Assessment of the Effects of Methylene Blue on Cellular Bioenergetics in H9c2 Cells

OANA M. DUICU[1,2], ILEANA SCURTU[1], ROXANA POPESCU[3], ADRIAN STURZA[1,2], DORINA CORICOVAC[4], MARIA D. DANILA[1,2], ANDREEA PRIVISTIRESCU[1], DANINA M. MUNTEAN[1,2,*]

[1]"Victor Babes" University of Medicine and Pharmacy Timisoara, Department of Pathophysiology, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

[2]"Victor Babes" University of Medicine and Pharmacy Timisoara, Center for Translational Research and Systems Medicine, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

[3]"Victor Babes" University of Medicine and Pharmacy Timisoara, Department of Cellular and Molecular Biology, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

[4]"Victor Babes" University of Medicine and Pharmacy Timisoara, Department of Toxicology, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

Methylene blue (MB), a tricyclic phenothiazine compound, has been reported to exert beneficial effects in experimental models of cerebral, hepatic, and intestinal ischemia/reperfusion injury via the modulation of mitochondrial function and the promotion of cell survival. The present study was purported to assess the acute effects of MB on the bioenergetic profile of the H9c2 rat cardiomyoblast cells. To this aim, H9c2 cells were incubated for 60 minutes with progressive concentrations of MB (0.05, 0.1, 1, 5, and 10 μM, respectively). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse Bioscience XF-24e extracellular flux analyzer. Analysis of mitochondrial function was performed in the presence of the classic modulators of the electron transport chain: oligomycin, FCCP, and antimycin. All OCR linked parameters (i.e., basal respiration, proton leak, ATP turnover, maximal respiration, spare reserve capacity), as well as ECAR were increased by 0.1 μM MB and decreased by the higher tested concentrations; the lowest concentration tested (0.05 μM) had no effect on respiratory and metabolic parameters. In conclusion, methylene blue in submicromolar concentrations elicited an overall improvement of cellular bioenergetics in the H9c2 cell line.

Keywords: methylene blue, H9c2 cell line, mitochondrial function, bioenergetics, glycolysis

In the past decades, several lines of evidence unequivocally proved the central role of mitochondrial dysfunction in the pathogenesis of ageing [1], the non-communicable chronic diseases [2], as well as of conditions associated with acute ischemia/reperfusion (I/R) injury [3]. Therefore it is not surprisingly that modulation of mitochondrial function has become nowadays an important therapeutic target [4-6]. Indeed, mitochondrial function protection has become synonim with cellular energy preservation and identifying mitochondria-targeted therapeutic agents represents currently a priority in basic and translational research. In this regard, pharmacological agents able to prevent reactive oxygen species (ROS) generation via a redox effect (i.e., transfer of electrons between the redox cytosolic and mitochondrial centers) and/or to improve mitochondrial metabolic function are of particular interest [7-8]. One of such agents is methylene blue (MB), a tricyclic phenothiazine compound, used for more than a century in the treatment of disorders such as poisoning, toxic methemglobinemia, cyclophosphamide-induced encephalopathy, hypotension in septic shock, or vasoplegia following cardiopulmonary bypass [9]. Recently, a couple of studies have demonstrated that MB, in nanomolar concentrations, increased the activity of mitochondrial complex IV, heme synthesis, cell resistance to oxidants, and oxygen consumption [8].

MB protective effects have been studied in pathological states associated with cerebral, hepatic, and intestinal I/R injury [10-13]. To the best of our knowledge, there are no data describing the effects of MB on mitochondrial function in normal and pathological heart. Accordingly, the present study was aimed at assessing the concentration-dependent effects of MB on bioenergetic and metabolic parameters in H9c2 rat cardiomyoblasts.

Experimental part

Material and methods

Reagents

All chemicals were from Sigma-Aldrich.

Cell culture

H9c2 rat cardiomyoblast cells were purchased from ECACC and maintained in a humidified incubator gassed with 5% CO_2 at 37°C. Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-Glutamine, 100U/mL penicillin, and 100 ng/mL streptomycin.

Bioenergetic and metabolic effects of MB were assessed in H9c2 cells using the XF24 Flux Analyzer (Seahorse Biosciences) according to a protocol modified after (Nicholls, Darley-Usmar et al. 2010) that allows the simultaneous measurement of the oxygen consumption rate (OCR) as an indicator of mitochondrial respiration, and the extracellular acidification rate (ECAR), as a measure of the glycolysis that generates lactic acid, respectively.

H9c2 cells were seeded as 25 K cells/well in Seahorse XF-24 cell culture plates (in duplicate). Twenty-four hours later, the incubation media was replaced by unbuffered XF assay medium (provided by Seahorse Co.) containing 25 mM glucose. Sixty minutes prior to the initiation of OCR

* *email: daninamuntean@umft.ro*

and ECAR measurements, cells were treated with 5 concentrations of MB (0.05, 0.1, 1, 5, and 10μM, respectively) and compared to controls (Ctrl, untreated cells). OCR and ECAR rates were measured over the time to establish the baseline rates. Afterwards the cells were metabolically challenged (in order to shift their bioenergetic profile) by three successive additions: oligomycin (1 μg/mL), FCCP (3μM), and antimycin A (5 μM). Previous studies were performed to optimize the cell seeding density and the FCCP concentrations, respectively (data not shown). Background correction wells (i.e., wells that were not seeded with cells) were included in the assay to normalize the data to the background plate noise.

The following OCR parameters were recorded (fig. 1): (1) the basal respiration of the cells; (2) the percent of O_2 consumption required for the ATP production (ATP turnover); (3) the amount of O_2 consumption devoted to maintain the proton gradient (proton leak); (4) the maximal respiratory rate under conditions of uncoupled respiration; (5) the spare reserve capacity (the difference between the maximal respiratory rate and the basal respiration), also called the reserve capacity; (6) the amount of O_2 consumption not due to mitochondrial processes (non-mitochondrial respiration) [14]. For further analysis, mitochondrial parameters were corrected to the non-mitochondrial respiration and instrumental background.

OCR was reported in units of pmoles/minute and ECAR in mpH/minute.

Statistical analysis

All values are presented as mean ± SEM. Group comparisons were performed by one-way analysis of variance (ANOVA) and Dunnett's post-hoc multiple comparison test (GraphPad Prism version 5.0). Values for $p < 0.05$ are considered statistically significant.

Results and discussions

The dynamic of the experimental protocol and the effects of the MB on OCR and ECAR plotted against time are presented in figure 2. The following injections have been performed: firstly, oligomycin (Omy) injection, by blocking the proton channel of the ATP synthase (complex V) induced a decrease in OCR (upper panel). Since intracellular ATP synthesis via OXPHOS was blocked in the presence of Omy, energy production shifted to glycolysis, therefore ECAR significantly increased (lower panel). Second, in the presence FCCP (a classic uncoupler of OXPHOS), the cells had to overcome the proton leak across the inner mitochondrial membrane; the collapse of the mitochondrial membrane potential lead to a rapid consumption of energy and O_2 without the generation of ATP. Accordingly, both OCR and ECAR increased, OCR due to uncoupling, and ECAR as the cells attempted to maintain their energy balance by using glycolysis to generate ATP. Finally, injection of antimycin A (Ama) – a classic inhibitor of mitochondrial complex III, induced an important decrease of OCR, while ECAR was not significantly modified, since cells shifted to a glycolytic state, in order to maintain their energy balance [15].

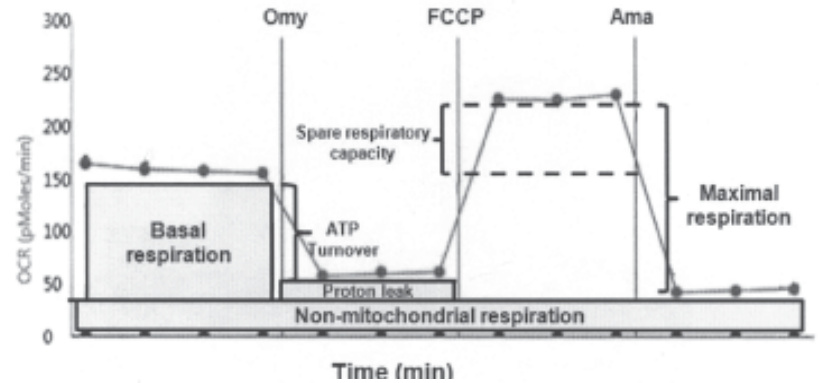

Fig. 1. Schematic representation of the bioenergetic and metabolic parameters recorded during the experimental protocol. Basal respiration represents OCR before compound injections. Proton leak is the difference in OCR after oligomycin and antimycin A injection. ATP turnover is the difference between basal respiration and proton leak. Maximal respiration is the OCR after FCCP injection. Spare respiratory capacity is the difference between maximal and basal OCR. The non-mitochondrial rate was subtracted from all other rates.

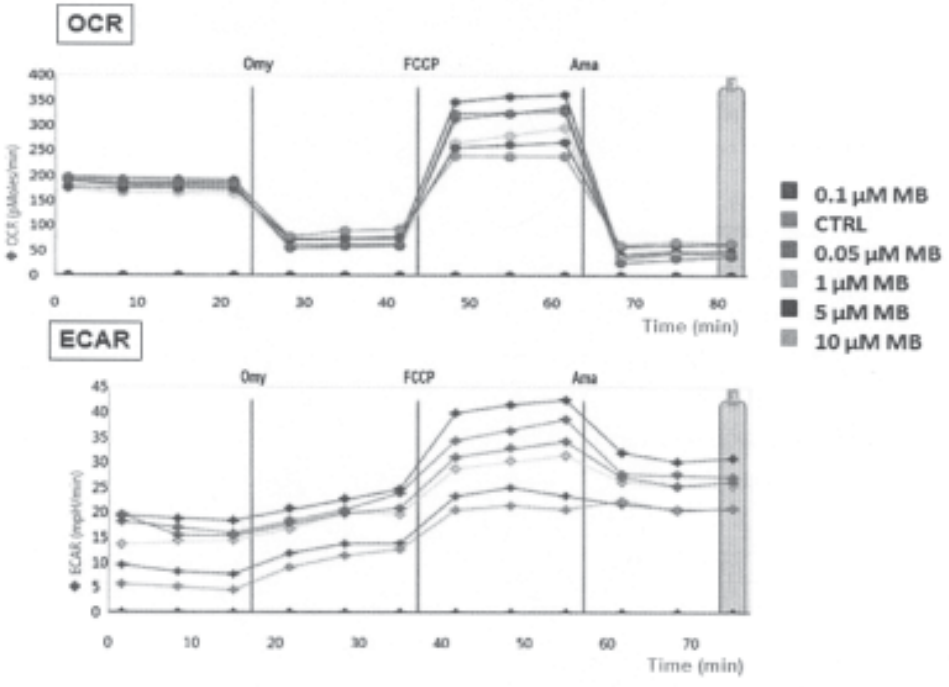

Fig. 2. Graphic representation of the experimental protocol in dynamic and the dose-dependent effects of MB on OCR and ECAR. Increasing concentrations of MB (0.05, 0.1, 1, 5, and 10 μM) have been tested and compared with the untreated Ctrl group (n = 6-8/group). OCR and ECAR measurements were performed in the presence of successive injections of oligomycin (Omy,1 μg/mL), FCCP (3 μM), and antimycin A (Ama, 5 μM)

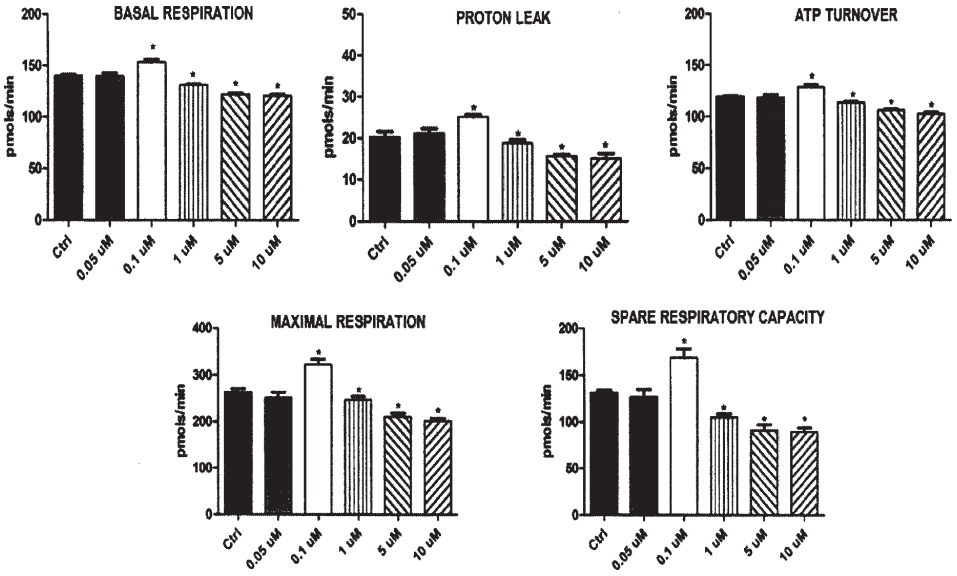

Fig. 3. Acute effects of MB on OCR linked parameters. (n = 6–8/group. *p < 0.05 vs. untreated controls).

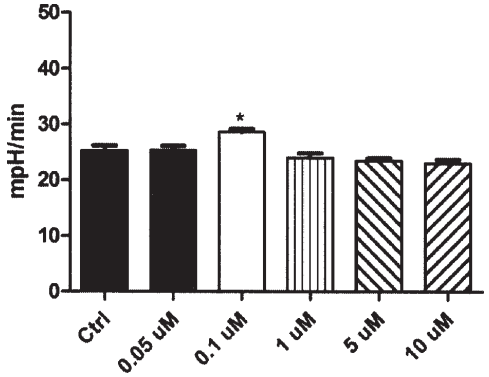

Fig. 4. Acute effects of MB on ECAR. (n = 6–8/group. *p < 0.05 vs. untreated controls)

The effects of 5 progressive concentrations of MB (0.05, 0.1, 1, 5, and 10 μM, respectively) on OCR linked parameters (fig. 3) and ECAR (fig. 4) were assessed.

Our data showed that 0.1μM MB exhibited a significant increase of basal respiration, proton leak, ATP turnover, maximal respiration, and spare respiratory capacity of H9c2 myoblasts as compared to the non-treated cells. All OCR linked parameters were decreased in a concentration-dependent manner when MB was administered in the higher concentrations (1, 5, and 10 μM, respectively), whereas 0.05 μM MB had no effect on the OCR (fig. 3).

An important parameter of cell bioenergetics is the spare respiratory capacity that reflects the ability of substrate supply and electron transport to respond to an increased energy demand [16-17]; in other words it indicates how close to its bioenergetic limit is a cell operating [18]. In this respect, our study demonstrated that cells treated with 0.1 μM MB presented a higher capacity to adapt to stress as compared to untreated cells, since the spare respiratory capacity was significantly increased vs. Ctrl (fig. 3).

When we evaluated the compound effect on glycolysis, we found (as in the case of OCR) a significant increase of ECAR vs. controls when cells were treated with 0.1 μM MB. Conversely, higher concentrations of MB induced a dose-dependent decrease of ECAR (that did not reach statistic significance). The lowest concentration of MB (i.e., 0.05 μM) had no effect on ECAR (fig. 4).

In the past decade several groups attempted to identify pharmaceutical or phytochemical compounds able to modulate mitochondrial function as potential therapeutic target. We joined that quest and demonstrated a modulator effect of total extract of Glycyrrhiza glabra L [19], and of a porphyrin compound [20], respectively on respiratory function of isolated rat liver mitochondria. However, redox agents with mild redox potential (- 0.1 V; 0.1 V) able to readily donate or accept electrons (thus increasing the cellular metabolic activity and preventing ROS production), such as MB, have also been reported to improve mitochondrial function in different experimental settings [8]. In line with this observation, the effects of MB have been systematically reevaluated since it is a redox active agent with mild redox potential (~ 10 mV) [21]. Our results are in agreement with these observations, showing a nanomolar beneficial effect on both respiratory and metabolic mitochondrial function in a cardiac cell line. The limit of the present study is that we did not asses the reactive oxygen species production so far. This aspect and whether the beneficial effects of MB can be recapitulated in pathological conditions, such as diabetes and ischemia/reperfusion injury of the heart, are currently under investigation in our center.

Conclusions

In H9c2 myoblasts acute administration of 0.1 μM methylen blue elicited an increase in O_2 consumption (OCR) and extracellular acidification (ECAR) rates, indicating an overall stimulatory impact on cellular bioenergetics.

Acknowledgements: The work was supported by the POSDRU grant no. 159/1.5/S/136893 titled: "Strategic partnership for the increase of the scientific research quality in medical universities through the award of doctoral and postdoctoral fellowships – DocMed.Net_2.0".

References

1.DUICU, O. M., et al. Canadian Journal of Physiology and Pharmacology 91(8), 2013, p. 593-600.
2.HERNANDEZ-AGUILERA A. et al. Mediators of Inflammation, 2013, 2013, ID 135698, 13 pages.
3.CAMARA A. K. S., et al. Frontiers in Physiology 2:13, 2011.
4.DAVIDSON S. M., et al. 171(8), 2014, p.1795-1797.
5.OLSZEWSKA A., SZEWCZYK A. IUBMB Life 65(3), 2013, p. 273-281.
6.HERNANDEZ-RESENDIZ S., et al. Current Drug Targets 14(5), 2013, p. 586-600.
7.WEN Y., et al. Journal of Biological Chemistry 286(18), 2011, p. 16504-16515.
8.ATAMNA H., et al. Biofactors **38(2),** 2012, p. 158-166.
9.MICLESCU A., WIKLUND L. Jurnalul Roman de Anestezie Terapie Intaensiva. **17:1**, 2010, p. 35-41.
10.POTEET E., et al. PloS One **7(10)**, 2012, e48279.

11.LIN A. L., et al. PloS One **7(10)**, 2012, e46585.
12.ROSIQUE R. G., et al. Transplantation Proceedings **43(10)**, 2011, p. 3643-3651.
13.COLLANGE O., et al. European Journal of Vascular and Endovascular Surgery **45(2)**, 2013, p. 168-175.
14.HILL B. G., et al. Biochemical Journal **424(1)**, 2009, p. 99-107.
15.NICHOLLS D. G., et al. J Vis Exp **46**, 2010
16.YADAVA N., NICHOLLS D. G. Journal of Neuroscience **27(27)**, 2007, p. 7310-7317.
17.CHOI S. W., et al. Journal of Neurochemistry **109(4),** 2009, p. 1179-1191.
18.BRAND M. D., NICHOLLS D. G. Biochemical Journal **435(Pt 2),** 2011, p. 297-312.
19.CORICOVAC, D., et al. Rev. Chim. (Bucharest), **65,** no. 5, 2014, p. 608
20.KERTI, V., et al. Rev. Chim.(Bucharest), **65**, no. 4, 2014, p. 447
21.KAMAT P. V., et al. The Journal of Physical Chemistry **91(2)**, 1987, p. 396-401

Manuscript received: 25.08.2014