were energized with glutamate plus malate (5 mM each) or succinate (5 mM) or α-glycerophosphate (α-GP) (20 mM).

Measurement of mitochondrial ATP synthesis

Synthesis of ATP was measured by a coupled enzymatic assay [94]. Standard assay medium was supplemented with $NADP^+$ (1.5 mM), hexokinase (2 U/ml), glucose 6-phosphate dehydrogenase (3.84 U/ml), 2.5 mM glucose, and 50 μM P^1,P^5-di(adenosine-5′) pentaphosphate (inhibitor of adenylate kinase). In the medium ATP phosphorylated glucose to glucose 6-phosphate in the presence of hexokinase, then glucose 6-phosphate was converted by glucose-6-phosphate dehydrogenase to 6-phosphogluconate with the concomitant reduction of $NADP^+$ to NADPH. Absorbance of NADPH was measured at 340 nm using a GBC-UV double-beam spectrophotometer. Measurements were calibrated with known amounts of ATP.

Measurement of membrane potential

$\Delta\Psi_m$ was determined using the cationic dye safranin O, which is accumulated and quenched in energized mitochondria [4]. The dye concentration was 2 μM. The excitation and emission wavelengths were 495 and 586 nm, respectively, as described previously [40]. Measurements were performed at 37 °C with 0.1 mg/ml mitochondrial protein using a Hitachi F-4500 spectrofluorimeter (Hitachi High Technologies, Maidenhead, UK).

Measurement of Ca^{2+} uptake

Mitochondria (0.1 mg/ml) were added to the incubation medium in the presence of ADP and glutamate plus malate (5 mM each) and then Ca^{2+} pulses were given in 100-s intervals. The free Ca^{2+} concentration at each added concentration of Ca^{2+} was calculated and measured. Ca^{2+} uptake by mitochondria was followed by measuring Calcium Green-5N (100 nM) fluorescence at 505 nm excitation and 535 nm emission wavelengths at 37 °C using a Hitachi F-4500 spectrofluorimeter.

Detection of H_2O_2 formation

The assay is based on the detection of H_2O_2 in the medium using the Amplex Red fluorescent dye [54]. Horseradish peroxidase (5 U per 2 ml) and Amplex UltraRed reagent (1 μM), and then mitochondria, were added to the incubation medium. H_2O_2 formation was initiated by the addition of glutamate plus malate or α-GP or succinate, in the concentrations indicated, and fluorescence was detected at 37 °C in a PTI Deltascan fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ, USA). The excitation wavelength was 550 nm and the fluorescence emission was detected at 585 nm. A calibration signal was generated with known quantities of H_2O_2 at the end of each experiment.

Measurement of NAD(P)H steady state

The matrix NAD(P)H autofluorescence was measured in parallel with the Amplex assay using the double-excitation and double-emission mode of the PTI Deltascan fluorescence spectrophotometer.

Mitochondria were incubated at 37 °C as described above and the fluorescence was measured using 344 nm excitation and 460 nm emission wavelengths. Changes in the NAD(P)H level were expressed in photon count × 10^3.

Measurement of H_2O_2 elimination

H_2O_2 elimination was detected with an H_2O_2-sensitive electrode (World Precision Instruments) [24]. Electrode signal was calibrated with additions of known concentrations of H_2O_2. In the presence of glutamate plus malate (5 mM), 300 s after addition of ADP (2 mM) and H_2O_2 (5 μM), a 0.1 mg/ml mitochondrial suspension was obtained and the amount of H_2O_2 was detected. At the end of each measurement catalase (33 IU/ml) was added. H_2O_2 elimination was characterized by the half-time (τ) of H_2O_2 disappearance from the medium.

Chemicals

Standard laboratory chemicals were obtained from Sigma (St. Louis, MO, USA). The Amplex UltraRed reagent and Calcium Green-5N were from Molecular Probes (Eugene, OR, USA).

Statistics

Statistical differences were evaluated with ANOVA (Sigmastat) for multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

The effects of MB on the respiration of mitochondria supported by three different substrates

Given the hormetic pharmacological effect of MB, meaning that it could have opposite effects at high and low doses [65], we used MB in a concentration range (100 nM–1 μM) that is relevant to cellular studies, in particular to those reporting neuroprotective effects of MB [10,60,93].

When mitochondria are respiring on glutamate plus malate, electrons enter the respiratory chain from NADH via complex I, whereas with succinate or α-GP electrons from succinate dehydrogenase or α-glycerophosphate dehydrogenase (α-GPDH), respectively, reduce CoQ, bypassing complex I. The rate of State 4 respiration of isolated brain mitochondria supported by glutamate plus malate in the absence of added ADP was stimulated from 375 ± 20.6 to 618.3 ± 36.2 pmol/s/mg protein upon addition of 1 μM MB (Fig. 1); the effect of MB was significant already at the 300 nM concentration (463 ± 24.6 pmol/s/mg protein). The ADP-stimulated (State 3) respiration was unaffected by MB, but the stimulation of the State 4-like respiration was again evident after the addition of carboxyatractylozide (CAT), which inhibits the adenine nucleotide translocator (ANT), preventing the effect of ADP; therefore mitochondria behave as if in the absence of ADP (Fig. 1).

The rate of respiration, as expected, was reduced by rotenone and addition of ADP was without an effect, but MB was capable of stimulating respiration also under this condition (Fig. 1, bars on the right).

Essentially similar results were obtained with mitochondria respiring on succinate or α-GP (Table 1). It is well documented that with succinate in the absence of ADP, high $\Delta\Psi_m$ is generated, allowing the flux of electrons from coenzyme Q (CoQ) in reverse via complex I, a phenomenon called reverse electron transport (RET) [11,34], resulting in the reduction of NAD^+ to NADH by complex I. NADH might reduce MB, which could then shortcut electrons to cytochrome *c*, contributing to the MB-induced stimulation of respiration. RET and the related NADH generation in succinate-supported mitochondria are prevented by the complex I inhibitor rotenone [20,32,42,90], but this did not alter the MB-evoked stimulation of respiration (Table 1). Resting oxygen consumption was higher with succinate (1094 ± 69 pmol/s/mg protein) than with glutamate plus malate and the effect of MB was less spectacular, but significant both in the absence and in the presence of rotenone. It is noteworthy that independent of RET, NADH could also be generated during the oxidation of the carbon skeleton of succinate subsequent to the succinate dehydrogenase reaction in the Krebs cycle. This ambiguity is lacking when α-GP is

Table 1
The effect of methylene blue on the rate of oxygen consumption in mitochondria supported by succinate or α-GP in the absence or presence of rotenone.

	Rate of oxygen consumption (pmol/s/mg protein)		
	Basal	+ADP	+ADP + CAT
Succinate	1094 ± 69 (5)	2668 ± 254 (5)	790 ± 45 (5)
Succinate + MB	1440 ± 65 (5)*	2464 ± 272 (5)	1136 ± 67 (5)*
Succinate + rotenone	1024 ± 61 (5)	4232 ± 395 (5)	738 ± 21 (5)
Succinate + rotenone + MB	1232 ± 41 (5)*	3894 ± 280 (5)	944 ± 25 (5)*
α-GP	335 ± 19 (7)	822 ± 22 (7)	446 ± 16 (7)
α-GP + MB	550 ± 24 (5)*	906 ± 43 (5)	602 ± 21 (5)*
α-GP + rotenone	328 ± 11 (4)	795 ± 46 (4)	402 ± 10 (4)
α-GP + rotenone + MB	472 ± 15 (5)*	854 ± 49 (5)	499 ± 16 (5)*

MB, methylene blue; α-GP, α-glycerophosphate. Experiments were performed as for Fig. 1 except that mitochondria were supported with either 5 mM succinate or 20 mM α-GP. Concentrations: MB, 1 μM; rotenone, 5 μM; ADP, 2 mM; carboxyatractylozide (CAT), 2 μM.

* $P < 0.05$; significant difference from the corresponding data obtained under MB-free conditions.

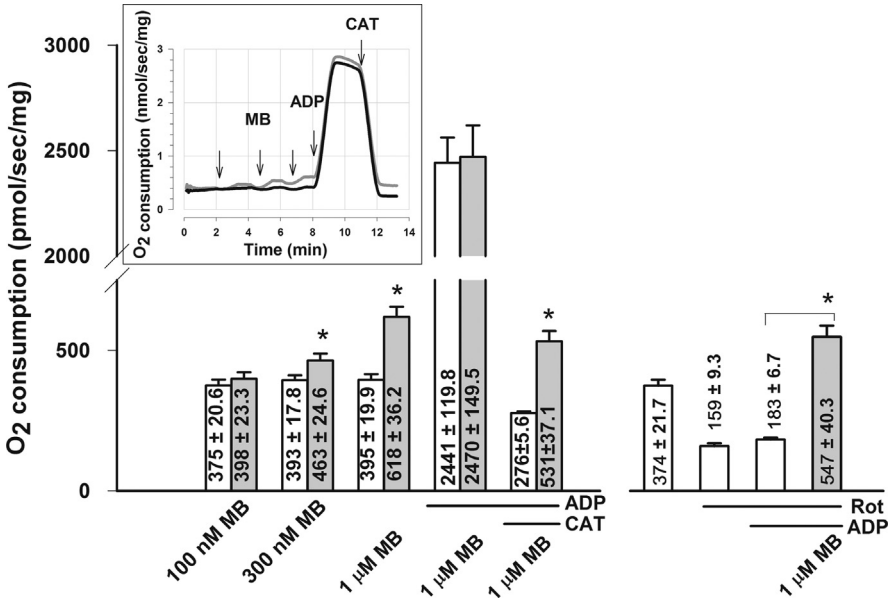

Fig. 1. The effect of MB on the rate of oxygen consumption in glutamate plus malate-supported mitochondria. Mitochondria (0.05 mg/ml) were incubated in the standard medium as described under Materials and methods. MB (100 nM, 300 nM, and 1 μM final concentrations), ADP (2 mM), and then carboxyatractylozide (CAT; 2 μM) were given as indicated in the inset (gray trace; no MB addition for black trace). The effects of MB (gray bars) are compared to controls (white bars; no MB, only buffer added) measured for each individual experiment. Data from similar experiments performed in the presence of rotenone (0.5 μM) are shown separated on the right. Results are expressed as mean oxygen consumption in pmol/s/mg protein ± SEM ($n > 4$) and written on the bars. *Significant difference ($P < 0.05$) from the corresponding MB-free controls.

used as a substrate, which is oxidized on the outer surface of the mitochondrial inner membrane by α-GPDH transferring the electrons to CoQ and in 20 mM concentration generating sufficient proton-motive force to drive RET [31,84]. Like in succinate-supported mitochondria, NADH generation by RET is prevented by rotenone, but unlike with succinate, no additional NADH generation should be considered with α-GP as a substrate. Table 1 demonstrates that in mitochondria energized with 20 mM α-GP addition of MB stimulated basal respiration by 64% (from 335 ± 19 to 550 ± 24 pmol/s/mg protein), which was also observed in the presence of CAT (446 ± 16 versus 602 ± 21 pmol/s/mg protein), but no significant effect of MB was seen in ADP-stimulated mitochondria. In the presence of rotenone (Table 1) the effects of MB were similar, both qualitatively and quantitatively, to those observed in the absence of complex I inhibition.

These results indicate that only the basal mitochondrial respiration is stimulated but the accelerated oxygen consumption in ATP-synthesizing mitochondria is unaffected by MB. Likewise, reduced respiration in mitochondria lacking complex I function is improved by MB. In addition, experiments with different respiratory substrates show that no matter whether electrons enter the respiratory chain via complex I or downstream at CoQ, MB remains a powerful stimulant of basal respiration, strongly suggesting that MB is able to accept electrons not only from NADH but also from the flavin group of succinate dehydrogenase or α-GPDH.

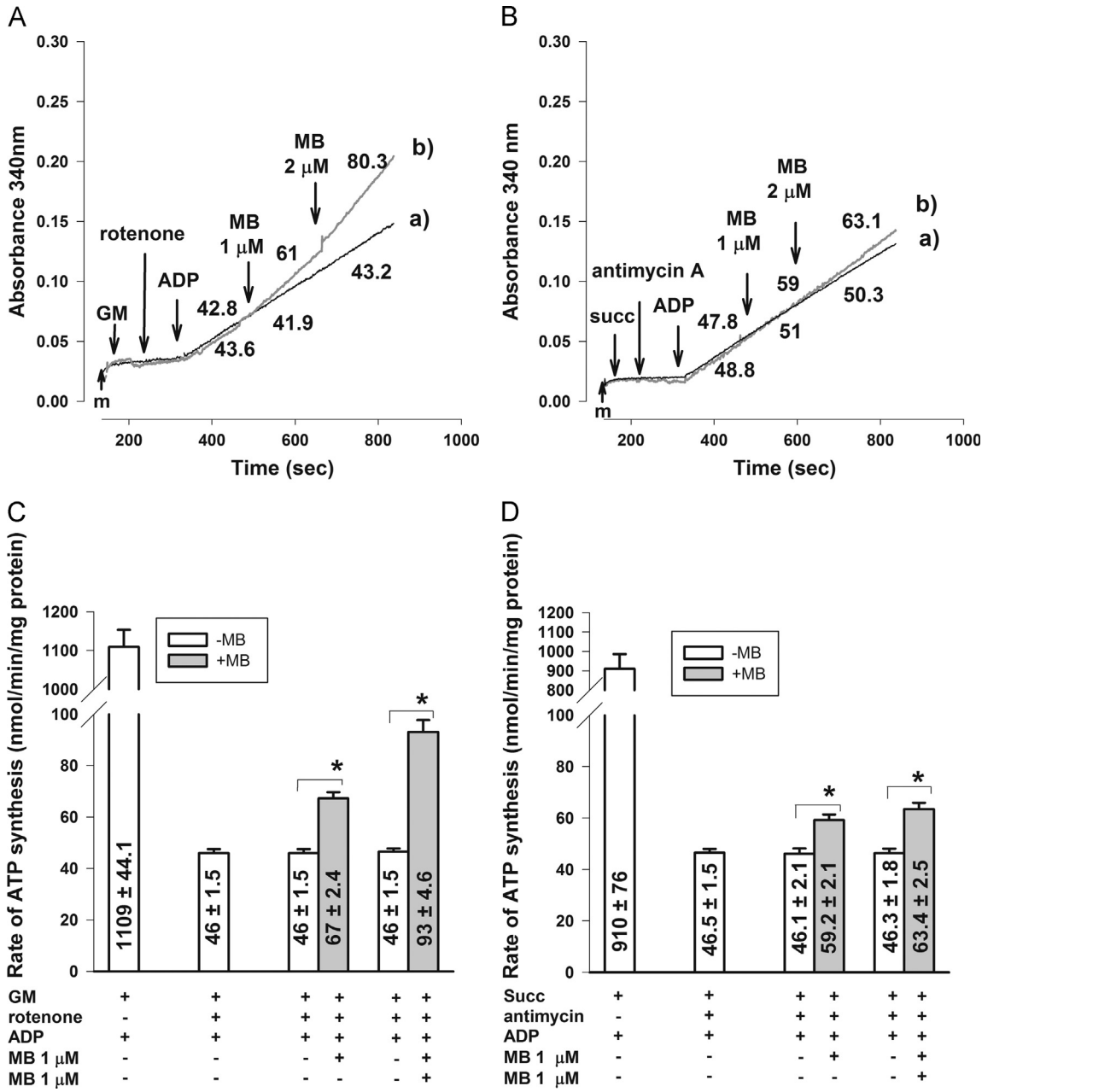

Fig. 2. The effects of MB on the rate of ATP production in respiration-impaired mitochondria. (A, C) In glutamate plus malate-supported mitochondria rotenone (0.5 μM) was used to inhibit complex I; (B, D) in succinate-supported mitochondria complex III was inhibited by antimycin A (0.1 μM). Original traces are shown in (A) and (B) with numbers calculated as the rate of ATP synthesis in nmol/min/mg in these particular experiments; additions were as indicated (MB 1 and 2 μM final concentrations). For traces (a) no MB was given. In (C) and (D) bars represent the average ATP production rates in nmol/min/mg protein ± SEM from at least three experiments. *Significant difference ($P < 0.05$) from the corresponding controls measured in the absence of MB.

ATP production is partially restored in respiration-impaired mitochondria

Stimulation of respiration itself is not informative as to the bioenergetic competence of mitochondria. To address this, ATP production in mitochondria respiring on glutamate plus malate or succinate was measured. In fully functional mitochondria ATP generation was unaffected by MB applied in 2 μM concentration (data not shown); however, significant effects were observed in respiration-compromised mitochondria. As expected, the rate of ATP generation initiated by addition of ADP in glutamate plus malate-supported mitochondria was drastically decreased by rotenone (from 1100 ± 44 to 46 ± 1.5 nmol/min/mg protein). The slow rate of ATP generation was significantly increased by 1 or 2 μM MB (67 ± 2.4 or 93 ± 4.6 nmol/min/mg protein, respectively; Figs. 2A and C). In succinate-supported mitochondria the rate of ATP generation was reduced by the complex III inhibitor, antimycin A, from 910 ± 76 to 46.5 ± 1.5 nmol/min/mg protein. ATP generation from this low level was slightly but significantly stimulated by 1 or 2 μM MB (to 59.2 ± 2.1 or 63.4 ± 2.5 nmol/min/mg protein, respectively; Figs. 2B and D).

The effect of MB on $\Delta\Psi_m$ in resting and respiration-compromised mitochondria

$\Delta\Psi_m$ is another key parameter that is essential for the bioenergetic performance of mitochondria. In highly energized mitochondria in the presence of glutamate plus malate (5 mM each) $\Delta\Psi_m$ was unchanged by MB as detected by safranin fluorescence (Fig. 3, traces a and b). With insufficient amount of substrates, in the presence of 50 or 10 μM glutamate plus malate, mitochondria cannot be fully energized because of the limited availability of NADH as indicated by $\Delta\Psi_m$ set at a depolarized value (Fig. 3, traces c and e, respectively). Diversion of electrons from NADH to MB under these conditions is probably responsible for the evident further loss in $\Delta\Psi_m$ (traces d and f), which is not apparent when substrates are abundant (traces a and b). Similar results were obtained with mitochondria respiring on succinate (not shown).

In glutamate plus malate-supported mitochondria rotenone induced an abrupt drop in $\Delta\Psi_m$, which recovered after addition of 1 μM MB, and then these mitochondria, similar to competent ATP-synthesizing mitochondria, reacted with depolarization to the addition of ADP followed by recovery of $\Delta\Psi_m$ in response to the ANT inhibitor, CAT (Fig. 4A, trace b). $\Delta\Psi_m$ was not rescued by MB when complex IV was inhibited by KCN (Fig. 4A, trace c). ATP synthesis can also be blocked by oligomycin, instead of CAT, given after ADP (Fig. 4B, trace b), but with this, smaller repolarization was observed than with CAT (compare traces b in Fig. 4A versus Fig. 4B). In mitochondria without MB addition, oligomycin given after ADP resulted in a small further depolarization (Fig. 4B, trace a), whereas CAT slightly repolarized $\Delta\Psi_m$ (Fig. 4A, trace a). The explanation lies in the different reversal potentials of the adenine nucleotide translocase and the ATP synthase. At low $\Delta\Psi_m$ ATP synthase can operate in reverse, but ANT still works in the forward mode. Under this condition application of oligomycin depolarizes $\Delta\Psi_m$ because it stops proton pumping, but inhibition of ANT would hyperpolarize mitochondria [18].

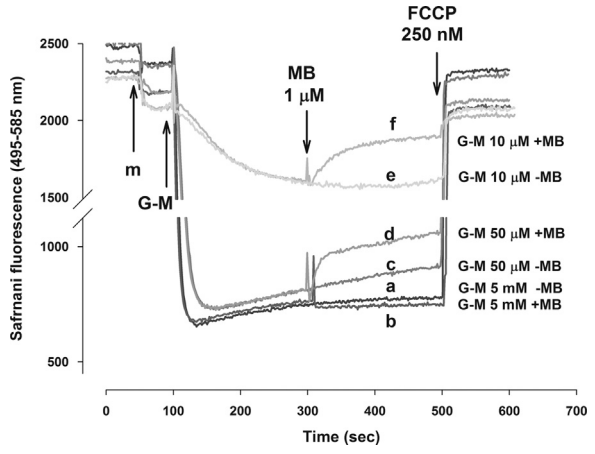

Fig. 3. The effects of MB on safranin fluorescence indicating $\Delta\Psi_m$ in mitochondria respiring on glutamate plus malate (G-M; 5 mM for traces a and b, 50 μM for traces c and d, and 10 μM for traces e and f). MB (1 μM) was added as indicated for traces b, d, and f. Traces are representative of three parallel experiments.

Similarly, rescue of $\Delta\Psi_m$ by MB, though to a smaller degree, was observed with succinate (Fig. 4C, trace b) or α-GP (Fig. 4D, trace b) as a substrate, when respiration was inhibited and $\Delta\Psi_m$ was highly depolarized with the complex III inhibitor, antimycin. In contrast to this, in mitochondria poisoned by cyanide, MB was unable to rescue $\Delta\Psi_m$ (Figs. 4A, C, and D, traces c), indicating that MB donates electrons to the respiratory chain proximal to cytochrome oxidase. These results indicate that MB supports the maintenance of $\Delta\Psi_m$ in mitochondria subjected to inhibitors of complex I or complex III.

Ca^{2+} uptake capacity is increased by MB in energetically compromised mitochondria

Ca^{2+} uptake is also a key function of mitochondria and it is well established that mitochondrial Ca^{2+} uptake is an energy- and $\Delta\Psi_m$-dependent process [17,64]. The effect of MB on the mitochondrial Ca^{2+} uptake was investigated in resting and energetically compromised mitochondria. The rate of Ca^{2+} uptake was decreased in the presence of 1 μM MB in resting mitochondria energized with glutamate plus malate (Fig. 5A, upper trace). In contrast to this, Ca^{2+} uptake reduced in rotenone-treated mitochondria was stimulated by MB (Fig. 5B, lower trace), consistent with the partially restored $\Delta\Psi_m$ observed under similar conditions.

Enhanced H_2O_2 generation by MB in both energized and respiration-impaired mitochondria

Given the controversy in the available data as to the pro-oxidant versus antioxidant response of various cells to treatment with MB [5,10,26,27,29,35,93], we compared the H_2O_2 generation in brain mitochondria in the absence and presence of MB. The use of various substrates to support respiration in these experiments is justified by the fact that, owing to the different sites of entry of electrons into the respiratory chain, ROS are generated by distinct mechanisms. H_2O_2 formation was measured using the Amplex UltraRed horseradish peroxidase (HRP) fluorescence system as described under Materials and methods.

H_2O_2 formation in mitochondria energized with glutamate plus malate

In mitochondria fueled with NADH-linked substrates, complex I, the major site of entry of electrons into the respiratory chain [7], and/or α-ketoglutarate dehydrogenase [6,61,77,80] appears to be the predominant site of ROS generation. Complex III in these mitochondria contributes to ROS production mainly when the respiratory chain is blocked at a downstream site [2].

As demonstrated in Fig. 6, MB caused a remarkably large stimulation of H_2O_2 production in the presence of glutamate plus malate. The stimulation of H_2O_2 formation by MB was evident both in resting mitochondria (in the absence of ADP), in which the rate of H_2O_2 generation was increased from 378 ± 12 to

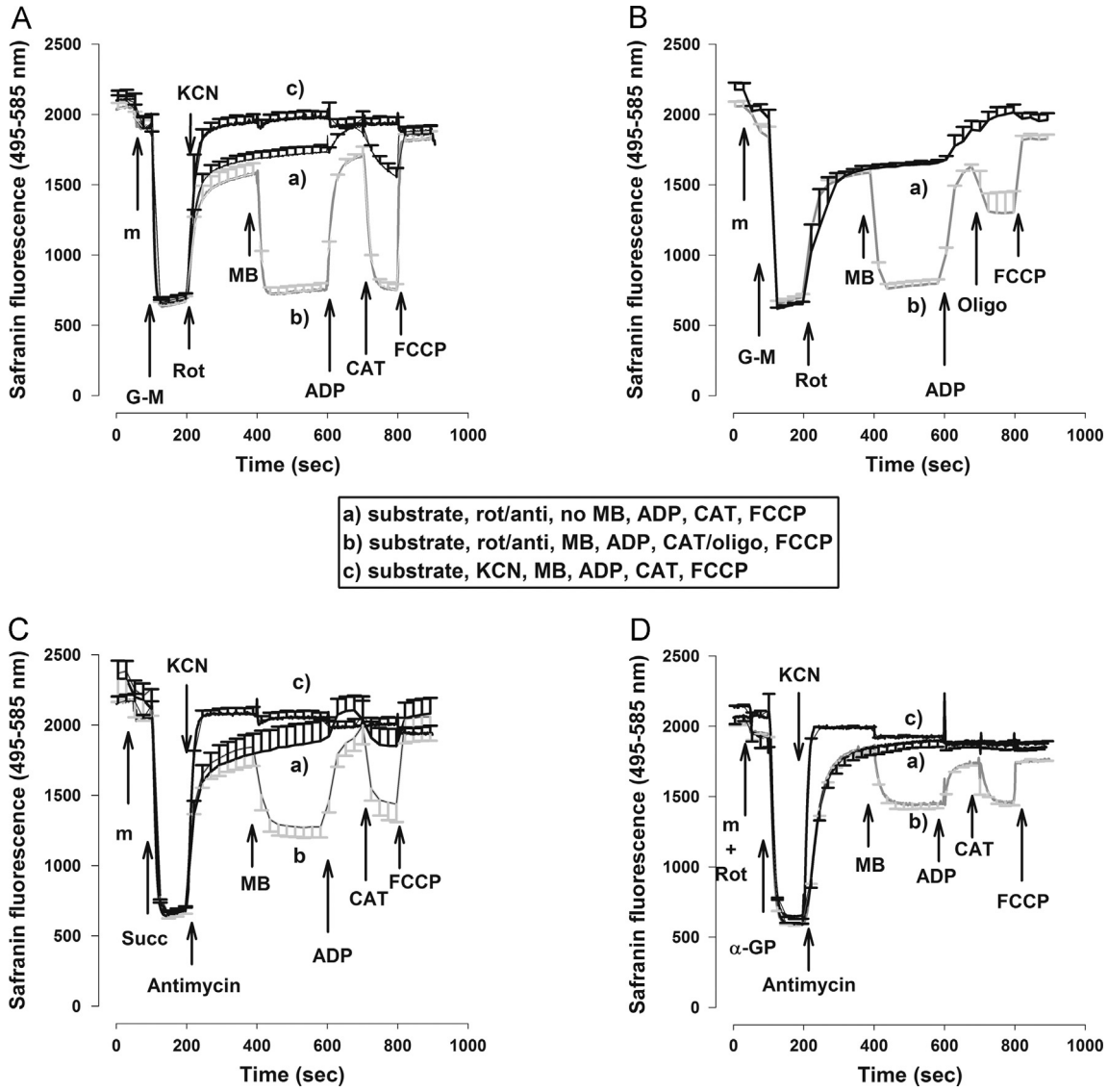

Fig. 4. The effect of MB (1 μM) on $\Delta\Psi_m$ in respiration-impaired mitochondria supported by (A, B) glutamate plus malate (5 mM each), (C) succinate (5 mM), or (D) α-GP (20 mM). Additions are indicated by arrows and the order of additions for traces a, b, and c is also shown in the frame. For traces a, no MB was given. For each trace mitochondria (m) and then respiratory substrate were given, then the order of additions was as follows: traces a, rotenone (0.5 μM, for (A) and (B)) or antimycin (0.1 μM, for (C) and (D)), ADP (2 mM), CAT (2 μM), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 250 nM); traces b, rotenone ((A) and (B)) or antimycin ((C) and (D)), MB (2 μM), ADP, CAT ((A), (C), and (D)) or oligomycin (2 μM, (B)), FCCP; traces c, KCN (2 mM), MB, ADP, CAT, FCCP. Each trace represents an average ± SEM of at least three independent experiments.

1792 ± 53 pmol/min/mg protein by 1 μM MB (not shown), and in ATP-synthesizing mitochondria (in the presence of ADP, in which the rate of H_2O_2 generation is smaller than that without ADP owing to depolarization of $\Delta\Psi_m$; Figs. 6A and C). The rate of H_2O_2 formation under the latter condition was significantly increased by as low as 100 nM MB from 147.8 ± 21 to 660 ± 16 pmol/min/mg protein and further increased to 1516 ± 47 pmol/min/mg by 1 μM MB. NAD(P)H fluorescence was decreased by MB in parallel to the stimulation of H_2O_2 generation (Fig. 6D, trace c); an effect less apparent in the presence of ADP, when the level of NAD(P)H was already low (Fig. 6D, trace b, compared to trace a, for which no MB was given). To address H_2O_2 formation in energetically impaired mitochondria, rotenone (0.5 μM) was given after ADP, followed by addition of MB (100 nM, 300 nM, or 1 μM). Rotenone, as documented earlier [2,32,78,86,90] resulted in a huge stimulation of H_2O_2 formation (from 138 ± 8 to 1268 ± 18 pmol/min/mg protein) and 1 μM MB was able to induce a significant further increase in the rate of H_2O_2 generation to 2069 ± 28 pmol/min/mg protein (Fig. 6B).

H_2O_2 formation in mitochondria energized with succinate

In succinate-supported mitochondria succinate dehydrogenase [62] and complex III could contribute to the overall ROS generation (see [1]) and when sufficient proton-motive force is generated, RET is considered a major ROS-forming mechanism [7,32]. The RET-related high rate of H_2O_2 generation observed in fully polarized mitochondria (in the presence of 5 mM succinate) [7,31,43,45] in the present experiment was 2219 ± 180 pmol/min/mg protein (Fig. 7A). Addition of 1 μM MB induced an additional increase in the rate of H_2O_2 formation to 3321 ± 113 pmol/min/mg protein. When RET was prevented by rotenone the rate of H_2O_2 generation was dropped to 451 ± 15.9 pmol/min/mg protein, but MB also under this condition resulted in a large stimulation of H_2O_2 generation (to 1887 ± 83 pmol/min/mg protein). In parallel experiments the level of NAD(P)H was moderately decreased by MB (Fig. 7C, trace b). The sharp drop in the NAD(P)H fluorescence by rotenone (Fig. 7C, trace b) reflects the elimination of RET and NADH generation by complex I and was parallel with a decrease in H_2O_2 formation as demonstrated in Fig. 7A. At low succinate concentration (50 μM), which is

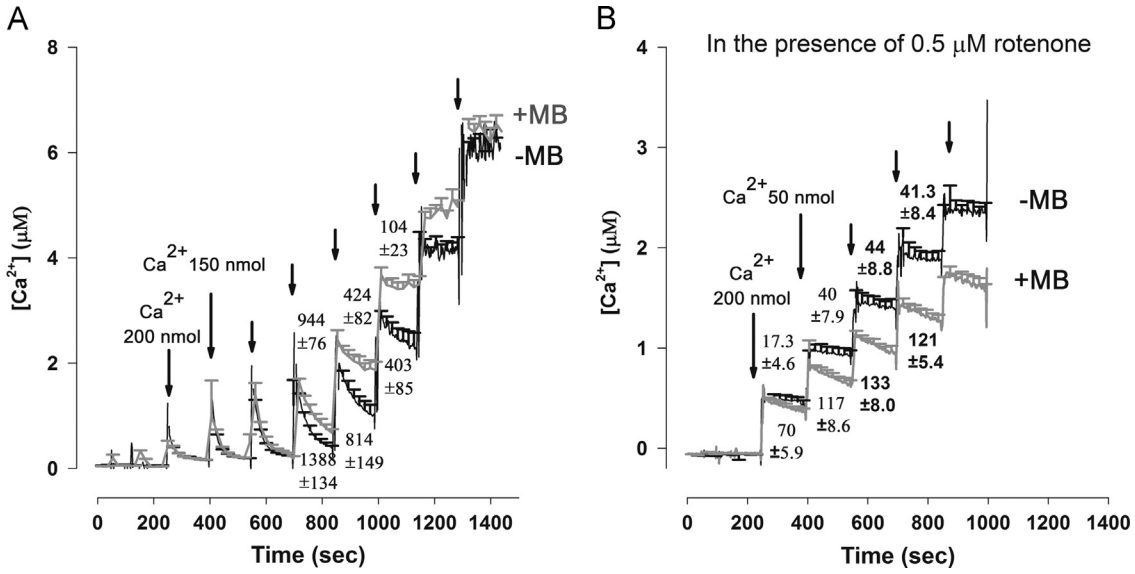

Fig. 5. The effect of MB on the Ca^{2+} uptake (A) in energized and (B) in rotenone-treated mitochondria respiring on glutamate plus malate. Ca^{2+} pulses (150 nmol each for (A) except the first addition, which was 200 nmol; 50 nmol each for (B) except the first addition, which was 200 nmol) were given every 100 s in the absence (lower trace in (A); upper trace in (B)) or in the presence of 1 μM MB (upper trace (gray line) in (A); lower trace (gray line) in (B)). Lower Ca^{2+} concentrations in the presence of rotenone were used to avoid permeability transition pore opening under this condition. At the end of the experiments Ca^{2+} was added at saturating concentrations. Numbers on the traces represent average rates of changes in Ca^{2+} concentration in the medium (nM/min). Traces are averages ± SEM from at least four independent experiments.

insufficient to support RET, the rate of ROS formation was low but addition of 1 μM MB produced a sixfold elevation in the rate of H_2O_2 generation (from 41.3 ± 4 to 274 ± 21 pmol/min/mg protein, which was further stimulated by rotenone (Fig. 7B). There was only a small and slow NAD(P)H formation here (Fig. 7C, traces c and d), unrelated to RET, as addition of rotenone significantly increased NAD(P)H level (Fig. 7C, trace c), suggesting that NADH generation due to succinate oxidation in the Krebs cycle is reflected in the NAD(P) signal under this condition.

To demonstrate that with Amplex UltraRed fluorescence H_2O_2 is specifically detected, we added catalase to the incubation medium containing MB. The MB-induced rise in the fluorescence signal was abruptly halted by catalase, which then reacted to the addition of HRP. This shows that the elevated signal in the presence of MB indeed reflects H_2O_2 generation (Fig. 7D). It is noteworthy that in this experiment lower activity of horseradish peroxidase was used to allow an efficient competition between HRP and catalase for scavenging H_2O_2, explaining the smaller rate of H_2O_2 formation found in this particular experiment. H_2O_2 generation in the experiments with MB in mitochondria respiring on glutamate plus malate or α-GP was also verified with catalase (not shown).

H_2O_2 generation in α-GP-supported mitochondria

As previously characterized [53,82,84,85] ROS generation at high α-GP concentration (20 mM) can be attributed mainly to complex I receiving electrons from the oxidation of α-GP via RET [31,82,84]. Oxidation of α-GP at a low α-GP concentration (5 mM) is unable to generate sufficient proton-motive force for RET; electrons for H_2O_2 generation under this condition were suggested to originate largely from α-GPDH or, in the case of a blockage of electrons at complex III, they could be provided by CoQ or complex III [52,59,84].

In mitochondria respiring on 20 mM α-GP, MB induced a large stimulation of H_2O_2 generation (Fig. 8A); the rate of H_2O_2 formation was increased from 554 ± 40 to 1368 ± 31 pmol/min/mg protein (Fig. 8C). Consistent with the involvement of RET and RET-related NADH generation in this effect, both rotenone and ADP, which decreased α-GP-supported ROS generation by preventing RET [84,85], decreased the MB-induced H_2O_2 formation (Fig. 8C). H_2O_2 generation stimulated by MB was paralleled by a drop in the NAD(P)H fluorescence (Fig. 8B, trace b), suggesting that for H_2O_2 formation MB takes electrons from NAD(P)H generated by RET. MB, however, remained capable of enhancing H_2O_2 formation in the total absence of RET (Fig. 8C). This is evident not only because MB increased H_2O_2 formation in 20 mM α-GP-supported mitochondria in the presence of rotenone or ADP, but also because mitochondria respiring on 5 mM α-GP, when no RET is possible, also responded with a huge increase in H_2O_2 generation to a treatment with MB (from 67 ± 10.9 to 459 ± 51 pmol/min/mg; Fig. 8D). This shows that for MB-mediated H_2O_2 generation electrons could be provided by α-GPDH as well.

The rate of H_2O_2 elimination in mitochondria is decreased by MB

Mitochondria not only produce H_2O_2 but also participate in the elimination/detoxification of H_2O_2 [24,69,75,98], and we investigated whether this function is influenced by MB. Elimination of 5 μM H_2O_2 from the medium was followed in the presence of mitochondria supported with glutamate plus malate and stimulated by ADP (Fig. 9). Half-life of H_2O_2 (τ) in the medium was 54.5 ± 5.4 s, which increased to 139.2 ± 22.5 s when MB (1 μM) was also present in the medium. MB at 300 nM concentration was already effective at increasing the half-life of H_2O_2 in the medium (not shown). MB also decreased the rate of H_2O_2 elimination in mitochondria treated with rotenone (0.5 μM) (not shown).

Enhanced H_2O_2 signal in the presence of MB is unrelated to illumination during the experiment

MB is used in photodynamic therapy and MB-mediated phototoxicity was suggested to be, at least partially, ROS-dependent [27,51,58]. It is important to demonstrate that the enhanced H_2O_2 signal in the presence of MB presented above is not due to the illumination during excitation in our experiments. Thus, to exclude false positive results with MB, key experiments were repeated without using continuous illumination of the samples. For this, the excitation light of the PTI Deltascan fluorescence

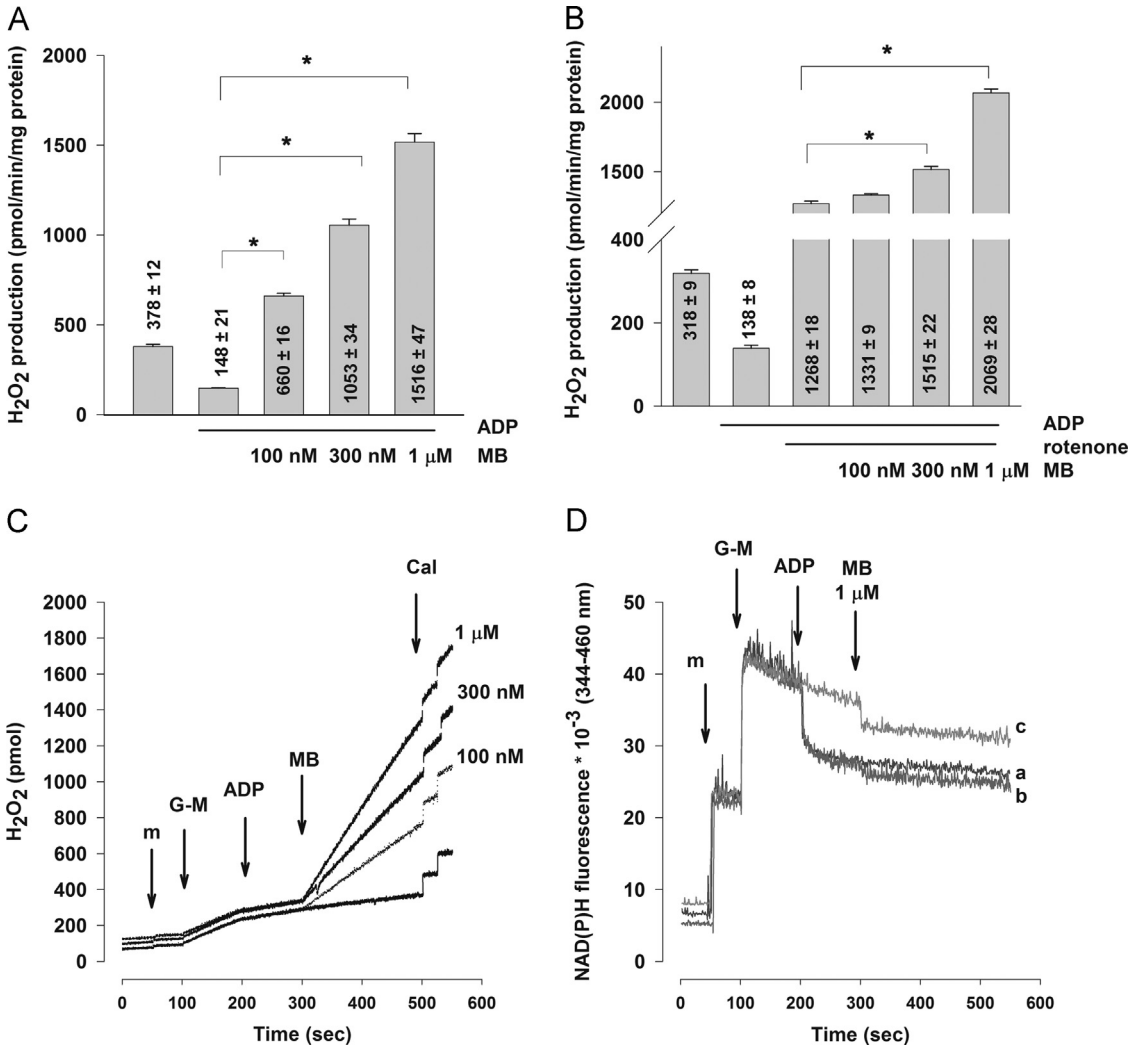

Fig. 6. The effects of MB on (A, B, C) H_2O_2 production and (D) NAD(P)H steady state in glutamate plus malate-supported mitochondria. Mitochondria (m; 0.1 mg/ml), ADP (2 mM), MB, and 0.5 μM rotenone (for (B)) were given where indicated. Representative experiments with original traces are shown in (C) and (D). Data on H_2O_2 generation in pmol/min/mg protein in (A) and (B) represent the average ± SEM from at least four experiments. *Significant difference.

spectrophotometer was switched on and off using a shutter. When the illumination time was reduced to 1/8 of the control, the Amplex UltraRed fluorescence signal in response to MB decreased only by 9% and there was no difference in the mitochondrial NAD(P)H fluorescence, either (Fig. 10). We can safely conclude that more than 90% of H_2O_2 production observed in the presence of MB is independent of the illumination of the samples. Similarly, measurements on mitochondrial oxidation in the presence of MB were also repeated in the dark, providing results essentially similar to those presented above (not shown).

Discussion

Addressing several key bioenergetic parameters in this study, we aimed to dissect the mitochondrial effects of MB, which could determine its beneficial effects found in several pathological conditions [5,10,14,15,21,26,60]. In addition, release as well as elimination of H_2O_2 was addressed to reveal the pro-oxidant/antioxidant effect of MB that accompanies the mitochondrial bioenergetic alterations.

(i) We established that MB stimulated respiration in isolated brain mitochondria and, by this, confirmed several earlier reports demonstrating MB-stimulated oxygen consumption in different mitochondria and cellular models [10,44,88,89,93]. Stimulation of respiration by MB has been assigned to its ability to shunt electrons in the respiratory chain. It was first described in the 1960s and 1970s that MB could be reduced in isolated mitochondria by electrons from NADH and transfer them to cytochrome *c*, bypassing coenzyme Q in the respiratory chain [70,92]. Although reduction of MB by flavoenzymes such as xanthine oxidase, NADH cytochrome *c* reductase, and NADPH cytochrome *c* reductase has been reported [39,50], generally NADH has been considered a major electron donor for MB in mitochondria [10,93,60]; recently it has also been found in isolated rat heart mitochondria that cytochrome *c* reduction was increased with NADH as an electron donor but not with succinate [44,60]. It is evident from our study that respiration was improved by MB not only with NADH-linked substrates but also with succinate or α-GP, suggesting that electrons from succinate dehydrogenase or α-GPDH could also reduce MB. This conclusion is supported by data obtained with diphenyleneiodonium (DPI), which was shown to inhibit complex I by keeping the flavin groups reduced [46]; MB in DPI-treated succinate-supported mitochondria significantly increased (by 10%) the State 3 respiration of mitochondria (not shown). Importantly, our study shows that in functional

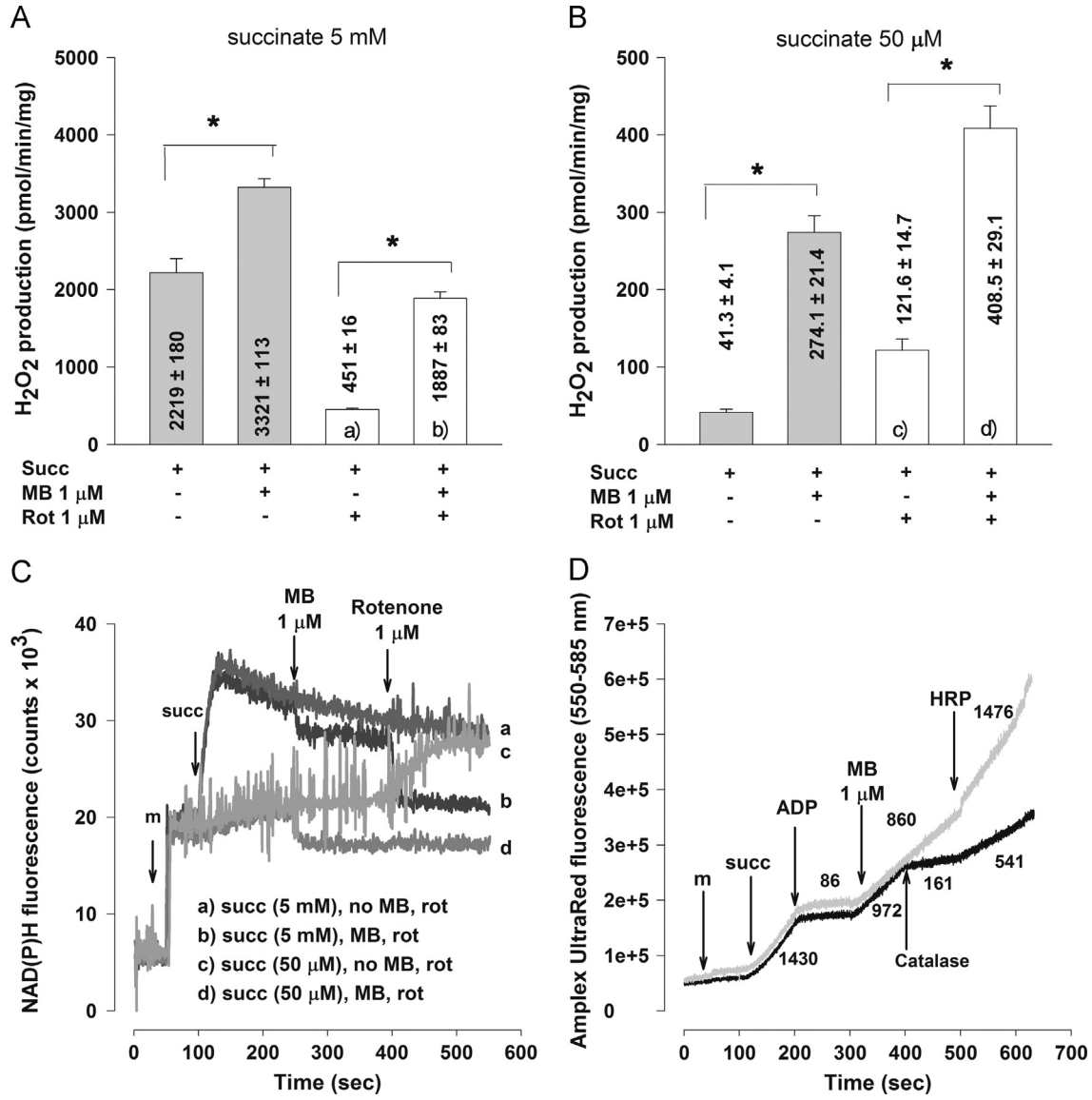

Fig. 7. The effects of MB on succinate-supported (A, B) H_2O_2 production and (C) NAD(P)H level. Succinate (5 mM for (A) and for traces a and b in (C); 50 μM for (B) and for traces c and d in (C)) was given to mitochondria, then 300 s later 1 μM MB was added where indicated, followed by addition of rotenone (0.5 μM) after 300 s, where indicated. Numbers on bars in (A) and (B) indicate the average rate of H_2O_2 production in pmol/min/mg ± SEM from at least four independent experiments. (D) The effect of catalase on the MB-induced Amplex UltraRed fluorescence. Horseradish peroxidase activity at the beginning of these experiments was 20 times lower than in other Amplex experiments. Numbers indicate the rate of fluorescence changes per second. Traces are representative of three similar measurements. *Significant difference.

mitochondria only the resting oxygen consumption is stimulated; the ADP-stimulated respiration is unaffected by MB. Because mitochondria in vivo are functioning in the presence of ADP, this finding allows the conclusion that in vivo MB might not influence the respiration of normal ATP-synthesizing mitochondria. However, the significant stimulation of oxygen consumption in respiration-impaired mitochondria supports the suggestion that MB could partially restore respiration when the flux of electrons in the respiratory chain is inhibited either at complex I or at complex III, evident in this study with NADH-linked substrates in the presence of rotenone (Fig. 1) or with succinate in the presence of antimycin (not shown).

(ii) Accelerated respiration alone would not imply an improved bioenergetic performance of mitochondria, but only when this would result in an increased ATP generation. We report here, as a new finding, that in respiration-impaired mitochondria, in the presence of complex I or complex III inhibitors, the severely inhibited ATP synthesis was increased by MB (Fig. 2). The rate of ATP synthesis in rotenone-inhibited mitochondria was doubled in the presence of 2 μM MB, but was still modest compared to that observed in mitochondria not treated with rotenone. Importantly, ATP synthesis in normally respiring isolated brain mitochondria was unchanged by MB. This finding seems to be contradictory to that by Wen et al. [93] showing an increased cellular ATP level in HT-22 cells. However, in this report the ATP level in the transformed HT-22 cells was only moderately decreased by strong mitochondrial drugs such as the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone or the complex IV inhibitor cyanide [93], implicating a largely nonmitochondrial ATP generation.

(iii) It is also revealed in this study that MB, although not influencing $\Delta\Psi_m$ in fully respiring functional mitochondria, supports the maintenance of $\Delta\Psi_m$ when complex I or complex III is inhibited (Fig. 4). The significance of this finding is that with a partially maintained $\Delta\Psi_m$ mitochondria become more resistant against PTP induction [13,56,57,63]; therefore, the chances for survival of these mitochondria are highly

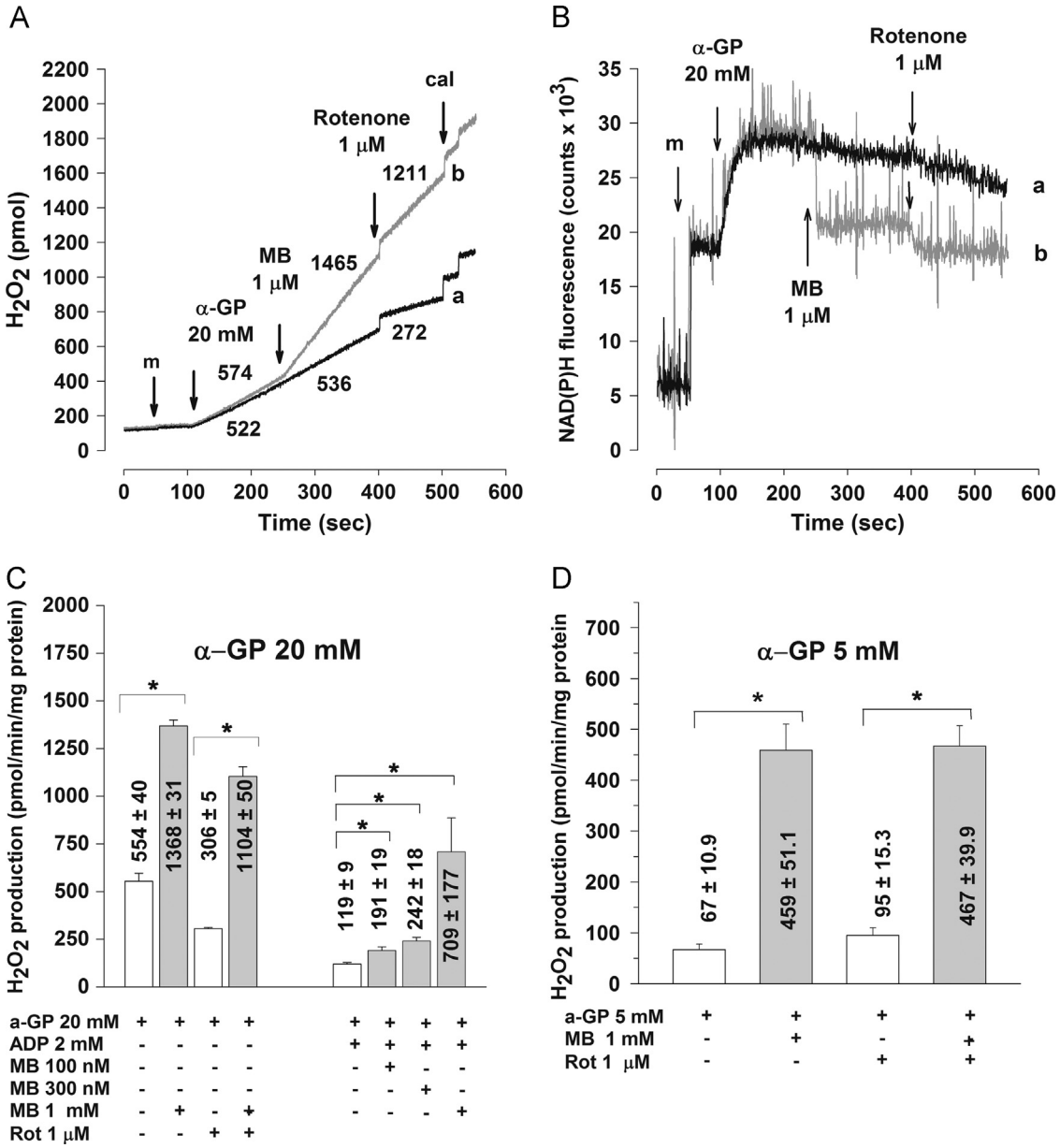

Fig. 8. The effect of MB on (A, C, D) H_2O_2 production and (B) NAD(P)H level in α-GP-supported mitochondria. Mitochondria (m), MB (1 μM; for traces b), or rotenone was given as indicated. Original traces from one experiment are shown in (A) and (B). Experiments with rotenone were as shown for (A), but in the experiments with ADP, this was given 150 s after α-GP followed by MB addition 150 s later. Quantitative data from at least four experiments observed in the presence of 20 mM (C) or 5 mM α-GP (D) in pmol/min/mg ± SEM are shown. *Significant difference.

increased. This suggestion is supported by data from Ca^{2+}-uptake measurements in this study (Fig. 5) showing an improved Ca^{2+} uptake capacity in MB-treated mitochondria. Rescued $\Delta\Psi_m$ by MB in rotenone-treated mitochondria could contribute to a decreased rotenone-induced neurodegeneration observed in HT-22 cells [60,93] and retinal ganglion cells [96]. The finding that ADP depolarized $\Delta\Psi_m$ in rotenone-treated mitochondria in the presence of MB (Figs. 4A and B) indicates that the rescued $\Delta\Psi_m$ is able to drive ATP synthesis. Depolarization of HT-22 cells by a neurotoxic amount of glutamate was also attenuated by MB [60], but HeLa cells were depolarized in the presence of MB [58]. Depolarization in our study was seen only when substrates were used in suboptimal concentrations (Fig. 3), decreasing the amount of NADH available for complex I, and under this condition diversion of electrons from NADH by MB could critically decrease proton pumping.

Our results show that mitochondria modestly but significantly benefit from the ability of MB to transfer electrons between NADH and cytochrome *c* or between α-GPDH or succinate dehydrogenase and cytochrome *c*, bypassing blocks in the respiratory chain at either complex I or complex III. The beneficial effects of MB are consistent with the modestly improved ATP synthesis and maintained $\Delta\Psi_m$ in respiration-impaired mitochondria shown in this report. The polarizing action of CAT in rotenone- and MB-treated mitochondria (Fig. 4) indicates that MB prevents ANT from functioning in reverse, which could be critical to save the glycolytic ATP from entering mitochondria and being hydrolyzed by ANT. The reversal of ANT is dependent upon $\Delta\Psi_m$ and the concentrations of ADP and ATP on both sides of the inner membrane [18]. These data extend our understanding of the bioenergetic consequences of stimulated respiration by MB reported earlier by many studies [10,15,16,88,93,96], which alone would be insufficient to suggest improved mitochondrial function.

(iv) We found in this study a remarkable increase in the rate of H_2O_2 release from mitochondria by MB. The extent of stimulation of H_2O_2 release by MB was unusually large; a greater than fourfold increase was induced already at 100 nM MB in ATP-synthesizing glutamate plus malate-supported mitochondria (Fig. 6A). The enhanced H_2O_2 generation was observed in respiring, as well as in respiration-impaired mitochondria, under resting as well as ATP-synthesizing conditions, and with each substrate combination. These observations were somewhat surprising considering literature reports on the antioxidant effects of MB [10,12,22,33,60,93]. MB, in the presence of cytochrome *c*, decreased the paraquat-induced superoxide production [39] and suppressed the superoxide generation in the xanthine oxidase reaction [68]. These effects were clearly related to a competition between MB and oxygen for electrons resulting with the two-electron reduction of MB to MBH_2, rather than the one-electron reduction of O_2 to $O_2^{\cdot-}$. As an explanation for the suppressed superoxide generation in the xanthine oxidase reaction by MB the possibility of reduction of superoxide to H_2O_2 by MBH_2 has been raised [68]. Similarly, Poteet et al. [60] assumed that MBH_2 might directly scavenge superoxide, contributing to the decrease in the glutamate-evoked ROS generation observed with the nonselective fluorescent ROS indicator H_2DCF-DA in HT-22 cells, and then H_2O_2 could be eliminated by catalase or peroxidases, without the accumulation of harmful reactive oxygen species. However, the actual H_2O_2 generation or accumulation in the presence of MB has never been addressed in a biological system, in particular in mitochondria, where MB exerts its major cellular effects. Furthermore it has not been considered that H_2O_2 is one of the reactive oxygen species having its own damaging effect due to interaction with iron–sulfur centers and protein SH groups, though with less reactivity than superoxide (see [95]). H_2O_2 has sensitive targets within mitochondria [19,36,55,76,79,83,97] and because it is a membrane-permeative ROS its effect in vivo is likely to extend beyond mitochondria.

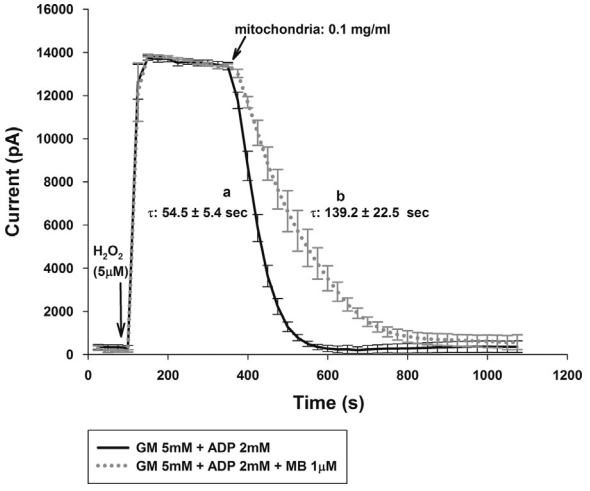

Fig. 9. Removal of exogenous H_2O_2 in the presence and absence of MB (1 μM) as measured by H_2O_2-sensitive electrode. H_2O_2 (5 μM) was added to the medium containing glutamate plus malate (5 mM each) and ADP (2 mM) with (trace b) or without (trace a) MB. 300 s later 0.1 mg/ml mitochondria were applied as shown. Average half-life (sec) of H_2O_2 is given as mean ± SEM (n=6).

For the ROS measurements in cellular studies suggesting antioxidant effects of MB, DCF-DA [60,93], a nonselective ROS sensor, or MitoSOX [93], detecting superoxide, has been used. With these dyes H_2O_2 formation is not detected or could be masked by a reduced superoxide generation in the presence of MB. In fact, when we used MitoSOX to repeat crucial experiments done with Amplex red no increase in superoxide signal was observed in the presence of MB (not shown). H_2O_2 measurements with Amplex fluorescence in mitochondria are generally done with the understanding that it reflects primarily generation of superoxide, which is dismutated to H_2O_2. In the particular case with MB, however, H_2O_2 could be generated without superoxide formation. Our results are in line with the effect

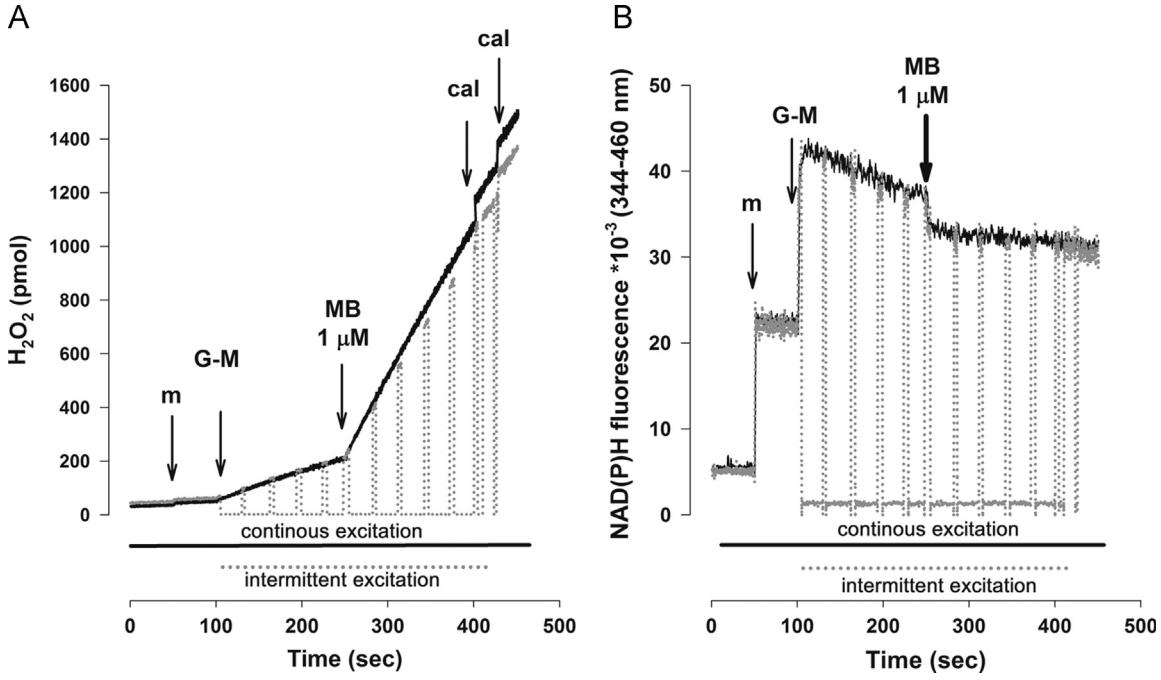

Fig. 10. The effect of intermittent excitation light on MB-induced changes in (A) H_2O_2 production and (B) NAD(P)H level. Under control conditions (black continuous line) mitochondrial samples in the cuvette were exposed to continuous excitation light (550 nm for (A) and 340 nm for (B)). In the period from 100 to 400 s (gray, dotted line) samples were subjected to cycles of 35 s of dark and 5 s of illumination by closing and opening the shutter for excitation light. Additions were as indicated on the graphs. Traces are representative of three experiments.

of MB as an alternative electron acceptor in mitochondria taking electrons from complex I [10], but also from complex II and α-GPDH, as shown in this study, resulting in the formation of MBH_2. We suggest that MBH_2 would reduce not only cytochrome *c* but also O_2, generating H_2O_2 and recycling back to MB. This interpretation is consistent with the standard redox potential of MB → MBH_2 (E'_o +10 mV) favoring electron donation for the ½O_2 → H_2O_2 reaction (E'_o +300 mV) under standard conditions. The remarkably large H_2O_2 generation implies that a significant number of electrons from MBH_2 reduce O_2 instead of cytochrome *c*, decreasing the efficiency of the improvement in the bioenergetic competence of MB-treated mitochondria. This could explain the relatively modest increase in the ATP synthesis in MB-treated respiration-impaired mitochondria (1.5-fold increase; Fig. 2C), whereas the respiration under the same conditions is stimulated 3-fold (Fig. 1). In addition to stimulating the generation of H_2O_2 MB also attenuates the elimination of H_2O_2 (Fig. 9). This could also result, at least partly, from the electron-acceptor property of MB oxidizing glutathione directly (E'_o 2GSH → GSSG −230 mV) as demonstrated in red blood cells [38]. Furthermore, the decrease in NAD(P)H level by MB could impair the glutathione peroxidase and thioredoxin system, the major mechanisms responsible for H_2O_2 elimination. In a mitochondrion-free experiment we could indeed demonstrate an MB-dependent oxidation of NADPH, but no direct inhibition of glutathione reductase by MB (data not shown).

The huge H_2O_2 release from mitochondria in the presence of MB observed in our study clearly indicates an oxidative burden, which should be balanced when MB is applied in vivo. The need for an increased resistance to H_2O_2 may be reflected in the enhanced expression of thioredoxin reductase measured in HepG2 cells treated with MB [10].

In MB-treated piglets increased transcription of antioxidant enzymes in the brain was also found in vitro [49]. Likewise in a recent study MB improved among others the oxidative damage in P301S mice and upregulated the prosurvival Nrf2/ARE genes [74]. As Nrf2 is known to be upregulated by H_2O_2 [48,74] it is likely that the stimulated H_2O_2 production demonstrated in the present study contributes to the upregulation of Nrf2/ARE genes.

In summary, our results demonstrate significant bioenergetic improvement by MB in isolated respiration-impaired mitochondria, in particular a modest, but significant increase in ATP synthesis and a restoration of $\Delta\Psi_m$, which could be important in the beneficial in vivo neuroprotective and cognitive-enhancing action of MB. However, the highly elevated H_2O_2 generation observed in our in vitro study has to be considered in the estimation of the overall oxidative state in vivo in mitochondria under treatment with MB.

Acknowledgments

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