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Full Length Research Paper

Effects of hydrogen sulfide (H_2S) on respiration control of state 3/4 in mitochondria from bovine heart

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Hydrogen sulfide (H_2S) could availably regulate electron transport in the inner membrane of mitochondria from bovine heart when succinate as substrate and rotenone as complex I inhibitor at 37°C were used. H_2S increased to a certain extent the respiratory rate of state 4. It also increased first and then decreased the respiratory rate of state 3, respiratory control ratio and ADP/O ratio. In addition, it quicken first and then delayed recovery time from state 3 to state 4. The effects of H_2S as aforementioned in normoxic condition were more obvious than those in hypoxic condition. Experimental results indicated that more than 10 μM H_2S brought about uncoupling of mitochondrial electron transport and the opening of mitochondrial K_{ATP} channel located in complex III-IV, and less than 10 μM H_2S unexpectedly facilitated this course, which might be via sulfide-quinone oxidoreductase. The finding that H_2S was closely related with sulfide-quinone oxidoreductase, however, requires in depth investigation.

Key words: Respiration, hydrogen sulfide, mitochondria, bovine heart.

INTRODUCTION

Hydrogen sulfide (H_2S) is produced by cystathionine γ-lyase and cystathionine β-synthase (Wang 2002a, b; Zhao et al., 2001b) enzymes in the human body. H_2S, as a physiologic vasorelaxant (Yang et al., 2008), significantly dilates coronary arteries and increases coronary blood flow. In patients with coronary heart disease, plasma H_2S levels are reduced from 50 to 25 μM (Jiang et al., 2005). Spontaneously hypertensive rats have substantially lower plasma levels of H_2S (Du et al., 2003). The vasorelaxant effect of H_2S is mediated by an interaction of H_2S with vascular smooth muscle cells (Zhao and Wang, 2002). H_2S may protect heart via activating K_{ATP} channels (Geng et al., 2004; Pan et al., 2006). By ligating to the ferric heme iron of cytochrome a_3 in complex IV (Nicholls, 1975), H_2S causes vaso-relaxation via ATP depletion and K_{ATP} channel opening (Brayden, 2002). H_2S could diffuse past cytochrome c oxidase (CcO) into the mitochondria matrix where it deprotonates to HS^- and H^+ (O'Brien and Vetter, 1990).

Mitochondrial dysfunction is a characteristic of myocardial ischemia injure (Lesnefsky et al., 2001). Inhibition of CcO is not detected, but H_2S could have important biological effects on CcO at lower H_2S concentrations, such as rapidly reducing Fe (III) and Cu (II) in the oxidized form. Therefore, H_2S might serve as source of electrons (Collman et al., 2009), regulate the mitochondrial electron transport, and involved in the opening mitochondrial K_{ATP} channel. If mitochondrial respiration is inhibited, electron transport in CcO could be blocked (Wagner et al., 2009), resulting in the opening of the mitochondrial K_{ATP} channel. To test this mechanism, effects of H_2S on respiration control related with state 3/4 of mitochondria from bovine heart in normoxic and

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hypoxic conditions were researched.

MATERIALS AND METHODS

Chemicals

Sodium hydrosulfide (Aladdin), $ADPNa_2$ (Amresco), rotenone (Sigma), bovine serum albumin (Aladdin), and sodium succinic hexahydrate (Sigma) were used. All other reagents including sucrose, ethylenediaminetetraacetic acid disodium, potassium phosphate, mannitol and sodium hydroxide were analytical reagent. Mitochondria were obtained from bovine hearts obtained from Changchun Haoyue Halal Meat Co., Ltd., China.

Experimental instruments

Mini MACS anaerobic workstation (Don Whitley Scientific Limited, United Kingdom), OXY5401S (Guiyang Xuetong Instrument and Meter Co., Ltd., China) and PHS-3D (Shanghai Precision & Scientific Instrument Co., Ltd. China) were used. Other instruments used in the experiments included 6S auto high speed tissue homogenizer (Jiangsu Salt City Experiment Instrument Plant, China), high speed refrigerated centrifuge (Hitachi, Japan), Beckman ultra high speed and low temperature refrigerated centrifuge (U.S.A.), ultra-low temperature freezer (Sanyo Electric Co., Ltd., Japan), Sartorius electronics scales (Sartorius Instrument Co., Ltd.) and 90-3 thermostat two-way magnetism mixer (Shanghai Zhengrong Scientific Instrument Co., Ltd., China).

Isolation of mitochondria

Isolation of mitochondrial from bovine heart according to the modified method of Shen et al. (2007) and Zhang et al. (2008) was as follows: the ratio between sample and isolation medium was 1:4; the first and second centrifugal forces and centrifugal times were 2,500 g for 10 min and 7,000 g for 20 min, respectively. The mitochondrial protein concentration was determined (Lowry et al., 1951) using bovine serum albumin as the standard. Optical microscope (×200) and electron microscopy were used for observing isolated mitochondria.

Experimental conditions and procedures

Under normoxic and hypoxic conditions, respiration rate of mitochondria were measured using bioxygraph (Reynafarje et al., 1985) equipped with closed cell at 37°C. In a chamber volume of 3.0 ml containing D-mannitol (225 mM), sucrose (70 mM), potassium phosphate (10 mM), ethylenediaminetetraacetic acid (EDTA, 1 mM), bovine serum albumin (0.1%) and pH 7.4, 20 µL (0.2 M) succinic sodium and 8 µL (50 mM) $ADPNa_2$ was added. Next, mitochondria (0.1 ml) with 5 µM rotenone (complex I inhibitor) were added for 6 min. After a short equilibration period, the oxygen electrode was inserted into the reaction vessel, and 0.2 M succinic sodium 20 µL as substrate was added. Respiratory rate of state 4 was determined after adding substrate to the reaction vessel. Respiration of state 3 was initiated by adding 50 mM (8 µL) adenosine diphosphate to the assay medium. Respiratory control ratio (RCR) named as the ratio between respiration of state 3 and respiration of state 4, and adenosine diphosphate/oxygen ratio (ADP/O) were calculated. Mitochondrial respiration was expressed as nmoles of oxygen utilized per minute per milligram of protein. Mitochondria protein concentration was 2.0 mg/ml. The mitochondrial respiratory rate was measured using a polarographic electrode (OXY5401S). A change in O_2 consumption rate before and after adding H_2S was therefore interpreted as the effect of H_2S.

The handling of H_2S and groups

In the experiments, sodium hydrogen sulfide (NaHS) which is a stable H_2S donor, was at 10^{-5} to 10^{-4} mol/L (Pan et al., 2006) because the concentration of H_2S produced by NaHS solution is approximately 30 to 33% of the original concentration of NaHS. When NaHS is dissolved in water, HS^- is released and forms H_2S with H^+ (Dombkowski et al., 2004; Reiffenstein et al., 1992). The concentrations of H_2S at 5 to 40 µM are sufficient to relax vessels under physiological O_2 conditions (Koenitzer et al., 2007) and H_2S produced by a solution containing 10^{-4} mol/L NaHS are within the physiological range (~4.6×10^{-5} mol/L) in the serum (Zhao et al., 2001a). The experimental concentrations of H_2S were chosen as 1, 10, 20, 30, 40 and 50 µM coming from 3, 30, 60, 90, 120 and150 µM NaHS, respectively. According to the differences in normoxic condition (Bhambhani and Singh, 1991; Trizno et al., 1993) and hypoxic condition, each condition was divided into 7 groups including the control group. The control group did not contain H_2S (Bhambhani et al., 1996; Richardson, 1995).

Experimental paradigm model

The mitochondria from isolated bovine heart exposed to normoxic or hypoxic conditions (Mini MACS anaerobic workstation, United Kingdom) served as the experimental paradigm models to define respiratory regulatory mechanisms.

Data analysis

Data were obtained from 5 different preparations, approximately normally distributed, and expressed as mean ± SD. Statistical significance of changes in different groups was evaluated by one-way analysis of variances (ANOVA) and differences were considered statistically significant at values of $p < 0.05$. The Origin 8.0 professional software was also used.

RESULTS

Effects of H_2S on respiratory rate of state 4

Influences of H_2S on respiratory rate of state 4 in mitochondria under normoxic and hypoxic conditions are shown in Figure 1. The changes of respiratory rate of state 4 by H_2S increased in both conditions. Results show that the change in magnitude in normoxic condition was similar to that in hypoxic, and all the data indicated uncouple appearing with H_2S concentration increase.

Effects of H_2S on respiratory rate of state 3

Changes of respiratory rate of state 3 by H_2S are indicated in Figure 2. Influence on respiratory rate of state 3 by H_2S increased first and then decreased remarkably in a concentration dependent manner ($p < 0.05$) compared with normoxic and hypoxic control groups,

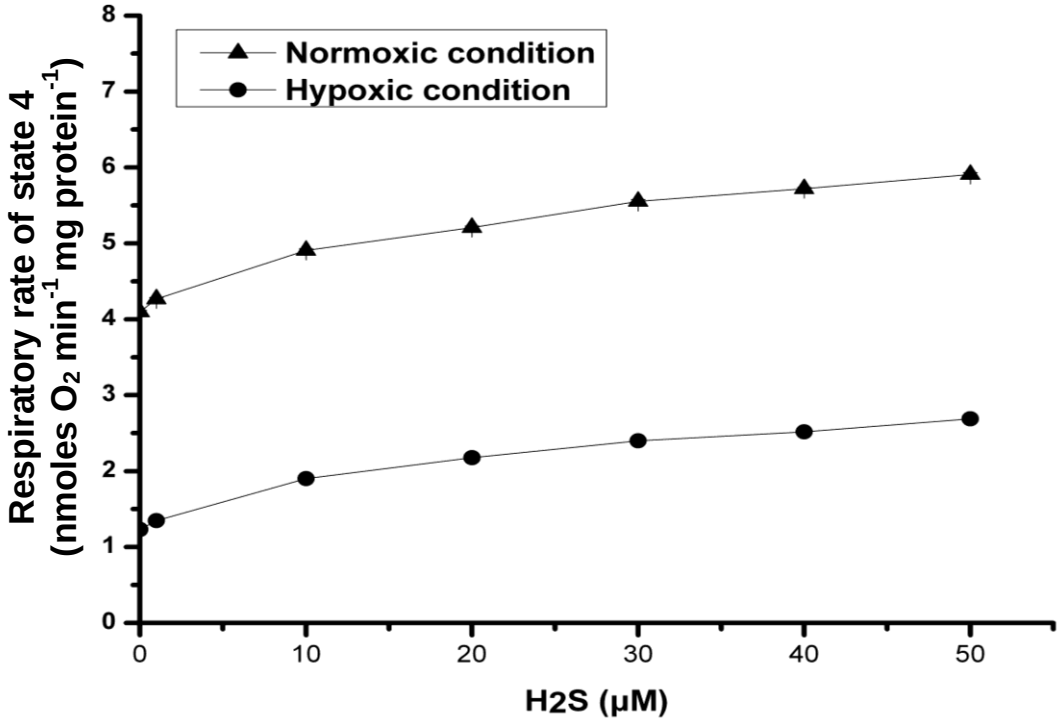

Figure 1. Effects of H_2S on respiratory rate of state 4.

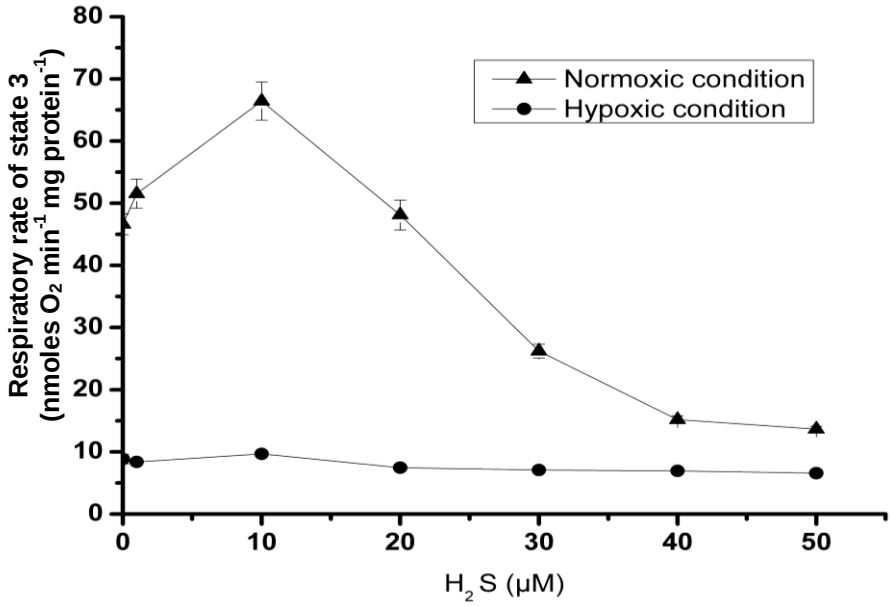

Figure 2. Effects of H_2S on respiratory rate of state 3.

respectively. In normoxic condition, less than or equal to 10 μM of H_2S, respiratory rates of state 3 increased, while respiratory rates of state 3 in more than 10 μM decreased. With the increasing concentration of H_2S, effects of H_2S on respiratory rate of state 3 showed markedly decrease characteristic. Within the range of 40 to 50 μM H_2S, changes were very small. In hypoxic condition, effects of H_2S on respiratory rate of state 3 were similar to those in the normoxic condition, but changes in magnitudes of respiratory rate of state were small. Results show that the oxygen utilization by higher concentration of H_2S decreased in the mitochondrial

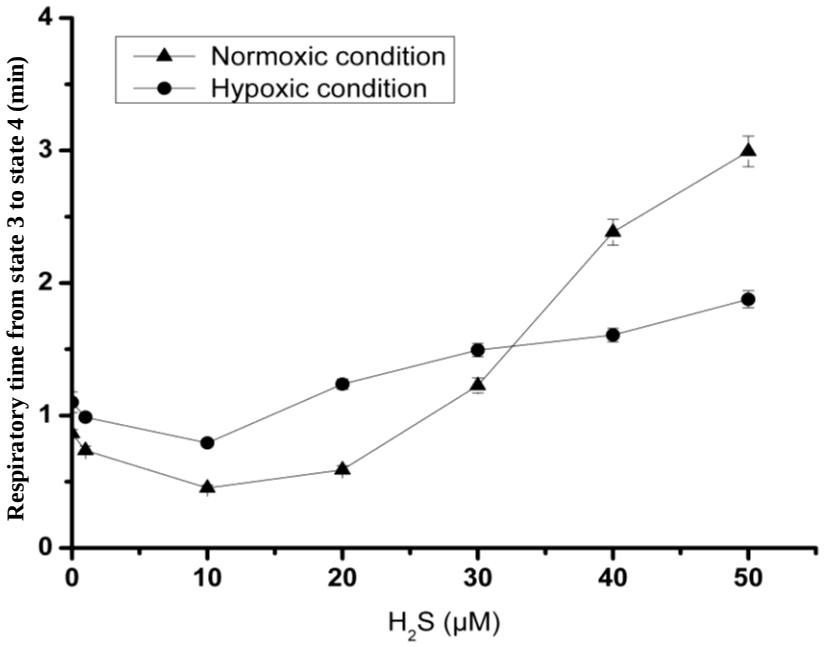

Figure 3. Effects of H_2S on respiratory time from state 3 to state 4.

respiration of state 3.

Changes of H_2S on recovery time from state 3 to state 4

Figure 3 shows the result obtained on the recovery time from state 3 to state 4 after adding H_2S. Recovery time from state 3 to state 4 first quicken and then delayed with the increasing of H_2S concentration at $p < 0.05$, compared within normoxic and hypoxic control groups, respectively. Moreover, the recovery time from state 3 to state 4 in normoxic condition was markedly delayed, especially in the higher concentration of H_2S. Changes of H_2S on recovery time from state 3 to state 4 in normoxic condition were more obvious than those in hypoxic condition. Delay of recovery time from state 3 to state 4 indicated that efficiency in mitochondrial respiration of state 3 deteriorated with increasing H_2S concentration.

Effects of H_2S on respiratory control ratio (RCR)

There were marked changes in RCR by H_2S. Changes of RCR by H_2S showed upward and then downward characteristics in a concentration-dependent manner at $p<0.05$, compared with normoxic and hypoxic control groups, respectively (Figure 4). Control intensity of RCR by H_2S was stronger in normoxic condition than in hypoxic condition. In normoxic condition, decreasing changes of RCR by more than 10 μM H_2S were distinct, while decreasing changes of RCR by more than 1 μM H_2S in hypoxic condition were obvious. Within the range of 40 to 50 μM, RCR was similar both in normoxic condition and hypoxic. Experimental data displayed that H_2S could regulate RCR, and H_2S regulating concentrations of RCR in hypoxic condition were lower than those in normoxic.

Influences of H_2S on ADP/O ratio

Figure 5 presents the ADP/O ratio by H_2S. Changes of ADP/O ratio also indicated an increase first and then decrease in both conditions. ADP/O ratio in more than 20 μM H_2S under normoxic condition dropped, while that in more than 10 μM H_2S under hypoxic condition decreased obviously compared with normoxic or hypoxic control groups at $p<0.05$. The downtrend of ADP/O ratio displayed oxygen utilization status in mitochondria which supported the opening of K_{ATP} channel.

DISCUSSION

Effects of H_2S on mitochondrial respiratory rate of state 4 in both conditions indicated uncouple appearing. Changes of respiratory rate of state 3 by H_2S increased first and then decreased remarkably. Recovery time from state 3 to state 4 was first quickened and then delayed, showing deteriorating mitochondrial respiration of state 3. Control intensity of RCR by H_2S displayed first upward and then downward characteristics. Changes of ADP/O ratio also increased first and then decreased. The

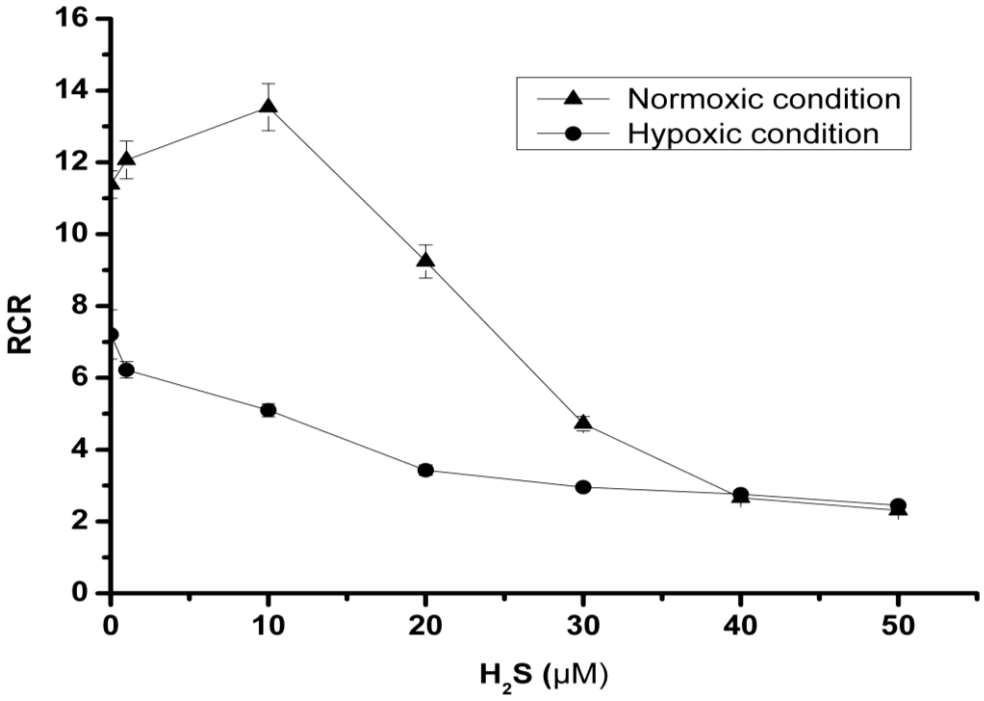

Figure 4. Effects of H_2S on RCR.

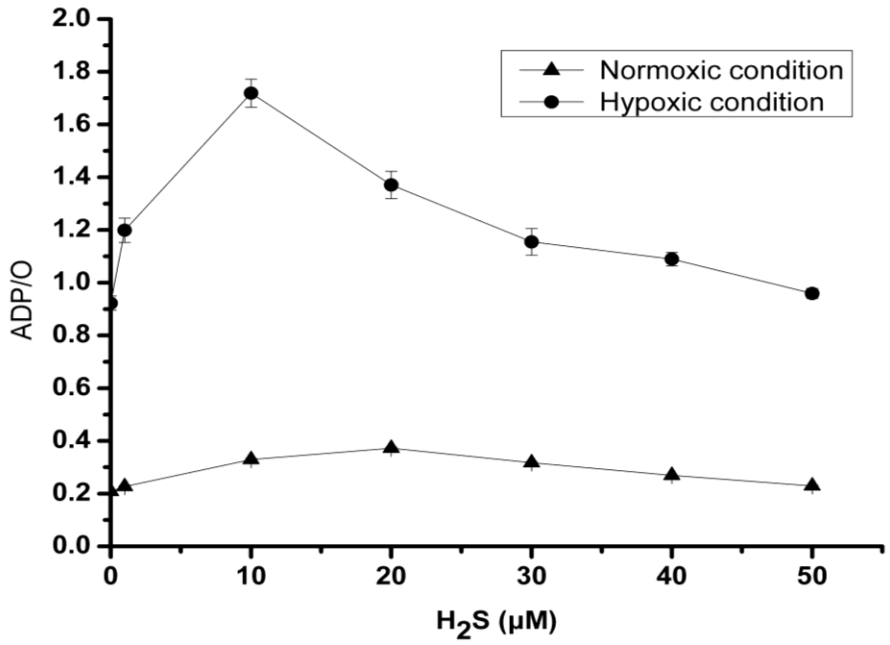

Figure 5. Effects of H_2S on ADP/O.

downtrend of ADP/O highlighted the uncoupling status of oxygen utilization with opening of K_{ATP} channel in the mitochondria.

The experimental hypothesis tested was that brief cardiac ischemia, duration as brief as *ca.*4 min, induced an increase in electron transport chain activity within the complex III and IV segment of mitochondrial respiratory chain. Mitochondria exposed to normoxic or hypoxic environments via adding different concentration of H_2S were expected to control over RCR in mitochondrial inner

membrane. Some studies showed that complex I produced superoxide predominantly on the matrix side of the inner membrane, while complex III produces superoxide towards both the inner membrane space and matrix (St-Pierre et al., 2002). Therefore, complex III-derived free radicals may be expected to have a greater impact on redox signaling pathways outside of the mitochondrial matrix. In light of this, the segment between complexes III and IV in mitochondrial inner membrane respiratory chain may be a very important respiratory regulative sites in brief cardiac ischemia (Matsuzaki et al., 2009). Mitochondria are important organelles for aerobic oxidation. Adequate blood supply, normal diffusion and exchange of gas and effective utilization of oxygen contribute to the normal mitochondrial respiratory function. State 3 means fast oxidation period in the presence of ADP and respiratory substrate. When ADP is exhausted, mitochondrial respiration enters into state 4. Respiration in state 4 could be used to reflect the mitochondrial permeability (Madesh et al., 2000). The decrease of RCR means uncoupling of respiration from oxidative phosphorylation in the mitochondria.

Longer ischemic durations, *ca.*24 h, was accompanied by loss of complex I activity (Matsuzaki et al., 2009), while brief cardiac ischemia induced an increase in electron transport chain activity within the complex III and IV segment of the mitochondrial respiratory chain. Complex III produces superoxide at the coenzyme-Q (CoQ) binding site via the production of ubisemiquinone (Turrens et al., 1985). In cardiac ischemia, elevated rates of superoxide production and increase in complex III and IV activity with brief ischemia are observed (Czerski et al., 2003). It is possible that cytochrome *c* disassociation via increasing osmolarity enhances complex IV activity and superoxide production in brief cardiac ischemia (Matsuzaki et al., 2009) and implicates it as a potentially important site of mitochondrial regulation. Both succinate as substrate and rotenone as complex I inhibitor were chosen to observe the effects of H_2S on RCR in mitochondrial inner membrane related to activities of sulfide-quinone oxidoreductase (SQR) and cytochrome c oxidase (CcO). The time of treatment with rotenone in the experiment was 6 min because the effects of rotenone are expected to be fast within minutes (Matsuzaki et al., 2009).

Furthermore, the changes by H_2S on mitochondrial respiratory rate of the state 4 and state 3 give us the experiment thought to explore the mechanism of H_2S in regulating electron transport in the inner membrane in mitochondria. Respiratory rate of the state 4 is generally assumed to reflect the summed leak processes across the inner mitochondrial membrane. This experimental result displayed that effects of adding different concentration of H_2S meant increasing respiratory rate of state 4 in normoxic and hypoxic conditions. The respiratory rate of state 3 in both conditions decreased markedly in more than 10 μM H_2S, which displayed very important significance. H_2S delayed recovery time from state 3 to state 4, and also decreased RCR and ADP/O ratio, respectively which probably supported that H_2S in appropriate concentrations facilitated the opening of mitochondrial K_{ATP} channel.

Moreover, the respiratory rate of state 3 can be limited by the rate of electron flow, the rate of entry of ADP or by the rate of phosphorylation, whereas state 4 rates are limited by the back leakage of the pH gradient or electrochemical potential. Therefore, change of respiratory rate of state 4 affects the RCR and not the ADP/O ratio. The degree of intactness of isolated mitochondria in respiratory function can be obtained from RCR. The control of respiration by ADP is a very sensitive criterion of the metabolic state of isolated mitochondria. A greater RCR values results from a greater degree of coupling between respiration and oxidative phosphorylation and suggests an increased efficiency of electron transfer. These experimental results showed gradual uncoupling between respiration and oxidative phosphorylation and suggested a decreasing controlled influence of electron transfer.

Mitochondrion is the location of ATP energy production in the cell. H_2S as exogenous substrate determines alternative pathways for oxygen metabolism. There are two sites for oxygen consumption when H_2S is oxidized by mitochondria: the formation of water in mitochondrial complex IV (CcO) and the formation of sulfur which at present requires in depth investigation. H_2S dose-dependently inhibits cardiac mitochondrial respiration and preserves mitochondrial function *in vitro*. A dose-dependent reduction of oxygen consumption compared with the control group was observed. Inhibition of CcO by H_2S is an important mechanism for the regulation of cellular oxygen consumption and likely involves the reduction of intracellular ferric iron to ferrous iron (Leschelle et al., 2005). While H_2S reaches a concentration sufficient to inhibit CcO, the electrons are redirected toward mitochondrial complex II (succinate dehydrogenase) which participates in the Krebs cycle and is situated on the matrix side of the inner membrane. In these conditions, H_2S acts in reverse mode and reduces fumarate into succinate. In hypoxia oxidation, H_2S yields electrons to the cytosolic side of the inner membrane and its products are released and oxidized further on the matrix side (Goubern et al., 2007).

In this experiment, less than or equal to 10 μM H_2S in both conditions on respiratory rate of state 4, state 3, recovery time from state 3 to state 4, RCR, and ADP/O displayed contrasting values compared with those of more than 10 μM H_2S in both conditions on all items. This phenomenon was closely related to the formation of sulfur. During the formation of sulfur-containing oxygenated products, H_2S as sulfide-quinone oxidoreductase (SQR) substrate (Vande Weghe and Ow, 1999) donates electrons to the mitochondrial respiratory chain between the complexes I and III. Coenzyme Q is the intermediate and acceptor of the electrons between complex I and complex III. Reduction of quinone, coenzyme Q by H_2S

requires that the two hydrogen atoms of H_2S (two electrons and two protons) would produce polysulfide or elemental sulfur. The oxidation of H_2S results in the accumulation of oxygenated products: mainly thiosulfate ($S_2O_3^{2-}$), sulfites (SO_3^{2-}) and sulfates (SO_4^{2-}), which implies the use of some oxygen (Volkel and Grieshaber, 1996). These are two separate processes from H_2S to its oxidation products via mitochondrial respiratory chain, which indicates electrons from H_2S enter the mitochondrial respiratory chain at the level of coenzyme Q and continue through proton pumping complexes III and IV to reach oxygen (Turrens et al., 1985).

SQR is a flavoprotein containing binding sites for H_2S and quinone. Electron transfer between H_2S and quinone is mediated by flavin adenine dinucleotide (FAD). Three cysteine residues have shown to be essential for the reductive half-reaction by site-directed mutagenesis. The catalytic activity is nearly completely abolished after mutation of each of the cysteines to serine. Mutation of a conserved valine residue to aspartate within the third flavin-binding domain leads to a drastically reduced substrate affinity for both H_2S and quinone. Two conserved histidine residues have mutated individually to alanine. The formations of polysulfide with sulfide-dependent reduction and quinone-dependent oxidation of the enzyme (Griesbeck et al., 2002), SQR displayed a shift in the pH dependence. SQR releases its oxidation products in the inter-membrane space where they are oxidized further. The formation of oxygenated products occurs in the inter-membrane space or the cytosol. The two sites of oxygen consumption have important consequences with regard to the possibility of an uncoupled state of mitochondria when H_2S is oxidized. On the one hand, mitochondrial respiration appears to be uncoupled when sulfide is oxidized. Furthermore, mitochondrial respiration may be incompletely uncoupled because oxygen consumption in the presence of H_2S is not uniquely due to the circulation of electrons from coenzyme Q to CcO. Proton circulation between mitochondrial respiratory complexes III and IV on the basis of oxygen consumption when sulfide is oxidized may be an expected situation. In brief, first of all, H_2S accumulates and leads to inhibition of CcO. Then electrons from H_2S would be directed toward the reduction of malate to succinate by reversing the succinate dehydrogenase complex (complex II) to regulate activity of SQR. The capacity of mitochondria to oxidize H_2S with activity change of SQR might be physiologically significant.

H_2S oxidation in the mitochondria is a phenomenon shared by all mitochondria (Yong and Searcy, 2001). Electrons from H_2S could be given to the mitochondrial respiratory chain at the level of coenzyme Q (Ouml and 1997) and cytochrome *c* (Powell and Somero, 1986). When H_2S is oxidized by mitochondria, the coupling of electron transport to proton pumping and/or ATP production is observed (Ouml et al., 1997; Powell and Somero, 1986). In these experiments, elemental sulfur in reacting chamber was detected by colorimetric determination. The elemental sulfur content in mitochondria sample was certainly positive.

In addition, mitochondria appeared to produce three ATPs per electron via the “long” route and 2 via the “short” (succinate) route (data not shown). It is a known fact that no one could demonstrate the presence of the putative chemical intermediate. However, the nature of interaction of H_2S and flavoprotein enzyme provides the opportunity to unearth new facts. The flavoprotein enzyme is located in the inner membrane of the mitochondrion and closely associated with the electron transport system. H_2S as a small molecule combined with metal ion suggests that H_2S may be potential candidate for regulating electron transport in isolated mitochondria from bovine heart.

Conclusion

H_2S could availably regulate respiratory process in the inner membrane of mitochondria and was certainly a potential drug in anti-ischemia. H_2S increased to a certain extent, the respiratory rate of state 4; increased first and then decreased the respiratory rate of state 3, respiratory control ratio, and ADP/O ratio; and finally quicken first and then delayed recovery time from state 3 to state 4. The effects of H_2S on mitochondrial respiratory control in normoxic condition were more obvious than those in hypoxic condition. Experimental results displayed that higher H_2S brought about uncoupling of the mitochondrial electron transport and the opening of mitochondrial K_{ATP} channel located in complex III – IV. Moreover, lower concentration of H_2S unexpectedly facilitated electron transport in mitochondrial inner membrane, which might be via sulfide-quinone oxidoreductase. The relationship between H_2S and sulfide-quinone oxidoreductase requires in depth investigated.

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