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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.

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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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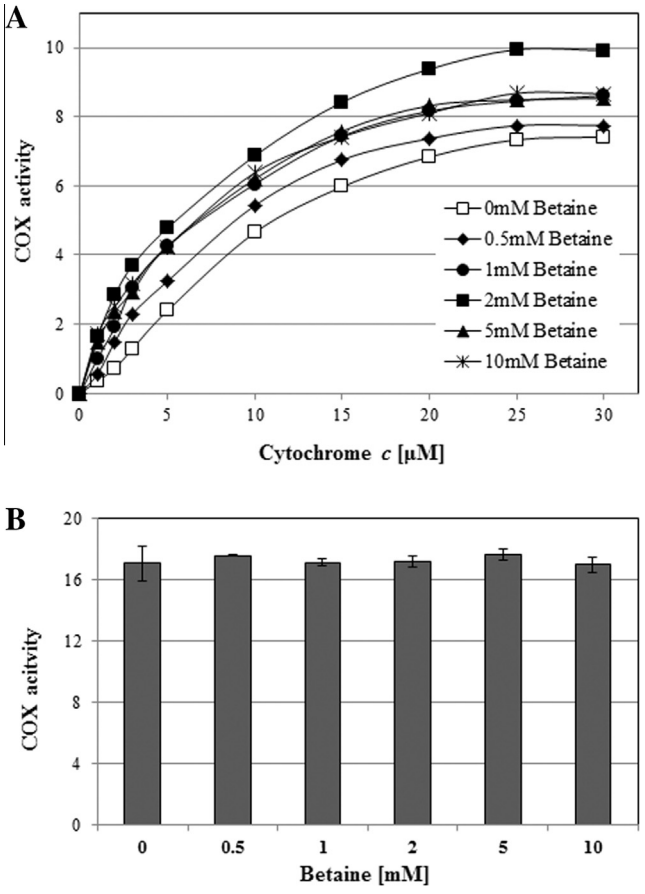

Fig. 1. COX activity is increased in betaine treated cells. (A) COX activity of H2.35 cells was analyzed by addition of increasing amounts of cytochrome *c*. The cells were incubated with 0 (□), 0.5 (♦), 1 (●), 2 (■), 5 (▲), and 10 mM (∗) betaine for 30 min. COX specific activity is defined as consumed 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).

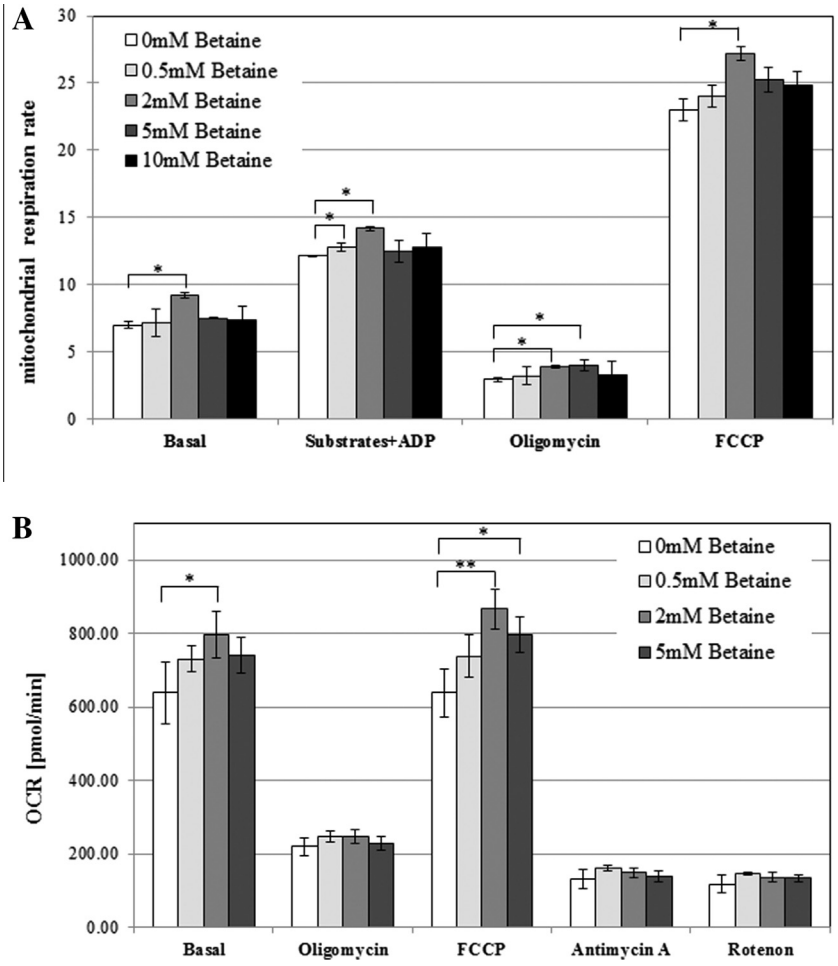

Fig. 2. Betaine treatment stimulates mitochondrial respiration and total cell respiration. (A) Cells were incubated with 0–10 mM betaine, permeabilized with digitonin, and mitochondrial respiration was determined. Respiration was initiated by addition of complex I and II substrates and state 3 respiration was induced by addition of ADP. Mitochondria were challenged using oligomycin and FCCP subsequently. $n = 3$, $^{*}p < 0.05$. (B) Oxygen consumption rate (OCR) of intact cells was determined on a Seahorse analyzer. Basal OCR was measured following treatment with oligomycin, FCCP, antimycin A, and rotenone. $n = 4$, $^{*}p < 0.05$, $^{**}p < 0.001$.

2.7. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). One way analysis of variance (ANOVA) was used to determine statistical significance between groups.

3. Results

3.1. Betaine increases cytochrome c oxidase (COX) activity

To investigate whether betaine directly targets mitochondria we first measured COX activity, since it is the proposed rate limiting enzyme of the ETC. Intact cells were incubated with betaine for 30 min. COX specific activity of H2.35 cells was increased by betaine treatment compared to controls in a concentration dependent manner, and the highest activity was obtained with 2 mM betaine (about 20% higher compared to the control at 30 μM cytochrome *c*) (Fig. 1A). Since allosteric regulation was previously reported for COX with ADP and ATP, we tested a possible direct effect of betaine on the enzyme. When betaine was directly added to purified cow liver COX the activity was not altered (Fig. 1B). These results demonstrate that upregulation of COX activity by betaine is not mediated by direct allosteric regulation, but by an indirect mechanism, such as via cell signaling.

3.2. Betaine stimulates mitochondrial and cellular respiration

The elevated COX activity may affect mitochondrial function and whole cellular respiration. Betaine treated cells were permeabilized using digitonin and the ETC substrates for complexes I and II were directly added to the mitochondria. Oxygen consumption by the ETC was monitored to assess mitochondrial respiration rates. Two millimolar betaine was able to enhance mitochondrial respiration by 17% in the presence of ADP (state 3 respiration) compared to controls (Fig. 2A). The stimulatory effect of betaine was still observed when the ETC was challenged by inhibitors such as oligomycin (state 4 respiration) and the uncoupler FCCP.

The observed change in mitochondrial respiration after betaine treatment might affect respiration of intact cells. Oxygen consumption rate (OCR) of H2.35 cells utilizing glucose as substrate was analyzed using a Seahorse XF^{e}24 instrument. The cells were subsequently challenged with oligomycin, FCCP, antimycin A, and rotenone. Basal OCR was higher in the betaine treated cells, and an increase of about 24% was obtained with 2 mM betaine compared to controls (Fig. 2B). The enhanced OCR was also observed

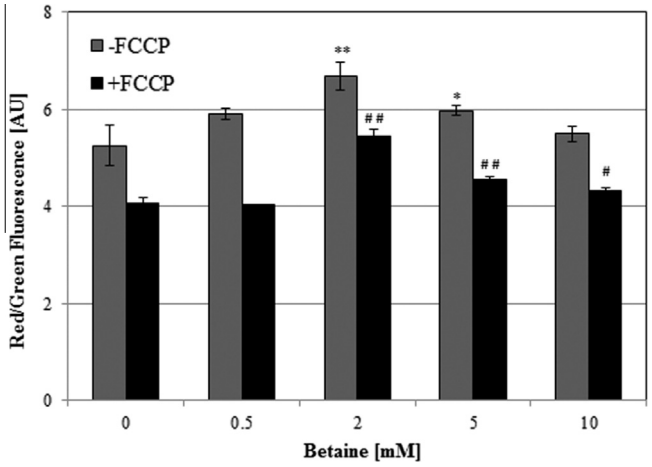

Fig. 3. Mitochondrial membrane potential is increased by betaine treatment. Mitochondrial membrane potential ΔΨm was measured using the probe JC-1. The ratio of red to green fluorescence is reported and an increase of the ratio indicates higher ΔΨm levels. As a negative control 1 μM FCCP was added to dissipate ΔΨm. $n = 3$, $^{*}p < 0.05$ compared to 0 mM betaine without FCCP, $^{**}p < 0.001$ to 0 mM betaine without FCCP, $^{\#}p < 0.05$ to 0 mM betaine with FCCP, $^{\#\#}p < 0.001$ to 0 mM betaine with FCCP.

when FCCP was added, a measure sometimes referred to as the excess capacity of the ETC.

3.3. Mitochondrial membrane potential is increased in betaine treated cells

The mitochondrial membrane potential ΔΨm generated by ETC complexes I, III, and COX is essential for ATP synthesis. Since COX specific activity and mitochondrial and intact cellular respiration was increased after betaine treatment we expected that this would translate into increased ΔΨm levels. JC-1 was used to determine ΔΨm and increased fluorescence ratios of red aggregates to green monomer of JC-1 indicate higher ΔΨm values. ΔΨm was elevated by the betaine treatment and the highest value was shown at 2 mM betaine (Fig. 3).

3.4. ATP level is elevated by betaine treatment

The majority of cellular energy is produced by mitochondrial ATP synthase. It utilizes ΔΨm to produce ATP from ADP and P_i. We investigated whether the increase in ΔΨm by betaine translated into higher ATP levels in the cells. Indeed, betaine treatment improved cellular energy status (Fig. 4). At 5 mM betaine the ATP concentration was increased by 100%.

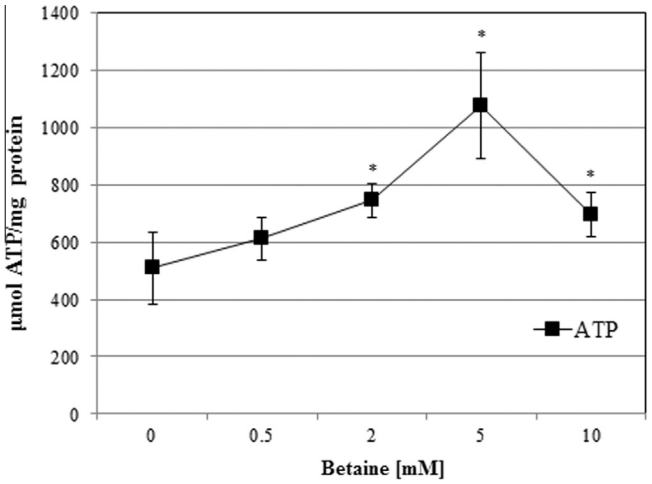

Fig. 4. Betaine increases cellular ATP levels. Total cellular ATP content was determined with the bioluminescent method. ATP concentration was normalized to total cell protein concentration. $n = 4$, $^{*}p < 0.05$ compared to 0 mM betaine.

4. Discussion

Mitochondrial dysfunction has been reported in many human pathological conditions, such as neurodegenerative diseases, aging, and cancer. Often, therapeutic approaches target improvement of mitochondrial OXPHOS.

Betaine has been proposed to have a protective effect against alcoholic and nonalcoholic liver damage. Chronic alcohol consumption causes disruption in OXPHOS, decrease in respiration, and inability to maintain liver ATP levels, and it can lead to cell death [8–11,36]. Decreased mitochondrial OXPHOS and ATP levels were observed in nonalcoholic steatohepatitis as well [37,38]. The cytoprotective effect of betaine on those conditions might be explained by the elevated mitochondrial respiration and improved ATP levels that might be critical for recovery from cellular damage.

Cancer is another pathological condition that is associated with decreased OXPHOS activity. More than 90% of cancers show the Warburg effect with suppressed mitochondrial respiration and increased aerobic glycolysis. This phenomenon is more pronounced in aggressive cancers [39,40]. Alteration of OXPHOS influences cell apoptosis [41,42] and cancer metastasis [43]. Cancer cell growth and metastasis could be inhibited by elevating OXPHOS content and its activity [44]. Previous studies showed that betaine delays cancer cell growth and its intake has an inverse correlation with cancer incidence in humans [11,21–25]. We show here with the hepatocyte-derived transformed H.2.35 cell line that betaine treatment leads to improved COX activity and mitochondrial and intact cellular respiration. The maximal effect is obtained with betaine concentrations of 2–5 mM. Thus, the anti-oncogenic effect of betaine might in part be explained by reversal of the Warburg effect.

It was suggested that betaine induces the activation of AKT [45]. AKT is a protein kinase that plays an important role in cell function, proliferation, and protein synthesis, and the AKT signaling pathway is known to be associated with mitochondrial OXPHOS. For example, it was shown that AKT activation stimulates mitochondrial biogenesis, OXPHOS complexes, and ATP levels in cardiac muscle, skeletal muscle, renal proximal tubules, and macrophages [46–49]. It is therefore possible that the improved mitochondrial function and COX activity after betaine treatment shown in this study might be mediated by the AKT signaling pathway. Since betaine shows a fast response, as analyzed after 30 min, which cannot be explained by changes in gene expression and increased mitochondrial biogenesis that occur over a longer time period, it is likely that OXPHOS is targeted by cell signaling. Therefore, future work should investigate changes in the phosphorylation state of the OXPHOS complexes following betaine treatment.

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