The Effect of Methylene Blue and Its Metabolite—Azure I—on Bioenergetic Parameters of Intact Mouse Brain Mitochondria

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Abstract—Methylene blue, a phenothiazine dye, that is widely used in medicine and is under clinical trials as an agent for treatment of Alzheimer’s disease. One of the factors of the unique therapeutic effect of methylene blue is its redox properties, allowing implementation of alternative electron transport: the dye accepts electrons from reducing equivalents in mitochondria and transfer them to other components of the respiratory chain or molecular oxygen. Azure I, an N-dimethylated metabolite of methylene blue, is potentially a more effective compound than methylene blue, but its ability for alternative electron transport has not been studied yet. We have shown that in contrast to methylene blue, azure I is unable to restore the membrane potential in isolated mouse brain mitochondria, inhibited by rotenone and, therefore, is unable to perform bypass of the respiratory chain complex I. Moreover, addition of azure I does not affect the rate of mitochondrial respiration in contrast to methylene blue, which increases the rate of non-phosphorylation respiration. At the same time, both dyes stimulate an increase in H2O2 production. Thus, only methylene blue is capable of alternative electron transport, while azure I does not produce complex I bypass. This limits its therapeutic application only as a mitochondrial-targeted agent, but does not question its antidepressant effects.

Keywords: methylene blue, azure I, mitochondria, alternative electron transport

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INTRODUCTION

Mitochondria are the most important center of the cell where not only metabolic but also signaling pathways determining cell fate (cell survival or death) converge. Mitochondria are the main source of energy and the site of production of large quantities of reactive oxygen species (ROS). ATP production requires a high membrane potential, at which the maximum ROS production is observed [1]. Animal mitochondria have systems for uncoupling the coupled respiratory chain, but they are not characteristic of all tissues and are less efficient than, for example, alternative plant respiratory chains [2]. Therefore, pharmacological approaches to uncoupling the mitochondrial electron transport chain (ETC) represent a promising approach to treat metabolic diseases.

2,4-Dinitrophenol was one of the first metabolic modulators widely used in medicine, particularly, for the treatment of obesity. However, a wide range of side effects was identified, which led to its complete ban in some countries, for example, in the USA and UK [3]. During the last decade, the concept of “alternative electron transport” was formulated, which, to some extent, could be also considered as the way to uncouple coupled respiration [4]. Methylene blue (Fig. 1a) belongs to the class of phenothiazines; due to its unique redox properties methylene blue can accept electrons from reducing equivalents (e.g. NADH or FADH2) and transfer them to other components of the respiratory chain (for example, complex III or cytochrome c) or molecular oxygen. Using this approach it is possible to bypass damaged or inhibited site of the respiratory chain [4]. In contrast to 2,4-dinitrophenol, methylene blue is a safe compound lacking serious side effects. It is widely used to treat methemoglobinemia, malaria, and cyanide poisoning. The photodynamic activity of the compound makes it possible to use methylene blue in the treatment of oncological diseases and as an antiviral agent [5]. Certain evidence exists that the dye is effective against Covid-19 [6].

However some side effects of methylene blue have been also described. Methylene blue treatment of methemoglobinemia in patients with glucose-6-phosphate dehydrogenase deficiency leads to a sharp decrease in NADPH levels and can cause hemolysis [7], which can also occur in newborns treated with this compound [8]. Intra-amniotic administration of methylene blue causes hemolytic anemia [9] and liver
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MATERIALS AND METHODS

Research Objects

Eight male C57BL/6 mice were used in experiments. The animals were kept under standard vivarium conditions at 25°C and air humidity of at least 40%. Mice received a standard laboratory diet and drinking water ad libitum. Animals were sacrificed by rapid cervical dislocation followed by decapitation without the use of anesthetics, since the use of anesthetics can cause changes in the bioenergetic characteristics of mitochondria, in particular, uncoupling of oxidative phosphorylation [21]. All bioenergetic parameters of mitochondria were measured in at least six technical repeats.

Isolation of Brain Mitochondria

After decapitation, the mouse brain was removed and homogenized using a KIMBLE Dounce tissue grinder (Sigma-Aldrich, USA) in buffer A, containing 225 mM mannitol (Sigma-Aldrich), 75 mM sucrose (Dia-M, Russia), 5 mM Hepes (BioClot, Germany), 1 mM ethylene glycol tetraacetic acid (EGTA) (Sigma-Aldrich), pH 7.4 with the addition of 2 mg/mL fatty acid-free bovine serum albumin (BSA) (Dia-M). The wash buffer (buffer B) used in the centrifugation step had the same composition except BSA. The resulting homogenate was centrifuged using a Z 36 HK centrifuge (Hermle Labortechnik, Germany) for 5 min at 900 \( g \). The supernatant was transferred into clean tubes and centrifuged for 10 min at 14000 \( g \). After that, the supernatant was removed, and the pellet was resuspended in 100 \( \mu \)L of buffer B and after addition of digitonin (Sigma-Aldrich) (0.02% final concentration) the suspension was incubated on ice for 2 min. The tubes were centrifuged for 15 min at 14000 \( g \). The supernatant was removed again, the pellet was resuspended in 100 \( \mu \)L of buffer B, and then centrifuged for 10 min at 14000 \( g \). The last step was repeated twice. The resulting pellet was resuspended in 20 \( \mu \)L of buffer B [22].

Measurement of Bioenergetic Parameters

All subsequent experiments, performed in six independent measurements, were carried out in buffer A (its composition was described above). The rate of mitochondrial respiration was measured using an Oxygraph high-resolution respirometric system (Han-
The membrane potential was measured using a Hitachi F-7000 fluorescent spectrophotometer (Hitachi High Technologies, Japan). The value of the transmembrane potential was measured by the fluorescence intensity of the cationic lipophilic probe safranin O (Sigma-Aldrich). The excitation wavelength was 495 nm, and the emission wavelength was 586 nm. The substrate, 5 mM pyruvate + 5 mM malate, 4 mM phosphate (KH₂PO₄), 1 μM safranin O, and 10 μL of mitochondrial suspension (concentration 100 μg/mL), were added to 1 mL of buffer A. Changes in respiration intensity were recorded after the addition of 1 μM methylene blue (Sigma-Aldrich) and 1 μM azure I (Interkhim, Russia) in separate measurements (independently of each other).

The rate of H₂O₂ production in mitochondria was measured using an Amplex Ultra Red fluorescent marker (Invitrogen, USA). The excitation wavelength was 530 nm, the emission wavelength was 590 nm. The substrate, 5 mM pyruvate + 5 mM malate, 4 mM phosphate (KH₂PO₄), 1 U Amplex Ultra Red, 4 U horseradish peroxidase (Amresco, USA) and 10 μL of mitochondrial suspension (100 μg/mL) were added to 1 mL of buffer A. Changes in the rate of H₂O₂ production were recorded after addition of 1 μM methylene blue and 1 μM azure I in separate measurements (independently of each other). The H₂O₂ concentration was measured as the fluorescence intensity of resorufin formed in the reaction of Amplex Ultra Red oxidation.

The protein concentration was measured using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA).

Statistical Data Processing

Statistical analysis was performed using STATIS-TICA 10 software. Data are presented as means ± error of the mean. Distribution normality was tested using the Shapiro–Wilk test (W-test). Differences between groups were assessed using the Mann–Whitney test (U-test). The paper discusses only statistically significant differences at p < 0.05.

RESULTS AND DISCUSSION

Mitochondrial Membrane Potential

Methylene blue has a wide range of functions within the cell; one of the most discussed is alternative electron transport in the mitochondria ETC. Earlier, it has been repeatedly shown that methylene blue bypasses complex I block [4, 23]. Rotenone is a classic inhibitor of complex I, which inhibits the movement of electrons in the immediate vicinity of the ubiquinone binding site [24]. Figure 2 shows that this contributed to the suppression of the membrane potential as evidenced by an increase in the level of safranin O fluorescence. The subsequent addition of 1 μM methylene blue led to a partial restoration of the membrane potential, as shown by a decrease in the level of safranin O fluorescence (Fig. 2). The addition of 1 μM azure I did not cause changes in the safranin O fluorescence, therefore, there was no restoration of the membrane potential, which was inhibited by rotenone (Fig. 2). Thus, in contrast to methylene blue, azure I is not capable of bypassing mitochondrial ETC components, at least complex I.

Respiration of Isolated Mitochondria

The rate of mitochondrial respiration using NADH-dependent substrates without the addition of ADP was 21.59 ± 1.81 nmol O₂ min⁻¹ mg⁻¹ of protein. The addition of 1 μM methylene blue stimulated an increase in the respiratory rate to 30.67 ± 3.77 nmol O₂ min⁻¹ mg⁻¹ of protein (p < 0.05) (Fig. 3a). These data are consistent with results showing that methylene blue increases the rate of ADP-unstimulated respiration with succinate as the respiration substrate of isolated rat liver mitochondria [25]. At the same time, methylene blue did not affect the rate of ADP-stimulated respiration thus suggesting that methylene blue could uncouple oxidative phosphorylation [25]. We have shown that methylene blue addition to mitochondria in the presence of ADP did not affect the rate of respiration (165.39 ± 11.07 nmol O₂ min⁻¹ mg⁻¹ of protein in the control and 182.57 ± 19.83 nmol O₂ min⁻¹ mg⁻¹ of protein in mitochondria after the methylene blue addition) (Fig. 3b). Similar data (an increase in the respiratory rate without ADP addition and the absence of stimulation of mitochondrial respiration in the presence of ADP) were obtained [23] using succinate, α-glycerophosphate, and malate + glutamate as the respiration substrates.

We have shown that the addition of 1 μM azure I did not affect the rate of either ADP-unstimulated mitochondrial respiration (21.58 ± 2.74 nmol O₂ min⁻¹ mg⁻¹ protein) (Fig. 3a) or the rate of ADP-stimulated respiration of mitochondria (140.23 ± 35.99 nmol O₂ min⁻¹ mg⁻¹ protein) (Fig. 3b). Thus, azure I does not exhibit the properties of an oxidative phosphorylation uncoupler.
The Rate of H$_2$O$_2$ Production by Isolated Mitochondria

The rate of H$_2$O$_2$ production by isolated mitochondria was 45.39 ± 5.95 pmol H$_2$O$_2$ min$^{-1}$ mg$^{-1}$. The addition of 1 μM methylene blue more than tripled the rate of H$_2$O$_2$ production (177.6 ± 14.99 pmol H$_2$O$_2$ min$^{-1}$ mg$^{-1}$; $p < 0.01$). The addition of 1 μM azure I also stimulated H$_2$O$_2$ production by isolated mitochondria (150.23 ± 7.34 pmol H$_2$O$_2$ min$^{-1}$ mg$^{-1}$, $p < 0.01$) (Fig. 4). Thus, on the one hand, the data suggests that Azur I produces 15% less H$_2$O$_2$. However, if we calculate the proportion of H$_2$O$_2$ formed from O$_2$ consumed, the results will be opposite. Mitochondria without the addition of phenothiazines produced 0.21% H$_2$O$_2$. The addition of methylene blue resulted in an H$_2$O$_2$ content to 0.58%, while the addition of azure I increases this proportion to 0.7%. These data partially correlated with LD$_{50}$ values. The LD$_{50}$ value for Azure I administered intravenously to mice is 65 mg/kg [26], which is slightly lower than for methylene blue (77 mg/kg) [27].

However, these results cannot indicate that Azur I may be potentially more toxic to the body. Certain evi-
cence exists that azure I penetrates into tissues 14 times better [16], and the therapeutic effect of 4 mg/kg azure I corresponds to the dose 20 mg/kg of methylene blue [20].

CONCLUSIONS

Thus, both methylene blue and azure I can increase the rate of H₂O₂ production by isolated mitochondria in vitro. This is not entirely consistent with the notion that they can be mild uncouplers, since an increase in the rate of H₂O₂ production is impossible at a high membrane potential [1]. We can speculate that alternative electron transport is a form of noncoupled respiration, but it is not associated with a decrease in ROS production. In this case, only methylene blue is able to perform alternative electron transport, bypassing ETC components. Azur I, the demethylated form of methylene blue, does not bypass complex I. This limits its functionality as a mitochondria-targeted drug, but does not question its therapeutic potential as a potent inhibitor of monoamine oxidase, inflammatory and apoptotic processes.

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COMPLIANCE WITH ETHICAL STANDARDS

Authors declare that they have no conflict of interest. The maintenance and euthanasia of laboratory animals was carried out according to the rules established by the Committee for the Care and Use of Animals of the Voronezh State University, which comply with the directive established by the European Union 2010/63/EU regarding experiments using animals.

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