Stimulation of respiration by methylene blue in rat liver mitochondria

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Abstract The effect of methylene blue on isolated rat liver mitochondria in the presence and absence of chloroacetaldehyde was investigated. Fatty acid oxidation was inhibited by chloroacetaldehyde and subsequently stimulated by methylene blue. Assessment of tightly coupled mitochondria revealed decreasing respiratory control ratios induced by increasing concentrations of methylene blue and methylene blue provoked mitochondrial swelling. In uncoupled mitochondria, methylene blue promoted a concentration-dependent stimulation of respiration. These findings provide evidence that methylene blue, the redox dye currently used as an antidote for encephalopathy associated with alkylating chemotherapy, uncouples oxidative phosphorylation and acts as an electron transfer mediator to stimulate mitochondrial respiration.

Key words: Mitochondrion; Oxidative Phosphorylation; Uncoupling; Methylene Blue; Ifosfamide; Chloroacetaldehyde

1. Introduction

Chloroacetaldehyde (CAA) is a potent mutagen, capable of impairing an array of normal cellular functions [1–3]. It is a reactive metabolite of many industrial chemicals [4,5] and of the alkylating, cytostatic drugs ifosfamide (IFO) and cyclophosphamide [6,7]. CAA is presumed to be neurotoxic [8–10], and its presence is thought to potentiate IFO associated encephalopathies. Presently, methylene blue (MB) is prophylactically or acutely used as an antidote for IFO induced encephalopathy [11–14], although its mechanism of action and relation to CAA toxicity are undefined. Since an inhibition of normal hepatic fatty acid oxidation may trigger an encephalopathic event [15–17], the relations between mitochondrial fatty acid metabolism, CAA and MB were investigated.

The effect of MB on cellular redox status is well established. MB has been shown to non-enzymatically oxidize NADH to NAD and reverse some of the redox state changes in ethanol-fed animals [18–21]. Others have demonstrated its inhibition of aldehyde dehydrogenase in cytosol and mitochondria from human and rat, respectively [22,23]. However, MB induced alterations in mitochondrial oxidative metabolism as a consequence of its redox character remain to be described.

The present study was designed to test the hypothesis that CAA alters hepatic mitochondrial oxidative metabolism of long chain fatty acids and application of MB corrects for CAA induced metabolic perturbations. Since impairment of hepatic β-oxidation is a known marker of hepatotoxicity [24], we used isolated rat liver mitochondria and selected palmitoyl-L-carnitine (PLC), as a substrate for β-oxidation. It follows that monitoring fluctuations in fatty acid oxidation and thus, cellular energetics, may be useful in predicting mitochondrial fitness, and in turn indicate a primary step in the cascade of events leading to encephalopathy, which may be prevented or rectified due to the biophysical activity of MB.

2. Materials and methods

2.1. Materials

Methylene Blue was purchased from Hansler, AG, Herisau, Switzerland. All other chemicals were obtained from either Merck, Darmstad, Germany, or from Sigma Chemical Corp., St. Louis, MO, and were of the highest grade available.

2.2. Isolation and incubation of mitochondria

Mitochondria from the livers of male Wistar rats (200–250 g) were isolated as previously described [25]. Briefly, livers were quickly removed from decapitated rats and immediately transferred to ice-cold mannitol [0.25 M]-sucrose [0.07 M] buffer (MSB). Livers were finely minced and EDTA [2 mmol/L] was added prior to homogenisation. Mitochondria were isolated by means of differential centrifugation, suspended in MSB at a concentration of 1 g original liver weight per ml and stored on ice. Protein concentrations in mitochondrial preparations were determined with the biuret method using bovine serum albumin as standard [26].

Oxygen consumption by intact mitochondria was measured at 37°C in a chamber equipped with a Clark-type oxygen electrode. Buffer for all mitochondrial incubations contained potassium phosphate [0.1 M], triethanolamine [0.1 M], magnesium sulfate [0.15 M] in addition to MSB, pH 7.4.

The respiratory control ratio (RCR = state 3/state 4) in mitochondria energized with succinate was used to control the quality of each preparation. For RCR evaluations in the presence of MB, the redox dye was introduced to the incubation chamber before addition of succinate.

In incubations to assess the effect of CA and MB on fatty acid oxidation, rotenone [2 μM] was used to inhibit the oxidation of endogenous NAD-linked substrates. Uncoupling of mitochondria was achieved by the addition of 2,4-dinitrophenol (DNP) [150 μM]. Mitochondria uncoupled with DNP were used in all experiments with PLC since fatty acids are potent uncouplers in themselves. Addition of PLC [100 μM], allowed for the assessment of mitochondrial β-oxidation.

2.3. Mitochondrial swelling

Experiments with freshly isolated, tightly coupled mitochondria were carried out in KCl [0.175 M]-Tris-HCl [0.025 M] buffer at pH 7.4. Approximately 0.5 mg of mitochondrial protein were diluted in working buffer and swelling was followed by the decrease in turbidity or absorbance (A_{520}) in a Perkin Elmer Lambda 16 spectrophotometer. Readings were taken every 60 s.

2.4. Mathematical and statistical analysis

Data were fitted to the Michaelis-Menten equation in order to calculate the kinetic parameters of K_m and V_{max}. Results are expressed as mean ± S.E.M. of measurements. Statistically significant differences between means were first evaluated by a one-way analysis of variance. When significant '*F*-values' were obtained, within group comparisons

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were assessed by a Student's t-test. Two-tailed probabilities of less than 0.05 were considered to be significant and Bonferroni criteria were applied, where applicable.

3. Results and discussion

Essential to the maintenance of normal mitochondrial function is an intact cascade of reactions resulting in the β-oxidation of fatty acids. We have observed the correlation between CAA and mitochondrial dysfunction. Specifically, we have explored the relationships between mitochondrial β-oxidation, CAA and subsequent exposure to MB. To assess this, uncoupled mitochondria energized with PLC were exposed first to CAA [500 μM] followed by MB [1 or 2 μM]. CAA induced a 4-fold decrease in oxidative metabolism, while the addition of MB [1 μM] to the PLC+CAA mitochondrial incubation stimulated oxygen consumption by 2-fold. PLC+CAA incubations receiving MB [2 μM] produced respiratory rates comparable to mitochondria respiring on substrate previous to CAA intoxication (Fig. 1). Since electrons from reduced MB are known to re-enter the electron transport chain near cytochrome c, and low concentrations [2 μM] can be used to create a bypass of the point where antimycin A blocks electron transport [27,28], it is conceivable that CAA may exert its toxic effect directly on elements of the respiratory chain, inhibiting electron transfer at or before cytochrome c. The presence of MB would therefore not prevent CAA induced local toxicity, but rather compensate for reduced function. Alternatively, CAA may lead to the formation of chloracetic acid and chloroacetyl-coenzyme A [29] and thus, depletion of CoASH pools. In this event, β-oxidation would consequently be slowed, and MB would act not only as a shuttle for electrons, but more importantly, as a donor.

To better estimate the electron donating capacity of MB and subsequent flux through the respiratory chain, we exposed uncoupled mitochondria, without substrate, to rotenone [2 μM] and MB [0.5–45 μM]. A concentration-dependent, saturable response to MB was realized which, when fit to Michaelis-Menten conditions yielded a K_m of 2.2±0.3 μM and V_{max} of 233.8±8.0 nmol/min per mg protein (Fig. 2A). In the presence of MB+PLC, a similar relation was observed and the associated K_m and V_{max} calculated values were 4.0±0.7 μM and 2456±131 nmol/min·mg protein, respectively (Fig. 2B). The control rate of oxidation of PLC in uncoupled mitochondria in the presence of rotenone was 245.7±15.6 nmol/min·mg protein marking a maximum 10-fold increase in fatty acid oxidation induced by MB. V_{max}/K_m values indicate a 6-fold increase in the metabolic capacity when PLC is added to mitochondria in the presence of MB as compared to MB alone.

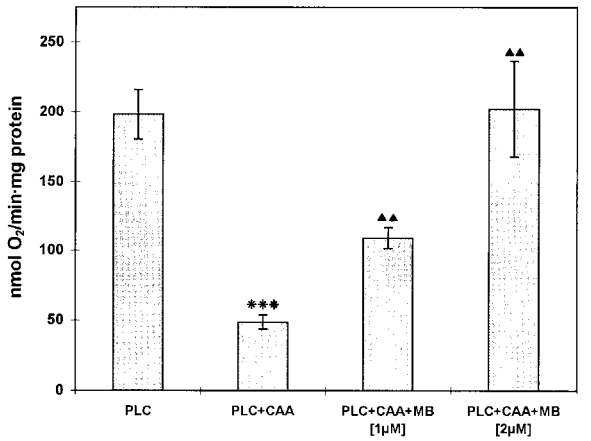

Fig. 1. Inhibition of β-oxidation by CAA and subsequent stimulation with MB. Mitochondria in the presence of rotenone [2 μM] and DNP [150 μM] with substrate PLC were sequentially exposed to CAA [500 μM] and MB [1 or 2 μM]. CAA induced significant impairment of long chain fatty acid oxidation. Addition of MB rapidly stimulated respiration. ***Significant difference in comparison with PLC ($p<0.001$). ▲▲Significant difference in comparison with PLC+CAA ($p<0.01$).

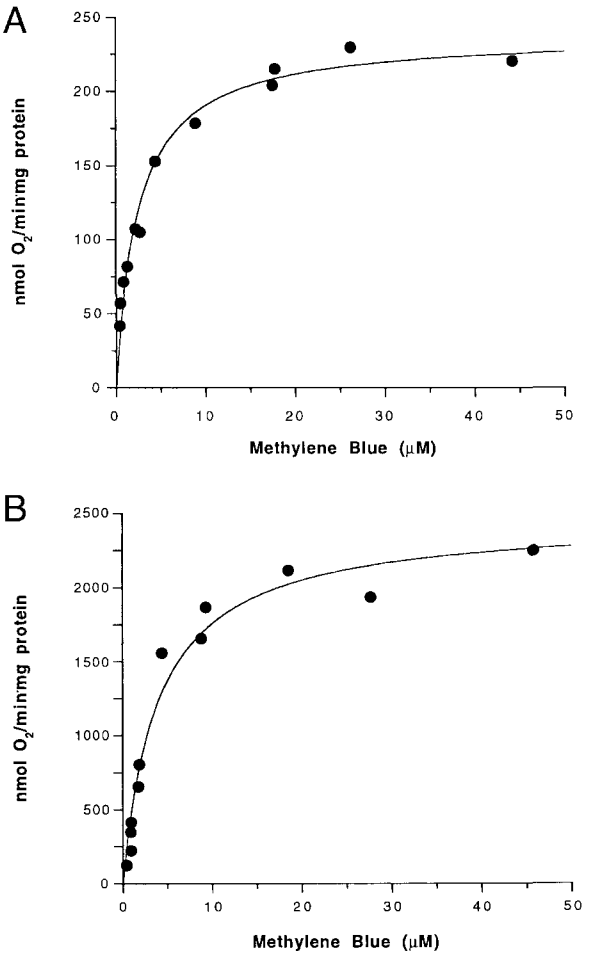

Fig. 2. Stimulation of respiration by MB in uncoupled mitochondria. (A) Mitochondria in the presence of rotenone [2 μM] and DNP [150 μM] but absence of substrate. (B) Effect of MB on β-oxidation. Mitochondria in the presence of rotenone [2 μM], DNP [150 μM] and PLC [100 μM].

In tightly coupled, succinate-respiring mitochondria, addition of MB resulted in an acceleration of state-4 respiration, resulting in the overall decrease of RCR values, indicating the uncoupling of oxidative phosphorylation (OXPHOS) (Fig. 3). Significant inhibition of OXPHOS was apparent after the addition of just 0.5 μM MB, whereas, mitochondria exposed to 5 μM MB demonstrated a much stronger inhibition. Our selected range of MB was based on recently published data reporting a mean concentration of MB [5 μM] attained in plasma after a single 100 mg intravenous bolus injection [30], a typical daily dose administered to patients receiving IFO.

To determine if ultrastructural changes induced the uncoupling of OXPHOS, we investigated the effect of MB on mitochondrial swelling. Appreciable swelling occurred in mitochondria exposed to MB [5 μM], while mitochondria receiving MB [0.5, 1 or 2 μM], were not significantly different from controls (Fig. 4). Since the onset of swelling occurs in mitochondria incubated with MB [5 μM], while inhibition of OXPHOS begins with just 0.5 μM MB, structural permutations, although supportive of the uncoupling effect, are secondary to MB promoted derangements in oxidative metabolism.

Mitochondrial respiration and OXPHOS play a major role in cellular bioenergetics. We describe substantial modulation of OXPHOS and respiration in coupled and uncoupled rat liver mitochondria, respectively. Analysis of tightly coupled mitochondria provided evidence that sub-micromolar concentrations of MB could significantly impair OXPHOS. Higher concentrations, in the range attainable in plasma, uncouple OXPHOS and induce mitochondrial swelling. In uncoupled mitochondria, MB effectively stimulates respiration in the presence or absence of PLC or CAA, due to its multiple actions, namely, rapid oxidation of NADH providing reducing equivalents necessary for β-oxidation and electron donating/shuttling capacity, with respect to the respiratory chain. We demonstrate empirically that CAA dramatically reduces mitochondrial respiratory rates and MB affects both OXPHOS and the oxidation of long chain fatty acids resulting in compensatory, increased mitochondrial respiration. It has not escaped our attention that MB induced stimulation of fatty acid oxidation may be useful in a wide variety of applications where the metabolism of long chain fatty acids is desired. However, whether the impairment of OXPHOS or β-oxidation is an important event in the onset of IFO induced neurotoxicity remains to be established, but the fact that MB is currently used to protect from or acutely treat encephalopathic events implies an important link.

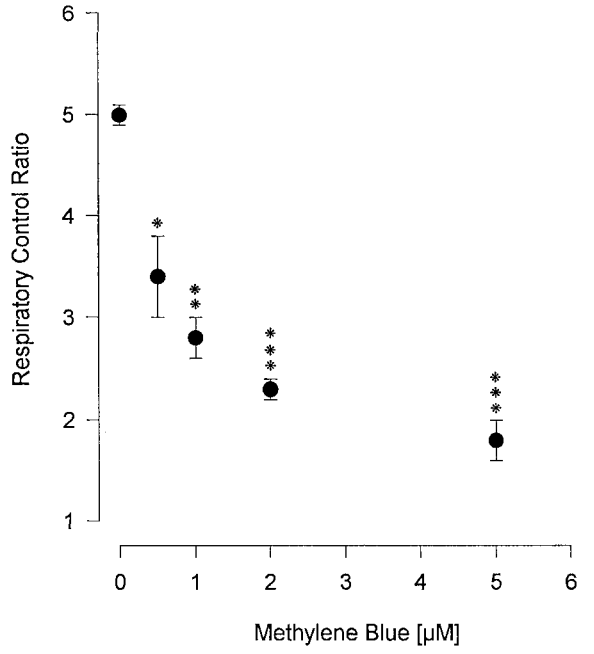

Fig. 3. Uncoupling of oxidative metabolism by MB. RCRs for succinate were measured in tightly coupled mitochondria exposed to varying concentrations of MB previous to succinate and ADP addition. Significant difference in comparison to control RCR: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

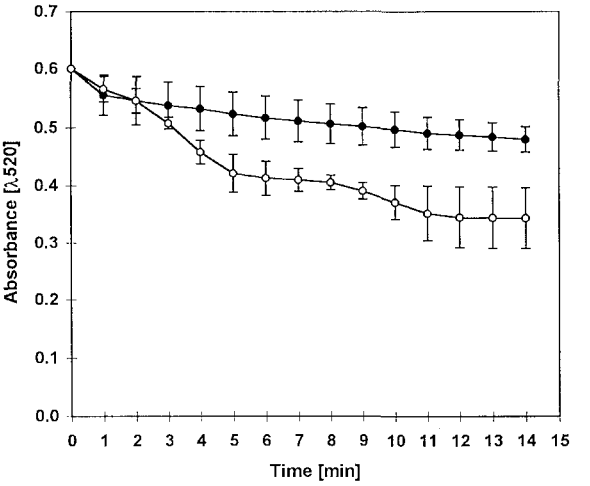

Fig. 4. MB induced mitochondrial swelling. Tracings of the change in absorbance [λ 520] of control mitochondria (filled circles), and those in the presence of MB [5 μM] (open circles). Incubations with MB [0.5, 1 or 2 μM] were not significantly different from control and thus do not appear in the figure. MB [5 μM] ($p < 0.001$) in comparison to control.

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