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Oxidative stress suppresses the cellular bioenergetic effect of the 3-mercaptopyruvate sulfurtransferase/hydrogen sulfide pathway

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ABSTRACT

Recent data show that lower concentrations of hydrogen sulfide (H_2S), as well as endogenous, intramitochondrial production of H_2S by the 3-mercaptopyruvate (3-MP)/3-mercaptopyruvate sulfurtransferase (3-MST) pathway serves as an electron donor and inorganic source of energy to support mitochondrial electron transport and ATP generation in mammalian cells by donating electrons to Complex II. The aim of our study was to investigate the role of oxidative stress on the activity of the 3-MP/3-MST/ H_2S pathway *in vitro*. Hydrogen peroxide (H_2O_2 , 100–500 μM) caused a concentration-dependent decrease in the activity of recombinant mouse 3-MST enzyme. In mitochondria isolated from murine hepatoma cells, H_2O_2 (50–500 μM) caused a concentration-dependent decrease in production of H_2S from 3-MP. In cultured murine hepatoma cells H_2O_2 (3–100 μM), did not result in overall cytotoxicity, but caused a partial decrease in basal oxygen consumption and respiratory reserve capacity. The positive bioenergetic effect of 3-MP (100–300 nM) was completely abolished by pre-treatment of the cells with H_2O_2 (50 μM). The current findings demonstrate that oxidative stress inhibits 3-MST activity and interferes with the positive bioenergetic role of the 3-MP/3-MST/ H_2S pathway. These findings may have implications for the pathophysiology of various conditions associated with increased oxidative stress, such as various forms of critical illness, cardiovascular diseases, diabetes or physiological aging.

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1. Introduction

Studies over the last decade have recognized multiple regulatory roles of endogenously produced H_2S in mammals [1–3]. Mitochondrial function, oxidative phosphorylation and cellular bioenergetics are under multiple levels of regulatory control by H_2S . Two opposite effects on cellular bioenergetics have been described: a stimulatory role at Complex II, which occurs in response to lower concentrations of exogenously administered H_2S , and/or in response to endogenously produced H_2S from 3-mercaptopyruvate (3-MP) via the mitochondrial enzyme 3-mercaptopyruvate sulfurtransferase (3-MST), and is considered

physiologically relevant [4–6], and an inhibitory role at Complex IV, which occurs in response to higher concentrations of exogenously administered H_2S , and/or in response to higher concentrations of endogenously produced H_2S from 3-MP, and may be predominant at pathophysiologically elevated H_2S concentrations [4–9].

Oxidative stress plays a pluripotent regulatory role in mammalian bioenergetics. Low-levels of oxidants are produced from the mitochondrial respiration, and are usually effectively neutralized by the endogenous antioxidant systems of cells. However, higher levels of oxidative stress (due to overproduction of oxidants and/or the impairment of antioxidant defense systems) can induce cell dysfunction or cell death [10–12]. The role of oxidative stress is well established in the pathogenesis of many conditions, including critical illness, cardiovascular diseases, diabetic complications and physiological aging [10–17]. The goal of the current studies was to determine, in recombinant 3-MST enzyme, in isolated mitochondria and in cultured murine hepatoma cells *in vitro*, whether oxidative stress affects 3-MST-mediated H_2S production, and of so, then whether this effect influences the bioenergetic regulatory role of mitochondrially produced H_2S .

Abbreviations: ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; CcOX complex IV, cytochrome c oxidase mitochondrial complex IV; Cyt C, cytochrome C; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; ECAR, extracellular acidification rate; 3-MST, 3-mercaptopyruvate sulfurtransferase; 3-MP, 3-mercaptopyruvate; OCR, oxygen consumption rate; PPR, proton production rate; SQR, sulfide:quinone oxidoreductase.

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2. Materials and methods

2.1. 3-MST expression and purification

Escherichia coli BL21(DE3) Codon Plus was used as the host strain for the expression vector pGEX-Kg. Mouse 3-MST was cloned into pGEX-Kg to create an N-terminal GST fusion. The expression vectors were transformed on Luria–Bertani (LB)-agar plates, supplemented with ampicillin (100 µg/ml). BL21(DE3) Codon Plus cells containing the expression vector pGEX-Kg/GST-3-MST were grown at 37 °C and 180 rpm in Luria–Bertani (LB) broth medium containing 100 µg/ml ampicillin to an absorption of 0.6–0.8 at 600 nm. Then protein expression was induced by addition of 0.1 mM IPTG (isopropyl- β -D-thiogalactopyranoside final concentration). Cells were harvested and sonicated in lysis buffer PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.8) containing the protease inhibitors cocktail (Sigma). The soluble fraction containing the GST-3-MST recombinant protein was loaded onto a GSTrap FF 1 ml affinity column and eluted from the beads with elution buffer (50 mM Tris–HCl, 10 mM reduced glutathione, pH 8.0) and then dialyzed and concentrated in 10 mM sodium phosphate buffer pH 8.2 and DTT 1 mM. Fig. 1A shows a representative SDS–PAGE gel stained with Coomassie blue in which the major band corresponds to recombinant GST-3-MST following purification.

2.2. Measurement of H₂S production by recombinant 3-MST

H₂S production by recombinant mouse 3-MST enzyme was measured according to Stipanuk and Beck [18] with some modifications. Briefly, each test consisted of 100 µl reaction mixture containing 5 µg of the purified GST-3-MST enzyme, 15 µM 3-mercaptopyruvate and 50 mM sodium phosphate buffer pH 8.2. H₂O₂ was added to the reaction 15 min before 3-mercaptopyruvate was added to the solution. Reaction was initiated by transferring the Eppendorf tubes from ice to a 37 °C shaking water bath. After 60 min of incubation at 37 °C, the reaction was terminated with 1% zinc acetate solution (100 µl/tube) was added followed by the administration of 10% trichloroacetic acid solution (100 µl/tube). Subsequently, *N,N*-dimethylphenylendiamine sulfate (DPD, 20 mM in 7.2 M HCl, 49 µl/tube) and FeCl₃ (30 mM in 1.2 M HCl, 49 µl/tube) were added and the optical absorbance of the solutions was measured after 15 min of incubation at a wavelength of 650 nm. All samples were assayed in triplicate and

H₂S concentration was calculated against a calibration curve of NaHS.

2.3. Mitochondrial isolation and measurement of H₂S production

Mitochondria were isolated from cultured Hepa1c1c7 mouse hepatoma cell line as previously described [19]. Briefly, cells were washed with PBS thoroughly and then were detached in PBS using a cell scraper. They were centrifuged at 600 g, at 4 °C for 10 min. The pellet was resuspended in mitochondrial isolation buffer (pH 7.4) containing 10 mM Tris–MOPS, 1 mM EGTA/Tris and 200 mM sucrose at final concentration. The cell suspension was homogenized using Glass Teflon Potter homogenizers applying 32 strokes for Hepa1c1c7 murine hepatocytes. The homogenate was centrifuged at 600g for 10 min at 4 °C. The supernatant was discarded and centrifuged 7000g for 10 min at 4 °C. The pellet contained the mitochondrial fraction, which were washed once with the mitochondrial isolation buffer applying the same centrifugation step, 7000g for 10 min at 4 °C. The presence of 3-MST in the isolated mitochondria was confirmed by Western blotting as described [6]. Complex IV served as loading control and specific mitochondrial marker. Mitochondria in lysis buffer were suspended in potassium phosphate buffer (100 mM pH = 7.4) with protease inhibitors and 1 mM DTT. Mitochondrial samples were incubated in a reaction mixture (total volume 500 µl/tube) 3-mercaptopyruvate (at 10, 30 or 100 µM final concentration) with or without H₂O₂ (at 50, 100 or 500 µM final concentration) in sealed Eppendorf tubes. The reaction was initiated by transferring tubes from ice to a water bath at 37 °C. After 45 min incubation, 1% zinc acetate solution (250 µl/tube) was added followed by the administration of 10% trichloroacetic acid solution (250 µl/tube), and H₂S production was measured as for the experiments with recombinant 3-MST (see above). All samples were assayed in triplicate and H₂S concentration was calculated against a calibration curve of NaHS. To determine basal production of H₂S, 3-MP was not added to the reaction mixture. Results were expressed as nmol H₂S produced per milligram of protein.

2.4. Cell culture and pharmacological treatments

The Hepa1c1c7 murine hepatoma cell line was purchased from ATCC, and maintained in MEM α GlutaMAX™ with 10% FBS, 2 mmol/l glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C/5% CO₂. 35,000 cells per well were plated into

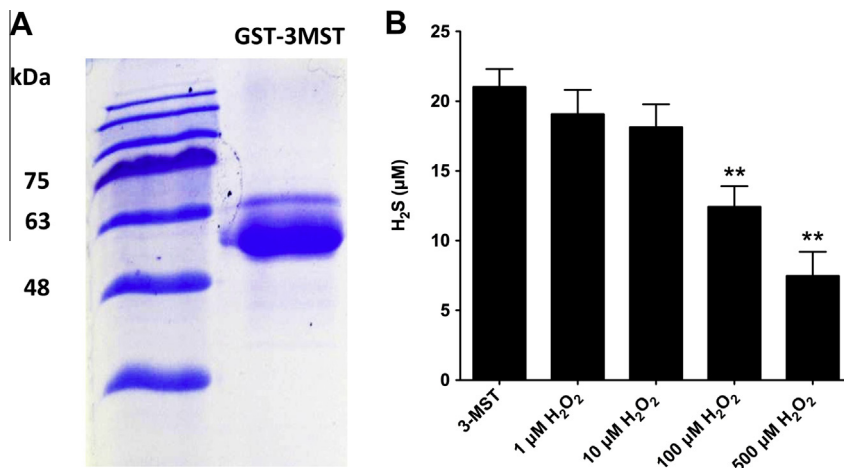


Fig. 1. Oxidative stress elicited by hydrogen peroxide (H₂O₂) inhibits H₂S production from 3-MST. (A) Representative SDS–PAGE gel of purified GST-3-MST. Visualization was made using Nippon Genetics Protein Marker. (B) Concentration-dependent inhibitory effect of hydrogen peroxide (H₂O₂) on H₂S production by recombinant mouse 3-MST. ***p* < 0.01 shows significant inhibition of H₂S production after H₂O₂ exposure; *n* = 4.

24-well XF24 V7 plates [6]. 3-MP, the substrate of 3-MST, was added to the cells at increasing concentrations (30, 100 or 1000 nM) in the absence or presence of 30-min pretreatment with hydrogen peroxide (3, 10, 30, 50 or 100 μ M).

2.5. MTT cell viability assay

After 45-min H_2O_2 exposure, in order to estimate the number of viable cells 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the cells at a final concentration of 0.5 mg/ml and cultured at 37 °C at 5% CO_2 atmosphere for 1 h. Cells were washed with PBS and the converted formazan dye was dissolved in isopropanol and measured at 570 nm with background measurement at 690 nm on a SpectraMax M2 reader (Molecular Devices Corp., Sunnyvale, CA, USA). A calibration curve was created by measuring the converting capacity of MTT of serial dilutions of Hepa1c1c7 cells. Viable cell count was calculated using Gen5 data reduction software [20].

2.6. LDH cytotoxicity assay

Cell culture supernatant (30 μ l) was mixed with 100 μ l freshly prepared LDH assay reagent to reach final concentrations of 85 mM lactic acid, 1040 mM nicotinamide adenine dinucleotide (NAD), 224 mM *N*-methylphenazonium methyl sulfate (PMS), 528 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) and 200 mM Tris (pH 8.2). The changes in absorbance were read kinetically at 492 nm for 15 min. LDH activity values are shown as V_{max} for kinetic assay in mOD/min [20].

2.7. Bioenergetic analysis in cultured hepatoma cells

The XF24 Analyzer was used to measure bioenergetic function in intact Hepa1c1c7 cells as described [6,20]. The XF24 creates a transient 7- μ l chamber in specialized microplates that allows for the measurement of OCR (oxygen consumption rate) and glycolytic capacity, as measured by PPR (proton production rate, pMoles/min) in real time. Prior to the bioenergetic measurements, the culture medium was changed to unbuffered 5 mM glucose containing DMEM (pH 7.4) with 1 mM *L*-glutamine and 0.1 mM sodium pyruvate. In preliminary studies, the optimum number of cells per well was determined as 35,000/0.32 cm². Next, a protocol was applied to measure indices of mitochondrial function. Oligomycin, FCCP, and antimycin A were injected sequentially through ports of the Seahorse Flux Pak cartridges to yield final concentrations of 1 μ g/ml, 0.4 μ M, and 2 μ g/ml, respectively. After recording basal OCR and PPR, oligomycin was used to block the mitochondrial ATP synthesis. The resulting compensatory switch to glycolysis yields a bioenergetic parameter termed ‘cellular glycolytic capacity’. The OCR value measured after FCCP injection was taken as a representation of the ‘maximal mitochondrial respiratory capacity’. From this latter parameter, by subtracting the basal and non-mitochondrial respiration values ‘mitochondrial reserve (or spare) respiratory capacity’, a parameter that characterizes the overall mitochondrial function of the cell) was calculated. Finally, antimycin A was used to inhibit the flux of electrons through complex III, to detect residual non-mitochondrial activity related to cytosolic oxidases. Bioenergetic parameters were normalized to cell count, as the same amount of cells was seeded in each well.

2.8. Statistical analysis

Data are shown as means \pm SEM. Student's *t* tests, one-way and two-way ANOVA with Bonferroni post-hoc test were used to detect

differences between groups; **p* < 0.05 or ***p* < 0.01 considered statistically significant.

3. Results

3.1. H_2O_2 -mediated oxidative stress suppresses H_2S production by recombinant mouse 3-MST

Exposure of 3-MST to 1 or 10 μ M H_2O_2 failed to significantly affect H_2S production. However, at 100 and 500 μ M H_2O_2 , a concentration-dependent decrease in H_2S production was observed (Fig. 1B).

3.2. H_2O_2 -mediated oxidative stress suppresses H_2S production by mitochondria isolated from cultured murine hepatoma cells

In accordance with previous studies [6,17,21], mitochondrial isolates contained 3-MST (Fig. 2A), and produced H_2S when incubated with its substrate, 3-MP in a concentration-dependent fashion (Fig. 2). In response to 100 μ M 3-MP, 258 \pm 51 nmol H_2S /mg protein was detected (*n* = 3). H_2O_2 exposure concentration-dependently attenuated the 3-MP-induced H_2S production; the inhibition was already significant at 50 μ M H_2O_2 (173 \pm 39 nmol H_2S /mg protein, (*n* = 3), amounted to 54% inhibition at 100 μ M (118 \pm 20 nmol H_2S /mg protein, (*n* = 3), and became near-

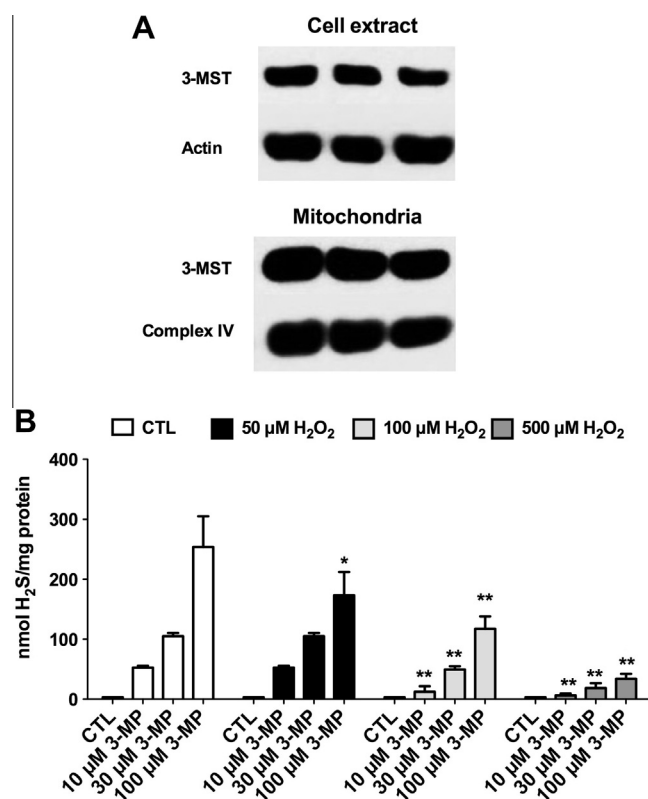


Fig. 2. Concentration-dependent inhibitory effect of hydrogen peroxide (H_2O_2) on H_2S production in mitochondria isolated from cultured murine hepatoma cells. (A) Western blot analysis confirming the presence of 3-MST in cell lysates and in isolated mitochondria collected from Hepa1c1c7 cells. Each of the 3 lanes represents a cell extract or mitochondrial isolate obtained on a different experimental day. 3-MST enzyme was detected at 33 kDa, Complex IV, used as a mitochondrial marker and loading control, was detected at 17 kDa; actin, used as a cytosolic marker and loading control was detected at 43 kDa. In panel B, **p* < 0.05 and ***p* < 0.01 represent significant inhibition of the 3-MP-induced H_2S production in isolated mitochondria harvested from Hepa1c1c7 cells by H_2O_2 , when compared to the H_2S production elicited by the same concentration of 3-MP in the absence of H_2O_2 pretreatment; *n* = 3.

complete, 87% inhibition at 500 μM H_2O_2 (34 ± 8 nmol $\text{H}_2\text{S}/\text{mg}$ protein, ($n = 3$), (Fig. 2).

3.3. H_2O_2 -induced oxidative stress suppresses respiratory reserve capacity in cultured hepatoma cells

Exposure of the hepatocytes to 3 or 10 μM hydrogen peroxide (H_2O_2) failed to affect any of the bioenergetic parameters recorded (Fig. 3). Increasing the concentration of H_2O_2 to 30 μM and 50 μM induced a slight, but significant suppression of the basal oxygen consumption rate (OCR) values, and resulted in a partial, dose-dependent suppression of respiratory reserve capacity (Fig. 3A and B), without having an impact on cell viability and cell count, assessed by the MTT method (Fig. 3C and D) and LDH assays (data not shown). Likewise, glycolytic parameters remained unchanged (Fig. 3E). Even at the highest concentration of H_2O_2 tested (100 μM) there were no changes in these remarkable indices of cell viability, even though at this concentration, respiratory reserve capacity was significantly diminished by below 50%. These findings are consistent with previous studies [20,22,23] suggesting that a suppression of OCR is an early and sensitive indicator of the impairment of cellular bioenergetics that precedes oxidant-induced cell death, and, according to our data (Fig. 3E) is not associated with a compensatory alteration in glycolysis.

3.4. Oxidative stress abrogates the bioenergetic effect of 3-MP in cultured hepatoma cells

From the above experiments, we have selected the concentration of 50 μM H_2O_2 as a concentration that is non-toxic to investigate the interactions of oxidative stress with the 3-MP/3-MST pathway. In accordance with previous studies showing that 3-MP at low concentration (100–300 nM) has a stimulating effect on oxygen consumption and subsequent ATP production, without affecting glycolytic parameters [6], we have observed a stimulatory effect of 3-MP (100–300 nM) on respiratory capacity of the cells (Fig. 4), without affecting glycolytic parameters (data not shown). Pre-exposure of the hepatocytes to 50 μM H_2O_2 slightly reduced basal cellular respiration (Fig. 4A) and abrogated the 3-MP-induced stimulatory bioenergetic response (Fig. 4B). At 1000 nM and above, 3-MP begins to inhibit mitochondrial respiration due to the fact that high local concentrations H_2S inhibit the activity of Complex IV [5,6]. Accordingly, at 1000 nM, 3-MP induced a suppression of respiratory reserve capacity, and this effect was even more pronounced in the cells that were pretreated with 50 μM H_2O_2 (Fig. 4B).

4. Discussion

Three enzymes present in mammalian tissues: (a) cystathionine β -synthase (CBS), (b) cystathionine γ -lyase (CSE) and (c) 3-mercaptopyruvate sulfurtransferase (3-MST) in conjunction with cysteine (aspartate) aminotransferase (CAT), are capable of the production of H_2S -generators [1–3,18]. Recent work began to explore the regulatory roles of 3-MST (sometimes referred to as the “third” H_2S -producing enzyme) in the regulation of mammalian biology. It is now well established that 3-MST is expressed in most cells and tissues; its localization is partially mitochondrial and partially cytosolic, and the formation of H_2S depends on a cooperative interaction between cysteine (aspartate) aminotransferase (CAT) and 3-MST, where CAT catalyses the transamination between L-cysteine and α -ketoglutarate to produce 3-mercaptopyruvate and L-glutamate, and then 3-MST then transfers sulfur from 3-mercaptopyruvate to sulfurous acid to ultimately produce H_2S [2,24].

The specific physiological role of the 3-MP/3-MST/ H_2S pathway has been a subject of several recent investigations. H_2S produced via this pathway has been proposed to serve as a vasodilator [25] and neurotransmitter [21]. However, it is not immediately apparent why these functions would require mitochondrial localization. An intramitochondrial antioxidant role for intracellular H_2S , in general, [26,27], and for mitochondrially produced, 3-MST-derived H_2S , in specific, [28], has been proposed recently. To further explore the specific mitochondrial function of 3-MST-derived H_2S , we have recently conducted a series of studies in cultured hepatoma cells and mitochondria isolated from them, and demonstrated that H_2S produced from mitochondrial 3-MST acts as a positive bioenergetic regulator, by donating electrons to the mitochondrial electron transport chain, and contributing to mitochondrial ATP generation [6]. During these experiments, we have performed transient or permanent silencing of 3-MST, and observed that these interventions markedly attenuate the positive bioenergetic effects of 3-MP [6].

A relevant aspect of the biochemistry of 3-MST is that its activity is regulated intra- and inter-molecular cysteines in an oxidant-sensitive fashion. Using recombinant rat 3-MST and measurement of rhodanese activity (which is another catalytic function of the enzyme, whereby the enzyme converts cyanide to thiocyanate), it has previously been demonstrated that an intermolecular switch consisting of Cys¹⁵⁴ and Cys²⁶³ can be oxidized to form a dimer (an inactive form) between Cys¹⁵⁴ and Cys¹⁵⁴, Cys¹⁵⁴ and Cys²⁶³, and/or Cys²⁶³ and Cys²⁶³, thereby rendering the enzyme inactive [29]. Moreover, higher degrees of oxidative stress can affect an intramolecular switch, whereby a catalytic site Cys²⁴⁷, is oxidized to form a cysteine sulfenate, which also results in an inhibition of the rhodanese activity of the enzyme [29]. We hypothesize that similar oxidative modifications may also be responsible for the inhibition of the H_2S -synthesizing activity of 3-MST by H_2O_2 in our current experiments. Another level of oxidative modification (especially in mitochondrial preparations or in living cells) may affect the endogenous cofactors of 3-MST. Several lines of studies have established that endogenous reducing substrates (such as thioredoxin and lipoic acid) are required to maintain the catalytic activity of 3-MST, at least in part because they affect cysteine oxidation states, thereby 3-MST monomer/dimer equilibrium [30,31]. It is conceivable that oxidative stress may impair these regulatory factors as well.

Because of the redox regulation of the 3-MP/3-MST/ H_2S pathway, and because of the role of this pathway in cellular bioenergetics, in the current studies we have tested whether (a) 3-MST-derived H_2S production is affected by oxidative stress, whether (b) mitochondrial H_2S production from 3-MP is affected by oxidative stress and (c) whether the 3-MP-associated, H_2S -mediated positive bioenergetic response is affected by oxidative stress in cultured cells. The prototypical oxidant H_2O_2 was used to generate different levels of oxidative stress. The results demonstrated that H_2O_2 exerts a concentration-dependent inhibitory effect on 3-MST-mediated H_2S production, on mitochondrial H_2S production from 3-MP, and abrogates the bioenergetic stimulatory effect of 3-MP in cultured hepatoma cells. The concentrations of H_2O_2 where these effects occurred were apparent in the 50–100 μM concentration range of H_2O_2 , which corresponds to mild-to-moderate degree of oxidative stress. In fact, this concentration of H_2O_2 failed to show any detectable effects on cellular viability (e.g. MTT or LDH) assays. These findings are consistent with the hypothesis that the impairment of the 3-MST/ H_2S -associated positive bioenergetic response by H_2O_2 occurs at non-cytotoxic levels of oxidative stress, and may cause a degree of cellular dysfunction, which, then, may or may not progress into cell death, depending on the circumstances (duration of the oxidative stress, secondary insults, etc).

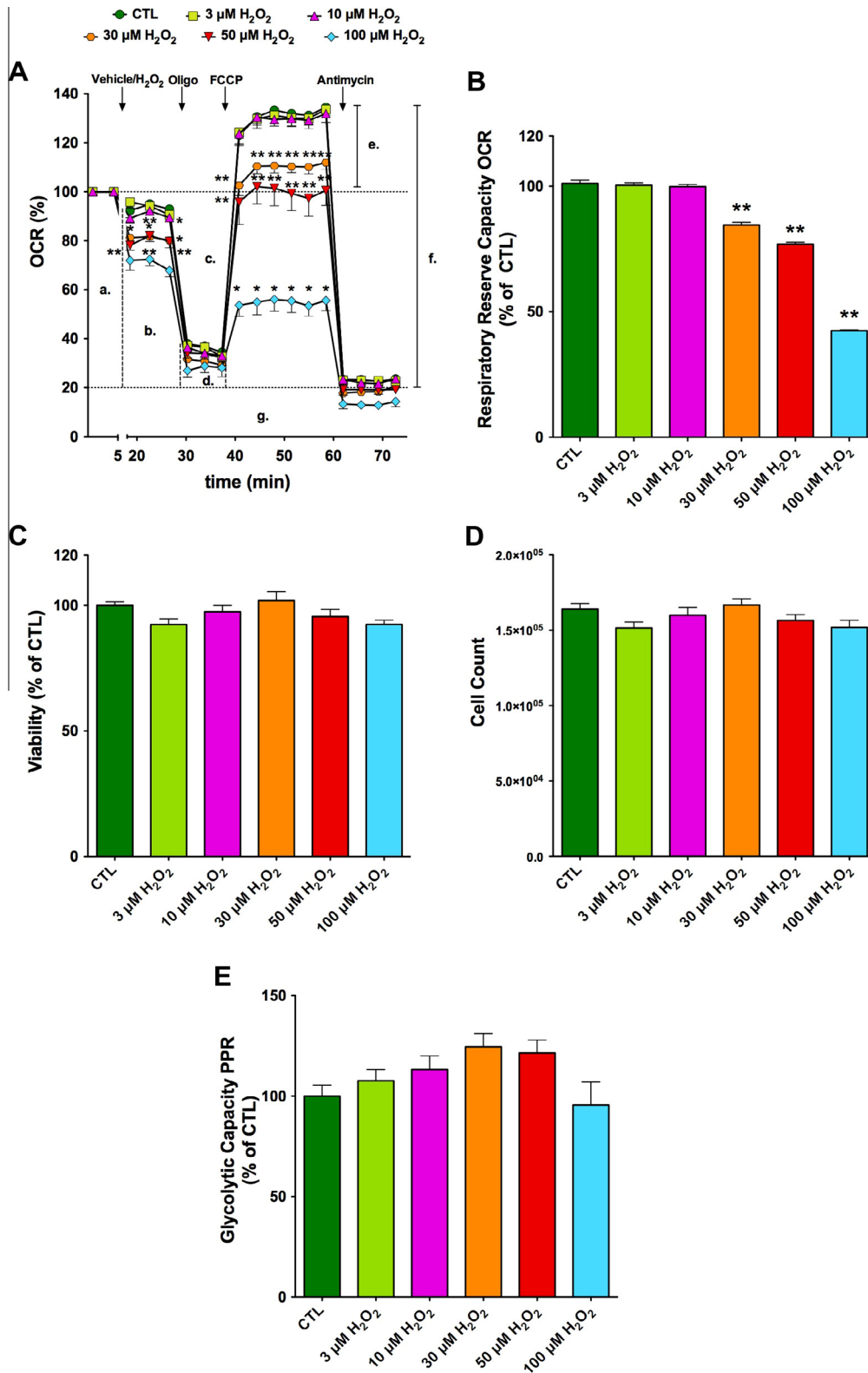


Fig. 3. Effect of moderate degree of oxidative stress on cellular viability and bioenergetic parameters in cultured murine hepatoma cells. Hydrogen peroxide (H_2O_2 , 30, 50 or 100 μM) significantly attenuated the basal oxygen consumption rate (OCR) (A) and the respiratory reserve capacity (A and B), without affecting cell viability (C), and calculated cell count (D) determined by MTT assay. Moreover, oxidative stress had no effect on glycolytic parameters (E). In panel A, the following parameters are indicated by lower case letters: (a), basal respiration; (b), respiration after the addition of H_2O_2 or its vehicle (c), calculated ATP turnover; (d), proton leak; (e), mitochondrial reserve capacity (f), maximal respiratory capacity (g), non-mitochondrial respiration. * $p < 0.05$ and ** $p < 0.01$ represent significant inhibition of OCR by H_2O_2 ; $n = 12$.

The mechanism of H_2O_2 's action in the isolated enzyme may be due to a direct effect on 3-MST (e.g. cysteine modification), while in cultured cells, the suppression of the 3-MP-mediated cellular

bioenergetics may involve additional effects (e.g. interactions with the reducing co-factors of the enzyme, or with other intracellular redox regulators).

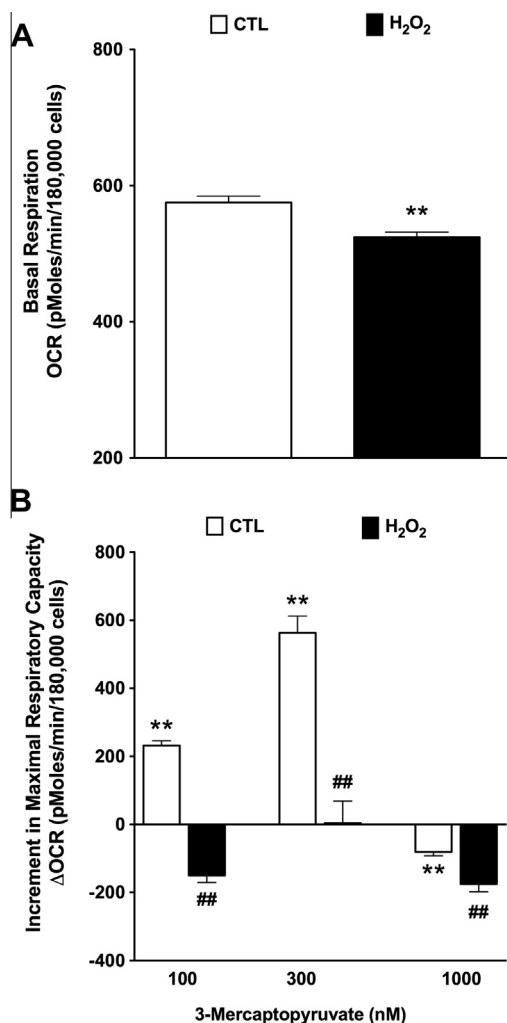


Fig. 4. Suppression of the stimulatory bioenergetic effect of 3-MP by a moderate degree of oxidative stress in cultured murine hepatoma cells. (A) Slight attenuation by H₂O₂ (50 μ M) of the basal oxygen consumption rate (OCR, pMoles/min/180,000 cells) in cultured murine hepatoma cells (** p < 0.01, n = 7). (B) Suppression by H₂O₂ (50 μ M) of the 3-MP (100, 300 or 1000 nM) induced increment in maximal respiratory capacity of cultured murine hepatocytes. ** p < 0.01 shows significant effect of 3-MP on cellular bioenergetics, when compared to normal control cells (CTL). ## p < 0.01 shows significant inhibition of basal or 3-MP-stimulated oxygen consumption rate in cells pretreated with 50 μ M H₂O₂, as compared to control cells; n = 7.

In conclusion, the current study demonstrates that the endogenous intramitochondrial H₂S-producing pathway, governed by 3-MST, is markedly impaired during intermediate degree of oxidative stress. We hypothesize that such impairment may attenuate the bioenergetic/cytoprotective potential of H₂S, thereby contributing to the pathogenetic role of oxidative stress in various pathophysiological conditions.

Acknowledgments

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