Marine Environmental Research 92 (2013) 110–119

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Oxygen radical formation in anoxic transgression and anoxia-reoxygenation: Foe or phantom? Experiments with a hypoxia tolerant bivalve

Georgina A. Rivera-Ingraham [a], Iara Rocchetta [a, b], Stefanie Meyer [a], Doris Abele [a, *]

[a] Department of Functional Ecology, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany
[b] Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, INQUIBICEN-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Pab. II, Ciudad Universitaria, 1428 Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 19 July 2013
Received in revised form
10 September 2013
Accepted 12 September 2013

Keywords:
Blue mussel
Oxidative stress
Reoxygenation
Live imaging

ABSTRACT

Intertidal blue mussels, *Mytilus edulis*, experience hypoxia reoxygenation during tidal emersion and resubmersion cycles, and this is often suggested to represent a major stress for the animals, especially for their respiratory tissues, the gills. We exposed mussels to experimental short and prolonged anoxia and subsequent reoxygenation and analyzed the respiratory response in excised gill tissue and the effects of treatment on reactive oxygen species (mainly ROS: superoxide anion, $O_2^{\cdot-}$ and hydrogen peroxide, H_2O_2), formation using live imaging techniques and confocal microscopy. Our aim was to understand if this "natural stress" would indeed produce oxidative damage and whether antioxidant defenses are induced under anoxia, to prevent oxidative damage during reoxygenation. Exposure to declining pO_2 in the respiration chamber caused an increase of gill metabolic rate between 21 and 10 kPa, a pO_2 range in which whole animal respiration is reported to be oxyregulating. Exposure of the animals to severe anoxia caused an onset of anaerobiosis (succinate accumulation) and shifted high and low critical p_c values (p_{c1}: onset of oxyregulation in gills, p_{c2}: switch from oxyregulation to oxyconformity) to higher pO_2. Concentrations of both ROS decreased strongly during anoxic exposure of the mussels and increased upon reoxygenation. This ROS burst induced lipid peroxidation in the mantle, but neither were protein carbonyl levels increased (oxidative damage in the protein fraction), nor did the tissue glutathione concentration change in the gills. Further, analysis of apoptosis markers indicated no induction of cell death in the gills. To our knowledge, this is the first paper that directly measures ROS formation during anoxia reoxygenation in mussels. We conclude that hypoxia tolerant intertidal mussels do not suffer major oxidative stress in gill and mantle tissues under these experimental conditions.

1. Introduction

Marine and fresh water bivalves are among the most hypoxia tolerant macrofauna, and some benthic infauna species survive days and even weeks in complete anoxia (Abele et al., 2009). Intertidal, epibenthic bivalves, like blue mussels *Mytilus edulis*, experience complete anoxia when they close their shells during low tides to prevent desiccation, and shell water pO_2 rapidly falls to zero. Whereas some intertidal bivalves prevent severe anoxia by opening the valves for air gaping, this behavior is not common in *M. edulis*. Instead the strategy of the blue mussel is to keep the shell closed and depress energy expenditures and, as a consequence, ATP turnover and dissipation of heat (Wang and Widdows, 1993). Exposing mussels to declining oxygen levels in seawater, the authors recorded pO_2-independent respiration rates (oxyregulation) between 20 kPa (normoxia) and 10 kPa (moderate hypoxia). Below 10 kPa, respiration and heat dissipation diminished as the mussels entered into an energy saving state of suspended animation, characterized by reduced filtration and partial arrest of protein synthesis and degradation, to enable metabolic rate depression and, at the same time, minimize the energy and oxygen debt. Further reduction of pO_2 to near anoxia (1–2 kPa) induces anaerobic glycolysis in mussels and causes rapid accumulation of succinate within hours, with the highest concentrations measurable in

* Corresponding author. Tel.: +49 471 4831 1517; fax: +49 471 4831 1149.
E-mail addresses: grivera@us.es (G.A. Rivera-Ingraham), Doris.Abele@awi.de (D. Abele).

0141-1136/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.marenvres.2013.09.007

the gills. In prolonged anoxia (>16 h), succinate is converted to propionate and, to a lesser extent, to acetate in anaerobically working mussel mitochondria (Zurburg and Kluytmans, 1980 for mussels held in anoxia). Altogether these strategies render *M. edulis* relatively hypoxia tolerant, and median mortality time of 30–35 mm mussels is 9.6 days (Wang et al., 1992). Under intertidal conditions, re-immersion usually happens after 6 h so that tidal exposure poses no major problem to the mussels from the point of view of energetics. Hence this species qualifies as an euryoxic, hypoxia tolerant model to study the physiological effects of exposure to anoxia and subsequent reoxygenation.

Exposure of non-hypoxia tolerant animals to anoxia and subsequent reoxygenation has major consequences at the level of the cellular prooxidant/antioxidant balance and cellular redox homeostasis (Li and Jackson, 2002; Pamplona and Costantini, 2011; Tarpey et al., 2004). Small amounts of reactive oxygen species (ROS) are permanently formed in aerobically working cells through incomplete reduction of oxygen in the mitochondria. As tissue oxygen concentrations decline, the respiratory chain intermediates, especially at complex 1 (NADH–FADH2 oxidoreductase) and complex III (ubiquinone-cytochrome c-oxidoreductase), become increasingly reduced. Reduced intermediates of respiratory electron transport, such as ubisemiquinone, are charged with loosely bound electrons that are rapidly transferred to oxygen during reoxygenation. This produces oxygen derivatives with odd numbers of electrons (ROS), not all of them radicals, but many of them highly reactive and potentially damaging. This is why tissue oxygenation in animals is usually controlled low and constant in air or water breathers (for review see Massabuau and Abele, 2012).

For a long time since their discovery in the 1950s, ROS were exclusively regarded as damaging molecules that cause deterioration of cells and subcellular structures, and are centrally involved in cancer development and aging. Indeed, some ROS (superoxide and hydroxyl radical, $OH^{\bullet}$) are highly reactive prooxidants and interact rapidly by subtracting electrons from all kinds of cellular macromolecules, such as membrane fatty acids, protein amino acids and also DNA molecules. Other radical species are less reactive and these are produced in controlled quantities, e.g. as antimicrobial defense (hydrogen peroxide) in the blood of marine invertebrates with open circulatory systems (Carballal et al., 1997; Dikkeboom et al., 1987; Husmann et al., 2011). Cells counteract the damaging effects of ROS with effective antioxidant systems, including a suite of non-enzymatic radical scavengers (e.g. vitamins A, C, E and K, glutathione) and enzymatic antioxidants such as superoxide dismutase (SOD, converting superoxide anion into hydrogen peroxide), catalase (CAT, transforming hydrogen peroxide into water) or selenium-dependent glutathione peroxidase (Se-GPX, removing organic peroxides).

The present study deals with the formation of ROS and the resulting oxidative damage in gills of blue mussels under control conditions (normoxia), after exposure to different periods of anoxia (48 h and 72 h), and following reoxygenation. The idea is by no means a new one, but earlier works addressing the effects of oxygen deprivation and reoxygenation in marine animals exclusively report the response of the antioxidant defense system and the resulting oxidative damage (Almeida and Bainy, 2006; Irato et al., 2007; Viarengo et al., 1989). Contrary, the intensity of ROS formation itself is difficult to assess in living animals or intact organs. Without a direct measurement of the changes of ROS levels in response to changing oxygenation levels, conclusions may however be biased. Alterations of the antioxidant system, e.g. antioxidant enzyme activities, are only secondary indicators of oxidative stress, which often results from a lack of a proper antioxidant defense and the failure to induce gene transcription and synthesis of stress proteins under conditions of critical stress. Thus, absence of an increase in cellular antioxidants does not necessarily indicate the absence of oxidative stress, and vice versa. Contrary, oxidative damage products such as protein carbonyls as markers for oxidative protein damage or TBARS (thiobarbituric acid reactive substances) as a measure for lipid peroxidation are well accepted indicators of oxidative stress. However, their accumulation also depends on cell turnover and autophagic or apoptotic damage removal. Hence, the best method is to directly measure radical formation, and one elegant approach to do this is live imaging of cells, tissues and even small intact animals, using confocal microscopy combined with ROS-sensitive fluorescent dyes (Rivera-Ingraham et al., 2013).

Here we present our analysis of the effects of anoxia and reoxygenation on mussel gills, the organ which is most active in mussels and, therefore, reacts most sensitive to oxygen deprivation. We recorded the respiratory response of isolated gill pieces to declining oxygen tension and compared ROS formation, antioxidant defense and oxidative damage after exposing whole animals to normoxia, anoxia and anoxia-reoxygenation. Succinate was measured as indicator of anaerobic energy production. To understand whether elevated ROS or changes in tissue redox balance under any of the treatments induce apoptotic activity in gill tissues, we measured caspase activity in gill homogenates.

Our expectation was that ROS production would increase under anoxia reoxygenation treatment in mussels, and that this would have an inducing effect on the antioxidant enzyme activities and possibly cause ROS damage, alter the glutathione redox state, and apoptotic activity in the gills.

2. Materials and methods

2.1. Animal collection and maintenance

Blue mussels *M. edulis* were collected at the intertidal at the island of Sylt (Germany) in December 2012. Animals were transported to the Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research (AWI) in Bremerhaven, where they were maintained in aquaria at constant temperature of 10 °C, 33 PSU and >99% air saturation, and totally submersed. Animal shells were cleaned from epibiontic growth and allowed to adapt to the aquarium conditions for 3 weeks. Mussels were fed weekly with living phytoplankton, Fa. Plankton Farm, Sycamore, USA. Water circulation in the aquaria was stopped for four hours to allow feeding. Water quality was assessed weekly, using Nanocolor® Tube Tests (Macherey–Nagel GmbH & Co. KG, Germany) for ammonium and nitrate. Water in the aquaria was changed when values exceeded 0.4 mg/l and 0.2 mg/l of ammonium and nitrate, respectively. Prior the start of the experiment, animals were kept without food for 1 week to avoid the impact of specific dynamic action on gill respiration (Bayne et al., 1976).

2.2. Experimental design

A total of 4 treatments were considered in our study: anoxia (<0.6% air saturation): for 48 h, anoxia for 72 h and anoxia 72 h followed by a 24 h period of reoxygenation in normoxia (20 kPa). In each experiment, three 3L-aquaria with 5 individuals each were used to obtain independent parallel samples for each treatment. An addition, three 3L-aquaria with 5 other individuals were kept at normoxia for the same timespan as control group.

A total of 60 animals were used in the study. These had an average (±SEM) shell length of 4.50 ± 0.04 cm, a height of 2.22 ± 0.02 cm and a width of 1.99 ± 0.03 cm. Based on their size, and the works of Ottway & Ross (in Gosling, 2003) and Sprung (1983), we consider these animals as adults. No differences were made regarding sex or state of maturity. Mussels were sacrificed by