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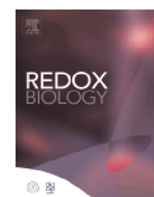

Combined activation of the energy and cellular-defense pathways may explain the potent anti-senescence activity of methylene blue

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ABSTRACT

Methylene blue (MB) delays cellular senescence, induces complex-IV, and activates Keap1/Nrf2; however, the molecular link of these effects to MB is unclear. Since MB is redox-active, we investigated its effect on the NAD/NADH ratio in IMR90 cells. The transient increase in NAD/NADH observed in MB-treated cells triggered an investigation of the energy regulator AMPK. MB induced AMPK phosphorylation in a transient pattern, which was followed by the induction of PGC1α and SURF1: both are inducers of mitochondrial and complex-IV biogenesis. Subsequently MB-treated cells exhibited >100% increase in complex-IV activity and a 28% decline in cellular oxidants. The telomeres erosion rate was also significantly lower in MB-treated cells. A previous research suggested that the pattern of AMPK activation (i.e., chronic or transient) determines the AMPK effect on cell senescence. We identified that the anti-senescence activity of MB (transient activator) was 8-times higher than that of AICAR (chronic activator). Since MB lacked an effect on cell cycle, an MB-dependent change to cell cycle is unlikely to contribute to the anti-senescence activity. The current findings in conjunction with the activation of Keap1/Nrf2 suggest a synchronized activation of the energy and cellular defense pathways as a possible key factor in MB's potent anti-senescence activity.

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1. Introduction

The process of cell senescence is considered to be protective against cancer because senescent cells have limited proliferative capacity [1]. Furthermore, accumulating evidence indicates that senescent cells increase in aging and they are believed to contribute to the process of aging [1–15]. A significant slowing in the age-related deterioration has been observed in mouse model engineered for eliminating senescent cells [2,3]. As the cell senesces it accumulates biochemical and morphological changes and loses its function [16]. Furthermore, senescent cells secrete specific factors (e.g., cytokines) that could affect the integrity of the extracellular matrix and may also interfere with the adjacent cells in their vicinity [1,17–19]. Thus, preventing or delaying the formation of senescent cells may provide a viable strategy for modulating the process of cellular senescence and the deleterious effects of senescent cells [20,21]. Along the line of this hypothesis, strategies such as genetic manipulations or development of specific drugs that lack side effects (e.g., promoting cancer) could be promising for reducing the load of senescent cells and modulating the deleterious effects of senescent cells in vivo.

Several compounds have been reported with various abilities of delaying cell senescence in vitro [20,22]. Recent findings show that methylene blue (MB, a redox active agent) is very effective in delaying cell senescence [23]. At very low concentration (100 nM) MB prolonged the lifespan of human embryonic lung fibroblasts (IMR90) by ≈50% [23]. The anti-senescence activity of MB was demonstrated in the large gain in Population Doubling Level (PDL) as well as in prolonging the replicative capacity of the cells by four to six weeks beyond the control [23]. These observations are intriguing since MB has been used in clinical settings for many years to treat a variety of ailments not related to aging [24,25]. MB could serve as a candidate drug to test anti-senescence therapy in research models. Therefore, the more we understand the effect of MB on the intermediary metabolism of the cell the better we understand the process of cellular senescence delay by MB.

MB is mostly used for treating congenital or poison-induced

Abbreviations: MB, methylene blue; NAD, nicotinamide adenine dinucleotide; NAO, 10-nonyl acridine orange; AMPK, AMP-activated protein kinase; NAMPT, Nicotinamide phosphoribosyltransferase; AICAR, 5-Aminoimidazole-4-carboxamide riboside

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methemoglobinemia [26]. In this regard MB serves as an electron carrier that transfers electrons from nicotinamide adenine dinucleotides (NAD(P)H) to methemoglobin (Fe^{+3}), which is then reduced to deoxyhemoglobin (Fe^{+2}). Thus, the electron shuttling by MB restores the ability of hemoglobin-heme to bind oxygen [26]. In this process MB oxidizes NAD(P)H to NAD (or NADP) via cellular dehydrogenases (e.g., cytochrome b5 reductase). Additional studies suggest that the effect of MB on cell metabolism is concentration dependent. At low concentrations (nanomolar range) MB improves mitochondrial respiration [23,27] and delays cellular senescence [23]. It increases subunit II of cytochrome *c* oxidase (complex IV) by $>30\%$; the rate of heme synthesis; iron uptake; cell resistance to oxidants (e.g., H_2O_2); induces the cytoprotective pathway Keap1/Nrf2 [23, 28]; and prevents cellular senescence induced by chronic exposure to oxidants [23]. At high concentration ($>$ micromolar) MB inhibits nitric oxide synthase [29], guanylyl cyclase [30], and causes down regulation of complex IV [23].

At low concentration MB induces complex IV and activates Keap1/Nrf2 (cytoprotective pathway) [23,28], however the molecular mechanism that drives these changes and their connection to cell senescence is not clear. Mitochondrial dysfunction, impairment to energy metabolism, telomeres erosion, and oxidative stress are factors that contribute to cellular senescence [31–35]. In the current study we concentrated on the effect of MB on complex IV. We measured the effect of MB on NAD/NADH, which led to investigating the energy-sensing kinase AMP-activated Protein Kinase (AMPK). MB induces PGC1α and SURF1, which is important for mitochondrial and complex IV biogenesis. The findings of this study in conjunction with the activation of Keap1/Nrf2 pathway by MB [23,28] led to investigating the status of reactive oxygen species and telomeres erosion in MB-treated cells. The current study provides molecular correlates to the effect of low concentration of MB on complex IV, energy metabolism, redox metabolism, and telomeres.

2. Materials and methods

2.1. Material

Cell culture reagents (DMEM, FBS, PenStrep, and trypsin-EDTA), as well as western blot reagents include NuPAGE 4–12% Bis-Tris Gel and MOPS SDS Running Buffer (20X) were from Life-Technologies (Grand Island, NY). NAO and DAPI were from Molecular Probes (Eugene, Oregon). DCFH, cytochrome *c*, n-Dodecyl-beta-maltoside, and DTT were from Sigma (St. Louis, MO). 5-Aminoimidazole-4-carboxamide riboside (AICAR) was from Cayman chemical company (Ann Arbor, MI). The antibodies for AMPK (AMP-activated protein kinase) and pAMPK (rabbit monocloncal anti-Phospho-Thr172-AMPKα), PGC1α, SIRT1, phospho-Ser47-SIRT1 (p-SIRT1) were from Cell Signaling Technology (Beverly, MA). Antibodies for SURF1 and Actin were from Santa Cruz Biotechnology (Dallas, TX). Protease inhibitors, phosphatase inhibitors, and RIPA buffer with EDTA were from Boston BioProducts (Ashland, MA). Pierce ECL Western blotting Substrate and Restore Western blot Stripping Buffer were from Thermo Scientific (Rockford, IL). Quick Start Bradford Dye Reagent for protein quantification was from Bio-Rad (Hercules, CA).

2.2. IMR90 cell culture, treatment with MB, and preparing cellular lysate

Normal human lung fibroblasts (IMR90) were obtained from Coriell Institute for medical research (Camden, NJ). The cells were cultured in DMEM supplemented with 10% FBS [23]. IMR90 cells can be maintained under tissue culture conditions for about three months until the replicative senescence is reached, which is usually at population doubling level (PDL) around 60. The optimal concentration of MB that delays cell senescence was established as 100 nM [23]. Thus, in the current study we used 100 nM MB. Newly seeded cell cultures were treated with 100 nM MB for the specified intervals as indicated below. At the completion of the treatment with MB, the cultures were harvested and used to prepare cell lysates as described below or stored for later use in -80 °C.

2.3. Measuring the weekly gain in population doubling level (PDL)

Cellular replicative senescence was determined by measuring the weekly gain in PDL and the final cumulative PDL as described previously [23]. Briefly, a week from seeding, the cultures of IMR90 cells were harvested by trypsinization, and the cells were counted using Coulter Counter (BeckmanCoulter, Brea CA). The seeding of a new cell culture was performed by transferring 0.5 million cells from the current cell culture to a new plate. Several plates can be seeded at once. After a week from seeding, the new culture was harvested; the cells were counted and new cultures were seeded again as described above. Every week, the gain in PDL was calculated by using the initial (0.5 million) and the final cell counts (i.e. densities) as described previously [23]. The new weekly gain in PDL was added to the previous PDL to establish the cumulative PDL number. As the cumulative PDL of the specific IMR90 cell culture increases, the culture approaches senescence. At senescence the PDL plateaus. Thus, young cells have low PDL (between 20 and 30) and the final PDL of old-senescent cells usually plateaus at PDL ≥ 55. Senescence was defined when the cell culture stops gaining PDLs. IMR90 cells were contentiously maintained in culture until they senesce, which usually lasts three months.

2.4. Measuring the ratio oxidized to reduced nicotinamid adenine dinucleotides (NAD/NADH)

The levels of NAD and NADH were measured using EnzyChrom NAD/NADH Assay Kit (E2ND-100; BioAssay Systems, Hayward, CA). IMR90 cells were grown in the presence or absence of 100 nM MB for 0–24 h, harvested, and the cells were collected in ice-cold PBS. About 10^5 cells were used to assay NAD and NADH as described by the manufacturer. Briefly, two separate sets of 10^5 cells were used. One set was extracted by 100 μl NAD-extraction buffer for NAD determination while the other set was extracted by NADH-extraction buffer for NADH determination. Both extracts were heated for 5 min at 60 °C. The level of NAD and NADH was assayed using enzymatic recycling of MTT followed by measuring the change in absorbance at 565 nm at the time points 0 and 15 min using Molecular Devices Spectra MaxM5. In conjunction with the unknown samples a standard curve was similarly prepared as described by the manufacturer. NAD and NADH concentrations were calculated using the difference in absorbance at 0 and 15 min and the standard curve. The concentration of NAD and NADH were used to calculate the ratio NAD/NADH at each time point.

2.5. SDS-PAGE and western blot analysis for specific protein factors

At the harvesting time point, the IMR90 cell culture plates were placed on ice, the used media was removed, and the cells were rinsed three times with ice-cold PBS (Ca^{+2}, Mg^{+2}-free). The cells were then scrapped using cell scraper, collected using ice-cold centrifuge, the supernatant was discarded, and the pellet was resuspended into RIPA buffer that was previously supplemented with phosphatase and protease inhibitors. This cell suspension

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