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Malate-aspartate shuttle inhibitor aminooxyacetic acid leads to decreased intracellular ATP levels and altered cell cycle of C6 glioma cells by inhibiting glycolysis

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ABSTRACT

NADH shuttles, including malate-aspartate shuttle (MAS) and glycerol-3-phosphate shuttle, can shuttle the reducing equivalents of cytosolic NADH into mitochondria. It is widely accepted that the major function of NADH shuttles is to increase mitochondrial energy production. Our study tested the hypothesis that the novel major function of NADH shuttles in cancer cells is to maintain glycolysis by decreasing cytosolic NADH/NAD^+ ratios. We found that AOAA, a widely used MAS inhibitor, led to decreased intracellular ATP levels, altered cell cycle and increased apoptosis and necrosis of C6 glioma cells, without affecting the survival of primary astrocyte cultures. AOAA also decreased the glycolytic rate and the levels of extracellular lactate and pyruvate, without affecting the mitochondrial membrane potential of C6 cells. Moreover, the toxic effects of AOAA were completely prevented by pyruvate treatment. Collectively, our study has suggested that AOAA may be used to selectively decrease glioma cell survival, and the major function of MAS in cancer cells may be profoundly different from its major function in normal cells: The major function of MAS in cancer cells is to maintain glycolysis, instead of increasing mitochondrial energy metabolism.

Introduction

NADH, the reduced form of nicotinamide adenine dinucleotide (NAD), plays significant roles in not only the redox reactions in energy metabolism, but also calcium homeostasis and antioxidation [1]. Because the inner mitochondrial membrane is impermeable to cytosolic NADH, NADH shuttles, including malate-aspartate shuttle (MAS) and glycerol 3-phosphate shuttle [1], act to transfer the reducing equivalents of cytosolic NADH into mitochondria thus enhancing mitochondrial ATP production.

MAS is considered the major NADH shuttles in the brain [2]. Previous studies have suggested that MAS plays important roles in multiple biological processes, including glial synthesis of glutamate and glutamine [3], calcium-dependent regulation of mitochondrial respiration in intact cortical neurons [4], glucose-stimulated insulin secretion from β-islet cells [5], and proliferation of breast cancer cells [6]. Aspartate aminotransferase (AAT), a pyridoxal phosphate-dependent transaminase, is the rate-limiting enzyme of MAS which functions in tandem with malate dehydrogenase [7]. It has been reported that aminooxyacetic acid (AOAA), a most widely used AAT inhibitor of MAS [2,8], can selectively inhibit the proliferation of breast cancer cells and suppress the growth of breast adenocarcinoma in athymic mice, suggesting that AAT and MAS are required for breast cancer cell proliferation [6]. It is warranted to further investigate the roles of MAS in the biological functions of other types of cancer cells. More importantly, future studies are necessary to investigate the mechanisms underlying the roles of MAS in the biological functions of cancer cells.

It is widely accepted that the major function of NADH shuttles is to increase mitochondrial energy production by shuttling the reducing equivalents of cytosolic NADH into mitochondria [1,9,10]. So far there has been no study suggesting that the NADH shuttles may have differential biological roles in normal cells and cancer cells. For the following reasons, we proposed the hypothesis that the major function of NADH shuttles in cancer cells may be prevention of glycolytic inhibition produced by high levels of cytosolic NADH/NAD^+ ratios, instead of increasing mitochondrial ATP production: There are Warburg effects in cancer cells, in which glycolysis becomes a much more significant pathway for ATP production compared to its role in normal cells [11–13]. Because the much greater glycolytic rate in cancer cells can lead to much higher levels of cytosolic NADH/

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NAD^+ ratios that may inhibit glycolysis, NADH shuttles may play significant roles in maintaining glycolysis by decreasing the cytosolic NADH/NAD^+ ratios in cancer cells.

Glioma represents 80% of all malignant brain tumors, while the pathological mechanisms of the cancer remain unclear [14]. Due to the critical roles of glucose metabolism in cancer biology [11–13], it is necessary to investigate the mechanisms of the regulation of glucose metabolism of glioma cells. In our current study, we investigated the roles of MAS in the glucose metabolism, cell cycle and cell death of C6 glioma cells, with the aim of testing our abovementioned hypothesis regarding the novel roles of MAS in cancer cell metabolism. Our study has not only suggested that AOAA may be used to selectively decrease glioma cell survival, but also provided evidence suggesting that MAS may have the novel function of maintaining glycolysis in cancer cells.

Materials and methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except where noted.

Cell cultures

C6 glioma cells were plated into 24-well or 12-well cell culture plates at the initial density of 1×10^6 cells/mL in Dulbecco's Modified Eagle's Medium containing 4500 mg/L D-glucose, 584 mg/L L-glutamine (Thermo Scientific, Waltham, MA, USA), and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (PAA, Germany). The cells were maintained in a 5% CO_2 incubator at 37 °C.

Primary rat cortical astrocyte cultures were prepared as described previously [15]. In brief, cortices were harvested from 1-day-old rat (SLAC, Shanghai, China). The cortices were dissociated with trypsin, then plated in 24-well culture plates in Dulbecco's Modified Eagle's Medium containing 4500 mg/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate (Thermo Scientific, Waltham, MA, USA), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), and 10% fetal bovine serum (PAA, Germany), and maintained in a 5% CO2 incubator at 37 °C. After 12–15 days, the cells became confluent, which were treated for 48 h with 10 μM cytosine arabinoside. The cell cultures could be used two days after removal of cytosine arabinoside.

Intracellular lactate dehydrogenase (LDH) assay

Cell survival was quantified by measuring the intracellular LDH activity of the cells. Briefly, cells were lysed for 15 min in lysing buffer containing 0.04% Triton X-100, 2 mM HEPES and 0.01% bovine serum albumin (pH 7.5). Then 50 μL cell lysates were mixed with 150 μL 500 mM potassium phosphate buffer (pH 7.5) containing 0.34 mM NADH and 2.5 mM sodium pyruvate. The A_{340nm} changes were monitored over 90 sec. Percentage of cell survival was calculated by normalizing the LDH values of samples to LDH activity measured in the lysates of control (wash only) culture wells.

Flow cytometry-based Annexin V/7-AAD assay

The flow cytometry assay was performed to determine the levels of early-stage apoptosis, late-stage apoptosis, and necrosis by using ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) according to the manufacturer's protocol. In brief, cells were digested with 0.25% trypsin-EDTA, washed by cold PBS one time and resuspended in cold 1X binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2, 0.1% BSA) at concentrations between 1×10^6 and 1×10^7 cells/mL. Then 5 μL of labeled Annexin V was added into 100 μL of the cell suspension. After incubation on ice for 15 min in the dark, 200 μL 1X binding buffer and 5 μL 7-AAD solution were added into the cell suspensions. The number of stained cells was assessed immediately by a flow cytometer (FACSAria II, BD Biosciences).

Cell cycle analysis

The C6 glioma cells were collected by trypsinization, washed with PBS and assessed for cell cycle by flow cytometry as described with a modification [16]. In brief, the cells were fixed with 70% cold ethanol at 4 °C overnight. Then the fixed cells were washed with PBS, treated with 100 μg/mL RNase A for 30 min at 37 °C, and stained with 50 μg/mL propidium iodide (PI) in the dark. Subsequently, the cells were analyzed by flow cytometry (BD FACSCalibur). At least 10,000 cells in each sample were analyzed to obtain a measurable signal. The cell debris and fixation artifacts were gated out and the cell populations that were at the G0/G1, S and G2/M phases were quantified using the Modfit LT 4.0 software (BD Biosciences).

ATP assay

Intracellular ATP levels were quantified using an ATP Bioluminescence Assay Kit (Roche Applied Science, Mannheim, Germany) following the standard protocol provided by the vendor. Briefly, the cells were lysed with the Cell Lysis Reagent on ice, and 50 μL of the lysates was mixed with 50 μL of the Luciferase Reagent. Then the chemiluminescence of the samples was measured by using a plate reader (Biotek Synergy 2). The ATP concentrations of the samples were calculated using an ATP standard, and normalized to the protein concentrations of the samples, which were determined using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

FACS-based determinations of mitochondrial membrane potential ($\triangle\Psi m$)

A molecular probe, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) (Enzo Life Sciences, Plymouth Meeting, PA, USA), was used to measure mitochondrial membrane potential [17]. In healthy cells that have higher $\triangle\Psi$m, JC-1 is accumulated in the mitochondria matrix, forming J-aggregates that emit red fluorescence at the wavelength of 590 nm. When cells have decreased $\triangle\Psi$m, JC-1 monomers are generated, which emit green fluorescence at the wavelength of 529 nm. Briefly, C6 glioma cells were digested with 0.25% trypsin-EDTA, washed by PBS one time and resuspended in PBS. Then 10 μg/mL JC-1 was added to the PBS. After 15 min incubation in the dark at 37 °C, the cells were analyzed immediately by flow cytometer (FACSAria II, BD Biosciences). FACS was used to determine both the red and green fluorescence of a cell, and the ratio between the red fluorescence and the green fluorescence was calculated as a measurement of $\triangle\Psi$m.

Determination of mitochondrial membrane potential ($\triangle\Psi m$) by JC-1-based confocal imaging

A molecular probe, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) (Enzo Life Sciences, Plymouth Meeting, PA, USA), was used to measure mitochondrial membrane potential using confocal microscopy. In healthy cells that have relatively high $\triangle\Psi$m, JC-1 is accumulated in the mitochondria matrix, forming J-aggregates that emit red fluorescence. When cells have decreased $\triangle\Psi$m, JC-1 monomers are generated, which emit green fluorescence. Briefly, the cells were incubated with 0.1 μg/mL JC-1 at 37 °C for 15 min, then washed with PBS and analyzed immediately by confocal laser scanning microscopy (TCS SP5, Leica, Germany). Confocal images were taken with excitation at 488 nm and emission at (585 ± 21) nm for red fluorescence or (530 ± 15) nm for green fluorescence. The ratio between the red fluorescence and the green fluorescence was used for indicating the levels of $\triangle\Psi$m. The levels of red and green fluorescence were evaluated in a blinded fashion.

Glucose uptake assays in cells

For glucose uptake assays in C6 glioma cells, cells were incubated for 60 min at 37 °C in a growth medium containing 100 μM 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) (Invitrogen) [18], a fluorescent glucose analog, which typically displays excitation/emission maxima of ~465/540 nm. Then the cells were digested by 0.25% trypsin-EDTA, washed with PBS one time and suspended in PBS. The fluorescence of the samples was immediately measured by a flow cytometer (FACSAria II, BD Biosciences).

Extracellular pyruvate and lactate assay

Extracellular pyruvate and lactate were measured by NADH-coupled lactate dehydrogenase (LDH) spectrophotometric assay [19]. Briefly, for pyruvate assay, 100 μL supernatant samples were mixed with 56 μL 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM NADH and 4 units/mL LDH. The values of A_{340nm} were monitored immediately. After 15 min at RT, the values of A_{340nm} were monitored again. The pyruvate concentrations of the samples were calculated using the difference of the two values of A_{340nm} and the pyruvate standard, and normalized to the protein concentrations of the samples, which were determined using the Pierce® BCA Protein Assay Kit. For lactate assay, 100 μL samples (containing 50 μL supernatant samples and 50 μL DMEM medium) were mixed with 100 μL reaction buffer (pH 9.5) containing 1 M glycine, 0.56 M hydrazine sulfate and 0.1 M NAD^+. Five minutes later, 1 unit/μL LDH was added. The values of A_{340nm} were monitored immediately. After incubation for 60 min at 37 °C, the values of A_{340nm} were monitored again. The lactate concentrations of the samples were calculated using the difference of the two values of A_{340nm} and the lactate standard, and normalized to the protein concentrations of the samples, which were determined using the Pierce® BCA Protein Assay Kit.

Senescence-associated-β-gal (SA-β-gal) staining

Cell staining of SA-β-gal was performed using a Senescence-β-Galactosidase Staining Kit according to the standard protocol provided by the vendor (Beyotime, Jiangsu Province, China). Then the cells were photographed under a Leica inverted fluorescence microscope. A cell is considered SA-β-gal-positive when the cytoplasm of the cell is in blue color. The number of SA-β-gal-negative cells and SA-β-gal-positive cells