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Sulfide-inhibition of mitochondrial respiration at very low oxygen concentrations

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ABSTRACT

Our aim was to study the ability of an immortalized cell line (AMJ2-C11) to sustain aerobic cell respiration at decreasing oxygen concentrations under continuous sulfide exposure. We assumed that the rate of elimination of sulfide through the pathway linked to the mitochondrial respiratory chain and therefore operating under aerobic conditions, should decrease with limiting oxygen concentrations. Thus, sulfide's inhibition of cellular respiration would occur faster under continuous sulfide exposure when the oxygen concentration is in the very low range. The experiments were performed with an O2K-oxygraph (Oroboros Instruments) by suspending 0.5–1 × 10^6 cells in 2 ml of continuously stirred respiration medium at 37 °C and calculating the oxygen flux (JO_2) as the negative derivative of the oxygen concentration in the medium. The cells were studied in two different metabolic states, namely under normal physiologic respiration (1) and after uncoupling of mitochondrial respiration (2). Oxygen concentration was controlled by means of a titration-injection pump, resulting in average concentration values of 0.73 ± 0.05 μM, 3.1 ± 0.2 μM, and 6.2 ± 0.2 μM. Simultaneously we injected a 2 mM Na_2S solution at a continuous rate of 10 μl/s in order to quantify the titration-time required to reduce the JO_2 to 50% of the initial respiratory activity. Under the lowest oxygen concentration this effect was achieved after 3.5 [0.3;3.5] and 11.7 [6.2;21.2] min in the *uncoupled* and *coupled* state, respectively. This time was statistically significantly shorter when compared to the intermediate and the highest O_2 concentrations tested, which yielded values of 24.6 [15.5;28.1] min (*coupled*) and 35.9 [27.4;59.2] min (*uncoupled*), as well as 42.4 [27.5;42.4] min (*coupled*) and 51.5 [46.4;51.7] min (*uncoupled*). All data are medians [25%, and 75% percentiles]. Our results confirm that the onset of inhibition of cell respiration by sulfide occurs earlier under a continuous exposure when approaching the anoxic condition. This property may contribute to the physiological role of sulfide as an oxygen sensor.

1. Introduction

Albeit toxic at high concentrations, hydrogen sulfide (H_2S) has been recently recognized as a further "gasotransmitter" involved in the regulation of various physiological functions [1]. In particular, it is thought to play a role as an intracellular oxygen sensor. In most species and in humans [2] H_2S is released in the cytoplasm by the enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthase, as well as in mitochondria by the 3-mercaptopyruvate sulfur transferase (3-MST). Recently, thiosulfate has been recognized as a further source of sulfide, which is particularly involved in the oxygen sensing role exerted by this molecule [3]. Once released, however, H_2S is quickly degraded by a strictly aerobic pathway linked to the mitochondrial respiratory chain. This sulfide eliminating pathway is comprised by two enzymes: the sulfide–quinone oxidoreductase (SQR) and a dioxygenase. The former enzyme oxidises the sulfide and passes the electrons to the ubiquinone pool situated in the inner mitochondrial membrane, whereas the dioxygenase, which also requires oxygen as electron acceptor, further oxidises the two disulfides temporarily generated by the SQR [4]. The SQR's high capacity for sulfide degradation was recently proven and further suggested to play a crucial role in maintaining a non-toxic concentration of H_2S within the cells [5,6]. The putative oxygen sensing role of H_2S is linked to the oxygen dependency of its degradation pathway [2]. Specifically, under low oxygen conditions, the diminished rate of sulfide elimination through the SQR and dioxygenase pathway would lead to an accumulation of

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the compound at the cellular level. As demonstrated by Olson et al. hypoxic pulmonary vasodilation was associated with sulfide production in pulmonary artery smooth muscle cells [7]. Albeit under hypoxic conditions cells may still eliminate sulfide in other ways to compensate for the increased concentration. Therefore, a switch between aerobic and anaerobic conditions in sulfide elimination may serve as an indicator for a limiting oxygen concentration. Thus we studied the ability of immortalised cells derived from alveolar macrophages (AMJ2-C11) to maintain a constant rate of aerobic respiration under a continuous exogenous sulfide exposure at low oxygen concentrations nearing anoxia. We hypothesize that in case of a decreasing sulfide elimination rate through the SQR and dioxygenase pathway, the continuous addition of sulfide would lead to an earlier inhibition of aerobic respiration indicating the onset of sulfide accumulation. In order to test this assumption, we used a recently established experimental model [8] based on a high-resolution respirometry [9], which had been used to study the relationship between sulfide toxicity and infusion rate. In the present experiment the setup is modified to maintain stable low oxygen conditions throughout the sulfide titration.

2. Materials and methods

2.1. Cell cultures

AMJ2-C11 cells were cultured for 36–48 h at 37 °C in a standard medium (DMEM, Gibco, Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal calf serum, 1% non-essential amino acids, 1% glutamine, and 0.5% gentamicin and an atmosphere containing 95% air and 5% CO_2. We used this cell line for our experiments because they provided a reliable model to study the effects of sulfide in our previous investigation [8].

2.2. High-resolution respirometry

Cellular oxygen uptake was quantified by high-resolution respirometry using the Oroboros®Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria). This device allows for simultaneous recording of the O_2 concentration in two parallel chambers calibrated for 2 ml of respiration medium equilibrated with 21% O_2 in N_2 at 37 °C. For our present scope the medium was supplemented with catalase, which allowed to maintain the oxygen concentration by adding small volumes of hydrogen peroxide without re-opening the chamber (see below for more details). The partially adherent cultured cells were scraped, centrifuged at 150 × *g* for 5 min, and then suspended in the respiration medium at a concentration of 0.5–1 × 10^6 cells/ml. The cell suspensions were continuously stirred at 750 rpm. Cellular respiration was quantified in terms of oxygen flux (JO_2) based on the rate of change of the O_2 concentration in the chambers.

The continuous sulfide titration as well as the control of the oxygen concentration in the chamber were performed using a TIP-2K® titration-injection micropump (Oroboros Instruments). Control for the TIP-2K® as well as data acquisition and analysis were performed with the DatLab® software, version 4.3 (Oroboros Instruments). This enables continuous monitoring and recording of the oxygen concentration in the chambers as well as of the derived oxygen flux over time, normalized for the amount of cells at rates of 0.5–1 Hz.

2.3. Respiration under sulfide-exposure

Aerobic respiration under Na_2S exposure was assessed using a similar experimental setup as previously described [8]. Briefly, this technique consists of measuring the JO_2 while continuously injecting a 2 mM Na_2S solution at a rate of 10 nl/s into the oxygraph chambers containing the cell suspension. We performed the experiments on intact cells *coupled* respiration (1) and *uncoupled* respiration (2). The latter condition was achieved by sequentially injecting 2.5 μM oligomycine to inhibit the ATP-synthase, and 1–1.5 μM of the uncoupler p-trifluoromethoxy-carbonyl-cyanide phenylhydrazone (FCCP) into the respiration medium before starting the sulfide-injection. The reason to test both coupling states was to exclude the influence of the added sulfide on ATP-consuming processes, which may indirectly modify the level of cellular respiration independent from any effect on the cytochrome-*c* oxidase. Regardless of the coupling state, in both cases the cells were intact, and cell respiration was therefore sustained by endogenous substrates without the need for exogenous supplementation. Simultaneous to the sulfide injection, specific oxygen concentrations were maintained in the oxygraph chambers by a closed loop task of the DatLab® software. This tool allowed controlling the TIP-2K® for titrating 50–100 nl of a 20 mM hydrogen peroxide solution, thus maintaining the oxygen level in the respiration medium close within three predefined concentration ranges yielding mean values of 6.2 ± 0.2 μM, 3.1 ± 0.2 μM, and 0.73 ± 0.04 μM, and oscillating between average minima of 5.8 ± 0.1 μM, 2.7 ± 0.3 μM, and 0.62 ± 0.05 μM, and average maxima of 6.5 ± 0.5 μM, 3.3 ± 0.4 μM, and 0.82 ± 0.05 μM, respectively (see Fig. 1). The JO_2 throughout the sulfide-injection was then obtained calculating the linear decay of the oxygen concentration from each maximum to the next minimum (see Fig. 1 for more details), which corresponds to the standard method used for respirometry as mentioned above. The oxygen concentration ranges chosen for the experiments were determined based on the average results of five preliminary experiments performed according to previously published methods [9] recording the JO_2 of the AMJ2-C11 cells throughout the full range between normoxia and anoxia (“anoxic transition”), and allowed to limit mitochondrial respiration to 95%, 90%, and 70% of the maximum JO_2, respectively. These values were chosen in order to obtain measurements near to maximum (95% and 90%) and approaching half maximum respiratory activity (70%). In the latter case 50% would have been more appropriate, but at cost of a respiratory activity below the range of detection when using the same amount of cells in the chamber. We therefore preferred to perform these measurements at 70% JO_2, which yield a good signal quality without varying the cells content. Finally, each sulfide titration experiment was quantified in terms of the mean duration of the sulfide injection required to reduce the JO_2 to 50% of its value preceding the addition of sulfide. Since the titration rate was constant, this corresponds exactly to the total amount of sulfide injected into the chamber. For each specific oxygen concentration and cell respiration state we performed five separate experiments in order to obtain average results for mathematical and statistical calculations.

Furthermore, we performed two additional experiments in order to demonstrate in principle the effects of low oxygen concentration on sulfide degradation. For these experiments we used intact AMJ2-C11 cells in the FCCP-induced *uncoupled* state, and added of 0.5 μM rotenone to inhibit mitochondrial respiration by blocking complex I. Then we injected two sequential 4 μM boluses of the sulfide donor Na_2S. Since complex I had been blocked before, the increase in JO_2 induced by Na_2S was now exclusively attributable to sulfide turnover. After the second sulfide bolus the medium in the chamber was re-oxygenated to determine the JO_2 after restoration of the initial oxygen concentration.

2.4. Mathematical data processing and statistics

The resulting data obtained from each titration experiment were fitted to polynomial functions mathematically describing