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Betaine is a positive regulator of mitochondrial respiration

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ABSTRACT

Betaine protects cells from environmental stress and serves as a methyl donor in several biochemical pathways. It reduces cardiovascular disease risk and protects liver cells from alcoholic liver damage and nonalcoholic steatohepatitis. Its pretreatment can rescue cells exposed to toxins such as rotenone, chloroform, and LiCl. Furthermore, it has been suggested that betaine can suppress cancer cell growth *in vivo* and *in vitro*. Mitochondrial electron transport chain (ETC) complexes generate the mitochondrial membrane potential, which is essential to produce cellular energy, ATP. Reduced mitochondrial respiration and energy status have been found in many human pathological conditions including aging, cancer, and neurodegenerative disease. In this study we investigated whether betaine directly targets mitochondria. We show that betaine treatment leads to an upregulation of mitochondrial respiration and cytochrome *c* oxidase activity in H2.35 cells, the proposed rate limiting enzyme of ETC *in vivo*. Following treatment, the mitochondrial membrane potential was increased and cellular energy levels were elevated. We propose that the anti-proliferative effects of betaine on cancer cells might be due to enhanced mitochondrial function contributing to a reversal of the Warburg effect.

1. Introduction

Betaine (trimethylglycine) obtained from foods and from synthesis through choline metabolism is abundant in liver and kidney [1,2]. It plays an important role in cellular protection against environmental stress, such as high temperature, osmotic imbalance, or high salinity in microorganisms, plants, and animals and by serving as a methyl-group donor in several biochemical pathways [3–7].

Betaine has been suggested to have several therapeutic benefits. It decreases plasma homocysteine concentration in hyperhomocysteinemia, which is a risk factor of atherosclerotic diseases [8,9]. It has been shown that betaine has a hepatoprotective function against alcoholic and nonalcoholic liver damage. Nonalcoholic steatohepatitis and fibrosis were improved by betaine administration in humans and rodents [10–12]. Betaine also attenuated hepatosteatosis and steatohepatitis caused by alcohol intake [13–15]. Betaine treatment was able to alleviate damage to the mitochondrial oxidative phosphorylation (OXPHOS) system and oxidative stress caused by alcohol administration in rats, mice, and HepG2 cells [14,16,17]. It was also shown that pretreatment with betaine protected from liver damage caused by toxin exposure such as chloroform and LiCl [18,19]. Betaine has potential as a neuroprotective agent since it attenuated mitochondrial dysfunction and increased cell viability of PC12 cells treated with rotenone [20]. In addition, betaine might suppress cancer cell proliferation. After addition of betaine, cell growth of HepG2 human liver cancer cells was inhibited [21] and tumorigenesis was delayed in the liver of rodents [22]. Interestingly, an inverse correlation between dietary intake of betaine and the risk of lung, colon, and breast cancers in humans has been reported [23–25].

Mitochondria provide the majority of cellular energy in the form of ATP through OXPHOS. The mitochondrial electron transport chain (ETC) complexes pump protons across the inner mitochondrial membrane generating the mitochondrial membrane potential, $\Delta\Psi m$, which is utilized by ATP synthase for the synthesis of ATP. Dysregulation of mitochondrial respiration and cellular energy status has been reported in many pathological conditions in humans, such as neurodegenerative diseases, autoimmune diseases, diabetes mellitus, aging, and cancer [26,27]. Cytochrome *c* oxidase (COX) is the terminal enzyme of the ETC and contributes to the generation of $\Delta\Psi m$. COX is regulated by allosteric regulators (nucleotides, thyroid hormones), isoform expression, and phosphorylation. This enzyme has been proposed to be the rate limiting step of the ETC *in vivo* [26,28,29].

Abbreviations: COX, cytochrome *c* oxidase; ETC, electron transport chain; OXPHOS, oxidative phosphorylation.

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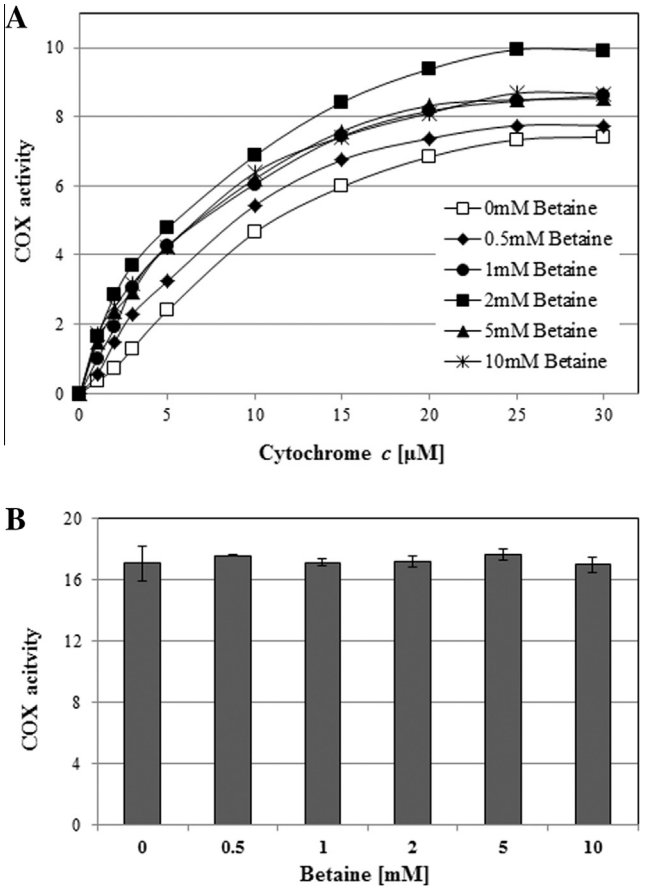

Fig. 1. COX activity is increased in betaine treated cells. (A) COX activity of H2.35 cells was analyzed by addition of increasing amounts of cytochrome *c*. The cells were incubated with 0 (□), 0.5 (♦), 1 (●), 2 (■), 5 (▲), and 10 mM (∗) betaine for 30 min. COX specific activity is defined as consumed O_2 (nmol)/min · total protein (mg). Shown are representative measurements ($n = 3$). (B) Increasing amount of betaine was added to the purified cow liver COX and enzymatic activity was monitored after addition of 5 μM cytochrome *c*. No significant change was observed ($n = 3$). COX activity is defined as [s^{-1}].

In this study we examined whether the beneficial properties of betaine are the result of a direct effect on mitochondria. H2.35 cells incubated with betaine showed enhanced mitochondrial respiration and elevated COX activity. ΔΨm and ATP levels increased following the treatment. These results support the concept that betaine exerts beneficial effects and improves survival of the cells under stress conditions. In addition, we propose that the reported cancer suppressive effect of betaine might be explained at least in part by improved respiration, reversing the Warburg effect, which is a typical phenomenon in most cancer cells.

2. Materials and methods

2.1. Cell culture and betaine treatment

All chemicals were purchased from Sigma unless stated otherwise. Mouse hepatocyte H2.35 cells were cultured in DMEM Medium (high glucose with pyruvate, Gibco) supplemented with 10% fetal bovine serum (Gibco), 100U/mL penicillin and 100 mg/mL streptomycin (Gibco) under standard conditions at 37 °C and 5% CO_2.

Cells were washed once with phosphate buffered saline (PBS) and treated with the indicated concentrations of betaine or PBS as a control in phenol red free DMEM medium (Hyclone) without supplements for 30 min in the cell culture incubator.

2.2. COX activity measurement

COX activity was analyzed with the Oxygraph system (Hansatech) at 25 °C. The cells were harvested by scraping and following solubilization as described previously [30]. Measurements were performed in the presence of 20 mM ascorbate by addition of increasing amounts of cow heart cytochrome *c* and analyzed with the Oxygraph plus software. Protein concentration was determined with the DC protein assay kit (Bio-Rad) and COX activity is defined as consumed O_2 (nmol)/min · total protein (mg).

Cow liver COX was purified, dialyzed, and its activity was measured as described with modifications [30,31]. Increasing amounts of betaine were added up to 10 mM to the dialyzed COX and changes in enzymatic activity were monitored in the presence of 5 μM cytochrome *c*. COX activity is expressed as [s^{-1}].

2.3. Mitochondrial respiration measurement

Mitochondrial respiration was measured as described with modifications [32]. Cells were permeabilized using digitonin (8 μg/mg protein) and mitochondrial respiration was measured in the presence of substrates for ETC complexes I and II (10 mM pyruvate + 3 mM malate and 10 mM succinate, respectively) at 30 °C. The mitochondria were activated by addition of 1 mM ADP following addition of ATP synthase inhibitor oligomycin (200 μM) and oxidative phosphorylation uncoupler FCCP (200 μM, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone). Non-mitochondrial oxygen consumption as determined after addition of 300 μM KCN was subtracted from the respiration data. The mitochondrial respiration rate is defined as consumed O_2 (nmol)/min - · total protein (mg).

2.4. Intact cell respiration measurement

Oxygen consumption rate (OCR) was measured using a Seahorse XF^e24 analyzer (Seahorse Bioscience) [33]. Fifty thousand cells were plated per well. Treatment with betaine was performed in unbuffered DMEM medium (pH 7.4, 10 mM glucose) and OCR was measured under basal conditions.

2.5. Mitochondrial membrane potential measurement

The mitochondrial membrane potential (ΔΨm) was determined using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, Molecular Probes) [34]. It is a lipophilic green fluorescent probe that accumulates in mitochondria ΔΨm-dependently, forming red fluorescent aggregates. Cells were grown on 96 well plates, treated with betaine, and incubated with 0.5 μM JC-1 for 30 min. The JC-1 containing medium was removed and fluorescence was measured at excitation/emission 485 nm/527 nm for the green monomers and at excitation/emission 485 nm/590 nm for red aggregates using a plate reader (Fluoroskan Ascent FL, Labsystems). The ratio of red to green fluorescence serves as an indicator for relative changes of ΔΨm. As a control 1 μM uncoupler FCCP was added prior to the JC-1 incubation.

2.6. Cellular ATP level measurement

To measure ATP concentration the cells were collected and processed as described [35]. Cells were collected by scraping and immediately flash frozen to prevent ATP degradation. ATP concentration was determined using the ATP bioluminescence assay kit HS II (Roche).